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Biodetekční systémy pro studium endokrinně disruptivního potenciálu

Habilitační práce

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Seznam použitých zkratek

AEQ	androgenní ekvivalent
AhR	arylhydrokarbonový receptor (*arylhydrocarbon receptor*)
AOP	dráha škodlivého účinku (*adverse outcome pathway*)
AOP-KB	znalostní databáze drah škodlivého účinku (*AOP Knowledge Base*)
AR	androgenní receptor
ARE	androgen responzivní element
ARNT	translokátor AhR (*aryl hydrocarbon receptor nuclear translocator*)
ATRA	kyselina all-*trans* retinová (*all trans-retinoic acid*)
azaPAH	heterocyklické dusíkaté deriváty polycyklických aromatických sloučenin
CYP19	enzym komplexu cytochromu P450, aromatáza
CYP	enzymy z rodiny cytochromů P450
ČOV	čistírna odpadních vod
ČR	Česká republika
DNA	deoxyribonukleová kyselina
E2	17β-estradiol
EBT	nástroje/metody založené na sledování účinku (*effect-based tools*)
ED	endokrinní disrupce; endokrinně disruptivní
EDA	účinkem-řízená analýza (*effect directed analysis*)
EDC	endokrinně disruptivní látky (*endocrine disruptive compounds*)
EDSTAC	Konzultační komise pro skríning a testování endokrinních disruptorů pro US EPA (*Endocrine Disruptor Screening and Testing Advisory Comittee*)
EDSP	program na skrínink endokrinních disruptorů v USA (*Endocrine Disruptor Screening Program*)
EDTA	aktivita OECD zaměřená na testování a hodnocení endokrinních disruptorů (*Endocrine Disruptors Testing and Assessment*)
EEA	Evropská agentura pro životní prostředí (*European Environment Agency*)
EEQ	estrogenní ekvivalent
EEQ-SSE	bezpečné koncentrace estrogenních ekvivalentů v odtokových vodách z komunálních ČOV
EFSA	Evropský úřad pro bezpečnost potravin (*European Food Safety Authority*)
EK	Evropská komise (*European Commission*)
ER	estrogenní receptor
EROD	enzym ethoxyresorufin-*O*-deethyláza
EU	Evropská unie
FETAX	test embryotoxicity a teratogenity na embryích drápatky velké (*Frog Embryo Teratogenesis Assay: Xenopus*)
GR	glukokortikoidní receptor
IPCS	Mezinárodní program chemické bezpečnosti (*International Programme on Chemical Safety*)
LC-MS	kapalinová chromatografie s hmotnostní spektrometrií
LC-HRMS	kapalinová chromatografie s vysokorozlišovací hmotnostní spektrometrií
LXR	jaterní X receptor

MIE	molekulární iniciační události (*molecular initiating event*)
mRNA	mediátorová ribonukleová kyselina
OCP	organické chlorované pesticidy (*organic chlorinated pesticides*)
Oct-4	transkripční faktor Octamer-4, marker pluripotence
OECD	Organizace pro hospodářskou spolupráci a rozvoj (*Organisation for Economic Co-operation and Development*)
OV	odpadní vody
PAH	polycyklické aromatické uhlovodíky (*polycyclic aromatic hydrocarbons*)
PBDE	polybromované difenylethery
PCB	polychlorované bifenyly
PCDD/F	polychlorované dibenzo-*p*-dioxiny a dibenzofurany
PNEC	předpokládaná koncentrace bez účinku (*predicted no effect concentration*)
POCIS	pasivní vzorkovač polárních látek (*polar organic chemical integrative sampler*)
POP	persistentní organické polutanty
PPAR	receptor aktivovaný peroxizomálními proliferátory (*peroxisome proliferator-activated receptor*)
QSAR	modelování vztahů mezi strukturou a aktivitou látek (*quantitative structure – activity relationships*)
9cisRA	9-*cis* retinová kyselina
RAR	receptor kyseliny retinové
REACH	Nařízení Evropského parlamentu a rady 1907/2006/EC o registraci, hodnocení, povolování a omezování chemických látek (Registration, Evaluation, Authorisation and Restriction of Chemicals)
RECETOX	Výzkumné centrum pro chemii životního prostředí a ekotoxikologii
REQ	ekvivalenty kyseliny retinové (ATRA)
RXR	retinoidní X receptor
SPMD	pasivní vzorkovač hydrofobních látek (*semipermeable membrane devices*)
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEQs	toxické ekvivalenty
TR	thyroidní receptor
UNEP	Program organizace spojených národů pro životní prostředí (*United Nations Environment Programme*)
US EPA	Agentura na ochranu životního prostředí v USA (*Environmental Protection Agency*)
WFD	Rámcová směrnice na ochranu vod (*Water Framework Directive*)
WHO	Světová zdravotnická organizace (*World Health Organization*)
XRE	Xenobiotické responsivní elementy

English abstract

Endocrine disruption has in recent years become an important issue not only for research but also for regulatory authorities. There is a great concern related to the effects of endocrine disruptive compounds on both human health and ecosystems. Endocrine disruptive compounds are abundant and wide spread in many environmental compartments, where they are present in complex mixtures. This habilitation thesis presents a cross-section of 15 years of research dealing with endocrine disruption related topics. It includes 26 papers published in international impacted journals, which are commented in the main text. Our research represented by selected papers in this thesis was focused on the development and optimization of the approaches and biodetection tools based on mammalian, fish and yeast cells for the investigation of the interference of compounds and their relevant environmental mixtures with the signaling of several cellular receptors crucial for endocrine regulation and with steroidogenesis. These tools were applied in a number of studies concerned with significant pollutants and their mixtures. First part of the thesis is devoted to the introduction of endocrine disruptors issue and also to its current regulatory context. The second part focuses on the *in vitro* and *in vivo* methods for the assessment of endocrine disruptive potential of compounds and mixtures, the development of the methodology including also fast "ready-to-use" biodetection tools. It also includes studies investigating the potential of several important pollutant groups (polycyclic aromatic hydrocarbons and their derivatives, phthalates, pharmaceuticals) to interfere with endocrine signaling through the studied modes of action. The following chapter presents *in vivo* approaches for the evaluation of endocrine disruptive potency and results of studies focused on selected pollutants. The developed approaches contributed to the ecotoxicological characterization of studied pollutant groups. The next part summarizes a number of studies which brought the first information on the specific endocrine disruptive potencies in various environmental media in the Czech Republic and abroad. This thesis focuses on aquatic environment as the major recipient of many pollutants with endocrine disruptive potencies, where also numerous effects on organisms have been reported. The studies characterized the pollution of surface and waste waters and of sediments with compounds with specific toxic potencies (anti/estrogenicity, anti/androgenicity, dioxin-like toxicity, retinoid-like activity and disruption of steroidogenesis), their seasonal and regional variability, impact of floods. They also showed the removal efficiency of these compounds and residual pollution in waste water treatment plants. Several studies documented *in vivo* effects in model organisms caused by the exposure to environmental samples and their association with the *in vitro* potentials and presence of pollutants characterized by chemical analysis. The biodection tools have been proven very useful towards the characterization of the potency of compounds to act through important endocrine disruptive modes of action as well as for the assessment of complex samples from the environment.

Předmluva

Když jsme před patnácti lety začínali s výzkumem problematiky endokrinní disrupce na našem pracovišti, bylo o tomto tématu známo poměrně málo informací. Poslední desetiletí přinesla velký rozvoj výzkumu v této oblasti. V posledních letech se endokrinní disruptory dostaly do středu zájmů i u regulačních orgánů.

Předkládaná habilitační práce shrnuje výsledky dlouhodobějšího výzkumu, jehož hlavním cílem bylo získávat poznatky o kontaminaci životního prostředí látkami s potenciálními účinky na endokrinní systém organismů. Práce obsahuje v přílohách plné texty 26 vybraných vědeckých prací (označovány jako Článek I – XXVI) publikovaných v impaktových mezinárodních časopisech, které jsou detailněji komentovány v textu práce. Vlastní plné texty jsou přiloženy v kapitole 6, která také obsahuje jejich seznam a komentář autorského podílu. Vzhledem k velkému rozsahu příloh jsou další relevantní autorské publikace zařazeny formou referencí a uvedeny v seznamu v kapitole 6.2 Další publikace autorky relevantní k tématu habilitační práce.

Kapitola 1 představuje zastřešující úvod do problematiky endokrinní disrupce. Kapitola 2 se zaměřuje na *in vitro* i *in vivo* metody pro sledování potenciálu různých látek i vzorků z prostředí způsobovat endokrinní disrupci (ED). Zahrnuje také výsledky našich výzkumů potenciálu různých typů polutantů interferovat s důležitými mechanismy účinků ED. Následuje kapitola 3, která shrnuje výsledky řady studií zaměřených na sledování endokrinně disruptivního potenciálu směsí látek v různých matricích akvatického prostředí a studie propojující *in vitro* a *in vivo* přístupy ve studiu této problematiky. V kapitole 4 je závěr shrnující všechny hlavní poznatky, následuje seznam literatury (kapitola 5), přílohy a přiložené publikace (kapitola 6).

Výsledky shrnuté v habilitační práci vycházejí z podílu autorky na řešení řady projektů zabývajících se problematikou endokrinní disrupce či zatížení prostředí endokrinními disruptory. Část byla realizována během výzkumné stáže v rámci Fulbrightova stipendia a pak následně postdoktorantského pobytu v Aquatic Toxicology Lab, Michigan State University. Další výzkumné projekty pak byly realizovány na našem pracovišti RECETOX na Masarykově univerzitě. Studie zaměřené na další metodický rozvoj a zkoumání potenciálu různých typů látek narušovat fungování endokrinního systému a zejména na výskyt a osud těchto látek v různých složkách prostředí podpořily zejména následující projekty:

GAČR 525/03/0367 (2003-2005): Ekotoxikologie persistentních organických polutantů životního prostředí.

GAČR 525/05/P160 (2005-2007): In vitro modely pro studium endokrinní disrupce - účinky tradičních a nových typů persistentních organických polutantů.

Projekt US EPA (Contract No. GS-10F-0041L, 2002-2005): Optimization of the H295R Cell Line for Use in Evaluating Toxicant-induced Effects on Steroidogenesis.

Výzkumný záměr INCHEMBIOL 021622412 (2005-2011): INterakce mezi CHEMickými látkami, prostředím a BIOLogickými systémy a jejich důsledky na globální, regionální a lokální úrovni

Projekt MŠMT NPVII 2B08036 ENVISCREEN (2008 - 2011): Nové molekulárně biologické a biochemické metody pro monitoring estrogenů a dalších chemických endokrinních disruptorů

CETOCOEN - projekt vybudování Centra pro výzkum toxických látek v prostředí (2010-2014)

GAČR 524/08/0496 (2008-2010) - Mechanismy nádorové promoce metabolitů toxických sinic

GAČR P503/12/0553 (2012 - 2015): Metabolity sinic jako potenciální endokrinní disruptory

EU FP7 SOLUTIONS (grant agreement No. 603437, 2013-2018) - Solutions for present and future emerging pollutants in land and water resources management

1 Endokrinní disrupce

Důležitým problémem současnosti je kontaminace životního prostředí látkami, které mohou narušovat přirozené fungování endokrinního systému a působení hormonů jako klíčových signálních a řídících molekul v živých organismech. Endokrinní systém hraje zásadní roli v udržování rovnováhy v organismu. Komplexní a citlivá endokrinní regulace biologických procesů je společná charakteristika živočišného kmene a je fylogeneticky velmi konzervována zejména mezi obratlovci. Látky, které narušují přirozené funkce endokrinního systému organismů a citlivě řízené působení hormonů, tzv. endokrinní disruptory (EDC - z anglického endocrine disruptive compounds), zasahují do regulačních pochodů u bezobratlých živočichů a obratlovců včetně člověka, a chronicky tak působí na základní fyziologické procesy. Narušení hormonální regulace vede k poruchám normální buněčné diferenciace a růstu, vývoje, metabolismu, imunity, chování a reprodukce během života (Swedenborg et al., 2009; Baker, 2001).

Nejcitovanější definice toho, co je endokrinní disruptor, o kterou se opírá i řada regulatorních orgánů, pochází z reportu WHO/IPCS (2002). Endokrinní disruptory jsou zde definovány jako látky nebo směsi, které mění funkci endokrinního systému, následkem čehož vyvolávají škodlivé zdravotní účinky v organismu, potomstvu či (sub)populacích. Potenciální endokrinní disruptory pak jako látky nebo směsi, které mají vlastnosti, které mohou vést k endokrinní disrupci v organismu, jeho potomstvu nebo populacích (WHO/IPCS, 2002).
Výzkumy v posledních desetiletích přinesly řadu důkazů, že chemické látky používané v průmyslu i v mnoha výrobcích mohou mít vliv na endokrinní systém. Několik mezinárodních odborných zpráv zahrnujících rozsáhlé rešeršní podklady a zpracovaných pod dohledem a na zadání organizací jako mezinárodní Endokrinologická Společnost (Endocrine Society; Diamanti-Kandarakis et al., 2009), Evropská komise (Kortenkamp et al., 2011), Evropská agentura pro životní prostředí (European Environment Agency; EEA, 2012) či rozsáhlá zpráva WHO & UNEP (2013) vyjadřuje rostoucí obavy z vlivu endokrinních disruptorů na organismy v kontaminovaných ekosystémech i na lidskou populaci.

Hormony jsou chemické signály produkované v organismu, které působí ve velmi malých koncentracích v určitém konkrétním čase. Expozice endokrinním disruptorům během vývoje může mít vliv na jedince v průběhu celého života a dokonce následky pro budoucí generace. Zdravotní účinky se mohou projevit dlouhou dobu poté, co skončila expozice. Výzkumy prokázaly, že existují velmi citlivá období v průběhu prenatálního i postnatálního vývoje, kdy mohou EDC nebo jejich směsi mít silné a často nevratné účinky na vyvíjející se orgány, zatímco u dospělců mohou být účinky menší nebo žádné (WHO & UNEP, 2013). Velikost a délka trvání účinků jsou tedy velmi ovlivňovány načasováním expozice a stupněm vývoje, ve kterém byl jedinec exponován a mohou se lišit během doby života organismu (embryo vs. fetus vs. dospělec) (De Coster & Van Larebeke, 2012). Účinky EDC jsou často opožděné, kdy ke kompletním projevům nemusí dojít až do dospělosti.

Zpráva o současném stavu poznání ohledně endokrinní disruptorů (State of the Science of Endocrine Disrupting Chemicals; WHO & UNEP, 2013), kterou společně vydaly Program organizace spojených národů pro životní prostředí (UNEP) a Světová zdravotnická organizace (WHO) i Endokrinologická Společnost (Gore et al., 2015) zdůrazňují zvyšující se pravděpodobnost, že expozice chemickým látkám hraje významnou roli v onemocněních a poruchách spojených s endokrinním systémem. Vědci poukazují na nárůst výskytu řady zdravotních poruch, který nemůže být vysvětlen pouze genetickými faktory nebo životním stylem. U chlapců a mužů jsou diskutovány klesající kvalita spermatu, zvýšený výskyt malformací pohlavních orgánů i rakoviny varlat a prostaty. U dívek a žen pak časná puberta a

nádory prsu a vaječníků. EDC také mohou přispívat k rychlému nárůstu výskytu cukrovky, obezity, onemocnění štítné žlázy a některých neurologických a imunitních problémů (WHO & UNEP, 2013).

Kromě lidské populace, kde je problematika endokrinní disrupce v poslední době hodně diskutovaným tématem, existuje celá řada infomací ohledně jiných živočišných druhů, kde byla prokázána spojitost endokrinní disrupce s účinky *in vivo* případně i s ovlivněním celých populací či ekosystémů (Kortenkamp et al., 2011; WHO & UNEP, 2013). Mnoho environmentálních kontaminantů může působit jako disruptor endokrinního systému a napodobovat nebo antagonizovat funkce nebo ovlivňovat biosyntézu endogenních hormonů a tím působit negativně na hormonální regulaci u volně žijících organismů (WHO/IPCS, 2002). U řady látek přítomných v životním prostředí bylo prokázáno negativní působení na normální fyziologické fungování endokrinního systému savců, ptáků, ryb, plazů, obojživelníků i bezobratlých (Sumpter & Johnson, 2005; Kortenkamp et al., 2011). Endokrinní disrupce je u volně žijících živočichů spojena s mnoha *in vivo* účinky, jako reprodukční a vývojová toxicita, embryotoxicita, karcinogenita a další. Následky endokrinní disrupce ve volně žijících živočiších zahrnují také sníženou plodnost a líhnivost, zhoršenou kvalitu a kvantitu spermatu, změněný poměr pohlaví, demaskulinizaci a feminizaci samců, defeminizaci a maskulinizaci samic, snížené přežívání mláďat, poruchy imunitního systému, změny chování, malformace pohlavních orgánů, abnormální funkci a morfologii štítné žlázy, ale také účinky na vyšších úrovních biologické organizace včetně vymizení populací.
Kromě mnoha účinků u ryb a vodních bezobratlých diskutovaných podrobněji v kapitole 3, další známé příklady zahrnují například změny v pohlavních orgánech a poruchy reprodukce u aligátorů. EDC jsou také diskutovány v souvislosti se snížením počtu jedinců v populacích mořských savců či s poruchami reprodukčního traktu, funkce štítné žlázy a chování u ptáků (Kortenkamp et al., 2011; WHO & UNEP, 2013).

1.1 Řešení problematiky endokrinních disruptorů ve světě a v ČR

Počátky řešení problematiky endokrinních disruptorů na regulatorní úrovni spadají do závěru minulého století. V roce 1996 byla založena poradní komise pro skrínink a testování endokrinních disruptorů v USA (EDSTAC; Endocrine Disruptor Screening and Testing Advisory Comittee). Ve stejném roce byla zahájena speciální aktivita OECD zaměřená na testování a hodnocení endokrinních disruptorů (EDTA; Endocrine Disruptors Testing and Assessment). OECD v roce 2002 navrhlo koncepční rámec pro EDTA, který byl revidován v roce 2012. V rámci OECD byla od roku 2002 validována řada *in vitro* i *in vivo* testů pro detekci a charakterizaci endokrinně aktivních látek. Další takové testy jsou ve vývoji, či ve validační fázi (více kapitola 2).

Na základě doporučení EDSTAC připravila americká Agentura na ochranu životního prostředí (US EPA) dvoustupňový program na skrínink endokrinních disruptorů (EDSP; Endocrine Disruptor Screening Program). V roce 2009 byl ve Spojených státech vyhlášen první seznam vybraných prioritních látek k hodnocení na konkrétní sadě testů. V prvním stupni je požadováno 5 *in vitro* a 5 *in vivo* testů zaměřených především na estrogenní, androgenní a thyroidní dráhy a steroidogenezi. V druhém stupni jsou pak u vybraných látek požadovány některé dlouhodobější a vícegenerační studie (Marty et al., 2011). Tyto testy musí být prováděny dle platných norem US EPA, pro některé jsou dostupné OECD normy.

V roce 1999 uveřejnila Evropská komise (EK) Strategii Společenství pro endokrinní disruptory (COM(1999)706), kde byl vytyčen rámec a stanoveny krátkodobé, střednědobé a dlouhodobé aktivity s cílem řešení problematiky endokrinních disruptorů v Evropě.

Krátkodobé a střednědobé cíle zahrnovaly souhrny a syntézu nejaktuálnějších vědeckých poznatků, identifikaci chybějících informací a jejich doplnění, stanovení priorit pro další hodnocení a výzkum a vývoj v této oblasti (např. zavedení monitorovacích programů, vývoj a harmonizace nových testovacích metod, shromažďování informací o potenciálních endokrinních disruptorech a vypracování seznamu prioritních látek).

Zásadním dlouhodobějším cílem je adaptace a doplnění legislativních nástrojů v rámci EU týkajících se chemických látek a ochrany spotřebitelů, zdraví a životního prostředí tak, aby zohledňovaly možná rizika účinků na endokrinní systém.

Evropská unie vydala čtyři hlavní legislativní nařízení, které přímo obsahují požadavky ohledně hodnocení potenciálu látek či jejich směsí působit endokrinní disrupci:

Nařízení Evropského parlamentu a Rady

č.1907/2006 o registraci, hodnocení, povolování a omezování chemických látek (REACH)

č.1107/2009 o uvádění přípravků na ochranu rostlin na trh (Plant Protection Product Regulation)

č.528/2012 o dodávání biocidních přípravků na trh a jejich používání (Biocidal Product Regulation)

č.1223/2009 o kosmetických přípravcích (Cosmetics Products Regulation)

Tato nařízení podporují prodej a použití pouze chemických produktů, které nezpůsobují endokrinní disrupci v lidech či volně žijících zvířatech. Bohužel v rámci těchto legislativ stále neexistuje jednotný přístup k tomu, jak identifikovat a hodnotit potenciál látek a směsí způsobovat endokrinní disrupci. Nařízení EU jsou nadřazena národní legislativě, tudíž jsou obecně závazná a bezprostředně použitelná v každém členském státě včetně České republiky. Dle nařízení REACH měla Evropská Komise do června 2013 provést revizi autorizace endokrinních disruptorů po zohlednění nejnovějšího vývoje vědeckých poznatků. V nařízení pro biocidy a pesticidy byl požadavek do prosince 2013 stanovit vědecká kritéria pro určení vlastností vyvolávajících narušení endokrinní činnosti. Než budou tato kritéria přijata, jsou za látky s vlastnostmi, které narušují činnost endokrinního systému, považovány látky, které jsou nebo musí být podle nařízení č. 1272/2008 klasifikovány jako karcinogenní kategorie 2 a toxické pro reprodukci kategorie 2. V případě kosmetických přípravků měla být funkčnost legislativy ohledně endokrinních disruptorů přezkoumána, jakmile budou k dispozici na úrovni Společenství nebo na mezinárodní úrovni dohodnutá kritéria pro identifikaci látek s vlastnostmi, které narušují činnost žláz s vnitřní sekrecí (nejpozději do ledna 2015).

V současnosti se na problematice stanovení kritérií pro endokrinní disruptory ještě stále intenzivně pracuje, jak na úrovni OECD, tak na úrovni Komise. Mezitím poskytuje legislativa aplikovatelná provizorní kritéria. Evropská komise se snaží zajistit, aby byla kritéria založena na co možná nejlepších vědeckých poznatcích. Proto v roce 2012 pověřila dvě expertní skupiny vypracováním podkladových zpráv a doporučení přístupu k identifikaci a charakterizaci EDC a přezkoumáním, zda současné metody testování toxicity jsou vhodné pro identifikaci a charakterizaci potenciální endokrinní aktivity a/nebo endokrinní disrupce u lidí a v ekosystému. Jednu zprávu vypracovala expertní poradní skupina pro endokrinní disruptory pod vedením odborníků ze Společného výzkumného centra Evropské komise (Endocrine Disrupters Expert Advisory Group; Munn & Goumenou, 2013), druhou vědecká komise Evropského úřadu pro bezpečnost potravin (European Food and Safety Authority; EFSA, 2013). Obě byly publikovány v roce 2013 a společně s dřívější zprávou vypracovanou pro EK týmem profesora Andrease Kortenkampa o současném stavu poznání v této oblasti

(Kortenkamp et al., 2011) a závěry konferencí EK zaměřených na tuto problematiku slouží jako vědecký základ k navržení chybějících kritérií.

Další Evropská legislativa se vztahem k problematice endokrinní disrupce je např. nařízení o klasifikaci, označování a balení látek a směsí (1272/2008/ES) a Rámcová směrnice na ochranu vod (2000/60/ES). Cílem Rámcové směrnice na ochranu vod (WFD) je dosáhnout dobrého ekologického a chemického stavu vod v EU a redukovat znečištění. V příloze VIII směrnice, která zahrnuje indikativní seznam hlavních polutantů jsou uvedeny i látky a přípravky, které mohou ovlivňovat steroidogenní, thyroidní, reprodukční nebo jiné funkce spojené s endokrinním systémem v akvatickém prostředí a nebo jeho prostřednictvím. Na aktuálním seznamu prioritních polutantů, pro něž byly stanoveny limity v rámci WFD, je řada látek známých jako endokrinní disruptory.

I přes velkou pozornost věnovanou této problematice v posledních letech pro klasifikaci látek jako EDC zatím neexistují jasná kriteria ani politika v rámci EU ani v dalších oblastech světa. Stále dochází k vývoji nových koncepcí a nastavování systémů ochrany proti EDC. V Evropě probíhá mnoho aktivit výzkumných skupin a projektů, které se zabývají vývojem metod, monitoringem, inventarizací a shromažďováním dat o EDC, či zkoumají a vyvíjejí metodiku hodnocení rizik EDC. Velká skupina renomovaných mezinárodních vědců podepsala Berlaymontskou deklaraci o endokrinních disruptorech, která byla v létě 2013 předána Evropské komisi, v níž požadují rychlejší a přísnější postup při formování EU legislativy k efektivní regulaci těchto látek. Odborníci v této deklaraci vyjadřují obavy z vlivu těchto látek a poukazují na jejich možnou roli v řadě onemocnění. Vyzývají k cílenému výzkumu endokrinních disruptorů zaměřenému na identifikaci látek schopných narušovat hormonální systém, na zhodnocení expozice, na vývoj laboratorních modelů a na podporu studia jejich vlivu na lidské zdraví.

1.2 Endokrinní disruptory a jejich zdroje v prostředí

Rozvoj průmyslu a používání jeho produktů jsou spojeny s uvolňováním chemických látek do životního prostředí. Teprve po mnoha desetiletích masivního rozvoje používání různých chemických látek je prokazována schopnost některých z nich narušovat hormonální systém organismů. V současné době neexistuje žádný obecně přijímaný seznam, který by rozlišoval prokázané a potenciální endokrinní disruptory, neboť kritéria, která by jednoznačně rozlišila, co jsou negativní účinky způsobené primárně mechanismy endokrinní disrupce, a (eko)toxikologické testy, které by je zhodnotily, jsou stále ve vývoji a předmětem intenzivní diskuse (EFSA, 2013; Munn & Goumenou, 2013; OECD, 2012). Evropská komise na svých webových stránkách prezentuje seznam látek klasifikovaných do tří kategorií, s tím že v první kategorii jsou zahrnuty látky (194 látek v roce 2014), u nichž byla prokázána endokrinní disrupce *in vivo* alespoň v jednom organismu (European Commission, 2015). Do druhé kategorie patří látky, u nichž byla ED aktivita zjištěna *in vitro* a třetí skupinu tvoří látky, u nichž nebyl zjištěn potenciál působit ED nebo pro něž nejsou dostupná dostatečná data. Vedle toho mnoho informací ohledně látek s ED potenciálem a jejich účinků je mimo jiné shrnuto ve výše uvedených souhrných mezinárodních zprávách od WHO & UNEP (2013) a Evropské komise (Kortenkamp et al., 2011).

Environmentální kontaminanty, u nichž byly prokázány negativní účinky na endokrinní systém volně žijících organismů, zahrnují řadu persistentních organických polutantů (POP) jako polychlorované dioxiny a furany (PCDD/F) a příbuzné agonisty aryl hydrokarbonového receptoru (AhR) polychlorované bifenyly (PCB). I přes všechna regulační opatření jsou běžně

sledované, ale i nově prioritní POP, díky vysoké persistenci nadále přítomné v prostředí ve významných koncentracích a dochází k jejich bioakumulaci v potravním řetězci. Endokrinně disruptivní účinky byly také pozorovány u širokého spektra látek s nižší persistencí, jako jsou polycyklické aromatické uhlovodíky (PAH), pesticidy, syntetická analoga steroidů (např. 17α-ethinylestradiol používaný v hormonální antikoncepci), farmaka a látky z kosmetických přípravků a produkty osobní péče (např. krémy s UV filtrem, konzervanty), či přírodní produkty jako jsou fytoestrogeny (WHO & UNEP, 2013). Patří k nim také surfaktanty, různá aditiva průmyslových materiálů jako alkylfenoly či ftaláty (změkčovače plastů), zpomalovače hoření, těžké kovy, ale také přirozené hormony, které jsou uvolňovány do prostředí a vykazují schopnost narušovat endokrinní systém organismů a představují tak potenciální nebezpečí pro živočichy včetně člověka. Jednotlivé EDC se liší svým potenciálem narušovat endokrinní systém organismů i fyzikálně-chemickými vlastnostmi.

Řada těchto látek se může v prostředí vyskytovat jako tzv. pseudopersistentní. Tedy samy o sobě nevykazují vysokou persistenci, ale mají do prostředí stálý přísun a mohou dlouhodoběji dosahovat významných hladin s potenciálem negativních účinků. Navíc buněčné odpovědi, které mohou vést k narušení endokrinní rovnováhy organismu, jsou často indukovány při velmi nízkých koncentracích (Vandenberg et al., 2012).
EDC do životního prostředí mohou vstupovat během výroby, použití či likvidace různých materiálů, z bodových i plošných zdrojů, průmyslových a komunálních odpadů a odpadních vod, splachy ze zemědělských ploch (WHO & UNEP, 2013). Některé EDC pocházejí i z přírodních zdrojů (např. fytoestrogeny). Syntetické potenciální EDC mohou být uvolňovány do prostředí při výrobě a používání pesticidních přípravků, plastů, nábytku, elektroniky, produktů denní spotřeby či kosmetiky.
Kromě skupiny doposud identifikovaných endokrinních disruptorů je však zřejmé, že člověk do komplexních směsí v prostředí uvolňuje řadu dalších (doposud neidentifikovaných) kontaminantů, jejichž účinky lze očekávat a předpovědět, ale o jejichž chemické povaze a skutečných hladinách v životním prostředí není dostatek informací (Brack et al., 2015).

2 Metody pro studium endokrinně disruptivního potenciálu

Jak je výše uvedeno, hodnocení potenciálu látek a směsí působit mechanismy endokrinní disrupce je velmi aktuálním tématem mezinárodního vědeckého výzkumu i předmětem zájmu regulačních orgánů. Prioritou Evropské Komise, WHO, OECD i US EPA (WHO & UNEP, 2013) je vývoj, validace a revize testů pro detekci endokrinních disruptorů, zavádění skríningových programů a koordinace výzkumu a testování těchto látek na mezinárodní úrovni. Hodnocení potenciálu látek působit mechanismy endokrinní disrupce zahrnuje *in vitro*, *in vivo* a *in silico* (modelovací, prediktivní) přístupy. *In vitro* metody umožňují sledování mechanismů působení, charakterizaci potence jednotlivých látek i směsí působit určitým mechanismem ED, skríning velkého množství vzorků, zatímco *in vivo* testy poskytují informaci o celkové komplexní odpovědi organismu, ale jsou výrazně náročnější na provedení. V rámci zavádění principů 3R (Reduce = omezení, Refine = zjemnění, Replace = náhrada *in vivo* testů alternativními přístupy; Russell & Burch, 1959) je stále větší důraz kladen na využívání *in vitro* a *in silico* přístupů a omezení nutnosti testování *in vivo*.
Velmi intenzivně také probíhá výzkum a vývoj vhodných metod a biodetekčních systémů, které by umožnily efektivně sledovat a monitorovat výskyt látek vyvolávajících endokrinní

disrupci ve všech hlavních matricích životního prostředí (voda, sedimenty, půda, ovzduší a biota) i v biotických materiálech (tkáně organismů, potraviny). Účinné studium širokého spektra polutantů s potenciálem způsobovat endokrinní disrupci naráží z chemicky-analytického hlediska na některé překážky. Hlavním problémem je fakt, že chemická podstata je známa pouze u omezeného množství endokrinních disruptorů a do prostředí jsou prokazatelně vnášeny další látky, které zatím nebyly chemicky identifikovány a nebo nejsou běžně analyzovány, avšak mohou mít i ve spolupůsobení s dalšími polutanty škodlivé účinky na živé organismy. I přes velký rozvoj environmentální analytické chemie v posledních letech není možné sledovat a kvantifikovat všechny polutanty přítomné ve vzorcích v prostředí, zejména kvůli omezené kapacitě analýz, finanční a časové náročnosti a nedostupnosti analytických standardů (Jia et al., 2015). Cílené analytické metody mohou sledovat jen omezený počet analytů a výsledky dostatečně nereprezentují celkový toxický potenciál studovaného vzorku. Kontaminanty se navíc vyskytují ve směsích, kde jejich spolupůsobení je obtížně predikovatelné z výsledků chemických analýz pouze vybraných sledovaných polutantů (Carvalho et al., 2014). V některých případech mohou svou nebezpečnost ve vzájemných interakcích potencovat. Za této situace se jako velmi vhodné ukazuje komplementární využívání nejrůznějších specifických (eko)toxikologických metod, které poskytují významnou informaci o účincích složitých kontaminovaných směsí (Escher et al., 2014). Kombinací obou typů metod lze získat komplexní informace jak o chemickém složení z hlediska známých endokrinních disruptorů, tak o celkovém potenciálu vzorku vyvolávat škodlivé účinky (Neale et al., 2015).

Výzkum představený v této habilitační práci je možné shrnout do čtyř základních okruhů:

1. Vývoj, charakterizace a optimalizace *in vitro* biodetekčních systémů pro sledování potenciálu cizorodých látek a jejich směsí ovlivňovat endokrinní systém organismů prostřednictvím několika klíčových receptorových mechanismů a ovlivněním steroidogeneze

2. Stanovení schopnosti a potence důležitých skupin polutantů narušit signálování těchto receptorů či průběh steroidogeneze, popis vztahu struktury a účinku

3. Charakterizace *in vivo* účinků vybraných prioritních polutantů

4. Charakterizace potenciálu směsí látek přítomných v různých typech vzorků z akvatických ekosystémů působit sledovanými mechanismy endokrinní disrupce, studium souvislosti s *in vivo* účinky a kontaminací charakterizovanou pomocí analytických metod, zhodnocení možných rizik pro akvatické ekosystémy

   Studie z posledního tematického bloku se zaměřují na:

   – zatížení různých typů odpadních vod, povrchových vod a sedimentů

   – vliv různých typů zdrojů znečištění na zatížení akvatických ekosystémů

   – zbytkové znečištění v odpadních vodách, odbourávání ED potenciálu v průběhu čištění odpadních vod

   – prostorovou a sezónní variabilitu znečištění

   – vliv povodní na kontaminaci

Specifické dílčí cíle jsou vždy uvedeny u konkrétních studií a v přiložených publikacích.

2.1 In vitro metody

Endokrinní disruptory mohou působit řadou mechanismů, na různých cílových místech, v různých orgánech. Některé EDC interagují přímo jako agonisté či antagonisté vazbou na různé typy proteinových receptorů s následnou aktivací nebo inhibicí jejich přirozených funkcí. Tyto látky napodobují či antagonizují endogenní působení hormonů *in vivo* i *in vitro*. Látky také mohou ovlivňovat některé z řady proteinů, které kontrolují přísun hormonu k jeho cílové buňce či tkáni (tj. transport v krvi či hemolymfě). K dalším mechanismům patří narušení syntézy a sekrece endogenních hormonů, metabolismu nebo vylučování (Kortenkamp et al., 2011). EDC mohou vyvolat narušení křehké rovnováhy mezi regulačními mechanismy v endokrinním systému, které pak mají zásadní vliv na koncentrace hormonů v organismu. V endokrinní disrupci mohou hrát roli i další epigenetické mechanismy jako změny v metylaci DNA a modifikaci histonů (Casati et al., 2015; De Coster & Van Larebeke, 2012).

Část našeho výzkumu se dlouhodobě zaměřuje na vývoj a využití *in vitro* přístupů k hodnocení potenciálu jednotlivých látek i komplexních směsí působit některými klíčovými mechanismy endokrinní disrupce. *In vitro* biotesty zaměřené na konkrétní mechanismy ED slouží jako citlivé a specifické biodetekční systémy k hodnocení ED potenciálu, především díky miniaturizaci a možnosti relativně rychle získávat široké spektrum informací. Základní mechanismy endokrinní disrupce sledované pomocí *in vitro* biodetekčních systémů zahrnují především mechanismy mediované receptory, modifikace syntézy, inhibice nebo akcelerace metabolismu endogenních hormonů. Samozřejmě i další mechanismy endokrinní disrupce je možné zkoumat v *in vitro* modelech, ale ty zpravidla zatím nejsou využívány jako rychlé biodetekční systémy.

2.1.1 Receptorové mechanismy

Endokrinní systém zahrnuje mnoho signálních drah, které mohou být narušeny působením exogenních látek. Ty mohou vykazovat účinky na endokrinní a reprodukční systém prostřednictvím jaderných receptorů, nejaderných steroidních receptorů či nesteroidních receptorů (receptory neurotransmiterů jako serotonin, dopamin) (De Coster & Van Larebeke, 2012). Interakce s jadernými receptory hormonů studované v rámci našich výzkumů patří k velmi významným mechanismům ED. Jaderné receptory plní funkce transkripčních faktorů, které jsou fyziologicky aktivované ligandy s nízkou molekulovou hmotností (jako steroidní hormony), které mohou být mimikovány řadou strukturně podobných látek z životního prostředí. Navíc tyto receptory mohou být aktivovány už velmi nízkými koncentracemi potentních ligandů, endogenních hormonů, i některých cizorodých látek (Vandenberg et al., 2012). Interakce EDC s jadernými receptory patří k významným molekulárním iniciačním událostem, které vedou ke škodlivým účinkům spojeným s endokrinní disrupcí.

Jaderné receptory jsou aktivovány navázáním ligandu (např. estrogeny, androgeny, progesteron, glukokortikoidy) a aktivovaný komplex receptor-ligand se váže na specifické responzivní elementy v DNA, kde zapojením různých kofaktorů reguluje transkripci genů nebo postranskripční děje, čímž ovlivňuje hladiny specifických cílových mRNA a proteinů (Marty et al., 2011). Přirozený receptorový mechanismus může být ovlivněn buď přímým navázáním xenobiotika na receptor a jeho aktivací (agonista) nebo inhibicí (antagonista) nebo modulací přidružených signálních drah.
Narušení regulace signálních drah zejména estrogenního receptoru (ER), androgenního receptoru (AR), glukokortikoidního receptoru (GR), receptoru kyseliny retinové (RAR), retinoidního X receptoru (RXR), receptoru aktivovaného proliferátory peroxizomů (PPAR),

thyroidního receptoru (TR), nebo aryl hydrokarbonového receptoru (AhR) jsou považovány za velmi důležité mechanismy toxických projevů řady environmentálních polutantů. Signální dráhy jednotlivých jaderných receptorů se vzájemně ovlivňují, existují různé funkční interakce mezi receptory, důležitou roli mají koaktivátory a korepresory receptorů (tzv. "cross-talk"; Kortenkamp et al., 2011, Ohtake et al., 2011). Některé receptory mohou mít navíc společné ligandy, stejná látka může interagovat s více receptory s různou vazebnou silou.

Na téma jaderných receptorů a také možnosti využívání *in vitro* reporterových buněčných testů ke sledování interakcí látek a environmentálních směsí s těmito receptory jsme vypracovali dva detailní přehledové články (Hilscherova et al., 2000 a Janošek et al., 2006 – přidán v přílohách jako **Článek I**). **Článek I** pojednává o klíčových jaderných receptorech, mechanismech účinku zprostředkovaných těmito receptory, látkách které působí těmito mechanismy a *in vitro* i *in vivo* metodách k jejich sledování. Jako citlivé *in vitro* biodetekční systémy jsou vyvíjeny geneticky upravené buněčné linie s reporterovými geny (reporterové biotesty), které v přítomnosti látek působících přes tyto receptory po interakci aktivovaného receptorového komplexu s responsivními elementy v DNA syntetizují specifické do buňky uměle vnesené a snadno stanovitelné enzymy (např. luciferáza, beta-galaktosidáza apod.) nebo jiné proteiny (např. zelený fluoreskující protein). Změny v aktivitách či hladinách těchto reporterových proteinů po expozici EDC pak informují o schopnosti čisté látky (nebo celé směsi látek) působit daným mechanismem.
Specificky problematikou narušení signálování arylhydrokarbonového a estrogenního receptoru a možností sledování těchto mechanismů se zabývala naše publikace Hilscherova et al. (2000). Tato publikace také prezentuje přístupy k odvození relativních potencí látek a testování environmentálních směsí. Strategie hodnocení toxicity v komplexních směsích zahrnuje identifikaci aktivních látek či frakcí pomocí biotestem-řízené frakcionace (effect directed analysis; EDA) a hodnocení příspěvku sledovaných látek k celkové detekované aktivitě.

Důležitou roli v endokrinní regulaci hraje širší spektrum receptorů (De Coster & Van Larebeke, 2012). Zde podrobněji zmíním jen ty receptory, o kterých pojednávají další studie uvedené v této habilitační práci.

Estrogenní receptory

Estrogenní receptory (ER) zahrnují jaderné a membránové receptory (ty tvoří asi 5% všech ER), přičemž jaderné receptory (u savců 2 subtypy ERα, ERβ) regulují pomalejší genomickou odpověď a fungují jako ligandem-indukovatelné transkripční faktory, zatímco membránové receptory regulují rychlou negenomickou odpověď (Levin, 2015; Fu & Simoncini, 2008). *In vitro* reporterové biotesty využívají genomického mechanismu, který je pod kontrolou jaderných estrogenních receptorů. Ty jsou lokalizovány v cytosolu a v jádře, po aktivaci receptoru ligandem tvoří aktivované receptory dimery a celý komplex se následně přesune k DNA, kde se naváže na estrogen responzivní element a působí jako transkripční faktor, který spouští kaskádu dějů vedoucích k transkripci cílových genů.

Estrogeny regulují vývoj pohlaví, pohlavních buněk, sekundárních pohlavních znaků u samic, řízení reprodukce i reprodukční chování organismů. Ovlivňují také metabolismus, buněčnou proliferaci a diferenciaci, vývoj a aktivitu tkání podílejících se na reprodukci. Mají také vliv na tvorbu kostí, regulaci homeostázy, mohou hrát roli v karcinogenezi. Hlavní endogenní ligand, který se používá jako referenční látka v biotestech, je 17β-estradiol.

Ke xenobiotikům, které působí prostřednictvím estrogenního receptoru, mimo jiné patří alkylfenoly, bisfenol A, ftaláty, látky z antikoncepčních přípravků (17α-ethinylestradiol), některé léčiva, pesticidy, či fytoestrogeny (Shanle & Xu, 2011).

Androgenní receptory

Podobně jako estrogenní receptory, i androgenní receptory (AR) jsou jaderné i membránové (Foradori et al., 2008). Opět v *in vitro* reporterových biotestech je využívána genomická odpověď pod kontrolou jaderných receptorů, kde aktivované receptory mají roli transkripčních faktorů. Po aktivaci ligandem dimerizují a v komplexu s dalšími faktory nasedají na androgen responzivní elementy (ARE) v DNA a spouštějí transkripci cílových genů pod kontrolou aktivace androgenního receptoru.

Androgeny regulují vývoj pohlaví, zejména samčích pohlavních charakteristik, řízení reprodukce, aktivitu samčích pohlavních orgánů, spermatogenezi, růst, hrají roli v karcinogenezi. Jako referenční látka se v biotestech používá testosteron nebo dihydrotestosteron. Polutanty vykazují častěji antagonistické než agonistické působení. Antiandrogenní potenciál byl zjištěn u řady pesticidů, některých parabenů, antioxidantů, syntetických mošusových látek, UV-filtrů, perfluorovaných látek, polychlorovaných bifenyletherů, polybromovaných difenyletherů, bisfenolu A a dalších látek (Orton et al., 2014; Ermler et al., 2011).

Arylhydrokarbonový receptor (AhR)

AhR je cytosolový receptor. Po aktivaci ligandem translokuje aktivovaný komplex z cytosolu do jádra, kde tvoří heterodimer s proteinem ARNT (aryl hydrocarbon receptor nuclear transporter) a s dalšími kofaktory zvyšuje transkripci AhR-responsivních genů obsahujících v promotorové části xenobiotické responsivní elementy (XRE).

Nejznámějšími ligandy tohoto receptoru jsou koplanární aromatické látky, včetně persistentních organických polutantů. Nejpotentnější známý ligand je 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), proto bývá AhR-zprostředkovaná odpověď označována jako dioxinová aktivita (tak je pro zjednodušení označována i v rámci této habilitační práce) nebo aktivita dioxinového typu. TCDD se také používá jako referenční látka v *in vitro* biotestech.

In vivo toxicita potentních AhR ligandů, jako je TCDD, zahrnuje celou řadu negativních účinků, projevuje se v různých orgánech, včetně dermální toxicity-chlorakné, teratogenity, embryotoxicity, poškození jater, vývojové a reprodukční toxicity, imunotoxicity, karcinogeneze a promoce nádorů, vyčerpání organismu (Bradshaw & Bell, 2009). TCDD je Mezinárodní agenturou pro výzkum rakoviny a jinými uznávanými mezinárodními organizacemi klasifikován jako lidský karcinogen. Přes Ah receptor působí mimo jiné velmi významná skupina persistentních organických polutantů, některých polycyklických aromatických uhlovodíků (Pieterse et al., 2013), ale také řada dalších látek různé struktury (DeGroot et al., 2015). AhR nebývá klasifikován mezi jaderné receptory, ale jeho ligandy hrají důležitou roli v endokrinní disrupci (Kortenkamp et al., 2011). AhR reguluje klíčové enzymy metabolismu endogenních látek a xenobiotik, včetně cytochromů P450. Kromě indukce detoxifikačních enzymů také reguluje aktivitu různých jaderných receptorů prostřednictvím interakcí se signálními drahami estrogenního a androgenního receptoru (ER a AR) i receptoru kyseliny retinové (Ohtake et al., 2011; Murphy et al., 2007). Některé AhR a ER ligandy se mohou překrývat v účincích díky podobnosti v chemické struktuře. Mezi možné důsledky interakcí patří indukce enzymů P450, které metabolizují estrogen, utlumení transkripce genů buněčného cyklu, indukce proteasomální degradace ER, ovlivnění

koaktivátorů a další. Aktivace AhR také ovlivňuje např. signálování kyseliny retinové ovlivněním její syntézy, katabolismu, transportu a vylučování, i na úrovni ovlivnění aktivace či represe specifických genů (Murphy et al., 2007).

Retinoidní receptory

Retinoidní látky působí prostřednictvím jaderných receptorů kyseliny retinové (RAR) a retinoid X receptoru (RXR), které také fungují jako ligandem aktivované transkripční faktory. RAR jsou aktivovány kyselinou all-*trans* retinovou (ATRA) a kyselinou 9-*cis* retinovou (9cisRA), RXR jsou aktivovány pouze 9cisRA. Každý z nich má 3 subtypy (α, β, γ). Po aktivaci receptoru tvoří heterodimery RAR/RXR nebo homodimery RXR/RXR, které interagují se specifickými responsivními DNA elementy a se zapojením koregulátorů ovlivňují expresi cílových genů (Brtko & Dvorak, 2015; Evans & Mangelsdorf, 2014). RXR slouží jako heterodimerizační partner pro celou řadu dalších receptorů (např. PPAR, TR, LXR).

Role retinoidů v organismu, jejich metabolismus a signálování jsou společně s problematikou vlivu různých typů environmentálních polutantů na tyto procesy podrobně zpracovány v odborném přehledovém **Článku II.** (Novák et al., 2008). Tento článek diskutuje vědecké poznatky o interakcích xenobiotik se signálováním retinoidů se zaměřením na významné skupiny organických polutantů, zaměřuje se jak na *in vivo*, tak *in vitro* účinky environmentálních kontaminantů na signálování, metabolismus a transport retinoidů. Retinoidy jsou nesteroidní hormony, které hrají důležitou roli v kontrole některých životně důležitých procesů včetně embryonálního vývoje, růstu, morfogeneze, reprodukce a udržování homeostázy. Jsou důležité pro biologické funkce v embryogenezi, buněčné diferenciaci, apoptóze a vidění. Na rozdíl od steroidních hormonů nejsou čistě endogenní, vznikají z vitamínu A či jeho prekurzorů přijímaných s potravou, někdy jsou nazývány jako tzv. dietární hormony. K látkám, u kterých byla prokázána schopnost narušení metabolismu, transportu nebo přenosu signálu retinoidů patří pesticidy, polychlorované dioxiny, polychlorované bifenyly, polycyclické aromatické uhlovodíky a ftaláty. Působení xenobiotik na signální dráhy retinoidů či jejich metabolismus může narušit normální vývoj embrya a další procesy. Některé z nich jsou esenciálními živinami a jejich nedostatek nebo nadbytek může způsobit teratogenitu.

Využívané *in vitro* testy pro hodnocení ED potenciálu

V průběhu výzkumů shrnutých v této habilitační práci byla postupně zavedena, optimalizována a v řadě studií využita sada *in vitro* (eko)toxikologických testů pro charakterizaci účinků kontaminantů na důležité mechanismy endokrinní disrupce. Tyto *in vitro* biodetekční systémy umožňují výzkum a hodnocení ovlivnění endokrinního signálování na úrovni receptorově mediovaných mechanismů i na úrovni produkce hormonů:

1. Ovlivnění receptorově mediovaných mechanismů je hodnoceno v *in vitro* biotestech s reporterovou luciferázou pod kontrolou specifických receptorů (reporterové biotesty):

I. **Test interference látek/vzorků se signálováním estrogenního receptoru (ER)**
– sledováno estrogenní i antiestrogenní působení
- savčí buněčné modely
- kvasinkový model včetně rychlé imobilizované verze

II. **Test interference látek/vzorků se signálováním androgenního receptoru (AR)**
– sledováno androgenní i antiandrogenní působení

- savčí buněčné modely
- kvasinkový model včetně rychlé imobilizované verze

III. **Test interference látek/vzorků se signálování kyseliny retinové** – test na modulace RAR/RXR-zprostředkované aktivity, sledováno pro-retinoidní nebo anti-retinoidní působení
    - savčí buněčný model

IV. **Test interference látek/vzorků se signálováním aryl hydrokarbonového receptoru** – test na modulace AhR- zprostředkované (dioxinové) aktivity
    - savčí buněčné modely
    - rybí buněčné modely
    - kvasinkový model s reporterovým genem pod kontrolou AhR

2. **Test ovlivnění mechanismů steroidogeneze** – sledována produkce hormonů či ovlivnění exprese genů klíčových enzymů steroidogeneze
    - savčí buněčný model - linie H295R

Kromě reporterových systémů založených na savčích či rybích buněčných liniích mohou být vhodnou alternativou kvasinkové modely vytvořené zpravidla stabilní transfekcí kvasinek druhu *Saccharomycees cerevisie*. Ve spolupráci s Department of Applied Chemistry and Microbiology, University of Helsinky, Finsko, se nám podařilo pro receptorové mechanismy zavést rychlejší a poměrně citlivé testy na kvasinkových modelech. Jedná se o rekombinantní linie kvasinky *Saccharomyces cerevisiae* stabilně transfekované ER, AR či GR společně s reporterovým genem pro luciferázu. Byly optimalizovány a validovány reporterové testy na kvasinkovaných liniích a proběhlo srovnání kvasinkových a savčích buněčných modelů. V rámci naší společné studie Leskinen et al. (2008) byl vyvinut a charakterizován nový kvasinkovaný model pro studium interakce látek a směsí s AhR a ověřeno jeho využití na vzorcích říčních sedimentů. V posledních dvou letech se v rámci našeho výzkumu podařilo vyvinout metody pro přípravu takzvaných „ready-to-use“ testů založených na kvasinkových modelech pro hodnocení schopnosti látek a směsí interagovat s estrogenním a androgenním receptorem. Pomocí optimalizace metod imobilizace kvasinek a přístupů pro jejich dlouhodobé uchování byly vyvinuty nástroje, které velmi výrazně zkracují dobu potřebnou k hodnocení estrogenní a androgenní aktivity látek a vzorků, jsou méně nákladné než jiné přístupy a také méně náročné na speciální vybavení laboratoří a tudíž dostupnější k širšímu využití (**Článek III** - Bittner et al., 2015**,** **Článek IV** - Jarque et al., 2016). Imobilizovaná verze biotestu umožnila zkrátit čas nutný k realizaci testu z několika dní na pouhé 3 hodiny bez nutnosti práce ve sterilních podmínkách a s využitím přenosného luminometru možnost přímé aplikce testu v terénních podmínkách.

Popsané *in vitro* testy byly následně využity ve výzkumu účinků vybraných organických polutantů, huminových látek či komplexních vzorků z životního prostředí, které jsou uvedeny v dalších kapitolách.

2.1.2 Vliv modelových látek na receptorově mediované odpovědi

Výzkumy prezentované v této kapitole přinesly nové informace o potenciálu vybraných prioritních polutantů ovlivňovat endokrinní systém specifickými mechanismy. Studie byly zaměřeny mimo jiné na méně prozkoumané mechanismy účinku ovlivnění signálních drah retinoidů a steroidogeneze. Výsledky doplňují (eko)toxikologickou charakteristiku studovaných látek a přispívají k předpovědi rizik spojených s kontaminací prostředí.

Dioxinová a retinoidní aktivita dusíkatých heterocyklů polycyklických aromatických uhlovodíků

Heterocyklické dusíkaté deriváty polycyklických aromatických sloučenin (azaPAH) jsou důležité polutanty. Podobně jako nesubstituované polycyklické aromatické uhlovodíky (PAH) vznikají mimo přírodních zdrojů především během spalovacích procesů a jako vedlejší produkty průmyslových činností včetně zpracování uhlí, dehtů, odpadů, v těžařském a chemickém průmyslu (Bleeker et al., 2002, Wei et al., 2014). Byly detekovány ve všech složkách prostředí, v ovzduší, půdě, vodě i sedimentech, jejich koncentrace dosahují 1-10% nesubstituovaných polycyklických aromatických uhlovodíků (PAH), které jsou velmi rozšířené polutanty v prostředí. Avšak azaPAH jsou reaktivnější, polárnější a více rozpustné ve vodě než homocyklické aromatické uhlovodíky, s čímž souvisí jejich větší mobilita a biodostupnost. Jejich strukturní parametry (jako počet a vzájemné postavení benzenových jader, rozmístění funkčních skupin) mají významný vliv na jejich fyzikálně chemické vlastnosti, toxicitu i osud v prostředí. Po expozici azaPAH byly pozorovány karcinogenní, mutagenní a teratogenní účinky (Bleeker et al., 2002).

Naše studie zkoumaly zejména dioxinovou a retinoidní aktivitu těchto látek. Výsledky těchto studií jsou shrnuty ve třech odborných publikacích (**Článek V** - Sovadinová et al., 2006; **Článek VI** - Beníšek et al., 2008; **Článek VII** - Beníšek et al., 2011).

Výsledky první uvedené studie (**Článek V**) poukázaly na významnou indukci AhR-zprostředkované (dioxinové) odpovědi u azaPAH s vyšší molekulovou hmotností (dibenzakridiny), zejména vysokou potenci některých dibenzakridinů a dibenzokarbazolu, která byla významně vyšší než u nesubstituovaných PAH. Dokonce v některých případech, kde nesubstituovaný PAH neměl detekovatelnou AhR-potenci, jeho aza-analog ji vykázal.
Na základě studia širší řady látek (29 PAH a azaPAH) zahrnující jak parentální, tak substituované analogy PAH, mohl být studován vztah mezi strukturou a toxicitou/aktivitou těchto látek (QSAR). Byl prokázán vztah s jejich environmentálními a strukturními vlastnostmi. Rozdělovací koeficient n-oktanol/voda (logP) významně koreloval s AhR aktivitou sledovaných látek. Detailní QSAR model poukázal na tři základní parametry ovlivňující aktivitu látek: elipsoidní objem, molární refraktivitu a velikost molekuly. Tyto parametry hrají důležitou roli ve vstupu látky do buňky (hydrofobicita) a navázání na AhR (elipsoidní objem, velikost a hustota).

Velmi omezené jsou informace o působení polutantů na RAR/RXR signálování, přičemž RAR a RXR hrají zásadní roli v organogenezi, růstu a vývoji embryií. Ve studii zaměřené na vliv široké sady modelových kontaminantů PAH a jejich N-heterocyklických derivátů na retinoidní signálování (**Článek VI**) bylo zjištěno, že tyto polutanty vykazují schopnost narušovat přirozené signálování retinoidů *in vitro*. *In vitro* testy na zavedených modelech s jadernými receptory RAR/RXR prokázaly, že žádná z 26 testovaných látek nevykazovala samostatně schopnost aktivace retinoidních receptorů. Na druhou stranu, mnoho z testovaných PAH a azaPAH vykazovalo účinky ve spolupůsobení s modelovým ligandem ATRA – snižování či zvyšování účinku nebo dvoufázové účinky, kdy intenzita a charakter odpovědi závisely na koncentraci a délce expozice. Po 6 hodinové expozici většina látek snižovala signál ATRA, po 24 h docházelo u většiny látek k posílení účinků. S využitím technik QSAR byly identifikovány klíčové parametry struktury ovlivňující působení látek po kratší a delší době expozice.

V navazující studii byly podrobněji sledovány účinky vybraných PAH a azaPAH v koexpozici s ATRA na signálování retinoidních receptorů a na buněčnou diferenciaci (**Článek VII**). Benz[a]antracen a benz[c]acridin významně zvyšovaly odpověď v koexpozici s různými koncentracemi ATRA, včetně fyziologicky relevantních koncentrací, zatímco 1,7-

fenantrolin účinky snižoval a fenantren vykazoval bifázický efekt. Vliv na pluripotenci a diferenciační procesy byl sledován v myší embryonální nádorové linii P19 pomocí detekce hladin pluripotentního markeru Octameru-4 (Oct-4) (Wang et al., 2009). Bylo potvrzeno snížení hladin Oct-4 reflektující buněčnou diferenciaci po působení ATRA (Pacherník et al., 2005) a byly pozorovány účinky studovaných polutantů. Po působení PAH a azaPAH došlo ke zvýšení a/nebo snižování hladin Oct-4 v závislosti na době expozice, což oboje může narušit normální diferenciaci. Ze studovaných látek se jako nejvíce účinné ukázaly fenantren a jeho analog 1,7-fenantrolin. Výsledky studie ukazují, že PAH a azaPAH mohou mít vliv na proces diferenciace a embryonální vývoj narušením signálování ATRA a změnami hladin Oct-4. Celkově studie zaměřené na interakce azaPAH se signálními dráhami retinoidního a arylhydrokarbonového receptoru dokumentují, že tyto látky mohou významně ovlivňovat jejich signálování v *in vitro* experimentech s možnými důsledky v podmínkách *in vivo* zejména směrem k možnému embryotoxickému a teratogennímu působení. V *in vivo* expozici rybích embryí byla vývojová toxicita prokázána pro 4-azapyren (Hawliczek et al., 2012), k jiným azaPAH nejsou informace.

Huminové látky

V rámci našich studií byla také věnována pozornost působení huminových látek, které se v prostředí vyskytují přirozeně, a mohou také vykazovat biologickou aktivitu, stejně jako ovlivňovat působení různých typů kontaminantů. Byly studovány účinky širokého spektra huminových látek na odpovědi zprostředkované přes arylhydrokarbonový a estrogenní receptor. Mezi vzorky byly jak huminové kyseliny, tak fulvokyseliny i nerozdělená přírodní organická hmota izolovaná z povrchových vod. Byla zjištěna významná dioxinová aktivita a také antiestrogenní působení některých huminových látek (Janosek et al., 2007; Bittner et al., 2006) a také jejich schopnost působit aditivně či zvyšovat účinky při spolupůsobení s persistentními organickými polutanty (Bittner et al., 2009; 2011).

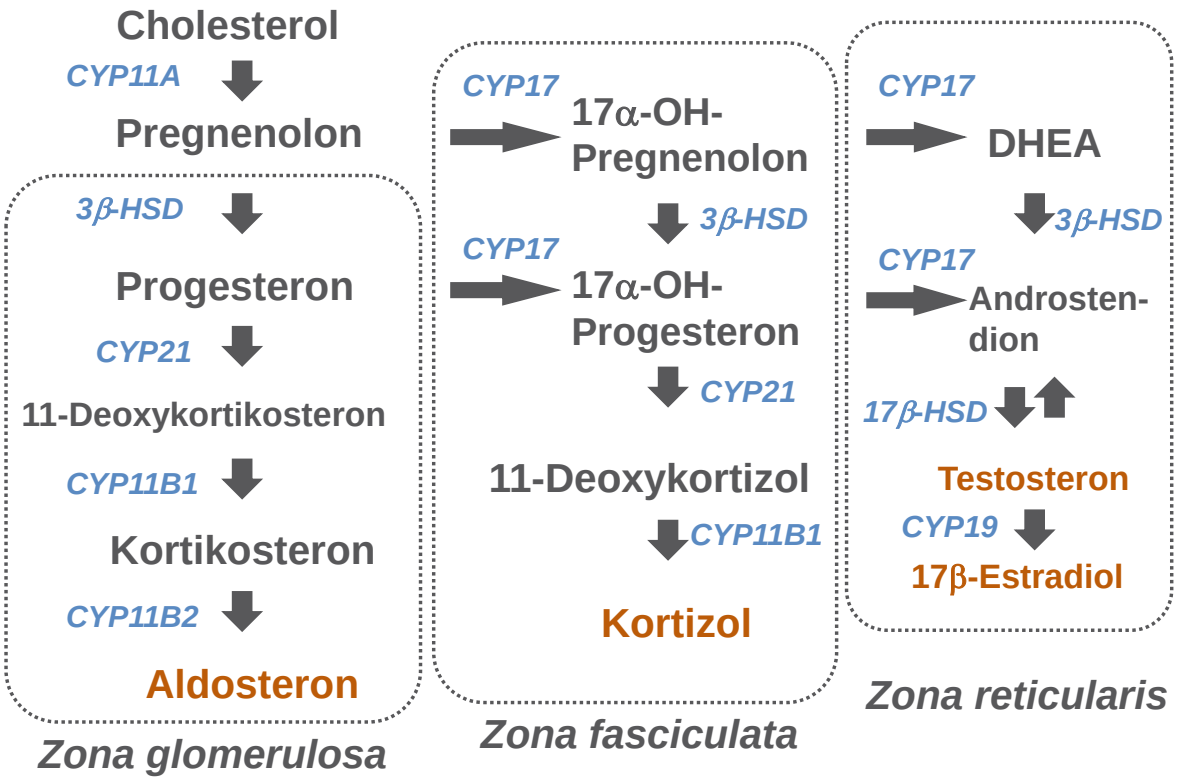
2.1.3 Steroidogeneze

V první fázi výzkumu endokrinních disruptorů byla největší pozornost upírána zejména na receptory pohlavních steroidních hormonů. Velmi důležitým mechanismem, kterým mohou endokrinní disruptory ovlivňovat endokrinní systém je však také narušení systému produkce steroidů. Syntéza steroidních hormonů (steroidogeneze) sestává z komplexní sítě citlivě regulovaných kroků, která může být narušena řadou látek schopných modulace aktivit enzymů steroidogeneze a tím modulace produkce steroidních hormonů (Harvey & Everett, 2003; Harvey et al., 2007).

Ke sledování vlivu látek i komplexních směsí na proces steroidogeneze byla v rámci našich studií vyvinuta a validována *in vitro* metoda využívající buněčnou linii karcinomu nadledvinek H295R (**Článek VIII -** Hilscherova et al., 2004). V těchto buňkách jsou aktivní všechny hlavní enzymy steroidogeneze (Obr.1), včetně všech enzymů nutných k produkci mineralkortikoidů, glukokortikoidů, androgenů a estrogenů, a proto mohou sloužit jako velmi vhodný systém pro detekci adrenokortikoidní toxicity a ovlivnění steroidogeneze. Tento test v sobě integruje přímé účinky látek na enzymy, stejně jako receptorové i nereceptorové odpovědi. Jako metoda pro hodnocení ovlivnění steroidogeneze xenobiotiky je možná detekce produkovaného množství steroidů (měření hladin estradiolu, testosteronu, progestronu a kortizolu z kultivačního média). Množství produkovaných hormonů je možno hodnotit imunochemickými metodami nebo metodami kapalinové chromatografie s hmotnostní spektrometrií (LC-MS; Tonoli et al., 2015). Tento model také umožňuje detailnější studie ovlivnění steroidogeneze na úrovni exprese a aktivit kritických enzymů steroidogeneze v

reakci na působení modelových látek a xenobiotik. Limitní pro průběh steroidogeneze v klasických endokrinních tkáních je aktivita enzymů StAR a CYP11A, které mají zásadní roli při transportu cholesterolu (prekursor pro všechny steroidní hormony produkované v nadledvinkách) z vnější k vnitřní membráně, rozštěpení jeho bočního řetězce a konverzi na pregnolon. Pokud není pregnolon metabolizován cytochromem CYP17, vznikají prekurzory mineralokortikoidů, pokud k metabolizaci dojde, vznikají steroidy, které slouží za substrát řadě dalších enzymů (jako CYP21, CYP11B1, CYP19) a jsou prekurzory glukokortikoidů (kortizol) a pohlavních steroidů (testosteron, estradiol) (Harvey et al., 2007). Jedním z kritických kroků ve steroidogenezi je ovlivnění aktivity enzymu aromatázy (CYP19), zodpovědného za konverzi testosteronu na estrogeny.

V rámci našich studií byly optimalizovány kritické podmínky provedení testu, analytická koncovka pro detekci ovlivnění steroidogeneze, zhodnocena citlivost a meze detekce kompetitivní imunochemické analýzy (ELISA) i hodnocení ovlivnění steroidogeneze na úrovni exprese genů steroidogenních enzymů ve studovaném modelu.



Obr.1. Schéma základních kroků syntézy hormonů ve třech zónách kůry nadledvin. DHEA= dehydroepiandrosteron, CYP = enzymy z rodiny cytochromu P450, HSD = hydroxysteroiddehydrogenáza.

S využitím tohoto modelu bylo ve spolupráci s Laboratoří akvatické toxikologie (Aquatic Toxicology Lab) na Michigan State University (MSU) v USA realizováno několik studií pro ověření efektivity modelu jako účinného nástroje zjišťování potenciálních účinků látek na steroidogenní dráhu. Byly vyvinuty a optimalizovány přístupy ke sledování ovlivnění exprese deseti klíčových enzymů steroidogeneze i produkce steroidních hormonů (**Článek VIII**). Bylo charakterizováno ovlivnění steroidogeneze modelovými induktory a inhibitory a časová dynamika odpovědi na expozici. V rámci další studie (**Článek IX -** Gracia et al., 2006) bylo studováno ovlivnění steroidogeneze modelovými látkami a jejich binárními směsmi. Studie indikuje různé mechanismy účinku modelových látek, které při spolupůsobení mohou

potenciálně zvyšovat celkový účinek směsi. Výsledky dokládají, že míra produkce hormonů neodpovídá vždy úrovni exprese genů steroidogenních enzymů**.** Následně byly realizovány studie s různými typy polutantů, modelových směsí i environmentálních vzorků. Optimalizovaný test byl poté podroben mezinárodnímu mezilaboratornímu srovnávání a byl validován v rámci validačního postupu OECD i US EPA. V současné době je zaveden jako jeden z testů pro sledování potenciálu endokrinní disrupce látek jak v rámci OECD normy 456 (OECD, 2011), tak i v US EPA, kde je zároveň jedním z požadovaných testů pro Tier 1 skríning v rámci Endocrine Disruptor Screening Program (US EPA, 2015).

Ve studii **Článek X** (Gracia et al., 2007) byl charakterizován vliv vybraných často užívaných léčiv, které mají potenciál dostávat se do prostředí, na produkci hormonů i na expresi genů klíčových enzymů steroidogeneze. Léčivům je v posledních letech věnována zvýšená pozornost jako významným kontaminantům životního prostředí, které byly dříve dlouhodobě přehlíženy. Řada farmak je nalézána zejména v odpadních a povrchových vodách. Léčiva jsou cíleně designována tak, aby měla biologický účinek, tudíž mohou mít nežádoucí účinky na necílové organismy v prostředí. Naše studie**,** která zkoumala vliv často užívaných farmak z různých terapeutických skupin na steroidogenezi, prokázala, že environmentálně relevantní koncentrace některých farmaceutik mohou narušovat přirozenou produkci steroidů i expresi klíčových enzymů steroidogeneze. Byl zjištěn vliv některých běžně užívaných farmak na produkci hormonů i vedlejší účinky, které mohou mít léčiva na průběh steroidogeneze. Největší účinky byly zjištěny zejména u antibiotik a hormonálních terapeutik, zatímco volně prodejná analgetika a protizánětlivé léky nevyvolávaly významné změny v produkci hormonů. Experimenty zaměřené na účinky binárních směsí farmak prokázaly různou míru spolupůsobení léčiv ve směsích, a také že účinky směsí mohou být odlišné od účinků jednotlivých látek, což poukazuje na interakce ve spolupůsobení farmak ovlivňující produkci hormonů.

Kromě našeho výzkumu účinků léčiv byl zavedený standardizovaný model následně využit v dalších studiích ke zkoumání ovlivnění steroidogeneze různými typy látek, polutantů i environmentálních vzorků. Ovlivnění exprese a aktivity steroidogeních enzymů bylo pomocí tohoto modelu prokázáno pro řadu kontaminantů životního prostředí, jako jsou pesticidy, ftaláty nebo bisfenol A (Mankidy et al., 2013; Thibeault et al., 2014; Zhang et al., 2011).

2.2 Hodnocení endokrinní disrupce *in vivo*

Pro hodnocení potenciálu látek způsobovat endokrinní disrupci a s ní spojené škodlivé účinky u různých druhů organismů byly vyvinuty a validovány některé testy a řada dalších je v současné době ve vývoji či validační fázi. Současně jsou také modifikovány standardní toxikologické testy tak, aby lépe podchytily možné endokrinně disruptivní působení látek. OECD realizuje speciální aktivitu zaměřenou na koordinaci vývoje přístupů k hodnocení a testování potenciálu látek působit endokrinní disrupci a validaci norem pro detekci endokrinních disruptorů a k harmonizaci přístupů pro hodnocení rizik těchto látek. V roce 2002 byl vytvořen koncepční rámec pro hodnocení endokrinně disruptivní aktivity látek (OECD, 2015). Byl také vydán normativní dokument s pokyny OECD 150 (OECD, 2012), který zastřešuje vytvářenou sadu norem pro hodnocení endokrinní disrupce, doporučuje postupy k používání, vyhodnocování a interpretaci výsledků z testů.

Koncepční rámec OECD organizuje hodnocení do pěti úrovní komplexity (Tab. 1) od charakterizace látek a *in silico* metod přes serii *in vitro* biotestů k *in vivo* přístupům. V *in vitro*

části se zaměřuje zejména na schopnost látek narušit signálování estrogenních, androgenních a thyroidních receptorů a steroidogeneze.

Tab.1. Koncepční rámec OECD pro hodnocení endokrinně disruptivního působení látek

Úroveň	Princip	Hodnocené údaje a příklady testů (v závorce uvedeno číslo OECD normy)
1	Existující data a netestovací informace	Fyzikálně-chemické vlastnosti Dostupná (eko)toxikologická data ze standardizovaných i nestandardizovaných testů *In silico* metody, predikce
2	*In vitro* testy poskytující informace o vybraných ED mechanismech působení	Vazba na estrogenní nebo androgenní receptor Transkripční aktivace ER (455, 457), AR, TR (reporterové buněčné testy) Steroidogeneze *in vitro* (H295R, 456) Test proliferace
3	*In vivo* testy poskytující informace o vybraných ED mechanismech působení	Uterotrofní test na hlodavcích (440) Hershbergerův test na hlodavcích (441) Test metamorfózy obojživelníků (231) Reprodukční skrínigový test na rybách (229)
4	*In vivo* testy poskytující informace o škodlivých účincích způsobených ED	28- a 90-denní toxicita u hlodavců (407, 408) 1-generační studie (415) Test chronické toxicity a karcinogenity (451) Test vývojové toxicity, neurotoxicity (426) Test pohlavního vývoje u ryb (234) Reprodukční test u ryb Reprodukční test u ptáků (206) Test na pakomárech (218)
5	*In vivo* testy poskytující informace o škodlivých účincích způsobených ED při dlouhodobějším působení v rámci životního cyklu organismů	Rozšířená jednogenerační studie reprodukční toxicity (443) Dvougenerační studie (416) Celoživotní test s pakomáry (233) Reprodukční test na dafniích (211)

Klasické (eko)toxikologické testy jsou doplňovány o sledování detailních parametrů spojených s endokrinní disrupcí. Zejména pro studium působení na volně žijící organismy v prostředí je zatím k dispozici jen malá sada testů omezená na několik málo modelových druhů. Další testy jsou aktuálně ve vývoji nebo ve validační fázi. Jedná se například o metody k hodnocení endokrinní disrupce a narušení reprodukce u měkkýšů, rané a vývojové toxicity u obojživelníků a ryb, celoživotní nebo vícegenerační studie u ryb a bezobratlých. Vedle těchto testů validovaných nebo procházejících validací jsou v literatuře publikovány přístupy zvláště s ohledem na další citlivé skupiny organismů (Schmitt et al., 2011; Nentwig, 2007), pro něž validované normované biotesty nejsou dostupné, ale které lépe reprezentují cílové organismy v prostředí. Podle sledované problematiky pokrývají druhy z různých skupin jak bezobratlých, tak obratlovců a parametry související s narušením endokrinního systému u konkrétních druhů.

2.2.1 *In vivo* účinky modelových látek

Endokrinní regulace je mnohem méně prozkoumána u bezobratlých živočichů a tudíž přímé spojení *in vivo* účinků s mechanismy endokrinní disrupce je obtížnější (Duft et al., 2007; Mazurová et al., 2008b). U modelových bezobratlých se pro studium možného vlivu endokrinních disruptorů používají často dlouhodobější reprodukční testy, jak je vidět v kategorii 4 a 5 koncepčního rámce OECD (Tab. 1). V několika našich pracech jsme přispěli k poznání účinků vybraných skupin EDC s využitím různých *in vivo* ekotoxikologických modelů.

Naše studie **Článek XI** (Haeba et al., 2008) se zaměřila na studium širší sady parametrů vzhledem k potenciálním endokrinně-disruptivním účinkům u bezobratlých. Byl zkoumán vliv čtyř modelových EDC (vinclozolinu, flutamidu, ketoconazolu a dicofolu) na korýše hrotnatku velkou (*Daphnia magna*). Konkrétně bylo sledováno přežívání, výskyt samců, růst, svlékání a reprodukce po akutní 48 h expozici, po sub-chronické 4-6 denní expozici a po chronické 21 denní expozici. Tato studie prokázala, že některé látky známé jako EDC u obratlovců vyvolávají endokrinně disruptivní účinky u studovaných bezobratlých a ovlivňují některé z vývojových procesů, které u nich nebyly dříve studovány (jako vývoj pohlaví, embryogeneze, svlékání a dospívání). Vinclozolin a dicofol měly vliv na poměr pohlaví, flutamid způsoboval opoždění vývoje vedoucí až k jeho přerušení. Ovlivnění poměru pohlaví některými látkami (vinclozolin a dicofol) odpovídalo známému působení těchto látek u obratlovců (i.e. antiandrogenita a antiestrogenita). Naše výsledky přinesly nové informace o citlivosti bezobratlých na působení EDC (Kortenkamp et al., 2011).

Test chronické toxicity u hrotnatky velké (*Daphnia magna*), který je zahrnut v koncepčním rámci OECD pro hodnocení endokrinně disruptivního působení látek (Tab.1), byl využit také ve studii *in vivo* účinků dusíkatých derivátů polycyklických aromatických uhlovodíků (**Článek XII -** Feldmannova et al., 2006). Zejména retinoidní aktivita a narušení diferenciace, ale i dioxinová aktivita, pozorované po působení azaPAH v *in vitro* testech (kapitola 2.1.2) mohou velmi úzce souviset se škodlivými účinky *in vivo*. V testu akutní a chronické toxicity u korýše hrotnatky velké měly studované dusíkaté deriváty polycyklických aromatických uhlovodíků významný vliv na přežívání, plodnost a rozmnožování. V chronickém testu byl nejtoxičtější 1,7 fenantrolin, který také vykazoval nejsilnější účinky na signálování ATRA a na diferenciaci v *in vitro* testech. Při chronické expozici řada azaPAH negativně ovlivnila reprodukci již v relativně nízkých koncentracích, kde byla pozorována také indukce oxidativního stresu. Některé látky také způsobily opoždění nástupu reprodukce u hrotnatek až úplnou inhibici reprodukce.

Naše další studie ukázala, že azaPAH, které ovlivňovaly signálování ATRA a diferenciaci *in vitro*, také narušovaly časný vývoj obojživelníků a způsobovaly teratogenitu a mortalitu u embryí žáby drápatky velké (*Xenopus laevis*) v testu FETAX. Nejčastěji docházelo ke vzniku otoků, narušení vývoje střeva, páteře a změnám v pigmentaci, i k indukci oxidativního stresu. Nejtoxičtější látkou v testu FETAX testu byl 1,7-fenatrolin, který také vykazoval nejsilnější účinky na signálování ATRA a na diferenciaci v *in vitro* testech (Burýšková et al., 2006).

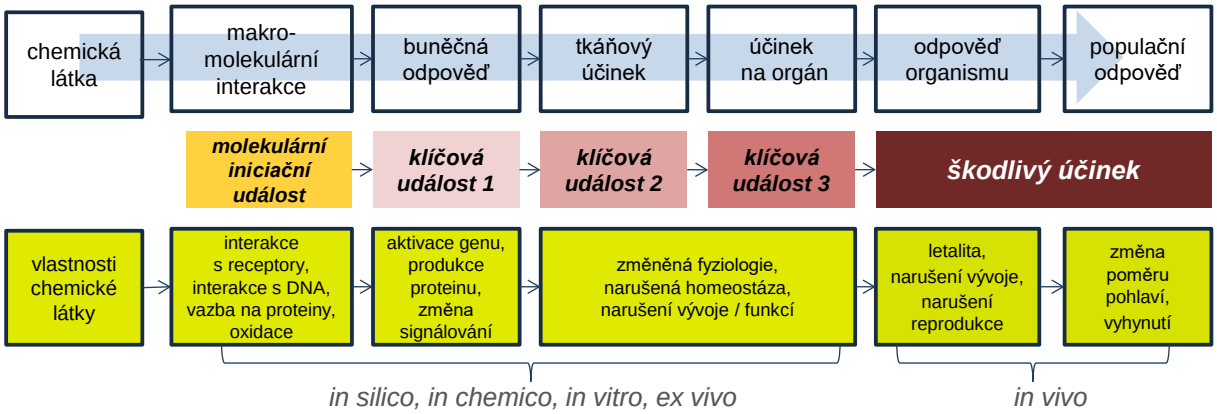
Zpravidla vyšší účinky azaPAH v porovnání s nesubstitovanými PAH a také jejich vyšší biodostupnost díky výrazně vyšší rozpustnosti ve vodě poukazují na možnou významnou roli azaPAH v toxicitě směsí PAH a jejich derivátů zejména ve vodním prostředí.

Několik našich studií zahrnutých v této habilitační práci zkoumalo souvislosti kontaminace sledované pomocí chemických analýz a *in vitro* biodetekčních systémů s *in vivo* účinky u relevantních exponovaných organismů (viz. Kapitola 3.3).

2.3 Dráhy škodlivého účinku

V posledních letech dochází k velkému rozvoji výzkumu a zájmu odborné veřejnosti i regulatorních orgánů o koncept tzv. drah škodlivého účinku (adverse outcome pathways, AOP, Obr. 2). Dráhy škodlivého účinku poskytují kauzální důkazy pro *in vivo* účinky a umožňují tedy propojit výsledky z *in vitro* studií a *in vitro* skríningu s *in vivo* ovlivněním a se škodlivými účinky na úrovni organismu nebo populací i s možnou predikcí chronické toxicity (Groh et al., 2015). AOP tvoří série definovaných kauzálně spojených událostí napříč různými úrovněmi biologické organizace, které vedou k poškození zdraví nebo (eko)toxicitě. Při jejich sestavování jsou využívány existující znalosti a informace k propojení dvou ukotvujících bodů: Molekulární iniciační události (molecular initiating event, MIE) a škodlivého účinku (adverse outcome, AO) přes sérií mezikroků, tzv. klíčové události (key events). Koncept AOP je rozvíjen a podporován v široké spolupráci řady mezinárodních organizací zejména OECD, US EPA, WHO a další (Garcia-Reyero, 2015).

Molekulární iniciační událost nebo klíčové události jsou zpravidla měřitelné *in vitro* na molekulární, buněčné nebo tkáňové úrovni a lze je hodnotit pomocí rychlých skríningových *in vitro* metod (Altenburger et al., 2015).



Obr.2. Dráha škodlivého účinku

AOP je možné využít jako základ pro extrapolace mezi chemickými látkami, extrapolace mezi druhy či mezi úrovněmi biologické organizace. Přispívají k charakterizaci rizika, umožňují jeho detailnější hodnocení (např. stanovení referenční dávky, koncentračních limitů) a prioritizaci chemických látek pro další testování. K podpoře zapojení mezinárodních expertů do tvorby a validace AOP vzniklo několik důležitých nástrojů, které jsou zastřešovány společnou znalostní základnou (AOP Knowledge Base, AOP-KB, https://aopkb.org; Garcia-Reyero, 2015). Důležitým nástrojem v rámci AOP-KB je AOP Wiki, platforma pro vývoj, sdílení, revize a doplňování AOP.

Řada diskutovaných AOP se zaměřuje na narušení endokrinního systému vedoucí ke škodlivým účinkům. Parametry sledované v *in vitro* biotestech ve studiích zahrnutých v této habilitační práci byly identifikovány jako molekulární iniciační události několika AOP, jak je shrnuto v následující tabulce (Tab. 2).

Tab.2. Příklady AOP ve vývoji, jejichž MIE jsou sledovány ve studiích v rámci této habilitační práce (z AOP Wiki; https://aopkb.org/aopwiki/index.php/Main_Page)

Molekulární iniciační událost	Škodlivý účinek
Agonismus/antagonismus k androgenímu receptoru	Narušení reprodukce Rakovina jater
Agonismus/antagonismus k estrogenímu receptoru	Narušení reprodukce (ryby, obojživelníci, ptáci) Změna poměru pohlaví
Inhibice aromatázy	Narušení reprodukce (ryby)
Aktivace AhR	Vývojová toxicita, embryotoxicita (ryby, ptáci) Poškození jater

3 Endokrinně disruptivní potenciál směsí látek z vodního prostředí

Výzkum a monitoring výskytu a účinků endokrinních disruptorů v různých složkách životního prostředí je v současnosti zohledňován a vyžadován řadou dokumentů a směrnic Evropské unie, ale také mezinárodních organizací (European Commission, 2011; WHO & UNEP, 2013) a mezinárodních expertních skupin (Kortenkamp et al., 2011). Také díky našim studiím byly získány důležité informace ohledně výskytu těchto kontaminantů v různých složkách životního prostředí ČR.

(Eko)toxikologické testování vzorků z prostředí vhodně doplňuje chemickou analýzu kontaminantů, protože ta je omezená jen na určité látky a nepostihuje jejich vzájemné působení. Pomocí *in vitro* biotestů je možné získat komplementární údaje o potenciálu směsi působit určitým toxickým mechanismem účinku a tudíž o možných škodlivých účincích pro biotu, které z chemické analýzy nelze získat (Leusch et al., 2010). Vzhledem k širokému spektru účinků a biochemických mechanismů spojených s endokrinní disrupcí je vhodná kombinace více *in vitro* metod, která poskytne komplexnější informaci o toxicitě studovaných vzorků. Tyto *in vitro* biotesty jsou rychlé, citlivé a relativně levné nástroje, které umožňují studium nejrůznějších matric prostředí (Altenburger et al., 2015; Kennedy et al., 2009; Šídlová et al., 2009). Jejich spojení s frakcionací a účinkem-řízenou analýzou umožňuje blíže identifikovat skupiny látek, které přispívají k detekovanému účinku (Brack et al., 2015).

Naše studie se zaměřovaly na výskyt látek s endokrinně disruptivním potenciálem v různých složkách životního prostředí. Pomocí *in vitro* biotestů byla hodnocena přítomnost látek se specifickými mechanismy účinku, zejména anti/estrogenity, anti/androgenity, dioxinové aktivity, ale také narušení retinoidního signálování a steroidogeneze. Celkový potenciál vzorků z prostředí či jejich frakcí působit určitým mechanismem je kvantifikován pomocí toxických ekvivalentů vyjádřených jako koncentrace standardní látky působící tímto mechanismem, která by způsobovala stejný účinek. Například - estrogenní aktivita se vyjadřuje jako estrogenní ekvivalent EEQ v koncentračních jednotkách endogenního ligandu 17β-estradiolu (E2), AhR-zprostředkovaná (dioxinová) aktivita jako TEQ (dioxinový ekvivalent) vyjádřený jako koncentrace TCDD, androgenní aktivita jako androgenní

ekvivalent (AEQ) odpovídající koncentraci testosteronu či dihydrotestosteronu a retinoidní aktivita jako retinoidní ekvivalent REQ v koncentraci endogenního ligandu kyseliny all-*trans*-retinové (ATRA).

Za pomoci *in vitro* biotestů jsme se zabývali m.j. problematikou výskytu ED látek v ovzduší (Érseková et al., 2014; Novák et al., 2014; 2013; 2009) či v půdách (Šídlová et al., 2009; Hilscherova et al., 2003) ovlivněných různými zdroji znečištění. V rámci habilitační práce se zaměřuji detailněji na akvatické prostředí, kterému jsme v našich studiích věnovali největší pozornost i vzhledem tomu, že endokrinní disrupce byla nejčastěji prokázána právě u akvatických organismů.

Vodní prostředí je příjemcem kontaminantů z řady zdrojů, především různých typů odpadních vod, splachů z povrchů, ze zemědělství i z atmosferické depozice. Povrchové vody i sedimenty obsahují široké spektrum látek přírodního i antropogenního původu s různými mechanismy účinku, často v relativně nízkých koncentracích, které mohou spolupůsobit (aditivní, synergistické nebo antagonistické spolupůsobení) a ovlivňovat organismy. Ve vodním prostředí se také vyvíjejí citlivá stadia organismů, která mohou být znečištěním negativně ovlivněna. S tím souvisí i mnoho známých případů endokrinní disrupce, která byla ve vodním prostředí pozorována především u ryb, ale také bezobratlých živočichů, obojživelníků, plazů i vodních savců v řadě oblastí světa (Sumpter & Johnson, 2008; Kortenkamp et al., 2011).

V laboratorních podmínkách i v prostředí, kde se výzkumy expertů celého světa zaměřovaly především na lokality pod výpustěmi odpadních vod s obsahem endokrinně disruptivních látek, byla zjištěna řada škodlivých účinků u různých druhů ryb (Burkhardt-Holm, 2010). Patří k nim především narušení pohlavního vývoje, počtu a kvality spermií a poruchy plodnosti, zvýšené hladiny proteinu vaječného žloutku vitelogeninu u samců, změny poměru pohlaví, vývoj intersexu, kdy gonády současně obsahují samčí i samiččí buňky, feminizace či maskulinizace jedinců dle charakteru expozice, narušení vývoje sekundárních pohlavních znaků (Kortenkamp et al., 2011; Tyler & Jobling, 2008). Tyto účinky mohou vést až k negativnímu ovlivnění celých populací i mezidruhových vztahů v prostředí (Kidd et al., 2007).

Endokrinní disrupce je významným problémem i u bezobratlých živočichů, i když u nich je endokrinní regulace mnohem méně prozkoumaná. Nejznámějším příkladem je narušení pohlavního vývoje u předožábrých plžů vedoucí až k vymizení populací měkkýšů (Oehlmann et al., 2007). V našem přehledovém článku Mazurova et al. (2008b) byly zpracovány dostupné informace ohledně endokrinní regulace s vlivem na reprodukci a určení pohlaví u korýšů. Mezi citlivé parametry studované v souvislosti s endokrinní disrupcí u korýšů patří také působení neurohormonů či parametry spojené se svlékáním, ovlivnění signálování ekdysteroidů. Publikované studie poukazují na reprodukční toxicitu a/nebo s ní spojené morfologické změny na pohlavních orgánech po působení směsí kontaminantů ze sedimentů či povrchových vod (Galluba & Oehlmann, 2012). Projevy ED u dalších bezobratlých zahrnují narušení reprodukce, narušení vývoje pohlavních orgánů, vznik imposexu, maskulinizace či vývoj tzv. supersamic u měkkýšů a změny v poměru pohlaví v populacích (Kortenkamp et al., 2011).

3.1 Povrchové a odpadní vody

Z hlediska hodnocení zatížení vodního prostředí jsou vedle chemických analýz stále častěji používány různé nástroje založené na sledování účinku (EBT, effect-based tools). Využití EBT je také uváděno v kontextu nové Společné Implementační strategie Rámcové směrnice na ochranu vod (European Commission, 2014). V roce 2014 byla publikována technická zpráva o EBT vypracovaná mezinárodní skupinou expertů pro podskupinu CMEP (Chemický monitoring a emergentní polutanty) pracovní skupiny zaměřené na chemické aspekty společné implementační strategie pro Rámcovou směrnici na ochranu vod (Working Group on Chemical Aspects under the CIS for the WFD; European Commission, 2014). Jako hlavní nástroje jsou zde zahrnuty *in vitro* biotesty, biomarkery v organismech *in vivo* a ekologické indikátory.

Naše výzkumná skupina, která se dlouhodobě zabývá využitím specifických *in vitro* biodetekčních systémů jako EBT pro hodnocení zatížení nejrůznějších matric životního prostředí, realizovala v této oblasti řadu studií. Tyto studie kombinovaly chemické analýzy a biodetekční systémy při výzkumu kontaminace odpadních vod (OV), říčních vod a sedimentů z vytipovaných prioritních oblastí, které reprezentují situaci v České republice, s cílem charakterizace zatížení vod a sedimentů látkami s endokrinně disruptivním potenciálem a odhadem možných účinků na biotu.

Vzhledem k dynamice kontaminace zejména tekoucích povrchových vod je klíčovým problémem výběr vhodného přístupu reprezentativního vzorkování. Bodové odběry běžně používané v řadě studií charakterizují okamžitou situaci v době odběru, tudíž v případě kdy míra kontaminace není stabilní (např. mění se v průběhu času v závislosti na zdroji, splachy při přívalových deštích apod.) nemusí poskytovat reprezentativní informaci ohledně kontaminace daného ekosystému (Coes et al., 2014; Vallejo et al., 2013). Reprezentativnější informaci mohou poskytnout směsné (kompozitní) vzorky, které bývají připraveny z několika bodových odběrů za určitou dobu. Ale i tyto kompozitní odběry zpravidla poskytují informaci o průměrné kontaminaci v omezeném časovém úseku a tento typ odběrů navíc není možné realizovat současně na větším počtu lokalit z hlediska náročnosti na obsluhu nebo speciální vybavení (automatické vzorkovače).

Jiný přístup - pasivní vzorkování - umožňuje charakterizovat dlouhodobou situaci na sledovaných lokalitách. Poskytuje průměrné koncentrace polutantů během delších vzorkovacích období, tudíž může podchytit i nárazové či periodické situace, které by jednorázové ani kompozitní vzorkování nemuselo zachytit. Pasivní vzorkovače bývají exponovány zpravidla v řádu několika týdnů a koncentrují látky z vody či sedimentů. Tento způsob vzorkování tak umožňuje zachytit i látky, které se vyskytují v nízkých koncentracích (Alvarez et al., 2014; Harman et al., 2012), které v případě ED látek mohou být toxikologicky relevantní. Další výhodou jsou relativně nízké náklady na vzorkování i zpracování vzorků.

Ve studiích zahrnutých v této habilitační práci byly dle charakteru studie použity kompozitní odběry odpadních vod a pasivní odběr povrchových i odpadních vod dvěma základními typy vzorkovačů. SPMD vzorkovače (semipermeable membrane devices) slouží ke vzorkování stopových množství hydrofobních polutantů ve vodách (Vrana et al., 2014). Používají se k účinnému vzorkování PAH, PCB, organických chlorovaných pesticidů (OCP), PCDD/F, alkylfenolů, středně polárních organofosfátových pesticidů, pyretroidů a některých heterocyclických aromatických látek (Charlestra et al., 2008; Stuer-Lauridsen, 2005). Vzorkovače POCIS (polar organic chemical integrative samplers) vzorkují hydrofilní

polutanty, jako polární pesticidy, farmaceutika, látky z kosmetických přípravků a výrobků denní spotřeby, přírodní a syntetické hormony (Long et al., 2014; Vallejo et al., 2013; Harman et al., 2012).

Studie **Článek XIII** **(**Jarosova et al., 2012**),** realizovaná ve spolupráci řady výzkumných institucí v ČR, zkoumala ED potenciál a koncentraci polárních organických polutantů v horních tocích sedmi potoků/řek tekoucích přes relativně neznečištěné oblasti v České republice. Konkrétně byl zjišťován vliv prvních větších obcí (o velikosti 1900-13000 obyvatel) s čistírnami odpadních vod (ČOV) na zatížení říčního ekosystému s tím, že tyto obce a ČOV byly prvním známým zdrojem znečištění na studovaných tocích. Voda byla vzorkována pomocí pasivního vzorkování s použitím dvou typů vzorkovačů POCIS (pro odběr pesticidů a farmaceutik) exponovaných několik kilometrů nad a několik desítek metrů pod výpustěmi ČOV. Ve většině vzorků byla detekována estrogenní a dioxinová aktivita (i v lokalitách nad městy považovaných za pozaďové), naopak nebyla zjištěna žádná detekovatelná antiestrogenní či anti/androgenní aktivita. Podobně byla estrogenní aktivita pozorována na referenčních lokalitách i v některých předchozích studiích (Nadzialek et al., 2010; Alvarez et al., 2013). I přes přítomnost funkčních komunálních ČOV došlo na všech sledovaných horních tocích pod městy s výpustěmi ČOV ke zvýšení estrogenní aktivity a většinou i aktivity dioxinového typu. Koncentrace EEQ přepočtené na odhadnuté vzorkované množství vody dosahovaly pod některými městy (Prachatice, Cvikov) 2,3 ng/L. Na lokalitě pod obcí Prachatice také zjistili výzkumníci z Fakulty rybářství a ochrany vod z Jihočeské univerzity v Českých Budějovicích významně zvýšené hladiny (více než 300000krát) žloutkového proteinu vitellogeninu u samců pstruha obecného (*Salmo trutta fario L.*) v porovnání s rybami z lokality nad obcí (**Článek XIII**), což indikuje působení estrogenních látek.

Výzkum prokázal, že i malé lokální zdroje mohou mít významný vliv na zatížení akvatických ekosystémů EDC, obzvláště v místech s nízkým zředěním odpadních vod povrchovou vodou. Nebyla zjištěna korelace velikosti sídel či ředícího poměru odpadní voda/povrchová voda s detekovanými toxickými ekvivalenty pod obcemi, což poukazuje na důležitou roli dalších faktorů jako je kapacita a technologie ČOV, či rozdílnost primárních zdrojů kontaminantů na lokalitách (v surové odpadní vodě i přímo v povrchové vodě). Koncentrace sledovaných polárních organických polutantů byly relativně nízké (Grabic et al., 2010; Vystavna et al., 2012). Na rozdíl od pesticidů bylo pozorováno zvýšení obsahu některých farmak pod obcemi, což koresponduje s rozptýleným charakterem zdrojů pesticidů, zatímco vstupy léčiv do vodních ekosystémů jsou spojeny především s obcemi, případně zemědělskými farmami.

Další studie realizovaná v širší spolupráci odborníků více pracovišť byla zaměřena na hodnocení vlivu velké městské a průmyslové aglomerace (Brno, 400tis. obyvatel) s moderní velkokapacitní ČOV na říční ekosystémy (**Článek XIV** - Jálová et al., 2013). V této studii byly využity dva typy pasivních vzorkovačů, SPMD pro hydrofobní polutanty a POCIS pro polární látky, ke vzorkování přítokových a odtokových vod z ČOV a také řek nad a pod městskou aglomerací a nad a pod ČOV. Design studie umožnil rozlišit přímo vliv hustě osídlené městské aglomerace s průmyslem a vliv ČOV na kontaminaci ve sledovaných řekách. Vedle toho byla také realizována dílčí studie celoroční variability cytotoxicity, estrogenní, androgenní a dioxinové toxicity kompozitních vzorků přítokových a odtokových vod z ČOV odebíraných v měsíčních intervalech. Výsledky prokázaly, že ČOV celoročně relativně účinně odstraňuje cytotoxické látky, xenoestrogeny a xenoandrogeny (míra odstranění většinou >95 %). Zbytková estrogenní aktivita v odtokové vodě se celoročně pohybovala v rozmezí 0,1 - 5,1 ng/L. V případě účinnosti odstraňování látek dioxinového typu byly zjištěny významné rozdíly v průběhu roku. Koncentrace ED látek a ekvivalentů zjištěné na vstupu i výstupu

studované ČOV odpovídají hladinám z jiných evropských ČOV s podobnou kapacitou a technologickým vybavením.

I přes vysokou účinnost odstraňování sledovaných biologických aktivit a většiny analyzovaných látek může sledovaná ČOV přispívat k hladinám EDC ve studovaných řekách. Zdroje z městské aglomerace (mimo ČOV) také přispívaly k zatížení řek některými skupinami látek. Pasivní vzorkovače hydrofobních (SPMD) i hydrofilních (POCIS) látek z říčních toků obsahovaly látky s dioxinovou, antiestrogenní a antiandrogenní aktivitou. Výsledky také poukázaly na zvýšení koncentrací léčiv, methyl/triclosanu, polybromovaných difenyletherů (PBDE) a nepolárních antiandrogenních látek pod ČOV a pokles hladin řady látek i biologických aktivit ve větší vzdálenosti dále po toku.

In vitro biotesty pro hodnocení estrogenního potenciálu byly s úspěchem aplikovány také v rámci celoevropského monitoringu bioaktivních látek v odpadních vodách v projektu koordinovaném EU JRC (Joint Research Centre), Ispra, Itálie (**Článek XV** - Jarošová et al., 2014b). V této studii byly analyzovány vzorky odpadních vod odebírané v řadě zemí EU pro charakterizaci výskytu širokého spektra polutantů (150 polárních organických a 20 anorganických látek) včetně estrogenních látek (Loos et al., 2013). Celkově byly hodnoceny vzorky odtokových vod z 75 ČOV ze 16 zemí Evropy, studie zahrnovala 24 h kompozitní i bodové vzorky poskytnuté vlastníky ČOV. S využitím našich *in vitro* biotestů byla ve 27 testovaných vzorcích detekována estrogenní aktivita v rozmezí 0,53 až 17,9 ng/L EEQ. U devíti vzorků byla zjištěna cytotoxicita/antiestrogenita. Odtokové vody ze zhruba třetiny komunálních ČOV a také z některých čistíren průmyslových odpadních vod obsahovaly více než 0,5 ng/L EEQ, což potvrzuje jejich možnou roli jako zdroj ED látek v povrchových vodách. V případě šesti komunálních ČOV z ČR zařazených do této studie se EEQ pohybovalo mezi <0,5 a 2,1 ng/L. Chemické analýzy neprokázaly přítomnost steroidních estrogenů nad detekčním limitem 10 ng/L v žádném ze vzorků. Nebyly zjištěny korelace naměřených EEQ s žádnou ze sledovaných skupin polutantů ani žádné významné rozdíly mezi EEQ odtokových vod z komunálních ČOV různé velikosti či průmyslových ČOV. Tato i předchozí studie prokazují schopnost *in vitro* biotestů účinně detekovat určité skupiny polutantů jako jsou estrogeny, které se vyskytují často ve velmi nízkých koncentracích, v kterých ovšem mohou mít škodlivé účinky na organismy, ale ve kterých jsou obtížně detekovatelné pomocí chemických analytických metod. Zapojení vyvíjených biodetekčních nástrojů do pan-evropského monitoringu ukazuje na jejich významný potenciál jako screeningového nástroje s vysokou citlivostí a selektivitou.

Aktuálně velmi diskutovaným tématem v EU i v jiných oblastech světa je možnost využití biodetekčních systémů k monitorovacím a regulatorním účelům a pro hodnocení ekologických i humánních rizik. K tomuto účelu je potřeba stanovit bezpečné limity pro celkové toxické potenciály stanovené v *in vitro* biotestech. Vzhledem k tomu, že potence jednotlivých endokrinních disruptorů v *in vitro* a *in vivo* modelech se mohou lišit, není možné přímo zhodnotit rizika *in vivo* expozice z *in vitro* stanovení ED potenciálu. V naší studii **Článek XVI** (Jarošová et al., 2014a) byly odvozeny bezpečné koncentrace estrogenních ekvivalentů (EEQ-SSE) v odtokových vodách z komunálních ČOV na základě zjednodušeného racionálního předpokladu, že u těchto typů OV jsou steroidní estrogeny zodpovědné za nejvýznamnější díl estrogenity stanovené v *in vitro* systémech. Tento předpoklad dokladuje také řada studií z celého světa shrnutých v naší publikaci, které v případě komunálních odpadních vod prokazují, že estron, 17β-estradiol, 17α-ethinylestradiol a v menší míře estriol zodpovídají za naprostou většinu estrogenního potenciálu. EEQ-SSE byly odvozeny z potence specifické pro konkrétní testovací protokol a použitý *in vitro* model, předpokládané koncentrace těchto látek bez negativního účinku (PNEC) odvozené z velkého počtu *in vivo* studií na rybách jako nejcitlivější skupině

organismů (Caldwell et al., 2012), a jejich relativního příspěvku k celkové estrogenitě detekované v odtocích komunálních odpadních vod. EEQ-SSE pro dlouhodobou expozici se pro 15 různých biotestů pohybovaly mezi 0,1 a 0,4 ng EEQ/L. Konkrétně v případě buněčného modelu MVLN používaného v řadě našich studií bylo odvozeno EEQ-SSE 0,3 ng/L pro dlouhodobější expozici a 1,4 ng/L pro krátkodobou expozici (do 60 dní). Vzhledem k tomu, že na většině lokalit dochází k naředění odpadní vody vodou povrchovou, EEQ-SSE v odpadní vodě bude vyšší o tento konkrétní ředící poměr. I když nejsou známy ředící faktory pro jednotlivé ČOV z pan-evropského monitoringu, srovnání s EEQ-SSE indikuje, že v případě některých z evropských ČOV by mohly hladiny vypouštěných estrogenních látek způsobovat riziko pro organismy ve vodách, kam jsou tyto odpadní vody vypouštěny. Podobně výsledky ze studie malých vodních toků (**Článek XIII**) a celoroční variability ED potenciálu odtokových vod z ČOV Brno (**Článek XIV**) poukazují na možnost překročení EEQ-SSE v případě zvýšené estrogenní aktivity a nízkého ředícího poměru v recipientu. V případě ČOV Brno, kde bylo realizováno celoroční sledování, bylo nejvyšší riziko spojeno s letními měsíci, kdy bylo vyšší zbytkové znečištění a navíc může být nižší ředící poměr z důvodu menších průtoků.

Vstupy EDC do akvatického prostředí bývají často spojovány s hustěji obydlenými a průmyslovými oblastmi, kde tyto látky mohou vstupovat zejména z komunálních i průmyslových odpadních vod (Luo et al., 2014; Loos et al., 2013). Jak ukázaly naše studie, významné mohou být i menší lokální zdroje. Vliv odtokových vod z čistíren odpadních vod na kontaminaci povrchových toků záleží na kapacitě a technologii ČOV (Gros et al., 2007) a naředění v recipientu a dalších faktorech ovlivňujících probíhající (bio)degradační pochody (Caliman & Gavrilescu, 2009). Účinnost odstraňování xenobiotik na komunálních ČOV je většinou poměrně vysoká, může dosahovat 88 - 99% a 96 - 99% v případě xenoestrogenů a xenoandrogenů (Roberts et al., 2015; Leusch et al., 2014; Svenson & Allard, 2004). Přesto často nedochází ke kompletnímu odstranění těchto látek z odpadních vod. Většina odtoků z ČOV stále obsahuje komplexní směsi látek včetně transformačních produktů vzniklých během čištění. Negativní účinky na populace ryb, jako narušení jejich endokrinních funkcí a reprodukce, feminizaci ryb po expozici odtokových vod z ČOV, byly zjištěny ve volně žijících populacích ryb i ve vzdálenosti několik km pod ČOV v mnoha oblastech světa (Fuzzen et al., 2015; Sumpter & Johnson, 2008, Vethaak et al., 2005) i v České republice (Peňáz et al., 2005, Randak et al., 2009). Podobné účinky byly pozorovány i v rybách exponovaných *in situ* v klecích pod výpustěmi odpadních vod (Chiang et al., 2015; Wang et al., 2013). Jak je výše diskutováno, v komunálních odpadních vodách jsou často nejpotentnějšími EDC steroidní estrogeny (Rutishauser et al., 2004; Aerni et al., 2004). Avšak v jiných typech odpadních vod a v povrchových vodách mohou v závislosti na typech zdrojů být nejvýznamnějšími EDC jiné skupiny látek (Thorpe et al., 2006; Vermeirssen et al., 2005).

Využitelnost spektra *in vitro* biotestů při hodnocení znečištění odpadních a povrchových vod byla dokumentována také v aktuálních rozsáhlých mezinárodních studiích s naším zapojením (Escher et al., 2014; Neale et al., 2015). V široké mezinárodní spolupráci byla provedena srovnávací studie zaměřená na zapojení různých typů *in vitro* biotestů do hodnocení efektivity procesu čištění odpadní vody a její recyklace až na pitnou vodu. V jejím rámci 20 laboratoří včetně naší zapojilo 103 různých *in vitro* testů pro hodnocení různých typů biologických potencí u vzorků odpadní vody v různých stupních čištění, povrchové i pitné vody. Každý vzorek vykazoval typický bioanalytický profil, který indikoval klíčové dráhy toxicity. Bylo dokumentováno odbourávání různých typů bioaktivních látek v průběhu čistírenského procesu. Biotesty zaměřené na aryl hydrokarbonový receptor a na hormonální receptory patřily k těm s nejvýraznější odpovědí a prokázaly velkou relevanci sledování potenciálu směsí v odpadních i povrchových vodách působit těmito mechanismy. Výsledky jasně

zdokumentovaly vhodnost *in vitro* testů jako citlivého nástroje pro sledování odbourávání znečištění a biologických směsí látek v odpadních i povrchových vodách (Escher et al., 2014).

V posledních letech dochází k velkému vývoji a zlepšování citlivosti analytických metod, které dříve nebyly dostupné. Komplementaritu *in vitro* biotestů a pokročilých analytických metod dokumentuje nová mezinárodní studie, ve které jsme se podíleli na hodnocení zatížení řeky Dunaje (Neale et al., 2015). V této studii bylo pomocí kapalinové chromatografie s vysokorozlišovací hmotnostní spektrometrií (LC-HRMS) analyzováno 264 polutantů a realizována sada *in vitro* testů a embryonální test na rybách s cílem zjistit, jak velkou část odpovědi v biotestech je možné vysvětlit přítomností sledovaných látek. Studie poukázala na nedostatek informací ohledně relativních potencí širokého spektra detekovaných látek v biotestech. Míra vysvětlitelnosti výsledků biotestů pomocí informací ohledně koncentrací látek z chemických analýz a jejich relativních potencí byla velmi nízká (obecně pod 1%) u nespecifických testů (adaptivní stresová odpověď a embryotoxicita *in vivo*), ale výrazně vyšší v případě aktivace AhR (3.3-71%) a ER (0.31-80%). Výsledky zdůrazňují skutečnost, že i v případě rozsáhlých analýz širokého spektra látek může být velká část biologické potence vzorků způsobená neanalyzovanými látkami, a tudíž nutnost doplnění chemické analýzy vhodně zvolenými biotesty.

Výsledky našich i zahraničních studií dokumentují velmi dobrou využitelnost *in vitro* biodetekčních systémů pro hodnocení zatížení různých typů vod EDC, stejně jako pro hodnocení efektivity odstraňování těchto látek během čistírenských procesů. V současné době jsou *in vitro* biotesty nejčastěji používány při sledování odbourávání estrogenních a androgenních látek na ČOV a jsou zpravidla jednodušší a citlivější než většina rutinních chemických analýz (Loos et al., 2013; Gerbersdorf et al., 2015).

3.2 Sedimenty

Sedimenty jsou důležitou složkou akvatických ekosystémů, která hraje klíčovou roli v osudu a účincích polutantů. Slouží jako životní substrát pro řadu bentických či bentofágních organismů, jejichž prostřednictvím se polutanty mohou dostávat do vodního sloupce a potravního řetězce. Mohou sloužit jako dlouhodobé ukládací medium i potenciální druhotný zdroj mnoha látek, včetně živin i environmentálních polutantů (Peck et al., 2004; Stachel et al., 2003). V některých oblastech mohou obsahovat vysoké dlouhodobě akumulované koncentrace polutantů. Důležitými faktory pro vazbu organických polutantů na sedimenty je specifický povrch částic i kvantita a charakter organického uhlíku. Kumulovány jsou především hydrofobní organické kontaminanty, které díky své persistenci mohou často v prostředí zůstávat po dlouhou dobu a mají tendenci k bioakumulaci a biomagnifikaci v organismech. Ovšem i další látky jako jsou průmyslová aditiva, změkčovače plastů, xenohormony, pesticidy, látky z kosmetických přípravků a léčiva (Brack et al., 2007; Jobling & Tyler, 2003; Ricking et al., 2003), mohou vstupovat do sedimentů a ovlivňovat akvatické organismy. Řada těchto polutantů není zařazena v rutinním monitoringu a jejich toxické účinky ještě nejsou zcela prozkoumány. V dynamických říčních systémech může vlivem významnějších změn průtoků či jiných zásahů do říčního koryta, například vlivem povodní či lidské činnosti, docházet k resuspendaci sedimentů a uvolnění látek do vodního sloupce, čímž se zvyšuje jejich biodostupnost.

Výsledky našich studií zaměřených na výzkum EDC v sedimentech byly publikovány v několika odborných publikacích, z nichž část je podrobněji diskutována v této kapitole a další v kapitole následující (Kaisarevic et al., 2011; Hilscherova et al., 2003).

Několik studií bylo zaměřeno na výskyt látek se specifickými mechanismy účinku v sedimentech řek ve Zlínském regionu, což je urbanizovaná oblast dlouhodobě zatížená průmyslem, strojírenstvím a zemědělstvím. Tato oblast byla vybrána jako modelová pro region-specifický přístup k hodnocení rizik a je dlouhodbě využívána pro řadu studií pracoviště RECETOX. Představuje vhodný modelový ekosystém pro výzkum distribuce polutantů na regionální úrovni i s ohledem na opakovaný výskyt povodní. Sledovány byly sedimenty povodí řek Dřevnice a Moravy. Na některých lokalitách byly v sedimentech překročeny limitní hodnoty sledovaných polycyklických aromatických uhlovodíků i kovů, což dokumentuje lokálně zvýšenou kontaminaci v těchto tocích (Hilscherova et al., 2007; Bednarova et al., 2013).

Studie **Článek XVII** (Hilscherova et al., 2001) zkoumala citlivost sledování dioxinové aktivity extraktů ze sedimentů ze 7 lokalit pomocí měření aktivity endogenního enzymu ethoxyresorufin *O*-deethylázy (EROD) v savčích a rybích buňkách a aktivity reporterového enzymu luciferázy v rekombinantních buněčných systémech. AhR-zprostředkovaná odpověď byla dobře detekovatelná ve všech studovaných modelech, přičemž lepší citlivost, opakovatelnost a větší intenzita odpovědi (indukční faktor) byla prokázána pro rekombinantní buněčné systémy. Hodnoty TEQ stanovené v rybích buňkách byly vyšší než v případě savčích buněk, což indikuje rozdíly mezi buněčnými liniemi v citlivosti k některým látkám ve směsi. Byla zjištěna vysoká korelace mezi hodnotami TEQ stanovenými ve všech modelových systémech, stejně jako s hodnotami TEQ vypočtenými na základě výsledků chemických analýz a relativních potencí stanovovaných látek. Mezi kontaminací před a po povodních nebyl zjištěn jednoznačný trend, jen na několika lokalitách byl zřejmý pokles kontaminace způsobený pravděpodobně redistribucí sedimentů. Frakcionace a přepočty příspěvku stanovovaných skupin látek k celkové dioxinové aktivitě prokázaly dominantní roli polycyklických aromatických uhlovodíků a jejich aditivní působení.

Studie **Článek XVIII** (Hilscherova et al., 2002) přinesla první informace ohledně přítomnosti estrogenních látek v sedimentech českých řek. Byly zkoumány stejné vzorky sedimentů jako ve výše uvedené studii. Všechny vzorky obsahovaly detekovatelné koncentrace estrogenních ekvivalentů, které se mezi lokalitami výrazně lišily (0,01-12 ng EEQ/g sedimentu). Frakcionace společně s biotestováním umožnila identifikaci estrogenní frakce, která obsahovala mezi jinými látkami také alkylfenoly, PAH a chlorované pesticidy. Studie také přinesla první informace ohledně koncentrací alkylfenolů v sedimentech v ČR v rozmezí 1,7-154 ng/g; jejich příspěvek k estrogenní aktivitě byl relativně nízký, podobně jako u pesticidů. Naopak významně přispívaly některé PAH, u nichž byla dříve prokázána estrogenní aktivita. Estrogenní ekvivalenty vypočtené na základě výsledků chemických analýz korelovaly s EEQ z biotestů. V některých případech byly hodnoty z biotestů nižší, což poukazuje na možný vliv antiestrogenních látek ve vzorku. Přítomnost antiestrogenů byla také prokázána při frakcionaci vzorku, kdy zejména nejpolárnější frakce vykazovala u řady vzorků antiestrogenní aktivitu. Trendy změn EEQ na jednotlivých lokalitách před a po povodních odpovídaly trendům pozorovaným u dioxinové aktivity i některých kontaminantů prezentovaným v předchozí publikaci.

Říční sedimenty reprezentují dynamický systém, zejména v oblastech častého výskytu povodní. Byly realizovány dvě studie zaměřené na prostorovou, dlouhodobou a sezónní dynamiku kontaminace říčních sedimentů prostřednictvím chemických a sedimentologických analýz a široké škály biotestů (**Článek XIX** - Hilscherova et al., 2010; **Článek XX** - Macikova et al., 2014). Sedimenty byly opakovaně vzorkovány během rozdílných hydrologických situací. V první studii byly odběry realizovány ve dvou letech po sobě na jaře po období vysokých průtoků a na podzim po delší době nízkých průtoků, ve druhé pak byly uskutečněny celoroční měsíční odběry. Ve všech testovaných sedimentech byla nalezena

významná dioxinová aktivita (TEQ 0,5–17,7 ng/g), v řadě z nich také estrogenní (EEQ 0,02-3,8 ng/g) či antiandrogenní aktivita. Nejvyšší koncentrace TEQ byly zjištěny v zimě, zejména na lokalitách pod městskou a průmyslovou aglomerací. Ve všech vzorcích byla zjištěna přítomnost antiandrogenních látek, zatímco androgenní aktivita (0,7–16,8 ng/g AEQ) byla detekována pouze ve 30 % vzorků. Byla prokázána vysoká výpovědní hodnota biotestů zaměřených na specifické mechanismy toxicity (ER, AhR, AR) v porovnání s méně specifickými testy toxicity (Microtox) či genotoxicity, které dávaly poměrně variabilní a obtížně interpretovatelné výsledky. Výsledky ukázaly sezónní dynamiku i prostorovou distribuci kontaminace a zdůraznily význam abiotických faktorů v distribuci a akumulaci polutantů. Dioxinová a antiandrogenní aktivita a koncentrace řady polutantů korelovaly s obsahem organického uhlíku a kationtovou výměnnou kapacitou. Odpovědi biotestů korelovaly s obsahem dominantních kontaminantů v sedimentech polycyklických aromatických uhlovodíků a částečně také s PCB. Jiné neanalyzované látky z více kontaminovaných lokalit přispívaly zejména k zjištěné antiandrogenitě a estrogenitě. Obecně studie z modelového regionu na Zlínsku poukazují na dlouhodobou přítomnost ED látek v sedimentech i na lokalitách mimo přímý vliv ČOV a na vliv dalších zdrojů. Výsledky demonstrují klíčovou roli designu odběru vzorků při studiu zatížení říčních systémů, který by měl brát v potaz hydrologii řeky a její sezónní změny, které ovlivňují prostorovou i sezónní variabilitu znečištění pro získání reprezentativních údajů pro analýzu rizik.

Ve spolupráci s finskými kolegy byla studována kontaminace vzorků sedimentů z řeky Kymi zatížené zejména papírenským průmyslem (**Článek XXI** - Novák et al., 2007). U všech vzorků z řeky Kymi byla zjištěna vysoká dioxinová aktivita (22-377 ng/g); na některých lokalitách až o dva řády vyšší než v sedimentech ze Zlínského regionu. Tato aktivita byla z velké části způsobena persistentními organickými polutanty, jež se v této řece nacházely ve velmi vysokých koncentracích. V případě retinoidní aktivity samotné extrakty nevykazovaly žádný účinek, ale ve spolupůsobení s ATRA bylo u většiny vzorků pozorováno velmi významné zvýšení aktivity. Nejvyšší účinek vykazoval vzorek, kterým měl současně nejvyšší dioxinovou aktivitu. Tuto schopnost potencovat působení retinoidů vykazovaly zejména nepersistentní kontaminanty ze studovaných sedimentů a také několik testovaných PAH. Tudíž PAH či jim příbuzné látky pravděpodobně přispívají k pozorované pro-retinoidní aktivitě sedimentů z řeky Kymi. Tato studie byla první, která poukázala na schopnost látek z kontaminovaných vodních ekosystémů ovlivnit signální dráhy kyseliny retinové.

3.3 Komplexní in vitro a in vivo studie ED potenciálu

Tato kapitola shrnuje několik studií zaměřených na propojení výsledků charakterizace kontaminace vodních ekosystémů pomocí *in vitro* metod a chemických analýz s *in vivo* účinky v organismech. V uvedených studiích byly zkoumány vztahy mezi hladinami polutantů, *in vitro* biologickými aktivitami a účinky na organismy v modelových expozicích nebo přímo v prostředí. První část kapitoly pojednává o využití tohoto přístupu ve výzkumu ED potenciálu v sedimentech a druhá se zabývá toxicitou vodních květů sinic.

3.3.1 Kontaminované sedimenty

První dvě studie (**Články XXII-XXIII**, Mazurová et al., 2008a; Mazurová et al., 2010) se zabývají kontaminací sedimentů z nádrže se zvýšeným výskytem intersexu u raků v Ostravském regionu a jejími účinky na organismy.

V rámci série prací byly detailně studovány účinky směsí látek z lokality Pilňok na severní Moravě, která v minulosti sloužila jako odkalovací nádrž. V nádrži byla pozorována populace ohroženého druhu korýše raka bahenního (*Pontastacus* (syn. *Astacus*) *leptodactylus*) s neobvykle zvýšenou frekvencí intersexu (až 18% jedinců). Ekotoxikologický potenciál odebraných sedimentů byl paralelně hodnocen v *in vitro* modelech a *in vivo* experimentech s vodními bezobratlými živočichy.

Výsledky *in vitro* studií poukázaly na přítomnost významného množství neznámých, zejména méně persistentních, organických látek s biologickou aktivitou. V sedimentech z lokality Pilňok byla zjištěna významná koncentrace nepersistentních AhR-ligandů, která dobře korelovala zejména se zvýšeným obsahem polycyklických aromatických uhlovodíků. V *in vitro* testech byla také zjištěna přítomnost estrogenních a antiandrogenních látek v těchto sedimentech. V naší další studii (Bláha et al., 2006) byly zkoumány účinky extraktů ze sedimentů z této nádrže na steroidogenezi. V této práci byl test založený na H295R buňkách poprvé použit pro hodnocení ovlivnění parametrů steroidogeneze komplexními vzorky z prostředí. Byly zjištěny výrazné změny v expresi kritických enzymů steroidogeneze, dokumentující schopnost organických extraktů modulovat expresi některých genů kódujících enzymy významné ve steroidogenezi a tím ovlivnit syntézu a metabolismus steroidních hormonů a posunout hormonální rovnováhu v organismu. Persistentní (chlorované POP) i nepersistentní (PAH) frakce extraktů z těchto sedimentů přispívaly k významnému vlivu směsi na steroidogenezi (významná upregulace CYP11B2 a downregulace CYP21 a 3βHSD2). Data z *in vitro* testů dokumentují vysoký endokrinně disruptivní potenciál studovaných sedimentů.

Pro výzkum potenciálu těchto sedimentů působit endokrinní disrupci *in vivo* byly vybrány dva relevantní modelové organismy zastupující skupiny bezobratlých citlivé na endokrinně disruptivní působení látek – předožábrý plž písečník novozélandský (*Potamopyrgus antipodarum*) a korýš blešivec potoční (*Gammarus fossarum*). U obou těchto druhů studie shrnuté ve **Článcích XXII** a **XXIII** ukázaly citlivost na působení modelových endokrinních disruptorů. Expozice sedimenty z lokality Pilňok (a jejich extrakty) významně ovlivnila plodnost a počet embryí v různých vývojových stupních u měkkýše písečníka novozélandského. V případě vyvinutých embryí došlo při expozici sedimentu ke stimulaci plodnosti v některých expozičních variantách po krátké době expozice (5 týdnů), zatímco dlouhodobější expozice (8 týdnů) vedla ke snížení plodnosti (**Článek XXII**). Chronická 12-ti týdenní expozice blešivce potočního kontaminovaným sedimentům vedla kromě poškození hepatopankreatu a mortality k ovlivnění reprodukčních parametrů - zejména k posunu v reprodukčním cyklu samic a dozrávání oocytů s vyšším zastoupením a zvětšením pozdně vitellogenních oocytů a vyšším podílem atretických oocytů, zvýšeným počtem vylíhlých jedinců a jejich větší velikostí (**Článek XXIII**). Podobné změny byly u těchto organismů pozorovány v předchozích studiích po expozici modelovými estrogeny 17α-ethinylestradiolem a bisfenolem A či odpadními vodami (Schirling et al., 2006; Schirling et al., 2005; Watts et al., 2002). Histopatologické vyšetření hepatopankreatu prokázalo u blešivců rozdílnost v citlivosti a toxických projevech u samců a samic. Data z *in vitro* studií společně s pozorovanými *in vivo* účinky indikujícími narušení reprodukce u modelových plžů a korýšů podporují hypotézu o chemicky-indukované endokrinní disrupci vedoucí ke zvýšení výskytu intersexu u populací raků vyskytujících se přirozeně na studované lokalitě.

Písečník novozélandský exponovaný *in situ* v říčním ekosystému byl také společně s *in vitro* a chemickými analýzami úspěšně využit v komplexní studii vlivu městské aglomerace na zatížení vodních ekosystémů (Zounkova et al., 2014)**.** V této studii zaměřené na kontaminaci sedimentů i vod charakterizovanou pomocí pasivního vzorkování byla prokázána přítomnost endokrinně disruptivních látek a demonstrovány účinky na životaschopnost a reprodukci

jedinců exponovaných po 4-8 týdnů přímo v říčních ekosystémech ovlivněných městskou aglomerací.

Ačkoli není možné vždy přímo spojovat *in vitro* detekovaný ED potenciál vzorků z prostředí s účinky na úrovni organismů, publikované studie prokázaly korelace *in vitro* a *in vivo* účinků a dobrou predikční hodnotu *in vitro* biodetekčních systémů směrem k *in vivo* účinkům (Sonneveld et al., 2006; Chakraborty et al., 2011; Leusch et al., 2014).
Nejvíce informací ohledně indikační hodnoty *in vitro* biodetekčních systémů směrem k *in vivo* účinkům je ze studií na rybách (Leusch et al., 2014), výrazně méně informací je dostupných směrem k bezobratlým živočichům. I když endokrinní systém měkkýšů a korýšů není dostatečně popsán, aby bylo možné určit přesný mechanismus účinku EDC, v řadě studií bylo dokumentováno, že expozice estrogenům ovlivňuje pohlavní diferenciaci a reprodukci u těchto skupin organismů, v některých případech už na environmetálně relevantních koncentracích (Henneberg et al., 2014; Oehlmann et al., 2007; Duft et al., 2007; Mazurová et al., 2008a). Na druhou stranu nespecifické odpovědi, zejména celkové cytotoxické působení komplexních environmentálních matric, mohou modulovat a překrývat působení EDC ze vzorku (Henneberg et al., 2014).

3.3.2 Sinicové vodní květy

Jedním ze známých důležitých zdrojů toxických látek do řady vodních ekosystémů jsou masové rozvoje vodních květů sinic. Toxické sinice představují celosvětově významný problém degradace vodního prostředí a také produkují široké spektrum bioaktivních látek, z nichž některé mohou mít negativní účinky na organismy. V rámci několika našich prací jsme uplatnili kombinovaný *in vitro* - *in vivo* výzkum jejich ED aktivit.

Některé z toxinů produkovaných sinicemi - cyanotoxinů (např. microcystiny) byly v minulosti intenzivně studovány a charakterizovány, ale řada studií ukázala, že celková toxicita komplexních směsí sinic může být vyšší než by odpovídalo koncentracím známých cyanotoxinů (Falconer, 2007; Teneva et al., 2003). Naše studie prokázaly schopnost metabolitů sinic ovlivňovat signální dráhy jaderných receptorů, zejména významný retinoidní potenciál (**Článek XXIV** - Jonas et al., 2014, **Článek XXV -** Jonas et al., 2015). Některé předchozí studie upozornily na možnou přítomnost retinoidů v akvatickém prostředí a jejich potenciální vliv na malformace pozorované u vodních obratlovců (Gardiner et al., 2003). Retinoidní látky byly detekovány jak v extraktech buněk některých druhů sinic, tak i v tzv. exudátech (tj. směsích látek produkovaných při růstu sinic do okolního prostředí). Ve studii **Článek XXIV** skríning exudátů z širšího spektra fytoplanktonních druhů poukázal na extracellulární produkci látek s retinoidní aktivitou u některých druhů sinic, zatímco žádný ze studovaných druhů zelených eukaryotických řas je neprodukoval do svého okolí v detekovatelném množství. Dva vzorky exudátů sinic s nejvyšší aktivitou (dosahující až jednotek µg ATRA-ekvivalentu/L) a jeden vzorek z řas bez prokazatelné aktivity byly následně společně s modelovou retinoidní látkou (ATRA) zkoumány v *in vivo* testu na embryích ryb zebřičky pruhované (*Danio rerio).* Tento embryonální test je velmi vhodný pro studium účinků retinoidních látek, které hrají klíčovou roli v raném vývoji obratlovců. Expozice sinicovými exudáty s retinoidní aktivitou i ATRA způsobovaly podobná narušení vývoje rybích embryií, zahrnující mimo jiné i malformace páteře, ocásku, hlavové části, otoky až úhyn embryí. Efektivní koncentrace i fenotypy malformací způsobené exudáty sinic odpovídaly výsledkům získaným po expozici modelovou látkou ATRA, což dokumentuje pravděpodobnou roli látek s retinoidní aktivitou produkovaných sinicemi v pozorovaných účincích.

Druhá studie zaměřená na extrakty z buněk sinic (**Článek XXV**) zkoumala relevanci *in vitro* detekované přítomnosti bioaktivních látek pro *in vivo* situaci s využitím transgenního modelu *Danio rerio* tg(*cyp19a1b*-GFP), který umožňuje *in vivo* detekci estrogenních látek. Ve třech zkoumaných druzích sinic byla zjištěna porovnatelná vysoká retinoidní aktivita v řádech µg ATRA-ekvivalentu/g suché váhy, zatímco estrogenní aktivita byla nízká, zvýšená pouze u druhu *Plankthotrix agardhii*. Extrakty při vyšším obsahu retinoidních látek způsobovaly teratogenitu a ovlivnily růst embryí. *In vivo* estrogenita byla pravděpodobně překryta toxicitou celkového extraktu. Navíc byl při subletálních koncentracích pozorován vliv na pohybovou aktivitu embryí. Tradičně sledované cyanotoxiny microcystiny nehrály významnou roli v pozorovaných *in vitro* a *in vivo* účincích u extraktů i exudátů.

Navazující nejnovější terénní studie také prokázaly schopnost komplexních sinicových vodních květů produkovat retinoidní látky do prostředí (**Článek XXVI** - Javůrek et al., 2015). Na některých lokalitách byly detekovány koncentrace dosahující až µg ATRA-ekvivalentu/L (publikace v přípravě). Tyto koncentrace jsou dostatečně vysoké, aby mohly způsobovat narušení vývoje citlivých stádií vodních obratlovců. Podařilo se také identifikovat některé látky přispívající k retinoidní aktivitě v laboratorních kultivacích i přímo ve vodních ekosystémech s masovým rozvojem sinic. Patří k nim např. kyselina all-*trans*-retinová (ATRA), 9-cis retinová a 13-cis retinová, all-*trans*-5,6-epoxy retinová kyselina, all-*trans*-4-keto retinová kyselina a retinal.

Výsledky ukazují na důležitost dalších sinicových metabolitů kromě známých sledovaných cyanotoxinů pro potenciální toxické působení zejména vzhledem k některým druhům a/nebo jejich vývojovým fázím. Sinice mohou produkovat retinoidní i další bioaktivní látky, kterým by měla být věnována pozornost, neboť mohou souviset s negativními účinky sinicových vodních květů na organismy. Tyto látky se vyskytují ve velmi komplexních směsích různých metabolitů, pro něž většinou nejsou dostupné analytické standardy, a tudíž by jejich celkové působení mělo být sledováno a charakterizováno pomocí *in vitro* biodetekčních systémů a *in vivo* metod.

3.4 Shrnutí výsledků terénních studií

Série prací, které se zabývají výskytem bioaktivních látek ovlivňujících signálování přes jaderné receptory a steroidogenezi v ekosystémech vodního prostředí (méně zatížené vodní toky, vliv čistíren odpadních vod, urbanizované oblasti, vliv velké městské aglomerace či průmyslu, kontaminované sedimenty, stojaté vody s rozvojem vodního květu sinic) demonstrovaly přínos *in vitro* biodetekčních systémů v charakterizaci zatížení různých typů vzorků (pasivní vzorkovače, odpadní voda, sedimenty, biomasa sinic). Studie také prokázaly velmi dobrou využitelnost kombinace pasivních vzorkovačů, které reflektují dlouhodobější situaci kontaminace prostředí, s používanými biotesty při hodnocení zatížení říčních ekosystémů. Zdokumentovaly významné trendy v efektech nad-pod zdroji znečištění a vhodnost *in vitro* nástrojů k hodnocení účinnosti odbourávání EDC v průběhu čištění na ČOV (Escher et al., 2014; **Článek XIV**). Studie také dokumentují dynamiku kontaminace v říčních ekosystémech a vliv povodní, které se projevují i v biologických odpovědích působení směsí látek z odebraných vzorků. Tyto nové údaje modifikují tradiční pohled na hodnocení účinků toxických látek v akvatickém prostředí, kde jsou často uplatňovány výsledky jednorázových měření a ukazují na potřebu zohlednění variability a sezónní dynamiky kontaminace v pravděpodobnostním hodnocení rizik.

Z hlediska zjištěných biologických aktivit se jako nejvýznamnější směrem k možným negativním účinkům ukazuje estrogenní aktivita vypouštěných odpadních vod či přímo povrchových vod. Kromě estrogenního potenciálu se ve vodách i sedimentech často setkáváme zejména s antiandrogenní a dioxinovou aktivitou. V sedimentech jsou vysoké hladiny dioxinové aktivity nacházeny především v souvislosti s akumulací hydrofobních kontaminantů. Rizika dalších typů endokrinně disruptivní aktivity ve vodách, ale i v sedimentech (androgeny, antiandrogeny, dioxinová toxicita), byly ve studovaných vodních ekosystémech ČR zpravidla srovnatelné nebo nižší v porovnání se zahraničím (Creusot et al., 2013). Řada studií prokázala důležitost sledování anti/estrogenní, anti/androgenní a dioxinové aktivity jako klíčových mechanismů působení směsí z odpadních i povrchových vod i sedimentů a jejich relevanci pro účinky ve vodních ekosystémech (Poulsen et al., 2011; Scott et al., 2014). Poměrně často detekovaná přítomnost antiandrogenních látek je mnohem méně prozkoumaná, ale může přispívat k ED účinkům u ovlivněných populací (Jobling et al., 2009). Směsi estrogenních a antiandrogenních látek mohou spolupůsobit a zvyšovat projevy endokrinní disrupce, jako je feminizace u ryb (Lange et al., 2015). Publikované studie poukazují i na ovlivnění signálování některých dalších receptorů, jako je glukokortikoidní a progesteronový, látkami v odpadních vodách i pod výpustěmi ČOV (Roberts et al., 2015; Scott et al., 2014). Mnohem méně informací je dostupných ohledně vlivu směsí látek z prostředí na signálování retinoidů, i když to je velmi důležité s ohledem na jejich roli v citlivě řízeném raném vývoji organismů. Naše studie upozornily na spolupůsobení látek ze sedimentů s retinoidy a na vodní květy sinic jako zdroj retinoidních látek do akvatických ekosystémů. Několik zahraničních studií detekovalo retinoidní aktivitu v odpadních vodách vypouštěných z ČOV v podobných hladinách, jako v našich studiích ve vodě s rozvojem vodního květu sinic (Allinson et al., 2011; Sawada et al., 2012).
Je poměrně málo informací ohledně možného příspěvku jiných zdrojů než ČOV k endokrinní aktivitě polutantů v povrchových vodách. Jak dokumentují naše studie, v urbanizovaných oblastech se látky s ED potenciálem běžně vyskytují i mimo lokality s bezprostředním vlivem ČOV, což dokládá příspěvek dalších rozptýlených nekontrolovaných zdrojů, jako splachy z povrchů, přepadové nádrže, velkochovy dobytka apod. Náš přehledový článek Jarošová et al. (2015) také poukázal na možný příspěvek fytoestrogenů a mykoestrogenů k estrogenní aktivitě vod na některých lokalitách.

4 Závěry

Jednou z hrozeb pro zachování dlouhodobé stability populací a dobré roprodukční kondice je přítomnost látek schopných narušovat fungování endokrinního systému organismů v prostředí. Pro řešení problematiky endokrinní disrupce jsou nezbytné kvalitní vědecké informace a nástroje, které umožní odhalit přítomnost látek s potenciálem působit endokrinní disrupci, charakterizují mechanismy působení kontaminantů (a jejich směsí v prostředí) a jejich účinky na různé druhy. Výsledky představené v habilitační práci i dalších výzkum na našem pracovišti včetně zapojení do řady mezinárodních aktivit přispívají k řešení problematiky ED v České republice i v širším kontextu.

Jak bylo ukázáno spojení *in vitro* a *in vivo* přístupů s chemickými analýzami má velkou přidanou hodnotu. *In vitro* biodetekční systémy byly využity k porozumění interakce xenobiotik s regulačními procesy na úrovni receptorů (estrogenní, androgenní, retinoidový, aryl hydrokarbonový) a mechanismy steroidogeneze.

Biodetekční systémy diskutované v habilitační práci mimo jiné umožňují:

1. hodnocení potenciálu cizorodých látek ovlivňovat endokrinní systém organismů konkrétními mechanismy, odvození relativních toxických potencí a identifikaci prioritních nebezpečných sloučenin;
2. specifikaci mechanismu působení, pochopení principiálních buněčných a biochemických reakcí, které hrají roli při interakci živého organismu s cizorodou chemickou látkou a při endokrinní disrupci;
3. skríningové hodnocení kontaminovaných vzorků nebo extraktů, odhad jejich nebezpečnosti z hlediska potenciálu pro endokrinní disrupci, prioritizaci vzorků pro podrobnější průzkum.
4. identifikaci látek přispívajících ke sledovanému specifickému potenciálu pomocí účinkem-řízené analýzy (EDA)
5. hodnocení efektivity odstraňování látek se specifickým mechanismem účinku v jednotlivých krocích technologických procesů na ČOV či úpravnách vod

Provedení těchto biotestů je zpravidla výrazně rychlejší a levnější než komplexní chemické analýzy širokého spektra látek a jejich výsledky reflektují i působení neanalyzovaných látek a spolupůsobení celé směsi.

S využitím sady biotestů byla realizována řada studií zaměřených na poznání mechanismů toxických účinků tradičních i nově prioritních organických environmentálních polutantů jako např. azaPAH a důležitých přírodních látek (huminové látky, metabolity sinic) a vytvořeny modely vztahu mezi chemickou strukturou a biologickými účinky. Byly získány nové vědecké poznatky o interakcích xenobiotik se studovanými receptory, i o účincích na steroidogenezi. Naše studie nově dokumentují působení polutantů a směsí látek na RAR/RXR signálování, kde je doposud poměrně málo informací. Výsledky byly doplněny *in vivo* studiemi, které potvrzují potenciál ED pozorovaný *in vitro*, doplňují (eko)toxikologický profil studovaných polutantů a jsou dále využitelné pro hodnocení jejich ekotoxikologické nebezpečnosti a rizik pro člověka i životní prostředí.

V rámci studií představených v habilitaci byly dále získány nové informace o zatížení různých složek prostředí látkami se specifickými mechanismy účinku. Velkou potřebou je validace biologických testovacích systémů a přístupů, které mohou být použity pro hodnocení rizik spojených s komplexní expozicí z prostředí. Jak bylo zdůrazněno v řadě publikací mezinárodních expertních týmů z poslední doby (Altenburger et al., 2015; European Commission, 2014), budoucí hodnocení rizik by mělo zahrnovat hodnocení parametrů (eko)toxicity společně s chemickými analýzami k identifikaci směsí a chemických látek, které představují riziko pro prostředí a lidské zdraví. Širší zapojení EBT do monitoringu povrchových vod je velmi aktuálním tématem diskutovaným v souvislosti s revizí Rámcové směrnice pro ochranu vod v roce 2019. Vývoj a zapojení optimalizované baterie relevantních EBT do monitoringu je i jednou z priorit v rámci aktuálně řešeného projektu EU FP7 SOLUTIONs (no.603437), na kterém se naše pracoviště podílí (Brack et al., 2015). Skríningové metody sledování toxického potenciálu vzorků jsou navrhovány jako důležitý doplněk chemických analýz při hodnocení odpadních, povrchových i pitných vod, i efektivity odstraňování toxických látek v průběhu čištění odpadních vod, a jsou diskutovány nejvhodnější přístupy jejich zapojení, optimální baterie biotestů i stanovení bezpečných limitů odvozených z biotestů (Escher et al., 2015; Poulsen et al., 2011). Důležitou prioritou je zohlednění širšího spektra mechanismů působení (nejen) EDC v těchto bateriích. K potenciálnímu širšímu využívání biotestů v praktickém monitoringu přispívají také adaptace a vývoj nových modelů s rychlou odpovědí, kterým se věnuje i náš aktuální výzkum.

5 Literatura

1272/2008/ES, 2008. Nařízení Evropského parlamentu a Rady (ES) č. 1272/2008 ze dne 16. prosince 2008 o klasifikaci, označování a balení látek a směsí, o změně a zrušení směrnic 67/548/EHS a 1999/45/ES a o změně nařízení (ES) č. 1907/2006. [online] [cit. 10. 2. 2015]. Dostupné z: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:353:0001:1355:cs:PDF

2000/60/ES, 2000. Směrnice Evropského parlamentu a Rady 2000/60/ES ze dne 23. října 2000, kterou se stanoví rámec pro činnost Společenství v oblasti vodní politiky. [online] [cit. 10. 2. 2015]. Dostupné z: http://eur-lex.europa.eu/legal-content/CS/TXT/PDF/?uri=CELEX:32000L0060&from=CS

Aerni, H.R., Kobler, B., Rutishauser, B.V., Wettstein, F.E., Fischer, R., Giger, W., Hungerbuhler, A., Marazuela, M.D., Peter, A., Schonenberger, R., Vogeli, A.C., Suter, M.J.F., Eggen, R.I.L., 2004. Combined biological and chemical assessment of estrogenic activities in wastewater treatment plant effluents. Anal. Bioanal. Chem. 378, 688–696.

Allinson, M., Shiraishi, F., Salzman, S.A., Allinson, G., 2011. In vitro assessment of retinoic acid and aryl hydrocarbon receptor activity of treated effluent from 39 wastewater-treatment plants in Victoria, Australia. Arch. Environ. Contam. Toxicol. 61, 539–546. doi:10.1007/s00244-011-9665-z

Altenburger, R., Ait-Aissa, S., Antczak, P., Backhaus, T., Barceló, D., Seiler, T.B., Brion, F., et al.., 2015. Future water quality monitoring — Adapting tools to deal with mixtures of pollutants in water resource management. Sci. Total Environ. 512-513, 540–551. doi:10.1016/j.scitotenv.2014.12.057

Alvarez, D.A., Shappell, N.W., Billey, L.O., Bermudez, D.S., Wilson, V.S., Kolpin, D.W., Perkins, S.D., Evans, N., Foreman, W.T., Gray, J.L., Shipitalo, M.J., Meyer, M.T., 2013. Bioassay of estrogenicity and chemical analyses of estrogens in streams across the United States associated with livestock operations. Water Res. 47, 3347–63. doi:10.1016/j.watres.2013.03.028

Alvarez, D.A., Maruya, K.A., Dodder, N.G., Lao, W., Furlong, E.T., Smalling, K.L., 2014. Occurrence of contaminants of emerging concern along the California coast (2009-10) using passive sampling devices. Mar. Pollut. Bull. 81, 347–354. doi:10.1016/j.marpolbul.2013.04.022

Baker, V.A., 2001. Endocrine disrupters -- testing strategies to assess human hazard. Toxicol. In Vitro 15, 413–419.

Bednarova, Z., Kuta, J., Kohut, L., Machat, J., Klanova, J., Holoubek, I., Jarkovsky, J., Dusek, L., Hilscherova, K., 2013. Spatial patterns and temporal changes of heavy metal distributions in river sediments in a region with multiple pollution sources. J. Soils Sediments 13, 1257–1269. doi:10.1007/s11368-013-0706-2

Beníšek, M., Bláha, L., Hilscherová, K., 2008. Interference of PAHs and their N-heterocyclic analogs with signaling of retinoids in vitro. Toxicol. In Vitro 22, 1909–1917. doi:10.1016/j.tiv.2008.09.009

Beníšek, M., Kubincová, P., Bláha, L., Hilscherová, K., 2011. The effects of PAHs and N-PAHs on retinoid signaling and Oct-4 expression in vitro. Toxicol. Lett. 200, 169–75. doi:10.1016/j.toxlet.2010.11.011

Bittner, M., Hilscherova, K., Giesy, J.P., 2009. In vitro assessment of AhR-mediated activities of TCDD in mixture with humic substances. Chemosphere 76, 1505–8. doi:10.1016/j.chemosphere.2009.06.042

Bittner, M., Janosek, J., Hilscherova, K., Giesy, J., Holoubek, I., Blaha, L., 2006. Activation of Ah receptor by pure humic acids. Environ. Toxicol. 21, 338–342.

Bittner, M., Jarque, S., Hilscherová, K., 2015. Polymer-immobilized ready-to-use recombinant yeast assays for the detection of endocrine disruptive compounds. Chemosphere 132, 56–62. doi:10.1016/j.chemosphere.2015.02.063

Bittner, M., Macikova, P., Giesy, J.P., Hilscherova, K., 2011. Enhancement of AhR-mediated activity of selected pollutants and their mixtures after interaction with dissolved organic matter. Environ. Int. 37, 960–4. doi:10.1016/j.envint.2011.03.016

Bláha, L., Hilscherová, K., Mazurová, E., Hecker, M., Jones, P.D., Newsted, J.L., Bradley, P.W., Gracia, T., Duris, Z., Horká, I., Holoubek, I., Giesy, J.P., 2006. Alteration of steroidogenesis in H295R cells by organic sediment contaminants and relationships to other endocrine disrupting effects. Environ. Int. 32, 749–57. doi:10.1016/j.envint.2006.03.011

Bleeker, E., Wiegman, S., de Voogt, P., Kraak, M., Leslie, H., de Haas, E., Admiraal, W., 2002. Toxicity of azaarenes. Rev. Environ. Contam. Toxicol. 173, 39–83.

Brack, W., Altenburger, R., Schüürmann, G., Krauss, M., López Herráez, D., van Gils, J., et al., 2015. The SOLUTIONS project: Challenges and responses for present and future emerging pollutants in land and water resources management. Sci. Total Environ. 503-504, 22–31. doi:10.1016/j.scitotenv.2014.05.143

Brack, W., Klamer, H.J.C., López de Alda, M., Barceló, D., 2007. Effect-directed analysis of key toxicants in European river basins a review. Environ. Sci. Pollut. Res. Int. 14, 30–8.

Bradshaw, T.D., Bell, D.R., 2009. Relevance of the aryl hydrocarbon receptor (AhR) for clinical toxicology. Clin. Toxicol. (Phila). 47, 632–642. doi:10.1080/15563650903140423

Brtko, J., Dvorak, Z., 2015. Triorganotin compounds - ligands for “rexinoid” inducible transcription factors: Biological effects. Toxicol. Lett. 234, 50–58. doi:10.1016/j.toxlet.2015.02.009

Burkhardt-Holm, P., 2010. Endocrine Disruptors and Water Quality: A State-of-the-Art Review. Int. J. Water Resour. Dev. 26, 477–493. doi:10.1080/07900627.2010.489298

Burýšková, B., Hilscherová, K., Bláha, L., Maršálek, B., Holoubek, I., 2006. Toxicity and modulations of biomarkers in Xenopus laevis embryos exposed to polycyclic aromatic hydrocarbons and their N-heterocyclic derivatives. Environ. Toxicol. 21, 590–598.

Caldwell, D.J., Mastrocco, F., Anderson, P.D., Länge, R., Sumpter, J.P., 2012. Predicted-no-effect concentrations for the steroid estrogens estrone, 17β-estradiol, estriol, and 17α-ethinylestradiol. Environ. Toxicol. Chem. 31, 1396–1406. doi:10.1002/etc.1825

Caliman, F.A., Gavrilescu, M., 2009. Pharmaceuticals, Personal Care Products and Endocrine Disrupting Agents in the Environment - A Review. CLEAN - Soil, Air, Water 37, 277–303. doi:10.1002/clen.200900038

Carvalho, R.N., Arukwe, A., Ait-Aissa, S., Bado-Nilles, A., Balzamo, S., Baun, A., Belkin, S., et al. 2014. Mixtures of Chemical Pollutants at European Legislation Safety Concentrations: How Safe are They? Toxicol. Sci. doi:10.1093/toxsci/kfu118

Casati, L., Sendra, R., Sibilia, V., Celotti, F., 2015. Endocrine disrupters: the new players able to affect the epigenome. Front. Cell Dev. Biol. 3, 37. doi:10.3389/fcell.2015.00037

Coes, A.L., Paretti, N.V., Foreman, W.T., Iverson, J.L., Alvarez, D.A., 2014. Sampling trace organic compounds in water: A comparison of a continuous active sampler to continuous passive and discrete sampling methods. Sci. Total Environ. 473-474, 731–741. doi:10.1016/j.scitotenv.2013.12.082

COM(1999)706. Commission of the European Communities. 1999. Community Strategy for Endocrine Disrupters, a range of substances suspected of interfering with the hormone systems of humans and wildlife. [online] [cit. 10. 2. 2015]. Dostupné z: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:1999:0706:FIN:EN:PDF

Creusot, N., Tapie, N., Piccini, B., Balaguer, P., Porcher, J.-M., Budzinski, H., Aït-Aïssa, S., 2013. Distribution of steroid- and dioxin-like activities between sediments, POCIS and SPMD in a French river subject to mixed pressures. Environ. Sci. Pollut. Res. Int. 20, 2784–94. doi:10.1007/s11356-012-1452-5

De Coster, S., Van Larebeke, N., 2012. Endocrine-disrupting chemicals: Associated disorders and mechanisms of action. J. Environ. Public Health 2012. doi:10.1155/2012/713696

DeGroot, D.E., Franks, D.G., Higa, T., Tanaka, J., Hahn, M.E., Denison, M.S., 2015. Naturally occurring marine brominated indoles are aryl hydrocarbon receptor ligands/agonists. Chem. Res. Toxicol. 28, 1176–1185. doi:10.1021/acs.chemrestox.5b00003

Diamanti-Kandarakis, E., Bourguignon, J.-P., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., Zoeller, R.T., Gore, A.C., 2009. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr. Rev. 30, 293–342. doi:10.1210/er.2009-0002

Duft, M., Schmitt, C., Bachmann, J., Brandelik, C., Schulte-Oehlmann, U., Oehlmann, J., 2007. Prosobranch snails as test organisms for the assessment of endocrine active chemicals - An overview and a guideline proposal for a reproduction test with the freshwater mudsnail Potamopyrgus antipodarum. Ecotoxicology 16, 169–182. doi:10.1007/s10646-006-0106-0

EEA, 2012. The impacts of endocrine disrupters on wildlife, people and their environments. The Weybridge+15 (1996-2011) report. 2014 [online] [cit. 10. 2. 2015]. Dostupné z: http://www.eea.europa.eu/publications/the-impacts-of-endocrine-disrupters

EFSA, 2013. Scientific Opinion on the hazard assessment of endocrine disruptors : Scientific criteria for identification of endocrine disruptors and appropriateness of existing test methods for assessing effects mediated by these substances on human health and the environment. [online] [cit. 10. 2. 2015]. Dostupné z: http://www.efsa.europa.eu/en/efsajournal/pub/3132

Ermler, S., Scholze, M., Kortenkamp, A., 2011. The suitability of concentration addition for predicting the effects of multi-component mixtures of up to 17 anti-androgens with varied structural features in an in vitro AR antagonist assay. Toxicol. Appl. Pharmacol. 257, 189–197. doi:10.1016/j.taap.2011.09.005

Érseková, A., Hilscherová, K., Klánová, J., Giesy, J.P., Novák, J., 2014. Effect-based assessment of passive air samples from four countries in Eastern Europe. Environ. Monit. Assess. 186, 3905–16. doi:10.1007/s10661-014-3667-z

Escher, B.I., Allinson, M., Altenburger, R., Bain, P.A., Balaguer, P., Busch, W., Crago, J., et al., 2014. Benchmarking organic micropollutants in wastewater, recycled water and drinking water with in vitro bioassays. Environ. Sci. Technol. 48, 1940–56. doi:10.1021/es403899t

Escher, B.I., Neale, P.A., Leusch, F.D.L., 2015. Effect-based trigger values for in vitro bioassays: Reading across from existing water quality guideline values. Water Res. 81, 137–148. doi:10.1016/j.watres.2015.05.049

European Commission. 2015. Endocrine Disruptors - What is being done?/ Priority list. 2014 [online] [cit. 10. 2. 2015]. Dostupné z: http://ec.europa.eu/environment/chemicals/endocrine/strategy/substances_en.htm

European Commission, 2011. 4th Report on the implementation of the “Community Strategy for Endocrine Disrupters” a range of substances suspected of interfering with the hormone systems of humans and wildlife (COM (1999) 706). Brussels. doi:10.1017/CBO9781107415324.004 [cit. 10. 2. 2015]. Dostupné z: http://ec.europa.eu/environment/chemicals/endocrine/pdf/sec_2011_1001.pdf

European Commission, 2014. Technical report on aquatic effect-based monitoring tools. Technical Report 2014 – 77. ISBN 978-92-79-35787-9. doi:10.2779/7260

Evans, R.M., Mangelsdorf, D.J., 2014. Nuclear receptors, RXR, and the big bang. Cell 157, 255–266. doi:10.1016/j.cell.2014.03.012

Falconer, I.R., 2007. Cyanobacterial toxins present in Microcystis aeruginosa extracts--more than microcystins! Toxicon 50, 585–8. doi:10.1016/j.toxicon.2007.03.023

Feldmannova, M., Hilscherova, K., Marsalek, B., Blaha, L., 2006. Effects of N-heterocyclic polyaromatic hydrocarbons on survival, reproduction, and biochemical parameters in Daphnia magna. Environ. Toxicol. 21, 425–431.

Foradori, C.D., Weiser, M.J., Handa, R.J., 2008. Non-genomic actions of androgens. Front. Neuroendocrinol. 29, 169–181. doi:10.1016/j.yfrne.2007.10.005

Fu, X.D., Simoncini, T., 2008. Extra-nuclear signaling of estrogen receptors. IUBMB Life 60, 502–510. doi:10.1002/iub.80

Fuzzen, M.L.M., Bennett, C.J., Tetreault, G.R., McMaster, M.E., Servos, M.R., 2015. Severe intersex is predictive of poor fertilization success in populations of rainbow darter (Etheostoma caeruleum). Aquat. Toxicol. 160, 106–116. doi:10.1016/j.aquatox.2015.01.009

Galluba, S., Oehlmann, J., 2012. Widespread endocrine activity in river sediments in Hesse, Germany, assessed by a combination of in vitro and in vivo bioassays. J. Soils Sediments 12, 252–264. doi:http://dx.doi.org/10.1007/s11368-011-0454-0

Garcia-Reyero, N., 2015. Are adverse outcome pathways here to stay? Environ. Sci. Technol. 49, 3–9. doi:10.1021/es504976d

Gardiner, D., Ndayibagira, A., Grun, F., Blumberg, B., 2003. Deformed frogs and environmental retinoids. Pure Appl. Chem. 75, 2263–2273.

Gerbersdorf, S.U., Cimatoribus, C., Class, H., Engesser, K.H., Helbich, S., Hollert, H., Lange, C., Kranert, M., Metzger, J., Nowak, W., Seiler, T.B., Steger, K., Steinmetz, H., Wieprecht, S., 2015. Anthropogenic Trace Compounds (ATCs) in aquatic habitats — Research needs on sources, fate, detection and toxicity to ensure timely elimination strategies and risk management. Environ. Int. 79, 85–105. doi:10.1016/j.envint.2015.03.011

Gore, A.C., Chappell, V.A., Fenton, S.E., Flaws, J.A., Nadal, A., Prins, G.S., Toppari, J., Zoeller, R.T., 2015. Executive Summary to EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. Endocr. Rev. 36, 593–602. doi:10.1210/er.2015-1093

Grabic, R., Jurcikova, J., Tomsejova, S., Ocelka, T., Halirova, J., Hypr, D., Kodes, V., 2010. Passive sampling methods for monitoring endocrine disruptors in the Svratka and Svitava Rivers in the Czech Republic. Environ. Toxicol. Chem. 29, 550–555. doi:10.1002/etc.85

Gracia, T., Hilscherova, K., Jones, P.D., Newsted, J.L., Higley, E.B., Zhang, X., Hecker, M., Murphy, M.B., Yu, R.M.K., Lam, P.K.S., Wu, R.S.S., Giesy, J.P., 2007. Modulation of steroidogenic gene expression and hormone production of H295R cells by pharmaceuticals and other environmentally active compounds. Toxicol. Appl. Pharmacol. 225, 142–53. doi:10.1016/j.taap.2007.07.013

Gracia, T., Hilscherova, K., Jones, P.D., Newsted, J.L., Zhang, X., Hecker, M., Higley, E.B., Sanderson, J.T., Yu, R.M.K., Wu, R.S.S., Giesy, J.P., 2006. The H295R system for evaluation of endocrine-disrupting effects. Ecotoxicol. Environ. Saf. 65, 293–305. doi:10.1016/j.ecoenv.2006.06.012

Groh, K.J., Carvalho, R.N., Chipman, J.K., Denslow, N.D., Halder, M., Murphy, C.A., Roelofs, D., Rolaki, A., Schirmer, K., Watanabe, K.H., 2015. Development and application of the adverse outcome pathway framework for understanding and predicting chronic toxicity: II. A focus on growth impairment in fish. Chemosphere 120, 778–92. doi:10.1016/j.chemosphere.2014.10.006

Gros, M., Petrovic, M., Barcelo, D., 2007. Wastewater treatment plants as a pathway for aquatic contamination by pharmaceuticals in the Ebro River basin (Northeast Spain). Environ. Toxicol. 26, 1553–1562.

Haeba, M.H., Hilscherova, K., Mazurova, E., Blaha, L., 2008. Selected endocrine disrupting compounds (vinclozolin, flutamide, ketoconazole and dicofol): Effects on survival, occurrence of males, growth, molting and reproduction of Daphnia magna. Environ. Sci. Pollut. Res. 15, 222–227.

Harman, C., Allan, I.J., Vermeirssen, E.L.M., 2012. Calibration and use of the polar organic chemical integrative sampler-a critical review. Environ. Toxicol. Chem. 31, 2724–2738. doi:10.1002/etc.2011

Harvey, P.W., Everett, D.J., 2003. The adrenal cortex and steroidogenesis as cellular and molecular targets for toxicity: Critical omissions from regulatory endocrine disrupter screening strategies for human health? J. Appl. Toxicol. 23, 81–87. doi:10.1002/jat.896

Harvey, P.W., Everett, D.J., Springall, C.J., 2007. Adrenal toxicology: a strategy for assessment of functional toxicity to the adrenal cortex and steroidogenesis. J. Appl. Toxicol. 27, 103–115.

Hawliczek, A., Nota, B., Cenijn, P., Kamstra, J., Pieterse, B., Winter, R., Winkens, K., Hollert, H., Segner, H., Legler, J., 2012. Developmental toxicity and endocrine disrupting potency of 4-azapyrene, benzo[b]fluorene and retene in the zebrafish Danio rerio. Reprod. Toxicol. 33, 213–223. doi:10.1016/j.reprotox.2011.11.001

Henneberg, A., Bender, K., Blaha, L., Giebner, S., Kuch, B., Kohler, H.R., Maier, D., Oehlmann, J., Richter, D., Scheurer, M., Schulte-Oehlmann, U., Sieratowicz, A., Ziebart, S., Triebskorn, R., 2014. Are in Vitro methods for the detection of endocrine potentials in the aquatic environment predictive for in Vivo effects? Outcomes of the projects SchussenAktiv and SchussenAktiv plus in the Lake Constance Area, Germany. PLoS One 9. doi:10.1371/journal.pone.0098307

Hilscherova, K., Dusek, L., Kubik, V., Cupr, P., Hofman, J., Klanova, J., Holoubek, I., 2007. Redistribution of organic pollutants in river sediments and alluvial soils related to major floods. J. Soils Sediments 7, 167–177.

Hilscherova, K., Dusek, L., Sidlova, T., Jalova, V., Cupr, P., Giesy, J.P., Nehyba, S., Jarkovsky, J., Klanova, J., Holoubek, I., 2010. Seasonally and Regionally Determined Indication Potential of Bioassays in Contaminated River Sediments. Environ. Toxicol. Chem. 29, 522–534.

Hilscherova, K., Jones, P.D., Gracia, T., Newsted, J.L., Zhang, X., Sanderson, J.T., Yu, R.M.K., Wu, R.S.S., Giesy, J.P., 2004. Assessment of the effects of chemicals on the expression of ten steroidogenic genes in the H295R cell line using real-time PCR. Toxicol. Sci. 81, 78–89. doi:10.1093/toxsci/kfh191

Hilscherova, K., Kannan, K., Holoubek, I., Giesy, J.P., 2002. Characterization of estrogenic activity of riverine sediments from the Czech Republic. Arch. Environ. Contam. Toxicol. 43, 175–185.

Hilscherova, K., Kannan, K., Kang, Y.S., Holoubek, I., Machala, M., Masunaga, S., Nakanishi, J., Giesy, J.P., 2001. Characterization of dioxin-like activity of sediments from a Czech river basin. Environ. Toxicol. Chem. 20, 2768–77.

Hilscherova, K., Kannan, K., Nakata, H., Hanari, N., Yamashita, N., Bradley, P.W., McCabe, J.M., Taylor, A.B., Giesy, J.P., 2003. Polychlorinated dibenzo-p-dioxin and dibenzofuran concentration profiles in sediments and flood-plain soils of the Tittabawassee River, Michigan. Environ. Sci. Technol. 37, 468–74. doi: 10.1021/es020920c

Hilscherova, K., Machala, M., Kannan, K., Blankenship, A.L., Giesy, J.P., 2000. Cell bioassay for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples - review. Environ. Sci. Pollut. Res. 7, 159–171.

Chakraborty, T., Katsu, Y., Zhou, L.Y., Miyagawa, S., Nagahama, Y., Iguchi, T., 2011. Estrogen receptors in medaka (Oryzias latipes) and estrogenic environmental contaminants: An in vitro-in vivo correlation. J. Steroid Biochem. Mol. Biol. 123, 115–121. doi:10.1016/j.jsbmb.2010.11.015

Charlestra, L., Courtemanch, D.L., Amirbahman, A., Patterson, H., 2008. Semipermeable membrane device (SPMD) for monitoring PCDD and PCDF levels from a paper mill effluent in the Androscoggin River, Maine, USA. Chemosphere 72, 1171–1180. doi:10.1016/j.chemosphere.2008.03.057

Chiang, G., Barra, R., Díaz-Jaramillo, M., Rivas, M., Bahamonde, P., Munkittrick, K.R., 2015. Estrogenicity and intersex in juvenile rainbow trout (Oncorhynchus mykiss) exposed to Pine/Eucalyptus pulp and paper production effluent in Chile. Aquat. Toxicol. 164, 126–134. doi:10.1016/j.aquatox.2015.04.025

Jálová, V., Jarošová, B., Bláha, L., Giesy, J.P., Ocelka, T., Grabic, R., Jurčíková, J., Vrana, B., Hilscherová, K., 2013. Estrogen-, androgen- and aryl hydrocarbon receptor mediated activities in passive and composite samples from municipal waste and surface waters. Environ. Int. 59, 372–83. doi:10.1016/j.envint.2013.06.024

Janosek, J., Bittner, M., Hilscherová, K., Bláha, L., Giesy, J.P., Holoubek, I., 2007. AhR-mediated and antiestrogenic activity of humic substances. Chemosphere 67, 1096–101. doi:10.1016/j.chemosphere.2006.11.045

Janošek, J., Hilscherová, K., Bláha, L., Holoubek, I., 2006. Environmental xenobiotics and nuclear receptors-Interactions, effects and in vitro assessment. Toxicol. In Vitro 20, 18–37.

Jarosova, B., Blaha, L., Vrana, B., Randak, T., Grabic, R., Giesy, J.P., Hilscherova, K., 2012. Changes in concentrations of hydrophilic organic contaminants and of endocrine-disrupting potential downstream of small communities located adjacent to headwater. Environ. Int. 45, 22-31.

Jarošová, B., Bláha, L., Giesy, J.P., Hilscherová, K., 2014a. What level of estrogenic activity determined by in vitro assays in municipal waste waters can be considered as safe? Environ. Int. 64, 98–109. doi:10.1016/j.envint.2013.12.009

Jarošová, B., Erseková, A., Hilscherová, K., Loos, R., Gawlik, B.M., Giesy, J.P., Bláha, L., 2014b. Europe-wide survey of estrogenicity in wastewater treatment plant effluents: the need for the effect-based monitoring. Environ. Sci. Pollut. Res. Int. 21, 10970–82. doi:10.1007/s11356-014-3056-8

Jarošová, B., Javůrek, J., Adamovský, O., Hilscherová, K., 2015. Phytoestrogens and mycoestrogens in surface waters — Their sources, occurrence, and potential contribution to estrogenic activity. Environ. Int. 81, 26–44. doi:10.1016/j.envint.2015.03.019

Jarque, S., Bittner, M., Hilscherova, K., 2016. Freeze-drying as suitable method to achieve ready-to-use yeast biosensors for androgenic and estrogenic compounds. Chemosphere 148, Accepted. doi:10.1016/j.chemosphere.2016.01.038

Javůrek, J., Sychrová, E., Smutná, M., Bittner, M., Kohoutek, J., Adamovský, O., Nováková, K., Smetanová, S., Hilscherová, K., 2015. Retinoid compounds associated with water blooms dominated by Microcystis species. Harmful Algae 47, 116–125. doi:10.1016/j.hal.2015.06.006

Jia, A., Escher, B.I., Leusch, F.D.L., Tang, J.Y.M., Prochazka, E., Dong, B., Snyder, E.M., Snyder, S.A., 2015. In vitro bioassays to evaluate complex chemical mixtures in recycled water. Water Res. 80, 1–11. doi:10.1016/j.watres.2015.05.020

Jobling, S., Burn, R.W., Thorpe, K., Williams, R., Tyler, C., 2009. Statistical modeling suggests that antiandrogens in effluents from wastewater treatment works contribute to widespread sexual disruption in fish living in english rivers. Environ. Health Perspect. 117, 797–802. doi:10.1289/ehp.0800197

Jobling, S., Tyler, C.R., 2003. Endocrine disruption in wild freshwater fish. Pure Appl. Chem. 75, 2219–2234.

Jonas, A., Buranova, V., Scholz, S., Fetter, E., Novakova, K., Kohoutek, J., Hilscherova, K., 2014. Retinoid-like activity and teratogenic effects of cyanobacterial exudates. Aquat. Toxicol. 155, 283–290. doi:10.1016/j.aquatox.2014.06.022

Jonas, A., Scholz, S., Fetter, E., Sychrova, E., Novakova, K., Ortmann, J., Benisek, M., Adamovsky, O., Giesy, J.P., Hilscherova, K., 2015. Endocrine, teratogenic and neurotoxic effects of cyanobacteria detected by cellular in vitro and zebrafish embryos assays. Chemosphere 120C, 321–327. doi:10.1016/j.chemosphere.2014.07.074

Kaisarevic, S., Hilscherova, K., Weber, R., Sundqvist, K.L., Tysklind, M., Voncina, E., Bobic, S., Andric, N., Pogrmic-Majkic, K., Vojinovic-Miloradov, M., Giesy, J.P., Kovacevic, R., 2011. Characterization of dioxin-like contamination in soil and sediments from the "hot spot" area of petrochemical plant in Pancevo (Serbia). Environ. Sci. Pollut. Res. Int. 18, 677–86. doi:10.1007/s11356-010-0418-8

Kennedy, K., Macova, M., Leusch, F., Bartkow, M.E., Hawker, D.W., Zhao, B., Denison, M.S., Mueller, J.F., 2009. Assessing indoor air exposures using passive sampling with bioanalytical methods for estrogenicity and aryl hydrocarbon receptor activity. Anal. Bioanal. Chem. 394, 1413–21. doi:10.1007/s00216-009-2825-6

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. U. S. A. 104, 8897–8901.

Kortenkamp, A.A., Martin, O., Faust, M., Evans, R., Mckinlay, R., Orton, F., Rosivatz, E., 2011. State of the art asesment of endocrine disruptors. Final Report Project Contract Number 070307 / 2009 / 550687 / SER / D3. [online] [cit. 15. 2. 2016]. Dostupné z: http://ec.europa.eu/environment/chemicals/endocrine/pdf/sota_edc_final_report.pdf

Lange, A., Sebire, M., Rostkowski, P., Mizutani, T., Miyagawa, S., Iguchi, T., Hill, E.M., Tyler, C.R., 2015. Environmental chemicals active as human antiandrogens do not activate a stickleback androgen receptor but enhance a feminising effect of oestrogen in roach. Aquat. Toxicol. 168, 48–59. doi:10.1016/j.aquatox.2015.09.014

Leskinen, P., Hilscherova, K., Sidlova, T., Kiviranta, H., Pessala, P., Salo, S., Verta, M., Virta, M., 2008. Detecting AhR ligands in sediments using bioluminescent reporter yeast. Biosens. Bioelectron. 23, 1850–1855. doi:10.1016/j.bios.2008.02.026

Leusch, F.D.L., De Jager, C., Levi, Y., Lim, R., Puijker, L., Sacher, F., Tremblay, L.A., Wilson, V.S., Chapman, H.F., 2010. Comparison of five in vitro bioassays to measure estrogenic activity in environmental waters. Environ. Sci. Technol. 44, 3853–60. doi:10.1021/es903899d

Leusch, F.D.L., Khan, S.J., Gagnon, M.M., Quayle, P., Trinh, T., Coleman, H., Rawson, C., Chapman, H.F., Blair, P., Nice, H., Reitsema, T., 2014. Assessment of wastewater and recycled water quality: a comparison of lines of evidence from in vitro, in vivo and chemical analyses. Water Res. 50, 420–31. doi:10.1016/j.watres.2013.10.056

Levin, E.R., 2015. Extranuclear Steroid Receptors Are Essential for Steroid Hormone Actions*. Annu. Rev. Med. 66, 271–280. doi:10.1146/annurev-med-050913-021703

Long, M., Strand, J., Lassen, P., Kruger, T., Dahllof, I., Bossi, R., Larsen, M.M., Wiberg-Larsen, P., Bonefeld-Jorgensen, E.C., 2014. Endocrine-disrupting effects of compounds in Danish streams. Arch. Environ. Contam. Toxicol. 66, 1–18. doi:10.1007/s00244-013-9959-4

Loos, R., Carvalho, R., António, D.C., Comero, S., Locoro, G., Tavazzi, S., Paracchini, B., Ghiani, M., Lettieri, T., Blaha, L., Jarosova, B., Voorspoels, S., Servaes, K., Haglund, P., Fick, J., Lindberg, R.H., Schwesig, D., Gawlik, B.M., 2013. EU-wide monitoring survey on emerging polar organic contaminants in wastewater treatment plant effluents. Water Res. 47, 6475–6487. doi:10.1016/j.watres.2013.08.024

Luo, Y., Guo, W., Ngo, H.H., Nghiem, L.D., Hai, F.I., Zhang, J., Liang, S., Wang, X.C., 2014. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Sci. Total Environ. 473-474, 619–641. doi:10.1016/j.scitotenv.2013.12.065

Macikova, P., Kalabova, T., Klanova, J., Kukucka, P., Giesy, J.P., Hilscherova, K., 2014. Longer-term and short-term variability in pollution of fluvial sediments by dioxin-like and endocrine disruptive compounds. Environ. Sci. Pollut. Res. Int. 21, 5007–22. doi:10.1007/s11356-013-2429-8

Mankidy, R., Wiseman, S., Ma, H., Giesy, J.P., 2013. Biological impact of phthalates. Toxicol. Lett. 217, 50–8. doi:10.1016/j.toxlet.2012.11.025

Marty, M.S., Carney, E.W., Rowlands, J.C., 2011. Endocrine disruption: historical perspectives and its impact on the future of toxicology testing. Toxicol. Sci. 120 Suppl , S93–108. doi:10.1093/toxsci/kfq329

Mazurová, E., Hilscherová, K., Jálová, V., Köhler, H.-R., Triebskorn, R., Giesy, J.P., Bláha, L., 2008a. Endocrine effects of contaminated sediments on the freshwater snail Potamopyrgus antipodarum in vivo and in the cell bioassays in vitro. Aquat. Toxicol. 89, 172–9. doi:10.1016/j.aquatox.2008.06.013

Mazurová, E., Hilscherová, K., Šídlová-Štěpánková, T., Köhler, H.-R., Triebskorn, R., Jungmann, D., Giesy, J.P., Bláha, L., 2010. Chronic toxicity of contaminated sediments on reproduction and histopathology of the crustacean Gammarus fossarum and relationship with the chemical contamination and in vitro effects. J. Soils Sediments 10, 423–433. doi:10.1007/s11368-009-0166-x

Mazurova, E., Hilscherova, K., Triebskorn, R., Köhler, H.R., Marsalek, B., Blaha, L., 2008b. Endocrine regulation of the reproduction in crustaceans: Identification of potential targets for toxicants and environmental contaminants. Biologia. 63, 139–150. doi:10.2478/s11756-008-0027-x

Munn, S., Goumenou, M., 2013. Key scientific issues relevant to the identification and characterisation of endocrine disrupting substances Report of the Endocrine Disrupters Expert Advisory Group. doi:10.2788/8659. [online] [cit. 10. 2. 2015]. Dostupné z: http://publications.jrc.ec.europa.eu/repository/bitstream/JRC79981/lbna25919enn.pdf

Murphy, K.A.., Quadro, L.., White, L.A., 2007. The intersection between the aryl hydrocarbon receptor (AHR)-and retinoic acid-signaling pathways. Vitam. A B. Ser. Vitam. Horm. 75, 33–67.

Nadzialek, S., Vanparys, C., Van der Heiden, E., Michaux, C., Brose, F., Scippo, M.-L., De Coen, W., Kestemont, P., 2010. Understanding the gap between the estrogenicity of an effluent and its real impact into the wild. Sci. Total Environ. 408, 812–21. doi:10.1016/j.scitotenv.2009.09.002

Neale, P.A., Ait-Aissa, S., Brack, W., Creusot, N., Denison, M.S., Deutschmann, B., Hilscherova, K., Hollert, H., Krauss, M., Novák, J., Schulze, T., Seiler, T.B., Serra, H., Shao, Y., Escher, B.I., 2015. Linking in vitro effects and detected organic micropollutants in surface water using mixture toxicity modeling. Environ. Sci. Technol. 49, 14614-24. doi:10.1021/acs.est.5b04083

Nentwig, G., 2007. Effects of pharmaceuticals on aquatic invertebrates. Part II: the antidepressant drug fluoxetine. Arch. Environ. Contam. Toxicol. 52, 163–70. doi:10.1007/s00244-005-7190-7

Novák, J., Benísek, M., Hilscherová, K., 2008. Disruption of retinoid transport, metabolism and signaling by environmental pollutants. Environ. Int. 34, 898–913. doi:10.1016/j.envint.2007.12.024

Novák, J., Beníšek, M., Pacherník J., Janošek J., Šídlová T., Kiviranta H., Verta M., Giesy J.P., Bláha L., Hilscherová K., 2007. Interference of contaminated sediment extracts and environmental pollutants with retinoid signaling. Environ. Toxicol. Chem. 26, 1591-1599.

Novák, J., Giesy, J.P., Klánová, J., Hilscherová, K., 2013. In vitro effects of pollutants from particulate and volatile fractions of air samples-day and night variability. Environ. Sci. Pollut. Res. Int. 20, 6620–7. doi:10.1007/s11356-013-1726-6

Novák, J., Hilscherová, K., Landlová, L., Čupr, P., Kohút, L., Giesy, J.P., Klánová, J., 2014. Composition and effects of inhalable size fractions of atmospheric aerosols in the polluted atmosphere. Part II. In vitro biological potencies. Environ. Int. 63, 64–70. doi:10.1016/j.envint.2013.10.013

Novák, J., Jálová, V., Giesy, J.P., Hilscherová, K., 2009. Pollutants in particulate and gaseous fractions of ambient air interfere with multiple signaling pathways in vitro. Environ. Int. 35, 43–9. doi:10.1016/j.envint.2008.06.006

OECD, 2011. OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. Test No. 456: H295R Steroidogenesis Assay [online] [cit. 10. 2. 2016]. Dostupné z: http://www.oecd-ilibrary.org/docserver/download/9745601e.pdf?expires=1465564808&id=id&accname=guest&checksum=7C9DF95D345FDF3FEA299660F6B486C3

OECD, 2012. Series on Testing and Assessment: No 150: Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption. ENV/JM/MONO(2012)22. [online] [cit. 10. 2. 2016]. Dostupné z: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO%282012%2922&doclanguage=en

OECD, 2015. OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals [online] [cit. 10. 2. 2016]. Dostupné z: http://www.oecd.org/env/ehs/testing/oecdconceptualframeworkforthetestingandassessmentofendocrinedisruptingchemicals.htm.

Oehlmann, J., Di Benedetto, P., Tillmann, M., Duft, M., Oetken, M., Schulte-Oehlmann, U., 2007. Endocrine disruption in prosobranch molluscs: evidence and ecological relevance. Ecotoxicology 16, 29–43. doi:10.1007/s10646-006-0109-x

Ohtake, F., Fujii-Kuriyama, Y., Kawajiri, K., Kato, S., 2011. Cross-talk of dioxin and estrogen receptor signals through the ubiquitin system. J. Steroid Biochem. Mol. Biol. 127, 102–7. doi:10.1016/j.jsbmb.2011.03.007

Orton, F., Ermler, S., Kugathas, S., Rosivatz, E., Scholze, M., Kortenkamp, A., 2014. Mixture effects at very low doses with combinations of anti-androgenic pesticides , antioxidants , industrial pollutant and chemicals used in personal care products. Toxicol. Appl. Pharmacol. 278, 201–208. doi:10.1016/j.taap.2013.09.008

Pacherník, J., Bryja, V., Esner, M., Kubala, L., Dvorák, P., Hampl, A, 2005. Neural differentiation of pluripotent mouse embryonal carcinoma cells by retinoic acid: inhibitory effect of serum. Physiol. Res. 54, 115–22.

Peck, M., Gibson, R.W., Kortenkamp, A., Hill, E.M., 2004. Sediments are major sinks of steroidal estrogens in two United Kingdom rivers. Environ. Toxicol. Chem. 23, 945–52.

Peňáz, M., Svobodová, Z., Baruš, V., Prokeš, M., Drastichová, J., 2005. Endocrine disruption in a barbel, Barbus barbus population from the River Jihlava, Czech Republic. J. Appl. Ichthyol. 21, 420–428. doi:10.1111/j.1439-0426.2005.00663.x

Pieterse, B., Felzel, E., Winter, R., Van Der Burg, B., Brouwer, A., 2013. PAH-CALUX, an optimized bioassay for AhR-mediated hazard identification of polycyclic aromatic hydrocarbons (PAHs) as individual compounds and in complex mixtures. Environ. Sci. Technol. 47, 11651–11659. doi:10.1021/es403810w

Poulsen, A., Chapman, H., Leusch, F., Escher, B., 2011. Application of Bioanalytical Tools for Water Quality Assessment- Urban Water Security Research Alliance Technical Report No. 41 76.

Randak, T., Zlabek, V., Pulkrabova, J., Kolarova, J., Kroupova, H., Siroka, Z., Velisek, J., Svobodova, Z., Hajslova, J., 2009. Effects of pollution on chub in the River Elbe, Czech Republic. Ecotoxicol. Environ. Saf. 72, 737–746. doi:10.1016/j.ecoenv.2008.09.020

Ricking, M., Schwarzbauer, J., Franke, S., 2003. Molecular markers of anthropogenic activity in sediments of the Havel and Spree Rivers (Germany). Water Res. 37, 2607–17. doi:10.1016/S0043-1354(03)00078-2

Roberts, J., Bain, P.A., Kumar, A., Hepplewhite, C., Ellis, D.J., Christy, A.G., Beavis, S.G., 2015. Tracking multiple modes of endocrine activity in Australia's largest inland sewage treatment plant and effluent-receiving environment using a panel of in vitro bioassays. Environ. Toxicol. Chem. 34, 2271–2281. doi:10.1002/etc.3051

Russell, W.M.S., Burch, R.L., 1959. The Principles of Humane Experimental Technique. Methuen, London.

Rutishauser, B. V, Pesonen, M., Escher, B.I., Ackermann, G.E., Aerni, H.-R., Suter, M.J.F., Eggen, R.I.L., 2004. Comparative analysis of estrogenic activity in sewage treatment plant effluents involving three in vitro assays and chemical analysis of steroids. Environ. Toxicol. Chem. 23, 857–64.

Sawada, K., Inoue, D., Wada, Y., Sei, K., Nakanishi, T., Ike, M., 2012. Detection of retinoic acid receptor agonistic activity and identification of causative compounds in municipal wastewater treatment plants in Japan. Environ. Toxicol. Chem. 31, 307–315. doi:10.1002/etc.741

Scott, P.D., Bartkow, M., Blockwell, S.J., Coleman, H.M., Khan, S.J., Lim, R., McDonald, J.A., Nice, H., Nugegoda, D., Pettigrove, V., Tremblay, L.A., Warne, M.S.J., Leusch, F.D.L., 2014. An assessment of endocrine activity in Australian rivers using chemical and in vitro analyses. Environ. Sci. Pollut. Res. Int. 21, 12951–67. doi:10.1007/s11356-014-3235-7

Shanle, E.K., Xu, W., 2011. Endocrine Disrupting Chemicals Targeting Estrogen Receptor Signaling: Identification and Mechanisms of Action. Chem. Res. Toxicol. 24, 6–19.

Schirling, M., Jungmann, D., Ladewig, V., Ludwichowski, K.U., Nagel, R., Köhler, H.R., Triebskorn, R., 2006. Bisphenol A in artificial indoor streams: II. Stress response and gonad histology in Gammarus fossarum (Amphipoda). Ecotoxicology 15, 143–56. doi:10.1007/s10646-005-0044-2

Schirling, M., Jungmann, D., Ladewig, V., Nagel, R., Triebskorn, R., Köhler, H.R., 2005. Endocrine effects in Gammarus fossarum (Amphipoda): Influence of wastewater effluents, temporal variability, and spatial aspects on natural populations. Arch. Environ. Contam. Toxicol. 49, 53–61. doi:10.1007/s00244-004-0153-6

Schmitt, C., Vogt, C., Machala, M., de Deckere, E., 2011. Sediment contact test with Potamopyrgus antipodarum in effect-directed analyses-challenges and opportunities. Environ. Sci. Pollut. Res. Int. 18, 1398–404. doi:10.1007/s11356-011-0497-1

Sonneveld, E., Riteco, J.A.C., Jansen, H.J., Pieterse, B., Brouwer, A., Schoonen, W.G., van der Burg, B., 2006. Comparison of in vitro and in vivo screening models for androgenic and estrogenic activities. Toxicol. Sci. 89, 173–87. doi:10.1093/toxsci/kfj009

Sovadinová, I., Bláha, L., Janošek, J., Hilscherová, K., Giesy, J., Jones, P.D., Holoubek, I., 2006. Cytotoxicity and Aryl Hydrocarbon Receptor-Mediated Activity of N-heterocyclic Polycyclic Aromatic Hydrocarbons - Structure-Activity Relationships. Environ. Toxicol. Chem. 25, 1291–1297.

Stachel, B., Ehrhorn, U., Heemken, O.P., Lepom, P., Reincke, H., Sawal, G., Theobald, N., 2003. Xenoestrogens in the River Elbe and its tributaries. Environ. Pollut. 124, 497–507. doi:10.1016/S0269-7491(02)00483-9

Stuer-Lauridsen, F., 2005. Review of passive accumulation devices for monitoring organic micropollutants in the aquatic environment. Environ. Pollut. 136, 503–524. doi:10.1016/j.envpol.2004.12.004

Sumpter, J.P., Johnson, A.C., 2005. Lessons from endocrine disruption and their application to other issues concerning trace organics in the aquatic environment. Environ. Sci. Technol. 39, 4321–4332.

Sumpter, J.P., Johnson, A.C., 2008. 10th Anniversary Perspective: Reflections on endocrine disruption in the aquatic environment: from known knowns to unknown unknowns (and many things in between). J. Environ. Monit. 10, 1476–85. doi:10.1039/b815741n

Svenson, A., Allard, A.S., 2004. Occurrence and Some Properties of the Androgenic Activity in Municipal Sewage Effluents. J. Environ. Sci. Heal. Part A 39, 693–701. doi:10.1081/ESE-120027735

Swedenborg, E., Ruegg, J., Makela, S., Pongratz, I., 2009. Endocrine disruptive chemicals: mechanisms of action and involvement in metabolic disorders. J. Mol. Endocrinol. 43, 1–10.

Šídlová, T., Novák, J., Janošek, J., Anděl, P., Giesy, J.P., Hilscherová, K., 2009. Dioxin-like and endocrine disruptive activity of traffic-contaminated soil samples. Arch. Environ. Contam. Toxicol. 57, 639–50. doi:10.1007/s00244-009-9345-4

Teneva, I., Asparuhova, D., Dzhambazov, B., Mladenov, R., Schirmer, K., 2003. The freshwater cyanobacterium Lyngbya aerugineo-coerulea produces compounds toxic to mice and to mammalian and fish cells. Environ. Toxicol. 18, 9–20.

Thibeault, A.A.H., Deroy, K., Vaillancourt, C., Sanderson, J.T., 2014. A unique co-culture model for fundamental and applied studies of human fetoplacental steroidogenesis and interference by environmental chemicals. Environ. Health Perspect. 122, 371–7. doi:10.1289/ehp.1307518

Thorpe, K.L., Gross-Sorokin, M., Johnson, I., Brighty, G., Tyler, C.R., 2006. An assessment of the model of concentration addition for predicting the estrogenic activity of chemical mixtures in wastewater treatment works effluents. Environ. Health Perspect. 114, 90–97. doi:10.1289/ehp.8059

Tonoli, D., Fürstenberger, C., Boccard, J., Hochstrasser, D., Jeanneret, F., Odermatt, A., Rudaz, S., 2015. Steroidomic Footprinting Based on Ultra-High Performance Liquid Chromatography Coupled with Qualitative and Quantitative High-Resolution Mass Spectrometry for the Evaluation of Endocrine Disrupting Chemicals in H295R Cells. Chem. Res. Toxicol. 28, 955–966. doi:10.1021/tx5005369

Tyler, C.R., Jobling, S., 2008. Roach, Sex, and Gender-Bending Chemicals: The Feminization of Wild Fish in English Rivers. Bioscience 58, 1051. doi:10.1641/B581108

US EPA, 2015. Endocrine Disruptor Screening Program - Tier 1 Battery of Assays. [online] [cit. 10. 2. 2016]. Dostupné z: https://www.epa.gov/endocrine-disruption/endocrine-disruptor-screening-program-tier-1-battery-assays#assays-included

Vallejo, A., Prieto, A., Moeder, M., Usobiaga, A., Zuloaga, O., Etxebarria, N., Paschke, A., 2013. Calibration and field test of the Polar Organic Chemical Integrative Samplers for the determination of 15 endocrine disrupting compounds in wastewater and river water with special focus on performance reference compounds (PRC). Water Res. 47, 2851–2862. doi:10.1016/j.watres.2013.02.049

Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs, D.R., Lee, D.H., Shioda, T., Soto, A.M., vom Saal, F.S., Welshons, W. V., Zoeller, R.T., Myers, J.P., 2012. Hormones and Endocrine-Disrupting Chemicals: Low-Dose Effects and Nonmonotonic Dose Responses. Endocr. Rev. 33, 378–455. doi:10.1210/er.2011-1050

Vermeirssen, E.L.M., Körner, O., Schönenberger, R., Suter, M.J.F., Burkhardt-Holm, P., 2005. Characterization of environmental estrogens in river water using a three pronged approach: Active and passive water sampling and the analysis of accumulated estrogens in the bile of caged fish. Environ. Sci. Technol. 39, 8191–8198. doi:10.1021/es050818q

Vethaak, A.D., Lahr, J., Schrap, S.M., Belfroid, A.C., Rijs, G.B.J., Gerritsen, A., de Boer, J., Bulder, A.S., Grinwis, G.C.M., Kuiper, R. V, Legler, J., Murk, T.A.J., Peijnenburg, W., Verhaar, H.J.M., de Voogt, P., 2005. An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands. Chemosphere 59, 511–524.

Vrana, B., Klučárová, V., Benická, E., Abou-Mrad, N., Amdany, R., Horáková, S., Draxler, A., Humer, F., Gans, O., 2014. Passive sampling: An effective method for monitoring seasonal and spatial variability of dissolved hydrophobic organic contaminants and metals in the Danube river. Environ. Pollut. 184, 101–112. doi:10.1016/j.envpol.2013.08.018

Vystavna, Y., Huneau, F., Grynenko, V., Vergeles, Y., Celle-Jeanton, H., Tapie, N., Budzinski, H., Le Coustumer, P., 2012. Pharmaceuticals in rivers of two regions with contrasted socio-economic conditions: Occurrence, accumulation, and comparison for Ukraine and France. Water. Air. Soil Pollut. 223, 2111–2124. doi:10.1007/s11270-011-1008-1

Wang, R., Liu, J., Yang, X., Lin, C., Huang, B., Jin, W., Pan, X., 2013. Biological response of high-back crucian carp (Carassius auratus) during different life stages to wastewater treatment plant effluent. Environ. Sci. Pollut. Res. 20, 8612–8620. doi:10.1007/s11356-013-1817-4

Wang, X., Zhao, Y., Xiao, Z., Chen, B., Wei, Z., Wang, B., Zhang, J., Han, J., Gao, Y., Li, L., Zhao, H., Zhao, W., Lin, H., Dai, J., 2009. Alternative translation of OCT4 by an internal ribosome entry site and its novel function in stress response. Stem Cells 27, 1265–75. doi:10.1002/stem.58

Watts, M.M., Pascoe, D., Carroll, K., 2002. Population responses of the freshwater amphipod Gammarus pulex (L.)to an environmental estrogen, 17α-ethinylestradiol. Environ. Toxicol. Chem. 21, 445–450.

Wei, C., Bandowe, B.M., Han, Y., Cao, J., Zhan, C., Wilcke, W., 2014. Polycyclic aromatic hydrocarbons (PAHs) and their derivatives (alkyl-PAHs, oxygenated-PAHs, nitrated-PAHs and azaarenes) in urban road dusts from Xi'an, Central China. Chemosphere 134, 512–520. doi:10.1016/j.chemosphere.2014.11.052

WHO, UNEP, 2013. State of the Science of Endocrine Disrupting Chemicals - 2012. p. 1–296. [online] [cit. 15. 2. 2016]. Dostupné z: http://www.who.int/ceh/publications/endocrine/en/

WHO/IPCS, 2002. Global Assessment of the State-of-the-science of Endocrine Disruptors. [online] [cit. 15. 2. 2016]. Dostupné z: http://www.who.int/ipcs/publications/en/toc.pdf.

Zhang, X., Chang, H., Wiseman, S., He, Y., Higley, E., Jones, P., Wong, C.K.C., Al-Khedhairy, A., Giesy, J.P., Hecker, M., 2011. Bisphenol a disrupts steroidogenesis in human H295R cells. Toxicol. Sci. 121, 320–327. doi:10.1093/toxsci/kfr061

Zounkova, R., Jalova, V., Janisova, M., Ocelka, T., Jurcikova, J., Halirova, J., Giesy, J.P., Hilscherova, K., 2014. In situ effects of urban river pollution on the mudsnail Potamopyrgus antipodarum as part of an integrated assessment. Aquat. Toxicol. 150, 83–92. doi:10.1016/j.aquatox.2014.02.021

6 Přílohy

6.1 Seznam plných textů přiložených k habilitační práci

Článek I:

Janošek, J., Hilscherová, K., Bláha, L., Holoubek, I., 2006. Environmental xenobiotics and nuclear receptors - Interactions, effects and in vitro assessment. Toxicology In Vitro 20 (1), 18-37.

KH se podílela na designu článku, na sběru, zpracování a kompletaci informací i na finalizaci článku a přípravě k odeslání (20%).

Článek II:

Novák, J., Beníšek, M., Hilscherová, K., 2008. Disruption of retinoid transport, metabolism and signaling by environmental pollutants. Environment International 34 (6), 898-913.

KH byla korespondenční autor, připravila design článku, vedla postup jeho zpracování a sběru informací, prováděla finalizaci článku a přípravu k odeslání (40%).

Článek III:

Bittner, M., Jarque, S., Hilscherová, K., 2015. Polymer-immobilized ready-to-use recombinant yeast assays for the detection of endocrine disruptive compounds. Chemosphere 132, 56–62.

KH byla korespondenční autor, konzultovala design a postup řešení se spoluautory, podílela se na vyhodnocení dat, sepsání a finalizaci článku (20%).

Článek IV:

Jarque, S., Bittner, M., Hilscherová, K., 2016. Freeze-drying as suitable method to achieve ready-to-use yeast biosensors for androgenic and estrogenic compounds. Chemosphere 148, 204–210.

KH byla korespondenční autor, konzultovala design a postup řešení se spoluautory, podílela se na vyhodnocení dat, sepsání a finalizaci článku (30%).

Článek V:

Sovadinová, I., Bláha, L., Janošek, J., Hilscherová, K., Giesy, J.P., Jones, P.D., Holoubek, I., 2006. Cytotoxicity and aryl hydrocarbon receptor-mediated activity of N-heterocyclic polycyclic aromatic hydrocarbons - Structure-activity relationships. Environmental Toxicology and Chemistry 25 (5), 1291-1297.

KH konzultovala design a postup řešení se spoluautory, podílela se na vyhodnocení a interpretaci dat, finalizaci článku (15%).

Článek VI:

Beníšek, M., Bláha, L., Hilscherová, K., 2008. Interference of PAHs and their N-heterocyclic analogs with signaling of retinoids *in vitro*. Toxicology in Vitro 22 (8), 1909-1917.

KH byla korespondenční autor, pracovala na designu studie, konzultovala realizaci laboratorních experimentů, podílela se na zpracování, analýze a interpretaci dat, prováděla finalizaci článku a přípravu k odeslání (40%).

Článek VII:

Beníšek, M., Kubincová, P., Bláha, L., Hilscherová K., 2011. The effects of PAHs and N-PAHs on retinoid signaling and Oct-4 expression *in vitro*. Toxicology Letters 200 (3), 169-175.

KH byla korespondenční autor, podílela se na designu studie, konzultovala realizaci laboratorních experimentů, prováděla finalizaci článku a přípravu k odeslání (30%).

Článek VIII:

Hilscherova, K., Jones, P.D., Gracia, T., Newsted, J.L., Zhang, X., Sanderson, J.T., Yu, R., Wu, R., Giesy, J.P., 2004. Assessment of the effects of chemicals on the expression of ten steroidogenic genes in the H295R cell line using real-time PCR. Toxicological Sciences 81 (1), 78-89.

KH vyvinula metody a realizovala experimenty uvedené v publikaci, podílela se na designu studie, zpracovala a interpretovala získaná data, sepsala publikaci (70%).

Článek IX:

Gracia, T., Hilscherova, K., Jones, P.D., Newsted, J.L., Zhang, X., Hecker, M., Higley, E.B., Sanderson, T., Yu, R.M.K., Wu, R.S.S., Giesy J. P., 2006. The H295R system for evaluation of endocrine-disrupting effects. Ecotoxicology and Environmental Safety 65, 293-305.

KH se podílela na designu studie, vývoji metodik, interpretaci dat a sepsání publikace (20%)

Článek X:

Gracia, T., Hilscherova, K., Jones, P.D., Newsted, J.L., Higley, E.B., Zhang, X., Hecker, M., Murphy, M., Yu, R.M.K., Lam, P.K.S., Wu, R.S.S., Giesy, J.P., 2007. Modulation of steroidogenic gene expression and hormone production of H295R cells by pharmaceuticals and other environmentally active compounds. Toxicology and Applied Pharmacology 225, 142-153.

KH se podílela na designu studie, vývoji metodik, na finalizaci textu článku (10%)

Článek XI:

Haeba, M.H., Hilscherová, K., Mazurová, E., Bláha, L., 2008. Selected endocrine disrupting compounds (vinclozolin, flutamide, ketoconazole and dicofol): Effects on survival, occurrence of males, growth, molting and reproduction of *Daphnia magna*. Environmental Science and Pollution Research 15, 222–227.

KH se podílela na designu studie, vývoji metodik, interpretaci dat a finalizaci publikace (20%)

Článek XII:

Feldmannová, M., Hilscherová, K., Maršálek, B., Bláha, L., 2006. Effects of N-heterocyclic polyaromatic hydrocarbons on survival, reproduction, and biochemical parameters in *Daphnia magna*. Environmental Toxicology 21, 425–431.

KH se podílela na designu studie, vývoji metodik, analýze a interpretaci dat a finalizaci publikace (20%)

Článek XIII:

Jarosova, B., Blaha, L., Vrana, B., Randak, T., Grabic, R., Giesy, J.P., Hilscherova, K., 2012. Changes in concentrations of hydrophilic organic contaminants and of endocrine-disrupting potential downstream of small communities located adjacent to headwaters. Environment International 45, 22-31.

KH byla korespondenční autor, vedla realizaci laboratorních experimentů zaměřených na studium endokrinně-disruptivního potenciálu, vedla zpracování publikace, provedla její finalizaci a přípravu k odeslání (30%)

Článek XIV:

Jálová, V., Jarošová, B., Bláha, L., Giesy, J.P., Ocelka, T., Grabic, R., Jurčíková, J., Vrana, B., Hilscherová, K., 2013. Estrogen-, androgen- and aryl hydrocarbon receptor mediated activities in passive and composite samples from municipal waste and surface waters. Environment International 59, 372–383.

KH byla korespondenční autor, vedla realizaci laboratorních experimentů zaměřených na studium endokrinně-disruptivního potenciálu, vedla zpracování publikace, provedla její finalizaci a přípravu k odeslání (30%)

Článek XV:

Jarošová, B., Erseková, A., Hilscherová, K., Loos, R., Gawlik, B. M., Giesy, J. P., Bláha, L., 2014. Europe-wide survey of estrogenicity in wastewater treatment plant effluents: the need for the effect-based monitoring. Environmental Science and Pollution Research 21(18), 10970–82.

KH se podílela zejména na interpretaci dat a zpracování a finalizaci publikace (10%)

Článek XVI:

Jarošová, B., Bláha, L., Giesy, J.P., Hilscherová, K., 2014. What level of estrogenic activity determined by *in vitro* assays in municipal waste waters can be considered as safe? Environment International 64, 98–109.

KH byla korespondenční autor, vedla zpracování publikace, provedla její finalizaci a přípravu k odeslání (40 %)

Článek XVII:

Hilscherova, K., Kannan, K., Kang, Y.S., Holoubek, I., Machala, M., Masunaga, S., Nakanishi, J., Giesy, J.P., 2001. Characterization of dioxin-like activity of sediments from a Czech river basin. Environmental Toxicology and Chemistry 20 (12), 2768-2777.

KH byla korespondenční autor, podílela se na designu studie, realizovala odběry a zpracování vzorků, experimenty uvedené v publikaci, zpracovala a interpretovala získaná data, sepsala publikaci (80%)

Článek XVIII:

Hilscherova, K., Kannan, K., Holoubek, I., Giesy, J.P., 2002. Characterization of estrogenic activity of riverine sediments from the Czech Republic. Archives of Environmental Contamination and Toxicology 43 (2),175-185.

KH byla korespondenční autor, realizovala experimenty uvedené v publikaci, podílela se na designu studie, zpracovala a interpretovala získaná data, sepsala publikaci (80%)

Článek XIX:

Hilscherová, K., Dušek, L., Šídlová T., Jálová V., Čupr P., Giesy J.P., Nehyba S., Jarkovský J., Klánová J., Holoubek I., 2010. Seasonally and regionally determined indication potential of bioassays in contaminated river sediments. Environmental Toxicology and Chemistry 29 (3), 522-534.

KH byla korespondenční autor, podílela se na designu studie, koordinovala realizaci části laboratorních experimentů, sběr rozsáhlého datového souboru z biotestů a chemických analýz, podílela se na interpretaci, sepsala publikaci (60%)

Článek XX:

Macikova, P., Kalabova, T., Klanova, J., Kukucka, P., Giesy, J. P., Hilscherova K., 2014. Longer-term and short-term variability in pollution of fluvial sediments by dioxin-like and endocrine disruptive compounds. Environmental Science and Pollution Research 21 (7), 5007-5022.

KH byla korespondenční autor, podílela se na designu studie, koordinovala realizaci laboratorních experimentů, podílela se na analýze dat a interpretaci rozsáhlého datového souboru, podílela se na psaní publikace (40%)

Článek XXI:

Novák, J., Beníšek, M., Pacherník, J., Janošek, J., Šídlová, T., Kiviranta, H., Verta, M., Giesy, J.P., Bláha L., Hilscherová K., 2007. Interference of contaminated sediment extracts and environmental pollutants with retinoid signaling. Environmental Toxicology and Chemistry 26(8), 1591-1599.

KH byla korespondenční autor, zpracovala design studie, vedla realizaci laboratorních experimentů, koordinaci se zahraničními partnery, prováděla finalizaci článku a přípravu k odeslání (30%)

Článek XXII:

Mazurová, E., Hilscherová, K., Jálová, V., Kohler, H.R., Triebskorn, R., Giesy, J.P., Bláha, L., 2008. Endocrine effects of contaminated sediments on the freshwater snail *Potamopyrgus antipodarum in vivo* and in the cell bioassays *in vitro*. Aquatic Toxicology 89, 172-179.

KH koordinovala část laboratorních analýz, podílela se na vyhodnocení a intepretaci dat i na psaní publikace (20%)

Článek XXIII:

Mazurová, E., Hilscherová, K., Šídlová-Štěpánková, T., Kohler, H.R., Triebskorn, R., Jungmann, D., Giesy, J.P., Bláha, L., 2010. Chronic toxicity of contaminated sediments on reproduction and histopathology of the crustacean *Gammarus fossarum* and relationship with the chemical contamination and in vitro effects. Journal of Soils and Sediments 10, 423-433.

KH koordinovala všechny in vitro analýzy, konzultovala průběh in vivo testů, provedla analýzu dat a intepretaci dat z biotestů, podílela se na psaní publikace (20%)

Článek XXIV:

Jonas, A., Buranova, V., Scholz, S., Fetter, E., Novakova, K., Kohoutek, J., Hilscherova, K., 2014. Retinoid-like activity and teratogenic effects of cyanobacterial exudates. Aquatic Toxicology 155, 283–290.

KH byla korespondenční autor, připravila design studie, konzultovala realizaci, podílela se na zpracování a interpretaci výsledků, sepsání a finalizaci manuscriptu (25%)

Článek XXV:

Jonas, A., Scholz, S., Fetter, E., Sychrova, E., Novakova, K., Ortmann, J., Benisek, M., Adamovsky, O., Giesy, J., Hilscherova, K., 2015. Endocrine, teratogenic and neurotoxic effects of cyanobacteria detected by cellular *in vitro* and zebrafish embryos assays. Chemosphere 120, 321–327.

KH byla korespondenční autor, připravila design studie, konzultovala realizaci s prvním autorem a se zahraničními partnery, podílela se na zpracování a interpretaci výsledků, sepsání a finalizaci manuscriptu (25%)

Článek XXVI:

Javůrek, J., Sychrová, E., Smutná, M., Bittner, M., Kohoutek, J., Adamovský, O., Nováková, K., Smetanová, S., Hilscherová, K., 2015. Retinoid compounds associated with water blooms dominated by *Microcystis* species. Harmful Algae 47: 116–125.

KH byla korespondenční autor, vymyslela design, byla zapojena v odběrech vzorků, koordinovala zpracování a analýzu vzorků včetně biotestů, podílela se na interpretaci dat a sepsání publikace (40%)

6.2 Další publikace autorky relevantní k tématu habilitační práce

V níže uvedeném seznamu jsou uvedeny další publikace, na kterých se předkladatelka podílela, a které jsou relevantní k tématu habilitační práce. Tyto publikace z důvodu rozsahu habilitační práce nejsou zařazeny v plných přílohách, ale je na ně odkazováno v textu.

Altenburger, R., Ait-Aissa, S., Antczak, P., Backhaus, T., Barceló, D., Seiler, T.-B., et al. 2015. Future water quality monitoring — Adapting tools to deal with mixtures of pollutants in water resource management. Science of the Total Environment 512-513, 540–551. doi:10.1016/j.scitotenv.2014.12.057

Bittner, M., Hilscherova, K., Giesy, J.P., 2009. In vitro assessment of AhR-mediated activities of TCDD in mixture with humic substances. Chemosphere 76, 1505–8. doi:10.1016/j.chemosphere.2009.06.042

Bittner, M., Janosek, J., Hilscherova, K., Giesy, J., Holoubek, I., Blaha, L., 2006. Activation of Ah receptor by pure humic acids. Environmental Toxicology 21, 338–342.

Bittner, M., Macikova, P., Giesy, J.P., Hilscherova, K., 2011. Enhancement of AhR-mediated activity of selected pollutants and their mixtures after interaction with dissolved organic matter. Environment International 37, 960–4. doi:10.1016/j.envint.2011.03.016

Brack, W., Altenburger, R., Schüürmann, G., Krauss, M., López Herráez, D., van Gils, J., et al., 2015. The SOLUTIONS project: Challenges and responses for present and future emerging pollutants in land and water resources management. Science of the Total Environment 503-504, 22–31. doi:10.1016/j.scitotenv.2014.05.143

Érseková, A., Hilscherová, K., Klánová, J., Giesy, J.P., Novák, J., 2014. Effect-based assessment of passive air samples from four countries in Eastern Europe. Environmental Monitoring and Assessment 186, 3905–16. doi:10.1007/s10661-014-3667-z

Escher, B.I., Allinson, M., Altenburger, R., Bain, P.A., Balaguer, P., Busch, W., et al. 2014. Benchmarking organic micropollutants in wastewater, recycled water and drinking water with in vitro bioassays. Environmental Science & Technology 48, 1940–56. doi:10.1021/es403899t

Hilscherova, K., Machala, M., Kannan, K., Blankenship, A.L., Giesy, J.P., 2000. Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. Environmental Science and Pollution Research 7, 159–171.

Hilscherova, K., Dusek, L., Kubik, V., Cupr, P., Hofman, J., Klanova, J., Holoubek, I., 2007. Redistribution of organic pollutants in river sediments and alluvial soils related to major floods. Journal of Soils and Sediments 7, 167–177.

Hilscherova, K., Kannan, K., Nakata, H., Hanari, N., Yamashita, N., Bradley, P.W., McCabe, J.M., Taylor, A.B., Giesy, J.P., 2003. Polychlorinated dibenzo-p-dioxin and dibenzofuran concentration profiles in sediments and flood-plain soils of the Tittabawassee River, Michigan. Environmental Science & Technology 37, 468–74. doi: 10.1021/es020920c

Janosek, J., Bittner, M., Hilscherová, K., Bláha, L., Giesy, J.P., Holoubek, I., 2007. AhR-mediated and antiestrogenic activity of humic substances. Chemosphere 67, 1096–101. doi:10.1016/j.chemosphere.2006.11.045

Jarošová, B., Javůrek, J., Adamovský, O., Hilscherová, K., 2015. Phytoestrogens and mycoestrogens in surface waters — Their sources, occurrence, and potential contribution to estrogenic activity. Environment International 81, 26–44. doi:10.1016/j.envint.2015.03.019

Kaisarevic, S., Hilscherova, K., Weber, R., Sundqvist, K.L., Tysklind, M., Voncina, E. et al., 2011. Characterization of dioxin-like contamination in soil and sediments from the “hot spot” area of petrochemical plant in Pancevo (Serbia). Environmental Science and Pollution Research 18, 677–86. doi:10.1007/s11356-010-0418-8

Leskinen, P., Hilscherova, K., Sidlova, T., Kiviranta, H., Pessala, P., Salo, S., Verta, M., Virta, M., 2008. Detecting AhR ligands in sediments using bioluminescent reporter yeast. Biosensors and Bioelectronics 23, 1850–1855. doi:10.1016/j.bios.2008.02.026

Mazurova, E., Hilscherova, K., Triebskorn, R., Kohler, H.R., Marsalek, B., Blaha, L., 2008b. Endocrine regulation of the reproduction in crustaceans: Identification of potential targets for toxicants and environmental contaminants. Biologia 63, 139–150. doi:10.2478/s11756-008-0027-x

Neale, P.A., Ait-Aissa, S., Brack, W., Creusot, N., Denison, M.S., Deutschmann, B., Hilscherova, K., Hollert, H., Krauss, M., Novák, J., Schulze, T., Seiler, T.B., Serra, H., Shao, Y., Escher, B.I., 2015. Linking in vitro effects and detected organic micropollutants in surface water using mixture toxicity modeling. Environmental Science & Technology 49, 14614-14624. doi:10.1021/acs.est.5b04083

Novák, J., Giesy, J.P., Klánová, J., Hilscherová, K., 2013. In vitro effects of pollutants from particulate and volatile fractions of air samples-day and night variability. Environmental Science and Pollution Research 20, 6620–7. doi:10.1007/s11356-013-1726-6

Novák, J., Hilscherová, K., Landlová, L., Čupr, P., Kohút, L., Giesy, J.P., Klánová, J., 2014. Composition and effects of inhalable size fractions of atmospheric aerosols in the polluted atmosphere. Part II. In vitro biological potencies. Environment International 63, 64–70. doi:10.1016/j.envint.2013.10.013

Novák, J., Jálová, V., Giesy, J.P., Hilscherová, K., 2009. Pollutants in particulate and gaseous fractions of ambient air interfere with multiple signaling pathways in vitro. Environment International 35, 43–9. doi:10.1016/j.envint.2008.06.006

Šídlová, T., Novák, J., Janošek, J., Anděl, P., Giesy, J.P., Hilscherová, K., 2009. Dioxin-like and endocrine disruptive activity of traffic-contaminated soil samples. Archives of Environmental Contamination and Toxicology 57, 639–50. doi:10.1007/s00244-009-9345-4

Zounkova, R., Jalova, V., Janisova, M., Ocelka, T., Jurcikova, J., Halirova, J., Giesy, J.P., Hilscherova, K., 2014. In situ effects of urban river pollution on the mudsnail Potamopyrgus antipodarum as part of an integrated assessment. Aquatic Toxicology 150, 83–92. doi:10.1016/j.aquatox.2014.02.021

Přílohy (Článek I – Článek XXVI):

Článek I:

Janošek, J., Hilscherová, K., Bláha, L., Holoubek, I., 2006. Environmental xenobiotics and nuclear receptors - Interactions, effects and in vitro assessment. Toxicology In Vitro 20 (1), 18-37.

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Review

Environmental xenobiotics and nuclear receptors—Interactions, effects and in vitro assessment

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Abstract

A group of intracellular nuclear receptors is a protein superfamily including arylhydrocarbon AhR, estrogen ER, androgen AR, thyroid TR and retinoid receptors RAR/RXR as well as molecules with unknown function known as orphan receptors. These proteins play an important role in a wide range of physiological as well as toxicological processes acting as transcription factors (ligand-dependent signalling macromolecules modulating expression of various genes in a positive or negative manner). A large number of environmental pollutants and other xenobiotics negatively affect signaling pathways, in which nuclear receptors are involved, and these modulations were related to important in vivo toxic effects such as immunosuppression, carcinogenesis, reproduction or developmental toxicity, and embryotoxicity. Presented review summarizes current knowledge on major nuclear receptors (AhR, ER, AR, RAR/RXR, TR) and their relationship to known in vivo toxic effects. Special attention is focused on priority organic environmental contaminants and experimental approaches for determination and studies of specific toxicity mechanisms.

Keywords: Nuclear receptors; Environmental xenobiotics; Toxicity; Mechanisms; Effects; In vitro assessment

Abbreviations: AHH, aryl hydrocarbon hydroxylase; AhR, aryl hydrocarbon receptor; AR, androgen receptor; ARNT, AhR-nuclear translocator; ATRA, *all-trans*-retinoic acid; BROD, benzyloxyresorufin-*O*-deethylase; CYP, cytochrome P450; CRBP, cellular retinol binding protein; CRABP, cellular retinoic acid binding protein; D-MEM, Dulbecco's modified minimum essential medium; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; DHT, dihydrotestosterone; DNA, deoxyribonucleic acid; DRE, dioxin responsive element; EC_{50}, compound concentration causing 50% of the maximum effect; ER, estrogen receptor; ERE, estrogen responsive element; EROD, ethoxyresorufin-*O*-deethylase; GFP, green fluorescent protein; GR, glucocorticoid receptor; GST, glutathion-*S*-transferase; HSP 70, heat shock protein, molecular weight 70 kDa; HSP 90, heat shock protein, molecular weight 90 kDa; ILP, immunophilin-like protein; LD_{50}, dose causing lethal effect in 50% of experimental animals; MR, mineralglucocorticoid receptor; NADPH, nicotinadenindinucleotid phosphate-reduced form; PAS, Per-ARNT-Sim; PCBs, polychlorinated biphenyls; PCNs, polychlorinated naphthalenes; PCR, polymerase chain-reaction; POPs, persistent organic pollutants; PPAR, peroxisome prolifelator activated receptor; PR, progestin receptor; PROD, 7-pentoxyresorufin-*O*-deeethylase; RAR, retinoic acid receptor; RARE, retinoic acid responsive element; REP, relative potencies; RXR, retinoid X receptor; SPMDs, semipermeable membrane devices; TBP, thyroid-binding proteins; TEF, toxic equivalency factor; TEQ, toxic equivalents; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TH, thyroid hormones; TR, thyroid receptor; TRE, thyroid hormone response element; TSH, thyroid-stimulating hormone; VDR, vitamin D receptor; Vtg, vitellogenin; YES, yeast estrogen screen; Zrp, zona radiata protein.

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1. Nuclear receptor superfamily

Nuclear receptor superfamily is a common name for a large group of receptors that are involved in regulation of a wide range of physiological functions in eukaryotic organisms including cell growth and proliferation, differentiation or maintaining of homeostasis.

They are called "nuclear receptors" due to their common mode of action. After binding of a specific ligand their structural conformation is changed and the receptor (often after dimerization with a modulator) is transferred into the nucleus, binds to corresponding responsive element on DNA and triggers gene expression (Fig. 1).

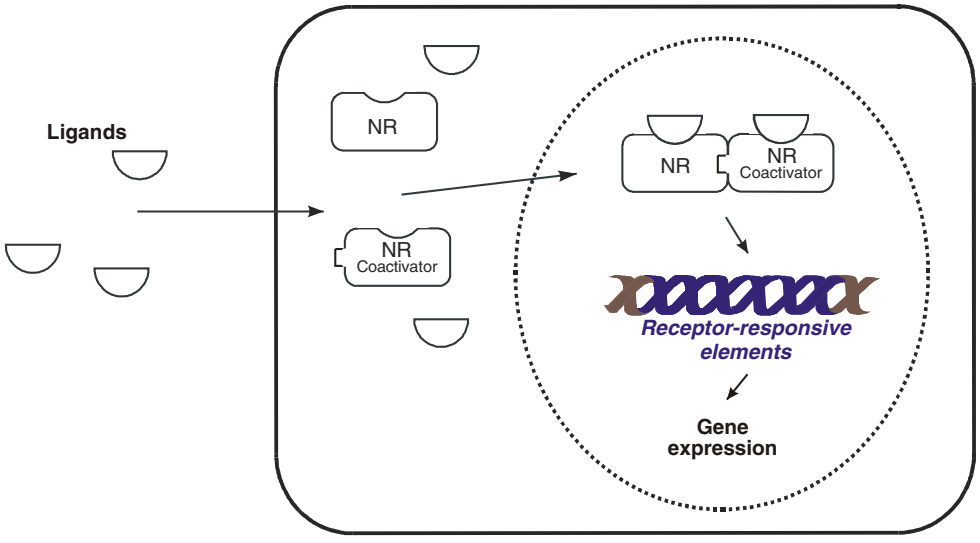

Fig. 1. General mechanism of nuclear receptors signalling; note that formation of homodimers or no coactivator binding are also possible; NR—nuclear receptor.

About 48 nuclear receptors have been identified so far and they are commonly divided into three subclasses with respect to corresponding ligands (Jacobs et al., 2003):

- type I receptors—for steroid hormones including progestins (progestin receptor, PR), estrogens (ER), androgens (AR), glucocorticoids (GR) and mineralcorticoids (MR),
- type II receptors—thyroid receptor (TR), vitamin D receptor (VDR), receptors for retinoids generally (RXR) and *all-trans*-retinoic acid (RAR), peroxisome proliferator activated receptor (PPAR) and aryl hydrocarbon receptor (AhR),
- type III receptors—orphan receptors—still awaiting recognition of specific ligands.

Numerous interactions of environmental pollutants with signaling pathways of nuclear receptors were described and include either direct binding of xenobiotics to receptor or indirect effects mediated via modulation of associated signaling pathways. Many nuclear receptors are physiologically activated by low molecular weight ligands (steroid hormones; vitamin A derivatives; thyroid hormones). These ligands often display substantial structural similarities to many environmental contaminants such as polychlorinated dibenzodioxins and dibenzofurans (PCDD/Fs), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) or phthalates e.g. (Hilscherova et al., 2000; Gray, 1998). Correspondingly, nuclear receptors become unfortunately highly susceptible to non-physiological modulations by anthropogenic contaminants and resulting disruption of signaling pathways was related to numerous in vivo effects like immunosuppressions, endocrine disruption, carcinogenesis, developmental toxicity etc.

The complexity of biochemical toxicity mechanisms of contaminant-induced nuclear receptor modulations is documented by example of anti/estrogenity. In vitro, numerous environmental pollutants like phthalates were shown to bind non-physiologically to ER and activate its function (Nakai et al., 1999; Balaguer et al., 1999). On the other hand, other environmental contaminants, e.g. some hydroxylated PCBs can act as ''anti-hormones'' by competitive binding to active site without activation of ER, thus inhibiting the function of natural estrogens (Moore et al., 1997). Furthermore, other compounds like ethanol or epidermal growth factor act as ''estrogen-like'' hormones (i.e. activating transcription of ER-controlled genes) by modulating upstream signalling without binding to ER (Combes, 2000). Additionally, ER-independent ''anti-estrogenity'' of AhR ligands (as TCDD and/or PAHs) was described revealing substantial cross-talk between signaling pathways of different nuclear receptors (Zacharewski et al., 1991). Environmental relevance of the estrogen-like or anti-estrogen biochemical mechanisms was experimentally proven by causal linking to several in vivo effects such as male feminization or reproduction disorder in various organisms (Combes, 2000).

This type of interference among nuclear receptors is not rare. Various mechanisms of interaction have been described, from induction of enzymes involved in decomposition of other hormones to depletion of coactivators levels (Klinge et al., 2001). A well-known example are retinoid X receptors that are able to form heterodimers with various other receptors and thus to influence their activity (e.g. Cai et al., 2002; Harvey and Williams, 2002). However, this matter is too wide for our study and there are other reviews concerning this matter (Tuohimaa et al., 1996; Gottlicher et al., 1998; Kato et al., 2000; Flototto et al., 2001; Harvey and Williams, 2002).

Multiple and contradictory modulations of other nuclear receptors by xenobiotics were also described and highlight our limited understanding of possible mechanisms and consequences of chemically induced disruption of signaling pathways. Generally, the action of endocrine disrupting chemicals can be mediated through receptor and/or non-receptor mechanisms. The first mechanism involves binding to receptors that can lead to activation of their responsive elements in nuclear DNA, which results in increased expression of target genes (i.e. estrogenity). On the other hand, interaction of some compounds with receptors can negatively affect binding of receptors to responsive elements on DNA, and thus suppress receptor action. Non-receptor and indirect mechanisms of chemically induced effects on nuclear receptor signalling have been proposed such as modulations of tissue levels of enzymes involved in synthesis or catabolism of natural ligands (Machala and Vondracek, 1998). Additionally, interactions of xenobiotics with hormone-binding proteins (disruption of transport and free hormone levels) or cross-talk between receptors are other important mechanisms of endocrine disruption (Gillesby and Zacharewski, 1998). Another target for disruption is often hypothalamo-pituitary axis and thus regulation of steroid hormone production (Combes, 2000).

In the present review we summarize existing information on the function of major nuclear receptors, their role in chemically induced adverse effects in living organisms, and the methods for studies of specific toxicity mechanisms.

2. Aryl hydrocarbon receptor

2.1. Mechanism of action

As a primary target for coplanar aromatic substances (including many persistent organic pollutants—POPs—and other environmental xenobiotics), the aryl hydro-

carbon receptor belongs among the most extensively studied nuclear receptors.

AhR is a cytosolic helix-loop-helix/PAS protein (Korkalainen et al., 2003) associated with heat-shock-proteins of molecular weight of 90 kDa (HSP90) and imunophilin-like proteins (ILP). Ligand binding to this complex causes conformational changes resulting in its transport into the nucleus. Here the AhR dissociates from the complex and after dimerization with Ah-receptor nuclear translocator (ARNT) binds to dioxin responsive elements regulating expression of specific genes (Pollenz, 2002).

The primary known biochemical responses to AhR activation are induction of drug metabolising monooxygenases such as cytochrome P450 1A1 (CYP1A1), CYP1A2 and CYP1B1 (enzymes participating in biotransformation phase I) as well as phase II enzymes like glutathione-*S*-transferase (GST), UDP-glucuronyltransferase, NADPH-quinone oxidoreductase, xanthinoxidase etc. (Reen et al., 2002). However, CYP enzymes are playing a key role not only in xenobiotics detoxication but may also greatly enhance their toxic and/or mutagenic potency (e.g. metabolic activation of PAHs; Machala et al., 2001b). Beside activation of CYPs, other effects like modulation of specific cellular signaling pathways are considered another molecular mechanism of AhR-mediated toxicity (Berghard et al., 1993). Numerous chronic adverse health effects of xenobiotics such as changes in cellular proliferation, neurotoxicity, embryotoxicity, immunotoxicity as well as carcinogenicity were experimentally related to AhR-dependent events (Parzefall, 2002).

2.2. AhR-active compounds and toxicity equivalency factors

Most of known Ah-receptor ligands are coplanar aromatic compounds, the most potent so far recognized is 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD). A toxicity equivalency factor (TEF) concept was accepted for better comparison of AhR-mediated effects (McLachlan, 1993; van den Berg et al., 2000) and is used for risk assessment purposes. TEF is a number representing the toxic potency of a particular compound to induce AhR-mediated effects related to the reference substance—TCDD. The TEFs of TCCD and some other highly toxic congeners of polychlorinated dibenzo-*p*-dioxins and dibenzofurans were set to 1.0. The toxic potencies of coplanar PCBs correspond to TEF values ranging from 10^{-5} to 10^{-1}. Substantial variability in the sensitivities to AhR-active (=dioxin-like) substances in various species was recognized and correspondingly separate sets of TEFs for human, fish and birds were accepted (van den Berg et al., 2000).

While the TEFs recommended by international bodies include PCDDs, PCDFs and PCBs, other environmentally important groups of compounds (PAHs or polychlorinated naphthalenes, PCNs) were also shown to activate AhR and act as dioxin-like compounds (van den Berg et al., 2000, 1998). Regulatory TEFs for PAHs or PCNs have not yet been accepted, although several studies described their dioxin-like effects and the relative potencies (REPs) based on in vitro comparisons have been suggested (Machala et al., 2001b; Behnisch et al., 2001b) ranging from 10^{-6} to 10^{-3} with median values about 10^{-5}. However, other chemicals like hexachlorobenzene (van Birgelen, 1998) and derivatives of compounds mentioned above, e.g. aza- and nitro- (Fent, 2001), oxo- and alkylated PAHs (Villeneuve et al., 2002) or hydroxylated PCB derivatives (Machala et al., 2004) are also able to activate AhR. Other "non-typical" ligands such as natural flavonoids, carotenoids or even endogenous ligands (e.g. tryptophan or arachidonic acid metabolites) also activate AhR signaling pathway (Denison et al., 2002).

To compare and quantify the dioxin-like toxic potency of environmental samples, TEFs are used for calculation of toxic equivalents (TEQs). Chemical analyses of environmental samples provide data on concentrations (c_i) of individual congeners of PCDDs, PCDFs, PCBs or other compounds such as PAHs or PCNs. The concentration of individual chemicals multiplied by their TEFs or REPs (TEF_i) represent the amount of reference compound 2,3,7,8-TCDD having the same AhR-mediated activity. Total toxic potency of the sample is calculated as a sum of individual TEQ-values:

$$TEQ = \sum TEF_i \times c_i$$

TEF approach for AhR-mediated effects therefore combines both the toxic potency and actual levels of contamination and it was shown that weak but abundant AhR-activating compounds such as PAHs e.g. (Machala et al., 2001b) or non-*ortho* substituted coplanar PCBs (e.g. Focant et al., 2002) substantially contribute to the overall dioxin-like potency of the sample. A TEF approach for dioxin-like effects is a potent tool for simple quantification and evaluation of toxicities of different samples by comparison of a single value of reference compound (2,3,7,8-TCDD in case of AhR-mediated effects). Similar concepts of relative-potencies for evaluation of environmental contamination were recently proposed also for other specific toxicity mechanisms mediated by nuclear receptors (such as for ER—estrogenic potency factors; Safe et al., 1998) or other processes as genotoxicity (Machala et al., 2001b) or tumor promotion (Blaha et al., 2002).

2.3. Toxicity assessment—in vivo and in vitro methods

Numerous in vivo studies with dioxin-like compounds were conducted with laboratory animals reviewed e.g. in van der Berg et al. (1998) or Behnisch

et al. (2001a) and describe specific endpoints like liver enlargement, reduction of thymus weight, reproductive and developmental disorders (number of offsprings, malformations) or wasting syndrome (progressive weight loss until death). The quantification of CYP1A1 activity (ethoxyresorufin-*O*-deethylase, EROD or aryl hydrocarbon hydroxylase, AHH) in exposed organisms is another possible endpoint used as biomarker of dioxin-like effects (Besselink et al., 1996).

Recently, a new approach to determine dioxin-like effects in vivo was suggested by Carvan et al. (2000). They developed transgenic zebrafish (*Brachydanio rerio*) with firefly luciferase and green fluorescent protein under the transcriptional control of AhR and demonstrated simple quantification of light emission and fluorescence after exposure to dioxin-like compounds.

However, routine application of in vivo tests for studies of AhR-mediated toxicities is limited due to both high time and cost expenses as well as to substantial ethical limitations.

Therefore, a variety of different in vitro assays for detection and studies of AhR-mediated toxicities were suggested and due to advantages including small scale, relative simplicity, time and cost efficiency, they are used for larger studies of numbers of chemicals (Behnisch et al., 2001a), and toxicity screenings of environmental samples (Behnisch et al., 2001b).

Several experimental in vitro setups were employed to characterize the AhR-mediated toxic potencies. One of the most important methods, also widely used for study of other receptors, is competitive ligand-binding assay (Meek, 1998). Other methods include detection and quantification of AhR-triggered mRNA (Xu et al., 2000) or proteins (Diaz-Ferrero et al., 1997). The quantification of protein products has become the most widely used approach employing protein electrophoretic methods (Drahushuk et al., 1998), immunoassays (Diaz-Ferrero et al., 1997; Roy et al., 2002), or quantification of enzymatic activities (Fent and Batscher, 2000).

In vitro assessment of AhR-mediated induction of monooxygenase activity of cytochromes P450 was generally the most widely employed approach for estimation of dioxin-like toxicities. For these purposes, particularly hepatic cells are often used because of their high content of AhR. However, the presence of AhR in breast, lung, uterus, nervous or cardiovascular cells has also been described (Jacobs et al., 2003). Examples of cell lines used for in vitro tests of toxicity are shown in Table 1.

The most frequently used assay is the fluorimetric determination of 7-ethoxyresorufin-*O*-deethylase (EROD assay; Fent and Batscher, 2000) or AHH activity (Piskorska-Pliszczynska et al., 1986). Synthesis of these enzymes is AhR dependent and linearly corresponds to concentrations of dioxin-like substances and their affinity to AhR. EROD activity can be measured in fact in any cell containing AhR and the method was employed for studies with fish (Fent and Batscher, 2000; Clemons et al., 1997; Bols et al., 1999), rat (Koistinen et al., 1996; Sanderson et al., 1996), mouse (Paton and Renton, 1998) and human hepatic cell lines (Jones and Anderson, 1999; Wiebel et al., 1996). Primary hepatocyte cultures from birds (Sanderson et al., 1998; Bosveld et al., 1997), monkeys and castrated pigs (Andersson et al., 2000) were also used as a model.

Additionally to EROD, chemical potencies to induce other AhR-dependent enzymatic activities were used for characterization of dioxin-like effects (aromatic hydrocarbon hydroxylase (AHH), methoxyresorufin-*O*-demethylase (MROD), 7-pentoxy-resorufin-*O*-deethylase (PROD), benzyloxyresorufin-*O*-alkylase (BROD), or 7-ethoxycoumarin-*O*-deethylase (Behnisch et al., 2001b; Diaz-Ferrero et al., 1997).

Since AhR as well as other nuclear receptors act as transcription factors, reporter gene assays for assessment of their activities have become widespread during the last decade. The principle is based on the incorporation of a gene for synthesis of a specific reporter protein (enzyme) to cellular DNA under the control of specific transcription factor (nuclear receptor), e.g. under the control of dioxin-responsive element (DRE).

Several cells either stably (Murk et al., 1996) or transiently (Merchant and Safe, 1995) transformed were constructed for different nuclear receptors including AhR. The most common reporter genes include those for alkaline phosphatase, β-galactosidase, chloramphenicol acetyl transferase (Hilscherova et al., 2000), green fluorescent protein (GFP; Naylor, 1999) or firefly luciferase (Murk et al., 1996). The latter approach became the most popular due to several advantages such as high sensitivity of the luminescence assay or linear proportion between the intensity of emitted light and the amount of newly synthesised protein (luciferase), after the activation of desired promoter.

Cellular models employing luciferase reporter gene assays were developed also for characterization of AhR-mediated activities and determination of dioxin-like substances. Among the stably transfected cell lines, rat hepatoma cells H4IIE-luc are the best characterized model and were used for determination of dioxin-like potential of pure substances, e.g. PCDD/Fs, PCBs (Murk et al., 1996), PAHs (Machala et al., 2001b), PCNs (Blankenship et al., 1999), as well as characterization of dioxin-like effects in environmental samples of sediments (Murk et al., 1996; Machala et al., 2001a), air particulate matter (Hamers et al., 2000) or biota (Murk et al., 1998). The assay was shown to be relatively good standardized screening tool for rapid and sensitive determination of AhR-mediated toxicities and a variant of the in vitro assay (CALUX analysis—Chemically Activated LUciferase eXpression) is currently registered

Table 1
Some cell lines used in research of nuclear receptors

	Cell line	Parent tissue	Endpoint	Reference
AhR activity	101L	Human hepatocarcinoma	RR-L	Chen and Tukey (1996)
	H4IIE	Rat hepatocarcinoma	EA, RR-L	Sanderson et al. (1996) and Villeneuve et al. (2000)
	Hepa1	Mouse hepatocarcinoma	EA	DeHaan et al. (1996)
	HepG2	Human hepatocarcinoma	EA	Guigal et al. (2001) and Wiebel et al. (1996)
	PLHC-1	Topminnow hepatocarcinoma	EA	Fent and Batscher (2000)
	RTG-2	Rainbow trout gonad cell line	EA	Fent (2001)
	RTL-W1	Rainbow trout hepatocarcinoma	EA	Bols et al. (1999)
Anti/estrogenic activity	BG-1	Human ovarian adenocarcinoma (ERα)	RR-L	Rogers and Denison (2000)
	HeLa	Human breast carcinoma (ERα, ERβ)	RR-L	Balaguer et al. (1999)
	Ishikawa(+), Ishikawa(−)	Human endometrial adenocarcinoma (ERα, ERβ)	O	Frigo et al. (2002)
	KPL-1	Human breast carcinoma (ERα, ERβ)	P	Kurebayashi et al. (1998)
	MCF-7	Human breast carcinoma (ERα)	P, RR-L	Moore et al. (1997) and Diel et al. (2002)
	T47D	Human breast carcinoma (ERβ)	P, RR-L	Lebail et al. (1998) and Legler et al. (1999)
	ZR-75	Human breast carcinoma (ERα)	P	Poulin et al. (1987) and Schafer et al. (1999)
Anti/androgenic activity	COS-7	Monkey kidney cell line	T+O	Terouanne et al. (2002)
	CHO	Chinese hamster ovary cell line	RR-L	Paris et al. (2002b)
	L929	Mouse fibroblast cell line	RR-L	Zhang et al. (2000) and Paris et al. (2002b)
	LNCaP	Human prostatic adenocarcinoma	P, RR-L	Grigoryev et al. (2000) and Yamabe et al. (2000)
	MFM-223	Human mammary adenocarcinoma	O	Hackenberg et al. (1992)
	PC-3	Human prostatic adenocarcinoma	RR-L	Haendler et al. (2001)
	MDA-kb2	Human breast cancer	RR-L	Wilson et al. (2002)
	SC115	Mouse mammary carcinoma	P	Kizu et al. (2000)
Retinoid system activity	HSG	Human salivary gland adenocarcinoma	RR-L, O	Kyakumoto et al. (1997)
	MCF-7	Human breast carcinoma	T+P, O	Dietze et al. (2002) and Kogai et al. (2000)
	NRP-152	Prostate epithelial non-carcinoma	P, O	Richter et al. (1999)
	NRP-154	Prostate epithelial carcinoma	P, O	Richter et al. (1999)
	P19	Murine embryonic carcinoma	O	Seeley and Faustman (1998)
	RTG-2	Rainbow trout gonads	O	Miller et al. (2000)
	SCC4	Human keratinocyte carcinoma	O	Krig et al. (2002)
Thyroid system (T3, T4 or TSH) activity	LNCaP	Human prostatic adenocarcinoma	P	Esquenet et al. (1995)
	FRTL-5	Fischer rat thyroid	P	Medina and Santisteban (2000)
	CHO	Chinese hamster ovary cell line	RR-L	Sendak et al. (2002)
	PC C13	Rat thyroid	O	Pacifico et al. (1999)
	TE671	Human cerebellar meduloblastoma	RR-L	Iwasaki et al. (2002)
	GH3	Rat pituitary tumor	P, O	Kitamura et al. (2002)
	WRT	Wistar rat thyroid	P, O	Brandi et al. (1987) and Kimura et al. (2001)

Abbreviations: RR, receptor–reporter system (L, luciferase, GFP, green fluorescent protein); EA, enzymatic activity (e.g. EROD); P, proliferation; T, transient transfection; O, other endpoints.

trademark for US and European markets (Gray et al., 2003).

Detailed discussion on advantages and limitations of in vitro assays for AhR-mediated effects could be found in specialized reviews (Hilscherova et al., 2000; Behnisch et al., 2001a,b).

3. Estrogen receptor

3.1. Mechanism of action

Estrogens are a group of steroid hormones that play a key role in female hormone regulation and signalling. The major endogenous hormones are 17-β-estradiol, estrone and estriol, which are produced to the greatest extent in ovarian cells, lesser amounts are produced in placenta, cortex of adrenal gland and peripheral fatty tissues. They are responsible for metabolic, behavioural and morphologic changes occurring during various stages of reproduction. In general, they influence cell proliferation and differentiation, development and activity of tissues participating in process of reproduction. Estrogens also control the bone formation, regulation of organism homeostasis, cardiovascular system and behaviour. To lesser extent they are produced in males, regulating production, transport and concentration of testicular liquid and anabolic activity of androgens (Hess et al., 2001; Murray et al., 1993).

Although estrogens are almost exclusively produced in female organisms, estrogen receptors were localized in both sexes in numerous tissues (breast, ovaries, brain, liver, bone, cardiovascular system, adrenals, testis, prostate, urogenital or gastrointestinal tract; Jacobs et al., 2003). Consequently, abnormal presence of exogenous estrogen-like acting molecules (such as environmental contaminants) in males could cause a large spectrum of negative effects.

Production of estrogens is regulated by hypothalamic-pituitary axis. Hypothalamus excretes gonadotropin-releasing hormones that further increase or decrease follicle stimulating hormone (FSH) and luteinising hormone (LH) that are directly regulating estrogen (and androgen) hormone production (Murray et al., 1993).

The mechanism of ER action is similar to that of AhR. In fact, it differs only in chaperon proteins presence—instead of HSP 90 and ILP (for AhR), the DNA binding domain of ER is masked by proteins like HSP70 and/or p60 (Massaad et al., 2002). At least two structurally different subtypes of estrogen receptors were described in mammals (ERα and ERβ forming homo or heterodimers in cells). Another subtype ERγ possibly exists in fish (Drummond et al., 2002).

Xenoestrogenic action of xenobiotics is mediated mostly via binding to ER combined with activation of ERE (Estrogen Responsive Element in nuclear DNA). In contrast, some xenobiotics act as anti-estrogens by disruption of binding of ER to responsive elements on DNA. Non-receptor mechanisms include modulations of tissue levels or activities of enzymes participating in biosynthesis or catabolism of estradiol, such as CYP11A (an enzyme cleaving the side chain of cholesterol), CYP19 (an enzyme converting testosterone to estrogens), or CYP1A (group of enzymes involved in estradiol catabolism; Machala and Vondracek, 1998). Interactions of xenobiotics with estrogen-binding plasmatic proteins or cross-talk between ER and other receptors as well as disruption of hypothalamo-pituitary axis have also been described (Gillesby and Zacharewski, 1998; Combes, 2000).

3.2. Anti/estrogenic compounds

Anti/estrogenic activity of a variety of compounds was tested using in vitro and/or in vivo tests (Table 2). Numerous chemicals have been found to elicit either estrogenity or anti-estrogenity reviewed in Coldham et al. (1997), Combes (2000), Mantovani et al. (1999) or Vondracek et al. (2002). Extensive studies were performed with such chemicals like toxaphene, alkylphenol ethoxylates, phthalates, some pharmaceuticals, hydroxylated chlorinated biphenyls, methoxychlor, *o*,*p*′-DDT, some PCBs, PAHs or natural flavonoids and phytoestrogens or PCDD/Fs, PCBs.

Table 2
Comparison of REP (RElative Potencies) of selected compounds related to 17-β-estradiol derived from different assays (Fang et al., 2000; Legler et al., 1999, 2002a; Gutendorf and Westendorf, 2001; Machala et al., 2004)

Compound (group)	REP related to 17-β-estradiol			
	Ligand binding assay	YES	E-screen	Mammalian receptor–reporter systems
Estradiol (hormones)	1	1	1	1
Estriol (hormones)	7×10^{-2}–1.9×10^{-1}	3.6×10^{-3}–6.3×10^{-3}		8×10^{-2}
Coumestrol (phytoestrogens)	2.8×10^{-2}–9.3×10^{-1}	6.8×10^{-3}–1.3×10^{-2}	1.1×10^{-1}	1.3×10^{-1}
o,*p*′-DDT (pesticides)	8.9×10^{-4}	1.1×10^{-6}	1.7×10^{-5}	3×10^{-6}–1×10^{-4}
OH-PCBs	5.4×10^{-2}	1×10^{-2}	2.5×10^{-4}	1.7×10^{-5}
4-Octylphenol (alkylphenols)		3×10^{-5}	5.5×10^{-5}	1.4×10^{-6}
Butylbenzylphthalate (phthalates)	3.4×10^{-5}	4×10^{-6}	2.5×10^{-6}	1.4×10^{-6}
Bisphenol A (monomers)	1.8×10^{-3}–2.3×10^{-4}	5×10^{-5}–6.6×10^{-5}	1.7×10^{-5}–2.5×10^{-2}	2.5×10^{-2}

Exposure to specific chemicals as well as biochemical toxicity processes were related to numerous adverse health effects by both laboratory experiments and field studies. The major impairments include reproduction toxicity, increased incidence of breast cancer, male testis and uterus tumors, delayed male puberty, decreased semen quality and volume, increases of developmental anomalies of the male reproductive system including reduced secondary sex organs size, hypospadias, cryptochordism and enhanced susceptibility to seminomas (Mantovani et al., 1999; Gillesby and Zacharewski, 1998).

3.3. Toxicity assessment—in vivo and in vitro methods

In vivo assays of xenoestrogenity focus mostly on reproductive system dysfunctions. The most commonly used in vivo bioassays with laboratory rodents are uterotrophic (uterine wet weight) and vaginal cornification assay (Safe et al., 1998; Baker, 2001; Gillesby and Zacharewski, 1998). Several test procedures were suggested for assessment of endocrine disruption related to reproductive and developmental toxicity and are summarized elsewhere (Combes, 2000).

Beside mammals, other organisms including fish (Knudsen et al., 1998), amphibians (Kloas et al., 1999) or birds (Berg et al., 1998) were also shown to be highly susceptible to xenoestrogens and they were used as ecotoxicological models for assessment of ER-mediated toxicities. Production of yolk protein (vitellogenin, Vtg; Kloas et al., 1999; Tyler et al., 1999) and/or eggshell zona radiata proteins (Zrp; Machala and Vondracek, 1998) are also useful in vivo parameters that may be quantified and showed in oviparous organisms a good correlation to xeno/estrogen exposure. Both groups of estrogen-inducible proteins are synthesized by females during oogenesis and their abnormal production in males is a significant marker of exposure to estrogenic compounds (Celius et al., 1999). These biomarkers were used in numerous ecotoxicological studies (Celius et al., 1999; Knudsen et al., 1998; Latonnelle et al., 2002; Gagne and Blaise, 1998).

A wide variety of in vitro bioassays (Table 1) have been developed for screening of ER-mediated anti/estrogenic effects (Hilscherova et al., 2000; Legler et al., 2002b) and employed for assessment of pure chemicals (Villeneuve et al., 2002; Vakharia and Gierthy, 2000; Zacharewski et al., 1998) or complex environmental mixtures and samples (Legler et al., 1996; Balaguer et al., 1999). Further discussion focuses on estrogenity assays. However, as already mentioned, several xenobiotics were also shown to negatively modulate ER (anti-estrogenity). Experimental assessment of anti-estrogenic effects uses the same methods as described above for estrogenity. The only difference is simultaneous exposure to xenobiotic and 17-β-estradiol. Decrease in ER-mediated responses is then used as a measure of anti-estrogenity.

Among the oldest assays a cell proliferation test (so-called E-screen) is well characterized. It is based on measurement of ER-dependent proliferation in certain cell lines such as human breast carcinoma MCF-7, T47D or ZR-75 (Combes, 2000; Gupta et al., 1998). ER-dependent induction of cell number is measured as ^{3}H-thymidine incorporation into the cellular DNA, measurement of metabolic activity or staining of cells with fluorescent dyes (Combes, 2000).

In vitro induction of specific transcripts and proteins controlled by ER-activities were suggested as suitable cellular assays for estrogenity. Immunochemical determinations of Vtg (Kim and Takemura, 2003; Bon et al., 1997; Celius et al., 2000) or determinations of Vtg mRNA (Gagne and Blaise, 1998) in primary hepatocytes of rainbow trout (Pelissero et al., 1993) or African clawed frog (*Xenopus laevis*; Marilley et al., 1998) are the most widely employed techniques. Besides Vtg, other proteins like pS2, prolactine or catepsin D were shown to sensitively respond to ER and their quantification with radioimmunoassays, PCR or Northern or Western blotting was documented (Zacharewski, 1997; Combes, 2000). Additionally, inductions of prostaglandin H synthase (Degen, 1990) or ornithine decarboxylase (Qiu et al., 2003) were correlated with exposure to xeno/estrogens. However, many of these parameters are often tissue and species-specific (Machala and Vondracek, 1998) and are not strictly related to ER but can be controlled also by other signaling pathways (Zacharewski, 1997; Combes, 2000).

Several reporter gene assays for assessment of ER-mediated effects have been developed (Giesy et al., 2002). Human breast adenocarcinoma stably transfected with the firefly luciferase under the control of ERE (MCF-7—MVLN, T47D—ER-CALUX; Hilscherova et al., 2000; Lebail et al., 1998; Legler et al., 1999) became the most popular. Luciferase-reporter gene assays with HeLa (Balaguer et al., 1999) or ovarian carcinoma BG-1 (Rogers and Denison, 2000) were also proposed. The assays were successfully used for characterization of estrogenic potential of pure compounds reviewed in Hilscherova et al. (2000) as well as determination of xenoestrogens in environmental samples reviewed by Giesy et al. (2002).

Beside luciferase, the gene for green fluorescent protein (GFP) was introduced into human breast carcinoma MCF7 cells and used as reporter system for assessment of xeno/estrogenity (Kuruto-Niwa et al., 2002). However, the lack of enzymatic amplification makes this method less sensitive than enzymatic assays (Naylor, 1999). Mammalian steroid receptors along with β-galactosidase and luciferase (Combes, 2000) were also transfected into the yeast cells and the constructs used for detection of estrogenic activities (YES; Lascombe

et al., 2000; Jungbauer and Beck, 2002). Although yeasts have several advantages (easy culturing, steroid-free media, easy genetic manipulations, and absence of other possibly interfering nuclear receptors), other aspects must be considered, such as variability between the strains and individual investigators or presence of cell wall which could substantially affect the results of the test (Combes, 2000; Zacharewski, 1997).

4. Androgen receptor

4.1. Mechanism of action

With respect to environmental xenobiotics, there is much less information available on AR, RAR/RXR or TR compared to previously discussed AhR and ER. However, different nuclear receptors can interact with each other and form complicated networks with other signalling systems. Therefore, complex characterization of endocrine modulations by various xenobiotics is crucial for understanding consequences of chronic contaminant exposures.

Role of the androgen receptor (AR) in male organism is very similar to that of estrogen receptor in females. Androgens (agonists of AR) play a key role in the development of male primary and secondary sexual characteristics, act as anabolics stimulating protein synthesis, growth of bones and muscular mass. Androgens also affect cell differentiation, spermatogenesis and male type behaviour (Wang and Fondell, 2001; Murray et al., 1993). However, androgens were also shown to participate in adverse processes such as formation of benign prostate hyperplasia and carcinomas (Wang and Fondell, 2001). According to structure, two subtypes of androgen receptor called α and β were recognized. These receptors are present at the greatest extent in testis but significant levels have been found in prostate, adrenals, kidneys, brain or pituitary gland (Ikeuchi et al., 2001).

The main endogenous androgen hormone is testosterone. Its synthesis is controlled, similarly to the estrogens, by pituitary LH. Testosterone is produced mainly in testis; lesser amounts are formed in adrenals. Testosterone is the basic androgen that may be further transformed to other androgens such as 5α-dihydrotestosteron (DHT). While DHT shows higher affinity to AR compared to testosterone, the other derivatives are much weaker androgens (Murray et al., 1993). Furthermore, testosterone may also be converted by aromatase (CYP19) to 17-β-estradiol. This reaction takes place to the greatest extent in fatty tissues, but other tissues like bones (Simpson, 2003), liver or skin (Murray et al., 1993) also show aromatase activity. Despite of their major function in male organism, androgens (DHEA, androstenedione and testosterone) are produced in women as well and formed in ovaria and/or adrenals (Davison and Davis, 2003).

Several mechanisms of androgen signaling pathway disruption were described such as binding to AR with/without activating it (Wong et al., 1995), reducing of AR levels (List et al., 2000), changes in metabolism of androgens (Massaad et al., 2002) or FSH/LH signalling disruption (Massaad et al., 2002).

Effects of xenobiotics modulating AR-function are dependent on the development stage. In males exposed during prenatal development, anti-androgens may cause malformations of the reproductive tract like reduced anogenital distance, hypospadias, nipple and even vagina development, undescendent ectopic testes, atrophy of seminal vesicles and prostate gland etc. (Gray et al., 1994). Exposure in prepubertal age to both anti-androgenic and estrogenic substances leads to delay in male puberty, reduced seminal vesicles, ventral prostate and epididymal weight. Exposure of adult males to anti-androgens may result in oligospermia, azoospermia and libido diminution (Chapin et al., 1996; Massaad et al., 2002).

4.2. Xenobiotics affecting the AR function

Both androgen-like and anti-androgenic action of xenobiotics may lead to significant adverse effects. However, as far as environmental xenobiotics are concerned, anti-androgenic modes of action seem to be of particular importance (Kelce and Wilson, 1997; Gray et al., 1994; Kelce et al., 1994).

Although some xenobiotics like metabolites of a fungicide vinclozoline can act as weak AR-agonists in absence of natural ligands, in presence of testosterone they have antagonistic effects (Wong et al., 1995).

Also DDT and its metabolites (*o*,*p*′-DDT or *p*,*p*′-DDE), and fungicide procymidon bind to AR and act as competitive inhibitors (Kelce et al., 1997; Gray et al., 1997). Anti-androgenic activity of other compounds like bisphenol-A, 3-hydroxy-phenylphenol or 4-hydroxy-phenylphenol was documented in stably transfected cell lines (Paris et al., 2002a).

Other compounds (e.g. *o*,*p*′-DDT, Endosulfan, Mirex) are able to mobilize monooxygenases participating in androgen degradation (Dai et al., 2001).

Important environmental contaminants such as some PAHs (Kizu et al., 2000; Vinggaard et al., 2000) as well as PCBs (Schrader and Cooke, 2003) were shown to act as anti-androgens in vitro in micromolar concentrations, but the mode of action of these compounds is not clearly described yet. Also environmental contaminant of uncertain origin, tris-(4-chlorophenyl)-methanol has been found to be a potent AR-antagonist. This probable metabolite of tris-(4-chlorophenyl)-methane (a contaminant of commercial DDT mixtures) is up to 50-times more potent anti-androgen than vinclozolin

Table 3
Antiandrogenic effects—IC_{50} of some important environmental contaminants

Compound	IC_{50} (μM)	Reference
Benz[*a*]anthracene	3.2	Vinggaard et al. (2000)
Benzo[*a*]pyrene	3.9	Vinggaard et al. (2000)
7,12-Dimethylbenz[*a*]anthracene	10.4	Vinggaard et al. (2000)
Chrysene	10.3	Vinggaard et al. (2000)
Dibenz[*a*,*h*]anthracene	Activation in range 0.1–10 μM	Vinggaard et al. (2000)
Bisphenol A	7.0	Paris et al. (2002a)
3′,5′-Dichloro-2-hydroxy-2-methylbut-3-enanilide (vinclozolin metabolite)	9.7	Kelce et al. (1994)
Hydroxyflutamide	5.0	Wong et al. (1995)
Aroclor typical values	0.25–1.11	Schrader and Cooke (2003)
Individual PCBs typical values	64–87	Schrader and Cooke (2003)
tris-(4-chlorophenyl)-methanol	0.2	Schrader and Cooke (2002)

or *p*,*p*′-DDE (Schrader and Cooke, 2002). Furthermore, its effective concentrations (200 nM) are close to the levels found in human serum (55 nM). Some examples of chemicals acting as anti-androgens are shown in Table 3.

4.3. Toxicity assessment—in vivo and in vitro methods

The most widely used in vivo test for assessing androgenic effects is Hershberger assay. Endpoint of the assay, conducted in castrated rats, is a weight of the ventral and dorso-lateral prostate, seminal vesicles with coagulating glands, glans penis, Cowper's glands and levator ani plus bulbocavernosus muscles, measured 4–10 days after treatment with the studied substance (Baker, 2001; Yamada et al., 2003). Another method is measurement of androgen levels in serum since administration of some compounds elevates luteinizing hormone and testosterone concentrations (Massaad et al., 2002). However, this effect was not observed after exposure to certain chemicals with known anti-androgenic activity, what limits the value of this assay (Gray et al., 1997). Other methods for identification of anti-androgenic substances may be derived from classical toxicological in vivo tests, especially from developmental and reproductive assays (Baker, 2001).

For anti/androgenity assessment a wide range of specific in vitro tests has been established (Table 1). The cell lines derived from prostate carcinomas are the most common due to relatively high levels of AR (Berns et al., 1986; Terouanne et al., 2000; Veldscholte et al., 1994; Tilley et al., 1995), but other cell lines derived from breast cancer (Wilson et al., 2002; Hackenberg et al., 1992), ovary (Paris et al., 2002b) or even kidney (Terouanne et al., 2002) and fibroblasts (Zhang et al., 2000) were also successfully used for anti/androgenity testing. Methods were successfully used for determination of anti/androgenic activity of environmental xenobiotics (Shimamura et al., 2002), pharmaceuticals (Joly-Pharaboz et al., 2000; Esquenet et al., 1995), or complex environmental mixtures (Kizu et al., 2000).

Proliferation tests using the same principle as assays for xenoestrogenity with mammary and prostatic carcinoma cell lines are available and were used in the studies with environmental samples (crude extract of C-heavy oil; Kizu et al., 2000). However, anti/androgenity reporter gene assays (as well as for AhR or ER) become a popular in vitro testing system.

PALM cell line (Terouanne et al., 2000; Sultan et al., 2001) and AR-LUX assay (Blankvoort et al., 2001)—in fact prostatic carcinoma PC3 and breast carcinoma T47D cell lines stably transfected with firefly luciferase gene—are well accepted. Additionally, numerous other cell lines have been transfected by luciferase reporter gene such as prostatic adenocarcinoma LNCaP (Blok et al., 1992; Yamabe et al., 2000), Chinese hamster ovary CHO 515 (Paris et al., 2002b; Vinggaard et al., 2000) or mouse fibroblasts L929 (Zhang et al., 2000). Beside luciferase, monkey kidney COS-7 (Terouanne et al., 2002), human prostatic PNT1A and DU-145 cells stably transfected with GFP were also used for anti-androgenity assessment of environmental chemicals and pharmaceuticals (Avances et al., 2001; Sultan et al., 2001). Recombinant yeast strain stably transfected with β-galactosidase under transcriptional control of AR were also developed for anti/androgenity screening (Lee et al., 2003; Baker et al., 1990).

Other in vitro and ex vivo assays for anti/androgenity are based on the measurement of testosterone production in Leydig cells after exposure to tested substances (Combes, 2000). Determination of folicle-stimulating hormone (FSH) in pituitary cells as a model has also been described, but the methods using primary cultures are quite laborious and provide variable response with poor standardization (Baker, 2001).

5. Retinoid receptors

5.1. Mechanism of action

Natural retinoids—vitamin A and its metabolites—are mediators of various important processes in

eukaryotic organisms. They are necessary for vision, play an important role in controlling growth, apoptosis and differentiation of embryonic cells, epithelial cells of gastrointestinal tract, skin and bones. Furthermore, they affect nervous and immunity system, act as anti-oxidative agents, are involved in biosynthesis of another antioxidant, coenzyme Q (Bentinger et al., 2003), and their suppressive effects in cancer development (oral, skin, bladder, lung, prostate and breast cancer) have been described (Sun and Lotan, 2002).

The most active forms of retinoids are retinol (vitamin A) and retinoic acid while retinyl esters (especially retinyl palmitate) serve as storage forms. Beside retinyl esters, plant carotenoids such as β-carotene are the most important sources of retinoids (particularly retinoic acid; Murray et al., 1993). Three basic structural types of retinoic acid (*all-trans*-retinoic acid (ATRA), 9-*cis*- and 13-*cis*-retinoic acid) are recognized and were shown to have distinct functions (Allenby et al., 1994; Weiler et al., 1999).

Retinoids act via two basic nuclear receptors RXR and RAR. *All-trans*-retinoic acid binds selectively to RAR while the 9-*cis* isomer activates both receptor types (Shago et al., 1997). Both RAR and RXR have three basic subtypes α, β and γ with numerous isoforms that differ in amino- and carboxy-terminal domains (Sun and Lotan, 2002). All receptor variants may form homo and heterodimers and RXR was shown to form dimers with other nuclear receptors (like thyroid, vitamin D or PPAR receptors; Altucci and Gronemeyer, 2001). There are 48 possible RAR–RXR heterodimer complexes that may trigger distinct gene expression (Napoli, 1999).

There are specific differences in tissue localization of retinoid receptors that affect the final mechanisms of retinoid actions. RAR/RXR are able to regulate homeostasis of the whole organism due to different affinities to ligands and/or RAR/RXR-responsive elements on DNA (RAREs) and due to tendency of retinoid receptors to interact with other receptors (Klinge et al., 2001). While RARα and RXRβ seem to be expressed generally in all tissues, RARβ is specific to neural tissues (or skin at lesser extent), skin is dominant in RARγ expression, RXRα is abundant in the kidneys, liver, skin and spleen, while RXRγ is restricted to muscle and brain (Sun and Lotan, 2002).

An important role in retinoid regulation is attributed also to cellular retinol-binding proteins (CRBP) and cellular retinoic acid-binding proteins (CRABP), which sensitively regulate intracellular levels of different retinoid forms. Hence, modulation of levels of these proteins is another sensitive and potent tool of retinoid action autoregulation (Napoli, 1999).

Relatively little is known about mechanisms of disruption of retinoid signaling pathway by xenobiotics. Well-known effects of lack of vitamin A are eye keratinization, xerophthalmia and even blindness (Murray et al., 1993). Reduced levels of retinoids increase the risk of cancer development (Sun and Lotan, 2002). Three mechanisms of retinoid signalling disruption were suggested (Palace et al., 1997). Firstly, the levels and function of retinoids may directly be affected by metabolisation with phases I and II biotransformation enzymes (modulated by xenobiotics, e.g. after AhR activation). Secondly, metabolites of certain compounds like hydroxylated PCBs (van der Plas et al., 2001) may disrupt binding of retinoids to retinoid binding proteins. Finally, the levels of retinoids as known anti-oxidant agents may be disrupted by xenobiotic-induced oxidative stress (Palace et al., 1997).

5.2. Compounds affecting retinoid signalling system

Although relationship between the exposure to POPs and changes in retinoid homeostasis is known for relatively long time (Spear et al., 1992; Brouwer et al., 1989), the mode of action has not yet been exactly elucidated.

Retinoids act as important agents in embryonic cell differentiation and development and any decrease or elevation in their levels may cause adverse effects. A well-known fact is direct teratogenic effect of increased levels of retinoic acid (Kochhar et al., 1996; Deluca, 1991). On the other hand, decrease in embryonal retinoid concentrations in yolk sac of amphibians (Gutleb et al., 1999) and birds (Murk et al., 1994; Boily et al., 2003) as well as in tissues of neonatal rats (Morse and Brouwer, 1995) has been observed after exposures to PCBs and these modulations were related to observed developmental abnormalities.

Exposure of rats in vivo to 2,3,7,8-TCDD leads to mobilization of retinol storage forms in liver while the kidney lecithin:retinol acyltransferase (the key enzyme in retinol transformation to retinyl esters) is greatly increased. This modulation results in the increase of retinyl esters, retinol and retinoic acid levels in kidneys (Nilsson et al., 2000). Mobilization of hepatic retinyl esters increases retinol and retinoic acid levels in serum (Nilsson et al., 2000; Hoegberg et al., 2003). Similar observations were also reported in lake trout after exposure to non-*ortho* PCB 126 (Lind et al., 2000). Other described toxicity mechanisms could involve downregulation of retinoic acid dependent growth factor TGF-β (Lorick et al., 1998) and transglutaminase (Krig et al., 2002) as observed in vitro with 2,3,7,8-TCDD.

As documented, existing research focused only on effects of a few prototypal polyhalogenated hydrocarbons, particularly 2,3,7,8-TCDD or co-planar PCB126. In spite of observed in vivo effects, detailed characterization of other POPs and their mixtures as well as clear description of biochemical toxicity mechanisms is still missing.

5.3. Toxicity assessment—in vivo and in vitro methods

In vivo experimental setups for determination of retinoid-targeted toxicity are mostly derived from standard toxicity tests—developmental, chronic or acute bioassays. Modulation of retinoid levels (i.e. analytical approach) in different tissues of rats and fish after oral exposures to selected environmental contaminants was reported (Palace et al., 1997; Hoegberg et al., 2003; Ndayibagira and Spear, 1999). Also ecotoxicological in vivo studies confirmed the results with rodents and revealed adverse effects of POPs contamination on retinoid levels in wildlife like otters (Simpson et al., 2000), herons (Jenssen et al., 2001), swallows (Martinovic et al., 2003) or fish (Nacci et al., 2001). Carvan et al. (2000) reported progress in development of RAR-responsive transgenic clones of zebrafish (GFP and luciferase reporter genes).

Only few in vitro models were used for assessment of the effects of xenobiotics on RAR/RXR-mediated signalling (Table 1). These included particularly epithelial cells like human keratinocytes SCC-12F (Lorick et al., 1998) or SCC4 (Krig et al., 2002) with the high abundance of RAR, particularly RARα. In vitro modulations of growth factor TGF-β (Lorick et al., 1998) or transglutaminase gene expression (Krig et al., 2002) by TCDD were observed in human keratinocytes. Mouse embryonic P19 cells are often mentioned as suitable model for assessment of both developmental processes and toxicity effects. These pluripotent cells are able to differentiate into cardiac (van der Heyden and Defize, 2003; van der Heyden et al., 2003) or neuronal cells (Seeley and Faustman, 1998) after exposure to ATRA.

6. Thyroid receptors

6.1. Mechanism of action

Thyroid hormones (TH) tetraiodothyronine (thyroxin, T4) and triiodothyronine (T3) belong among the most important metabolic modulators in the living organisms. They act not only as direct enhancers of metabolism via modulation of oxygen consumption, but they also affect activities of other hormones like insulin, glucagon, somatotropin or adrenalin. Their importance in cell differentiation and growth in various tissues as well as crucial role in development of gonads (Cooke et al., 2004) and bones (Abu et al., 1997) was described.

Hypothyroidism during prenatal development was shown to cause severe damage in central nervous system leading from behavioral changes to cretenism (Smith et al., 2002), while low levels of thyroids during early life stages cause megalotestis and increased sperm counts in males (DeVito et al., 1999).

The metabolism of thyroid hormones is quite complex. Their formation in thyroid gland is controlled by a pituitary thyroid-stimulating hormone (TSH). T4, synthetized in thyroid gland, is much less metabolically active than T3, which is formed by tissue specific enzymatic deiodation from T4 (Murray et al., 1993).

Five isoforms of thyroid receptor (TRα1, α2, β1–3) were described so far from which TRα1 does not bind triiodothyronine and seems to act as a repressor of TR action (Tagami et al., 1998). While TRα are expressed in all investigated tissues, TRβ were found primarily in liver, kidney, central nervous system and pituitary gland (Kawakami et al., 2003). After activation of TR, it forms homodimers and also heterodimers with other nuclear receptors, in particular with RXR (Kersten et al., 1998) and these active forms bind to thyroid hormone response elements (TRE) on nuclear DNA (Murray et al., 1993).

Besides TRs, an important role in thyroid cellular levels regulation is attributed to thyroid-binding proteins (TBP; such as thyroid-binding globulin, transthyretin, albumin), which were also shown to be substantially affected by specific xenobiotics (Shi et al., 2002).

6.2. Xenobiotics affecting thyroid signalling system

Many POPs and other compounds have been shown to cause adverse effects at multiple levels of thyroid signalling both in vitro and in vivo.

In vivo decrease of serum thyroid levels leads through negative feedback to TSH release and subsequent increase in weight and histological changes in thyroid gland. These effects of exposure to POPs have been described in mammals, birds, fish and humans (Morse et al., 1996; Langer, 1998), reviewed in Rolland (2000), Brouwer et al. (1998) or Colborn (2002).

Only few compounds have been shown to bind directly to TR. Tetrabromo and tetrachlorobisphenol A induced thyroid-dependent growth in pituitary GH3 cell line at concentrations four to six orders of magnitude higher than T3 (Kitamura et al., 2002). Similar thyroid-like activities were reported for some OH-PCBs as well (Cheek et al., 1999).

However, these compounds have been also shown to strongly disrupt binding of T4 and T3 to corresponding transport proteins. This (together with simultaneous induction of biotransformation hormones like UDP-glucuronosyl transferase; Kohn et al., 1996) results in increased susceptibility to degradation and accelerated depletion of hormones in body (Cheek et al., 1999; Lans et al., 1993). The main disrupting pathway of these compounds as well as of PCBs (Porterfield, 2000), their hydroxylated metabolites and brominated analogs (PBBs; Gerlienke Schuur et al., 1998) or polybrominated diphenylethers (Darnerud, 2003), lies also probably in interaction with TBP (Cheek et al., 1999).

Similar effects were reported for a large group of pesticides like DDT and its metabolites (Cheek et al., 1999), dieldrin (Rathore et al., 2002), pentachlorophenol (Jekat et al., 1994; Ishihara et al., 2003), Alachlor (Cheek et al., 1999; Wilson et al., 1996) or toxaphene (Waritz et al., 1996).

6.3. Toxicity assessment—in vivo and in vitro methods

Measurement of TH serum concentrations in exposed animals and/or human is an often employed screening method for assessment of thyroid system modulations. However, TH levels vary with time and age and caution must be taken in results interpretation, so histological changes in thyroid gland (particularly increased weight and follicular cell number) are better in vivo markers. Developmental toxicity assays evaluating e.g. delayed eye-opening, abnormalities in brain development, increased sperm counts or testes weight were also proposed (DeVito et al., 1999). Another in vivo method for determination of toxicity mediated via TH or TR is a perchlorate discharge test (Atterwill et al., 1987).

An important ex vivo parameter is hepatic UDP-glucuronosyltransferase activity (a marker of enhanced TH clearance from serum; Barter and Klaassen, 1994; Sewall et al., 1995; Kohn et al., 1996; Okazaki and Katayama, 2003).

Several in vitro assays have been proposed for studies of substances that may affect specific thyroid-related processes such as synthesis, metabolism, protein binding and downstream effects (transcription and translation; Table 1). With respect to multiple recognized toxicity mechanisms, battery of assays should be used to characterize chemical potencies to disrupt thyroid signalling.

In vitro methods for assessment of thyroid metabolism like thyroid peroxidase assays (reflecting the TH synthesis; Jones et al., 1996) or deiodinase activity (Hotz et al., 1996) are often employed. Another method is assessment of T4 binding to TBP (particularly transthyretin and thyroxin-binding globulin). Saturation and competitive ligand-binding assays have been conducted with a large number of xenobiotics to estimate and compare disruptive potential (Lans et al., 1994; Cheek et al., 1999; Darnerud et al., 1996).

Numerous in vitro models employing cell lines originating from thyroid or pituitary gland were developed and used for research of TH signalling disruption. Proliferation of a rat pituitary tumor cell line GH3, which is thyroid hormone dependent, was suggested for examination of thyroid-like or thyroid disrupting effects of chemicals (Kitamura et al., 2002). Rat thyroid tumor cell lines FRTL-5, WRT or PC C13 were also used for studies of thyroid signalling and examination of effects of xenobiotics. TSH-dependent growth, iodine uptake or peroxidase production in these cells is a useful tool for both mechanistic studies and toxicity screening (Medina and Santisteban, 2000).

As with several other nuclear receptors (AhR, ER, AR), luciferase reporter gene assays for thyroid signalling were developed and include Chinese hamster ovary cell line CHO transfected with luciferase gene under the transcriptional control of TSH (Sendak et al., 2002; Zimmermann-Belsing et al., 2002), human brain TE671, monkey kidney CV-1 or fall armyworm (insect) Sf9 cells with luciferase expression controlled by TR (Cheek et al., 1999; Iwasaki et al., 2002).

7. Conclusions

Chemicals in the environment which are able to affect signalling system, particularly endocrine disruptors, have been of growing concern for decades. Besides the classical toxicological in vivo tests, use of in vitro methods based mostly on cell lines is steadily increasing. Although these methods are not able to provide the information about behaviour of compounds in real organisms (e.g. pharmacokinetics), they are a strong tool for assessment of specific toxicity mechanisms and/or for screening of toxic potential of large numbers of chemicals (such as agrochemicals, pharmaceuticals or environmental contaminants). A big advantage of these methods is their applicability to evaluation of environmental samples. These methods are generally faster, cheaper and often more sensitive than chemical analysis (even crude extracts may be used for some of them). They provide information about the overall potential of the mixture to interact with the specific signaling pathways without requiring wide spectra of standards necessary for chemical analysis. The screening of complex mixtures from the environment enables prioritising of the samples of interest that require further detailed chemical analysis.

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References

Abu, E.O., Bord, S., Horner, A., Chatterjee, V.K.K., Compston, J.E., 1997. The expression of thyroid hormone receptors in human bone. Bone 21, 137–142.

Allenby, G., Janocha, R., Kazmer, S., Speck, J., Grippo, J.F., Levin, A.A., 1994. Binding of 9-*cis*-retinoic acid and *all-trans*-retinoic acid to retinoic acid receptor-alpha, receptor-beta, and receptor-gamma—retinoic acid receptor-gamma binds *all-trans*-retinoic acid

preferentially over 9-*cis*-retinoic acid. Journal of Biological Chemistry 269, 16689–16695.

Altucci, L., Gronemeyer, H., 2001. Nuclear receptors in cell life and death. Trends in Endocrinology and Metabolism 12, 460–468.

Andersson, P.L., Burght, A.S.A.M.v.d., Berg, M.v.d., Tysklind, M., 2000. Multivariate modelling of polychlorinated biphenyl-induced CYP1A activity in hepatocytes from three different species: ranking scales and species differences. Environmental Toxicology and Chemistry 19, 1454–1463.

Atterwill, C.K., Collins, P., Brown, C.G., Harland, R.F., 1987. The perchlorate discharge test for examining thyroid function in rats. Journal of Pharmacological Methods 18, 199–203.

Avances, C., Georget, V., Terouanne, B., Orio Jr., F., Cussenot, O., Mottet, N., Costa, P., Sultan, C., 2001. Human prostatic cell line PNT1A, a useful tool for studying androgen receptor transcriptional activity and its differential subnuclear localization in the presence of androgens and antiandrogens. Molecular and Cellular Endocrinology 184, 13–24.

Baker, M.A., Cerniglia, G.J., Zaman, A., 1990. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Analytical Biochemistry 190, 360–365.

Baker, V.A., 2001. Endocrine disrupters—testing strategies to assess human hazard. Toxicology in Vitro 15, 413–419.

Balaguer, P., Francois, F., Comunale, F., Fenet, H., Boussioux, A.-M., Pons, M., Nicolas, J.-C., Casellas, C., 1999. Reporter cell lines to study the estrogenic effects of xenoestrogens. The Science of the Total Environment 233, 47–56.

Barter, R.A., Klaassen, C.D., 1994. Reduction of thyroid hormone levels and alteration of thyroid function by four representative UDP-glucuronosyltransferase inducers in rats. Toxicology and Applied Pharmacology 128, 9–17.

Behnisch, P.A., Hosoe, K., Sakai, S., 2001a. Bioanalytical screening methods for dioxins and dioxin-like compounds—a review of bioassay/biomarker technology. Environment International 27, 413–439.

Behnisch, P.A., Hosoe, K., Sakai, S.-i., 2001b. Combinatorial bio/chemical analysis of dioxin and dioxin-like compounds in waste recycling, feed/food, humans/wildlife and the environment. Environment International 27, 495–519.

Bentinger, M., Turunen, M., Zhang, X.X., Wan, Y.J.Y., Dallner, G., 2003. Involvement of retinoid X receptor alpha in coenzyme Q metabolism. Journal of Molecular Biology 326, 795–803.

Berg, C., Halldin, K., Brunstrom, B., Brandt, I., 1998. Methods for studying xenoestrogenic effects in birds. Toxicology Letters 28, 103671–103676.

Berghard, A., Gradin, K., Pongratz, I., Whitelaw, M., Poellinger, L., 1993. Cross-coupling of signal transduction pathways—the dioxin receptor mediates induction of cytochrome P-4501A1 expression via a protein kinase-c-dependent mechanism. Molecular and Cellular Biology 13, 677–689.

Berns, E., de Boer, W., Mulder, E., 1986. The androgen responsive human prostate tumor cell line LNCaP: androgen receptors and the effect of androgens on the release of proteins. Journal of Steroid Biochemistry 25, 247–259.

Besselink, H.T., van Beusekom, S., Roex, E., Vethaak, A.D., Koeman, J.H., Brouwer, A., 1996. Low hepatic 7-ethoxyresorufin-*O*-deethylase (EROD) activity and minor alterations in retinoid and thyroid hormone levels in flounder (*Platichthys flesus*) exposed to the polychlorinated biphenyl (PCB) mixture, clophen A50. Environmental Pollution 92, 267–274.

Blaha, L., Kapplova, P., Vondracek, J., Upham, B., Machala, M., 2002. Inhibition of gap-junctional intercellular communication by environmentally occurring polycyclic aromatic hydrocarbons. Toxicological Sciences 65, 43–51.

Blankenship, A., Kannan, K., Villalobos, S., Villeneuve, D., Falandysz, J., Imagawa, T., Jakobsson, E., Giesy, J., 1999. Relative potencies of Hallowax mixtures and individual PCNs to induce Ah receptor-mediated responses in the rat hepatoma H4IIE-luc cell bioassay. Organohalogen Compound 42, 217–220.

Blankvoort, B.M.G., de Groene, E.M., van Meeteren-Kreikamp, A.P., Witkamp, R.F., Rodenburg, R.J.T., Aarts, J., 2001. Development of an androgen reporter gene assay (AR-LUX) utilizing a human cell line with an endogenously regulated androgen receptor. Analytical Biochemistry 298, 93–102.

Blok, L.J., Themmen, A.P.N., Peters, A.H.F.M., Trapman, J., Baarends, W.M., Hoogerbrugge, J.W., Grootegoed, J.A., 1992. Transcriptional regulation of androgen receptor gene expression in Sertoli cells and other cell types. Molecular and Cellular Endocrinology 88, 153–164.

Boily, M.H., Ndayibagira, A., Spear, P.A., 2003. Retinoid metabolism (lrat, reh) in the yolk-sac membrane of japanese quail eggs and effects of mono-*ortho*-PCBs. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 134, 11–23.

Bols, N.C., Schirmer, K., Joyce, E.M., Dixon, D.G., Greenberg, B.M., Whyte, J.J., 1999. Ability of polycyclic aromatic hydrocarbons to induce 7-ethoxyresorufin-*O*-deethylase activity in a trout liver cell line. Ecotoxicology and Environmental Safety 44, 118–128.

Bon, E., Barbe, U., Rodriguez, J.N., Cuisset, B., Pelissero, C., Sumpter, J.P., LeMenn, F., 1997. Plasma vitellogenin levels during the annual reproductive cycle of the female rainbow trout (*Oncorhynchus mykiss*): Establishment and validation of an elisa. Comparative Biochemistry and Physiology B—Biochemistry and Molecular Biology 117, 75–84.

Bosveld, A.T.C., Kennedy, S.W., Seinen, W., Berg, M.v.d., 1997. Erod inducing potencies of planar chlorinated aromatic hydrocarbons in primary cultures of hepatocytes from different development stages of the chicken. Archives of Toxicology 71, 746–750.

Brandi, M.L., Rotella, C.M., Mavilia, C., Franceschelli, F., Tanini, A., Toccafondi, R., 1987. Insulin stimulates cell growth of a new strain of differentiated rat thyroid cells*1. Molecular and Cellular Endocrinology 54, 91–103.

Brouwer, A., Hakansson, H., Kukler, A., van Den Berg, K.J., Ahlborg, U.G., 1989. Marked alterations in retinoid homeostasis of sprague–dawley rats induced by a single i.P. Dose of 10 μg/kg of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Toxicology 58, 267–283.

Brouwer, A., Morse, D.C., Lans, M.C., Schuur, A.G., Murk, A.J., Klasson-Wehler, E., Bergman, A., Visser, T.J., 1998. Interactions of persistent environmental organohalogens with the thyroid hormone system: Mechanisms and possible consequences for animal and human health. Toxicology and Industrial Health 14, 59–84.

Cai, Y., Konishi, T., Han, G., Campwala, K.H., French, S.W., Wan, Y.-J.Y., 2002. The role of hepatocyte RXRα in xenobiotic-sensing nuclear receptor-mediated pathways. European Journal of Pharmaceutical Sciences 15, 89–96.

Carvan 3rd, M.J., Dalton, T.P., Stuart, G.W., Nebert, D.W., 2000. Transgenic zebrafish as sentinels for aquatic pollution. Annals of The New York Academy of Sciences 919, 133–147.

Celius, T., Haugen, T.B., Grotmol, T., Walther, B.T., 1999. A sensitive zonagenetic assay for rapid in vitro assessment of estrogenic potency of xenobiotics and mycotoxins. Environmental Health Perspectives 107, 63–68.

Celius, T., Matthews, J.B., Giesy, J.P., Zacharewski, T.R., 2000. Quantification of rainbow trout (*Oncorhynchus mykiss*) zona radiata and vitellogenin mRNA levels using real-time PCR after in vivo treatment with estradiol-17 beta or alpha-zearalenol. Journal of Steroid Biochemistry and Molecular Biology 75, 109–119.

Chapin, R.E., Stevens, J.T., Hughes, C.L., Kelce, W.R., Hess, R.A., Daston, G.P., 1996. Endocrine modulation of reproduction. Fundamental and Applied Toxicology 29, 1–17.

Cheek, A.O., Kow, K., Chen, J., McLachlan, J.A., 1999. Potential mechanisms of thyroid disruption in humans: interaction of

organochlorine compounds with thyroid receptor, transthyretin, and thyroid-binding globulin. Environmental Health Perspectives 107, 273–278.

Chen, Y.H., Tukey, R.H., 1996. Protein kinase *c* modulates regulation of the CYP1A1 gene by the aryl hydrocarbon receptor. Journal of Biological Chemistry 271, 26261–26266.

Clemons, J.H., Dixon, D.G., Bols, N.C., 1997. Derivation of 2,3,7,8-TCDD toxic equivalent factors (TEFs) for selected dioxins, furans and PCBs with rainbow trout and rat liver cell lines and the influence of exposure time. Chemosphere 34, 1105–1119.

Colborn, T., 2002. Clues from wildlife to create an assay for thyroid system disruption. Environmental Health Perspectives 110, 363–367.

Coldham, N., Dave, M., Sivapathasundaram, S., McDonnell, D., Connor, C., Sauer, M., 1997. Evaluation of a recombinant yeast cell estrogen screening assay. Environmental Health Perspectives 105, 734–742.

Combes, R.D., 2000. Endocrine disruptors: A critical review of in vitro and in vivo testing strategies for assessing their toxic hazard to humans. ATLA—Alternatives to Laboratory Animals 28, 81–118.

Cooke, P.S., Holsberger, D.R., Witorsch, R.J., Sylvester, P.W., Meredith, J.M., Treinen, K.A., Chapin, R.E., 2004. Thyroid hormone, glucocorticoids, and prolactin at the nexus of physiology, reproduction, and toxicology. Toxicology and Applied Pharmacology 194, 309–335.

Dai, D., Cao, Y., Falls, G., Levi, P.E., Hodgson, E., Rose, R.L., 2001. Modulation of mouse P450 isoforms CYP1A2, CYP2B10, CYP2E1, and CYP3A by the environmental chemicals mirex, 2,2-bis(*p*-chlorophenyl)-1,1-dichloroethylene, vinclozolin, and flutamide. Pesticide Biochemistry and Physiology 70, 127–141.

Darnerud, P.O., 2003. Toxic effects of brominated flame retardants in man and in wildlife. Environment International 29, 841–853.

Darnerud, P.O., Morse, D., Klasson-Wehler, E., Brouwer, A., 1996. Binding of a 3,3′,4,4′-tetrachlorobiphenyl (CB-77) metabolite to fetal transthyretin and effects on fetal thyroid hormone levels in mice. Toxicology 106, 105–114.

Davison, S.L., Davis, S.R., 2003. Androgens in women. The Journal of Steroid Biochemistry and Molecular Biology 85, 363–366.

Degen, G.H., 1990. Role of prostaglandin-h synthase in mediating genotoxic and carcinogenic effects of estrogens. Environmental Health Perspectives 88, 217–223.

DeHaan, L.H.J., Halfwerk, S., Hovens, S.E.L., DeRoos, B., Koeman, J.H., Brouwer, A., 1996. Inhibition of intercellular communication and induction of ethoxyresorufin-*O*-deethylase activity by polychlorobiphenyls, dibenzo-*p*-dioxins and dibenzofurans in mouse Hepa1c1c7 cells. Environmental Toxicology and Pharmacology 1, 27–37.

Deluca, L.M., 1991. Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. Faseb Journal 5, 2924–2933.

Denison, M.S., Pandini, A., Nagy, S.R., Baldwin, E.P., Bonati, L., 2002. Ligand binding and activation of the Ah receptor. Chemico-Biological Interactions 141, 3–24.

DeVito, M., Biegel, L., Brouwer, A., Brown, S., Brucker-Davis, F., Cheek, A.O., Christensen, R., Colborn, T., Cooke, P., Crissman, J., Crofton, K., Doerge, D., Gray, E., Hauser, P., Hurley, P., Kohn, M., Lazar, J., McMaster, S., McClain, M., McConnell, E., Meier, C., Miller, R., Tietge, J., Tyl, R., 1999. Screening methods for thyroid hormone disruptors. Environmental Health Perspectives 107, 407–415.

Diaz-Ferrero, J., Rodriguez-Larena, M.C., Comellas, L., Jimenez, B., 1997. Bioanalytical methods applied to endocrine disrupting polychlorinated biphenyls, polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans. A review. Trends in Analytical Chemistry 16, 563–573.

Diel, P., Olff, S., Schmidt, S., Michna, H., 2002. Effects of the environmental estrogens bisphenol A, *o*,*p*′-DDT, *p*-*tert*-octylphenol and coumestrol on apoptosis induction, cell proliferation and the expression of estrogen sensitive molecular parameters in the human breast cancer cell line MCF-7. The Journal of Steroid Biochemistry and Molecular Biology 80, 61–70.

Dietze, E.C., Caldwell, L.E., Marcom, K., Collins, S.J., Yee, L., Swisshelm, K., Hobbs, K.B., Bean, G.R., Seewaldt, V.L., 2002. Retinoids and retinoic acid receptors regulate growth arrest and apoptosis in human mammary epithelial cells and modulate expression of cbp/p300. Microscopy Research and Technique 59, 23–40.

Drahushuk, A.T., McGarrigle, B.P., Larsen, K.E., Stegeman, J.J., Olson, J.R., 1998. Detection of CYP1A1 protein in human liver and induction by TCDD in precision-cut liver slices incubated in dynamic organ culture. Carcinogenesis 19, 1361–1368.

Drummond, A.E., Britt, K.L., Dyson, M., Jones, M.E., Kerr, J.B., O'Donnell, L., Simpson, E.R., Findlay, J.K., 2002. Ovarian steroid receptors and their role in ovarian function. Molecular and Cellular Endocrinology 191, 27–33.

Esquenet, M., Swinnen, J.V., Heyns, W., Verhoeven, G., 1995. Triiodothyronine modulates growth, secretory function and androgen receptor concentration in the prostatic carcinoma cell line LNCaP. Molecular and Cellular Endocrinology 109, 105–111.

Fang, H., Tong, W.D., Perkins, R., Soto, A.M., Prechtl, N.V., Sheehan, D.M., 2000. Quantitative comparisons of in vitro assays for estrogenic activities. Environmental Health Perspectives 108, 723–729.

Fent, K., 2001. Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples. Toxicology in Vitro 15, 477–488.

Fent, K., Batscher, R., 2000. Cytochrome P4501A induction potencies of polycyclic aromatic hydrocarbons in a fish hepatoma cell line: Demonstration of additive interactions. Environmental Toxicology and Chemistry 19, 2047–2058.

Flototto, T., Djahansouzi, S., Glaser, M., Hanstein, B., Niederacher, D., Brumm, C., Beckmann, M.W., 2001. Hormones and hormone antagonists: mechanisms of action in carcinogenesis of endometrial and breast cancer. Hormone and Metabolic Research 33, 451–457.

Focant, J.-F., Pirard, C., Thielen, C., De Pauw, E., 2002. Levels and profiles of PCDDs, PCDFs and cPCBs in belgian breast milk: estimation of infant intake. Chemosphere 48, 763–770.

Frigo, D.E., Burow, M.E., Mitchell, K.A., Chiang, T.C., McLachlan, J.A., 2002. DDT and its metabolites alter gene expression in human uterine cell lines through estrogen receptor-independent mechanisms. Environmental Health Perspectives 110, 1239–1245.

Gagne, F., Blaise, C., 1998. Estrogenic properties of municipal and industrial wastewaters evaluated with a rapid and sensitive chemoluminescent in situ hybridization assay (CISH) in rainbow trout hepatocytes. Aquatic Toxicology 44, 83–91.

Gerlienke Schuur, A., Brouwer, A., Bergman, A., Coughtrie, M.W.H., Visser, T.J., 1998. Inhibition of thyroid hormone sulfation by hydroxylated metabolites of polychlorinated biphenyls. Chemico-Biological Interactions 109, 293–297.

Giesy, J.P., Hilscherova, K., Jones, P.D., Kannan, K., Machala, M., 2002. Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. Marine Pollution Bulletin 45, 3–16.

Gillesby, B.E., Zacharewski, T.R., 1998. Exoestrogens: Mechanisms of action and strategies for identification and assessment. Environmental Toxicology and Chemistry 17, 3–14.

Gottlicher, M., Heck, S., Herrlich, P., 1998. Transcriptional cross-talk, the second mode of steroid hormone receptor action. Journal of Molecular Medicine 76, 480–489.

Gray Jr., L.E., 1998. Tiered screening and testing strategy for xenoestrogens and antiandrogens. Toxicology Letters 102-103, 677–680.

Gray, L.E., Ostby, J.S., Kelce, W.R., 1994. Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex

differentiation of the male rat. Toxicology and Applied Pharmacology 129, 46–52.

Gray, L.E., Kelce, W.R., Wiese, T., Tyl, R., Gaido, K., Cook, J., Klinefelter, G., Desaulniers, D., Wilson, E., Zacharewski, T., 1997. Endocrine screening methods workshop report: detection of estrogenic and androgenic hormonal and antihormonal activity for chemicals that act via receptor or steroidogenic enzyme mechanisms. Reproductive Toxicology 11, 719–750.

Gray, J.P., Leas, T.L., Obert, E., Brown, D., Clark, G.C., van den Heuvel, J.P., 2003. Evidence of aryl hydrocarbon receptor ligands in Presque Isle Bay of Lake Erie. Aquatic Toxicology 64, 343–358.

Grigoryev, D.N., Long, B.J., Njar, V.C.O., Brodie, A.H.M., 2000. Pregnenolone stimulates LNCaP prostate cancer cell growth via the mutated androgen receptor. The Journal of Steroid Biochemistry and Molecular Biology 75, 1–10.

Guigal, N., Seree, E., Nguyen, Q.B., Charvet, B., Desobry, A., Barra, Y., 2001. Serum induces a transcriptional activation of CYP1A1 gene in HepG2 independently of the AhR pathway. Life Sciences 68, 2141–2150.

Gupta, M., McDougal, A., Safe, S., 1998. Estrogenic and antiestrogenic activities of 16 alpha- and 2-hydroxy metabolites of 17 beta-estradiol in MCF-7 and T47D human breast cancer cells. The Journal of Steroid Biochemistry and Molecular Biology 67, 413–419.

Gutendorf, B., Westendorf, J., 2001. Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. Toxicology 166, 79–89.

Gutleb, A.C., Appelman, J., Bronkhorst, M.C., van den Berg, J.H.J., Spenkelink, A., Brouwer, A., Murk, A.J., 1999. Delayed effects of pre- and early-life time exposure to polychlorinated biphenyls on tadpoles of two amphibian species (*Xenopus laevis* and *Rana temporaria*). Environmental Toxicology and Pharmacology 8, 1–14.

Hackenberg, R., Hawighorst, T., Filmer, A., Slater, E.P., Bock, K., Beato, M., Schulz, K.-D., 1992. Regulation of androgen receptor mRNA and protein level by steroid hormones in human mammary cancer cells. The Journal of Steroid Biochemistry and Molecular Biology 43, 599–607.

Haendler, B., Schuttke, I., Schleuning, W.-D., 2001. Androgen receptor signalling: comparative analysis of androgen response elements and implication of heat-shock protein 90 and 14-3-3 eta. Molecular and Cellular Endocrinology 173, 63–73.

Hamers, T., van Schaardenburg, M.D., Felzel, E.C., Murk, A.J., Koeman, J.H., 2000. The application of reporter gene assays for the determination of the toxic potency of diffuse air pollution. Science of the Total Environment 262, 159–174.

Harvey, C.B., Williams, G.R., 2002. Mechanism of thyroid hormone action. Thyroid 12, 441–446.

Hess, R.A., Bunick, D., Bahr, J., 2001. Oestrogen, its receptors and function in the male reproductive tract—a review. Molecular and Cellular Endocrinology 178, 29–38.

Hilscherova, K., Machala, M., Kannan, K., Blankenship, A.L., Giesy, J.P., 2000. Cell bioassay for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples—review. Environmental Science and Pollution Research 7, 159–171.

Hoegberg, P., Schmidt, C.K., Nau, H., Ross, A.C., Zolfaghari, R., Fletcher, N., Trossvik, C., Nilsson, C.B., Hakansson, H., 2003. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin induces lecithin: retinol acyltransferase transcription in the rat kidney. Chemico-Biological Interactions 145, 1–16.

Hotz, C.S., Belonje, B., Fitzpatrick, D.W., L'abbe, M.R., 1996. A method for the determination of type i iodothyronine deiodinase activity in liver and kidney using ^{125}I-labelled reverse triiodothyronine as a substrate*1. Clinical Biochemistry 29, 451–456.

Ikeuchi, T., Todo, T., Kobayashi, T., Nagahama, Y., 2001. Two subtypes of androgen and progestogen receptors in fish testes. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 129, 449–455.

Ishihara, A., Sawatsubashi, S., Yamauchi, K., 2003. Endocrine disrupting chemicals: interference of thyroid hormone binding to transthyretins and to thyroid hormone receptors. Molecular and Cellular Endocrinology 199, 105–117.

Iwasaki, T., Miyazaki, W., Takeshita, A., Kuroda, Y., Koibuchi, N., 2002. Polychlorinated biphenyls suppress thyroid hormone-induced transactivation. Biochemical and Biophysical Research Communications 299, 384–388.

Jacobs, M.N., Dickins, M., Lewis, D.F.V., 2003. Homology modelling of the nuclear receptors: human oestrogen receptorβ (hERβ), the human pregnane-X-receptor (PXR), the Ah receptor (AhR) and the constitutive androstane receptor (CAR) ligand binding domains from the human oestrogen receptor α (hERα) crystal structure, and the human peroxisome proliferator activated receptor α (PPARα) ligand binding domain from the human PPARγ crystal structure. The Journal of Steroid Biochemistry and Molecular Biology 84, 117–132.

Jekat, F.W., Meisel, M.L., Eckard, R., Winterhoff, H., 1994. Effects of pentachlorophenol PCP) on the pituitary and thyroidal hormone regulation in the rat. Toxicology Letters 71, 9–25.

Jenssen, B.M., Nilssen, V.H., Murvoll, K.M., Skaare, J.U., 2001. PCBs, TEQs and plasma retinol in grey heron (*Ardea cinerea*) hatchlings from two rookeries in norway. Chemosphere 44, 483–489.

Joly-Pharaboz, M.-O., Ruffion, A., Roch, A.-M., Michel-Calemard, L., Andre, J., Chantepie, J., Nicolas, B., Panaye, G., 2000. Inhibition of growth and induction of apoptosis by androgens of a variant of LNCaP cell line. The Journal of Steroid Biochemistry and Molecular Biology 73, 237–249.

Jones, J.M., Anderson, J.W., 1999. Relative potencies of PAHs and PCBs and based on the response of human cells. Environmental Toxicology and Pharmacology 7, 19–26.

Jones, P.A., Pendlington, R.U., Earl, L.K., Sharma, R.K., Barratt, M.D., 1996. In vitro investigations of the direct effects of complex anions on thyroidal iodide uptake: identification of novel inhibitors. Toxicology in Vitro 10, 149–160.

Jungbauer, A., Beck, V., 2002. Yeast reporter system for rapid determination of estrogenic activity. Journal of Chromatography B 777, 167–178.

Kato, S., Masuhiro, Y., Watanabe, M., Kobayashi, Y., Takeyama, K., Endoh, H., Yanagisawa, J., 2000. Molecular mechanism of a cross-talk between oestrogen and growth factor signaling pathways. Genes to Cells 5, 593–601.

Kawakami, Y., Tanda, M., Adachi, S., Yamauchi, K., 2003. Characterization of thyroid hormone receptor α and β in the metamorphosing japanese conger eel, *Conger myriaster*. General and Comparative Endocrinology 132, 321–332.

Kelce, W.R., Wilson, E.M., 1997. Environmental antiandrogens: developmental effects, molecular mechanisms, and clinical implications. Journal of Molecular Medicine-JMM 75, 198–207.

Kelce, W.R., Monosson, E., Gamcsik, M.P., Laws, S.C., Gray, L.E., 1994. Environmental hormone disruptors: evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites. Toxicology and Applied Pharmacology 126, 276–285.

Kelce, W.R., Lambright, C.R., Gray, J., Earl, L., Roberts, K.P., 1997. Vinclozolin and *p,p′*-DDE alter androgen-dependent gene expression: In vivo confirmation of an androgen receptor-mediated mechanism. Toxicology and Applied Pharmacology 142, 192–200.

Kersten, S., Dong, D., Lee, W.-Y., Reczek, P.R., Noy, N., 1998. Autosilencing by the retinoid X receptor 1. Journal of Molecular Biology 284, 21–32.

Kim, B.H., Takemura, A., 2003. Culture conditions affect induction of vitellogenin synthesis by estradiol-17 beta in primary cultures of *Tilapia* hepatocytes. Comparative Biochemistry and Physiology B—Biochemistry and Molecular Biology 135, 231–239.

Kimura, T., van Keymeulen, A., Golstein, J., Fusco, A., Dumont, J.E., Roger, P.P., 2001. Regulation of thyroid cell proliferation by TSH and other factors: a critical evaluation of in vitro models. Endocrine Reviews 22, 631–656.

Kitamura, S., Jinno, N., Ohta, S., Kuroki, H., Fujimoto, N., 2002. Thyroid hormonal activity of the flame retardants tetrabromobisphenol A and tetrachlorobisphenol A. Biochemical and Biophysical Research Communications 293, 554–559.

Kizu, R., Ishii, K., Kobayashi, J., Hashimoto, T., Koh, E., Namiki, M., Hayakawa, K., 2000. Antiandrogenic effect of crude extract of C-heavy oil. Materials Science and Engineering: C 12, 97–102.

Klinge, C.M., Jernigan, S.C., Risinger, K.E., Lee, J.E., Tyulmenkov, V.V., Falkner, K.C., Prough, R.A., 2001. Short heterodimer partner (SHP) orphan nuclear receptor inhibits the transcriptional activity of aryl hydrocarbon receptor (AhR)/AhR nuclear translocator (ARNT). Archives of Biochemistry and Biophysics 390, 64–70.

Kloas, W., Lutz, I., Einspanier, R., 1999. Amphibians as a model to study endocrine disruptors. II. Estrogenic activity of environmental chemicals in vitro and in vivo. Science of the Total Environment 225, 59–68.

Knudsen, F.R., Arukwe, A., Pottinger, T.G., 1998. The in vivo effect of combinations of octylphenol, butylbenzylphthalate and estradiol on liver estradiol receptor modulation and induction of zona radiata proteins in rainbow trout: no evidence of synergy. Environmental Pollution 103, 75–80.

Kochhar, D.M., Jiang, H., Penner, J.D., Beard, R.L., Chandraratna, R.A.S., 1996. Differential teratogenic response of mouse embryos to receptor selective analogs of retinoic acid. Chemico-Biological Interactions 100, 1–12.

Kogai, T., Schultz, J.J., Johnson, L.S., Huang, M., Brent, G.A., 2000. Retinoic acid induces sodium/iodide symporter gene expression and radioiodide uptake in the MCF-7 breast cancer cell line. Proceedings of the National Academy of Sciences of the United States of America 97, 8519–8524.

Kohn, M.C., Sewall, C.H., Lucier, G.W., Portier, C.J., 1996. A mechanistic model of effects of dioxin on thyroid hormones in the rat. Toxicology and Applied Pharmacology 136, 29–48.

Koistinen, J., Sanderson, J.T., Giesy, J.P., 1996. Ethoxyresorufin-*O*-deethylase induction potency of polychlorinated diphenyl ethers in H4IIE rat hepatoma cells. Environmental Toxicology and Chemistry 15, 2028–2034.

Korkalainen, M., Tuomisto, J., Pohjanvirta, R., 2003. Identification of novel splice variants of ARNT and ARNT2 in the rat. Biochemical and Biophysical Research Communications 303, 1095–1100.

Krig, S.R., Chandraratna, A.S., Chang, M.M.J., Wu, R., Rice, R.H., 2002. Gene-specific TCDD suppression of RAR alpha- and RXR-mediated induction of tissue transglutaminase. Toxicological Sciences 68, 102–108.

Kurebayashi, J., Otsuki, T., Yamamoto, S., Kurosumi, M., Nakata, T., Akinaga, S., Sonoo, H., 1998. A pure antiestrogen, ICI 182,780, stimulates the growth of tamoxifen-resistant KPL-1 human breast cancer cells in vivo but not in vitro. Oncology 55, 23–33.

Kuruto-Niwa, R., Terao, Y., Nozawa, R., 2002. Identification of estrogenic activity of chlorinated bisphenol A using a GFP expression system. Environmental Toxicology and Pharmacology 12, 27–35.

Kyakumoto, S., Nemoto, T., Sato, N., Ota, M., 1997. Expression of retinoid X receptors and COUP-TFI in a human salivary gland adenocarcinoma cell line. Biochemistry and Cell Biology—Biochimie Et Biologie Cellulaire 75, 749–758.

Langer, P., 1998. Polychlorinated biphenyls and the thyroid gland—minireview. Endocrine Regulations 32, 193–203.

Lans, M.C., Klasson-Wehler, E., Willemsen, M., Meussen, E., Safe, S., Brouwer, A., 1993. Structure-dependent, competitive interaction of hydroxy-polychlorobiphenyls, -dibenzo-*p*-dioxins and -dibenzofurans with human transthyretin. Chemico-Biological Interactions 88, 7–21.

Lans, M.C., Spiertz, C., Brouwer, A., Koeman, J.H., 1994. Different competition of thyroxine-binding to transthyretin and thyroxine-binding globulin by hydroxy-PCBs, PCDDs and PCDFs. European Journal of Pharmacology-Environmental Toxicology and Pharmacology Section 270, 129–136.

Lascombe, I., Beffa, D., Ruegg, U., Tarradellas, J., Wahli, W., 2000. Estrogenic activity assessment of environmental chemicals using in vitro assays: identification of two new estrogenic compounds. Environmental Health Perspectives 108, 621–629.

Latonnelle, K., Le Menn, F., Kaushik, S.J., Bennetau-Pelissero, C., 2002. Effects of dietary phytoestrogens in vivo and in vitro in rainbow trout and siberian sturgeon: interests and limits of the in vitro studies of interspecies differences. General and Comparative Endocrinology 126, 39–51.

Lebail, J.C., Marrefournier, F., Nicolas, J.C., Habrioux, G., 1998. C-19 steroids estrogenic activity in human breast cancer cell lines: importance of dehydroepiandrosterone sulfate at physiological plasma concentration. Steroids 63, 678–683.

Lee, H.J., Lee, Y.S., Kwon, H.B., Lee, K., 2003. Novel yeast bioassay system for detection of androgenic and antiandrogenic compounds. Toxicology in Vitro 17, 237–244.

Legler, J., Bouwman, L., Murk, T., Brouwer, A., 1996. Determination of dioxin- and estrogen-like activity in sediment extracts using in vitro calux assay. Organohalogen Compounds 29, 347–352.

Legler, J., Brink, C.E., Brouwer, A., Murk, A.J., Saag, P.T., Vethaak, A.D., Burg, B., 1999. Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. Toxicological Sciences 48, 55–66.

Legler, J., Dennekamp, M., Vethaak, A.D., Brouwer, A., Koeman, J.H., van der Burg, B., Murk, A.J., 2002a. Detection of estrogenic activity in sediment-associated compounds using in vitro reporter gene assays. The Science of the Total Environment 293, 69–83.

Legler, J., Zeinstra, L.M., Schuitemaker, F., Lanser, P.H., Bogerd, J., Brouwer, A., Vethaak, A.D., De Voogt, P., Murk, A.J., van der Burg, B., 2002b. Comparison of in vivo and in vitro reporter gene assays for short-term screening of estrogenic activity. Environmental Science and Technology 36, 4410–4415.

Lind, P.M., Larsson, S., Oxlund, H., Hakansson, H., Nyberg, K., Eklund, T., Orberg, J., 2000. Change of bone tissue composition and impaired bone strength in rats exposed to 3,3′,4,4′,5-pentachlorobiphenyl (PCB126). Toxicology 150, 41–51.

List, H.-J., Smith, C.L., Martinez, E., Harris, V.K., Danielsen, M., Riegel, A.T., 2000. Effects of antiandrogens on chromatin remodeling and transcription of the integrated mouse mammary tumor virus promoter. Experimental Cell Research 260, 160–165.

Lorick, K.L., Toscano, D.L., Toscano, W.A., 1998. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin alters retinoic acid receptor function in human keratinocytes. Biochemical and Biophysical Research Communications 243, 749–752.

Machala, M., Vondracek, J., 1998. Estrogenic activity of xenobiotics. Veterinarni Medicina 43, 311–317.

Machala, M., Ciganek, M., Bláha, L., Minksová, K., Vondráèek, J., 2001a. Aryl hydrocarbon receptor-mediated and estrogenic activities of oxygenated polycyclic aromatic hydrocarbons and azaarenes originally identified in extracts of river sediments. Environmental Toxicology and Chemistry 20, 2736–2743.

Machala, M., Vondracek, J., Blaha, L., Ciganek, M., Neca, J., 2001b. Aryl hydrocarbon receptor-mediated activity of mutagenic polycyclic aromatic hydrocarbons determined using in vitro reporter gene assay. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 497, 49–62.

Machala, M., Blaha, L., Lehmler, H.J., Pliskova, M., Majkova, Z., Kapplova, P., Sovadinova, I., Vondracek, J., Malmberg, T., Robertson, L.W., 2004. Toxicity of hydroxylated and quinoid PCB metabolites: inhibition of gap junctional intercellular communication and activation of aryl hydrocarbon and estrogen

receptors in hepatic and mammary cells. Chemical Research in Toxicology 17, 340–347.

Mantovani, A., Stazi, A.V., Macri, C., Maranghi, F., Ricciardi, C.R., 1999. Problems in testing and risk assessment of endocrine disrupting chemicals with regard to developmental toxicology. Chemosphere 39, 1293–1300.

Marilley, D., Robyr, D., Schildpoulter, C., Wahli, W., 1998. Regulation of the vitellogenin gene B1 promoter after transfer into hepatocytes in primary cultures. Molecular Cell Endocrinology 141, 79–93.

Martinovic, B., Lean, D.R.S., Bishop, C.A., Birmingham, E., Secord, A., Jock, K., 2003. Health of tree swallow (*Tachycineta bicolor*) nestlings exposed to chlorinated hydrocarbons in the St Lawrence River basin. Part I. Renal and hepatic vitamin A concentrations. Journal of Toxicology and Environmental Health. Part A 66, 1053–1072.

Massaad, C., Entezami, F., Massade, L., Benahmed, M., Olivennes, F., Barouki, R., Hamamah, S., 2002. How can chemical compounds alter human fertility? European Journal of Obstetrics and Gynecology and Reproductive Biology 100, 127–137.

McLachlan, M.S., 1993. Exposure toxicity equivalents (ETEs)—a plea for more environmental chemistry in dioxin risk assessment. Chemosphere 27, 483–490.

Medina, D.L., Santisteban, P., 2000. Thyrotropin-dependent proliferation of in vitro rat thyroid cell systems. European Journal of Endocrinology 143, 161–178.

Meek, M.D., 1998. Ah receptor and estrogen receptor-dependent modulation of gene expression by extracts of diesel exhaust particles. Environmental Research 79, 114–121.

Merchant, M., Safe, S., 1995. In vitro inhibition of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced activity by α-naphthoflavone and 6-methyl-1,3,8-trichlorodibenzofuran using an aryl hydrocarbon (Ah)-responsive construct. Biochemical Pharmacology 50, 663–668.

Miller, M.G., Kapron, C.M., Metcalfe, C.D., Lee, L.E.J., 2000. Down-regulation of fibronectin in rainbow trout gonadal cells exposed to retinoic acid. Aquatic Toxicology 48, 119–125.

Moore, M., Mustain, M., Daniel, K., Chen, I., Safe, S., Zacharewski, T., Gillesby, B., Joyeux, A., Balaguer, P., 1997. Antiestrogenic activity of hydroxylated polychlorinated biphenyl congeners identified in human serum. Toxicology and Applied Pharmacology 142, 160–168.

Morse, D.C., Brouwer, A., 1995. Fetal, neonatal, and long-term alterations in hepatic retinoid levels following maternal polychlorinated biphenyl exposure in rats. Toxicology and Applied Pharmacology 131, 175–182.

Morse, D.C., Wehler, E.K., Wesseling, W., Koeman, J.H., Brouwer, A., 1996. Alterations in rat brain thyroid hormone status following pre- and postnatal exposure to polychlorinated biphenyls (aroclor 1254). Toxicology and Applied Pharmacology 136, 269–279.

Murk, A.J., Bosveld, A.T.C., van den Berg, M., Brouwer, A., 1994. Effects of polyhalogenated aromatic hydrocarbons (PHAHs) on biochemical parameters in chicks of the common tern (sterna hirundo). Aquatic Toxicology 30, 91–115.

Murk, A.J., Legler, J., Denison, M.S., Giesy, J.P., van de Guchte, C., Brouwer, A., 1996. Chemical-activated luciferase gene expression (CALUX): a novel in vitro bioassay for Ah receptor active compounds in sediments and pore water. Fundamental and Applied Toxicology 33, 149–160.

Murk, A.J., Leonards, P.E.G., van Hattum, B., Luit, R., van der Weiden, M.E.J., Smit, M., 1998. Application of biomarkers for exposure and effect of polyhalogenated aromatic hydrocarbons in naturally exposed european otters (*Lutra lutra*). Environmental Toxicology and Pharmacology 6, 91–102.

Murray, R.K., Granner, D.K., Mayes, P.A., Rodwell, V.W., 1993. Harper's Biochemistry. Appleton & Lange, a Publishing division of Prentice-Hall, Int. Inc., East Norwalk, Connecticut, pp. 405–582.

Nacci, D., Jayaraman, S., Specker, J., 2001. Stored retinoids in populations of the estuarine fish fundulus heteroclitus indigenous to PCB-contaminated and reference sites. Archives of Environmental Contamination and Toxicology 40, 511–518.

Nakai, M., Tabira, Y., Asai, D., Yakabe, Y., Shimyozu, T., Noguchi, M., Takatsuki, M., Shimohigashi, Y., 1999. Binding characteristics of dialkyl phthalates for the estrogen receptor. Biochemical and Biophysical Research Communication 254, 311–314.

Napoli, J.L., 1999. Interactions of retinoid binding proteins and enzymes in retinoid metabolism. Biochimica et Biophysica Acta (BBA)—Molecular and Cell Biology of Lipids 1440, 139–162.

Naylor, L.H., 1999. Reporter gene technology: the future looks bright. Biochemical Pharmacology 58, 749–757.

Ndayibagira, A., Spear, P.A., 1999. Esterification and hydrolysis of vitamin A in the liver of brook trout (*Salvelinus fontinalis*) and the influence of a coplanar polychlorinated biphenyl. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 122, 317–325.

Nilsson, C.B., Hoegberg, P., Trossvik, C., Azais-Braesco, V., Blaner, W.S., Fex, G., Harrison, E.H., Nau, H., Schmidt, C.K., van Bennekum, 2000. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin increases serum and kidney retinoic acid levels and kidney retinol esterification in the rat. Toxicology and Applied Pharmacology 169, 121–131.

Okazaki, Y., Katayama, T., 2003. Effects of dietary carbohydrate and myo-inositol on metabolic changes in rats fed 1,1,1-trichloro-2,2-bis (*p*-chlorophenyl) ethane (DDT). The Journal of Nutritional Biochemistry 14, 81–89.

Pacifico, F., Liguoro, D., Acquaviva, R., Formisano, S., Consiglio, E., 1999. Thyroglobulin binding and TSH regulation of the RHL-1 subunit of the asialoglycoprotein receptor in rat thyroid. Biochimie 81, 493–496.

Palace, V.P., Klaverkamp, J.F., Baron, C.L., Brown, S.B., 1997. Metabolism of ^{3}H-retinol by lake trout (*Salvelinus namaycush*) pre-exposed to 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126). Aquatic Toxicology 39, 321–332.

Paris, F., Balaguer, P., Terouanne, B., Servant, N., Lacoste, C., Cravedi, J.P., Nicolas, J.C., Sultan, C., 2002a. Phenylphenols, biphenols, bisphenol-A and 4-*tert*-octylphenol exhibit α and β estrogen activities and antiandrogen activity in reporter cell lines. Molecular and Cellular Endocrinology 193, 43–49.

Paris, F., Servant, N., Terouanne, B., Sultan, C., 2002b. Evaluation of androgenic bioactivity in human serum by recombinant cell line: preliminary results. Molecular and Cellular Endocrinology 198, 123–129.

Parzefall, W., 2002. Risk assessment of dioxin contamination in human food. Food and Chemical Toxicology 40, 1185–1189.

Paton, T.E., Renton, K.W., 1998. Cytokine-mediated down-regulation of CYP1A1 in Hepa1 cells. Biochemical Pharmacology 55, 1791–1796.

Pelissero, C., Flouriot, G., Foucher, J.L., Bennetau, B., Dunogues, J., Legac, F., Sumpter, J.P., 1993. Vitellogenin synthesis in cultured hepatocytes—an in vitro test for the estrogenic potency of chemicals. Journal of Steroid Biochemistry and Molecular Biology 44, 263–272.

Piskorska-Pliszczynska, J.P., Keys, B., Safe, S., Newman, M.S., 1986. The cytosolic receptor binding affinities and AHH induction potencies of 29 polynuclear aromatic hydrocarbons. Toxicological Letters 34, 67–74.

Pollenz, R.S., 2002. The mechanism of Ah receptor protein down-regulation (degradation) and its impact on Ah receptor-mediated gene regulation. Chemico-Biological Interactions 141, 41–61.

Porterfield, S.P., 2000. Thyroidal dysfunction and environmental chemicals—potential impact on brain development. Environmental Health Perspectives 108, 433–438.

Poulin, R., Poirier, D., Labrie, F., 1987. Androgens decrease the number of estrogen receptors in the ZR-75-1 human breast cancer cell line. Journal of Steroid Biochemistry 28, 157.

Qiu, C.H., Ohe, M., Matsuzaki, S., 2003. Effect of diethylstilbestrol on polyamine metabolism in hamster epididymis. Asian Journal of Andrology 5, 301–306.

Rathore, M., Bhatnagar, P., Mathur, D., Saxena, G.N., 2002. Burden of organochlorine pesticides in blood and its effect on thyroid hormones in women. The Science of the Total Environment 295, 207–215.

Reen, R.K., Cadwallader, A., Perdew, G.H., 2002. The subdomains of the transactivation domain of the aryl hydrocarbon receptor (AhR) inhibit AhR and estrogen receptor transcriptional activity. Archives of Biochemistry and Biophysics 408, 93–102.

Richter, F., Huang, H.F.S., Li, M.T., Danielpour, D., Wang, S.L., Irwin, R.J., 1999. Retinoid and androgen regulation of cell growth, epidermal growth factor and retinoic acid receptors in normal and carcinoma rat prostate cells. Molecular and Cellular Endocrinology 153, 29–38.

Rogers, J.M., Denison, M.S., 2000. Recombinant cell bioassays for endocrine disruptors: development of a stably transfected human ovarian cell line for the detection of estrogenic and anti-estrogenic chemicals. In Vitro and Molecular Toxicology—Journal of Basic and Applied Research 13, 67–82.

Rolland, R.M., 2000. A review of chemically-induced alterations in thyroid and vitamin A status from field studies of wildlife and fish. Journal of Wildlife Diseases 36, 615–635.

Roy, S., Mysior, P., Brzezinski, R., 2002. Comparison of dioxin and furan TEQ determination in contaminated soil using chemical, micro-EROD, and immunoassay analysis. Chemosphere 48, 833–842.

Safe, S., Connor, K., Gaido, K., 1998. Methods for xenoestrogen testing. Toxicology Letters 28, 103665–103670.

Sanderson, J.T., Aarts, J.M.M.J.G., Brouwer, A., Froese, K.L., Denison, M.S., Giesy, J.P., 1996. Comparison of Ah receptor-mediated luciferase and ethoxyresorufin-*O*-deethylase induction in H4IIE cells: implications for their use as bioanalytical tools for the detection of polyhalogenated aromatic hydrocarbons. Toxicology and Applied Pharmacology 137, 316–325.

Sanderson, J.T., Kennedy, S.W., Giesy, J.P., 1998. In vitro induction of ethoxyresorufin-*O*-deethylase and porphyrins by halogenated aromatic hydrocarbons in avian primary hepatocytes. Environmental Toxicology and Chemistry 17, 2006–2018.

Schafer, T.E., Lapp, C.A., Hanes, C.M., Lewis, J.B., Wataha, J.C., Schuster, G.S., 1999. Estrogenicity of bisphenol A and bisphenol A dimethacrylate in vitro. Journal of Biomedical Materials Research 45, 192–197.

Schrader, T.J., Cooke, G.M., 2002. Interaction between tris(4-chlorophenyl)methanol and the human androgen receptor in vitro. Toxicology Letters 136, 19–24.

Schrader, T.J., Cooke, G.M., 2003. Effects of Aroclors and individual PCB congeners on activation of the human androgen receptor in vitro. Reproductive Toxicology 17, 15–23.

Seeley, M.R., Faustman, E.M., 1998. Evaluation of p19 cells for studying mechanisms of developmental toxicity: application to four direct-acting alkylating agents. Toxicology 127, 49–58.

Sendak, R.A., Wang, F., Geagan, L.B., Armstrong, L.A., Thyne, C.D., Cole, E.S., Mattaliano, R.J., 2002. Comparison of two in vitro methods for the measurement of recombinant human tsh bioactivity. Biologicals 30, 245–254.

Sewall, C.H., Flagler, N., van den Heuvel, J.P., Clark, G.C., Tritscher, A.M., Maronpot, R.M., Lucier, G.W., 1995. Alterations in thyroid function in female Sprague–Dawley rats following chronic treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Toxicology and Applied Pharmacology 132, 237–244.

Shago, M., Flock, G., Hagesteijn, C.-Y.L., Woodside, M., Grinstein, S., Giguere, V., Dedhar, S., 1997. Modulation of the retinoic acid and retinoid X receptor signaling pathways in p19 embryonal carcinoma cells by calreticulin. Experimental Cell Research 230, 50–60.

Shi, Y.-B., Ritchie, J.W.A., Taylor, P.M., 2002. Complex regulation of thyroid hormone action: multiple opportunities for pharmacological intervention. Pharmacology and Therapeutics 94, 235–251.

Shimamura, M., Kodaira, K., Kenichi, H., Ishimoto, Y., Tamura, H., Iguchi, T., 2002. Comparison of antiandrogenic activities of vinclozolin and camphorquinone in androgen receptor gene transcription assay in vitro and mouse in utero exposure assay in vivo. Toxicology 174, 97–107.

Simpson, E.R., 2003. Sources of estrogen and their importance*1. The Journal of Steroid Biochemistry and Molecular Biology 86, 225–230.

Simpson, V.R., Bain, M.S., Brown, R., Brown, B.F., Lacey, R.F., 2000. A long-term study of vitamin A and polychlorinated hydrocarbon levels in otters (*Lutra lutra*) in south west England. Environmental Pollution 110, 267–275.

Smith, J.W., Evans, A.T., Costall, B., Smythe, J.W., 2002. Thyroid hormones, brain function and cognition: a brief review. Neuroscience and Biobehavioral Reviews 26, 45–60.

Spear, P.A., Bilodeau, A., Branchaud, A., 1992. Retinoids: From metabolism to environmental monitoring. Chemosphere 25, 1733–1738.

Sultan, C., Balaguer, P., Terouanne, B., Georget, V., Paris, F., Jeandel, C., Lumbroso, S., Nicolas, J.-C., 2001. Environmental xenoestrogens, antiandrogens and disorders of male sexual differentiation. Molecular and Cellular Endocrinology 178, 99–105.

Sun, S.Y., Lotan, R., 2002. Retinoids and their receptors in cancer development and chemoprevention. Critical Review Oncology/Hematology 41, 41–55.

Tagami, T., Kopp, P., Johnson, W., Arseven, O.K., Jameson, J.L., 1998. The thyroid hormone receptor variant alpha 2 is a weak antagonist because it is deficient in interactions with nuclear receptor corepressors. Endocrinology 139, 2535–2544.

Terouanne, B., Tahiri, B., Georget, V., Belon, C., Poujol, N., Avances, C., Orio Jr., F., Balaguer, P., Sultan, C., 2000. A stable prostatic bioluminescent cell line to investigate androgen and antiandrogen effects. Molecular and Cellular Endocrinology 160, 39–49.

Terouanne, B., Paris, F., Servant, N., Georget, V., Sultan, C., 2002. Evidence that chlormadinone acetate exhibits antiandrogenic activity in androgen-dependent cell line. Molecular and Cellular Endocrinology 198, 143–147.

Tilley, W.D., Bentel, J.M., Aspinall, J.O., Hall, R.E., Horsfall, D.J., 1995. Evidence for a novel mechanism of androgen resistance in the human prostate cancer cell line, PC-3. Steroids 60, 180–186.

Tuohimaa, P., Blauer, M., Pasanen, S., Passinen, S., Pekki, A., Punnonen, R., Syvala, H., Valkila, J., Wallen, M., Valiaho, J., Zhuang, Y.H., Ylikomi, T., 1996. Mechanisms of action of sex steroid hormones: basic concepts and clinical correlations. Maturitas 23, S3–S12.

Tyler, C.R., Vanaerle, R., Hutchinson, T.H., Maddix, S., Trip, H., 1999. An in vivo testing system for endocrine disruptors in fish early life stages using induction of vitellogenin. Environmental toxicology and chemistry 18, 337–347.

Vakharia, D.D., Gierthy, J.F., 2000. Use of a combined human liver microsome-estrogen receptor binding assay to assess potential estrogen modulating activity of PCB metabolites. Toxicology Letters 114, 55–65.

van Birgelen, A.P.J.M., 1998. Hexachlorobenzene as a possible major contributor to the dioxin activity of human milk-review. Environmental Health Perspectives 106, 683–688.

van den Berg, M., Peterson, R.E., Schrenk, D., 2000. Human risk assessment and TEFs. Food Additives and Contaminants 17, 347–358.

van der Berg, M., Birnbaum, L., Bosveld, A.T.C., Brunström, B., Cook, P., Feeley, M., Giesy, J.P., Hanberg, A., Hasegawa, R., Kennedy, S., Kubiak, T., Larsen, J.C., van Leeuwen, F.X.R., Djien Liem, A.K., Nolt, C., Peterson, R.E., Poellinger, L., Safe, S., Schrenk, D., Tillitt, D., Tysklind, M., Younes, M., Waern, F., Zacharewski, T., 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environmental Health Perspectives 106, 775–792.

van der Heyden, M.A.G., Defize, L.H.K., 2003. Twenty one years of p19 cells: What an embryonal carcinoma cell line taught us about cardiomyocyte differentiation. Cardiovascular Research 58, 292–302.

van der Heyden, M.A.G., van Kempen, M.J.A., Tsuji, Y., Rook, M.B., Jongsma, H.J., Opthof, T., 2003. P19 embryonal carcinoma cells: a suitable model system for cardiac electrophysiological differentiation at the molecular and functional level. Cardiovascular Research 58, 410–422.

van der Plas, S.A., Lutkeschipholt, I., Spenkelink, B., Brouwer, A., 2001. Effects of subchronic exposure to complex mixtures of dioxin-like and non-dioxin-like polyhalogenated aromatic compounds on thyroid hormone and vitamin A levels in female Sprague–Dawley rats. Toxicological Sciences: An Official Journal of the Society of Toxicology 59, 92–100.

Veldscholte, J., Berrevoets, C.A., Mulder, E., 1994. Studies on the human prostatic cancer cell line LNCaP. The Journal of Steroid Biochemistry and Molecular Biology 49, 341–346.

Villeneuve, D.L., Kannan, K., Khim, J.S., Falandysz, J., Nikiforov, V.A., Blankenship, A.L., Giesy, J.P., 2000. Relative potencies of individual polychlorinated naphthalenes to induce dioxin-like responses in fish and mammalian in vitro bioassays. Archives of Environmental Contamination and Toxicology 39, 273–281.

Villeneuve, D.L., Khim, J.S., Kannan, K., Giesy, J.P., 2002. Relative potencies of individual polycyclic aromatic hydrocarbons to induce dioxinlike and estrogenic responses in three cell lines. Environmental Toxicology 17, 128–137.

Vinggaard, A.M., Hnida, C., Larsen, J.C., 2000. Environmental polycyclic aromatic hydrocarbons affect androgen receptor activation in vitro. Toxicology 145, 173–183.

Vondracek, J., Kozubik, A., Machala, M., 2002. Modulation of estrogen receptor-dependent reporter construct activation and G(0)/G(1)-S-phase transition by polycyclic aromatic hydrocarbons in human breast carcinoma MCF-7 cells. Toxicological Sciences 70, 193–201.

Wang, Q., Fondell, J.D., 2001. Generation of a mammalian cell line stably expressing a tetracycline-regulated epitope-tagged human androgen receptor: implications for steroid hormone receptor research. Analytical Biochemistry 289, 217–230.

Waritz, R.S., Steinberg, M., Kinoshita, F.K., Kelly, C.M., Richter, W.R., 1996. Thyroid function and thyroid tumors in toxaphene-treated rats. Regulatory Toxicology and Pharmacology 24, 184–192.

Weiler, R., He, S.G., Vaney, D.I., 1999. Retinoic acid modulates gap junctional permeability between horizontal cells of the mammalian retina. European Journal of Neuroscience 11, 3346–3350.

Wiebel, F.J., Wegenke, M., Kiefer, F., 1996. Bioassay for determining 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TEs) in human hepatoma HepG2 cells. Toxicology Letters 88, 335–338.

Wilson, A.G.E., Thake, D.C., Heydens, W.E., Brewster, D.W., Hotz, K.J., 1996. Mode of action of thyroid tumor formation in the male Long-Evans rat administered high doses of Alachlor*1. Fundamental and Applied Toxicology 33, 16–23.

Wilson, V.S., Bobseine, K., Lambright, C.R., Gray, L.E., 2002. A novel cell line, MDA-kb2, that stably expresses an androgen- and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists. Toxicological Sciences 66, 69–81.

Wong, C.I., Kelce, W.R., Sar, M., Wilson, E.M., 1995. Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. Journal of Biological Chemistry 270, 19998–20003.

Xu, L., Li, A.P., Kaminski, D.L., Ruh, M.F., 2000. 2,3,7,8 Tetrachlorodibenzo-*p*-dioxin induction of cytochrome P4501A in cultured rat and human hepatocytes. Chemico-Biological Interactions 124, 173–189.

Yamabe, Y., Hoshino, A., Imura, N., Suzuki, T., Himeno, S., 2000. Enhancement of androgen-dependent transcription and cell proliferation by tributyltin and triphenyltin in human prostate cancer cells. Toxicology and Applied Pharmacology 169, 177–184.

Yamada, T., Ueda, S., Yoshioka, K., Kawamura, S., Seki, T., Okuno, Y., Mikami, N., 2003. Lack of estrogenic or (anti-)androgenic effects of d-phenothrin in the uterotrophic and Hershberger assays. Toxicology 186, 227–239.

Zacharewski, T., 1997. In vitro bioassays for assessing estrogenic substances. Environmental Science and Technology 31, 613–623.

Zacharewski, T., Harris, M., Safe, S., 1991. Evidence for the mechanism of action of the 2,3,7,8-tetrachlorodibenzo-para-dioxin-mediated decrease of nuclear estrogen-receptor levels in wild-type and mutant mouse Hepa-1c1c7 cells. Biochemical Pharmacology 41, 1931–1939.

Zacharewski, T.R., Meek, M.D., Clemons, J.H., Wu, Z.F., Fielden, M.R., Matthews, J.B., 1998. Examination of the in vitro and in vivo estrogenic activities of eight commercial phthalate esters. Toxicological Sciences 46, 282–293.

Zhang, Z., Lundeen, S.G., Zhu, Y., Carver, J.M., Winneker, R.C., 2000. In vitro characterization of trimegestone: a new potent and selective progestin. Steroids 65, 637–643.

Zimmermann-Belsing, T., Rasmussen, A.K., Feldt-Rasmussen, U., Bog-Hansen, T.C., 2002. The influence of alpha1-acid glycoprotein (orosomucoid) and its glycoforms on the function of human thyrocytes and CHO cells transfected with the human TSH receptor. Molecular and Cellular Endocrinology 188, 241–251.

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Review article

Disruption of retinoid transport, metabolism and signaling by environmental pollutants

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Abstract

Although the assessment of circulatory levels of retinoids has become a widely used biomarker of exposure to environmental pollutants, the adverse effects caused by imbalance of the retinoid metabolism and signaling in wildlife are not known in detail. Retinoids play an important role in controlling such vital processes as morphogenesis, development, reproduction or apoptosis. Unlike other signaling molecules, retinoids are not strictly endogenous but they are derived from dietary sources of vitamin A or its precursors and thus they are sometimes referred to as 'dietary' hormones. Some environmental pollutants that affect embryogenesis, immunity or epithelial functions were also shown to interfere with retinoid metabolism and signaling in animals. This suggests that at least some of their toxic effects may be related to interaction with the retinoid metabolism, transport or signal transduction. This review summarizes *in vivo* and *in vitro* studies on interaction of environmental complex samples, pesticides, polychlorinated dioxins, polychlorinated biphenyls, polycyclic aromatic compounds and other organic pollutants with physiology of retinoids. It sums up contemporary knowledge about levels of interaction and mechanisms of action of the environmental contaminants.

Keywords: Vitamin A; All-trans retinoic acid; Pesticide; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Polychlorinated biphenyls; Retinoids

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Abbreviations: 9cRA, 9-*cis* retinoic acid; AhR, aryl hydrocarbon receptor; APGWamide, amidated tetrapeptide Ala-Pro-Gly-Trp-NH2; ARAT, acyl-CoA: retinol acyltransferase; atRA, all-*trans* retinoic acid; CRABP, cellular retinoic acid binding protein; CRBP, cellular retinol binding protein; CYP, cytochrome P450; DBP, di-n-butyl phthalate; DDE, 1,1-bis-(4-chlorophenyl)-2,2-dichloroethene; DDT, 1,1-bis-(4-chlorophenyl)-2,2,2-trichloroethane; EBP, ethyl-n-butyl phthalate; EROD, ethoxyresorufin-*O*-deethylase; ER, estrogen receptor; LRAT, lecithin:retinol acyltransferase; MEHP, mono-(2-ethylhexyl)phthalate; N-CoR, nuclear receptor corepressor; OCP, organochlorine pesticide; PCB, polychlorinated biphenyl; PCDD/Fs, polychlorinated dibenzo-*p*-dioxins and furans; PCP, pentachlorophenol; PEPCK, phosphoenolpyruvate carboxykinase; PP, peroxisome proliferator; PPAR, peroxisome proliferator activated receptor; PXR, pregnenolon X receptor; RA, retinoic acid; RALDH, retinaldehyde dehydrogenase; RAR, retinoic acid receptor; RARE, retinoic acid response element; RBP, retinol binding protein; REH, retinylester hydrolase; REs, retinyl esters; ROLDH, retinol dehydrogenase; RXR, retinoid X receptor; RXRE, retinoid X response element; SMRT, silencing mediator of retinoid and thyroid receptors; T4, thyroxin; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PAHs, polycyclic aromatic hydrocarbons; TEQ, toxic equivalent; TGF β, transforming growth factor β; TTNPB, (*E*)-4-(2-[5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl]-1-propenyl)benzoic acid; TTR, transthyretin; UDP, uridine diphosphate; UV, ultra violet radiation; Wy-14,643, 4-chloro-6(2,3-xylindino)-2-pyrimidinylthioacetic acid.

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1. Introduction

1.1. Role of retinoids

Recently, there has been increasing number of studies assessing endocrine disrupting effects as an endpoint relevant to endocrine function in animals following exposure to synthetic compounds. There is an increasing evidence that also environmental contaminants could disrupt endocrine processes, which may result in reproductive problems, carcinogenesis and other toxic effects related to differentiation, growth and development in animal populations. So far, research has focused mainly on the interactions of xenobiotics with steroid hormone system. However, the pollutants could interfere also with signaling of other hormones producing severe effects in exposed animals (Harvey and Everett, 2006; Harvey and Johnson, 2002). One of such targets is retinoid signaling system, which plays an essential role in the regulation of development and homeostasis of tissues in both vertebrates and invertebrates through control of cell differentiation, proliferation and apoptosis (Linan-Cabello et al., 2002; Reichrath et al., 2007; Zile, 2001). Besides antioxidative functions (Ciaccio et al., 1993; Shiota et al., 2006), retinoids affect many vital processes such as growth and development (Hofmann and Eichele, 1994), epithelial maintenance (Rosenthal et al., 1994), immune function (Ross and Hammerling, 1994), vision (Rando, 1994) or reproduction (Eskild and Hansson, 1994). Both excess and deficiency of retinoids have been associated with embryotoxicity and/or teratogenicity in vertebrates (Tzimas and Nau, 2001; Zile, 2001). Although the role of retinoids in invertebrates is not known as thoroughly as in vertebrates, diverse groups of invertebrates including insects (De Luca, 1991), gastropods (Nishikawa, 2006) or ascidians (DeBernardi et al., 1994; Katsuyama et al., 1995) possess retinoid metabolism and signaling pathway similar to vertebrates (Maden, 1993) and retinoids take part also in regulation of reproduction in crustaceans (Linan-Cabello and Paniagua-Michel, 2004) or embryogenesis in ascidians (Katsuyama et al., 1995).

The effects of environmental pollutants on retinoid physiology were described in populations of animals living in contaminated areas, which displayed significant changes in levels of retinoids that could cause shift in malformation rate or reproduction success (Branchaud et al., 1995; Murk et al., 1996; Spear et al., 1992). Several reviews summarize the effect of pollution on levels of retinoids establishing it as a sensitive biomarker of pollution (Rolland, 2000; Simms and Ross, 2000). This review sums up the contemporary knowledge on the various modes of interaction of environmental pollutants with retinoid transport, metabolism and action both *in vivo* and *in vitro* with focus on mechanisms and molecular processes underlying the toxic effects.

For the purpose of this paper, retinoids are defined as natural compounds that are structurally and functionally related to retinol. The term ‘vitamin A’ is used in this review for retinol and its esters although the contemporary definition of vitamin A is much wider (IUPAC-IUB, 1982).

1.2. Metabolism of retinoids

Animals are not capable of *de novo* synthesis of retinoids, which thus must be obtained from diet. Because retinoids play a role that seems to be similar to classical hormones but do not have strictly endogenic origin, they are sometimes referred to as ‘dietary hormones’ (Bastien and Rochette-Egly, 2004; Simms and Ross, 2000). Most of the intake of retinoids is represented by retinyl esters (REs) from animal sources or retinoid-precursors carotenoids from autotrophic organisms (e.g. β-carotene). In vertebrates, both types of the source compounds are transformed during digestion to retinol, which is subsequently bound by cellular retinol binding protein II (CRBP II) in cells of intestinal mucosa (Fig. 1). The CRBP II-bound retinol is again esterified with long-chain fatty acids by lecithin:retinol acyltransferase (LRAT). When the capacity of CRBP II is saturated, the excess of retinol is esterified by acyl–CoA:retinol acyltransferase (ARAT). REs are afterwards transferred into chylomicrons (lipoproteins that transport mainly dietary cholesterol and triglycerides) released through lymph into the blood circulation and transported to liver, or in lesser extend to adipose tissue (Harrison and Hussain, 2001). REs are hydrolyzed by retinyl ester hydrolase (REH) to retinol in liver parenchyma cells and bound to cellular retinol binding protein I (CRBP I; Napoli, 1999). In case of sufficient vitamin A concentrations, most of the diet-derived retinol is converted mainly by LRAT to REs stored in liver stellate cells (Napoli, 1996, 1999; Simms and Ross, 2000). In case of low retinol levels in plasma, REs are cleaved by REH and retinol is released from liver to plasma. The hepatic retinol release includes its transfer from the complex with CRBP I to retinol binding protein (RBP) before secretion into plasma (Fig. 1). RBP solubilizes and transports the lipid retinol through the aqueous medium of plasma, prevents its oxidation and/or isomerization and protects cell membranes from its lytic effect. However, the role of RBP in transport of retinol differs between various vertebrate species. In carnivores, a great portion of retinol is transported in the form of retinyl esters bound to lipoproteins in the plasma (Burri et al., 1993; Kakela et al., 2003; Schweigert et al., 1990). RBP occurs in blood in complex

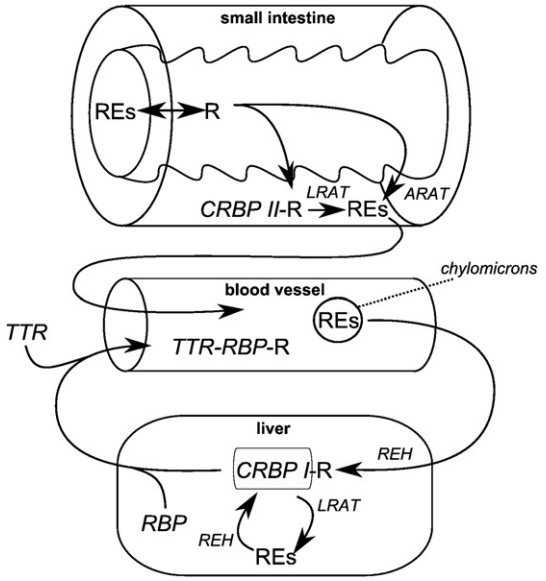

Fig. 1. Overview of the metabolism of retinoids. Retinyl esters (REs) are hydrolysed to retinol in lumen of the small intestine and absorbed by the cells of mucosa. Retinol (R) is bound by cellular retinol binding protein II (CRBP II) and re-esterified by lecithin retinol acyltransferase (LRAT) to retinyl esters that are released to the circulation in chylomicrons. In case of saturation of the CRBP II capacity, the excess of retinol is esterified by acyl–CoA:retinol acyltransferase (ARAT). REs from blood are transported to liver where they are hydrolysed by retinyl ester hydrolase (REH) into retinol which binds to CRBP I. When there is enough retinol in circulation, retinol is preferentially esterified by LRAT and stored in stellate cells in form of RE. If the levels of plasma retinol are low, retinol stores in liver are mobilized and released bound to retinol binding protein (RBP), which is associated with transthyretin (TTR) in the circulation (adapted from Simms et al., 2000).

with the 80 kDa transport protein for thyroid hormone T4 transthyretin (TTR), which is believed to help to protect the 21 kDa RBP from excretion by kidneys (Napoli, 1996; van Bennekum et al., 2001). The T4–TTR–RBP–retinol complex distributes retinol into various body tissues and helps to keep the retinol circulatory levels relatively stable even if the dietary intake fluctuates (Green and Green, 1994). However, the role of TTR–RBP complex in retinol transport is not clear because it has been shown that TTR-deficient mice that had very low RBP circulating levels did not display any dramatic changes in the levels of retinoids in the peripheral tissues (van Bennekum et al., 2001). Besides, there has been described an isoform of RBP in mammals that does not bind to TTR at all (Burri et al., 1993). This is also the case of fish RBP isoforms that also do not form TTR–RBP complex (Folli et al., 2003).

Retinol delivered to the extrahepatic tissue is bound by CRBP I and oxidized to retinal (Fig. 2), this reaction is reversible and it is catalyzed by diverse groups of enzymes such as alcohol dehydrogenases (e.g. retinol dehydrogenase ROLDH), short chain dehydrogenases or cytochromes P450 (CYP; Marill et al., 2003). The retinal is irreversibly converted to retinoic acid (RA) by retinaldehyde dehydrogenase (RALDH; Blaner and Olson, 1994; Marill et al., 2003). RA is a lipophilic, rapidly diffusing and low molecular weight (300 Da) molecule, which is generally considered the 'active' form of retinoids (Bastien and Rochette-Egly, 2004). It can adopt three conformations: all-*trans* retinoic acid (atRA), 9-*cis* retinoic acid (9cRA) and 13-*cis* retinoic acid that can be interchanged either spontaneously or by isomerases (Marill et al., 2003). In cells, it is bound by cellular retinoic acid binding protein (CRABP I or II) and either transferred to specific retinoid receptors in the nucleus or oxidatively inactivated by CYP system (Blaner and Olson, 1994; Marill et al., 2003; Noy, 2000). The control of RA levels in cells and tissues is regulated by the balance between its biosynthesis and metabolization. The inactivation of RA is catalyzed by several members of CYP families 1,2,3,4 and mainly CYP26, which is inducible by atRA. Their products (e.g. 4-oxo-RA, 4-OH-RA and 18-OH-RA) are more polar than RA and thus they are easier to excrete (Marill et al., 2003; Reijntjes et al., 2005). However, these RA metabolites do not loose completely their ability to induce RA-dependent transcription activity (Fig. 2) (Idres et al., 2002;

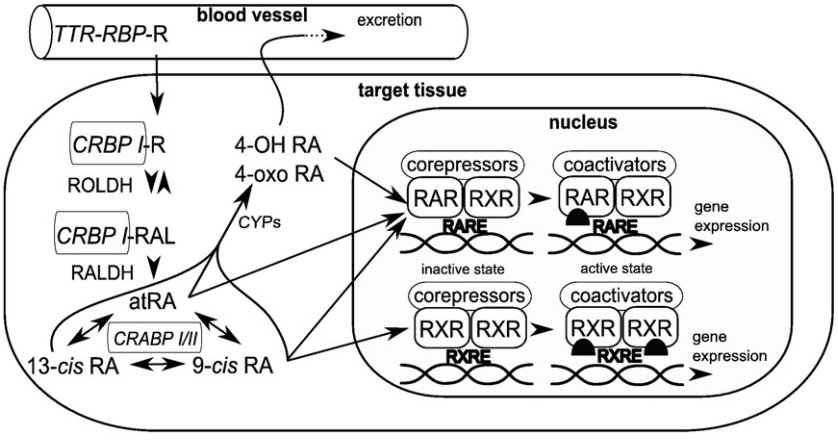

Fig. 2. Scheme of metabolism and signaling of retinoids in target tissues. Retinol (R) that enters the cell is bound by cellular retinol binding protein I (CRBP I). It is enzymaticaly converted to retinal (RAL) and then to three isomers of retinoic acid (RA), which bind to cellular retinoic acid binding protein I or II (CRABP I,II). RA is either metabolized by cytochromes P450 (CYPs) and excreted or transported to nucleus where it binds to its receptor. RAR and RXR receptors form heterodimers or homodimers, which are bound to retinoic acid response element (RARE) or retinoid X response element (RXRE), respectively. The receptor dimers are associated with corepressors (e.g. N-CoR and SMRT) in inactive state. After the binding of ligands, the corepressors are exchanged for coactivators (e.g. SRC/p160 complex, p300/CBP) and the complex starts the expression of associated genes (adapted from Simms et al., 2000; Marill et al., 2003; Bastien and Rochette-Egly, 2004).

Reijntjes et al., 2005). The excretion of retinoid metabolites is facilitated by glucuronidation (Marill et al., 2003). Retinoyl-glucuronides were also described to at least partially substitute the biological activity of RA in organism, despite the fact that they were not able to bind to RA-binding proteins or receptors (Barua and Sidell, 2004).

1.3. RAR/RXR system

In vertebrates, RA can modulate gene expression through binding to two families of nuclear receptors, retinoic acid receptors (RAR) and retinoid X receptors (RXR; Fig. 2). Both families consist of three isotypes of receptors (α, β and γ). While RARs are activated by all-*trans* retinoic acid (atRA) and 9-*cis* retinoic acid (9cRA), RXRs are activated only by higher levels of 9cRA (Bastien and Rochette-Egly, 2004; Chambon, 1996; Tzimas and Nau, 2001). The role of 13-*cis* retinoic acid is not clear; while some studies describe it can weakly activate RARs, it is possible that this effect is mediated by isomerization to the active isomers (Veal et al., 2002). RARs are active in form of RAR/RXR heterodimers where RXR is a silent partner that does not require any ligand (Vivat et al., 1997), while activated RXRs form homodimers. RAR and RXR act as transcriptional regulators via retinoic acid response elements (RARE) and retinoid X response elements (RXRE), respectively (Love and Gudas, 1994). In the basal state, retinoid receptors are bound to nuclear corepressors silencing mediator of retinoid and thyroid receptors (SMRT) or nuclear receptors corepressor (N-CoR; Marill et al., 2003; Widerak et al., 2006). Binding of the ligand leads to the conformational change of the complex, corepressors release, recruitment of coactivators such as SRC/p160 family or p300/CBP (Bastien and Rochette-Egly, 2004), and transcriptional activation of target genes via RARE or RXRE (Lemaire et al., 2005). Retinoids regulate expression of hundreds of genes and some of them are involved in retinoid metabolism and signaling e.g. RARβ, CYP26, CRABP, CRBP or in regulation of differentiation and morphogenesis e.g. jun, hox, or a gene for cytokine TGFβ (Balmer and Blomhoff, 2002; Bastien and Rochette-Egly, 2004; Eifert et al., 2006). A gene for phosphoenolpyruvate carboxykinase (PEPCK), which is involved in carbohydrate metabolism, was suggested as a model for studies of retinoid-regulated expression of genes because its expression seems to be directly regulated by retinoid signaling pathway (for review see McGrane, 2007). RXR is not specific just for retinoid signal transduction because it serves also as a heterodimeric partner for a wide range of other receptors such as vitamin D receptor, thyroid hormone receptor or peroxisome proliferator-activated receptor. This versatility could contribute to cross-talk among various hormone receptor networks (Chambon, 1996; Janosek et al., 2006; Tzimas and Nau, 2001). Also a number of receptors without currently known ligands (orphan receptors) have been implicated in the regulation of retinoid response (Blumberg and Evans, 1998; Lin et al., 2000). Besides receptor-dependent signaling, it was also shown that some of the effects of retinoids could be mediated by retinoylation of specific proteins (Marill et al., 2003).

There is only limited information on the system of retinoid signaling in invertebrates compared to vertebrates. While homologs of RAR family were not found in invertebrates, RXR-like nuclear receptor was described in porifera (Wiens et al., 2003), cnidaria (Kostrouch et al., 1998) and annelida (Aguinaldo et al., 1997) and functional RXR was described also in mollusca (Bouton et al., 2005). In arthropods, ultraspiracle is considered an ortholog of vertebrate RXR, though it was shown to bind only endogenous terpenoid-derived ligands and not 9cRA (Jones et al., 2006). Despite the differences in retinoid signaling system between invertebrates and vertebrates at least RXR seems to be conserved element present in diverse groups of animals, which participates in regulation of many vital processes either directly through RXRE or indirectly as a heterodimeric partner for other nuclear receptors.

2. Pollutants and retinoid system

2.1. Environmental complex samples

It has been well documented that environmental pollutants interfere with normal retinoid physiology and the change of retinoid levels in organism has been used as a sensitive biomarker of exposure to wide range of pollutants in wild animal populations (Boily et al., 1994; Champoux et al., 2006; Murk et al., 1996; Nilsson and Hakansson, 2002; Rolland, 2000; Simms and Ross, 2000; Zile, 1992). The studies that examined in detail the relationship of specific dominant pollutant groups in various environmental samples such as PCBs or pesticides with the retinoids transport, metabolism and signaling of the exposed species will be discussed in the following chapters.

Environmental pollutants bioaccumulate particularly well in the upper parts of aquatic ecosystem food chain. Murk et al. (1996) compared the impact of contamination and environmental factors on reproduction of fish-eating common tern (*Sterna hirundo*) colonies from relatively highly polluted areas of Belgium and Netherlands. They collected eggs in localities with different levels of contamination and hatched them artificially. The pollution indeed produced observable effects, such as prolonged incubation periods and smaller chicks and eggs, which correlated with decrease of yolk sac REs levels and increase of hepatic ethoxyresorufin-*O*-deethylase (EROD) activity, but the reproduction success has been influenced more by factors such as predation or flooding.

Several semi-field studies confirmed the effects of pollutants on retinoid system in mammals. A decrease of plasma retinol levels has been observed in captive common seal (*Phoca vitulina*) fed with fish from highly contaminated Wadden Sea (Brouwer et al., 1989b) and Baltic Sea (Swart et al., 1994) compared to seals fed with fish from cleaner North Atlantic Ocean. Similar results were obtained in mink fed with carp from substantially contaminated Saginaw River, Michigan, USA (Martin et al., 2006). Authors observed significant decrease of plasma retinol and hepatic REs levels as well as increase of hepatic retinol:REs ratio.

Many studies describe the effects of environment-derived complex samples on retinoid system in fish. Doyon et al. (1999)

described significantly increased malformation rate in Lake sturgeon (*Acipenser fulvescens*) from polluted St Lawrence River compared to that from relatively clean region of Abitibi and the effect seamed to be associated with increased metabolism of RA. The effect of heavily polluted sludge from Rotterdam harbor has been studied in mesocosm study with flounder (Besselink et al., 1998). The retinol levels in plasma and liver as well as liver REs were significantly reduced after three-year exposure. The authors described negative non-linear association between hepatic retinol concentrations and CYP1A protein levels, which suggests the involvement of substances with dioxin-like activity. Branchaud et al. (1995) observed that fish from river receiving pulp mill effluents had increased malformation rate and reduced hepatic levels of retinol and retinyl palmitate, while vitamin E levels were not affected.

The presence of contaminants able to interfere with retinoid signaling in samples from polluted aquatic environment has been also documented by *in vitro* studies. Alsop et al. (2003) have shown that some compounds from pulp mill effluents were able to bind to fish RAR and RXR and displace the natural ligands *in vitro*. The results of this study indicate that the RA receptor ligands may originate from the natural wood furnish and not from the chemical processes during bleaching.

Significant effects of paper mill effluents on signaling of retinoids have been also shown in murine teratocarcinoma cell line F9 stably transfected with reporter gene activated by RA (Schoff and Ankley, 2002). Even though no known retinoids were detected, the polar fraction of the effluent water decreased transcription of the genes stimulated by atRA or synthetic RAR-specific ligand TTNPB, while 9cRA-induced gene expression was not affected. It seemed that some RAR antagonists blocked the binding site for atRA, but they allowed either binding of 9cRA, or activation of RAR via allosteric interaction with ligand-bound RXR (Fig. 2).

Extracts from contaminated river sediments caused increase of atRA-induced differentiation of the HL-60 cells (Vondracek et al., 2001), but this effect did not correlate with the level of polycyclic aromatic hydrocarbons (PAHs) or phthalates, which were present in tested sediments at high concentrations. In another study, which used murine embryonic carcinoma cell line P19 transfected with luciferase reporter gene controlled by RARE, the extracts from river sediments highly contaminated by polychlorinated dioxins and furans (PCDD/Fs) and also PAHs did not display any effect when applied alone but they strongly potentiated the effect of atRA in co-exposure (Novak et al., 2007). The results also showed that both persistent and non-persistent pollutants contributed to the effect.

It is possible that oxidative stress might be involved in some of the effects on retinoid system caused by environmental pollution. Heavy metals are known oxidative stress inducers (Valko et al., 2005) and metal pollution has been described to cause decrease of retinoid levels due to oxidative stress. Payne et al. (1998) reported that fish, which lived in lakes receiving iron-ore runoffs, displayed increased level of DNA oxidative damage associated with depletion of retinoid levels. Similar effects have been also reported from experiments with zebrafish (*Danio rerio*) exposed to copper (Alsop et al., 2007). Anyway, it is possible that oxidative stress could be important mode of action for diverse classes of contaminants besides heavy metals.

Numerous pollutants including those discussed in the following chapters are known to possess also anti/estrogenic properties and some studies indicate that disruption of retinoid metabolism and signaling can be linked with estrogen receptor (ER) activation. Estradiol exposure was described to cause significant increase of plasma retinol levels and marginal decrease of hepatic REs in experiments with juvenile sturgeon (Palace et al., 2001). Li and Ong (2003) and Li et al. (2004) found out that estradiol is able to directly induce CRABP II and enzymes involved in RA biosynthesis (ROLDH, RALDH) via activation of ER in rat uterus. Moreover, increased levels of RARα and β were detected in developing and adult rat prostate exposed to estrogen during neonatal stage (Prins et al., 2002). These results indicate that retinoid system could be affected by some anti/estrogenic compounds in the complex environmental mixtures of pollutants.

2.2. Pesticides

Some studies report growing occurrence of deformed frogs in the environment. Many factors have been proposed as being responsible for the malformations including contaminants, ultraviolet radiation (UV) or parasites. Although some authors disprove the role of pollution in this phenomenon (Ankley et al., 2004; Johnson et al., 2004), others suggest that environmental pollutants could be at least partly involved (Bridges et al., 2004; Gardiner et al., 2003). The possible link between contamination by pesticides from agriculture and amphibian malformations has been also suggested by Taylor et al. (2005), who found relationship between malformation rate and proximity of intensive agriculture. It has been hypothesized that the contaminants present in surface waters may interfere with retinoic signaling pathway, which plays an important role in morphogenesis (Berube et al., 2005; Gardiner et al., 2003; La Clair et al., 1998). The influence of contamination on retinoid profiles and body weights was observed in bullfrogs (*Rana catesbeiana*) from areas with different degree of intensive agriculture (Berube et al., 2005; Boily et al., 2005). The plasma retinol levels were negatively correlated to body weight in males and the hepatic retinoid stores were significantly lower in localities with high concentration of pesticides in the water from Yamaska River basin, Quebec, Canada. However, experiments with exposure of European common frog (*Rana temporaria*) to p,p'-DDE, one of the most persistent metabolites of the pesticide DDT, have shown dose-dependent increase of hepatic retinol levels (Leiva-Presa et al., 2006). The expression and protein levels of CYP26 displayed the opposite trend, which suggests that the rise of retinol concentration could be explained by reduction of the activity of retinol-metabolizing enzymes caused by p,p'-DDE (Tables 1 and 2).

Several pesticides were shown to interact with the receptors of retinoid signaling pathway and/or to affect the interaction of the natural ligands with the receptors. Dorsey et al. (2002) reported a marginal induction of RARE promoter by organochlorine pesticide pentachlorophenol, but the induction was not

Table 1
Effects of environmental pollutants on levels of retinoids in studies with *in vivo* exposure

Contaminant	Species	Tissue	Effects	References
p, p'-DDE	Common frog	Liver	Retinol ↑	Leiva-Presa et al. (2006)
TCDD	Rat	Liver	REs ↓, RA ↑	Nilsson and Hakansson (2002);
		Kidney	RA ↑, REs ↑	Schmidt et al. (2003) and Hoegberg et al. (2003)
		Plasma	Retinol ↑	Nilsson and Hakansson (2002) and van der Plas et al. (2001)
	Mouse	Liver	REs ↓, RA ↓	Hoegberg et al. (2005) and Nishimura et al. (2005)
		Kidney	RA ↑, REs ↑	
PCB 77	Rainbow trout	Liver	RA metabolization ↑	Gilbert et al. (1995)
	Brook trout	Liver	REs ↓	Boyer et al. (2000)
		Plasma	Retinol ↓	Ndayibagira et al. (1995)
		Intestine	RE ↓	Ndayibagira et al. (1995)
	Eider ducklings	Liver	RE ↓	Murk et al. (1994b)
		Plasma	Retinol ↑	Murk et al. (1994b)
	Quail eggs (*in ovo* exposure)	Yolk-sac	Retinol ↓	Boily et al. (2003b)
	Quial eggs (maternal exposure)	Yolk-sac	Retinol ↑	Boily et al. (2003b)
PCB 77, 126, 153	Rat	Plasma	Retinol ↓	Brouwer and Vandenberg (1986); Chen et al. (1992); Morse and Brouwer (1995) and van der Plas et al. (2001)
PCB 156, 169	Rat	Plasma	Retinol ↑	Chen et al. (1992); van der Plas et al. (2001) and Vanbirgelen et al. (1994a)
Aroclor 1242[a]	Mink	Liver, plasma	Retinol ↓	Kakela et al. (1999)
Clophen A50[a]	Frogs	Plasma	Retinol:RE ratio ↓	Gutleb et al. (1999)
	Amphibian embryos	Homogenates	Retinol ↑, RE ↑, retinol:RE ratio ↓	Gutleb et al. (1999)
Estradiol	Juvenile sturgeon	Plasma	Retinol ↑	Palace et al. (2001)

[a] Technical mixture of PCBs.

statistically significant. Anyway, other organochlorine pesticides toxaphene and endosulfan were described to inhibit binding of tritiated atRA to RAR in human prostate or uterus, respectively (Paganetto et al., 2000). Another study reported that endosulfan, together with other pesticides chlordane, dieldrin, aldrin and endrin strongly induced CYP26 in HepG2 cells and activated RAR-mediated gene transcription via RARE (Table 2; Lemaire et al., 2005). This work showed that the pesticides were able to activate RARβ and γ, but not α in stable RARα, β and γ reporter HELN cell lines. Interestingly, only chlordane was confirmed to physically bind to RAR among the studied pesticides. Such discrepancy between ligand binding and receptor activation experiments were probably caused by different experimental conditions. Binding assays are able to measure only pure physicochemical interaction of potential ligand with the receptor, while the transactivation assays cover more mechanisms such as potential cross-talk with other signaling pathways, metabolical transformation of the potential ligand, interaction of receptors with corepressors or recruitment of coactivators. These factors could be involved in mediating the effect of the other pesticides (Lemaire et al., 2005).

Another pesticide that can interact with RA signaling is methoprene, which is an insect juvenile hormone agonist that blocks metamorphosis in insects. It is quickly degraded in the environment and it has been widely applied especially in wetlands and suburban areas to reduce mosquito populations. Stable methoprene metabolite methoxy-methoprene acid was described to bind to and activate RXR receptor (Table 2; Harmon et al., 1995). Consecutive studies have confirmed this effect and they also showed that methoprene *per se* is not potent enough to produce RA-like effects at environmentally relevant concentrations (for review see Ankley et al., 2004). However, it has been reported that UV and/or microbial degradation products of methoprene caused malformations in African clawed frog (*Xenopus laevis*) that were similar to those found in the environment (La Clair et al., 1998). Moreover, the photodegradation products were more stable than the parent compound because some of them were detected in sediments even several months after application. The sunlight-induced photolytic products of methoprene were teratogenic also in embryos of zebrafish (*Danio rerio*) and the phenotypical effects were similar to those observed in zebrafish embryos treated with retinol dehydrogenase inhibitor citral, which indicates that methoprene photoproducts could affect the conversion of retinol to retinal and thus the level of RA (Smith et al., 2003). Schoff and Ankley (2004) confirmed this effect of methoprene and methoxy-methoprene acid on ROLDH activity *in vitro* using murine F9-derived cell line.

Widely used azole antimycotic compounds are known teratogens in mammals (Landauer et al., 1971; Menegola et al., 2005a,b), marine ascidian embryos (Chordata, Ascidiacea; Pennati et al., 2006) and African clawed frog (Papis et al., 2006). Their antifungal effect is mediated by inhibition of cytochrome P450-catalyzed conversion of lanosterol to ergosterol, which results in faulty fungal cell wall synthesis (Menegola et al., 2006). This inhibitory activity affects also some other members of the mammalian CYPs including CYP26, one of the key enzymes in metabolism of RA (Menegola et al., 2006). The inhibition of RA-metabolizing enzymes would lead to increase of intracellular levels of RA, which is known to induce teratogenic changes in higher concentrations. This, together with the resemblance of the

Table 2
Modes of action of the pollutants in disruption of retinoid physiology

Mode of action	Active chemicals	Tissue/cell line	References
Effects on retinoid receptors			
9cRA-RXR binding inhibition	Pulp mill-produced compounds [b]	Isolated fish RXR	Alsop et al. (2003)
AhR-RAR crosstalk	TCDD	MCF-7	Widerak et al. (2006)
Decrease of atRA-mediated response	MEHP, Wy-14643	MSC-1	Dufour et al. (2003)
	Pulp mill-produced compounds [b]	F9	Schoff and Ankley (2002)
	TCDD	Murine palate cells	Weston et al. (1995)
		SCC12F	Lorick et al. (1998)
		SCC4	Krig and Rice (2000)
Increase of atRA-mediated response	PAHs	P19	Novak et al. (2007)
	Contaminated sediment extracts	HL60	Vondracek et al. (2001)
	TCDD	MCF-7	Widerak et al. (2006)
No effect on atRA-mediated response	TCDD	P19	Novak et al. (2007)
	Clofibrate	MSC-1	Dufour et al. (2003)
Inhibition of atRA-RAR binding	Endosulfan, EBP, 4-octylphenol	Human prostate	Paganetto et al. (2000)
	Toxaphene, MEHP, 4-nonylphenol	Human uterus	Paganetto et al. (2000)
	Pulp mill-produced compounds [b]	Isolated fish RAR	Alsop et al. (2003)
	TCDD	Mammalian RAR	Lorick et al. (1998)
No effect on atRA-RAR binding	di-(2-ethylhexyl)phthalate	Human uterus, prostate	Paganetto et al. (2000)
Increase of CRABPII expression	Estradiol, xenoestrogens	Rat uterus	Li and Ong (2003)
PPARα-RAR crosstalk	Peroxisome proliferators	MSC-1	Dufour et al. (2003)
No PPARα-RAR crosstalk	MEHP, Wy-14643 [c]	ML-457	Bhattacharya et al. (2005)
RARs binding	Chlordane	Mammalian RARβ,γ	Lemaire et al. (2005)
RXRs binding	Tributyltin, triphenyltin	Gastropodian RXR	Nishikawa et al. (2004)
RARs transactivation	Chlordane, dieldrin, aldrin, endrin, endosulfan	HELN	Lemaire et al. (2005)
RXR transactivation	Methoprene derivatives	CV-1, F9	Schoff and Ankley (2004) and Harmon et al. (1995)
	Tributyltin, triphenyltin	F9	Kanayama et al. (2005)
Increase of RARs/RXRs expression	Bisphenol A	Murine embryos	Nishizawa et al. (2005)
Increase of RARα expression	PCBs	Seal blubber	Mos et al. (2007)
Disruption of retinoid metabolism			
Induction of AhR-dependent CYPs	PCDD/Fs, PAHs, coplanar PCBs	Liver	(Khlood et al., 1999; Nilsson and Hakansson, 2002; Zile, 1992)
Increase of UDP-glucuronosyltransferase gene expression	TCDD	Liver	Nishimura et al., (2005)
Modulation of PXR-dependent CYPs	PCBs, PPs, pesticides	LLC-PK1	Schuetz et al. (1998) and Kretschmer and Baldwin (2005)
Decrease of CYP2C7 expression	Wy-14643 [c], gemfibrozil, di-n-butyl phthalate	Rat liver	Fan et al. (2004)
Inhibition of retinol dehydrogenase	Methoprene and derivatives	F9	Schoff and Ankley (2004)
CYP26 [a] downregulation	p,p'-DDE	Common frog liver	Leiva-Presa et al. (2006)
	Azole pesticides	Chordata [d] liver	Menegola et al. (2006)
CYP26 [a] upregulation	Chlordane, dieldrin, aldrin, endrin, endosulfan	HepG2	Lemaire et al. (2005)
Modulation of LRAT activity	TCDD	Rat kidney, liver	Nilsson et al. (2000) and Hoegberg et al. (2003)
	PCBs	Quail yolk sac	Boily et al. (2003a)
Modulation of REH activity	PCBs	Quail yolk sac	Boily et al. (2003a; 2003b)
	PCB 77	Fish liver	Ndayibagira and Spear (1999)
ROLDH, RALDH induction	Estradiol, xenoestrogens	Rat uterus	Li et al. (2004)
Disruption of retinoid transport			
Destabilization of TTR–RBP complex	PCBs	Plasma	Brouwer and Vandenberg (1986); Murvoll et al. (1999); Sormo (2005) and van der Plas et al. (2001)

[a] RA-metabolizing cytochrome P450.
[b] Compounds derived from pulp mill effluents.
[c] Peroxisome proliferator 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid.
[d] Mammals, amphibians, ascidians.

effects produced by azoles and higher doses of RA, indicates that retinoid system can be indeed involved in the teratogenicity of azole pesticides (Menegola et al., 2006). Many pesticides (e.g. chlordane, DDE) were describe to activate pregnenolon X receptor (PXR) and so they might induce some AhR-independent CYPs (e.g. CYP2B and CYP3A) and thus affect retinoid metabolism (Schuetz et al., 1998; Kretschmer and Baldwin, 2005).

Organotin compounds have been widely used in industry and agriculture as antifouling paints. They are very persistent and toxic to various groups of organisms and they have been linked to endocrine-disruptive effects such as imposex in molluscs (Gibbs and Bryan, 1986). There exists a number of hypotheses explaining the mechanism of imposex induction such as aromatase inhibition, the inhibition of testosterone excretion, functional disorder of the female cerebropleural ganglia or involvement of amidated tetrapeptide APGWamide (Oberdorster and McClellan-Green, 2002; Oehlmann et al., 2007; Ronis and Mason, 1996). Organotins tributyltin and triphenyltin were also shown to be able to activate mammalian RXR in F9 murine embryonic carcinoma cell line at the same concentrations as its physiological ligand 9cRA (Kanayama et al., 2005) and the same effect was observed with gastropod RXR (Nishikawa et al., 2004). These findings were supported by experiments *in vivo* when injection of 9cRA produced imposex in mollusc *Thais clavigera* (Nishikawa et al., 2004). Anyway, the injection exposures of another two molluscan species (*Nucella lapillus, Nassarius reticulatus*) failed to produce any intersex induction (Oehlmann et al., 2007). Although these results suggest species-specific differences in imposex induction, it is possible that organotin-induced imposex in some species could be mediated at least partly by activation of retinoid receptor RXR (for review see Nishikawa, 2006).

Thus, the effects of pesticides on retinoid signaling seems to be mediated by affecting activities of retinoid-metabolizing enzymes, interaction with retinoid receptors or it might be also possible that some pesticides could produce their effects on retinoid signaling by crosstalk with other receptors (e.g. ER).

2.3. TCDD

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the prototype compound for a class of coplanar halogenated aromatic hydrocarbons, which are known to share a common mechanism of action and induce similar toxic effects. TCDD achieved notoriety in the 1970's when it was discovered as a contaminant of herbicide Agent Orange and was shown to produce birth defects (Dwernychuk et al., 2002). As an environmental contaminant it continues to generate great concern because of its widespread distribution, persistence within the food chain, and great toxic potency (Boening, 1998; Janosek et al., 2006). TCDD is the most potent activator of aryl hydrocarbon receptor (AhR), a receptor that plays a key role in mediating toxic effects of many persistent organic pollutants such as PCDD/Fs, some polychlorinated biphenyls (PCBs) or PAHs. The contaminants that activate AhR were described to cause toxic effects reminding symptoms of vitamin A depletion such as respiratory tract and bile duct keratinization, dermal and epithelial lesions, thymus atrophy, immunodeficiency or impaired reproduction (Nilsson and Hakansson, 2002; Simms et al., 2000). Moreover it has been described that at least some of the negative effects caused by AhR ligands in exposed animals could be compensated by supplementation with vitamin A, which suggests interaction with system of retinoid transport, metabolism and signaling (Nilsson and Hakansson, 2002; Yang et al., 2005). Experiments with TCDD in rodents showed mobilization of retinoid-storage forms in liver, increase of renal REs levels and urinary excretion of retinoid metabolites (Table 1). It is generally accepted that this decrease in retinoid stores in liver results from decreased formation and increased metabolization of hepatic REs (Brouwer et al., 1989a; Hoegberg et al., 2003; Nilsson et al., 1996; for review see Nilsson and Hakansson, 2002).

The primary biochemical response to the AhR activation is an induction of monooxygenases CYP1A1, CYP1A2 and CYP1B1 and other drug metabolizing enzymes (Janosek et al., 2006; Soprano and Soprano, 2003). Some of these enzymes have been implicated in conversion of retinol to retinal, retinal to RA as well as in the conversion of RA to more polar metabolites (Nilsson and Hakansson, 2002; Schmidt et al., 2003). TCDD also increased the expression of UDP-glucuronosyltransferase and thus it could decrease the levels of hepatic vitamin A and increase excretion of retinoyl glucuronides (Nishimura et al., 2005). The reduction of hepatic levels of REs induced by TCDD seems to be mediated also by decrease of enzyme activity responsible for conversion of retinol to retinyl palmitate, such as LRAT in liver (Nilsson et al., 1996, 2000). However, a reverse trend with increased renal REs levels was observed in kidneys (Hoegberg et al., 2003). Despite the mobilisation of liver REs confirmed by kinetic studies (Kelley et al., 2000), no increase of REH activity was observed in liver of rats exposed to TCDD (Nilsson et al., 2000). The decrease of the hepatic LRAT activity may be responsible for the increase of retinol plasma levels described in rats exposed to TCDD (Fletcher et al., 2005; van der Plas et al., 2001).

Nilsson et al. (2000) described a significant increase of RA-levels in serum and kidneys of TCDD-treated rats. This phenomenon has been confirmed in a study of Schmidt et al. (2003) who monitored the activity and expression of CYPs that could be responsible for production or degradation of atRA (CYP1A1, 1A2, and 2B1/2) and reported that the TCDD-induced atRA synthesis seemed to be mediated by CYP1A1. Although hepatic atRA level increased after TCDD-exposure in rats (Schmidt et al., 2003), no such effect was observed in mice (Hoegberg et al., 2005, 2003). This discrepancy in TCDD-induced changes of atRA levels could be caused by some AhR-inducible enzyme that would posses different substrate specificity in these species (Hoegberg et al., 2005). The difference in both closely related mammalian species suggests that there could be also some substantial differences among other animal species.

Experiments with gene-knockout mice have indicated that the effects of TCDD on retinoid physiology are produced entirely via AhR because AhR-deficient mice did not display any changes of hepatic retinoid levels after TCDD treatment (Nishimura et al., 2005). Similarly, Hoegberg et al. (2005) showed that the effect of TCDD on retinoid physiology seems to be connected with RXRβ because RXRβ deficient-mice were non-responsive to TCDD treatment, while mice deficient in other isoforms of RARs and RXRs showed the same response as wild-type mice. CRBP I seems to be important in maintenance of retinoid balance, because CRBP I-deficient mice were more prone to the effects of TCDD on retinoid levels than mice deficient in other retinoid binding proteins (Hoegberg et al., 2005).

One of the symptoms of severe dioxin exposure in humans and some other species is chloracne, which is believed to be linked to retinoid disruption (Berkers et al., 1995). It has been shown that TCDD caused concentration and time-dependent decrease of atRA-binding to RARα and RARγ in cultured human keratinocytes SCC12F without a change of the transcription of the genes for the receptors (Lorick et al., 1998). Moreover, TCDD also decreased mRNA levels of anti-mitogenic cytokine TGFβ, whose regulation is at least partly regulated by atRA in a receptor-mediated manner. In another study, TCDD reduced amount of mRNA for transglutaminase, whose expression is also induced by atRA in malignant human keratinocytes (Krig and Rice, 2000). This effect was probably mediated indirectly because TCDD affected neither activity of RARE-linked reporter gene nor the availability of RAR-ligand.

Reciprocally, involvement of retinoids in the dioxin-signaling pathway was indicated by suppression of constitutive levels of AhR mRNA in the human keratinocytes or inhibition of AhR-induced expression of CYP1A1 in human colorectal adenocarcinoma Caco-2 cells after treatment with RA (Fallone et al., 2004; Nilsson and Hakansson, 2002).

Besides the changes in retinoid system, TCDD has been described to induce teratogenic effects, such as cleft palate, similar to those occurring after treatment with atRA in mice (Abbott and Birnbaum, 1989). Moreover, after coexposure of TCDD and atRA, the cleft palate had been produced with much lower median effective concentration of both compounds than in the individual exposures. The mechanism of action has been studied *in vitro* in murine embryonic palate mesenchymal cells and the effect seemed to be mediated by modulation of RA-signaling pathway by TCDD, which has caused inhibition of atRA-induced expression of RARβ and CRABII (Weston et al., 1995). Interestingly, Widerak et al. (2006) have reported the opposite effect in human breast cancer cell line MCF-7, where TCDD activated expression of RARE-linked reporter gene. The authors suggested that activated AhR have sequestrated corepressor SMRT from complex with RARα, which has then become active even without binding of any agonist (Widerak et al., 2006). The physical interaction between AhR and SMRT has been also described in human colorectal adenocarcinoma Caco-2 cells (Fallone et al., 2004), documenting yet another way of cross-talk between signaling pathways of retinoid and Ah receptors. However, no significant interaction of TCDD and RARE-linked reporter gene was found in malignant human keratinocytes (Krig and Rice, 2000) and murine embryonic carcinoma cell line P19 (Novak et al., 2007).

To conclude, it seems that AhR mediates all effects of TCDD on retinoid physiology and the activation of AhR could lead either to crosstalk with retinoid signaling pathway in some sensitive cell types or changes in activity of the enzymes responsible for transformation of retinoids.

2.4. Polychlorinated biphenyls

There are 209 congeners of PCBs and some of them (mainly the coplanar ones) are agonists of AhR inducing dioxin-like toxicity. Large quantities of PCBs had been produced and applied as dielectrics, plasticizers or adhesives in the past. Because of their high input to the environment, persistence and high bioaccumulating/biomagnifying potential, they have become an important subject for ecotoxicological studies (Schmitz et al., 1995). Exposure to PCBs is known to cause significant changes in retinoid circulatory levels, which are therefore considered as a sensitive biomarker of the exposure to organochlorine compounds (Fisk et al., 2005; Nilsson and Hakansson, 2002; Rolland, 2000; Simms and Ross, 2000).

Many studies showed that level of PCB contamination is dose-dependently associated with levels of retinoids in wild populations of fish (Doyon et al., 1999; Nacci et al., 2001), birds (Boily et al., 1994; Champoux et al., 2006; Kuzyk et al., 2003; Murk et al., 1996) or mammals (Murk et al., 1998; Simms et al., 2000).

Some of the retinoid disturbing effects seem to be produced through modulation of retinoid metabolizing enzymes, including AhR-dependent cytochromes (Zile, 1992). It has been shown that RA hydroxylation in fish could be accelerated by coplanar PCBs (Boyer et al., 2000). There was a significant increase in activity of atRA metabolizing CYPs after 56 days in rainbow trout (*Oncorhynchus mykiss*) exposed intraperitoneally with 5 μg/g of PCB-77, while no effect was observed after 7 days (Gilbert et al., 1995). Yet, the EROD activity was significantly higher in the PCB-treated group both at 7 and 56 days, which suggests that AhR-dependent cytochrome CYP1A1, which is responsible for the ethoxyresorufin-*O*-deethylase activity, does not participate significantly in atRA metabolization in fish. While the hepatic REs seemed to be unaffected by 5 μg/g PCB-77 in rainbow trout (Gilbert et al., 1995), the REs levels were significantly decreased at the same dose in brook trout (*Salvelinus fontinalis*) after the same exposure duration (56 days; Boyer et al., 2000). This dose has also caused decrease in growth rate as well as plasma retinol and retinyl and 3,4-retinyl palmitate in intestine wall of brook trout (Table 1; Ndayibagira et al., 1995). The REH activity in liver was dose-dependently inhibited by this PCB congener and this effect seems to be mediated probably on REH expression level, because the enzyme activity was not affected by exposure of control liver microsomes *in vitro*. The inhibition of REH may affect the uptake of REs from chylomicron remnants as well as the mobilization of stored REs in the brook trout (Ndayibagira and Spear, 1999).

In frogs (*Rana temporaria, Xenopus laevis*), maternal exposure to technical PCB mixture Clophen A50 has been associated with increase of malformation ratio (Gutleb et al., 1999). The homogenates from embryos of Clophen A50 exposed female frogs displayed increased levels of retinol as well as retinyl palmitate. Anyway, the molar ratio of retinol: REs was decreased mainly in early development stages.

There are many papers on effects of PCBs in birds. Many bird species accumulate pollutants by biomagnification due to their relatively high position in the food chain. The concentrations of pollutants in birds could therefore reach effective levels even when the contamination does not produce any observable effects in the lower part of the food chain. Field studies suggest that effects of PCB contamination on level of hepatic REs and retinol in plasma seem to be connected with extent of exposure but also species-specific differences should be taken to account. The

negative correlation of hepatic vitamin A levels and EROD activity ascribed to PCBs contamination has been shown in studies of black guillemots (*Cepphus grylle*) and tree swallows (*Tachycineta bicolor*) *in situ* on relatively highly polluted localities of Labrador or Great Lakes and St Lawrence river region, respectively (Bishop et al., 1999; Kuzyk et al., 2003 resp.). This indicates that this decrease could be produced by AhR-dependent mechanisms. Anyway, studies comparing hepatic REs with EROD activity in glaucous gulls (*Larus hyperboreus*) (Henriksen et al., 2000) or with PCBs levels in Brunnich's guillemot (*Uria lomvia*) and common eider (*Somateria mollissima*; Murvoll et al., 2007) have not proved any association either because of interspecies differences or, more likely, because of the relatively pristine character of the populations' habitat (Bjornoya in Barents Sea and Svalbard, respectively). Similar results are reported for plasma retinol levels. While the exposure to environmental PCBs mixtures has been described to decrease plasma retinol levels in blue heron (*Ardea herodias*) from highly polluted area of St. Lawrence River, Canada (Champoux et al., 2006) and in hatchlings of European shag (*Phalacrocorax aristotelis*) from more moderately polluted Norwegian coast (Murvoll et al., 2006), there was an increase of plasma retinol levels in artificially hatched common tern chicks (*Sterna hirundo*) from relatively highly contaminated localities in Wadden Sea, Netherlands (Murk et al., 1994a). However, no clear relationship between plasma retinol and PCBs levels was found in European shag hatchlings (Murvoll et al., 1999) from the coast of Central Norway and hatchlings and adult glaucous gulls from Svalbard in semi-field experiment (Henriksen et al., 1998), probably because the populations of arctic and sub arctic areas are much less contaminated. The discrepancy might be also partly explained by different metabolism of PCBs in some bird species. At least in case of common tern hatchlings, it has been suggested that their limited ability to produce hydroxylated metabolites of PCBs prevents the destabilization of retinol transporting TTR–RBP complex in plasma and so the plasma could accommodate more retinol from mobilization of the liver retinoid stores (Murk et al., 1994a; Murvoll et al., 1999). Anyway, the difference of contamination levels in the studied areas is probably more relevant in this case. Moreover, there could be a substantial difference between types of pollution in the studied areas of the *in situ* studies and although the effects are ascribed to PCBs, they might be mediated by interaction of various types of contaminants in the complex mixture.

Several studies with birds artificially exposed to pollutants have been reported. Murk et al. (1994b) observed significant effects in eider ducklings (*Somateria mollissima*) exposed intraperitoneally to PCB-77. Hepatic REs negatively correlated with PCB-77 while plasma retinol levels shown opposite trend. In eggs and embryos of hens fed for 7 weeks with fish from PCBs-contaminated localities of Great Lakes, the total amount of vitamin A was not affected (Zile et al., 1997). However, the proportion ratio of individual retinoid representatives had changed in yolks of eggs of the group with high PCB diet (lower all-*trans* retinol and 3,4-didehydroretinol levels and higher levels of retinyl palmitate), while the ratios of retinoids in the embryos were not affected. In Japanese quail (*Coturnix coturnix japonica*) eggs, which were exposed by injection of mono-ortho PCB congeners, both yolk retinol concentration and the retinol:retinyl palmitate molar ratio were decreased compared to control eggs (Boily et al., 2003a). REH and LRAT activities were elevated in yolk-sac membranes of the exposed eggs and the retinol concentration was negatively correlated with the LRAT activity.

Significant differences related to the way of exposure have been found in the effect of coplanar congener PCB-77 on retinol metabolism in the quail eggs (Boily et al., 2003b). The yolk retinol levels decreased and REH activity in yolk-sac membrane increased in eggs injected by PCB-77 (2–20 μg/g). On the other hand, after maternal exposure (3 bimonthly injections of 5 μg/g of PCB), eggs contained higher yolk levels of retinol and retinyl palmitate and REH activity was significantly inhibited. The difference between the maternal and *in ovo* exposure may be possibly related to transformation of the PCB to toxic metabolites by the adult organism or differences in post-transcriptional regulation of REH expression (Boily et al., 2003b). The results from experiments with birds and frogs indicate that maternal exposure to PCBs leads to increased deposition of retinol in form of REs in the eggs.

Similarly to birds, many mammals are on the top of the food chain and therefore some of them could be exposed to high levels of bioaccumulative contaminants. The effects of single congeners of PCBs are equivocal in rats. Although some congeners (PCB-169, PCB-156) were reported to increase levels of plasma retinol in a similar way as dioxins (Chen et al., 1992; van der Plas et al., 2001; Vanbirgelen et al., 1994a), a decrease of plasma retinol levels was reported after exposure to PCB-77, PCB-126, PCB-153 and PCB technical mixtures, which could represent complex mixture of PCBs in the environment (Brouwer and Vandenberg, 1986; Chen et al., 1992; Morse and Brouwer, 1995; van der Plas et al., 2001). This difference from the effect of TCDD, the strongest AhR agonist, could be related either to decrease of REH activity caused by some PCB congeners (Boily et al., 2003b; Zile, 1992) or effect of OH–PCB metabolites, that have been described to disrupt RBP–TTR complex and lead to subsequent retinoid losses due to the glomerular filtration (see 1.2 Metabolism of retinoids). This is in accordance with the fact that dioxin-like PCB congeners (e.g. PCB 156 and 169), which are not transformed to OH-metabolites easily, were described to increase plasma retinol levels in a similar fashion as TCDD in rodents and their effect is well predicted by TEQ concept (Vanbirgelen et al., 1994a,b). However, the complex environmental mixtures contain also other more biotransformable congeners that could cause the resulting decrease of plasma retinol levels that cannot be predicted by the TEQ (van der Plas et al., 2001).

Most studies focused just on rodent species and so the retinoid balance disrupting properties of PCBs are not known thoroughly in other mammalian species, yet, there are some papers on effects of PCBs in carnivores. Unlike other mammals, in carnivores a large percentage of retinol seems to be transported by other proteins than RBP and retinoids can be also transferred in form of REs in the circulation (Burri et al., 1993; Kakela et al., 2003; Schweigert et al., 1990).

The contamination of the environment by PCBs might be one of the factors that lead to decline of populations of Europeans otters (*Lutra lutra*) because there was found a strong negative correlation between hepatic retinol and PCB levels in liver together with higher susceptibility to infectious diseases (Murk et al., 1998).

The retinol levels in plasma decreased also in minks (*Mustela vision*) exposed to 1 mg of technical PCB mixture Aroclor 1242 daily for 28 days (Kakela et al., 1999). The minks were fed diets based on either freshwater or marine fish, which are richer in vitamin A and E. Hepatic level of retinol decreased in the freshwater diet variant, but not in the marine diet group. It was also shown that plasma retinyl esters levels in mink reflected the hepatic stores of retinoids and could serve as sensitive nondestructive indicator of total retinoid store (Kakela et al., 2003).

There are many *in situ* studies on retinoids in seals. Nyman et al. (2003) observed a negative correlation between hepatic REs levels and contamination (PCBs, DDT and heavy metals) in ringed and grey seal (*Halichoerus grypus*, *Pusa hispida* resp.) from highly contaminated Baltic Sea region and much cleaner polar areas. In these seals, the decrease of hepatic REs levels was correlated with dioxin-like TEQs suggesting the significant influence of dioxin-like PCBs as well as other dioxin-like compounds (Nyman et al., 2003). Routti et al., (2005) showed that there could be some differences in retinoid physiology of these seal species because there has been observed a negative association of hepatic REs and PCBs only in ring seals from the same seal populations as in work of Nyman et al. (2003). Anyway, it is possible that these differences could be explained by different vitamin A levels in the diet of both species. Interestingly, contamination was also associated with slightly elevated plasma retinol levels in grey seals (Nyman et al., 2003). Most other studies, however, report negative correlations between plasma retinol levels and total PCB loads in free-ranging grey seals from clean coast of Central Norway (Jenssen et al., 2003), relatively highly contaminated California sea lions (*Zalophus californianus*) from Californian coast (Debier et al., 2005), harbor seals (*Phoca vitulina*) from coast of Washington State, USA (Mos et al., 2007) and in captive seals exposed in semi-field experiments (Brouwer et al., 1989b; Swart et al., 1994). In the study of Simms et al. (2000), the lowest plasma retinol levels were reported in the most PCB contaminated population of free-ranging harbor seal pups coast of Washington State, USA. However, there was a big shift in the results when a nursing status of the pups has been taken into account because the nursed pups have much higher levels of retinoids than orphans or freshly weaned pups. The correction revealed that plasma retinol levels in both the high and low contaminated populations were positively correlated with PCBs and PCDD/Fs levels expressed by TEQs (Simms et al., 2000). This addresses a great problem of minimization of confounding factors in a study of retinoid-status in free-ranging populations of animals, which is in more detail discussed elsewhere (Simms and Ross, 2000). Anyway, all these contradictory results may be explained by the fact that different contaminant mixtures may induce antagonistic effects on plasma retinol levels in seals. The resulting effect (in accordance to the hypothesis of van der Plas et al., 2001) depends on the composition of the complex mixture of contaminants as well as on the proportion of each chemical or congeners in the mixture (Sormo, 2005; van der Plas et al., 2001). Noteworthy, in a recent study of Mos et al. (2007) a negative correlation between total PCBs and plasma retinol has been observed in the same harbor seal populations as in Simms et al. (2000), who described the positive correlation of plasma retinol levels with TEQs of total PCBs and PCDD/Fs. This finding shows that assessments using total PCB concentrations or TEQs (calculated from levels of PCBs and other dioxin-like compounds) may yield different conclusions with respect to plasma retinol levels even within the same population.

Thus, the weight of evidence suggests depleted plasma retinol levels in free-ranging animal populations following exposure to PCBs; with the possibility that high levels of persistent dioxin-like compounds may counteract this effect by increasing plasma retinol levels in some populations. Laboratory and *in situ* studies seem to indicate common patterns with respect to PCBs and dioxin-like compounds. While dioxin-like compounds may increase plasma retinol levels by decreased generation or increased mobilization of retinol storage forms, the total PCB load may deplete plasma retinol levels by disrupting RBP–TTR complex. Also an induction of CYPs (e.g. CYP2B and CYP3A) that are induced by different receptors than AhR (e.g. PXR) might be involved in these processes (Schuetz et al., 1998; Kretschmer and Baldwin, 2005). Noteworthy, Mos et al. (2007) observed positive correlation of RARα levels in blubber and levels of PCBs in harbor seal population on coast of Washington State, USA. Thus modulation of the receptors levels could represent another mechanism of effect of PCBs on retinoid signaling. Anyway it must be taken to account that environmental contaminants occur in complex mixtures of chemicals, some of which are probably still unknown (Schwarzenbach et al., 2006). Although PCBs present often very abundant part the environmental contamination, they do not have to be the only factor responsible for all the effects that are ascribed to them in the free ranging animals.

2.5. Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) represent another group of wide spread environmental pollutants. This class of toxic organic compounds consists of hundreds of compounds that are ubiquitous in the environment and foodstuffs. They are released into the environment mainly by incomplete combustion of fossil fuels or oil spills. PAHs have been shown to produce carcinogenicity in experimental animals (Miller and Ramos, 2001; Xue and Warshawsky, 2005) and some of their representatives act as AhR ligands (Machala et al., 2001). Although they are much less potent activators of AhR than PCDD/Fs or non-*ortho* PCBs, their importance is supported by their high levels in the environment. PAHs have been reported to be embryotoxic and teratogenic (Miller and Ramos, 2001), which makes them suspected from interfering with morphogenesis and thus with retinoid signaling. The teratogenic effects of PAH congener 3-methylcholanthrene and atRA were studied in

rat embryos. 3-methylcholanthrene was embryotoxic but did not elicit any teratogenicity when exposed alone. In co-exposure with atRA, it even decreased the teratogenic potential of atRA by induction of atRA-metabolizing enzymes, but the embryotoxic effect of the PAH congener was rather potentiated by atRA (Khlood et al., 1999).

There are also some indications that PAHs interact with atRA signaling *in vitro*. Sediment extracts contaminated mainly by PAHs were shown to significantly stimulate atRA-induced differentiation of HL-60 cells, although no correlation of the effect with PAHs levels has been observed (Vondracek et al., 2001). Moreover, PAHs congeners dibenzo[*a,h*]anthracene, benzo[*a*]anthracene and benzo[*a*]pyrene were described to increase atRA-induced activity of RARE-linked reporter gene in murine embryonic carcinoma cell line P19 (Novak et al., 2007). The mechanism of action is not known, but it does not seem to be mediated through AhR because TCDD did not produce any significant effect in this assay. Anyway, more studies are needed to prove if this retinoid system disruption plays a significant role in teratogenicity and embryotoxicity of PAHs *in vivo*.

2.6. Plasticizers and hypolipidemic drugs

The structure of some plasticizers and hypolipidemic drugs, which were proved to be environmental contaminants, is similar to structure of retinoids and they could therefore impair the activity of the signaling pathway of RA. Many plasticizers and hypolipidemic drugs share common mode of action and act as peroxisome proliferators (PPs; Cajaraville et al., 2003). PPs have been shown to act via peroxisome proliferator-activated receptors (PPARs), of which there are at least three subtypes: α, β, and γ and their signaling pathway intensively cross-talks with the signaling of retinoids (Dufour et al., 2003).

These compounds are probably able to disrupt retinoid system in several ways. It has been shown that PPs decrease levels of RA-metabolizing enzymes in rats. Hypolipidemic drugs Wy-14,643 and gemfibrozil or plasticizer di-n-butyl phthalate caused dose-dependent decrease of both expression and protein levels of retinoic acid 4-hydroxylase CYP2C7 in rat liver after 13 week exposure. The down-regulation of CYP2C7 activity is predicted to increase the local levels of RA and thus alter the activity of RA-signaling pathway (Fan et al., 2004). On the other hand, PPs were described to activate PXR and so they could induce AhR-independent retinoid-metabolising CYPs (Schuetz et al., 1998; Marill et al., 2003; Kretschmer and Baldwin, 2005).

The organ-specific effect of plasticizers phthalates, which are PPs, and alkylphenols that do not possess peroxisome proliferating properties, was shown in study of Paganetto et al. (2000) on *ex vivo* human tissues. While ethyl-n-butyl phthalate (EBP) and 4-octylphenol inhibited binding of tritiated atRA in uterus and not in prostate, mono-(2-ethylhexyl)phthalate (MEHP), a main metabolite of di-(2-ethylhexyl)phthalate, and 4-nonylphenol inhibited binding of atRA only in prostate. However, the parent compound di-(2-ethylhexyl)phthalate did not produce significant effect in either tissue (Paganetto et al., 2000).

Phthalates were also described to cause degeneration of testes and apoptosis of primary spermatocytes. This effect was linked with changes of retinoid signaling, which is crucial for normal function of testis (Dufour et al., 2003; Vo et al., 2001). Nishizawa et al. (2005) showed that common plasticizer bisphenol A significantly increased levels of mRNA for RARα and RXRα in murine embryos in doses much lower than putative environmentally relevant doses. Retinoids were also shown to be important in modulating the effect of bisphenol A. While negative effect of bisphenol A (decreased number of sperms of neonatally exposed males) was cancelled out by concurrent administration of retinol acetate, retinoid insufficiency accelerates this effect (Nakahashi et al., 2001).

MEHP and Wy-14,643 have disrupted the RA-induced nuclear localization of RARα in primary Sertoli cells and also inhibited RA-stimulated increase in transcriptional activity of a RA-responsive reporter gene in immortalized mouse Sertoli cells MSC-1. On the other hand clofibrate, which is another hypolipidemic drug with strong peroxisome proliferating activity in liver but only weak testicular toxicant, produced only weak effect and did not affect long-term expression of the RA-induced reporter gene. A possible biochemical mechanism for such disruption in the Sertoli cells may be the competition between RARα and PPARα for their heterodimerization partner RXR (Dufour et al., 2003). However, the effect of MEHP and Wy-14,643 on RARα activity was not observed in murine liver cell line ML-457 (Bhattacharya et al., 2005). The different response to PPs in cells derived from testes and liver may be connected with activity of mitogen-activated protein kinase, which was significantly increased in liver but reduced in testes.

3. Conclusions

The environmental contaminants are known to produce wide range of adverse effects in exposed animals and many of them interfere with processes such as development, embryogenesis, reproduction or function of the immune system, which are connected with action of retinoids. The toxic effects may be mediated by changes in metabolism, transport and/or signaling of retinoids. This hypothesis is supported by a number of studies reporting changes in levels of retinoids in populations of animals living at contaminated localities and works describing the interaction of diverse types of pollutants with system of retinoid regulation. Many pollutants have been shown to interact with multiple targets within retinoid regulated signaling pathway. The pollutants can produce the effect both directly and indirectly via metabolism of retinoids or cross-talk with other signaling pathways. However, further investigation is needed to prove that the mechanisms that were shown mainly *in vitro* are indeed participating in mediation of the toxic effects of the pollutants in real *in vivo* conditions.

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References

Abbott BD, Birnbaum LS. Cellular alterations and enhanced induction of cleft-palate after coadministration of retinoic acid and Tcdd. Toxicol Appl Pharmacol 1989;99:287–301.

Aguinaldo AMA, Turbeville JM, Linford LS, Rivera MC, Garey JR, Raff RA, et al. Evidence for a clade of nematodes, arthropods and other moulting animals. Nature 1997;387:489–93.

Alsop D, Hewitt M, Kohli M, Brown S, Van der Kraak G. Constituents within pulp mill effluent deplete retinoid stores in white sucker and bind to rainbow trout retinoic acid receptors and retinoid X receptors. Environ Toxicol Chem 2003;22:2969–76.

Alsop D, Brown S, Van der Kraak G. The effects of copper and benzo a pyrene on retinoids and reproduction in zebrafish. Aquat Toxicol 2007;82:281–95.

Ankley GT, Degitz SJ, Diamond SA, Tietge JE. Assessment of environmental stressors potentially responsible for malformations in North American anuran amphibians. Ecotoxicol Environ Saf 2004;58:7–16.

Balmer JE, Blomhoff R. Gene expression regulation by retinoic acid. J Lipid Res 2002;43:1773–808.

Barua AB, Sidell N. Retinoyl beta-glucuronide: a biologically active interesting retinoid. J Nutr 2004;134:286S–9S.

Bastien J, Rochette-Egly C. Nuclear retinoid receptors and the transcription of retinoid-target genes. Gene 2004;328:1–16.

Berkers JAM, Hassing I, Spenkelink B, Brouwer A, Blaauboer BJ. Interactive effects of 2,3,7,8-Tetrachlorodibenzo-P-Dioxin and retinoids on proliferation and differentiation in cultured human keratinocytes — quantification of cross-linked envelope formation. Arch Toxicol 1995;69:368–78.

Berube VE, Boily MH, DeBlois C, Dassylva N, Spear PA. Plasma retinoid profile in bullfrogs, *Rana catesbeiana*, in relation to agricultural intensity of sub watersheds in the Yamaska River drainage basin, Quebec, Canada. Aquat Toxicol 2005;71:109–20.

Besselink HT, Flipsen E, Eggens ML, Vethaak AD, Koeman JH, Brouwer A. Alterations in plasma and hepatic retinoid levels in flounder (*Platichthys flesus*) after chronic exposure to contaminated harbour sludge in a mesocosm study. Aquat Toxicol 1998;42:271–85.

Bhattacharya N, Dufour JM, Vo MN, Okita J, Okita R, Kim KH. Differential effects of phthalates on the testis and the liver. Biol Reprod 2005;72:745–54.

Bishop CA, Mahony NA, Trudeau S, Pettit KE. Reproductive success and biochemical effects in tree swallows (*Tachycineta bicolor*) exposed to chlorinated hydrocarbon contaminants in wetlands of the Great Lakes and St. Lawrence River basin, USA and Canada. Environ Toxicol Chem 1999;18: 263–71.

Blaner WS, Olson JA. Retinol and retinoic acid metabolism. In: Sporn MB, Roberts AB, Goodman DS, editors. The Retinoids: Biology, Chemistry, and Medicine. New York: Raven Press; 1994. p. 229–56.

Blumberg B, Evans RM. Orphan nuclear receptors-new ligands and new possibilities. Genes Dev 1998;12:3149–55.

Boening DW. Toxicity of 2,3,7,8-Tetrachlorodibenzo-p-dioxin to several ecological receptor groups: a short review. Ecotoxicol Environ Saf 1998;39: 155–63.

Boily MH, Champoux L, Bourbonnais DH, Desgranges JL, Rodrigue J, Spear PA. Beta-Carotene and retinoids in eggs of great-blue herons (*Ardea herodias*) in relation to St-Lawrence-River contamination. Ecotoxicology 1994;3:271–86.

Boily MH, Ndayibagira A, Spear PA. Retinoid metabolism (LRAT, REH) in the yolk-sac membrane of Japanese quail eggs and effects of mono-ortho-PCBs. Comp Biochem Physiol C 2003a;134:11–23.

Boily MH, Ndayibagira A, Spear PA. Retinoids, LRAT and REH activities in eggs of Japanese quail following maternal and in ovo exposures to 3,3',4,4'-tetrachlorobiphenyl. Ecotoxicology 2003b;12:9–21.

Boily MH, Berube VE, Spear PA, DeBlois C, Dassylva N. Hepatic retinoids of bullfrogs in relation to agricultural pesticides. Environ Toxicol Chem 2005;24: 1099–106.

Bouton D, Escriva H, de Mendonca RL, Glineur C, Bertin B, Noel C, et al. A conserved retinoid X receptor (RXR) from the mollusk *Biomphalaria glabrata* transactivates transcription in the presence of retinoids. J Mol Endocrinol 2005;34:567–82.

Boyer PM, Ndayibagira A, Spear PA. Dose-dependent stimulation of hepatic retinoic acid hydroxylation/oxidation and glucuronidation in brook trout, *Salvelinus fontinalis*, after exposure to 3,3',4,4'-tetrachlorobiphenyl. Environ Toxicol Chem 2000;19:700–5.

Branchaud A, Gendron A, Fortin R, Anderson PD, Spear PA. Vitamin-A stores, teratogenesis, and erod activity in white sucker, *Catostomus commersoni*, from Riviere-Des-Prairies near Montreal and a reference site. Can J Fish Aquat Sci 1995;52:1703–13.

Bridges C, Little E, Gardiner D, Petty J, Huckins J. Assessing the toxicity and teratogenicity of pond water in north-central Minnesota to amphibians. Environ Sci Pollut Res Int 2004;11:233–9.

Brouwer A, Vandenberg KJ. Binding of a Metabolite of 3,4,3',4'-Tetrachlorobiphenyl to transthyretin reduces serum vitamin-A transport by inhibiting the formation of the protein complex carrying both retinol and thyroxine. Toxicol Appl Pharmacol 1986;85:301–12.

Brouwer A, Hakansson H, Kukler A, Van Den Berg KJ, Ahlborg UG. Marked alterations in retinoid homeostasis of Sprague–Dawley rats induced by a single i.p. dose of 10 [mu]g/kg of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicology 1989a;58:267–83.

Brouwer A, Reijnders PJH, Koeman JH. Polychlorinated Biphenyl (Pcb)-contaminated fish induces vitamin-A and thyroid-hormone deficiency in the common seal (*Phoca vitulina*). Aquat Toxicol 1989b;15:99–105.

Burri BJ, Neidlinger TR, Zwick H. Comparison of the properties and concentrations of the isoforms of retinol-binding protein in animals and human-beings. Am J Vet Res 1993;54:1213–20.

Cajaraville MP, Cancio M, Ibabe A, Orbea A. Peroxisome proliferation as a biomarker in environmental pollution assessment. Microsc Res Tech 2003;61: 191–202.

Chambon P. A decade of molecular biology of retinoic acid receptors. FASEB J 1996;10:940–54.

Champoux L, Rodrigue J, Trudeau S, Boily MH, Spear PA, Hontela A. Contamination and biomarkers in the great blue heron, an indicator of the state of the St. Lawrence River. Ecotoxicology 2006;15:83–96.

Chen LC, Berberian I, Koch B, Mercier M, Azaisbraesco V, Glauert HP, et al. Polychlorinated and polybrominated biphenyl congeners and retinoid levels in rat-tissues-structure-activity-relationships. Toxicol Appl Pharmacol 1992;114:47–55.

Ciaccio M, Valenza M, Tesoriere L, Bongiorno A, Albiero R, Livrea MA. Vitamin-A inhibits doxorubicin-induced membrane lipid-peroxidation in rat-tissues in vivo. Arch Biochem Biophys 1993;302:103–8.

De Luca LM. Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. FASEB J 1991;5:2924–33.

DeBernardi E, Sotgia E, Ortolani G. Retinoic acid treatment of ascidian embryos: effects on larvae and metamorphosis. Anim Biol 1994;3.

Debier C, Ylitalo GM, Weise M, Gulland F, Costa DP, Le Boeuf BJ, et al. PCBs and DDT in the serum of juvenile California sea lions: associations with vitamins A and E and thyroid hormones. Environ Pollut 2005;134: 323–32.

Dorsey WC, Tchounwou PB, Ishaque AB, Shen E. Transcriptional activation of stress genes and cytotoxicity in human liver carcinoma (HepG2) cells exposed to pentachlorophenol. Int J Mol Sci 2002;3:992–1007.

Doyon C, Fortin R, Spear PA. Retinoic acid hydroxylation and teratogenesis in lake sturgeon (*Acipenser fulvescens*) from the St. Lawrence River and Abitibi region, Quebec. Can J Fish Aquat Sci 1999;56:1428–36.

Dufour JM, Vo MN, Bhattacharya N, Okita J, Okita R, Kim KH. Peroxisorne proliferators disrupt retinoic acid receptor alpha signaling in the testis. Biol Reprod 2003;68:1215–24.

Dwernychuk LW, Cau HD, Hatfield CT, Boivin TG, Hung TM, Dung PT, et al. Dioxin reservoirs in southern Viet Nam-A legacy of Agent Orange. Chemosphere 2002;47:117–37.

Eifert C, Sangster-Guity N, Yu LM, Chittur SV, Perez AV, Tine JA, et al. Global gene expression profiles associated with retinoic acid-induced differentiation of embryonal carcinoma cells. Mol Reprod Dev 2006;73: 796–824.

Eskild W, Hansson V. Vitamin A functions in the reproductive organs. In: R. B., editor. Vitamin A in health and disease. New York: Marcel Dekker; 1994. p. 531–59.

Fallone F, Villard PH, Seree E, Rimet O, Nguyen QB, Bourgarel-Rey W, et al. Retinoids repress Ah receptor CYP1A1 induction pathway through the SMRT corepressor. Biochem Biophys Res Commun 2004;322:551–6.

Fan LQ, Brown-Borg H, Brown S, Westin S, Mode A, Corton JC. PPAR alpha activators down-regulate CYP2C7, a retinoic acid and testosterone hydroxylase. Toxicology 2004;203:41–8.

Fisk AT, de Wit CA, Wayland M, Kuzyk ZZ, Burgess N, Letcher R, et al. An assessment of the toxicological significance of anthropogenic contaminants in Canadian arctic wildlife. Sci Total Environ 2005;351-352:57–93.

Fletcher N, Giese N, Schmidt C, Stern N, Lind PM, Viluksela M, et al. Altered retinoid metabolism in female Long–Evans and Han/Wistar rats following long term 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-treatment. Toxicol Sci 2005;86:264–72.

Folli C, Pasquato N, Ramazzina I, Battistutta R, Zanotti G, Berni R. Distinctive binding and structural properties of piscine transthyretin. FEBS Lett 2003;555: 279–84.

Gardiner D, Ndayibagira A, Grun F, Blumberg B. Deformed frogs and environmental retinoids. Pure Appl Chem 2003;75:2263–73.

Gibbs PE, Bryan GW. Reproductive failure in populations of the dog-whelk, *Nucella lapillus*, caused by imposex induced by tributyltin from antifouling paints. J Mar Biol Assoc UK 1986;66:767–77.

Gilbert ML, Cloutier MJ, Spear PA. Retinoic acid hydroxylation in rainbow-trout (*Oncorhynchus mykiss*) and the effect of a coplanar Pcb, 3,3',4,4'-Tetrachlorobiphenyl. Aquat Toxicol 1995;32:177–87.

Green MH, Green JB. Dynamics and control of plasma retinol. Vitamin A in Health and Disease. New York: Marcel Dekker; 1994. p. 119–33.

Gutleb AC, Appelman J, Bronkhorst MC, van den Berg JHJ, Spenkelink A, Brouwer A, et al. Delayed effects of pre- and early-life time exposure to polychlorinated biphenyls on tadpoles of two amphibian species (*Xenopus laevis* and *Rana temporaria*). Environ Toxicol Pharmacol 1999;8:1–14.

Harmon MA, Boehm MF, Heyman RA, Mangelsdorf DJ. Activation of mammalian retinoid-X receptors by the insect growth-regulator methoprene. Proc Natl Acad Sci U S A 1995;92:6157–60.

Harrison EH, Hussain MM. Mechanisms involved in the intestinal digestion and absorption of dietary vitamin A. J Nutr 2001;131:1405–8.

Harvey PW, Everett DJ. Regulation of endocrine-disrupting chemicals: critical overview and deficiencies in toxicology and risk assessment for human health. Best Pract Res Clin Endocrinol Metab 2006;20:145–65.

Harvey PW, Johnson I. Approaches to the assessment of toxicity data with endpoints related to endocrine disruption. J Appl Toxicol 2002;22:241–7.

Henriksen EO, Gabrielsen GW, Skaare JU, Skjegstad N, Jenssen BM. Relationships between PCB levels, hepatic EROD activity and plasma retinol in glaucous gulls, *Larus hyperboreus*. Mar Environ Res 1998;46: 45–9.

Henriksen EO, Gabrielsen GW, Trudeau S, Wolkers J, Sagerup K, Skaare JU. Organochlorines and possible biochemical effects in glaucous gulls (*Larus hyperboreus*) from Bjornoya, the Barents Sea. Arch Environ Contam Toxicol 2000;38:234–43.

Hoegberg P, Schmidt CK, Nau H, Ross AC, Zolfaghari R, Fletcher N, et al. 2,3,7,8-tetrachlorodibenzo-p-dioxin induces lecithin: retinol acyltransferase transcription in the rat kidney. Chem-Biol Interact 2003;145:1–16.

Hoegberg P, Schmidt CK, Fletcher N, Nilsson CB, Trossvik C, Schuur AG, et al. Retinoid status and responsiveness to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice lacking retinoid binding protein or retinoid receptor forms. Chem-Biol Interact 2005;156:25–39.

Hofmann C, Eichele G. Retinoids in development. In: Sporn MB, Roberts AB, a.G.D.S., editors. The Retinoids: Biology, Chemistry, and Medicine. New York: Raven Press; 1994. p. 387–441.

Idres N, Marill J, Flexor MA, Chabot GC. Activation of retinoic acid receptor dependent transcription by all-trans-retinoic acid metabolites and isomers. J Biol Chem 2002;277:31491–8.

IUPAC-IUB. Nomenclature of retinoids. Eur J Biochem 1982;129:1–5.

Janosek J, Hilscherova K, Blaha L, Holoubek I. Environmental xenobiotics and nuclear receptors-interactions, effects and in vitro assessment. Toxicol In Vitro 2006;20:18–37.

Jenssen BM, Haugen O, Sormo EG, Skaare JU. Negative relationship between PCBs and plasma retinol in low-contaminated free-ranging gray seal pups (*Halichoerus grypus*). Environ Res 2003;93:79–87.

Johnson PTJ, Sutherland DR, Kinsella JM, Lunde KB. Review of the trematode genus Ribeiroia (Psilostomidae): ecology, life history and pathogenesis with special emphasis on the amphibian malformation problem. Advances in Parasitology, vol. 57. ; 2004. p. 191–253.

Jones G, Jones D, Teal P, Sapa A, Wozniak M. The retinoid-X receptor ortholog, ultraspiracle, binds with nanomolar affinity to an endogenous morphogenetic ligand. FEBS J 2006;273:4983–96.

Kakela R, Kakela A, Hyvarinen H, Asikainen J, Dahl SK. Vitamins A(1), A(2), and E in minks exposed to polychlorinated biphenyls (Acoclor 1242((R))) and copper, via diet based on freshwater or marine fish. Environ Toxicol Chem 1999;18:2595–9.

Kakela A, Kakela R, Hyvarinen H, Nieminen P. Effects of Aroclor 1 and different fish-based diets on vitamins A(1) (retinol) and A(2) (3,4-didehydroretinol), and their fatty acyl esters in mink plasma. Environ Res 2003;91:104–12.

Kanayama T, Kobayashi N, Mamiya S, Nakanishi T, Nishikawa J. Organotin compounds promote adipocyte differentiation as agonists of the peroxisome proliferator-activated receptor gamma/retinoid x receptor pathway. Mol Pharmacol 2005;67:766–74.

Katsuyama Y, Wada S, Yasugi S, Saiga H. Expression of the labial group hox gene Hrhox-1 and its alteration induced by retinoic acid in development of the Ascidian *Halocynthia roretzi*. Development 1995;121:3197–205.

Kelley SK, Nilsson CB, Green MH, Green JB, Hakansson H. Mobilization of vitamin a stores in rats after administration of 2,3,7,8-tetrachlorodibenzo-p991 dioxin: a kinetic analysis. Toxicol Sci 2000;55:478–84.

Khlood EBM, Miyoshi H, Iwata H, Kazusaka A, Kon Y, Abou Hadid AH, et al. Effects of concurrent exposure to 3-methylcholanthrene and vitamin A on fetal development in rats. Jpn J Vet Res 1999;47:13–23.

Kostrouch Z, Kostrouchova M, Love W, Jannini E, Piatigorsky J, Rall JE. Retinoic acid X receptor in the diploblast, *Tripedalia cystophora*. Proc Natl Acad Sci U S A 1998;95:13442–7.

Kretschmer XC, Baldwin WS. CAR and PXR: xenosensors of endocrine disrupters? Chem Biol Interact 2005;155:111–28.

Krig SR, Rice RH. TCDD suppression of tissue transglutaminase stimulation by retinoids in malignant human keratinocytes. Toxicol Sci 2000;56: 357–64.

Kuzyk ZZA, Burgess NM, Stow JP, Fox GA. Biological effects of marine PCB contamination on black guillemot nestlings at Saglek, Labrador: liver biomarkers. Ecotoxicology 2003;12:183–97.

La Clair JJ, Bantle JA, Dumont J. Photoproducts and metabolites of a common insect growth regulator produce developmental deformities in Xenopus. Environ Sci Technol 1998;32:1453–61.

Landauer W, Sopher D, Salam N. Herbicide 3-Amino-1,2,4-Triazole (Amitrole) as Teratogen. Environ Res 1971;4:539.

Leiva-Presa A, Mortensen AS, Arukwe A, Jenssen BM. Altered hepatic retinol and CYP26 levels in adult European common frogs (*Rana temporaria*) exposed to p,p '-DDE. Mar Environ Res 2006;62:S10–5.

Lemaire G, Balaguer P, Michel S, Rahmani R. Activation of retinoic acid receptor dependent transcription by organochlorine pesticides. Toxicol Appl Pharmacol 2005;202:38–49.

Li XH, Ong DE. Cellular retinoic acid-binding protein II gene expression is directly induced by estrogen, but not retinoic acid, in rat uterus. J Biol Chem 2003;278:35819–25.

Li XH, Kakkad B, Ong DE. Estrogen directly induces expression of retinoic acid biosynthetic enzymes, compartmentalized between the epithelium and underlying stromal cells in rat uterus. Endocrinol 2004;145:4756–62.

Lin BZ, Chen GQ, Xiao DM, Kolluri SK, Cao XH, Su H, et al. Orphan receptor COUP-TF is required for induction of retinoic acid receptor beta, growth inhibition, and apoptosis by retinoic acid in cancer cells. Mol Cell Biol 2000;20:957–70.

Linan-Cabello MA, Paniagua-Michel J. Induction factors derived from carotenoids and vitamin A during the ovarian maturation of *Litopenaeus vannamei*. Aquac Int 2004;12:583–92.

Linan-Cabello MA, Paniagua-Michel J, Hopkins PM. Bioactive roles of carotenoids and retinoids in crustaceans. Aquac Nutr 2002;8:299–309.

Lorick KL, Toscano DL, Toscano WA. 2,3,7,8-tetrachlorodibenzo-p-dioxin alters retinoic acid receptor function in human keratinocytes. Biochem Biophys Res Commun 1998;243:749–52.

Love JM, Gudas LJ. Vitamin-A, differentiation and cancer. Curr Opin Cell Biol 1994;6:825–31.

Maden M. The effect of vitamin-A (retinoids) on pattern-formation implies a uniformity of developmental mechanisms throughout the animal kingdom. Acta Biotheor 1993;41:425–45.

Machala M, Vondracek J, Blaha L, Ciganek M, Neca J. Aryl hydrocarbon receptor-mediated activity of mutagenic polycyclic aromatic hydrocarbons determined using in vitro reporter gene assay. Mutat Res Genet Toxicol Environ Mutagen 2001;497:49–62.

Marill J, Idres N, Capron CC, Nguyen E, Chabot GG. Retinoic acid metabolism and mechanism of action: a review. Curr Drug Metab 2003;4:1–10.

Martin PA, Mayne GJ, Bursian S, Palace V, Kannan K. Changes in thyroid and vitamin A status in mink fed polyhalogenated-aromatic-hydrocarbon contaminated carp from the Saginaw River, Michigan, USA. Environ Res 2006;101: 53–67.

McGrane MM. Vitamin A regulation of gene expression: molecular mechanism of a prototype gene. J Nutr Biochem 2007;18:497–508.

Menegola E, Broccia ML, Di Renzo F, Massa V, Giavini E. Craniofacial and axial skeletal defects induced by the fungicide triadimefon in the mouse. Birth Defects Res Part B-Dev Reprod Toxicol 2005a;74:185–95.

Menegola E, Broccia ML, Di Renzo F, Massa V, Giavini E. Study on the common teratogenic pathway elicited by the fungicides triazole-derivatives. Toxicol In Vitro 2005b;19:737–48.

Menegola E, Broccia ML, Di Renzo F, Giavini E. Postulated pathogenic pathway in triazole fungicide induced dysmorphogenic effects. Reprod Toxicol 2006;22: 186–95.

Miller KP, Ramos KS. Impact of cellular metabolism on the biological effects of benzo a pyrene and related hydrocarbons. Drug Metab Rev 2001;33:1–35.

Morse DC, Brouwer A. Fetal, neonatal, and long-term alterations in hepatic retinoid levels following maternal polychlorinated biphenyl exposure in rats. Toxicol Appl Pharmacol 1995;131:175–82.

Mos L, Tabuchi M, Dangerfield N, Jeffries SJ, Koop BF, Ross PS. Contaminant associated disruption of vitamin A and its receptor (retinoic acid receptor alpha) in free-ranging harbour seals (*Phoca vitulina*). Aquat Toxicol 2007;81:319–28.

Murk AJ, Bosveld ATC, Vandenberg M, Brouwer A. Effects of polyhalogenated aromatic-hydrocarbons (PHAHs) on biochemical parameters in chicks of the common tern (Sterna-Hirundo). Aquat Toxicol 1994a;30:91–115.

Murk A, Morse D, Boon J, Brouwer A. In-vitro metabolism of 3,3',4,4'-tetrachlorobiphenyl in relation to ethoxyresorufin-o-deethylase activity in liver-microsomes of some wildlife species and rat. Eur J Pharm-Environ 1994b;270:253–61.

Murk AJ, Boudewijn TJ, Meininger PL, Bosveld ATC, Rossaert G, Ysebaert T, et al. Effects of polyhalogenated aromatic hydrocarbons and related contaminants on common tern reproduction: Integration of biological, biochemical, and chemical data. Arch Environ Contam Toxicol 1996;31:128–40.

Murk AJ, Leonards PEG, van Hattum B, Luit R, van der Weiden MEJ, Smit M. Application of biomarkers for exposure and effect of polyhalogenated aromatic hydrocarbons in naturally exposed European otters (*Lutra lutra*). Environ Toxicol Pharmacol 1998;6:91–102.

Murvoll KM, Skaare JU, Nilssen VH, Bech C, Østnes JE, Jenssen BM. Yolk PCB and plasma retinol concentrations in shag (*Phalacrocorax aristotelis*) hatchlings. Arch Environ Contam Toxicol 1999;V36:308–15.

Murvoll KM, Skaare JU, Anderssen E, Jenssen BM. Exposure and effects of persistent organic pollutants in European shag (*Phalacrocorax aristotelis*) hatchlings from the coast of Norway. Environ Toxicol Chem 2006;25:190–8.

Murvoll KM, Skaare JU, Jensen H, Jenssen BM. Associations between persistent organic pollutants and vitamin status in Brunnich's guillemot and common eider hatchlings. Sci Total Environ 2007;381:134–45.

Nacci D, Jayaraman S, Specker J. Stored retinoids in populations of the estuarine fish *Fundulus heteroclitus* indigenous to PCB-contaminated and reference sites. Arch Environ Contam Toxicol 2001;40:511–8.

Nakahashi K, Matsuda M, Mori T. Vitamin A insufficiency accelerates the decrease in the number of sperm induced by an environmental disruptor, bisphenol A, in neonatal mice. Zool Sci 2001;18:819–21.

Napoli JL. Biochemical pathways of retinoid transport, metabolism, and signal transduction. Clin Immunol Immunopathol 1996;80:S52–62.

Napoli JL. Interactions of retinoid binding proteins and enzymes in retinoid metabolism. Biochim Biophys Acta (BBA)-Mol Cell Biol Lipids 1999;1440: 139–62.

Ndayibagira A, Cloutier MJ, Anderson PD, Spear PA. Effects of 3,3',4,4'-Tetrachlorobiphenyl on the dynamics of vitamin-A in brook trout (Salvelinus-Fontinalis) and intestinal retinoid concentrations in Lake Sturgeon (Acipenser-Fulvescens). Can J Fish Aquat Sci 1995;52:512–20.

Ndayibagira A, Spear PA. Esterification and hydrolysis of vitamin A in the liver of brook trout (*Salvelinus fontinalis*) and the influence of a coplanar polychlorinated biphenyl. Comp Biochem Physiol C Toxicol Pharmacol 1999;122:317–25.

Nilsson CB, Hakansson H. The retinoid signaling system-A target in dioxin toxicity. Crit Rev Toxicol 2002;32:211–32.

Nilsson CB, Hanberg A, Trossvik C, Hakansson H. 2,3,7,8-tetrachlorodibenzo-p-dioxin affects retinol esterification in rat hepatic stellate cells and kidney. Environ Toxicol Pharmacol 1996;2:17–23.

Nilsson CB, Hoegberg P, Trossvik C, Azais-Braesco V, Blaner WS, Fex G, et al. 2,3,7,8-tetrachlorodibenzo-p-dioxin increases serum and kidney retinoic acid levels and kidney retinol esterification in the rat. Toxicol Appl Pharmacol 2000;169:121–31.

Nishikawa J. Imposex in marine gastropods may be caused by binding of organotins to retinoid X receptor. Mar Biol 2006;149:117–24.

Nishikawa J, Mamiya S, Kanayama T, Nishikawa T, Shiraishi F, Horiguchi T. Involvement of the retinoid X receptor in the development of imposex caused by organotins in gastropods. Environ Sci Technol 2004;38:6271–6.

Nishimura N, Yonemoto J, Miyabara Y, Fujii-Kuriyama Y, Tohyama C. Altered thyroxin and retinoid metabolic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin in aryl hydrocarbon receptor-null mice. Arch Toxicol 2005;79:260–7.

Nishizawa H, Morita M, Sugimoto M, Imanishi S, Manabe N. Effects of in utero exposure to bisphenol a on mRNA expression of arylhydrocarbon and retinoid receptors in murine embryos. J Reprod Dev 2005;51:315–24.

Novak J, Benisek M, Pachernik J, Janosek J, Sidlova T, Kiviranta H, et al. Interference of contaminated sediment extracts and environmental pollutants with retinoid signaling. Environ Toxicol Chem 2007;26.

Noy N. Retinoid-binding proteins: mediators of retinoid action. Biochem J 2000;348:481–95.

Nyman M, Bergknut M, Fant ML, Raunio H, Jestoi M, Bengs C, et al. Contaminant exposure and effects in Baltic ringed and grey seals as assessed by biomarkers. Mar Environ Res 2003;55:73–99.

Oberdorster E, McClellan-Green P. Mechanisms of imposex induction in the mud snail, *Ilyanassa obsoleta*: TBT as a neurotoxin and aromatase inhibitor. Mar Environ Res 2002;54:715–8.

Oehlmann J, Di Benedetto P, Tillmann M, Duft M, Oetken M, Schulte-Oehlmann U. Endocrine disruption in prosobranch molluscs: evidence and ecological relevance. Ecotoxicology 2007;16:29–43.

Paganetto G, Campi F, Varani K, Piffanelli A, Giovannini G, Borea PA. Endocrine-disrupting agents on healthy human tissues. Pharmacol Toxicol 2000;86:24–9.

Palace VP, Evans RE, Wautier K, Baron CL, Werner J, Klaverkamp JF, et al. Altered distribution of lipid-soluble antioxidant vitamins in juvenile sturgeon exposed to waterborne ethynylestradiol. Environ Toxicol Chem 2001;20:2370–6.

Papis E, Bernardini G, Gornati R, Prati M. Triadimefon causes branchial arch malformations in *Xenopus laevis* embryos. Environ Sci Pollut Res Int 2006;13:251–5.

Payne JF, Malins DC, Gunselman S, Rahimtula A, Yeats PA. DNA oxidative damage and vitamin a reduction in fish from a large lake system in Labrador, Newfoundland, contaminated with iron-ore mine tailings. Mar Environ Res 1998;46:289–94.

Pennati R, Groppelli S, Zega G, Biggiogero M, De Bernardi F, Sotgia C. Toxic effects of two pesticides, Imazalil and Triadimefon, on the early development of the ascidian *Phallusia mammillata* (Chordata, Ascidiacea). Aquat Toxicol 2006;79:205–12.

Prins GS, Chang WY, Wang Y, van Breeman RB. Retinoic acid receptors and retinoids are up-regulated in the developing and adult rat prostate by neonatal estrogen exposure. Endocrinology 2002;143:3628–40.

Rando RR. Retinoid isomerization reactions in the visual system. In: R. B., editor. Vitamin A in Health and Disease. New York: Marcel Dekker; 1994. p. 503–29.
Reichrath J, Lehmann B, Carlberg C, Varani J, Zouboulis CC. Vitamins as hormones. Horm Metab Res 2007;39:71–84.
Reijntjes S, Blentic A, Gale E, Maden M. The control of morphogen signalling: regulation of the synthesis and catabolism of retinoic acid in the developing embryo. Dev Biol 2005;285:224–37.
Rolland RM. A review of chemically-induced alterations in thyroid and vitamin A status from field studies of wildlife and fish. J Wildl Dis 2000;36:615–35.
Ronis MJJ, Mason AZ. The metabolism of testosterone by the periwinkle (*Littorina littorea*) in vitro and in vivo: effects of tributyl tin. Mar Environ Res 1996;42:161–6.
Rosenthal D, Lancillotti F, Darwiche N, Sinha R, De Luca LM. Regulation of epithelial differentiation by retinoids. In: Blomhoff R, editor. Vitamin A in Health and Disease. New York: Marcel Dekker; 1994. p. 425–50.
Ross AC, Hammerling UG. Retinoids and the immune system. In: Sporn MB, Roberts AB aGDS, editors. The Retinoids: Biology, Chemistry, and Medicine. New York: Raven Press; 1994. p. 521–43.
Routti H, Nyman M, Backman C, Koistinen J, Helle E. Accumulation of dietary organochlorines and vitamins in Baltic seals. Mar Environ Res 2005;60: 267–87.
Schmidt CK, Hoegberg P, Fletcher N, Nilsson CB, Trossvik C, Hakansson H, et al. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) alters the endogenous metabolism of all-trans-retinoic acid in the rat. Arch Toxicol 2003;77: 371–83.
Schmitz H-J, Hagenmaier A, Hagenmaier H-P, Bock KW, Schrenk D. Potency of mixtures of polychlorinated biphenyls as inducers of dioxin receptor regulated CYP1A activity in rat hepatocytes and H4IIE cells. Toxicology 1995;99:47–54.
Schoff PK, Ankley GT. Inhibition of retinoid activity by components of a paper mill effluent. Environ Pollut 2002;119:1–4.
Schoff PK, Ankley GT. Effects of methoprene, its metabolites, and breakdown products on retinoid-activated pathways in transfected cell lines. Environ Toxicol Chem 2004;23:1305–10.
Schuetz EG, Brimer C, Schuetz JD. Environmental xenobiotics and the antihormones cyproterone acetate and spironolactone use the nuclear hormone pregnenolone X receptor to activate the CYP3A23 hormone response element. Mol Pharmacol 1998;54:1113–7.
Schwarzenbach RP, Escher BI, Fenner K, Hofstetter TB, Johnson CA, von Gunten U, et al. The challenge of micropollutants in aquatic systems. Science 2006;313:1072–7.
Schweigert FJ, Ryder OA, Rambeck WA, Zucker H. The majority of vitamin-A is transported as retinyl esters in the blood of most carnivores. Comp Biochem Physiol, A-Physiol 1990;95:573–8.
Shiota G, Tsuchiya H, Hoshikawa Y. The liver as a target organ of retinoids. Hepatol Res 2006;36:248–54.
Simms W, Jeffries S, Ikonomou M, Ross PS. Contaminant-related disruption of vitamin A dynamics in free-ranging harbor seal (*Phoca vitulina*) pups from British Columbia, Canada, and Washington State, USA. Environ Toxicol Chem 2000;19:2844–9.
Simms W, Ross PS. Vitamin A physiology and its application as a biomarker of contaminant-related toxicity in marine mammals: a review. Toxicol Ind Health 2000;16:291–302.
Smith GD, Wilburn C, McCarthy RA. Methoprene photolytic compounds disrupt zebrafish development, producing phenocopies of mutants in the sonic hedgehog signaling pathway. Mar Biotechnol 2003;5:201–12.
Soprano DR, Soprano KJ. Pharmacological doses of some synthetic retinoids can modulate both the aryl hydrocarbon receptor and retinoid receptor pathways. J Nutr 2003;133:277S–81S.
Sormo, E.G., Organochlorine pollutants in grey seal (Halichoerus grypus) pups and their impact on plasma thyroid and vitamin A concentrations, vol 2007. Trondheim, Norway: Norwegian University of Science and Technology, 2005; PhD. thesis.
Spear PA, Bilodeau AY, Branchaud A. Retinoids-from metabolism to environmental monitoring. Chemosphere 1992;25:1733–8.
Swart RD, Ross PS, Vedder LJ, Timmerman HH, Heisterkamp S, Vanloveren H, et al. Impairment of immune function in harbor seals (*Phoca vitulina*) feeding on fish from polluted waters. Ambio 1994;23:155–9.
Taylor B, Skelly D, Demarchis LK, Slade MD, Galusha D, Rabinowitz PM. Proximity to pollution sources and risk of amphibian limb malformation. Environ Health Perspect 2005;113:1497–501.
Tzimas G, Nau H. The role of metabolism and toxicokinetics in retinoid teratogenesis. Curr Pharm Des 2001;7:803–31.
Valko M, Morris H, Cronin MTD. Metals, toxicity and oxidative stress. Curr Med Chem 2005;12:1161–208.
van Bennekum AM, Wei SH, Gamble MV, Vogel S, Piantedosi R, Gottesman M, et al. Biochemical basis for depressed serum retinol levels in transthyretin deficient mice. J Biol Chem 2001;276:1107–13.
van der Plas SA, Lutkeschipholt I, Spenkelink B, Brouwer A. Effects of subchronic exposure to complex mixtures of dioxin-like and non-dioxin-like polyhalogenated aromatic compounds on thyroid hormone and vitamin A levels in female Sprague–Dawley rats. Toxicol Sci 2001;59:92–100.
Vanbirgelen A, Vanderkolk J, Fase KM, Bol I, Poiger H, Brouwer A, et al. Toxic potency of 3,3',4,4',5-pentachlorobiphenyl relative to and in combination with 2,3,7,8-tetrachlorodibenzo-P-dioxin in a subchronic feeding study in the rat. Toxicol Appl Pharmacol 1994a;127:209–21.
Vanbirgelen A, Vanderkolk J, Fase KM, Bol I, Poiger H, Vandenberg M, et al. Toxic potency of 2,3,3',4,4',5-hexachlorobiphenyl relative to and in combination with 2,3,7,8-tetrachlorodibenzo-P-dioxin in a subchronic feeding study in the rat. Toxicol Appl Pharmacol 1994b;126:202–13.
Veal GJ, Errington J, Redfern CR, Pearson ADJ, Boddy AV. Influence of isomerisation on the growth inhibitory effects and cellular activity of 13-cis and all-trans retinoic acid in neuroblastoma cells. Biochem Pharmacol 2002;63:207–15.
Vivat V, Zechel C, Wurtz JM, Bourguet W, Kagechika H, Umemiya H, et al. A mutation mimicking ligand-induced conformational change yields a constitutive RXR that senses allosteric effects in heterodimers. EMBO J 1997;16:5697–709.
Vo MN, Dufour JM, Holden TD, Okita JR, Okita RT, Kim KH. Inhibition of retinoic acid receptor-mediated signaling by phthalates in the testis. Biol Reprod 2001;64:349.
Vondracek J, Machala M, Minksova K, Blaha L, Murk AJ, Kozubik A, et al. Monitoring river sediments contaminated predominantly with polyaromatic hydrocarbons by chemical and in vitro bioassay techniques. Environ Toxicol Chem 2001;20:1499–506.
Weston WM, Nugent P, Greene RM. Inhibition of retinoic-acid-induced gene-expression by 2,3,7,8-tetrachlorodibenzo-P-dioxin. Biochem Biophys Res Commun 1995;207:690–4.
Widerak M, Ghoneim C, Dumontier MF, Quesne M, Corvol MT, Savouret JF. The aryl hydrocarbon receptor activates the retinoic acid receptor alpha through SMRT antagonism. Biochimie 2006;88:387–97.
Wiens M, Batel R, Korzhev M, Muller WEG. Retinoid X receptor and retinoic acid response in the marine sponge *Suberites domuncula*. J Exp Biol 2003;206:3261–71.
Xue WL, Warshawsky D. Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: a review. Toxicol Appl Pharmacol 2005;206:73–93.
Yang YM, Huang DY, Liu GF, Zhong JC, Du K, Li YF, et al. Effects of 2,3,7,8-tetrachlorodibenzo-beta-dioxin on vitamin A metabolism in mice. J Biochem Mol Toxicol 2005;19:327–35.
Zile MH. Function of vitamin A in vertebrate embryonic development. J Nutr 2001;131:705–8.
Zile MH. Vitamin-A homeostasis endangered by environmental-pollutants. Proc Soc Exp Biol Med 1992;201:141–53.
Zile MH, Summer C, Aulerich R, Bursian SJ, Tillitt DE, Giesy JP, et al. Retinoids in eggs and embryos of birds fed fish from the Great Lakes. Environ Toxicol Pharmacol 1997;3:277–88.

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Polymer-immobilized ready-to-use recombinant yeast assays for the detection of endocrine disruptive compounds

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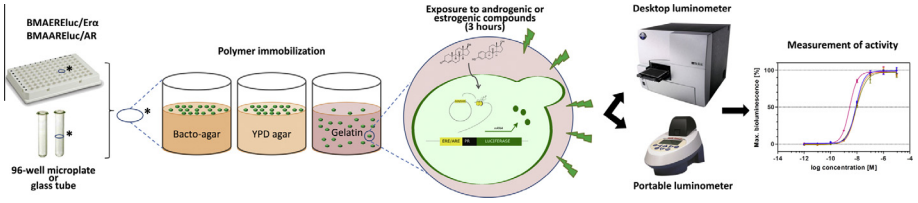
HIGHLIGHTS

- Immobilization techniques applied to develop rapid ready-to-use assays.
- Immobilized recombinant yeast used for detection of androgens and estrogens.
- Recombinant yeast cells were immobilized in gelatin, Bacto agar and YPD agar.
- Gelatin was the best immobilization matrix.
- Immobilized yeast stored in fridge maintained sensitivity for at least 90 d.

GRAPHICAL ABSTRACT

Scheme of recombinant yeast assays using three types of immobilizing matrices in a microplate or tubes.



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ABSTRACT

Recombinant yeast assays (RYAs) constitute a suitable tool for the environmental monitoring of compounds with endocrine disrupting activities, notably estrogenicity and androgenicity. Conventional procedures require yeast reconstitution from frozen stock, which usually takes several days and demands additional equipment. With the aim of applying such assays to field studies and making them more accessible to less well-equipped laboratories, we have optimized RYA by the immobilization of *Saccharomyces cerevisiae* cells in three different polymer matrices – gelatin, Bacto agar, and Yeast Extract Peptone Dextrose agar – to obtain a ready-to-use version for the fast assessment of estrogenic and androgenic potencies of compounds and environmental samples. Among the three matrices, gelatin showed the best results for both testosterone (androgen receptor yeast strain; AR-RYA) and 17β-estradiol (estrogen receptor yeast strain; ER-RYA). AR-RYA was characterized by a lowest observed effect concentration (LOEC), EC_{50} and induction factor (IF) of 1 nM, 2.2 nM and 51, respectively. The values characterizing ER-RYA were 0.4 nM, 1.8 nM, and 63, respectively. Gelatin immobilization retained yeast viability and sensitivity for more than 90 d of storage at 4 °C. The use of the immobilized yeast reduced the assay duration to only 3 h without necessity of sterile conditions. Because immobilized RYA can be performed either in multiwell microplates or glass tubes, it allows multiple samples to be tested at once, and easy adaptation to existing portable devices for direct in-field applications.

1. Introduction

Endocrine disrupting compounds (EDCs) are defined as exogenous substances that cause adverse effects in an organism, or its progeny, subsequent to changes in the endocrine system (European Commission, 1996). This definition covers a wide-range of substances, both man-made and natural, able to interfere with wildlife and human endocrine systems at very low concentrations, potentially leading to physiological anomalies (Sumpter and Johnson, 2005; Brander et al., 2013). EDCs are now widespread all over the world in various matrices (Rotchell and Ostrander,

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2003; Bainy, 2007; Novák et al., 2009; Jarque et al., 2014). Moreover, new chemical compounds that may show endocrine disrupting activity are produced and released into the environment every year, which demands new tools for their fast detection and subsequent risk assessment (Ezechiá et al., 2014).

Recombinant yeast assays (RYAs) have been demonstrated to be suitable tools for environmental monitoring (Brix et al., 2010; Jarošová et al., 2014; Mesquita et al., 2014). They consist of engineered yeast strains which respond to compounds with endocrine disrupting activities. They harbor two foreign elements: a vertebrate receptor able to recognize the analyte of interest, and a reporter gene under transcriptional control of the receptor (Michelini et al., 2005). Because the transcriptional response of the reporter gene is proportional to the receptor activation, it is possible to determine the equivalent concentration of standard ligand by measuring the activity of the reporter gene. In this work, the bioreporter yeast strains are based on the human androgen/estrogen receptor-mediated expression of *luc* reporter gene (Leskinen et al., 2005). Thus, the luminescence of recombinant yeast cells increases in the presence of compounds with estrogenic/androgenic activity.

Compared to other *in vitro* models, RYAs are easy to perform, are usually less time-consuming, show good sensitivity and high reproducibility, and are relatively inexpensive. Moreover, because yeast cells are relatively tolerant to environmental chemicals, they can be used for the detection of the hormonal activity of samples without any pre-treatment (Leskinen et al., 2005). However, the fact that yeast require previous reconstitution from frozen stock and cultivation in sterile conditions complicates the applicability of RYA in less well-equipped laboratories and in-field studies.

One of the critical steps for the development of ready-to-use whole-cell biosensors is the effective immobilization of living cells, which ideally should not affect the performance of the assay (Michelini et al., 2013). Biologically modified ceramics, also known as biocers, and cell arrays organized in defined patterns were developed to encapsulate unmodified cells (Böttcher et al., 2004; Krol et al., 2005). Recently, immobilizations to three-dimensional biocompatible gel matrices such as calcium alginate or polyvinyl alcohol (PVA) have been discussed as effective yeast cell entrapment methods (Fine et al., 2006). Similarly, polymeric matrices have been used as a support for cell immobilization with the aim of developing portable biosensors (Roda et al., 2011). Although they represented important advantages, most of these approaches significantly diminished the sensitivity of immobilized yeast cells by at least one order of magnitude compared to the non-immobilized versions (Fine et al., 2006; Roda et al., 2011). Moreover, cell viability after immobilization usually decreases due to the low stability and durability of the supporting matrices, resulting in lower yields after about one month (Fine et al., 2006). As a consequence, new immobilization strategies are needed.

The goal of this study was to develop an effective ready-to-use yeast bioassay that is easily applicable to field studies and in less well-equipped laboratories. This goal can be achieved by the immobilization of transgenic yeast in an appropriate matrix that holds the yeast responsive for several months, and is also compatible with commonly used microplates or tubes used in portable luminometers. We compare several novel approaches for the long-term immobilization of recombinant yeast cells by applying three different polymers, Yeast Extract-Peptone-Dextrose (YPD) agar, Bacto agar and gelatin. The sensitivities and durabilities of cells were compared among the bioassay versions using different immobilization strategies. Applicability of immobilized RYA in environmental samples was evaluated by the assessment of estrogenic and androgenic activities of extracts from river water, since presence of endocrine disrupting compounds in river water, especially in industrial or urbanized areas, is of high significance worldwide (Jálová et al., 2013; Gorga et al., 2014; Chou et al., 2015).

2. Experimental

2.1. Materials

Testosterone and 17β-estradiol, D-luciferin sodium salt, citric acid monohydrate and trisodium citrate dihydrate were purchased from Sigma–Aldrich (USA). Luciferin solution 1 mM was prepared by dissolving D-luciferin sodium salt into 0.1 M citric acid and 0.1 trisodium citrate dihydrate. Gelatin from porcine skin (No. 48724, Sigma–Aldrich, USA), Yeast Extract Peptone Dextrose (YPD) agar (No. Y1500, Sigma–Aldrich, USA) and Bacto agar (No. 214010, BD, USA) were used as immobilization polymers. Gelatin liquid solution was obtained by dissolving gelatin powder in synthetic dextrose (SD) complex medium to a final concentration of 20%. Agars were prepared according to the manufacturer's recommendations by dissolving the agar powder in SD complex medium.

2.2. Yeast cell cultures and standard assay

BMAEREluc/ERα (ER-RYA) contains the coding sequence of human estrogen receptor alpha (hERα) cloned into the constitutive expression vector pG-1 and a reporter plasmid carrying a truncated form of *Photinus pyralis* luciferase regulated by the estrogen responsive element (ERE), which serves as a reporter gene (Leskinen et al., 2003). BMAAREluc/AR (AR-RYA) has a similar construction but contains human androgen receptor (hAR) and androgen responsive element (ARE) in the reporter plasmid (Michelini et al. 2005).

The detection of EDCs is based on the measurement of firefly luciferase luminescence from intact living yeast cells (Leskinen et al., 2003). Estrogenic or androgenic compounds diffuse into the cell and bind to the hormone receptor. The resulting activated receptor complex translocates into the nucleus and activates the specific responsive promoter, which results in the expression of the *luc* reporter gene. By the external addition of D-luciferin, light is emitted and measured by a luminometer.

The standard RYA was performed according to the protocol from Michelini et al. (2008) with minor changes. Briefly, yeast from frozen stock (stored at −80 °C) were reconstituted on agar plates and incubated for three days at 30 °C. One colony was picked and grown overnight in complex SD medium at 30 °C and 180 rpm. The OD_{600} of the grown culture was adjusted to 0.4 and the culture was re-grown again for 2 h to reach an OD_{600} of 0.65, which is the mid-exponential phase, when cells are more sensitive to environmental stressors. 100 µl of yeast culture were transferred per well onto a 96-well microplate (Grainer Bio-One GmbH, Germany) and subsequently exposed to the tested chemicals. 17β-estradiol (1.5×10^{-11}–3.3×10^{-8} M) and testosterone (1×10^{-12}–1×10^{-5} M) in methanol (1% v/v) were used as positive induction controls. Methanol was used as the vehicle control. The microplates were incubated for 2.5 h at 30 °C and shaking at 160 rpm. After incubation, 100 µl of D-luciferin solution were added into each well by using an automatic dispenser, and the plates were briefly shaken. After one minute, luminescence was measured using a luminometer (BioTek, Winooski, Vermont, USA) with a controlled temperature of 30 °C.

With the aim of making RYA more accessible to *in situ* measurements, we adapted the assay to a portable luminometer. The procedure was the same as described for microplates with the following modifications: 200 µl of yeast culture with an OD_{600} of

0.65 were transferred to each tube (Macherey–Nagel GmbH & Co. KG, Germany) and exposed to 2 μl of standard hormone solutions in methanol. Before luminescence measurement, 200 μl of D-luciferin solution were pipetted into the tubes. The tubes were shaken and, after one minute, activity was measured by using a portable luminometer (Biofix Lumi-10, Macherey–Nagel GmbH & Co. KG, Germany).

2.3. Yeast cell immobilization strategies

In our ready-to-use approaches, we immobilized both androgen- and estrogen-responsive yeast strains in 96-well microplates either in gelatin or on two types of agar, Bacto agar and YPD agar.

To prepare yeast immobilized in gelatin, a sterile 20% liquid gelatin solution was mixed with yeast suspension in a 1:1 ratio to reach a final OD_{600} of 0.65. The yeast–gelatin suspension was dosed 50 μl per well into a microplate, sustaining a temperature of 35 °C during the dosing to maintain the gelatin liquid. The microplates were sealed with parafilm and stored at 4 °C. Immobilized yeasts were exposed for 3 h to 100 μl of tested samples diluted in complex SD media. The rest of the protocol was the same as described for standard RYA.

For immobilization in two types of agar, Bacto and YPD agar, 25 μl of warm agar (60 °C) per well were pipetted and allowed to solidify at room temperature for subsequent yeast cultivation. One yeast colony was diluted in complex media and grown overnight at 30 °C. Grown yeast was diluted to an OD_{600} of 0.8 using complex media without glucose to avoid shortening of the lifespan due to an environment rich in calories, which may compromise the long-term applicability of immobilized yeast (Lamming et al., 2004). Later, 25 μl of yeast suspension was added onto the agar per well, and the microplate was left uncovered in sterile conditions for 1 h to facilitate liquid evaporation. The microplates were finally incubated for three days at 30 °C to allow the yeast to grow. This incubation was performed for two variants of each type of agar; "wet" variants were obtained by covering and sealing the microplates with parafilm to prevent further drying, and stored at 4 °C after incubation; "dry" variants were incubated without sealing to allow additional drying before the final sealing and upside down storage at 4 °C. Because the outer wells dried faster, only the 60 inner wells were used for testing. Exposure to chemical compounds and final measurements of luminescence were performed according to the same protocol described above for gelatin immobilized yeast.

Similarly to the standard RYA, the immobilized version was also adapted to glass tubes to transfer the methodology to portable luminometers for its potential use in on-site applications. In accordance with the preliminary results obtained using microplates, only immobilization in gelatin was considered for this last approach. Immobilization in tubes was the same as that described for microplates with the following minor changes: 100 μl of yeast–gelatin solution were added per tube, immobilized yeast were exposed to tested samples diluted in 200 μl of SD media, and final measurements were performed using a portable luminometer after adding 200 μl of D-luciferin.

2.4. Long-term stability experiment

The long-term stability of immobilized yeast cells was assessed for each immobilization strategy by measuring AR/ER-mediated response at seven different time points. Immobilized yeast in microplates stored at 4 °C were tested after 0, 24, 42, 62, 90, 114 and 146 d of storage, and the performances of the different strategies, characterized by dose–response curves, were compared. In the case of yeast immobilized in gelatin or on agars, time zero measurements were done three days after yeast immobilization in order to allow the yeast to grow sufficiently before testing.

2.5. Assessment of environmental samples by immobilized RYA

The applicability of immobilized RYA was tested by exposing cells in gelatin to environmental samples. All samples were extracts from river water obtained from the project "Bosna River Survey – monitoring program within NATO – Science for Peace and Security Project Nr. ESP.EAP.SFP 984073", whose primary objective was to assess contamination by various types of pollutants of the Bosna River in Bosnia and Herzegovina. Contaminants in the water originated from various industrial activities in the Bosna River basin. Description and GPS localization of localities is in Supplementary material 1. As a background reference sample, extract from the spring of Bosna River was used. Sampling was done using a Polar Organic Chemicals Integrated Sampler (POCIS). Assessment using immobilized RYAs was carried out both on microplates and tubes three days after yeast immobilization in gelatin; assessment using non-immobilized RYAs was carried out on microplates. All samples were assessed in two to three independent assessments, each done in triplicate.

2.6. Data analysis

Measurements of activity for standard RYA and RYA immobilized in gelatin were performed in three independent experiments, while measurements of activity for RYAs in both agars were performed in two independent experiments. The response to each hormone concentration was measured in triplicate in each case. Sensitivity of the assays were determined as lowest observed effect concentration (LOEC), which was the hormone concentration causing a significantly different response from the solvent control in the ANOVA test with Dunnett's post hoc test (GraphPad Prism 5, GraphPad Software, Inc., CA, USA). Dose–response curves were plotted to determine hormone median effective concentration (EC_{50}) values. The obtained relative luminescence units (RLU) were expressed as a percentage of the maximum luminescence response induced by 33 nM 17β-estradiol for ER-RYA, and 1000 nM testosterone for AR-RYA, respectively, for easier comparison among experiments. These were the lowest concentrations that reached the upper plateau of respective dose–response curves, and RLU of their induction was set as 100%. The induction factor (IF), calculated as the fold induction of the maximum response induced by the hormone over that of the vehicle control, was calculated according to the equation $IF = L_S/L_B$, where L_S is the RLU value of the highest response induced by the hormone and L_B is the RLU value of the vehicle control variant.

Estradiol-equivalents (EEQ – ng/POCIS) were determined in extracts of river water that were sampled by Polar Organic Chemicals Integrative Samplers (POCIS). Measured RLU, as the responses of both non-immobilized and immobilized yeast to exposure to environmental samples, were normalized to a percentage of the maximum 17β-estradiol response. EEQ values for water extracts were calculated by the interpolation of the response of environmental samples into the calibration curve for 17β-estradiol, characterized by the logistic dose–response function in GraphPad Prism 5 software.

3. Results and discussion

3.1. Standard recombinant yeast assay

Standard AR-RYA and ER-RYA performed in microplates are characterized by LOEC, EC_{50}, as depicted in Table 1. Mean IF values were 66 and 77 for standard AR-RYA and ER-RYA, respectively. Results obtained from RYA performed in glass tubes were comparable to those obtained in microplates, the only difference being

a slightly lower IF in tubes. LOEC, EC_{50}, and IF values for AR-RYA in tubes were 1 nM, 2.2 nM, and 54, respectively, and the corresponding values for ER-RYA were 0.1 nM, 0.8 nM, and 40. These results for both AR-RYA and ER-RYA are comparable to analogous dose–response curves that were not corrected to the vitality control signal described by Michelini et al. (2005, 2008) and Leskinen et al. (2003).

3.2. Immobilization in various polymers

Yeast cells were successfully immobilized in all polymers tested in the study. The results in terms of sensitivity (LOEC, EC_{50}) for the different immobilization strategies are summarized in Table 1; induction factors and their changes over time are depicted in Fig. 1A and B. Immobilization in gelatin did not affect the response of AR-RYA in microplates, while ER-RYA showed slightly lower sensitivity compared to the standard ER-RYA (Fig. 2, Table 1). By contrast, immobilization on both agars, YPD and Bacto agar, generally lead to lower sensitivity for both receptors compared to the standard RYA (Fig. 2, Table 1). More specifically, immobilization on dry and wet YPD agar variants led to an 3–13-fold increase in EC_{50}, although the LOEC was affected more in the dry variant (Table 1). Similarly, immobilization in both variants of Bacto agar adversely affected the EC_{50} values for AR- and ER-RYA. However, the LOEC for AR-RYA was the same as in the case of non-immobilized RYA. Overall, immobilization in gelatin was shown as the most efficient strategy among all the tested variants. This method was further tested in glass tubes for adaptation to portable luminometers, yielding similar results as in microplates: LOEC, EC_{50}, and IF values for AR-RYA in gelatin in tubes were 1 nM, 2.1 nM and 44, respectively; analogous values for ER-RYA were 0.4 nM, 1.3 nM and 60.

Fine et al. (2006) reported that the process of immobilization of ER-RYA in PVA decreased sensitivity by about ten times compared to non-immobilized assay. Similarly, the immobilization of AR-RYA in a complex mixture of agarose, polyvinylpyrrolidone and collagen also significantly reduced the sensitivity of the assay compared to the standard AR-RYA (Michelini et al., 2008; Roda et al., 2011). On the other hand, in our approach using immobilization in gelatin, the sensitivity of AR-RYA was not affected, and ER-RYA was affected to a lesser extent compared to other immobilization matrices.

In general, ER-RYA was more likely to exhibit a decrease in sensitivity arising from immobilization in the tested polymers than AR-RYA, which is in agreement with the lower stability observed for the BMAEREluc/ERα strain in standard conditions. Gelatin was shown as the most efficient matrix for the immobilization of yeast cells in terms of sensitivity, not only among the polymers tested in the present study (Table 1), but also compared to similar existing matrices reported in literature so far (Fine et al., 2006; Michelini et al., 2008; Roda et al., 2011). Immobilization on agars significantly affected LOEC and EC_{50} values for both receptors, with particularly sharp increases in the case of YPD agar, maybe due to the brownish color of the polymer, which could partially interfere with the luminescence signal. In this case, RLU were about two times lower compared to RLU for corresponding concentrations (including solvent control and blank) from both gelatin and Bacto agar immobilized AR-RYA and ER-RYA. The lower sensitivity compared to gelatin could also be explained by the fact that cultures immobilized on agars were close to the stationary phase, when yeast cells are typically less sensitive to environmental stressors including the tested hormones. Thus, further experiments to optimize the immobilization methods may consider optimizing the initial cell density which could enable to maintain the sensitivity of assays for a longer period of storage. Further optimization of the volume and density of matrices could enhance yeast viability via the change in nutrients availability and matrices durability, and finally RYAs sensitivity could be affected as well via the change of uptake rate of tested compounds (Mitchell and Gu, 2006; Han et al., 2012).

3.3. Evaluation of the long-term applicability of immobilized RYA

LOEC and EC_{50} values for the seven different long-term storage time points as well as dose–response curves for four selected time points are shown in Table 1 and Fig. 1C and D, respectively. In agreement with results obtained with freshly immobilized yeast, gelatin was shown to be the best polymer to retain cell activity after long-term storage, showing significant increases in LOEC only after 90 d of storage. EC_{50} values mostly remained constant (Table 1), while IF values gradually decreased over the time of storage (Fig. 1A and B). Therefore, immobilization in gelatin was able to maintain comparable sensitivity for up to three months of storage at 4 °C. Although not tested in the present study, the durability of gelatin, and therefore cell viability, could increase at lower storage temperatures, as was observed for alginate beads, which showed better results when stored at −80 °C compared to −20 °C or 4 °C (Fine et al., 2006). Roda et al. (2011) described a 15% loss of viability in AR-yeast immobilized in a complex mixture of agarose, polyvinylpyrrolidone and collagen stored for one month at 4 °C.

Table 1
Summary of results from the evaluation of dose–response curves for testosterone (AR-yeast) and 17β-estradiol (ER-yeast) assessments performed in microplates. The first and seventh types of assay represent standard assays without immobilization. Values represent results from two to three long-term storage experiments. Coefficients of variation for EC_{50} values were below 33% (mean 17%) for standard and gelatin variants, and below 83% (mean 33%) for agar variants.

Yeast bioassay	EC50 (nM)							LOEC (nM)						
	t0	t24	t42	t62	t90	t114	t146	t0	t24	t42	t62	t90	t114	t146
AR, standard RYA	2.8							1						
AR, YPD agar-wet	20.6	11.5	16.1	14.7	57.6	13.8	9.7	1	1	1	10	10	10	10
AR, YPD agar-dry	39.6	25.2	a.	24.3	a.	n.r.	–	10	10	10	100	100	n.r.	–
AR, Bacto agar-wet	9.3	9.3	13.8	8.7	a.	n.r.	–	1	1	1	10	100	n.r.	–
AR, Bacto agar-dry	9.9	28.8	25.9	a.	n.r.	–	–	1	10	10	10	n.r.	–	–
AR, gelatin	2.2	5.4	7.3	7.7	5.9	4.5	4.9	1	1	1	1	1	10	10
ER, standard RYA	0.9							0.1						
ER, YPD agar-wet	6.0	4.4	4.7	3.9	19.5	n.r.	–	0.4	1.2	3.7	3.7	3.7	n.r.	–
ER, YPD agar-dry	2.9	5.0	2.2	a.	n.r.	–	–	1.2	3.7	3.7	n.r.	n.r.	–	–
ER, Bacto agar-wet	2.9	4.3	2.9	2.0	4.5	n.r.	–	0.4	0.4	1.2	1.2	3.7	n.r.	–
ER, Bacto agar-dry	4.5	1.5	5.2	2.8	n.r.	–	–	0.4	0.4	1.2	1.2	n.r.	–	–
ER, gelatin	1.8	3.1	2.9	1.9	1.7	2.2	2.1	0.4	1.2	1.2	1.2	1.2	3.7	3.7

n.r. – no significant response.
a. – ambiguous results unsuitable for a logistic dose–response fit (sufficient for LOEC determination).
– (dash) – no measurement at this time point, because the yeast from the previous time period showed no significant response.

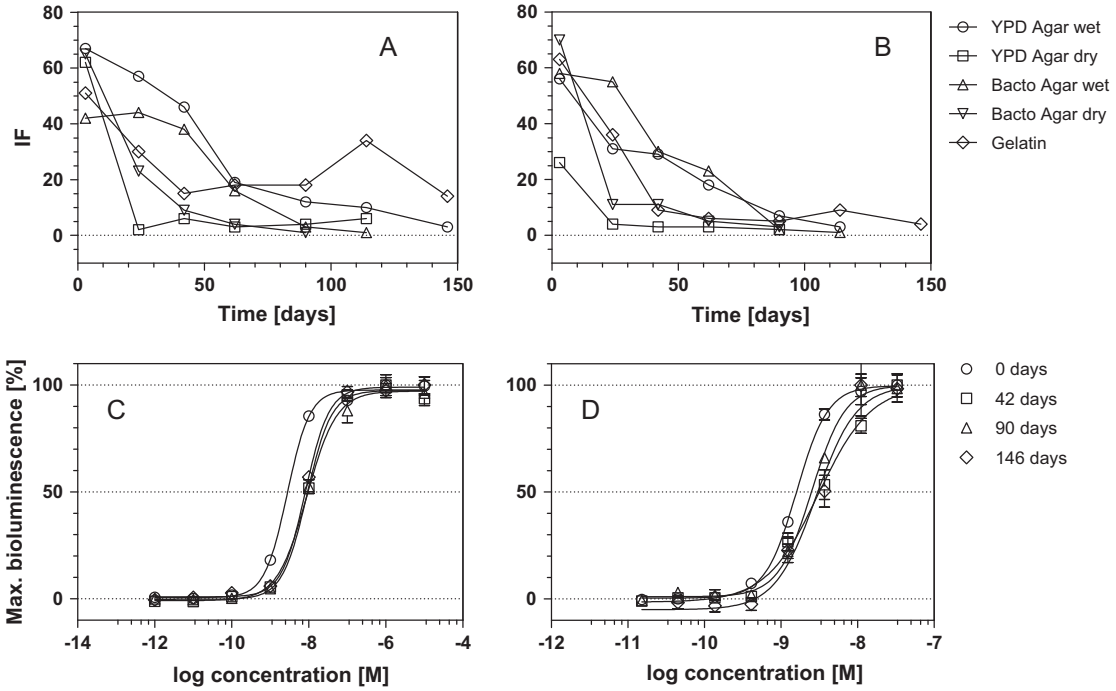

Fig. 1. Upper graphs show changes in induction factors over time for androgenicity assessment (A) and estrogenicity assessment (B) using ready-to-use assays with different immobilization strategies. Values represent the mean from three replicates for gelatin assessment and two replicates for agars assessment. Lower graphs show dose response curves for testosterone (C) and 17β-estradiol (D), respectively, using AR-yeast or ER-yeast immobilized in gelatin. Microplates with immobilized yeasts were stored for up to 146 d, and measurements were conducted in seven distinct time points (results for 24, 62, 114 d are not shown, but described in Table 1). The figure shows results from one set of measurements (plates that were prepared all at once). Values represent the mean ± SE of triplicate determination.

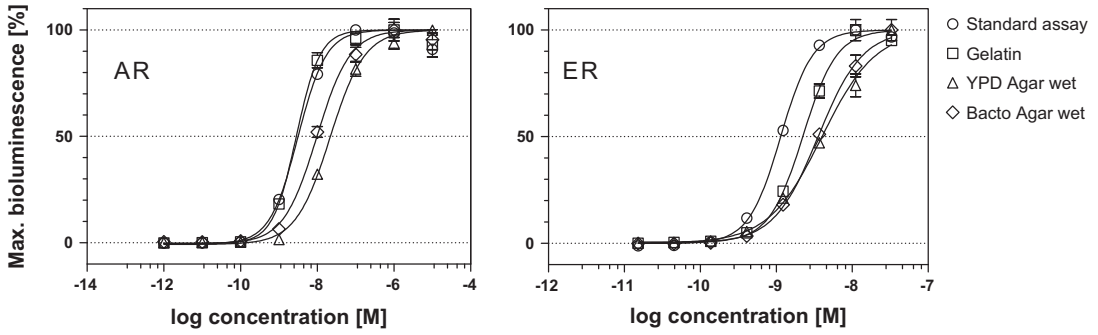

Fig. 2. Logistic dose–response curves for testosterone (AR) and 17β-estradiol (ER) in standard assay, and using the immobilization matrices gelatin, YPD agar-wet variant, and Bacto agar-wet variant. Dose–response curves for YPD agar-dry variant and Bacto agar-dry variant are not depicted, because the results were similar or worse compared to the respective wet variants. Values represent the mean ± SE of triplicate determination.

A possible explanation may be that lower temperatures together with efficient immobilization may contribute to the preservation of dehydrated conditions and cell viability (Borovikova et al., 2014).

The yeast immobilized by the other strategies, with the exception of AR-RYA immobilized in the wet variant of YPD agar, showed no activity after 90 d of storage at 4 °C (Table 1). Moreover, all determined parameters were significantly worse (i.e. higher EC_{50} and LOEC values, and lower IF values) than those recorded for both yeast strains in gelatin. Dry variants of YPD and Bacto agar were particularly unstable, showing higher variability among triplicates and a loss of activity between 42 and 62 d after storage because of the partial cracking of the matrices.

3.4. Assessment of environmental samples using immobilized RYA

The assessment of estrogenic activity using ER-RYA immobilized in gelatin was performed in microplates and tubes, obtaining comparable values for both methods and also the non-immobilized version (Fig. 3). All three types of ER-RYA clearly identified the samples with high ER-mediated activity (in the range of 14–34 ng EEQs/POCIS), and distinguished them from those with low or no activity. Androgenicity for the samples was also assessed using both immobilized and non-immobilized AR-RYA, but no activity was recorded in any sample, which is in agreement with the negative results from measurements in the "Bosna River Survey" using mammalian cells bioassay (data not shown).

These results confirm immobilized ER-RYA as a suitable tool for the detection of estrogenic activity in environmental samples, delivering comparable results to non-immobilized ER-yeast assay, which have already been proven to be suitable for the monitoring of environmental contamination (Novák et al., 2009; Jálová et al., 2013). In addition, our results also demonstrate that ER-RYA immobilized in tubes can be a potential method for use in direct in-field applications.

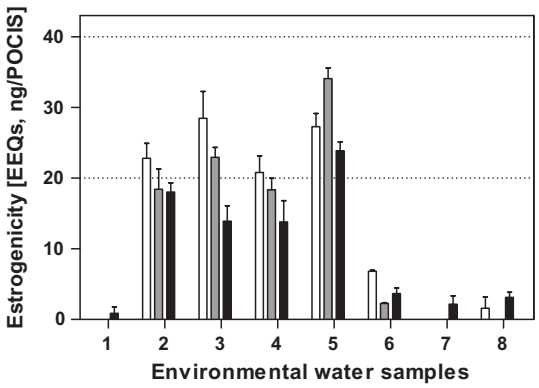

Fig. 3. Estrogenic activity of environmental water samples from the "Bosna River Survey" project. The sampling sites are described in Supplementary material 1. EEQ values were calculated per one POCIS sampler; white columns – values for gelatin immobilized ER-RYA performed in microplates; grey columns – values for gelatin immobilized ER-RYA performed in tubes; black columns – values for standard non-immobilized ER-RYA. Values represent the mean ± SE of two to three independent assessments, each done in triplicate.

4. Conclusions

In the present work, we developed ready-to-use versions of AR- and ER-RYAs. The comparison of dose response curves, characterized by LOEC, EC_{50} and IF values, showed immobilization in gelatin as the most efficient immobilization strategy.

Immobilized transgenic yeast does not require previous overnight reconstitution, which shortens the assay to a total time of several minutes for sample dosing and three hours for exposure. Moreover, by using sterile microplates with immobilized yeast, the assay can be performed without the need for sterile conditions; thus, flow boxes and other special equipment for sterile work are not needed, which greatly increases the applicability of the assay.

Multi-well microplates allow the fast screening of different samples in various concentrations at once. In addition, they may be easily adapted to recently developed portable devices based on interchangeable multi-well cartridges (Roda et al., 2011). Similarly, we succeeded in applying the gelatin immobilization strategy to sterile standard glass tubes, which enabled its adaptation to existing commercial battery powered portable luminometers. Both of these functionalities together make the new immobilized RYA version an easily accessible tool for the in-field detection of compounds with androgenic and estrogenic activities. Indeed, our study showed that the immobilized RYA could be applied to the assessment of environmental samples.

Although the immobilized versions presented here were developed for the assessment of androgenic and estrogenic potencies, we believe that the same methodologies could be potentially used for testing antagonistic effects in co-exposure with standard ligands, or transferred to other existing yeast cell-based biosensors harboring other endocrine receptors, e.g. thyroid receptor or progesterone receptor.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2015.02.063.

References

Bainy, A.C.D., 2007. Nuclear receptors and susceptibility to chemical exposure in aquatic organisms. Environ. Int. 33, 571–575. http://dx.doi.org/10.1016/j.envint.2006.11.004.

Borovikova, D., Rozenfelde, L., Pavlovska, I., Rapoport, A., 2014. Immobilisation increases yeast cells' resistance to dehydration-rehydration treatment. J. Biotechnol. 184C, 169–171. http://dx.doi.org/10.1016/j.jbiotec.2014.05.017.

Böttcher, H., Soltmann, U., Mertig, M., Pompe, W., 2004. Biocers: ceramics with incorporated microorganisms for biocatalytic, biosorptive and functional materials development. J. Mater. Chem. 14, 2176. http://dx.doi.org/10.1039/b401724b.

Brander, S.M., Connon, R.E., He, G., Hobbs, J.A., Smalling, K.L., Teh, S.J., White, J.W., Werner, I., Denison, M.S., Cherr, G.N., 2013. From 'omics to otoliths: responses of an estuarine fish to endocrine disrupting compounds across biological scales. PLoS ONE 8, e74251. http://dx.doi.org/10.1371/journal.pone.0074251.

Brix, R., Noguerol, T.-N., Piña, B., Balaam, J., Nilsen, A.J., Tollefsen, K.-E., Levy, W., Schramm, K.-W., Barceló, D., 2010. Evaluation of the suitability of recombinant yeast-based estrogenicity assays as a pre-screening tool in environmental samples. Environ. Int. 36, 361–367. http://dx.doi.org/10.1016/j.envint.2010.02.004.

Chou, P.-H., Liu, T.-C., Lin, Y.-L., 2015. Monitoring of xenobiotic ligands for human estrogen receptor and aryl hydrocarbon receptor in industrial wastewater effluents. J. Hazard. Mater. http://dx.doi.org/10.1016/j.jhazmat.2014.02.049.

European Commission, 1996. European Workshop on the Impact of Endocrine Disruptors on Human Health and Wildlife. Weybridge, 2–4 Dec. 1996, Report Eur 17549, Environment and Climate Research Program, DG XII.

Ezechiá, M., Covino, S., Cajthaml, T., 2014. Ecotoxicity and biodegradability of new brominated flame retardants: a review. Ecotoxicol. Environ. Saf. http://dx.doi.org/10.1016/j.ecoenv.2014.08.030.

Fine, T., Leskinen, P., Isobe, T., Shiraishi, H., Morita, M., Marks, R.S., Virta, M., 2006. Luminescent yeast cells entrapped in hydrogels for estrogenic endocrine disrupting chemical biodetection. Biosens. Bioelectron. 21, 2263–2269. http://dx.doi.org/10.1016/j.bios.2005.11.004.

Gorga, M., Insa, S., Petrovic, M., Barceló, D., 2014. Occurrence and spatial distribution of EDCs and related compounds in waters and sediments of Iberian rivers. Sci. Total Environ. http://dx.doi.org/10.1016/j.scitotenv.2014.06.037.

Han, J., Lee, D., Cho, J., Lee, J., Kim, S., 2012. Hydrogen production from biodiesel byproduct by immobilized Enterobacter aerogenes. Bioprocess Biosyst. Eng. 35, 151–157. http://dx.doi.org/10.1007/s00449-011-0593-0.

Jálová, V., Jarošová, B., Bláha, L., Giesy, J.P., Ocelka, T., Grabic, R., Jurčíková, J., Vrana, B., Hilscherová, K., 2013. Estrogen-, androgen- and aryl hydrocarbon receptor mediated activities in passive and composite samples from municipal waste and surface waters. Environ. Int. 59, 372–383. http://dx.doi.org/10.1016/j.envint.2013.06.024.

Jarošová, B., Bláha, L., Giesy, J.P., Hilscherová, K., 2014. What level of estrogenic activity determined by in vitro assays in municipal waste waters can be considered as safe? Environ. Int. 64, 98–109. http://dx.doi.org/10.1016/j.envint.2013.12.009.

Jarque, S., Bosch, C., Casado, M., Grimalt, J.O., Raldúa, D., Piña, B., 2014. Analysis of hepatic deiodinase 2 mRNA levels in natural fish lake populations exposed to different levels of putative thyroid disrupters. Environ. Pollut. 187, 210–213. http://dx.doi.org/10.1016/j.envpol.2014.01.010.

Krol, S., Nolte, M., Diaspro, A., Mazza, D., Magrassi, R., Gliozzi, A., Fery, A., 2005. Encapsulated living cells on microstructured surfaces. Langmuir 21, 705–709. http://dx.doi.org/10.1021/la047715q.

Lamming, D.W., Wood, J.G., Sinclair, D.A., 2004. Small molecules that regulate lifespan: evidence for xenohormesis. Mol. Microbiol. 53, 1003–1009. http://dx.doi.org/10.1111/j.1365-2958.2004.04209.x.

Leskinen, P., Virta, M., Karp, M., 2003. One-step measurement of firefly luciferase activity in yeast. Yeast 20, 1109–1113. http://dx.doi.org/10.1002/yea.1024.

Leskinen, P., Michelini, E., Picard, D., Karp, M., Virta, M., 2005. Bioluminescent yeast assays for detecting estrogenic and androgenic activity in different matrices. Chemosphere 61, 259–266. http://dx.doi.org/10.1016/j.chemosphere.2005.01.080.

Mesquita, S.R., van Drooge, B.L., Reche, C., Guimarães, L., Grimalt, J.O., Barata, C., Piña, B., 2014. Toxic assessment of urban atmospheric particle-bound PAHs: relevance of composition and particle size in Barcelona (Spain). Environ. Pollut. 184, 555–562. http://dx.doi.org/10.1016/j.envpol.2013.09.034.

Michelini, E., Leskinen, P., Virta, M., Karp, M., Roda, A., 2005. A new recombinant cell-based bioluminescent assay for sensitive androgen-like compound detection. Biosens. Bioelectron. 20, 2261–2267. http://dx.doi.org/10.1016/j.bios.2004.10.018.

Michelini, E., Cevenini, L., Mezzanotte, L., Leskinen, P., Virta, M., Karp, M., Roda, A., 2008. A sensitive recombinant cell-based bioluminescent assay for detection of androgen-like compounds. Nat. Protoc. 3, 1895–1902. http://dx.doi.org/10.1038/nprot.2008.189.

Michelini, E., Cevenini, L., Calabretta, M.M., Spinozzi, S., Camborata, C., Roda, A., 2013. Field-deployable whole-cell bioluminescent biosensors: so near and yet so far. Anal. Bioanal. Chem. 405, 6155–6163. http://dx.doi.org/10.1007/s00216-013-7043-6.

Mitchell, R.J., Gu, M.B., 2006. Characterization and optimization of two methods in the immobilization of 12 bioluminescent strains. Biosens. Bioelectron. 22, 192–199. http://dx.doi.org/10.1016/j.bios.2005.12.019.

Novák, J., Jálová, V., Giesy, J.P., Hilscherová, K., 2009. Pollutants in particulate and gaseous fractions of ambient air interfere with multiple signaling pathways in vitro. Environ. Int. 35, 43–49. http://dx.doi.org/10.1016/j.envint.2008.06.006.

Roda, A., Cevenini, L., Michelini, E., Branchini, B.R., 2011. A portable bioluminescence engineered cell-based biosensor for on-site applications. Biosens. Bioelectron. 26, 3647–3653. http://dx.doi.org/10.1016/j.bios.2011.02.022.

Rotchell, J.M., Ostrander, G.K., 2003. Molecular markers of endocrine disruption in aquatic organisms. J. Toxicol. Environ. Health B. Crit. Rev. 6, 453–496. http://dx.doi.org/10.1080/10937400306476.

Sumpter, J.P., Johnson, A.C., 2005. Lessons from endocrine disruption and their application to other issues concerning trace organics in the aquatic environment. Environ. Sci. Technol. 39, 4321–4332. http://dx.doi.org/10.1021/es048504a.

Článek IV:

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Freeze-drying as suitable method to achieve ready-to-use yeast biosensors for androgenic and estrogenic compounds

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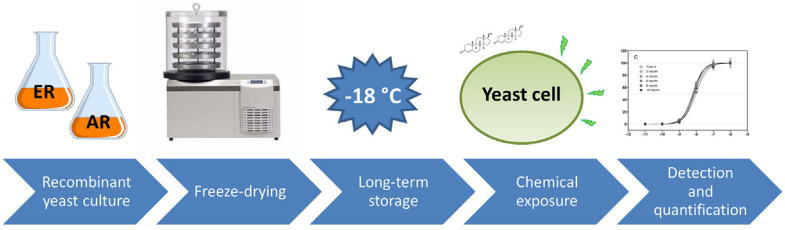
HIGHLIGHTS

- Freeze-drying immobilization to obtain "ready-to-use" versions of yeast biosensors.
- Immobilized yeast cells stored at −18 °C retained viability at least up to 10 months.
- Sensitivity towards androgens and estrogens was comparable to standard assays.
- The new method shortens conventional procedures from 3–4 days to 6 h in non-sterile conditions.

GRAPHICAL ABSTRACT



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ABSTRACT

Recombinant yeast assays (RYAs) have been proved to be a suitable tool for the fast screening of compounds with endocrine disrupting activities. However, ready-to-use versions more accessible to less equipped laboratories and field studies are scarce and far from optimal throughputs. Here, we have applied freeze-drying technology to optimize RYA for the fast assessment of environmental compounds with estrogenic and androgenic potencies. The effects of different cryoprotectants, initial optical density and long-term storage were evaluated. The study included detailed characterization of sensitivity, robustness and reproducibility of the new ready-to-use versions, as well as comparison with the standard assays. Freeze-dried RYAs showed similar dose-responses curves to their homolog standard assays, with Lowest Observed Effect Concentration (LOEC) and Median effective Concentration (EC_{50}) of 1 nM and 7.5 nM for testosterone, and 0.05 nM and 0.5 nM for 17β-estradiol, respectively. Freeze-dried cells stored at 4 °C retained maximum sensitivity up to 2 months, while cells stored at −18 °C showed no decrease in sensitivity throughout the study (10 months). This ready-to-use RYA is easily accessible and may be potentially used for on-site applications.

1. Introduction

Many chemicals and natural products are able to interact with the endocrine system by mimicking or counteracting natural hormones, which may result in the alteration of the correct physiological functioning and, thus, lead to deleterious effects (Duntas, 2014; Patisaul and Adewale, 2009; Waye and Trudeau, 2011). These substances, known as endocrine disrupting compounds (EDCs), are now widespread all over the globe and can exert their action at very low concentrations, representing a real threat for living organisms, including humans (Elsworth et al., 2015; Hu et al., 2009; Jarque et al., 2015; Kidd et al., 2007; Kinch et al., 2015). Effective tools for the fast detection of EDCs are

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consequently needed. The European Regulation No 1907/2006 for Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) calls for the development, validation and acceptance of alternative approaches for further replacement, reduction and refinement of animal use in testing of chemicals (OJL396, 2006). Given the high reproducibility and sensitivity, *in vitro* models are pointed out as good alternatives to *in vivo* testing (Brix et al., 2010; Thibeault et al., 2014).

Recombinant yeast assays (RYA) have been proved to be suitable tools for the fast detection and quantification of EDCs either alone or in environmental samples (Brix et al., 2010; Fernandez et al., 2009; Layton et al., 2002; Leskinen et al., 2005). Unlike bacteria, yeast are eukaryotic organisms with folding and post-translational processes similar to vertebrate cells, which result in the correct expression of transfected mammalian receptors. Compared to other more sensitive *in vitro* eukaryotic models, e. g. mammalian or fish cell lines, RYAs are easy to perform, usually less time-consuming, show good sensitivity and high reproducibility, represent relatively low costs and are compatible with immobilization strategies for in-field testing. They are obtained by introducing two foreign elements in a yeast cell, (i) a receptor able to recognize and to bind the ligand of interest, and (ii) a reporter gene whose expression is under control of specific sequences in the gene promoter. Thus, when the ligand binds to the receptor, the new complex receptor-ligand is able to recognize the specific sequences and activates the expression of the gene reporter, which is typically detected by chromogenic (García-Reyero et al., 2001), fluorogenic (Noguerol et al., 2006b) or luminometric methods (Michelini et al., 2008).

The topic of the effective cell immobilization, long-term storage and subsequent fast recovery to achieve ready-to-use versions of yeast-based systems has been widely studied in the past years (Cha et al., 2012; Diniz-Mendes et al., 1999; Lodato et al., 1999). However, while this task has been partially addressed in some industrial processes, e. g. for use in biocatalysts or commercial products, no optimal solutions have been found when applying to biosensors (reviewed in Michelini et al., 2013). Recently, several matrices such as hydrogels (Fine et al., 2006) and polymers (Bittner et al., 2015; Ponamoreva et al., 2015; Roda et al., 2011) were used for cell entrapment with the aim of obtaining ready-to-use versions of standard RYAs to broaden RYAs applicability. Nevertheless, most of these strategies significantly diminished the performance of the assays mainly because of affecting sensitivity compared to regular assays. In addition, relevant parameters to long-term storage, namely stability and durability of cells, usually showed lower performances relatively short time after immobilization.

Freeze-drying is a two-step dehydration process used for the long-term preservation of perishable materials, including living cells. This method has been relatively well characterized and successfully applied in some bacteria-based portable biosensors (Camanzi et al., 2011; Choi and Gu, 2002; Gu et al., 2001; Wenfeng et al., 2013), but almost no information is available for similar approaches in yeast. In this work, we characterized the applicability of freeze-drying methods in yeast biosensors and subsequently optimized existing RYAs to obtain simple and fast ready-to-use versions with high long-term stability and comparable sensitivity to the standard counterparts.

2. Materials and methods

2.1. Chemicals

All reagents were purchased from Sigma-Aldrich (St. Louis, USA). Stocks of trehalose and maltose were prepared in concentrations of 40% w/v. Luciferin solution 1 mM was prepared by dissolving D-luciferin sodium salt into 0.1 M citric acid and 0.1 M trisodium citrate dihydrate.

2.2. Strains and plasmids

Saccharomyces cerevisiae strains BMAEREluc/ERα and BMAAREluc/AR were obtained from BMA64-1A (MATa, ura 3-52, trp1Δ2 leu2-3 112his3-11 ade2-1, can1-100, wild type strain W303 (Baudin-Baillieu et al., 1997)). BMAEREluc/ERα (ER-RYA) contains the coding sequence of human estrogen receptor alpha (ERα) cloned into the constitutive expression vector pG-1 and a reporter plasmid carrying a truncated form of *Photinus pyralis* luciferase regulated by the estrogen responsive element (ERE), which serves as a reporter gene (Leskinen et al., 2003). BMAAREluc/AR (AR-RYA) presents similar construction but containing human androgen receptor (hAR) and androgen responsive element (ARE) in the reporter plasmid (Leskinen et al., 2005).

2.3. Recombinant yeast assay (RYA)

Detailed protocol for RYA was described elsewhere (Michelini et al., 2008). Briefly, yeast from frozen stocks stored at −80 °C were reconstituted for three days on agar plates incubated at 30 °C. Transformed clones were grown overnight in complex synthetic dextrose (SD) medium at 30 °C and 160 rpm. Culture OD_{600} was adjusted to 0.4 and grown again to reach OD_{600} of 0.6, the exponential phase. Aliquots of 100 μl were transferred on to a 96-well plate and 1 μl of tested chemical was added in 5 replicates. Testosterone and 17β-estradiol (E2) concentrations ranged from 10^{-11} to 10^{-6} and 1.5×10^{-11} to 3.3×10^{-8} M, respectively, using DMSO (1% v/v) as solvent. DMSO was used as vehicle control. Plates were incubated at 30 °C for 2.5 h. After incubation, 100 μl of luciferin were dispensed in each well and luminescence measured with a Synergy™ multifunctional microplate reader (BioTek, Winooski, Vermont, USA).

2.4. Freeze-drying procedure and RYA with freeze-dried yeast cells

A single colony from an agar plate containing SD medium was grown overnight in liquid SD medium. Two cryoprotectants and several culture densities were tested in the optimization of the freeze-drying process. Culture OD_{600} was subsequently adjusted to 8. Aliquots of yeast culture were mixed with cryoprotectants (trehalose or maltose dissolved in water, 40% w/v) in proportion 1:1 v/v reaching final OD_{600} of 4, and transferred into petri dishes. Petri dishes were shaken to homogenize the mix and frozen at −32 °C for 3 h. Frozen cultures were freeze-dried at 0.120 mbar (−40 °C) for 24 h with Christ™ Gamma 1-16 LSC (Martin Christ, Osterode, Germany). After the freeze-drying process, yeast were reconstituted into complex medium in the volume initially used (culture + cryoprotectant) during 3 h at 30 °C. Aliquots of 100 μL of resuspended yeast were transferred onto each well of a 96-well microplate and subsequently exposed to 1 μl of chemical during 2.5 h at 30 °C. After the incubation, luminescence was measured as in the case of the standard RYA.

2.5. Long-term storage

Yeast cells freeze-dried onto petri dishes were vacuum-sealed (Bag sealer ETA162, ETA a.s., Prague, Czech Republic) and stored at 4 and −18 °C, and long-term stability was assessed by measuring viability and activity at different time points. Yeast stored at 4 °C were tested each month until obtaining no signal (5 months), while yeast stored at −18 °C were tested every two months (2, 4, 6, 8 and 10 months). Results were compared with time point 0 (freeze-dried yeast used immediately after the freeze-drying process). In order to

estimate the cell viability, induction factors for each time point were calculated throughout the storage period. The values characterizing yeast performance after different storage durations were also compared to values derived from time point 0 (see *data analysis* section).

2.6. Freeze-drying in multi-well microplate

Yeast cells were further directly freeze-dried onto 96-well plates in a similar procedure to that described for petri dishes. Briefly, transformed clones were grown overnight in complex SD medium. Culture OD_{600} was adjusted to 8 in the same medium. Yeast aliquots were mixed with trehalose (40% w/v) in proportion 1:1 v/v reaching final OD_{600} of 4, and 100 μl of the mixture were transferred into each well. Plates were shaken to homogenize the mix and frozen at −32 °C for 3 h. Frozen plates were freeze-dried at 0.120 mbar (−40 °C) for 18 h with Christ™ Gamma 1-16 LSC. After the freeze-drying process, plates were immediately vacuum sealed and stored at 4 °C. At the day of assay, yeast were resuspended with 100 μl of complex medium, incubated at 30 °C for 3 h and subsequently exposed to 1 μl of test chemical for 2.5 h at the same temperature. Luminescence was measured in the same way as in the regular RYA.

2.7. Data analysis

The measurements of activity for all RYAs variants including long-term storage time points were performed in two independent experiments with five replicates for each hormone concentration. Accordingly, values representing the obtained dose-response curves (shown in the Figures) were calculated as means ± standard errors of the five replicates. Lowest observed effect concentrations (LOEC) were determined as the hormone concentration causing a significantly different response from the vehicle control in ANOVA test with Dunnett's posttest (GraphPad Prism 5, GraphPad Software, Inc., CA, USA). Dose-response curves were plotted to determine median effective hormone concentration (EC_{50}) values. Relative luminescence units (RLU) were expressed as percentage of maximum luminescence response for easier comparison. The induction factor (IF) was calculated as the fold difference between the maximum response induced by hormone and vehicle control response according to this equation: $IF = L_S/L_B$ where L_S is the RLU value of the measured hormone samples of the highest response, and L_B the RLU value of the measured vehicle control.

3. Results

3.1. Effect of different cryoprotectants

Two different disaccharides, trehalose and maltose, were evaluated as cryoprotectants since efficient protective properties for both of them have been reported in other yeast freeze-drying studies (Cerrutti et al., 2000; Diniz-Mendes et al., 1999; Lodato et al., 1999). Our preliminary results disclosed better protective efficiencies for trehalose and maltose alone compared to their mixtures or in combination with skimmed milk (data not shown), so only single cryoprotectants were considered in the final experiments.

Solutions of 40% trehalose (T) or maltose (M) (mixed in proportion 1:1 with yeast culture) were shown as efficiently cryoprotective, since dose-response curves after freeze-drying showed no significant difference compared to non-freeze dried yeast. LOEC (both T and M, AR: 1 nM; ER: 0.05 nM) and EC_{50} (AR T: 7.84 nM, AR M: 9.14 nM; ER T: 0.46 nM, ER M: 0.67 nM) were the same or similar, respectively, in both cases (Fig. 1, Table 1), although yeast

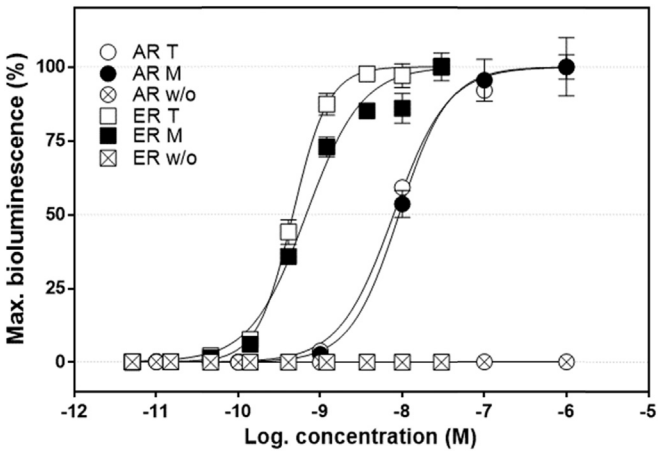

Fig. 1. Effect of cryoprotectants on the performance of the RYAs after cells freeze-drying shown as dose-response curves for testosterone in AR-RYA (circles) and 17β-estradiol in ER-RYA (squares). White symbols: trehalose; black symbols: maltose; crossed symbols: no cryoprotectant.

freeze-dried in trehalose tended to show slightly higher IF for both yeast strains (data not shown). No other difference was observed between both cryoprotectants. On the contrary, yeast cells freeze-dried without cryoprotectant showed no activity, which pointed out the indispensability of using cryoprotectant during the freeze-drying process. Accordingly with our results, trehalose was chosen as cryoprotectant for the rest of the experiments presented in this study.

3.2. Effect of different optical densities

While standard RYA is typically performed after adjusting OD_{600} to 0.6, where cultured yeast is considered to be in mid-logarithmic growth phase (Michelini et al., 2008), there are no available data about optimal OD_{600} for freeze-dried RYAs. In order to find the best OD_{600} of the yeast culture prior to freeze drying for optimal response of the assay reconstituted after freeze-drying, yeast cultures were grown until reaching OD_{600} ranging from 1 to 10 and mixed with trehalose in proportion 1:1. Cultures freeze-dried at initial OD_{600} of 8 and 10 (OD_{600} 4 and 5 after dilution in trehalose) showed after reconstitution similar dose-response curves to the standard assay for both yeast strains (Fig. 2). Initial OD_{600} of 6 was also sufficient to reproduce equivalent dose-response characteristics, although IF were significantly lower than those corresponding to OD_{600} of 8 and 10. Recovery after freeze-drying of the yeast at lower OD_{600} was less reliable since LOEC, EC_{50} and IF were significantly affected, particularly for ER strain. Therefore, initial OD_{600} of 8 was considered optimal and used in subsequent experiments.

3.3. Standard RYA vs freeze-dried variant

The responses of standard RYA and RYA with directly reconstituted yeast which was freeze-dried under optimized conditions (trehalose, OD_{600} 8) were characterized and compared by their LOEC, EC_{50} and IF values. Standard RYA showed LOEC, EC_{50} and IF of 1 nM, 3.5 nM and 207 for testosterone (AR-RYA), and 0.05 nM, 0.93 nM and 121 for 17β-estradiol (ER-RYA), respectively. Similar values were obtained for both receptors (1 nM, 7.5 nM and 201 for AR, and 0.05 nM, 0.46 nM and 64 for ER, respectively) in the variant freeze-dried in trehalose (Fig. 3, Table 1).

Table 1
Dose-response characterization of standard RYA and RYA with freeze-dried cells. Values represent results from two independent experiments.

Time point (month)	EC_{50} (nM)						LOEC (nM)					
	AR-RYA	AR-FD 4C	AR-FD -18C	ER-RYA	ER-FD 4C	ER-FD -18C	AR-RYA	AR-FD 4C	AR-FD -18C	ER-RYA	ER-FD 4C	ER-FD -18C
t0	3.5	7.5	7.5	0.9	0.5	0.5	1.0	1.0	1.0	0.05	0.05	0.05
t1		11.9	–		0.8	–		1.0	–		0.05	–
t2		11.2	7.0		0.6	0.3		1.0	1.0		0.05	0.05
t3		12.0	–		0.5	–		1.0–10.0	–		0.1	–
t4		22.0	6.1		0.9	0.2		10.0	1.0		0.1	0.05
t5		n.a.	–		n.a.	–		n.a.	–		n.a.	–
t6			6.7			0.3			1.0			0.05
t7			–			–			–			–
t8			9.7			0.4			1.0			0.05
t9			–			–			–			–
t10			7.9			0.5			1.0			0.05

RYA, standard assay; FD, freeze-dried RYA; 4C, storage at 4 °C; −18C, storage at −18 °C; -, not measured; n.a., no activity detected.

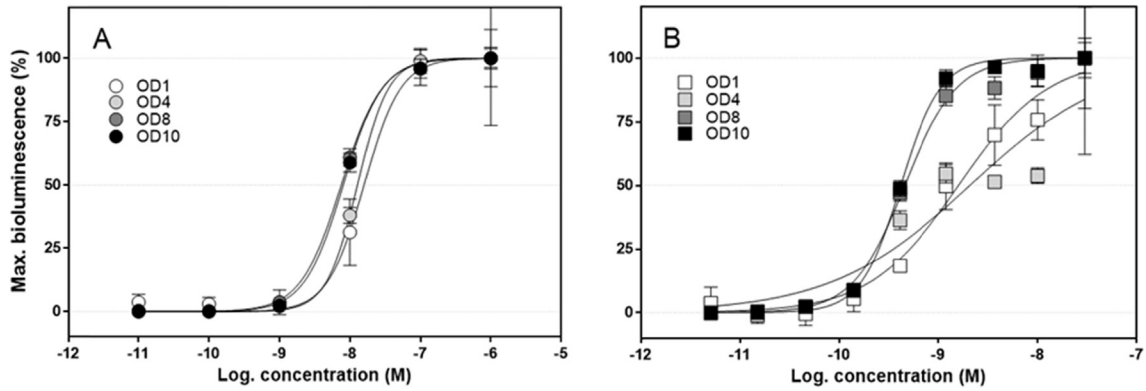

Fig. 2. Effect of the concentration of yeast expressed as OD_{600} prior to freeze-drying process on the performance of the RYAs after reconstitution from freeze-dried stock shown as dose-response curves for testosterone in AR-RYA (A) and 17β-estradiol in ER-RYA (B). For better clarity, OD of 2 and 6 are omitted in both graphs.

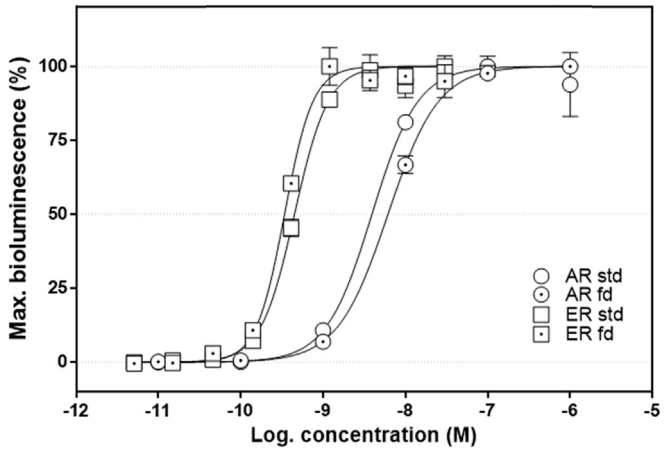

Fig. 3. Dose-response curves for testosterone (circles) and 17β-estradiol (squares) obtained with standard RYA (white) and RYA with freeze-dried cells using trehalose as cryoprotectant and OD_{600} of 8 (dotted white). The freeze-dried yeast was tested immediately after the freeze-drying process.

3.4. Stability during long-term storage

Freeze-dried yeast performances were evaluated after long-term storage at 4 and −18 °C. Dose-response curves, LOEC and EC_{50} for the different time points considered in the present study are shown in Fig. 4 and Table 1, respectively. Freeze-dried yeast showed very different stability depending on storage temperature. Yeast stored at 4 °C showed gradual increases in EC_{50}, resulting in no detectable activity for both strains after 5 months of storage (Fig. 4A, B). LOEC values were not affected until the third month. By contrast, yeast stored at −18 °C showed no significant worsening in any of the evaluated parameters (LOEC, EC_{50} and IF) throughout the ten months in which the experiment was performed (Fig. 4C, D).

The induction factor (IF) was used as a measure corresponding to the cell viability during the long-term storage (Bittner et al., 2015). In agreement with the long-term dose-response evaluation experiments, gradual decrease in IF was recorded in assays performed with yeast cells stored at 4 °C, with no detectable induction after 5 months of storage (Fig. 5A). By contrast, IF in freeze-dried yeast stored at −18 °C showed not only no significant decrease during the 10 months of storage, but even slight increases compared to time 0 in some months (Fig. 5B). Since dose-response curves showed no significant differences, we consider that these increases reflect the intrinsic natural variability in the number of colonies that may survive during the freeze-drying process.

3.5. Direct freeze-drying in multi-well microplate

To further evaluate the potential applicability of the freeze-dried yeast to more standardized formats and, in turn, the adaptability to more automated procedures, yeast cells were freeze-dried directly in 96-well microplates. Both strains showed similar performances to those registered for their standard counterparts, although variability among replicates increased in some cases (Fig. 6). The variability was well position-independent. Dose-response curves disclosed mean values for EC_{50} and LOEC of 10.3 nM and 1 nM for

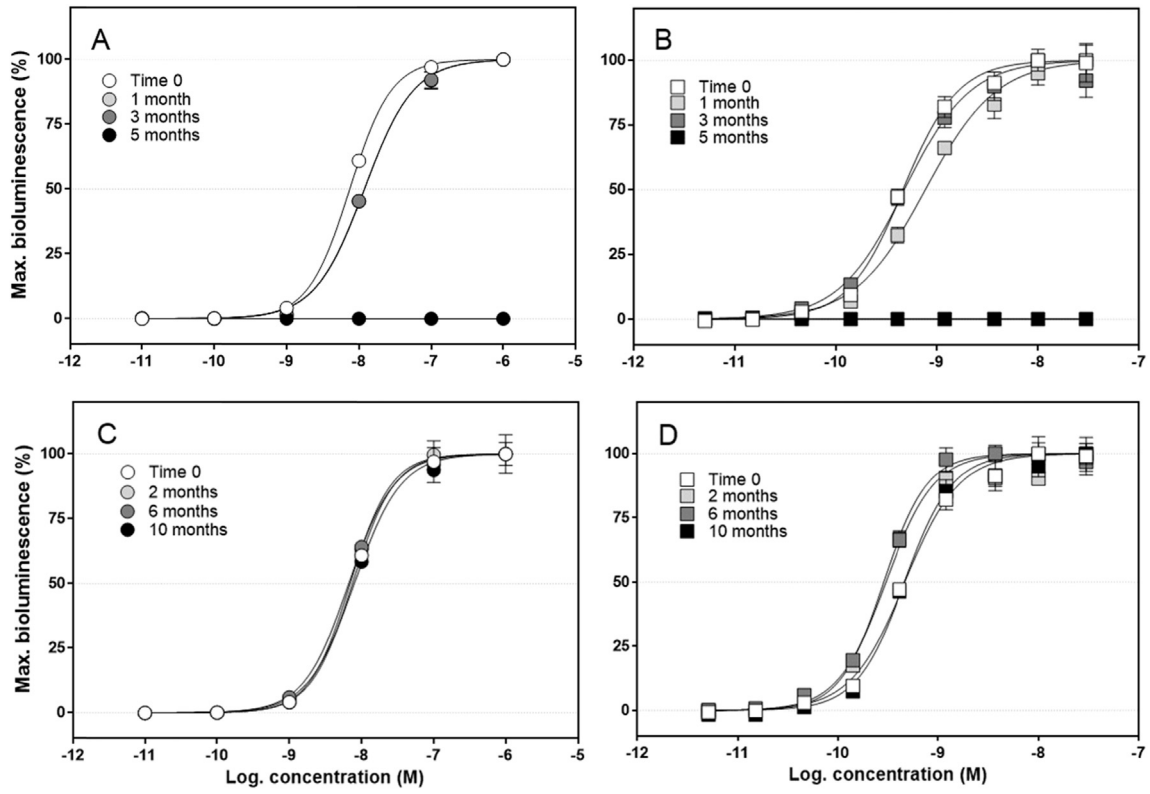

Fig. 4. Dose-response curves for testosterone in AR-RYA (A and C) and 17β-estradiol in ER-RYA (B and D) obtained after freeze-drying and subsequent long-term storage at 4 °C (A–B) and −18 °C (C–D). For better clarity, only results for time 0 and months 1, 3 and 5 (4 °C), and 2, 6 and 10 (−18 °C) are shown.

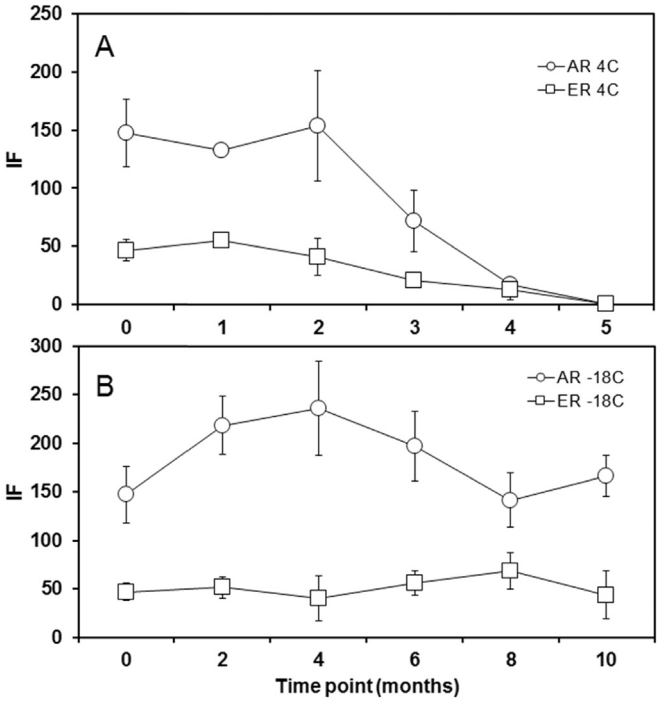

Fig. 5. Induction factor reflecting cell viability after long term storage at 4 °C (A) and −18 °C (B). Values represent mean ± SE of two independent experiments.

testosterone in AR-RYA, and 0.42 nM and 0.05 nM for 17β-estradiol in ER-RYA, respectively.

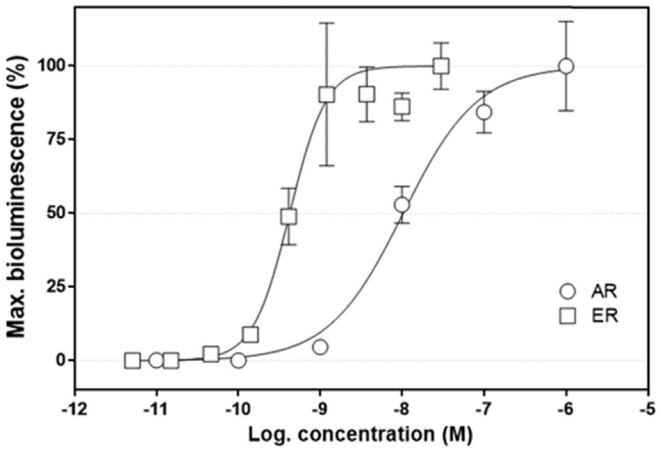

Fig. 6. Dose-response curves for testosterone (AR-RYA) and 17β-estradiol (ER-RYA) obtained with yeast cells directly immobilized in 96-well microplates.

4. Discussion

The efficient immobilization of living cells is a crucial step for the development of ready-to-use whole-cell biosensors. Several immobilization strategies have been recently suggested, but none of them offered optimal throughputs compared to non-immobilized versions. In the present study, two different yeast strains harboring androgen and estrogen receptors, respectively, were successfully freeze-dried. The use of cryoprotectant together with a relatively high initial cell density were key factors in the process (Figs. 1 and 2). Several compounds with protective properties, such as trehalose, maltose, skimmed milk (Bekatorou et al.,

2001), sodium glutamate (Polomska et al., 2012), polyethylene glycol (Wenfeng et al., 2013) or maltodextrin (Lodato et al., 1999), have been used in freeze-drying studies. In addition, it has been reported that mixtures of different cryoprotectants, e. g. trehalose and skimmed milk, may be more efficient than single compounds because of their complimentary effect in the protective role (Lodato et al., 1999; Polomska et al., 2012). In our experiments, trehalose and maltose were the compounds tested since they were usually reported as the most efficient cryoprotectants when used alone. Moreover, their solutions are transparent, which may help to avoid interference during bioluminescence readings. Since our preliminary results disclosed no additional protective role for mixtures, only single cryoprotectants were considered. Both disaccharides efficiently protected cells during freeze-drying, as it was disclosed by the similar dose-response curves compared to the standard RYAs. However, certain ratio of mortality was recorded after the process, likely due to the low pressure conditions required to totally sublimate the water content during lyophilization. Accordingly, high cell densities were needed to ensure maximum response and sensitivity after short reconstitution time (Fig. 2).

Although cell viability was high enough to ensure proper response after freeze-drying, gradual decrease in the cell responsiveness was recorded every month in yeast cells stored at 4 °C, resulting in the total activity loss after 5 months. By contrast, cells stored at −18 °C retained viability and activity up to 10 months, which was the longest period tested in the present study (Fig. 4, Table 1). In both cases, plates were stored in vacuum-sealed plastic bags, but probably full vacuum conditions were not achieved because of the device limitations. It is also very possible that residual water remained in freeze-dried cultures (Patel and Pikal, 2011). As a consequence, despite adequate sealing and storing, partial rehydration was observed in cells stored at 4 °C, affecting cell viability after long-term storage. In conclusion, storage at freezing temperature was shown to be better for preservation of the cells from humidity, which have direct influence on the long-term cell viability.

With the aim of adapting freeze-dried RYA to standardized high-throughput formats, yeast cells were further lyophilized directly in 96-well microplates. This approach showed similar dose-response curves to RYA freeze-dried on petri dishes. Nevertheless, higher variability among replicates was observed. Most plausible explanation for such variability is the lack of homogeneity of freeze-drying process across the microplate, which may be due to the different heat transfer among wells. The small capacity and particular geometry of the wells may contribute to this effect (Patel and Pikal, 2011). Thus, during freeze-drying, some wells would be randomly affected by different microconditions, diminishing their cell survival percentage and, in turn, slightly decreasing the luminescent signal. However, despite the increased variability among replicates, direct freeze-drying in multiwell microplate was shown as a valid high-throughput approach for environmental monitoring since freeze-dried cells were able to respond in dose-response manner to hormone exposures with sensitivity comparable to regular RYAs.

The preparation of freeze-dried yeast biosensors allows the application of ready-to-use assays for EDC detection, avoiding three-days lasting reconstitution from −80 °C frozen stocks and later overnight growing of cultures. This significantly shortens RYA from several days (3–4 days) to less than 6 h. In addition, since cells after freeze-drying require no sterile conditions, new RYA version could be easily performed in less equipped laboratories, and potentially adapted to portable devices designed for on-site biosensing applications (Choi and Gu, 2002; Roda et al., 2011). Freeze-dried biosensors also represent significant advantages compared to other immobilization strategies. Yeast cells encapsulated or immobilized into/onto polymers showed lower sensitivity (at least one order of magnitude) compared to conventional assays (Fine et al., 2006). Long-term stability was also an issue since polymeric matrices degenerated after 1–3 months of storage, affecting yeast biosensors performance (Bittner et al., 2015; Fine et al., 2006). Freeze-dried yeast biosensors described here retained stability and comparable sensitivity to non-immobilized yeast cells for at least 10 months (the longest time point tested) when stored at −18 °C. It is very possible that activity of cells may last even longer periods. Although our data is based on AR and ER recombinant yeast strains, it is very possible that this freeze-drying method could be also applied to other existing yeast-based systems constructed to detect ligands to other receptors, e. g. thyroid receptor (Li et al., 2014; Shiizaki et al., 2010), aryl hydrocarbon receptor (Noguerol et al., 2006a), or progesterone receptor (Chatterjee et al., 2008), contributing to the easier accessibility of these *in vitro* assays.

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References

Baudin-Baillieu, A., Guillemet, E., Cullin, C., Lacroute, F., 1997. Construction of a yeast strain deleted for the TRP1 promoter and coding region that enhances the efficiency of the polymerase chain reaction-disruption method. Yeast 13, 353–356. http://dx.doi.org/10.1002/(SICI)1097-0061(19970330)13:4<353::AID-YEA86>3.0.CO;2-P.

Bekatorou, A., Koutinas, A., Kaliafas, A., Kanellaki, M., 2001. Freeze-dried Saccharomyces cerevisiae cells immobilized on gluten pellets for glucose fermentation. Process Biochem. 36, 549–557. http://dx.doi.org/10.1016/S0032-9592(00)00246-6.

Bittner, M., Jarque, S., Hilscherová, K., 2015. Polymer-immobilized ready-to-use recombinant yeast assays for the detection of endocrine disruptive compounds. Chemosphere 132, 56–62. http://dx.doi.org/10.1016/j.chemosphere.2015.02.063.

Brix, R., Noguerol, T.-N., Piña, B., Balaam, J., Nilsen, A.J., Tollefsen, K.-E., Levy, W., Schramm, K.-W., Barceló, D., 2010. Evaluation of the suitability of recombinant yeast-based estrogenicity assays as a pre-screening tool in environmental samples. Environ. Int. 36, 361–367. http://dx.doi.org/10.1016/j.envint.2010.02.004.

Camanzi, L., Bolelli, L., Maiolini, E., Girotti, S., Matteuzzi, D., 2011. Optimal conditions for stability of photoemission and freeze drying of two luminescent bacteria for use in a biosensor. Environ. Toxicol. Chem. 30, 801–805. http://dx.doi.org/10.1002/etc.452.

Cerrutti, P., Segovia de Huergo, M., Galvagno, M., Schebor, C., del Pilar Buera, M., 2000. Commercial baker's yeast stability as affected by intracellular content of trehalose, dehydration procedure and the physical properties of external matrices. Appl. Microbiol. Biotechnol. 54, 575–580.

Cha, C., Kim, S.R., Jin, Y.-S., Kong, H., 2012. Tuning structural durability of yeast-encapsulating alginate gel beads with interpenetrating networks for sustained bioethanol production. Biotechnol. Bioeng. 109, 63–73. http://dx.doi.org/10.1002/bit.23258.

Chatterjee, S., Kumar, V., Majumder, C.B., Roy, P., 2008. Screening of some anti-progestin endocrine disruptors using a recombinant yeast based in vitro bioassay. Toxicol. In Vitro 22, 788–798. http://dx.doi.org/10.1016/j.tiv.2007.12.006.

Choi, S.H., Gu, M.B., 2002. A portable toxicity biosensor using freeze-dried recombinant bioluminescent bacteria. Biosens. Bioelectron. 17, 433–440.

Diniz-Mendes, L., Bernardes, E., de Araujo, P.S., Panek, A.D., Paschoalin, V.M., 1999. Preservation of frozen yeast cells by trehalose. Biotechnol. Bioeng. 65, 572–578. http://dx.doi.org/10.1002/(SICI)1097-0290(19991205)65:5<572::AID-BIT10>3.0.CO;2–7.

Duntas, L.H., 2014. Chemical contamination and the thyroid. Endocrine. http://dx.doi.org/10.1007/s12020-014-0442-4.

Elsworth, J.D., Jentsch, J.D., Groman, S.M., Roth, R.H., Redmond, E.D., Leranth, C., 2015. Low circulating levels of bisphenol-A induce cognitive deficits and loss of asymmetric spine synapses in dorsolateral prefrontal cortex and hippocampus of adult male monkeys. J. Comp. Neurol. 523, 1248–1257. http://dx.doi.org/10.1002/cne.23735.

Fernandez, M.P., Noguerol, T.-N., Lacorte, S., Buchanan, I., Piña, B., 2009. Toxicity

identification fractionation of environmental estrogens in waste water and sludge using gas and liquid chromatography coupled to mass spectrometry and recombinant yeast assay. Anal. Bioanal. Chem. 393, 957–968. http://dx.doi.org/10.1007/s00216-008-2516-8.

Fine, T., Leskinen, P., Isobe, T., Shiraishi, H., Morita, M., Marks, R.S., Virta, M., 2006. Luminescent yeast cells entrapped in hydrogels for estrogenic endocrine disrupting chemical biodetection. Biosens. Bioelectron. 21, 2263–2269. http://dx.doi.org/10.1016/j.bios.2005.11.004.

García-Reyero, N., Grau, E., Castillo, M., De Alda, M.J.L., Barceló, D., Piña, B., 2001. Monitoring of endocrine disruptors in surface waters by the yeast recombinant assay. Environ. Toxicol. Chem. 20, 1152–1158. http://dx.doi.org/10.1002/etc.5620200603.

Gu, M.B., Choi, S.H., Kim, S.W., 2001. Some observations in freeze-drying of recombinant bioluminescent Escherichia coli for toxicity monitoring. J. Biotechnol. 88, 95–105.

Hu, J., Zhang, Z., Wei, Q., Zhen, H., Zhao, Y., Peng, H., Wan, Y., Giesy, J.P., Li, L., Zhang, B., 2009. Malformations of the endangered Chinese sturgeon, Acipenser sinensis, and its causal agent. Proc. Natl. Acad. Sci. U. S. A. 106, 9339–9344. http://dx.doi.org/10.1073/pnas.0809434106.

Jarque, S., Quirós, L., Grimalt, J.O., Gallego, E., Catalan, J., Lackner, R., Piña, B., 2015. Background fish feminization effects in European remote sites. Sci. Rep. 5, 11292. http://dx.doi.org/10.1038/srep11292.

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. U. S. A. 104, 8897–8901. http://dx.doi.org/10.1073/pnas.0609568104.

Kinch, C.D., Ibhazehiebo, K., Jeong, J.-H., Habibi, H.R., Kurrasch, D.M., 2015. Low-dose exposure to bisphenol A and replacement bisphenol S induces precocious hypothalamic neurogenesis in embryonic zebrafish. Proc. Natl. Acad. Sci. U. S. A. 112, 1475–1480. http://dx.doi.org/10.1073/pnas.1417731112.

Layton, A.C., Sanseverino, J., Gregory, B.W., Easter, J.P., Sayler, G.S., Schultz, T.W., 2002. In vitro estrogen receptor binding of PCBs: measured activity and detection of hydroxylated metabolites in a recombinant yeast assay. Toxicol. Appl. Pharmacol. 180, 157–163. http://dx.doi.org/10.1006/taap.2002.9395.

Leskinen, P., Michelini, E., Picard, D., Karp, M., Virta, M., 2005. Bioluminescent yeast assays for detecting estrogenic and androgenic activity in different matrices. Chemosphere 61, 259–266. http://dx.doi.org/10.1016/j.chemosphere.2005.01.080.

Leskinen, P., Virta, M., Karp, M., 2003. One-step measurement of firefly luciferase activity in yeast. Yeast 20, 1109–1113. http://dx.doi.org/10.1002/yea.1024.

Li, J., Ren, S., Han, S., Li, N., 2014. A yeast bioassay for direct measurement of thyroid hormone disrupting effects in water without sample extraction, concentration, or sterilization. Chemosphere 100, 139–145. http://dx.doi.org/10.1016/j.chemosphere.2013.11.054.

Lodato, P., Se govia de Huergo, M., Buera, M.P., 1999. Viability and thermal stability of a strain of Saccharomyces cerevisiae freeze-dried in different sugar and polymer matrices. Appl. Microbiol. Biotechnol. 52, 215–220.

Michelini, E., Cevenini, L., Calabretta, M.M., Spinozzi, S., Camborata, C., Roda, A., 2013. Field-deployable whole-cell bioluminescent biosensors: so near and yet so far. Anal. Bioanal. Chem. 405, 6155–6163. http://dx.doi.org/10.1007/s00216-013-7043-6.

Michelini, E., Cevenini, L., Mezzanotte, L., Leskinen, P., Virta, M., Karp, M., Roda, A., 2008. A sensitive recombinant cell-based bioluminescent assay for detection of androgen-like compounds. Nat. Protoc. 3, 1895–1902. http://dx.doi.org/10.1038/nprot.2008.189.

Noguerol, T.-N., Boronat, S., Casado, M., Raldúa, D., Barceló, D., Piña, B., 2006a. Evaluating the interactions of vertebrate receptors with persistent pollutants and antifouling pesticides using recombinant yeast assays. Anal. Bioanal. Chem. 385, 1012–1019. http://dx.doi.org/10.1007/s00216-006-0476-4.

Noguerol, T.-N., Boronat, S., Jarque, S., Barceló, D., Piña, B., 2006b. Detection of hormone receptor ligands in yeast by fluorogenic methods. Talanta 69, 351–358. http://dx.doi.org/10.1016/j.talanta.2005.09.044.

OJL396, 2006. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 Concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), Establishing a European Chemicals Agency, Amending Directive 1999/4.

Patel, S.M., Pikal, M.J., 2011. Emerging freeze-drying process development and scale-up issues. AAPS PharmSciTech 12, 372–378. http://dx.doi.org/10.1208/s12249-011-9599-9.

Patisaul, H.B., Adewale, H.B., 2009. Long-term effects of environmental endocrine disruptors on reproductive physiology and behavior. Front. Behav. Neurosci. 3, 10. http://dx.doi.org/10.3389/neuro.08.010.2009.

Polomska, X., Wojtatowicz, M., Zarowska, B., Szoıtysik, M., Chrzanowska, J., 2012. Freeze-drying preservation of yeast adjunct cultures for Cheese production. Pol. J. Food Nutr. Sci. 62, 143–150.

Ponamoreva, O.N., Kamanina, O.A., Alferov, V.A., Machulin, A.V., Rogova, T.V., Arlyapov, V.A., Alferov, S.V., Suzina, N.E., Ivanova, E.P., 2015. Yeast-based self-organized hybrid bio-silica sol-gels for the design of biosensors. Biosens. Bioelectron. 67, 321–326. http://dx.doi.org/10.1016/j.bios.2014.08.045.

Roda, A., Cevenini, L., Michelini, E., Branchini, B.R., 2011. A portable bioluminescence engineered cell-based biosensor for on-site applications. Biosens. Bioelectron. 26, 3647–3653. http://dx.doi.org/10.1016/j.bios.2011.02.022.

Shiizaki, K., Asai, S., Ebata, S., Kawanishi, M., Yagi, T., 2010. Establishment of yeast reporter assay systems to detect ligands of thyroid hormone receptors alpha and beta. Toxicol. In Vitro 24, 638–644. http://dx.doi.org/10.1016/j.tiv.2009.10.001.

Thibeault, A.-A.H., Deroy, K., Vaillancourt, C., Sanderson, J.T., 2014. A unique co-culture model for fundamental and applied studies of human fetoplacental steroidogenesis and interference by environmental chemicals. Environ. Health Perspect. 122, 371–377. http://dx.doi.org/10.1289/ehp.1307518.

Waye, A., Trudeau, V.L., 2011. Neuroendocrine disruption: more than hormones are upset. J. Toxicol. Environ. Health. B. Crit. Rev. 14, 270–291. http://dx.doi.org/10.1080/10937404.2011.578273.

Wenfeng, S., Gooneratne, R., Glithero, N., Weld, R.J., Pasco, N., 2013. Appraising freeze-drying for storage of bacteria and their ready access in a rapid toxicity assessment assay. Appl. Microbiol. Biotechnol. 97, 10189–10198. http://dx.doi.org/10.1007/s00253-013-4706-3.

Článek V:

Sovadinová, I., Bláha, L., Janošek, J., Hilscherová, K., Giesy, J.P., Jones, P.D., Holoubek, I., 2006. Cytotoxicity and aryl hydrocarbon receptor-mediated cctivity of N-heterocyclic polycyclic aromatic hydrocarbons - Structure-activity relationships. Environmental Toxicology and Chemistry 25 (5), 1291-1297.

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CYTOTOXICITY AND ARYL HYDROCARBON RECEPTOR–MEDIATED ACTIVITY OF N-HETEROCYCLIC POLYCYCLIC AROMATIC HYDROCARBONS: STRUCTURE–ACTIVITY RELATIONSHIPS

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Abstract—Toxic effects of many persistent organic pollutants (e.g., polychlorinated biphenyls or polychlorinated dibenzo-*p*-dioxins and furans) are mediated via the aryl hydrocarbon receptor (AhR). Although polycyclic aromatic hydrocarbons (PAHs) and their derivatives also activate AhR, their toxic effects remain to be fully elucidated. In the present study, we used the in vitro H4IIE-luc transactivation cell assay to investigate cytotoxicity and potencies to activate AhR by 29 individual PAHs and their N-heterocyclic derivatives (aza-PAHs). The aza-PAHs were found to be significantly more cytotoxic and more potent inducers of AhR than their unsubstituted analogues. Several aza-PAHs, such as dibenz[*a*,*h*]acridine or dibenz[*a*,*i*]acridine, activated AhR within picomolar concentrations, comparable to the effects of reference 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Ellipsoidal volume, molar refractivity, and molecular size were the most important descriptors derived from the modeling of quantitative structure–activity relationships for potencies to activate AhR. Comparable relative toxic potencies (induction equivalency factors) for individual aza-PAHs are derived, and their use for evaluation of complex contaminated samples is discussed.

Keywords—Aza-arenes Polycyclic aromatic hydrocarbons Dioxin-like toxicity Aryl hydrocarbon receptor Quantitative structure–toxicity relationships

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a major class of organic contaminants in industrial and urban regions worldwide, and they are ubiquitous in the environment. Sixteen priority PAHs are monitored by the U.S. Environmental Protection Agency, but many compounds remain overlooked in monitoring programs. These include, for example, high-molecular-weight mutagenic PAHs [1,2], nitroderivatives and oxygenated PAHs [3,4], and N-heterocyclic aromatic compounds, such as aza-PAHs or aza-arenes [4,5]. The aza-PAHs may originate from natural sources, such as alkaloids, mycotoxins, or nucleotides. However, they are released predominantly as anthropogenic contaminants by incomplete combustion of fossil fuels, spills, or industrial effluents or as a result of oil drilling and refining, wood preservation, and tobacco smoking [6–8]. The aza-PAHs are concomitantly widespread with their parent analogues, and they have been detected in the air [3], in water and sediments [4,9], and in soil [10]. However, our understanding of their occurrence, environmental fate, biological metabolism, and effects is still limited. Although aza-PAHs outnumber the unsubstituted homocyclic PAHs, their environmental concentrations are lower than those of the parent compounds (1–10% of the total PAH concentrations [11]). However, greater polarity of aza-PAHs, along with higher water solubility and bioavailability may result, in more significant effects, even at lower environmental concentrations [12].

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The effects of a limited number of aza-PAHs, particularly low-molecular-weight compounds, have been investigated with algae, invertebrates, and fish [13–15]. Some benzacridines and dibenzacridines were found to be mutagens and carcinogens and to cause nongenotoxic effects, such as (anti)estrogenicity [4,16–18]. Modulation of the intracellular aryl hydrocarbon receptor (AhR) is one of the major toxicity mechanisms of many organic environmental contaminants, such as polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-*p*-dioxins and furans, and the in vivo effects related to the activation of AhR include porphyria, immunotoxicity, developmental, and reproductive failure or carcinogenicity. Polycyclic aromatic hydrocarbons and their derivatives also have been shown to modulate AhR [19], but their in vivo toxicity directly mediated by AhR remains disputable. The risk assessment of polychlorinated dibenzo-*p*-dioxins and furans and of PCBs uses the concept of toxic equivalency factors—that is, toxic potencies of individual chemicals related to reference 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [20]. A similar approach of relative toxic potencies also has been proposed for other nonhalogenated pollutants, such as PAHs [3,21]. Evidence also suggests that aza-PAHs modulate AhR and induce AhR-dependent hepatic microsomal mixed-function oxidases and cytochromes P450 (CYP450s) [4,5,22–24]. To date, however, only a limited number of aza-PAHs have been studied.

In the present study, we investigated the in vitro effects of 22 individual aza-PAHs and seven parent PAHs. The aim of the present study was to obtain principal information regarding

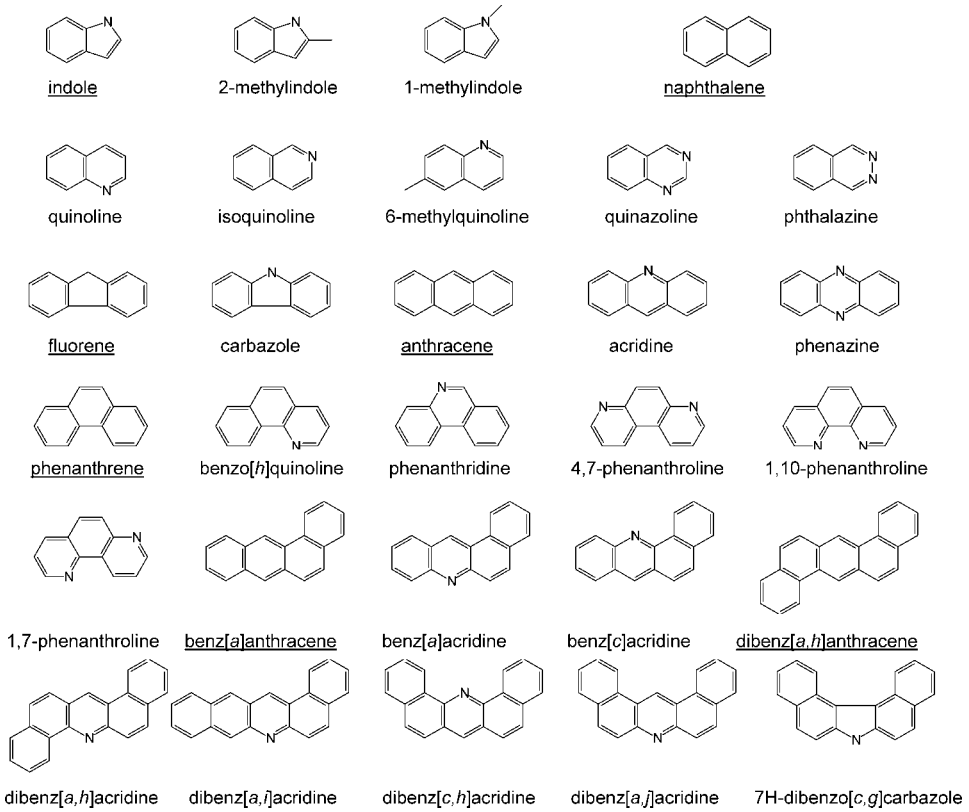

Fig. 1. Structures of the studied polycyclic aromatic hydrocarbons (PAHs) and their N-heterocyclic derivatives. The parent unsubstituted compounds are underlined.

the cytoxicity and the potencies to induce AhR of these poorly characterized xenobiotics. Additionally, we studied quantitative structure–activity relationships (QSAR), and we derived induction equivalency factors (IEFs) for evaluation of complex contaminated samples.

MATERIALS AND METHODS

Chemicals

Quinoline (Chemical Abstracts Service [CAS] no. 91-22-5; purity, 98%), benzo[*h*]quinoline (CAS no. 230-27-3; purity, 97%), acridine (CAS no. 260-94-6; purity, 97%), quinazoline (CAS no. 253-82-7; purity, 99%), isoquinoline (CAS no. 119-65-3; purity, 97%), phenanthridine (CAS no. 229-87-8; purity, 98%), 4,7-phenanthroline (CAS no. 230-07-9; purity, 98%), 1,10-phenanthroline (CAS no. 66-71-7; purity, 99%), carbazole (CAS no. 86-74-8; purity, 96%), indole (CAS no. 120-72-9; purity, 98%), 2-methylindole (CAS no. 95-20-5; purity, 98%), 1-methylindole (CAS no. 603-76-9; purity, 97%), 6-methylquinoline (CAS no. 91-62-3; purity, 98%), 1,7-phenanthroline (CAS no. 230-46-6; purity, 99%), phenazine (CAS no. 92-82-0; purity, 98%), phthalazine (CAS no. 253-52-1; purity, 98%), naphthalene (CAS no. 91-20-3; purity, 98%), anthracene (CAS no. 120-12-7; purity, 97%), benz[*a*]anthracene (CAS no. 56-55-3; purity, 99%), dibenz[*a,h*]anthracene (CAS no. 53-70-3; purity, 97%), fluorene (CAS no. 86-73-7; purity 98%), phenanthrene (CAS no. 85-01-8; purity 99%), dibenz[*a,j*]anthracene (CAS no. 224-41-9), and dibenzo[*a*]pyrene (CAS no. 50-32-8) were purchased from Sigma-Aldrich (Prague, Czech Republic). Benz[*a*]acridine (CAS no. 225-11-6; purity, 99.5%), benz[*c*]acridine (CAS no. 225-51-4; purity, 99.8%), dibenz[*a,i*]acridine (CAS no. 226-92-6; purity, 99.7%), dibenz[*a,j*]acridine (CAS no. 224-42-0; purity, 99%), dibenz[*a,h*]acridine (CAS no. 226-36-8; purity, 99.86%), dibenz[*c,h*]acridine (CAS no. 224-53-3; purity, 99.3%), and 7-H-dibenz[*c,g*]carbazole (CAS no. 194-59-2; purity, 99.7%) were obtained from Dr. Ehrenstorfer (Augsburg, Germany). The reference TCDD (CAS no. 1746-01-6) was from Ultra Scientific (North Kingstown, RI, USA). The structures of the studied compounds are shown in Figure 1.

Toxicity testing

Cytotoxicity and potency to activate AhR were investigated by the H4IIE-luc rat hepatoma cell line stably transfected with the pGudLuc 1.1 vector containing luciferase reporter gene under the transcriptional control of dioxin-responsive elements [25]. Assessment of cytotoxicity was based on conventional neutral red uptake bioassay, as described elsewhere [26]. Potencies to induce AhR were determined as reported previously [25,27]. Briefly, H4IIE-luc cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% (v/v) fetal calf serum and antibiotics (all from PAA Laboratories, Pasching, Austria) at 37°C and 5% CO_2. The cells were seeded into 96-well cell culture plates at a density of 2×10^4 cells/well. After 24 h of incubation (~75% cell confluence), tested chemicals diluted in dimethyl sulfoxide were added in three replicates (final concentration of the solvent did not exceed 0.5% v/v). Following 6 or 24 h of exposure, medium was removed, and the cells were washed with phosphate-buffered saline (pH 7.2). Reporter luciferase activity was then determined using a microplate luminometer GENios (Tecan, Mannedorf, Switzerland) with the Steady-Glo Luciferase Assay Kit (Promega, Madison, WI, USA). Blank, solvent control (dimethyl sulfoxide), and a standard curve of the TCDD (0.1–500 pM) were tested on each plate. At least five concentrations of each com-

pound were tested in two independent experiments. The resulting data were pooled and used for further evaluation; coefficient of variance was less than 20% for each individual treatment (concentration) tested.

Data analyses

All calculations and statistical analyses were performed with Microsoft Excel and Statistica® for Windows (Ver 6.0; StatSoft, Tulsa, OK, USA). For the assessment of cytotoxicity, data were compared with blank and solvent controls using analysis of variance followed by Dunnet's test. The lowest concentration that significantly ($p < 0.05$) affected cell viability was derived (experimental lowest-observed-effect concentration [$LOEC_{exp}$]). The H4IIE-luc bioassay data (relative luminescence units) were expressed as a percentage of the mean maximum TCDD response (% TCDD-max). Simple log-linear regression models were calculated for linear portions of the dose–response curves of TCDD and tested chemicals. Relative potencies (expressed as IEFs) were calculated using the equieffective approach [21]. Concentrations of the studied compounds inducing the 25 and 50% effect of the TCDD-max (C_{EQ-25} and C_{EQ-50}, respectively) were derived. The C_{EQ-50} values were compared with the 50% effective concentration of TCDD, and the IEFs of individual PAHs were derived (IEF = C_{EQ-50} for TCDD/C_{EQ-50} for PAH).

Quantitative structure–activity relationships

Structures of chemicals were built and optimized in the MOE software package (Ver 2003.2; Chemcomp, Montreal, PQ, Canada) and imported into TSAR (Ver 3.3; Accelrys, San Diego, CA, USA). Approximately 180 descriptors were calculated for each individual chemical (electronic and topological descriptors, parameters related to the molecular size and volume, and hydrophobicity descriptors). The bioassay results were expressed as log(1/$LOEC_{exp}$) and log(1/25% effective concentration [EC25]) for cytotoxicity and potencies to induce AhR, respectively. The relationships between the chemical descriptors and biological endpoints were analyzed with Statistica for Windows (Ver 6.0; StatSoft). Significant intercorrelations among the descriptors were first determined with principal component analysis, and the subsets of representative and easily interpretable parameters were selected for further analyses. The structure–activity relationships were, at first, described qualitatively, without any particular statistical evaluation (cytotoxic vs noncytotoxic and AhR-active vs nonactive compounds, respectively). Multivariate regression was used for quantitative modeling. Both forward-stepwise and backward-stepwise algorithms were applied to confirm the selection of significant descriptors. The multivariate correlation coefficient (r), the coefficient of multiple determination (R^2), and the Fisher's test (F value) were used as the quality criteria of calculated QSAR models. The models were validated with training sets there were randomly selected by both leave-one-out and leave-multiple-out techniques.

RESULTS

Viability of H4IIE-luc cells after the short-term, 6-h exposure was affected by only 2 of the 29 tested chemicals at the highest concentrations (200 μM of 1,10-phenanthroline and 7H-dibenzo[*c*,*g*]carbazole) (Table 1). Prolonged, 24-h exposures to most of the four- and five-ring aza-PAHs resulted in significant cytotoxicities ($LOEC_{exp}$ range, 7–30 μM) (Table 1). Low-molecular-weight compounds generally had weaker effects ($LOEC_{exp}$, ~100–200 μM), with the exception of 1,10-phenanthroline ($LOEC_{exp}$, 12.5 μM). In general, parent PAHs were less cytotoxic than their N-heterocyclic analogues, the $LOEC_{exp}$ values differed by more than one order of magnitude (compare, e.g., the 24-h $LOEC_{exp}$ for benz[*a*]anthracene [>200 μM] with those of the benzacridines [~8 μM]).

The potencies of 22 N-heterocyclic PAHs and seven homocyclic analogues to induce AhR-dependent luciferase in H4IIE-luc bioassay were investigated after 6 and 24 h. The dose–response curves for selected compounds that significantly induced reporter luciferase are shown in Figure 2. Effective concentrations and calculated IEFs are summarized in Table 1. In general, four- and five-ring PAHs were the most potent inducers of AhR in H4IIE-luc cells. The effects were more pronounced after shorter, 6-h exposure, followed by a decline after 24 h. Dibenz[*a*,*h*]acridine and dibenz[*a*,*i*]acridine had potencies comparable with that of reference TCDD after 6-h exposure (Fig. 2 and Table 1). The aza-PAHs generally were more toxic in comparison with the parent analogues, having IEFs up to three orders of magnitude higher (Table 1).

The evaluation of structure–activity relationships showed relatively poor correlation of cytotoxicity with hydrophobicity as represented by the octanol/water partition coefficient (log *P*) (Fig. 3). On the other hand, the combination of the molecule size (number of rings) with the molar refractivity (MR) qualitatively discriminated compounds that were cytotoxic from those with no effects up to 200 μM (when more than three rings and MR > 50 cm^3/mol $LOEC_{exp} \leq 200$ μM).

Interestingly, potencies to induce AhR in H4IIE-luc cells were significantly correlated with log *P* (Spearman's $r = 0.89$, $p < 0.001$, $n = 29$) (Fig. 3). Detailed selection of the chemical descriptors by stepwise multiple regression revealed that the potencies to induce AhR were best explained by ellipsoidal volume (EV) and/or the combinations of principal axes of inertia (molecular dimensions) with MR (Table 2). The QSAR models were validated with both the leave-one-out and leave-multiple-out algorithms. Each single compound was, first, sequentially removed from the training set, after which the model was recalculated and the log(1/EC25) of the eliminated individual was predicted. The leave-multiple-out validation was based on 10 repeated calculations, with five chemicals randomly removed in each step. Good stability of the calculated QSARs was confirmed, with the maximum relative deviations between the observed and predicted log(1/EC25) values being ±21%.

DISCUSSION

Polycyclic aromatic hydrocarbons and their derivatives are major pollutants in many areas worldwide, but our understanding of their toxic effects is still incomplete. For practical reasons, such as relatively high costs of standards and limited commercial availability, (eco)toxicological studies with aza-PAHs have focused mostly on lower-molecular-weight compounds [13–15]. Our study with 22 structurally diverse aza-PAHs and seven parent PAHs allowed comparative toxicological classification of these poorly characterized contaminants. In general agreement with the results of previous studies summarized by Bleeker et al. [7], our investigation confirmed significant toxicities of high-molecular-weight compounds. However, our results did not fully support the previously reported, simple linear correlations between the acute toxicity of aza-PAHs and the hydrophobicity of tested compounds [7,13,28]. Chemicals such as dibenz[*a*,*h*]acridine and dibenz[*a*,*i*]acridine,

Table 1. Cytotoxicity of the studied compounds and the potencies to induce aryl hydrocarbon receptor (AhR) in H4IIE-luc cell bioassay[a]

	$LOEC_{exp}$[b] (μM, 24 h)	C_{EQ-50}[c] (M)		IEF[d]	
		6 h	24 h	6 h	24 h
2,3,7,8-TCDD	>200	9.4×10^{-6}	3.5×10^{-6}	1.0	1.0
Indole	>200	NI[e]	NI	NA[f]	NA
2-Methylindole	>200	NI	NI	NA	NA
1-Methylindole	>200	NI	NI	NA	NA
Naphthalene		NI	NI	NA	NA
Quinoline	>200	NI	NI	NA	NA
Quinazoline	>200	NI	NI	NA	NA
Isoquinoline	>200	WI[g]	WI	NA	NA
Phthalazine	>200	WI	NI	NA	NA
6-Methylquinoline	>200	NI	NI	NA	NA
Fluorene	>200	NI	NI	NA	NA
Carbazole	200	NI	NI	NA	NA
Phenanthrene	100	90.0	NI	1.0×10^{-7}	NA
Phenanthridine	100	49.8	NI	1.9×10^{-7}	NA
Benzo[*h*]quinoline	200	WI	NI	NA	NA
4,7-Phenanthroline	200	WI	NI	NA	NA
1,10-Phenanthroline	12.5	NI	NI	NA	NA
1,7-Phenanthroline	200	NI	NI	NA	NA
Anthracene	>200	NI	NI	NA	NA
Acridine	>200	64.2	WI	1.5×10^{-7}	NA
Phenazine	>200	NI	NI	NA	NA
Benz[*a*]anthracene	>200	1.9×10^{-2}	2.5×10^{-1}	5.0×10^{-4}	1.4×10^{-5}
Benz[*a*]acridine	8.0	4.9×10^{-3}	2.5	1.9×10^{-3}	1.4×10^{-6}
Benz[*c*]acridine	8.0	2.0×10^{-1}	1.9	4.7×10^{-5}	1.8×10^{-6}
Dibenz[*a,h*]anthracene	>200	7.0×10^{-4}	1.9×10^{-3}	1.3×10^{-2}	1.9×10^{-3}
Dibenz[*a,h*]acridine	30.0	7.4×10^{-7}	3.2×10^{-3}	13	1.1×10^{-3}
Dibenz[*a,j*]acridine	7.0	8.8×10^{-3}	1.1×10^{-1}	1.1×10^{-3}	3.4×10^{-5}
Dibenz[*c,h*]acridine	20.0	4.0×10^{-3}	1.1×10^{-2}	1.3×10^{-3}	3.2×10^{-4}
Dibenz[*a,i*]acridine	30.0	7.5×10^{-6}	4.1×10^{-3}	1.3	8.6×10^{-4}
Dibenzo[*c,g*]carbazole	7.0	1.4	3.1×10^{-1}	6.7×10^{-6}	1.1×10^{-5}

[a] Parent unsubstituted polycyclic aromatic hydrocarbons are in italics.
[b] $LOEC_{exp}$ = lowest-observed-experimental concentrations (μmol/L) that significantly inhibited cell viability after 24 h of exposure.
[c] C_{EQ-50} = concentrations (mol/L) inducing AhR-dependent luciferase at levels equivalent to the 50% effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) after 6- and 24-h exposure periods.
[d] IEF = induction equivalency factors. Calculated as a ratio of C_{EQ-50} values of the TCDD and individual tested compounds.
[e] NI = no significant induction of AhR-dependent luciferase.
[f] NA = not available.
[g] WI = weak induction (< 50% of TCDD maximum).

which were not included in previously reported studies, had relatively lower cytotoxicities than those predicted from the log *P* (Fig. 3). On the other hand, 1,10-phenanthroline was significantly more cytotoxic than that predicted from log *P* (Fig. 3). Different effects of the outliers might be explained by simultaneous manifestation of multiple cellular mechanisms induced by these chemicals. It generally is accepted that log *P*, a parameter of hydrophobicity, correlates with basal narcotic toxicity of organic chemicals (i.e., nonspecific accumulation of the compounds into the cell membranes). However, we also observed significant activations of AhR by dibenzacridines. Consequently, cellular events following the activation of AhR, such as inductions of detoxification enzymes and increased cellular proliferation [29], may compensate the direct acute cytotoxic effects (i.e., cell death resulting from the nonspecific membrane damage). Alternatively, the greater toxicity of 1,10-phenanthroline can be explained by known ion-chelating properties of this chemical [30] that might potentiate the acute cytotoxic effects. In general, our results indicate that the acute cytotoxicity of aza-PAHs is better characterized by parameters related to the density of molecules (MR, >50 cm^3/mol [31]) and the molecular size (greater than three rings).

We also observed highly significant inductions of AhR-dependent luciferase in H4IIE-luc cells exposed to aza-PAHs. To the best of our knowledge, the present study provides new information regarding the effects of several compounds, such as dibenz[*a,i*]acridine, dibenz[*c,h*]acridine, and 7H-dibenzo[*c,g*]carbazole (Table 1). Significant activations of AhR by these compounds correspond to the effects of structurally related dibenz[*a,h*]acridine and dibenz[*a,j*]acridine that also have been observed previously [4,5,23]. The effects of reference TCDD increased with the exposure time (Fig. 2), but PAHs and their derivatives were more active after shorter, 6-h exposures, with decline after 24 h. These differences can be attributed to a greater susceptibility of PAHs to cellular biotransformation, as suggested by Machala et al. [4]. The aza-PAHs were more potent inducers of AhR-dependent luciferase than the parent compounds with IEFs by up to 1,000-fold (compare, e.g., dibenz[*a,h*]acridine [IEF_{6h}, >10] and dibenz[*a,h*]anthracene [IEF_{6h}, ~0.01]) (Table 1). Similarly, whereas anthracene did not modulate AhR, the N-heterocyclic analogue, acridine, showed significant inductions after 6 h of exposure. In agreement with the results of previous studies [4,5,23], we observed a high potency of dibenz[*a,h*]acridine to activate AhR that was comparable or even greater than that of TCDD after short periods. However, the relevance of the in vitro results should be explored by further in vivo toxicity studies.

Study of a wider set of individual chemicals allowed detailed investigation of the structure–toxicity relationships. Sig-

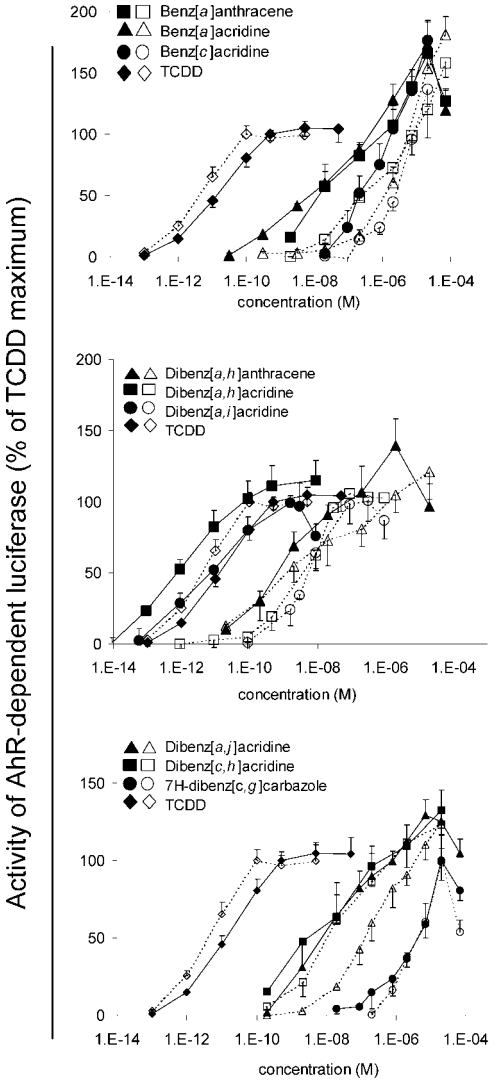

Fig. 2. Concentration–induction curves (6 h, full symbols; 24 h, open symbols) of hydrocarbon receptor (AhR)–dependent luciferase in H4IIE-luc cells. (**A**). Benzanthracene and its derivatives. (**B**) and (**C**). Effects of five-ring N-heterocyclic derivatives of polycyclic aromatic hydrocarbons (PAHs). Data points represent the mean ± standard deviation of three replicates. The effects of PAHs are compared with the reference 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).

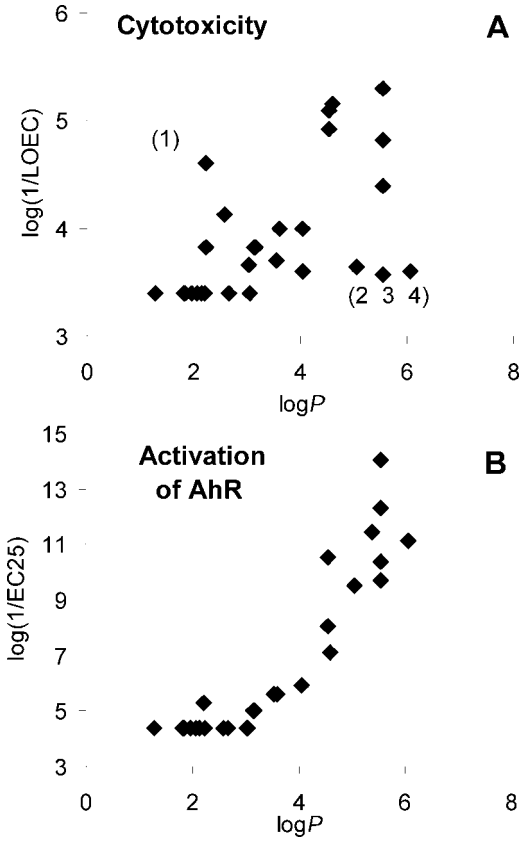

Fig. 3. Relationships between the hydrophobicity (log *P*) and the 24-h cytotoxicity (**A**) the lowest-observed concentration that significantly affected cell viability [LOEC]) and 6-h potencies to activate aryl hydrocarbon receptor (AhR) in H4IIE-luc cells (**B**) concentrations that induced 25% effects [EC25] of the reference 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD]). Selected individual compounds are labeled with numbers: 1 = 1,10-phenanthroline; 2 = dibenz[*a*,*h*]anthracene; 3 = dibenz[*a*,*i*]acridine; and 4 = dibenz[*a*,*h*]acridine.

nificant correlation between the activation of AhR and log *P* was observed (Fig. 3), thus confirming known potencies of hydrophobic toxicants to induce cellular defense mechanisms, including those mediated by AhR [32]. For those compounds that activated AhR, we performed stepwise selection of significant descriptors, and a single parameter (EV) best explained the potency to activate AhR (Table 2). Although EV is not often discussed in toxicological QSARs, a recent study [33] found correlations between EV and the protein-binding ca-

Table 2. Quantitative structure–activity relationships (QSARs) for activation of aryl hydrocarbon receptor (AhR) in H4IIE-luc cell bioassay[a]

QSAR model	*n*	*r*	R^2	*F*
All compounds that activated AhR				
Log(1/EC25) = 0.011·EV + 1.544	14	0.95	0.89	119
Subset of aza-PAHs that activated AhR				
Log(1/EC25) = 0.012·EV + 1.425	11	0.94	0.87	67
Log(1/EC25) = 0.34·length + 0.091 MR − 3.95	11	0.94	0.87	49
Log(1/EC25) = 1.14·length − 2.12·(l/w) + 2.82	11	0.95	0.88	54

[a] *n* = Number of chemicals in data set; *r* = multivariate correlation coefficient; R^2 = coefficient of multiple determination; *F* = Fisher's test, (variance ratio); EV = ellipsoidal volume; length = first principal axis of inertia; MR = molar refractivity; l/w = ratio of the length and width (i.e., ratio of the first and the second principal axes of inertia); aza-PAHs = N-heterocyclic derivatives of polycyclic aromatic hydrocarbons; EC25 = concentration that induced 25% of the maximum effect in H4IIE-luc cell bioassay.

pacity of low-molecular-weight compounds (unsaturated fatty acids). Other models for activation of AhR (Table 2) correspond to those previously published and summarized by Lewis et al. [34]. Significant positive correlations between the inductions of AhR and the length and planarity (area/depth-squared, a/d^2) as well as hydrophobicity (log *P*) were observed previously for data sets including PCBs and PAHs [34]. We observed correlations with MR (related to the density and the volume of the molecule) in combinations with molecular length and planarity (the first and second axes of inertia and their ratios; see the second and third equations in Table 2). The importance of molecular size for the activation of AhR by PAHs also was reported previously [4,21]. The derived descriptors explain well the basic steps of AhR activation, such as transport across the membrane (affected by the hydrophobicity) and binding to AhR (described by EV and/or size and density descriptors).

Numerous parent PAHs are routinely analyzed in environmental matrices, but information regarding the occurrence of aza-PAHs is rare, corresponding to the lack of appropriate analytical methods. Some aza-PAHs, such as benz[*c*]acridine, benz[*a*]acridine, quinoline, isoquinoline, carbazole, dibenz[*a,h*]acridine, dibenz[*a,j*]acridine, and dibenz[*c,h*]acridine, were identified in the air particulate matter or sediments at concentrations of 1 to 10% those of the parent PAHs [3,9,11]. Relatively lower concentrations can, however, be counterbalanced by properties of aza-PAHs, such as higher water solubility and bioavailability [12], lower biodegradability with a tendency to bioconcentrate [6], as well as higher toxicities in comparison with those of the parent PAHs [13,16,17].

To assess contaminated matrices, the toxic equivalency factor approach is well established for halogenated persistent contaminants [20,27], and methodologies based on relative potencies also have been proposed for dominant PAHs [35,36]. In the following paragraph, we demonstrate the potential use of IEFs derived in the present study (Table 1) for the evaluation of complex contaminated samples. The IEFs were compared with sediment concentrations of aza-PAHs published previously by Kozin et al. [9]: benz[*a*]acridine, 45 ng/g dry weight; benz[*c*]acridine, 95 ng/g dry weight; dibenz[*a,h*]acridine, 17.7 ng/g dry weight; dibenz[*a,j*]acridine, 3.7 ng/g dry weight; and dibenz[*c,h*]acridine, 7.6 ng/g dry weight. The final TCDD equivalent (226 ng/g dry wt) reflects the toxic contribution of five individual aza-PAHs, but the value corresponds to the total sediment TCDD equivalents published previously for PAH-contaminated samples. For example, Vondracek et al. [35] reported 24-h bioassay TCDD equivalents for nine sediments ranging from 5.9 to 48 ng/g dry weight. In a study of Hilscherova et al. [27], the bioassay TCDD equivalents for eight sediments ranged from 0.7 to 23 ng/g dry weight. A contribution of dibenz[*a,h*]acridine to the TCDD equivalents in river sediments also was published by Machala et al. [4]. Polycyclic aromatic hydrocarbons and their derivatives are dominant contaminants in urban areas in concentrations that highly exceed those of persistent chlorinated compounds. Although direct ''dioxin-like'' in vivo effects of PAHs remain unclear, PAHs and their derivatives certainly modulate AhR and induce CYP450 enzymes. Consequently, chronic exposures to PAHs and their derivatives might lead to increased formation of CYP450-mediated reactive metabolites or enhanced susceptibility to other contaminants that require metabolic activation [7].

In conclusion, the present study revealed significant in vitro toxicities of N-heterocyclic derivatives of PAHs. High potencies to induce AhR in vitro were observed, particularly for dibenzacridines. The principal QSAR descriptors correlated with the potencies to activate AhR were EV, MR, and molecular size. Individual IEFs for aza-PAHs are derived, and their use in evaluation of complex environmental samples is suggested.

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REFERENCES

1. Durant JL, Busby J, William F, Lafleur AL, Penman BW, Crespi CL. 1996. Human cell mutagenicity of oxygenated, nitrated and unsubstituted polycyclic aromatic hydrocarbons associated with urban aerosols. *Mutation Research—Genetic Toxicology* 371: 123–157.
2. Machala M, Vondracek J, Blaha L, Ciganek M, Neca J. 2001. Aryl hydrocarbon receptor–mediated activity of mutagenic polycyclic aromatic hydrocarbons determined using in vitro reporter gene assay. *Mutation Research—Genetic Toxicology* 497:49–62.
3. Durant JL, Lafleur AL, Plummer EF, Taghizadeh K, Busby WF, Thilly WG. 1998. Human lymphoblast mutagens in urban airborne particles. *Environ Sci Technol* 32:1894–1906.
4. Machala M, Ciganek M, Blaha L, Minksova K, Vondracek J. 2001. Aryl hydrocarbon receptor–mediated and estrogenic activities of oxygenated polycyclic aromatic hydrocarbons and azaarenes originally identified in extracts of river sediments. *Environ Toxicol Chem* 20:2736–2743.
5. Fent K, Jung DKJ. 2000. Nitrated polycyclic aromatic hydrocarbons and aza-arenes induce cytochrome P4501A in a fish hepatoma cell line. *Mar Environ Res* 50:545–552.
6. Yamauchi T, Handa T. 1987. Characterization of aza heterocyclic hydrocarbons in urban atmospheric particulate matter. *Environ Sci Technol* 21:1177–1181.
7. Bleeker EAJ, Wiegman S, de Voogt P, Kraak M, Leslie HA, de Haas E, Admiraal W. 2002. Toxicity of aza-arenes. *Rev Environ Contam Toxicol* 173:39–83.
8. Chen H-Y, Preston MR. 2004. Measurement of semivolatile azaarenes in airborne particulate and vapor phase. *Anal Chim Acta* 501:71–78.
9. Kozin IS, Larsen OFA, de Voogt P, Gooijer C, Velthorst NH. Isomer-specific detection of aza-arenes in environmental samples by Shpol'skii luminescence spectroscopy. *Anal Chim Acta* 354: 181–187.
10. Brooks LR, Hughes TJ, Claxton LD, Austern B, Brenner R, Kremer F. 1998. Bioassay-directed fractionation and chemical identification of mutagens in bioremediated soils. *Environ Health Perspect* 106:1435–1440.
11. Benestad C, Jebens A, Tveten G. 1987. Emission of organic micropollutants from waste incineration. *Chemosphere* 16:813–820.
12. Pearlman RS, Yalkowsky SH, Banerjee S. 1984. Water solubilities of polynuclear aromatic and heteroaromatic compounds. *Journal of Physico-Chemical Reference Data* 13:555–562.
13. Bleeker EAJ, Van der Geest HG, Klamer HJC, De Voogt P, Wind E, Kraak MHS. 1999. Toxic and genotoxic effects of aza-arenes: Isomers and metabolites. *Polycyclic Aromatic Compounds* 13: 191–203.
14. Kraak MHS, Wijnands P, Govers HAJ, Admiraal W, de Voogt P. 1997. Structural-based differences in ecotoxicity of benzoquinoline isomers to the zebra mussel (*Dreissena polymorpha*). *Environ Toxicol Chem* 16:2158–2163.
15. Van Vlaardingen PLA, Steinhoff WJ, de Voogt P, Admiraal WA. 1996. Property–toxicity relationships of aza-arenes to the green alga *Scenedesmus acuminatus*. *Environ Toxicol Chem* 15:2035–2042.
16. Yamada K, Suzuki T, Kohara A, Hayashi M, Mizutani T, Saeki K. 2004. In vivo mutagenicity of benzo[*f*]quinoline, benzo[*h*]quinoline, and 1,7-phenanthroline using the lacZ transgenic mice. *Mutat Res* 559:83–95.
17. Gabelova A, Farkasova T, Bacova G, Robichova S. 2002. Mutagenicity of 7H-dibenzo[*c,g*]carbazole and its tissue-specific de-

rivatives in genetically engineered Chinese hamster V79 cell lines stably expressing cytochrome P450. *Mutat Res* 517:135–145.
18. Fertuck KC, Kumar S, Sikka HC, Matthews JB, Zacharewski TR. 2001. Interaction of PAH-related compounds with the alpha and beta isoforms of the estrogen receptor. *Toxicol Lett* 121:167–177.
19. Till M, Riebniger D, Schmitz H-J, Schrenk D. 1999. Potency of various polycyclic aromatic hydrocarbons as inducers of CYP1A1 in rat hepatocyte cultures. *Chem-Biol Interact* 117:135–150.
20. Van den Berg M, Birnbaum L, Bosveld AT, Brunstrom B, Cook P, Feeley M, Giesy JP, Hanberg A, Hasegawa R, Kennedy SW, Kubiak T, Larsen JC, van Leeuwen FX, Liem AK, Nolt C, Peterson RE, Poellinger L, Safe S, Schrenk D, Tillitt D, Tysklind M, Younes M, Waern F, Zacharewski T. 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environ Health Perspect* 106:775–792.
21. Villeneuve DL, Khim JS, Kannan K, Giesy JP. 2002. Relative potencies of individual polycyclic aromatic hydrocarbons to induce dioxin-like and estrogenic responses in three cell lines. *Environ Toxicol* 17:128–137.
22. Ayrton AD, Trinick J, Wood BP, Smith JN, Ioannides C. 1988. Induction of the rat hepatic microsomal mixed-function oxidases by two aza-arenes. A comparison with their nonheterocyclic analogues. *Biochem Pharmacol* 37:4565–4571.
23. Jung DKJ, Klaus T, Fent K. 2001. Cytochrome P450 induction by nitrated polycyclic aromatic hydrocarbons, aza-arenes, and binary mixtures in fish hepatoma cell line PLHC-1. *Environ Toxicol Chem* 20:149–159.
24. Saeki K, Matsuda T, Kato T, Yamada K, Mizutani T, Matsui S, Fukuhara K, Miyata N. 2003. Activation of the human Ah receptor by aza-polycyclic aromatic hydrocarbons and their halogenated derivatives. *Biological & Pharmaceutical Bulletin* 26:448–452.
25. Sanderson JT, Aarts J, Brouwer A, Froese KL, Denison MS, Giesy JP. 1996. Comparison of Ah receptor-mediated luciferase and ethoxyresorufin-*O*-deethylase induction in H4IIE cells: Implications for their use as bioanalytical tools for the detection of polyhalogenated aromatic hydrocarbons. *Toxicol Appl Pharmacol* 137:316–325.
26. Babich H, Borenfreund E. 1990. Applications of the neutral red cytotoxicity assay to in vitro toxicology. *ATLA* 18:129–144.
27. Hilscherova K, Machala M, Kannan K, Blankenship AL, Giesy JP. 2000. Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. *Environ Sci Pollut Res Int* 7:159–171.
28. Konemann H. 1981. Quantitative structure–activity relationships in fish toxicity studies. Part 1: Relationship for 50 industrial pollutants. *Toxicology* 19:209–221.
29. Puga A, Xia Y, Elferink C. 2002. Role of the aryl hydrocarbon receptor in cell-cycle regulation. *Chem-Biol Interact* 141:117–130.
30. Zhu BZ, Chevion M. 2000. Mechanism of the synergistic cytotoxicity between pentachlorophenol and copper-1,10-phenanthroline complex: The formation of a lipophilic ternary complex. *Chem-Biol Interact* 129:249–261.
31. Padron JA, Carrasco R, Pellon RF. 2002. Molecular descriptor based on a Molar Refractivity partition using Randic-type graph-theoretical invariant. *J Pharm Sci* 5:267–274.
32. Safe S, Fujita T, Romkes M, Piskorska-Pliszczynska J, Homonko K, Denomme MA. 1986. Properties of the 2,3,7,8-TCDD receptor—A QSAR approach. *Chemosphere* 15:1657–1663.
33. Dobes P, Kmunicek J, Mikes V, Damborsky J. 2004. Binding of fatty acids to beta-cryptogein: Quantitative structure–activity relationships and design of selective protein mutants. *J Chem Inf Comput Sci* 44:2126–2132.
34. Lewis DFV, Jacobs MN, Dickins M, Lake BG. 2002. Quantitative structure–activity relationships for inducers of cytochromes P450 and nuclear receptor ligands involved in P450 regulation within the CYP1, CYP2, CYP3, and CYP4 families. *Toxicology* 176: 51–57.
35. Vondracek J, Machala M, Minksova K, Blaha L, Murk AJ, Kozubik A, Hofmanova J, Hilscherova K, Ulrich R, Ciganek M, Neca J, Svrckova D, Holoubek I. 2001. Monitoring river sediments contaminated predominantly with polyaromatic hydrocarbons by chemical and in vitro bioassay techniques. *Environ Toxicol Chem* 20:1499–1506.
36. Brack W, Schirmer K, Teneva I, Asparuhova D, Dzhambazov B, Mladenov R, Kind T, Schrader S, Schuurmann G, Segner H, Behrens A, Joyce EM, Bols NC. 2003. Effect-directed identification of oxygen and sulfur heterocycles as major polycyclic aromatic cytochrome P4501A-inducers in a contaminated sediment. *Environ Sci Technol* 37:3062–3070.

Článek VI:

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Interference of PAHs and their N-heterocyclic analogs with signaling of retinoids *in vitro*

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ABSTRACT

Retinoids are dietary hormones acting through nuclear receptors for retinoic acid, important especially during embryonic development. This study focuses on the disruption of signaling pathways of retinoids by polycyclic aromatic hydrocarbons (PAHs) and their N-heterocyclic analogs (N-PAHs), important environmental contaminants with numerous biological effects. *In vitro* test with P19/A15 cell line stably transfected with luciferase reporter gene under control of retinoic acid-responsive elements was used to investigate both direct activation of retinoic acid receptors and modulation of response induced by natural ligand all-*trans* retinoic acid (ATRA) by 26 PAHs and N-PAHs. While none of individual compounds alone activated retinoic acid receptors, many of them modulated ATRA-mediated activity both after 6 h and 24 h exposure. Majority of compounds active after 6 h downregulated ATRA-mediated activity (most effective were two analogs of dibenz[a,h]anthracene with LOECs about 185 nM), while most compounds active after 24 h upregulated the effects of ATRA (most effective benz[a]acridine and dibenz[a,i]acridine caused 400% induction of ATRA response). Quantitative structure-activity relationship analysis identified molecular volume and dipole moment as the most important descriptors of inhibitory effects after 6 h, while length, total molecular energy, gap-HOMO/LUMO and Van der Waals energy are important descriptors for stimulatory effects of PAHs and N-PAHs. This study demonstrates those abundant pollutants such as PAHs and their analogs interfere *in vitro* with retinoid signaling, which could play role in some *in vivo* effects of these organic contaminants such as teratogenicity.

1. Introduction

Retinoids, important non-steroidal dietary hormones, play an essential role in regulation of embryonic development and homeostasis of all vertebrate tissues through their effects on cell differentiation, proliferation and apoptosis (Zile, 2002).

The retinoid signal is transduced by two families of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), that work as RXR/RAR heterodimers or RXR/RXR homodimers (Bastien and Rochette-Egly, 2004). Retinoid receptors are ligand-dependent transcriptional regulators, repressing transcription in the absence of ligand and activating transcription in its presence. The different effects on transcription are carried out through recruitment of co-regulators: free receptors bind corepressors (NCoR and SMRT) that are found within a complex with histone deacetylase (HDAC) activity, whereas receptors with ligands recruit coactivators with histone acetylase activity (HATs) (Zile, 2002).

Natural ligands for RARs are all-*trans* retinoic acid (ATRA) and its 9-*cis* isomer, while RXRs are activated only by 9-*cis* retinoic acid (Bastien and Rochette-Egly, 2004). In addition to the RARs and RXRs, two types of cellular retinol (CRBP-I and -II) or retinoic

Abbreviations: 1,7-Pht, 1,7-phenanthroline; 1,10-Pht, 1,10-phenanthroline; 2-MeIn, 2-methylindole; 4,7-Pht, 4,7-phenanthroline; 6MeQ, 6-methylquinoline; Acr, acridine; AhR, aryl hydrocarbon receptor; Ant, anthracene; ATRA, all-*trans* retinoic acid; B[a]Acr, benz[a]acridine; B[a]A, benz[a]anthracene; B[c]Acr, benz[c]acridine; B[h]Q, benzo[h]quinoline; B[a]P, benzo[a]pyrene; CRABP, cellular retinoic acid binding protein; CRBP, cellular retinol binding protein; CYP450, cytochrome P450; DB[a,h]A, dibenz[a,h]anthracene; DB[a,h]Acr, dibenz[a,h]acridine; DB[a,j]Acr, dibenz[a,j]acridine; DB[a,i]Acr, dibenz[a,i]acridine; DB[c,h]Acr, dibenz[c,h]acridine; DB[c,g]C, 7-H-dibenzo[c,g]carbazole; DMEM, dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxid; EC50, compound concentration causing 50% of the maximum effect; FETAX, frog embryo teratogenesis assay-*Xenopus*; HAT, histone acetylase activity; HDAC, histone deacetylase activity; In, indole; IsQ, isoquinoline; In1Lng, molecular length; LOEC, experimental lowest observed effect concentration; MOEC, experimental maximal observed effect concentration; N-PAHs, N-heterocyclic polycyclic aromatic hydrocarbons; Nap, naphthalene; PAHs, polycyclic aromatic hydrocarbons; PCA, principle component analysis; PCBs, polychlorinated biphenyls; PCDDs/Fs, polychlorinated dibenzodioxins/furans; Phe, phenanthrene; Phd, phenanthridine; Phez, phenazine; POPs, persistent organic pollutants; QSAR, quantitative structure-activity relationship; Quin, quinoline; Quiz, quinazoline; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid responsive element; RXR, retinoid X receptor; SAR, structure-activity relationship; TOTEN, total energy of molecule.

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acid-binding proteins (CRABP-I and -II) are involved in the physiological activities of retinoids (Sonneveld et al., 1999). The complexity of the RAR/RXR system regulation makes the signal pathway vulnerable to disruption mediated by various xenobiotics that could lead to numerous adverse effects, especially during embryonic development (Degitz et al., 2003).

Among compounds able to disrupt retinoid signaling belong some pesticides (Lemaire et al., 2005), plasticizers (Bhattacharya et al., 2005), polychlorinated biphenyls (PCBs) (Mos et al., 2007) or polychlorinated dibenzodioxins and furans (PCDD/Fs) (Lorick et al., 1998). Our previous study also indicated interference of a few polycyclic aromatic hydrocarbons (PAHs) with retinoid signaling (Novak et al., 2007). Relationship between the exposure to some persistent organic pollutants (POPs) and changes of retinoic acid levels in the organism has been known for some time. Toxic action of many POPs is known to be related to their interaction with Aryl hydrocarbon receptor (AhR). It was shown that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent agonist of AhR, significantly suppresses all-*trans* retinoic acid (ATRA) action in diverse cell types (Lorick et al., 1998). Decreased retinoid levels have been observed in rat neonates exposed to PCB mixtures during embryonic development (Zile, 2002). Previous research of environmental contaminants focused mainly on few prototypal polyhalogenated hydrocarbons such as PCDD/Fs and PCBs, while effects of other contaminants (such as PAHs) on retinoid signaling are only poorly characterized (Janosek et al., 2006). This study thus focused on the interference of highly abundant environmental pollutants PAHs and some of their analogs with retinoid signaling *in vitro*.

PAHs and their derivatives and analogs are important environmental contaminants in many industrial and urban regions worldwide generated especially by the incomplete combustion of organic materials (Feng et al., 2007). They enter the environment from natural sources such as forest fires and seeps in ocean floors and also through anthropogenic activities, including combustion of fossil fuels and wood and petroleum products.

"PAH derivatives" include PAHs having an alkyl or other chemical group attached to a conjugated ring structure. "Heterocyclic aromatic compounds" include PAHs having one or more carbon atoms in a ring replaced by a nitrogen, oxygen, or sulfur atom (Xu et al., 2006). While homocyclic polyaromatics have been of a major concern since 1970s, heterocyclic PAHs have been studied only in recent years due to relatively lower concentrations in the environment (Machala et al., 2001; Sovadinova et al., 2006). Important group of PAH heterocycles are N-heterocyclic PAHs (N-PAHs). They are (similarly to PAHs) ubiquitous pollutants and they have been detected in air, soil, marine environment, and freshwater lake sediments (Chen and Preston, 2004; Jung et al., 2001). N-PAHs are more soluble in water than their homocyclic analogs, and consequently perhaps also more bioavailable (Bleeker et al., 2001).

Although information about biological effects of N-PAHs is limited, some studies demonstrated effects similar to their non-heterocyclic PAHs analogs including AhR-dependent inductions of cytochromes P 450 (CYP450s), carcinogenic and mutagenic potential (Arcaro et al., 1999; Jung et al., 2001; Sovadinova et al., 2006). Some results also indicate that N-PAHs have higher toxic and genotoxic potencies than their homocyclic analogs (Bartos et al., 2006; Sovadinova et al., 2006).

A growing body of literature also identifies PAHs as potential environmental endocrine disruptors. PAHs have been reported to possess both estrogenic and antiestrogenic properties in various experimental settings (Vondracek et al., 2002) and some PAHs also act as antiandrogens *in vitro* (Vinggaard et al., 2000). Interactions of PAHs with other hormonal signaling pathways, such as retinoid or thyroid, are poorly characterized. Recent study found that retinoid stores were depleted after benzo[a]pyrene exposure in female zebrafish adults (Alsop et al., 2007). Other *in vivo* studies with fish larvae (Wassenberg and Di Giulio, 2004) or amphibian embryos (Buryskova et al., 2006) found that several PAHs (including benzo[a]pyrene) and N-PAHs may cause malformations or embryotoxicity. With regard to the importance of retinoids and their receptors for embryonic development (Sucov et al., 1995), these observations raise the question of the potential interference of these compounds with retinoid signaling.

This study addresses potential of selected PAHs and N-PAHs to activate RAR/RXR signaling pathway or modulate ATRA-mediated response, which could possibly lead to disturbed embryonic development and other adverse effects (Degitz et al., 2003). The applied *in vitro* approach enables to directly characterize the potential interaction of chemicals with this crucial target within retinoid signaling. Additionally, quantitative structure-activity relationships were studied to determine key structural and physical–chemical features of the active compounds responsible for observed effects.

2. Materials and methods

2.1. Chemicals

Naphthalene (Nap) (CAS No. 91-20-3), quinoline (Quin) (CAS No. 91-22-5), benzo[h]quinoline (B[h]Q) (CAS No. 230-27-3), acridine (Acr) (CAS No. 260-94-6), quinazoline (Quiz) (CAS No. 253-82-7), 6-methylquinoline (6MeQ) (CAS No. 91-62-3), isoquinoline (IsQ) (CAS No. 119-65-3), phenanthridine (Phd) (CAS No. 229-87-8), 4,7-phenanthroline (4,7-Pht) (CAS No. 230-07-9), 1,10-phenanthroline (1,10-Pht) (CAS No. 66-71-7), indole (In) (CAS No. 120-72-9), 2-methylindole (2-MeIn) (CAS No. 95-20-5), 1,7-phenanthroline (1,7-Pht) (CAS No. 230-46-6), phenazine (Phez) (CAS No. 92-82-0), anthracene (Ant) (CAS No. 120-12-7), benz[a]anthracene (B[a]A) (CAS No. 56-55-3), dibenz[a,h]anthracene (DB[a,h]A) (CAS No. 53-70-3), phenanthrene (Phe) (CAS No. 85-01-8), benzo[a]pyrene (B[a]P) (CAS No. 50-32-8), and all-*trans* retinoic acid (ATRA) (CAS No. 302-79-4) were purchased from Sigma–Aldrich (Prague, CR). Benz[a]acridine (B[a]Acr) (CAS No. 225-11-6), benz[c]acridine (B[c]Acr) (CAS No. 225-51-4), dibenz[a,i]acridine (DB[a,i]Acr) (CAS No. 226-92-6), dibenz[a,j]acridine (DB[a,j]Acr) (CAS No. 224-42-0), dibenz[a,h]acridine (DB[a,h]Acr) (CAS No. 226-36-8), 7-H-dibenzo-[c,g]carbazole (DB[c,g]C) (CAS No. 194-59-2) and dibenz[c,h]acridine (DB[c,h]Acr) (CAS No. 224-53-3) were obtained from Dr. Ehrenstorfer, GmbH (Augsburg, Germany). TCDD (CAS No. 1746-01-6) was from Ultra Scientific (North Kingstown, USA). The purity of all compounds was 97% or higher. Structures of tested PAHs and N-PAHs are shown in Fig. 1.

2.2. Cell culture

For this study, murine embryonic carcinoma cell line P19 (European Collection of Cell Culture, Wiltshire, UK) transfected with luciferase reporter pRAREβ2-TK-luc plasmid (P19/A15 clone) was used (Novak et al., 2007). The plasmid contains reporter luciferase gene under the control of retinoic acid-responsive element. P19/A15 cells were cultured in tissue culture flasks (TPP, Austria) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum Mycoplex (PAA, Austria) at 37 °C in a humidified atmosphere of 5% CO_2.

Cells were split every third day to maintain cells in the undifferentiated state.

2.3. Cytotoxicity testing

Cytotoxicity of tested chemicals was measured by neutral red uptake assay as described by Freyberger and Schmuck (2005).

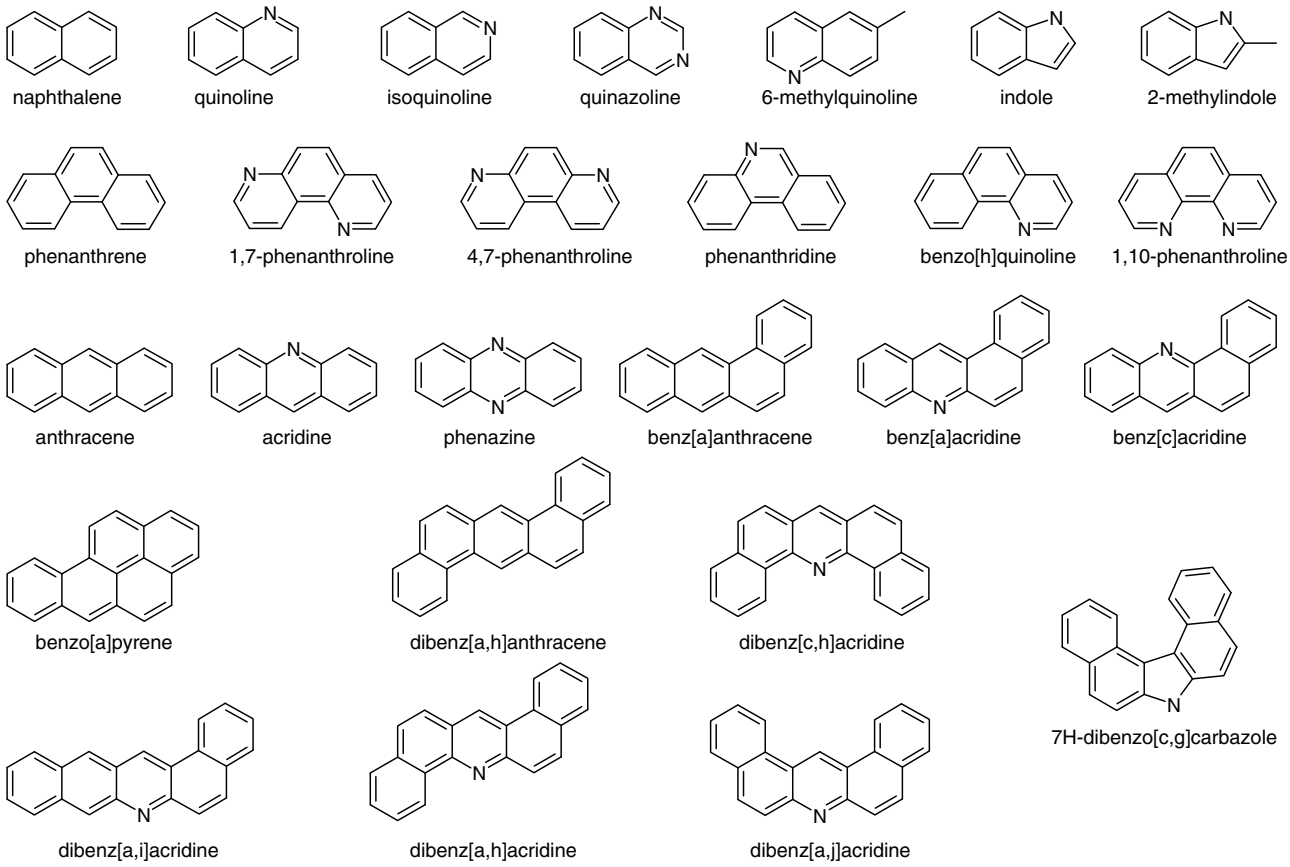

Fig. 1. Structures of studied polycyclic aromatic hydrocarbons (PAHs) and their N-heterocyclic analogs (N-PAHs).

Briefly, neutral red (0.5 mg/ml of medium) was added to each well and the microplate was incubated at 37 °C for 1 h. Medium was removed and cells were lysed with 1% acetic acid in 50% ethanol and absorbance at 570 nm was measured (only viable cells accumulated neutral red). Non-cytotoxic concentrations were used for further experiments.

2.4. Luciferase assay

For the RAR/RXR transactivation assay, 10 000 cells per well were seeded into 96-well microplates and incubated overnight. Then, the cells were exposed in three replicates to tested chemicals diluted in dimethyl sulphoxide alone, or simultaneously with endogenous ligand of retinoid receptor, all-*trans* retinoic acid (ATRA). Final concentration of the solvent did not exceed 1% v/v and it had no effect on the cell viability or RAR/RXR-dependent activity. The cells were exposed to various concentrations of model toxicant (TCDD), 6 parental PAHs and 20 N-heterocyclic PAHs either alone, or in co-exposure with ATRA. The activity of reporter luciferase induced in the presence of RAR/RXR ligands was measured after 6 or 24 h exposure using Promega Steady Glo Kit (Promega, Madison, WI, USA) and microplate luminometer Luminoskan Ascent (Thermo Electron Corp., USA).

For each compound at least two independent experiments were performed. Concentration range depended on cytotoxicity and compound solubility and varied between 12 nM and 200 μM. The tested concentrations of model toxicant TCDD ranged from 5 pM up to the highest non-cytotoxic concentration 5 nM. Based on the dose–response curves of model ATRA (Fig. 2), 32 nM was selected for co-exposure experiments of ATRA and tested compounds (concentration close to EC_{50} in both exposure times).

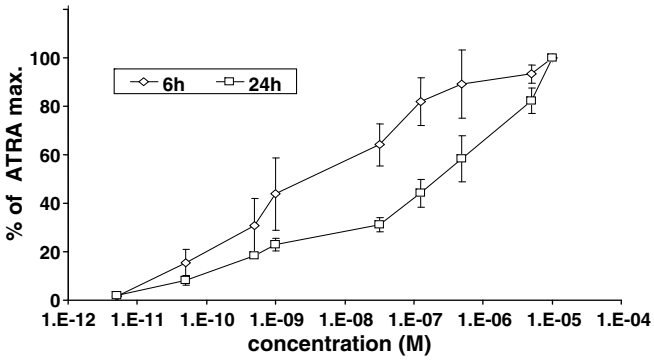

Fig. 2. Dose-response curve of luciferase activity after treatment with different all-*trans* retinoic acid (ATRA) concentrations in P19/A15 cells at two exposure times. Data are expressed as mean ± standard deviation (SD) of three independent experiments. Diamond – 6 h exposure. Square – 24 h exposure.

2.5. Statistical analyzes

All calculations and statistical analyzes were performed with Microsoft Excel and Statistica for Windows (Ver. 7.0, StatSoft, Tulsa, OK, USA). To determine significant difference from vehicle control, statistical analyzes were performed using one-way ANOVA followed by Dunnet's test. The lowest concentration that significantly ($p < 0.05$) modulated ATRA-mediated activity (experimental lowest observed effect concentration [LOEC]) and the concentration that caused maximal change of ATRA activity (experimental maximal observed effect concentration [MOEC]) were derived.

2.6. QSAR

Structures of chemicals were built and optimized in the MOE software package (Ver. 2003.2; Chemcomp, Montreal, PQ, Canada) and imported into TSAR (Ver. 3.3; Accelrys, San Diego, CA, USA). Approximately 180 descriptors were calculated for each compound (electronic and topological descriptors, parameters of the molecular size and volume, hydrophobicity descriptors). The bioassay results were expressed as log(1/LOEC) for potencies to modulate retinoic acid mediated activity. High concentration (1 mM) has been arbitrary set to non-active compounds to eliminate zero values from the calculations. The relationships between the chemical descriptors and biological endpoints were analyzed with Statistica for Windows (Ver. 7.0, StatSoft, Tulsa, OK, USA). Intercorrelations among the descriptors were first studied with principal component analysis (PCA) and Pearson correlation, and a subset of representative and easy to interpret parameters was selected for further analyzes. The structure-activity relationships (SARs) were, at first, described qualitatively (active versus non-active compounds for both exposure periods). Multiple regression analysis was than used for quantitative modeling of relationships between descriptors and biological activities. Both forward-stepwise and backward-stepwise algorithms were applied to add/remove parameters and to confirm the selection of significant descriptors. The multivariate correlation coefficient (r), the coefficient of multiple determination (R^2), and the Fisher's test (F value) were used as the quality criteria of calculated QSAR models.

3. Results

Overall 27 compounds were tested for retinoid or anti-retinoid activity using P19/A15 cell line. At first, studied compounds were tested for their ability to induce RAR/RXR-dependent luciferase expression in P19/A15 cells (i.e. without the endogenous ligand ATRA). However, none of the tested compounds significantly induced RAR/RXR-dependent activity (data not shown). On the other hand, most of the tested PAHs and N-PAHs significantly modulated RAR/RXR-dependent gene transcription when exposed simultaneously with ATRA. Dose-response curves for ATRA showed higher EC_{50} after 24 h (117 nM) than after 6 h exposure (3.34 nM) (Fig. 2). Based on these results, 32 nM ATRA was selected for simultaneous exposures. Potencies of individual compounds to stimulate or inhibit ATRA-mediated activity and corresponding values of LOEC, MOEC and maximum observed inhibitory/stimulatory effects (% of ATRA 32 nM treated control) are summarized in Table 1. Responses of the chemicals varied depending on the time of exposure. While most PAHs and N-PAHs downregulated ATRA-mediated response after 6 h, upregulation of ATRA-mediated response was the predominant effect after 24 h exposure.

The tested compounds can be divided into a few groups according to the potency to modulate ATRA response:

First group composed of six small 2-ring aza-PAHs, 3-ring Phe and one of its derivatives (4,7-Pht) significantly downregulated effects of ATRA after shorter 6 h period with no effects after 24 h exposure. Only two compounds (4,7-Pht and Isq) had effects stronger than 50% with LOECs about 12 μM (Fig. 3A), other compounds from this group showed weaker inhibitions at relatively high concentrations 50–200 μM (Fig. 3B). Interestingly, phenanthrene showed biphasic effect on ATRA-induced luciferase after 6 h: downregulations were observed only at 3.1 μM but the effect diminished at higher tested concentration (12.5 μM).

Two aza-derivatives of 3-ring anthracene (Acr, Phez) and two aza-derivatives of 4-ring B[a]A (B[a]Acr and B[c]Acr) form the second group of compounds that highly upregulated ATRA effects after 24 h (200–400% effect) but they had no effects at shorter 6 h exposure period (Fig. 3C). Further, B[a]Acr showed apparent bi-

Table 1
Modulation of ATRA-mediated luciferase activity in P19/A15 cells by tested chemicals

Chemical	No. of rings	6 h-effect	24 h-effect	LOEC (μM)		MOEC (μM)		% of ATRA 32 nM (MOEC)	
				6 h	24 h	6 h	24 h	6 h	24 h
TCDD	–	n.e.	n.e.	–	–	–	–	–	–
Naphthalene	2	n.e.	n.e.	–	–	–	–	–	–
Quinoline	2	↓	n.e.	200	–	200	–	70	–
Quinazoline	2	↓	n.e.	200	–	200	–	65	–
6-Methyl quinoline	2	↓	n.e.	50	–	200	–	55	–
Isoquinoline	2	↓	n.e..	12.5	–	100	–	40	–
Indole	2	↓	n.e.	200	–	200	–	70	–
2-Methylindole	2	↓	n.e.	50	–	200	–	80	–
Phenanthrene	3	↓[a]	n.e.	3.1/12.5[b]	–	3.1	–	65	–
Phenanthridine	3	↓	↓	12.5	12.5	100	100	35	45
Benzo[h]quinoline	3	n.e.	n.e.	–	–	–	–	–	–
1,7-Phenanthroline	3	↓	↓	3.1	3.1	150	150	10	45
4,7-Phenanthroline	3	↓	n.e.	12.5	–	200	–	35	–
1,10-Phenanthroline	3	n.e.	n.e.	–	–	–	–	–	–
Anthracene	3	n.e.	n.e.	–	–	–	–	–	–
Acridine	3	n.e.	↑	–	12.5	–	100	–	490
Phenazine	3	n.e.	↑	–	25	–	100	–	270
Benz[a]anthracene	4	↑	↑	3.1	3.1	12.5	12.5	140	260
Benz[a]acridine	4	n.e.	↑[a]	–	0.75;3.1[b]	–	2.1	–	400
Benz[c]acridine	4	n.e.	↑	–	3.1	–	7	–	290
Dibenz[a,h]anthracene	5	↓[a]	↑	0.047/12.5[b]	0.185	0.185	3.1	60	320
Dibenz[a,h]acridine	5	↓	↑[a]	0.047	0.75;25[b]	25	0.75	10	200
Dibenz[a,i]acridine	5	↓	↑	0.185	0.75	12.5	3.1	5	510
Dibenz[c,h]acridine	5	n.e.	n.e.	–	–	–	–	–	–
Dibenz[a,j]acridine	5	n.e.	n.e.	–	–	–	–	–	–
Dibenzo[c,g]carbazole	5	n.e.	n.e.	–	–	–	–	–	–
Benzo[a]pyrene	5	↑	↑	12.5	3.1	12.5	12.5	130	170

LOECs-lowest observable effect concentrations; MOECs-maximal observable effect concentration; n.e.-no effects; ↑-upregulation of ATRA-mediated activity; ↓-downregulation of ATRA-mediated activity.

[a] Biphasic effects.

[b] Concentration that diminished effects.

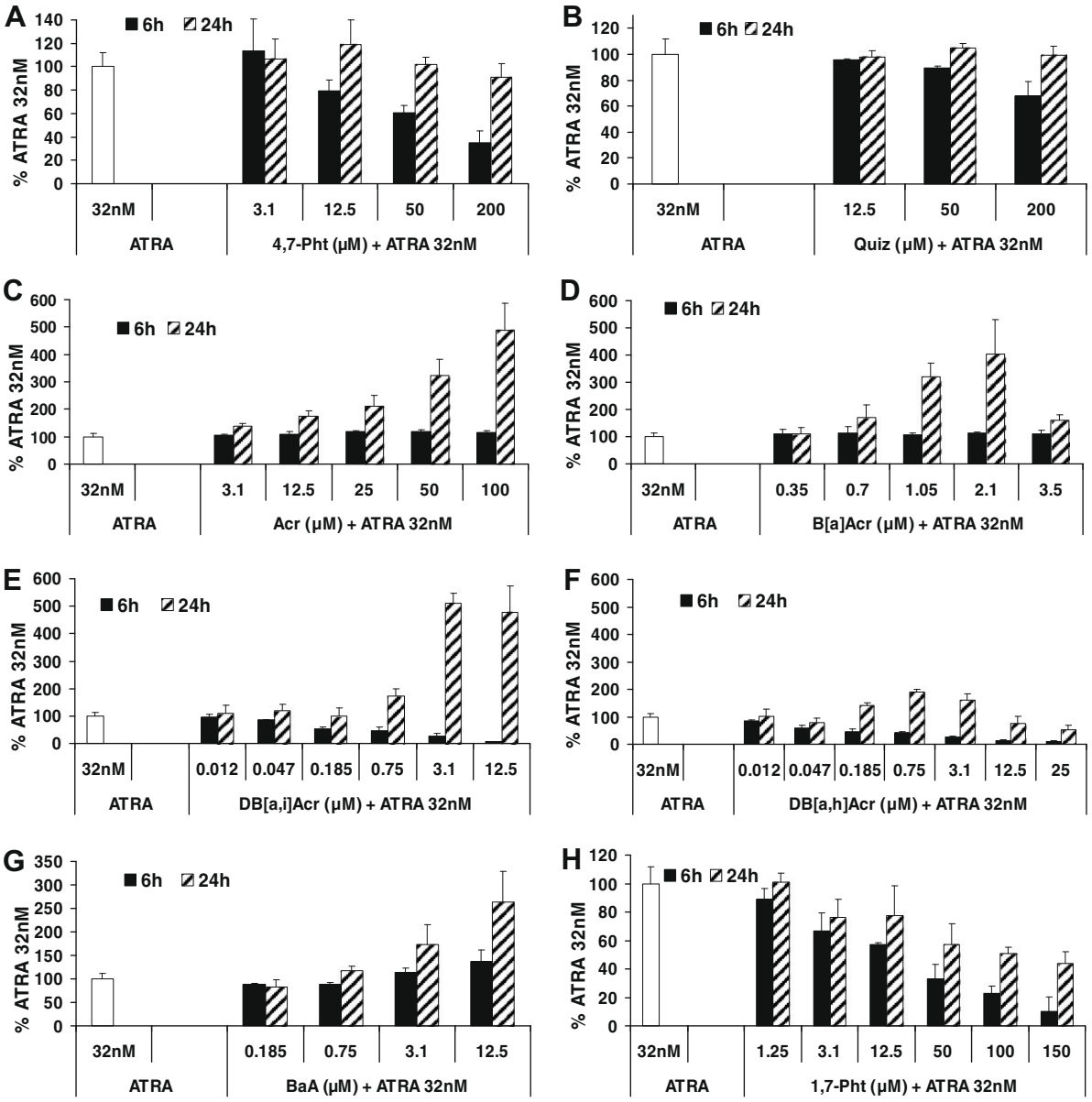

Fig. 3. Modulation of all-*trans* retinoic acid (ATRA) mediated activity by selected PAHs and N-PAHs. Each column is the mean ± standard deviation of three independent experiments. B[a]A – benz[a]anthracene; 1,7-Pht – 1,7-phenanthroline; 4,7-Pht – 4,7-phenanthroline; Quiz – quinazoline; Acr – acridine; B[a]Acr – benz[a]acridine; DB[a,i]Acr – dibenz[a,i]acridine; DB[a,h]Acr – dibenz[a,h]acridine.

phasic effect with stimulations (LOEC 0.7 μM) followed by no effects at higher tested non-cytotoxic concentrations (3.5 μM; Fig. 3D).

Other larger group of compounds consists of three high molecular weight 5-ring PAHs and N-PAHs (parental DB[a,h]A and two aza-heterocycles DB[a,h]Acr and DB[a,i]Acr), which strongly downregulated ATRA activity after 6 h (LOEC about 0.185 μM) but they had pronounced upregulatory effects after 24 h (up to 400% for DB[a,i]Acr with LOEC at 0.75 μM; Fig. 3E). Apparent biphasic effect similar to B[a]Acr showed also DB[a,h]Acr (Fig. 3F).

Only two parental PAHs (B[a]A and B[a]P) stimulated effects of ATRA after 6 h (LOEC 3.1–12.5 μM), all other compounds were inhibitory or had no effects at this short exposure period (see above). B[a]A and B[a]P had stimulatory effects also after 24 h with LOECs about 3.1 μM (Fig. 3G).

On the other hand, two 3 ring aza-derivatives of phenanthrene (1,7-Pht, Phd) were the only two compounds that downregulated ATRA-dependent luciferase activities after 24 h (Fig. 3H), all other compounds had either no or stimulatory effects at this period. These two compounds also suppressed effects of ATRA after shorter 6 h exposure.

No effects at 6 or 24 h were observed for two parent PAHs (Ant and Nap), two 3-ring aza-derivatives of phenanthrene (B[h]Q and 1,10-Pht) and also three 5-ring aza-PAHs (DB[c,g]C, DB[a,j]Acr and DB[c,h]Acr). Also model polychlorinated compound TCDD had no effect at either period of time.

3.1. QSAR results

Using the molecular descriptors derived in the TSAR package, we have firstly performed qualitative analysis on all compounds to examine if any parameters could discriminate "active" from "non-active" ones. Various parameters were examined but no clear general trend could be determined for the wide set of all

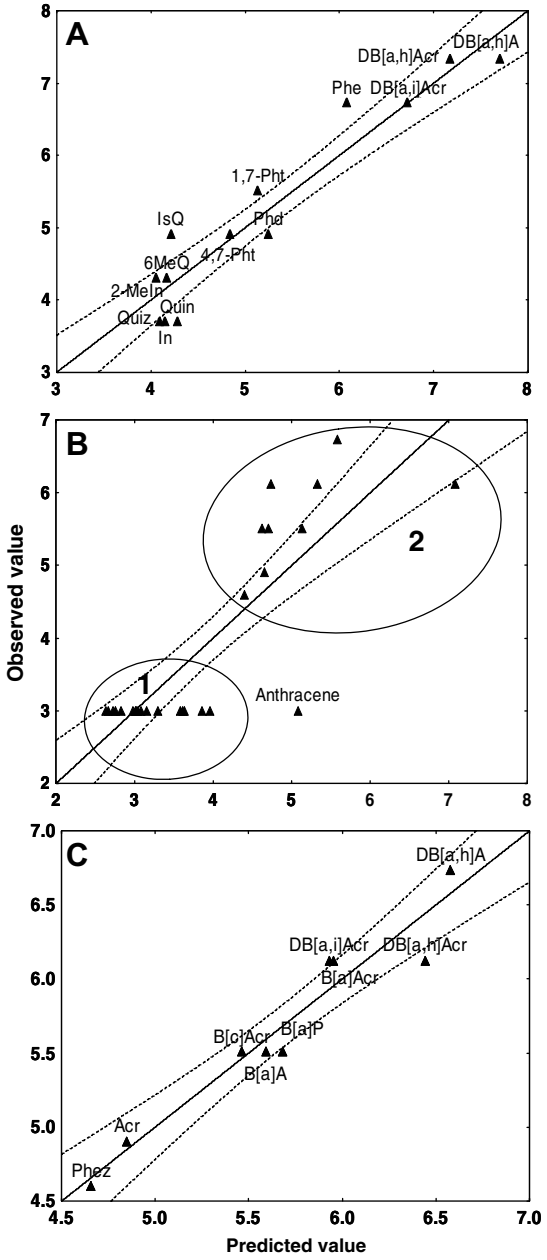

Fig. 4. Relationship between values predicted by the QSAR models for active compounds after 6 and 24 h and the observed log(1/LOEC) values determined by bioassay (see Table 2). (A) QSAR model for 6 h inhibitory effects of active compounds. (B) Qualitative discrimination between non-active or inhibitory chemicals (1) and chemicals able to upregulate ATRA-mediated activity (2) at 24 h based on combination of three descriptors. (C) QSAR model for 24 h stimulating effects of active compounds. Dashed lines represent 95% confidence interval.

compounds. The only observable pattern was greater dipole moment of a subset of compounds inhibitory at 6 h ($N = 9$ -small 2- and 3-ring N-PAHs) compared to all other compounds.

Secondly, we have used principle component analysis (PCA) to study multivariate correlations between effects and physicochemical descriptors. We have selected a subset of representative 21 descriptors that were not highly inter-correlated, and these were further analyzed along with the bioassay results (log(1/LOEC)). Out of numerous combinations of these 21 descriptors, we have found that a set of 6 descriptors (total energy, LUMO, Van der Waals energy, molecular volume, dipole moment and total lipole)

Table 2
Quantitative structure-activity relationship for modulation of ATRA-mediated activity

QSAR model	n	r	R^2	F
All compounds that down regulated ATRA-mediated activity after 6 h				
Log(1/LOEC) = 0.773 * MV − 0.277 * TDIP + 5.596	13	94	89	40
Qualitative discrimination of compounds that upregulated ATRA-mediated activity after 24 h from nonactive/inhibitory compounds				
Log(1/LOEC) = 0.850 * In1Lng + 0.484 * GHL + 0.566 * TOTEN + 3.770	27	84	70	18
All compounds that upregulated ATRA-mediated activity after 24 h				
Log(1/LOEC) = 1.10 * cVdW − 0.34 * GHL + 5.194	9	97	98	42

n = number of chemicals in data set; r = multivariate correlation coefficient; R^2 = coefficient of multiple determination; F = Fisher's test (variance ratio); LOEC-experimental lowest observed effect concentration; MV = molecular volume; TDIP = total dipole moment; cVdW = cosmic Van der Waals energy; GHL = gap-HOMO/LUMO; In1Lng = molecular length (first principle axis of inertia); TOTEN = total energy of molecule; ATRA-all-trans retinoic acid.

were able to provide multivariable discrimination of the compounds that were active after 6 h from those that had no effect (discrimination in the projection of the 1st and the 2nd PCs that covered 68% of overall variability). Similar parameters were also important when evaluating 24 h effects, but (instead of molecular volume) molecular length (first principal axis of inertia) and also gap between HOMO and LUMO were important parameters. Further, stepwise multiple regressions have been performed with individual descriptors. For compounds inhibitory after 6 h ($n = 13$), detailed quantitative analysis revealed highly significant relationships of log(1/LOEC) with molecular volume and dipole moment (Fig. 4A, Table 2). Qualitative evaluation for 24 h exposures showed that the combination of total energy, length and gap-HOMO/LUMO discriminated most of the non-active or inhibitory compounds from the compounds that upregulated ATRA-mediated activity (Fig. 4B, Table 2). The only exception was anthracene which was non-active in the bioassay but predicted as active compound by SAR. The potencies to upregulate ATRA-mediated activity at 24 h exposures were best explained by the combination of Van der Waals energy and gap-HOMO/LUMO descriptors (Fig. 4C, Table 2).

4. Discussion

In the present study, twenty one of 27 tested chemicals were able to modulate retinoic acid activity, while none of the individual chemicals alone directly activated RAR-mediated gene expression. Between chemicals that downregulated ATRA-mediated activity (especially during 6 h period) predominate two ring N-PAHs, and three ring analogs of phenanthrene. These compounds are more soluble in water and less lipophilic than other tested chemicals, and they are also probably easily metabolized (Bleeker et al., 1998). According to our SAR studies these compounds have higher dipole moment, which belongs among parameters important for binding of ligands to protein receptors (Lien et al., 1982). While all tested two ring N-PAHs were effective (inhibitory) during 6 h exposure, two of five tested analogs of phenanthrene (1,10-Pht and B[h]Q) were non-active. Interestingly, only these two compounds have the nitrogen atom inside the "bay" region of the polyaromatic structure.

Big group of chemicals composed of PAHs and N-PAHs was able to upregulate ATRA-mediated activity after 24 h. However, these compounds differed in their activity after 6 h exposure period (Table 1). Structurally similar PAHs with "bay" region (B[a]P and

B[a]A) upregulated retinoic acid mediated activity also at 6 h period (Fig. 3G). Further, three and four ring N-PAHs from this group did not change retinoic acid mediated activity at 6 h, whereas DB[a,h]Acr and DB[a,i]Acr strongly downregulated ATRA activity (Fig. 3C). However, other tested dibenzacridines and DB[c,g]C did not modulate ATRA activity at either time. Interestingly, in contrast to active 5-ring N-PAHs, these non-active high molecular weight N-PAHs have similar "U-shape" structures (Fig. 1). However, as documented in Fig. 3(E–F), also structurally similar active dibenzacridines differ in their activity after 24 h exposure.

Big differences between 6 h and 24 h activities of some compounds, especially DB[a,h]Acr and DB[a,i]Acr can be explained by possible biotransformation to metabolites with different effects. Biotransformation of PAHs and N-PAHs is joined with activation of CYP450s (Jung et al., 2001). Inducibility of CYP1A gene expression along with activation of AhR and xenobiotic response element in P19 cells was confirmed in a recent study with prototypical AhR ligand TCDD (Tonack et al., 2007). Interestingly, the CYP1A induction was faster (peak at 2 h) compared to HepG2 hepatoma cell line with maximum at 12 h. Most of the upregulating compounds, especially B[a]A, DB[a,h]Ant and their analogs are strong inducers of AhR and CYP1A (Jung et al., 2001; Sovadinova et al., 2006). Thus possibly not parent compounds, but their metabolites can be responsible for the observed effects. This theory may be supported by the non-linear dose-responses of B[a]Acr (Fig. 3D) and DB[a,-h]Acr (Fig. 3F) after 24 h that are similar to profiles of CYP1A enzyme activity induced by these compounds (Jung et al., 2001).

Also other studies reported that disruption of retinoid signaling pathways could be linked with activation of Ah receptor pathway (Murphy et al., 2007; Widerak et al., 2006). AhR ligands are (according to some studies) able to significantly change retinoic acid synthesis, catabolism, transport and excretion (Murphy et al., 2007), and also interact with retinoid signaling on levels of gene expression and coactivators and correpressors binding (Widerak et al., 2006). Several PAHs and also N-PAHs are known strong AhR ligands (Kawanishi et al., 2003; Saeki et al., 2003; Sovadinova et al., 2006) and CYP1A activators (Jung et al., 2001), but opposite to chlorinated compounds such as TCDD (Lorick et al., 1998; Widerak et al., 2006), there is only limited information about influence of PAHs or their analogs on retinoid signaling (Novak et al., 2007).

Comparing the present report with the study focused on N-PAHs modulation of AhR (Sovadinova et al., 2006), not all compounds that modulated retinoic acid activity were also AhR activators and vice versa. Especially TCDD, very strong ligand of AhR in general and a known teratogen (Blankenship et al., 1993; Wu et al., 2002) did not modulate ATRA activity in our assay. One explanation may be possible species-specific affinity of ligands to AhR. The mouse AhR (such as in P19/A15 cells) can be different from rat AhR (such as in H4IIE.luc) or human AhR (Garrison et al., 1996). According to a study with yeasts co-expressing human AhR and ARNT some compounds non-active in rat H4IIE.luc cells were able to weakly activate human AhR (Saeki et al., 2003). Among these compounds, Quin or 1,7-Pht were also able to modulate retinoic acid activity in our assay with mouse cells. Moreover, it is known that affinity of AhR to ligand (Maier et al., 1998; Garrison et al., 1996) as well as teratogenic potential of TCDD (Thomae et al., 2006) can differ among various mouse strains. Thus, it may be possible that higher TCDD concentrations (than used in our study) would cause some effects on RAR/RXR but these concentrations of TCDD were cytotoxic to P19/A15 cells.

One possible mechanism of RAR pathways disruption by AhR active compounds is activation of CYP450s followed by faster biotransformation of natural ligands such as ATRA (Janosek et al., 2006). On the other hand, increase of ATRA activity together with AhR activation and further sequestration of RARα correpresor SMRT by AhR was described (Widerak et al., 2006), and other examples of the cross-talk between AhR and retinoid signaling have also been confirmed (Fallone et al., 2004; Novak et al., 2007). Thus, further experiments are necessary to clarify the role of AhR in disruption of retinoid signaling pathways.

Several studies also reported disruption of retinoid signaling pathways upon exposure to complex environmental samples. In one study (Schoff and Ankley, 2002) water soluble fraction of paper mill effluents lowered reporter activity stimulated by ATRA. Water soluble fraction of creosote, which is widely used for wood preservatives (Galceran et al., 1994), is mainly composed of low molecular N-PAHs (Padma et al., 1998) and thus it is possible that these compounds contributed to the inhibitory effects of paper mill effluents. Our previous study with P19/A15 (Novak et al., 2007) found stimulating effects for ATRA-mediated activity, and another study with human HL-60 cells described increased ATRA-induced differentiation after treatment with extracts of sediments contaminated by PAHs (Vondracek et al., 2001).

As mentioned above, disruption of retinoid signaling by xenobiotics can lead to developmental anomalies (Degitz et al., 2003). Some *in vivo* studies found embryotoxicity of AhR ligands and CYP1A activators (Billiard et al., 1999; Hodson et al., 2007; Kim and Cooper, 1998), which could be potentially connected with disruption of retinoid signaling.

In a study of Wassenberg and Di Giulio (2004), synergistic embryotoxicity of some AhR active PAHs with antagonists of CYP1A for fish embryos was discovered. Other study with retene (alkyl derivative of phenanthrene) also found that embryotoxicity of this PAH was enhanced when co-exposed with low doses of CYP1A antagonist (alpha–naphtoflavone). The decreased CYP1A activity led to slower breakdown of generated specific low polar metabolites of retene that were shown to be responsible for the enhanced embryotoxicity (Hodson et al., 2007).

Though limited, there have also been some reports on studies of embryotoxicity with N-PAHs. The study of Buryskova et al. (2006) showed developmental toxicity of several N-PAHs and also their parental PAHs in FETAX assay (frog embryo teratogenesis assay-*Xenopus*). Acridine and phenazine, highly increasing ATRA-mediated activity in our assay (Fig. 3C), showed the highest potential to cause morfological abnormalities in *Xenopus laevis*. On the other hand, analogs of phenanthrene (inhibitory in our assay) were more embryotoxic for *Xenopus* embryo with lower potential to cause malformations. The importance of ATRA for embryonic development of *X. leavis* and also other amphibians confirmed the study of Degitz et al. (2000). It was found that excessive RA exposure leads to various malformations, and higher concentrations of RA were also toxic for *Xenopus* embryo. Thus, disruption of retinoic acid signaling pathways by studied PAHs and N-PAHs revealed in our assay could be possibly related to their embryotoxic and teratogenic effects in amphibians. Nevertheless, also other toxicity mechanisms such as oxidative stress, CYP1A induction or narcosis can be responsible for embryotoxicity of PAHs and N-PAHs (Wassenberg and Di Giulio, 2004). Differences between *in vitro* and *in vivo* tests such as different metabolism or binding of xenobiotics to serum proteins (Eertmans et al., 2003) evoke a question to what degree can studied compounds interfere with retinoid signaling *in vivo*. Chemicals can interfere *in vivo* with retinoid transport, metabolism and signaling on various levels as is summarized also in our recent review (Novak et al., 2008). Thus the used *in vitro* assay cannot be an overall predictive tool for the situation *in vivo*, but rather a mechanistic research tool able to characterize the potential interaction of chemicals with crucial target within retinoid signaling. Detailed studies focused on the disruption of retinoid signaling *in vivo* are necessary to confirm if these effects of PAHs and N-PAHs play an important role in *in vivo* situation.

According to our QSAR studies, several parameters were important for the potential of tested compounds to modulate ATRA-induced responses. Among these, total molecular energy, Van der Waals energy, molecular volume and length, dipole moment or HOMO and LUMO were the most important (Fig. 4, Table 2). According to the study of Douguet et al. (1999), some of these parameters, such as length or dipole moment were shown to be important for ability of synthetic retinoids to specifically bind RARα, RARβ or RARγ. Our study showed that length was an important parameter for upregulation of ATRA-mediated activity, while dipole moment has been more important for inhibitory effects. As previously reported, undifferentiated P19 cells constitutively express only RARα and RARγ, while RARβ expression is induced by retinoic acid (Pratt et al., 2000). Consequently, although PAHs and N-PAHs according to our study do not directly activate RAR-dependent signaling pathway in undifferentiated P19/A15 cells, exposure of these cells to ATRA could lead to RARβ expression and further activation of RARβ by tested chemicals.

Also selectivity of PAHs and N-PAHs as ligands for RXR can be responsible for significant effects only in the presence of ATRA. RXR-selective ligands do not directly induce RAR-mediated activity, however they can potentiate effects of RAR ligands (Minucci et al., 1997). QSAR studies with selective RXR receptor ligands found that majority of protein-ligand contacts are Van der Waals interactions (Egea et al., 2002), and Van der Waals energy was one of the most important parameters correlated with the upregulation of ATRA-mediated activity in our study. Thus, it is possible that PAHs and N-PAHs with higher Van der Waals energy can interact with ligand binding pocket of RXR and further contribute to activation of RAR/RXR-dependent genes (reporter luciferase in our assay).

In conclusion, our study characterizes the potencies of PAHs and N-PAHs to modulate ATRA-mediated activity *in vitro*. The effects differed between shorter 6 h exposures (mostly downregulations) and prolonged 24 h periods (mostly stimulations). Molecular parameters important for studied biological activities *in vitro* were also determined (molecular volume and length, Van der Waals energy, dipole moment, total energy of molecule or gap between HOMO and LUMO). Although the effective concentrations in our assay may seem relatively high, contribution of PAHs and N-PAHs to the chronic toxic effects of complex environmental mixtures (such as teratogenicity) is of general concern. Further studies should explore in detail the effects of PAHs and their derivatives as well as *in vivo* consequences of the interference with retinoid signaling.

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References

Alsop, D., Brown, S., Van der Kraak, G., 2007. The effects of copper and benzo[a]pyrene on retinoids and reproduction in zebrafish. Aquatic Toxicology 82, 281–295.

Arcaro, K.F., O'Keefe, P.W., Yang, Y., Clayton, W., Gierthy, J.F., 1999. Antiestrogenicity of environmental polycyclic aromatic hydrocarbons in human breast cancer cells. Toxicology 133, 115–127.

Bartos, T., Letzsch, S., Skarek, M., Flegrova, Z., Cupr, P., Holoubek, I., 2006. GFP assay as a sensitive eukaryotic screening model to detect toxic and genotoxic activity of azaarenes. Environmental Toxicology 21, 343–348.

Bastien, J., Rochette-Egly, C., 2004. Nuclear retinoid receptors and the transcription of retinoid-target genes. Gene 328, 1–16.

Bhattacharya, N., Dufour, J.M., Vo, M.N., Okita, J., Okita, R., Kim, K.H., 2005. Differential effects of phthalates on the testis and the liver. Biology of Reproduction 72, 745–754.

Billiard, S.M., Querbach, K., Hodson, P.V., 1999. Toxicity of retene to early life stages of two freshwater fish species. Environmental Toxicology and Chemistry 18, 2070–2077.

Blankenship, A.L., Suffia, M.C., Matsumura, F., Walsh, K.J., Wiley, L.M., 1993. 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) accelerates differentiation of murine preimplantation embryos in vitro. Reproductive Toxicology 7, 255–261.

Bleeker, E.A.J., van der Geest, H.G., Kraak, M.H.S., de Voogt, P., Admiraal, W., 1998. Comparative ecotoxicity of NPAHs to larvae of the midge *Chironomus riparius*. Aquatic Toxicology 41, 51–62.

Bleeker, E.A.J., Noor, L., Kraak, M.H.S., de Voogt, P., Admiraal, W., 2001. Comparative metabolism of phenanthridine by carp (*Cyprinus carpio*) and midge larvae (*Chironomus riparius*). Environmental Pollution 112, 11–17.

Buryskova, B., Hilscherova, K., Blaha, L., Marsalek, B., Holoubek, I., 2006. Toxicity and modulations of biomarkers in *Xenopus laevis* embryos exposed to polycyclic aromatic hydrocarbons and their N-heterocyclic derivatives. Environmental Toxicology 21, 590–598.

Chen, H.Y., Preston, M.R., 2004. Measurement of semi-volatile azaarenes in airborne particulate and vapor phases. Analytica Chimica Acta 501, 71–78.

Degitz, S.J., Kosian, P.A., Makynen, E.A., Jensen, K.M., Ankley, G.T., 2000. Stage- and species-specific developmental toxicity of all-*trans* retinoic acid in four native North American ranids and *Xenopus laevis*. Toxicological Sciences 57, 264–274.

Degitz, S.J., Holcombe, G.W., Kosian, P.A., Tietge, J.E., Durhan, E.J., Ankley, G.T., 2003. Comparing the effects of stage and duration of retinoic acid exposure on amphibian limb development: chronic exposure results in mortality, not limb malformations. Toxicological Sciences 74, 139–146.

Douguet, D., Thoreau, E., Grassy, G., 1999. Quantitative structure-activity relationship studies of RAR alpha, beta, gamma retinoid agonists. Quantitative Structure-Activity Relationships 18, 107–123.

Eertmans, F., Dhooge, W., Stuyvaert, S., Comhaire, F., 2003. Endocrine disruptors: effects on male fertility and screening tools for their assessment. Toxicology in Vitro 17, 515–524.

Egea, P.F., Mitschler, A., Moras, D., 2002. Molecular recognition of agonist Ligands by RXRs. Molecular Endocrinology 16, 987–997.

Fallone, F., Villard, P.H., Seree, E., Rimet, O., Nguyen, Q.B., Bourgarel-Rey, W., Fouchier, F., Barra, Y., Durand, A., Lacarelle, B., 2004. Retinoids repress Ah receptor CYP1A1 induction pathway through the SMRT corepressor. Biochemical and Biophysical Research Communications 322, 551–556.

Feng, C.L., Xia, X.H., Shen, Z.Y., Zhou, Z., 2007. Distribution and sources of polycyclic aromatic hydrocarbons in Wuhan section of the Yangtze River, China. Environmental Monitoring and Assessment 133, 447–458.

Freyberger, A., Schmuck, G., 2005. Screening for estrogenicity and anti-estrogenicity: a critical evaluation of an MVLN cell-based transactivation assay. Toxicology Letters 155, 1–13.

Galceran, M.T., Curto, M.J., Puignou, L., Moyano, E., 1994. Determination of acridine derived compounds in charcoal-grilled meat and creosote oils by liquid-chromatographic and gas-chromatographic analysis. Analytica Chimica Acta 295, 307–313.

Garrison, P.M., Tullis, K., Aarts, J., Brouwer, A., Giesy, J.P., Denison, M.S., 1996. Species-specific recombinant cell lines as bioassay systems for the detection of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin-like chemicals. Fundamental and Applied Toxicology 30, 194–203.

Hodson, P.V., Qureshi, K., Noble, C.A.J., Akhtar, P., Brown, R.S., 2007. Inhibition of CYP1A enzymes by alpha–naphthoflavone causes both synergism and antagonism of retene toxicity to rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology 81, 275–285.

Janosek, J., Hilscherova, K., Blaha, L., Holoubek, I., 2006. Environmental xenobiotics and nuclear receptors – Interactions, effects and in vitro assessment. Toxicology in Vitro 20, 18–37.

Jung, D.K.J., Klaus, T., Fent, K., 2001. Cytochrome P450 induction by nitrated polycyclic aromatic hydrocarbons, azaarenes, and binary mixtures in fish hepatoma cell line PLHC-1. Environmental Toxicology and Chemistry 20, 149–159.

Kawanishi, M., Sakamoto, M., Ito, A., Kishi, K., Yagi, T., 2003. Construction of reporter yeasts for mouse aryl hydrocarbon receptor ligand activity. Mutation Research-Genetic Toxicology and Environmental Mutagenesis 540, 99–105.

Kim, Y.C., Cooper, K.R., 1998. Interactions of 2, 3, 7, 8-tetrachlorodibenxo-p-dioxin (TCDD) and 3, 3 ' 4, 4 ', 5-pentachlorobiphenyl (PCB 126) for producing lethal and sublethal effects in the Japanese medaka embryos and larvae. Chemosphere 36, 409–418.

Lemaire, G., Balaguer, P., Michel, S., Rahmani, R., 2005. Activation of retinoic acid receptor-dependent transcription by organochlorine pesticides. Toxicology and Applied Pharmacology 202, 38–49.

Lien, E.J., Guo, Z.R., Li, R.L., Su, C.T., 1982. Use of dipole-moment as a parameter in drug receptor interaction and quantitative structure activity relationship studies. Journal of Pharmaceutical Sciences 71, 641–655.

Lorick, K.L., Toscano, D.L., Toscano, W.A., 1998. 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin alters retinoic acid receptor function in human keratinocytes. Biochemical and Biophysical Research Communications 243, 749–752.

Machala, M., Ciganek, M., Blaha, L., Minksova, K., Vondracek, J., 2001. Aryl hydrocarbon receptor-mediated and estrogenic activities of oxygenated polycyclic aromatic hydrocarbons and azaarenes originally identified in

extracts of river sediments. Environmental Toxicology and Chemistry 20, 2736–2743.

Maier, A., Micka, J., Miller, K., Denko, T., Chang, C.Y., Nebert, D.W., Puga, A., 1998. Aromatic hydrocarbon receptor polymorphism: development of new methods to correlate genotype with phenotype. Environmental Health Perspectives 106, 421–426.

Minucci, S., Leid, M., Toyama, R., SaintJeannet, J.P., Peterson, V.J., Horn, V., Ishmael, J.E., Bhattacharyya, N., Dey, A., Dawid, I.B., Ozato, K., 1997. Retinoid X receptor (RXR) within the RXR-retinoic acid receptor heterodimer binds its ligand and enhances retinoid-dependent gene expression. Molecular and Cellular Biology 17, 644–655.

Mos, L., Tabuchi, M., Dangerfield, N., Jeffries, S.J., Koop, B.F., Ross, P.S., 2007. Contaminant-associated disruption of vitamin A and its receptor (retinoic acid receptor alpha) in free-ranging harbour seals (*Phoca vitulina*). Aquatic Toxicology 81, 319–328.

Murphy, K.A., Quadro, L., White, L.A., 2007. The intersection between the aryl hydrocarbon receptor (AHR)- and retinoic acid-signaling pathways, In: Vitamin A. Elsevier Academic Press Inc, San Diego (pp. 33–67).

Novak, J., Benisek, M., Pachernik, J., Janosek, J., Sidlova, T., Kiviranta, H., Verta, M., Giesy, J.P., Blaha, L., Hilscherova, K., 2007. Interference of contaminated sediment extracts and environmental pollutants with retinoid signaling. Environmental Toxicology and Chemistry 26, 1591–1599.

Novak, J., Benisek, M., Hilscherova, K., 2008. Disruption of retinoid transport, metabolism and signaling by environmental pollutants. Environment International 34, 898–913.

Padma, T.V., Hale, R.C., Roberts, M.H., 1998. Toxicity of water-soluble fractions derived from whole creosote and creosote-contaminated sediments. Environmental Toxicology and Chemistry 17, 1606–1610.

Pratt, M.A.C., Crippen, C.A., Menard, M., 2000. Spontaneous retinoic acid receptor beta 2 expression during mesoderm differentiation of P19 murine embryonal carcinoma cells. Differentiation 65, 271–279.

Saeki, K., Matsuda, T., Kato, T., Yamada, K., Mizutani, T., Matsui, S., Fukuhara, K., Miyata, N., 2003. Activation of the human Ah receptor by aza-polycyclic aromatic hydrocarbons and their halogenated derivatives. Biological & Pharmaceutical Bulletin 26, 448–452.

Schoff, P.K., Ankley, G.T., 2002. Inhibition of retinoid activity by components of a paper mill effluent. Environmental Pollution 119, 1–4.

Sonneveld, E., van den Brink, C.E., van der Leede, B.J.M., Maden, M., van der Saag, P.T., 1999. Embryonal carcinoma cell lines stably transfected with mRAR beta 2-lacZ: sensitive system for measuring levels of active retinoids. Experimental Cell Research 250, 284–297.

Sovadinova, I., Blaha, L., Janosek, J., Hilscherova, K., Giesy, J.P., Jones, P.D., Holoubek, I., 2006. Cytotoxicity and aryl hydrocarbon receptor-mediated activity of N-heterocyclic polycyclic aromatic hydrocarbons: structure-activity relationships. Environmental Toxicology and Chemistry 25, 1291–1297.

Sucov, H.M., IzpisuaBelmonte, J.C., Ganan, Y., Evans, R.M., 1995. Mouse embryos lacking RXR alpha are resistant to retinoic-acid-induced limb defects. Development 121, 3997–4003.

Thomae, T.L., Stevens, E.A., Liss, A.L., Drinkwater, N.R., Bradfield, C.A., 2006. The teratogenic sensitivity to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin is modified by a locus on mouse chromosome 3. Molecular Pharmacology 69, 770–775.

Tonack, S., Kind, K., Thompson, J.G., Wobus, A.M., Fischer, B., Santos, A.N., 2007. Dioxin affects glucose transport via the arylhydrocarbon receptor signal cascade in pluripotent embryonic carcinoma cells. Endocrinology 148, 5902–5912.

Vinggaard, A.M., Hnida, C., Larsen, J.C., 2000. Environmental polycyclic aromatic hydrocarbons affect androgen receptor activation in vitro. Toxicology 145, 173–183.

Vondracek, J., Machala, M., Minksova, K., Blaha, L., Murk, A.J., Kozubik, A., Hofmanova, J., Hilscherova, K., Ulrich, R., Ciganek, M., Neca, J., Svrckova, D., Holoubek, I., 2001. Monitoring river sediments contaminated predominantly with polyaromatic hydrocarbons by chemical and in vitro bioassay techniques. Environmental Toxicology and Chemistry 20, 1499–1506.

Vondracek, J., Kozubik, A., Machala, M., 2002. Modulation of estrogen receptor-dependent reporter construct activation and G(0)/G(1)-S-phase transition by polycyclic aromatic hydrocarbons in human breast carcinoma MCF-7 cells. Toxicological Sciences 70, 193–201.

Wassenberg, D.M., Di Giulio, R.T., 2004. Synergistic embryotoxicity of polycyclic aromatic hydrocarbon aryl hydrocarbon receptor Agonists with cytochrome P4501A inhibitors in *Fundulus heteroclitus*. Environmental Health Perspectives 112, 1658–1664.

Widerak, M., Ghoneim, C., Dumontier, M.F., Quesne, M., Corvol, M.T., Savouret, J.F., 2006. The aryl hydrocarbon receptor activates the retinoic acid receptor alpha through SMRT antagonism. Biochimie 88, 387–397.

Wu, Q., Ohsako, S., Baba, T., Miyamoto, K., Tohyama, C., 2002. Effects of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) on preimplantation mouse embryos. Toxicology 174, 119–129.

Xu, P., Yu, B., Li, F.L., Cai, X.F., Ma, C.Q., 2006. Microbial degradation of sulfur, nitrogen and oxygen heterocycles. Trends in Microbiology 14, 398–405.

Zile, M.H., 2002. Function of vitamin A in vertebrate embryonic development Reprinted from vol 131, pg 705, 2001. Journal of Nutrition 132, 705A–708A.

Článek VII:

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The effects of PAHs and N-PAHs on retinoid signaling and Oct-4 expression *in vitro*

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) and their N-heterocyclic analogs (N-PAHs) are important environmental contaminants with negative effects in living organisms, including teratogenicity and embryotoxicity. Though most studies linked their embryotoxicity with aryl hydrocarbon receptor (AhR) and cytochrome P450 activation, the exact mechanism is not known. Other mechanisms such as disruption of retinoid signaling were recently suggested to be of importance. This study investigated PAHs and N-PAHs interference with retinoid signaling *in vitro* by modulating all-trans retinoic acid (ATRA) mediated response in a reporter gene assay using P19/A15 cell line. Further, effects on pluripotency and differentiation processes were evaluated by measuring octamer-4 (Oct-4), an important pluripotency marker and master differentiation factor. Two of the studied compounds, benz[a]anthracene and benz[c]acridine significantly up-regulated ATRA-mediated response in the co-exposure with a range of ATRA concentrations. Another structural N-PAH variant, 1,7-phenanthroline, downregulated ATRA-mediated response at most of tested ATRA concentrations and exposure times. Interesting concentration-dependent biphasic effects (i.e. downregulation with subsequent up-regulation to control levels) were observed at co-exposures of ATRA and parent PAH phenanthrene. Non significant Oct-4 modulation in co-exposure with ATRA was observed at compounds, which potentiated ATRA-mediated effects in the reporter gene assay. On the other hand, 1,7-phenanthroline and phenanthrene significantly suppressed Oct-4 levels in higher tested concentrations. Our results further extend the knowledge of PAH and N-PAH *in vitro* effects and indicate that these environmental toxicants may have influence on differentiation process and embryonic development by interfering with ATRA signaling and by modulating levels of Oct-4.

1. Introduction

Retinoids play an essential role in a wide variety of important biological processes, such as growth, vision, differentiation or embryonic development (Tzimas and Nau, 2001). They enter an organism through its diet mostly in the form of retinyl esters or carotenoids and they are enzymatically converted to retinol, which is released into the bloodstream, and transported bound to the plasma retinol-binding protein. After entering the cell, retinol is bound to the cellular retinol binding protein I (CRBP I) and is converted to retinal and retinoic acid (RA) (Blomhoff and Blomhoff, 2006). Retinoic acid, especially its all-trans and 9-cis isomers, acts as a ligand of retinoic acid receptors (RARs) or retinoid X receptors (RXRs). While RARs are activated by both all-trans retinoic acid (ATRA) and 9-cis retinoic acid (9-cis RA), RXRs are activated only by 9-cis RA (Bastien and Rochette-Egly, 2004). In the basal state, free receptors bind some co-repressors, while receptors activated by ligands recruit several co-activators (Widerak et al., 2006). Retinoid receptors then act via activation of retinoic acid response elements (RARE) or retinoid X response elements (RXRE) present in the promoter regions of retinoic acid responsive genes (Love and Gudas, 1994).

More than 500 genes have been suggested as targets controlled by RA. The regulation of these genes can be either direct (driven by a liganded RAR–RXR heterodimer bound to RARE), or indirect (reflecting the actions of intermediate transcription factors, non-classical associations of receptors with other proteins, or other mechanisms) (Fields et al., 2007). Some of these genes are involved

Abbreviations: 1,7-Pht, 1,7-phenanthroline; 9-cis RA, 9-cis retinoic acid; AhR, aryl hydrocarbon receptor; ATRA, all-trans retinoic acid; B[a]A, benz[a]anthracene; B[c]A, benz[c]acridine; COUP-TF I, chicken ovalbumin upstream promoter-transcription factor I; CRBP I, cellular retinol binding protein I; ERKs, extracellular signal-regulated kinases; LOEC, lowest observable effect concentration; N-PAHs, N-heterocyclic polycyclic aromatic hydrocarbons; Oct-4, octamer 4; PAHs, polycyclic aromatic hydrocarbons; PCBs, polychlorinated biphenyls; Phe, phenanthrene; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; RXR, retinoid X receptor; RXRE, retinoid X response element; SMRT, silencing mediator of retinoic acid and thyroide hormone receptor; TCDD, 2,3,7,8-tetrachlorodibenzodioxin; TR2, testicular receptor 2.

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in metabolism and signaling of retinoids (e.g. RARβ, CYP26, CRBP) and also in differentiation and morphogenesis (Oct-3/4, jun, hox, TGFβ) (Eifert et al., 2006; Love and Gudas, 1994).

Retinoic acid is a key factor during development of various vertebrate tissues and organs. It promotes cellular differentiation, regulates apoptosis and controls positioning of cells and tissue patterning (Blomhoff and Blomhoff, 2006). Several studies confirmed the importance of retinoic acid for many developmental processes including limb, eye, lung or central nervous system (Maden, 1999).

Experiments with embryonic carcinoma (Pachernik et al., 2005; Schoorlemmer et al., 1995) and embryonic stem cells (Faherty et al., 2005) also demonstrated that retinoic acid can modulate expression of Oct-4 (also called POU5f1 or Oct-3/4), an important pluripotency marker and master differentiation factor (Pesce and Scholer, 2001). Various concentrations of retinoic acid in pluripotent cells modulate Oct-4 protein levels and affect differentiation into various types of cells (Faherty et al., 2005; Pachernik et al., 2005).

Levels of Oct-4 change during different phases of embryonic development and the major function of Oct-4 is the maintenance of undifferentiated state of the inner cell mass and also the determination or establishment of the germline (Brehm et al., 1998). Experiments with Oct-4 knocked-out mice also found that embryos die due to the failure to form inner cell mass (Pesce and Scholer, 2001). The importance of Oct-4 for pluripotency has also been demonstrated by its role in reprogramming of mouse embryonic fibroblasts or adult mouse and human fibroblasts into embryonic stem cells (Kaji et al., 2009; Takahashi and Yamanaka, 2006).

The importance of retinoid receptors for embryonic development was confirmed in several studies. Interestingly, RAR single mutant mice developed normally, however, combined disruption of various genes of RAR family caused congenital defects (Tzimas and Nau, 2001). Targeted disruption of retinoid X receptors demonstrated that RXRα null mutant mice display ocular and cardiac malformations and die from cardiac failure (Maden, 1999), while RXRβ null mutant adult males are sterile (Tzimas and Nau, 2001). Some studies also indicate that retinoid teratogenicity is at least in part mediated via RAR/RXR signaling pathways and can be enhanced when both partners, RAR and RXR, are liganded (Tzimas and Nau, 2001).

Changes in levels of retinoid acid or its receptors during embryonic development can cause congenital defects or death of an embryo, and many environmental contaminants can interfere with retinoid system at various levels as summarized in our review (Novak et al., 2008). Many important environmental contaminants such as 2,3,7,8-tetrachlorodibenzodioxin (TCDD), polychlorinated biphenyls (PCBs) or polycyclic aromatic hydrocarbons (PAHs) are also known as teratogenic or embryotoxic compounds (Abbott and Birnbaum, 1989; Billiard et al., 2008; Lindenau and Fischer, 1996), and they were found to act mostly via activation of aryl hydrocarbon receptor (AhR) and cytochrome P450 induction (Billiard et al., 2008).

However, one study confirmed that also disruption of the retinoid system by TCDD and other compounds can lead to teratogenicity (Abbott and Birnbaum, 1989). Our previous study demonstrated that some PAHs and their N-heterocyclic analogs (N-PAHs) interfere with retinoid signaling *in vitro* and modulate gene expression mediated by all-trans retinoic acid (Benisek et al., 2008). PAHs and N-PAHs are important and ubiquitous environmental contaminants released into the environment from several sources (forest fires, combustion of fossil fuels or petroleum products) (Feng et al., 2007), and they can induce numerous adverse effects in biological organisms (carcinogenicity, mutagenicity, endocrine disruption, and embryotoxicity) (Buryskova et al., 2006; Santodonato, 1997; Sovadinova et al., 2006).

To further elucidate possible mechanisms and impacts of PAHs and N-PAHs on embryonic development, we analyzed interference of several structural PAH analogs with ATRA using the reporter gene assay with P19/A15 cells. Tested concentrations of ATRA (1 nM and 32 nM) are within the range of normal physiological levels in most mammalian tissues, while higher pharmacological doses 125 nM and 1000 nM were shown to induce different effects (Breems-de Ridder et al., 2000). Moreover, we investigated effects of PAHs on protein levels of Oct-4, an important marker of pluripotency with multiple regulatory roles. Thus, investigation of PAHs and N-PAHs brings more detailed insight into their toxic potencies on cellular differentiation. While ATRA alone is known to downregulate Oct-4 (Schoorlemmer et al., 1995), only rare co-exposure experiments with xenobiotics were performed. The present study is thus one of the first that investigated modulation of Oct-4 by toxic environmental contaminants.

2. Materials and methods

2.1. Chemicals

1,7-Phenanthroline (1,7-Pht) (CAS No. 230-46-6), benz[a]anthracene (B[a]A) (CAS No. 56-55-3), phenanthrene (Phe) (CAS No. 85-01-8) and all-trans retinoic acid (ATRA) (CAS No. 302-79-4) were purchased from Sigma–Aldrich (Prague, CR). Benz[c]acridine (B[c]A) (CAS No. 225-51-4) was obtained from Dr. Ehrenstorfer, GmbH (Augsburg, Germany). The purity of all compounds was 97% or higher.

2.2. Cell culture

For the study, we used murine embryonic carcinoma cell line P19 (European Collection of Cell Culture, Wiltshire, UK) either wild type or transfected with luciferase reporter pRAREβ2-TK-luc plasmid (P19/A15 clone) (Novak et al., 2007). The plasmid contains reporter luciferase gene under the control of retinoic acid-responsive element. Cells were cultured in plastic tissue culture flasks (TPP, Austria) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum Mycoplex (PAA, Austria) at 37 °C in a humidified atmosphere of 5% CO_2. Cells were split every third day to maintain undifferentiated state. Doubling time of P19 cells is usually between 18 and 22 h (Thier et al., 2000).

2.3. Luciferase reporter gene assay

For the RAR/RXR transactivation assay, 10,000 cells per well were seeded into 96-well microplates and incubated overnight. Then, the cells were exposed in three replicates to tested chemicals (concentration range 0.185–100 μM for 1,7-Pht and 0.185–12.5 μM for Phe, B[a]A, B[c]A) diluted in dimethyl sulfoxide (DMSO) simultaneously with various concentrations of endogenous ligand of retinoid receptor, all-trans retinoic acid (ATRA). Tested concentrations differed for different compounds with respect to lower solubility of Phe and B[a]A in the medium. Tested concentrations of ATRA in co-exposure were 1, 32, 125 and 1000 nM and the final data are standardized to ATRA 10 μM as this concentration represents the maximum (plateau) response for both 6 and 24 h exposure times. Final concentration of the solvent did not exceed 1% (v/v) and it had no effect on the cell viability, RAR/RXR-dependent activity or cellular differentiation (McBurney et al., 1982). The activity of reporter luciferase induced in the presence of RAR/RXR ligands was measured after 6 or 24 h exposure using Promega Steady Glo Kit (Promega, Madison, WI, USA) and microplate luminometer Luminoskan Ascent (Thermo Electron Corp., USA). At least three independent experiments in triplicates were performed for each exposure variant.

2.4. Cytotoxicity testing

Cytotoxicity of tested chemicals was measured by neutral red uptake assay (Freyberger and Schmuck, 2005). Briefly, neutral red (0.5 mg/ml of medium) was added to each well and the microplate was incubated at 37 °C for 1 h. Medium was removed and cells were lysed with 1% acetic acid in 50% ethanol and the absorbance at 570 nm was measured (only viable cells accumulated neutral red). Only non-cytotoxic concentrations were used for further experiments (data from cytotoxicity experiments are in supplementary material – Appendix 1).

2.5. Western blot analysis

P19 wild type (wt) cells were cultivated in plastic tissue culture Petri dish (500,000 cells per dish) overnight and then exposed to tested chemicals or solvent control (DMSO 1%) for either 6 or 24 h. Cells were briefly washed with phosphate buffered saline and lysed in sodium dodecyl sulfate lysis buffer (50 mM Tris–HCl, pH 7.5, 1% sodium dodecyl sulfate, 10% glycerol).

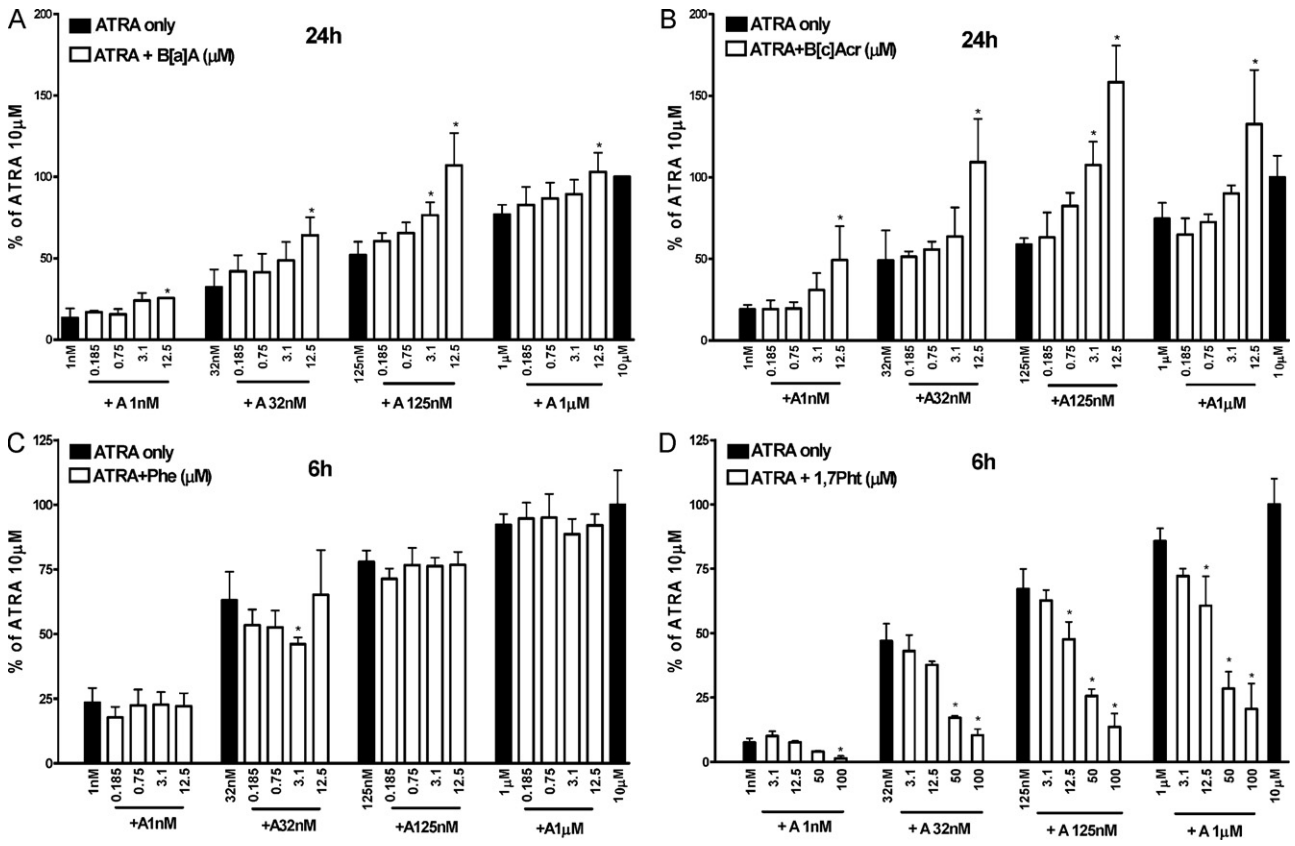

Fig. 1. Modulation of selected concentrations of all-trans retinoic acid (ATRA) activity by several PAHsand N-PAHs. Each column is the mean + standard deviation of at least three independent experiments. B[a]A – Benz[a]anthracene; B[c]Acr – Benz[c]acridine;1,7-Pht – 1,7-phenanthroline; Phe – phenanthrene; A – ATRA; * – effects statistically significantly different from control.

Protein concentrations were determined using the DC Protein assay kit (Bio-Rad, Hercules, CA, USA). Lysates were supplemented with bromphenol blue (0.01%) and β-mercaptoethanol (1%) and equal amounts of total protein (10 μg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis in 10% gel.

After electrotransfer onto a nitrocellulose membrane (SERVA, Heidelberg, Germany), proteins were immunodetected using rabbit anti-Oct-4 primary antibody (SC-9081; Santa Cruz Biotechnology, Heidelberg, Germany). Lamin B, a house keeping protein, was detected by goat primary SC-6217 antibodies (Santa Cruz Biotechnology). Horseradish peroxidase conjugate secondary antibodies were from Sigma–Aldrich (anti-rabbit A4914) and from Santa Cruz Biotechnology (anti-goat sc-2020).

Visualization was performed by enhanced chemiluminiscence using ECL-Plus kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. Image analysis was performed using Image J software (open source Image J software available on http://rsb.info.nih.gov/ij/).

2.6. Statistical analyses

All calculations and statistical analyses were performed with Microsoft Excel and Statistica for Windows (Ver 8.0, StatSoft, Tulsa, OK, USA). To determine significant differences from control, one-way analysis of variance (ANOVA) followed by Dunnet's test was used.

3. Results

3.1. PAHs and N-PAHs interfere with various concentrations of ATRA in reporter gene assay

Based on the results of our previous study (Benisek et al., 2008), we have investigated co-exposures of four compounds (2 PAHs and their 2 N-heterocyclic analogs) with a series of ATRA concentrations (1, 32, 125, 1000 nM) for 6 h or 24 h in the reporter gene assay with P19/A15 cell line. These compounds had no significant effects in RAR/RXR dependent reporter gene assay when tested alone (supplementary material – Appendix 2) but had diverse effects on the ATRA-mediated response, i.e. up-regulation (B[a]A and B[c]A), down-regulation (1,7-Pht) and biphasic effects (Phe). They were also structurally related (parental PAHs vs. their N-heterocyclic analogs).

As displayed in Fig. 1A, benz[a]anthracene significantly up-regulated ATRA-mediated response after 24 h exposure when co-exposed with tested ATRA concentrations. Stimulatory effects of benz[a]anthracene then did not significantly exceed effects of ATRA 10 μM (plateau) for all tested concentrations of ATRA in co-exposure (Fig. 1A). N-heterocyclic analog benz[c]acridine up-regulated ATRA-mediated effects after 24 h exposure at all tested ATRA concentrations. In contrast to B[a]A, some responses at higher concentrations (12.5 μM) exceeded the plateau observed at 10 μM ATRA alone (Fig. 1B). No statistically significant effects were observed after shorter 6 h exposure with these two 4-ring PAH compounds.

Three-ringed PAH phenanthrene did not show any effects after 24 h exposure. Similarly to our previous study (Benisek et al., 2008), biphasic effects in the co-exposure with ATRA 32 nM after 6 h were observed. However, co-exposure with other tested ATRA concentrations did not cause these biphasic effects, although phenanthrene co-exposed with higher ATRA concentration (1 μM) caused weak downregulation with subsequent up-regulation (Fig. 1C).

N-heterocyclic analog of phenanthrene – i.e. 1,7-phenanthroline – suppressed effects of most of tested concentrations of ATRA after both exposure times (Fig. 1D). The lowest observable effect concentrations (LOEC ~ 50 μM) determined after 24 h were the same for all co-exposed ATRA concentrations higher than 1 nM. Interesting results were found after 6 h exposures. At higher ATRA concentration (1 μM) 1,7-phenanthroline was more effective suppressor of

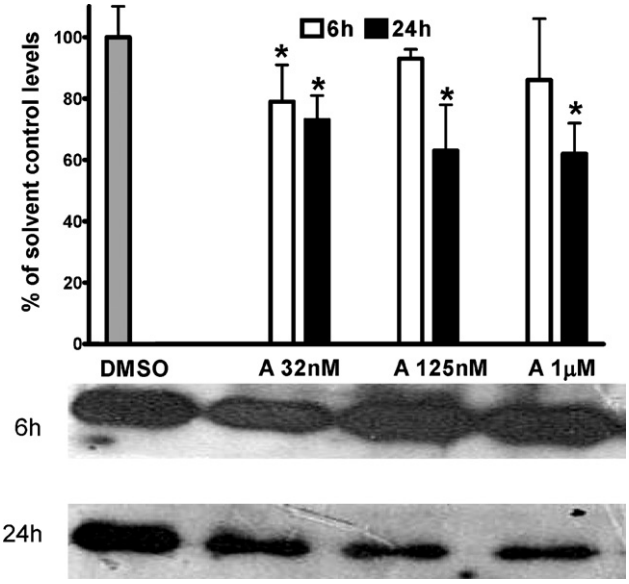

Fig. 2. Modulation of Oct-4 protein levels by several concentrations of all-trans retinoic acid (ATRA) in P19 cells after 6 h or 24 h exposure; each column is the mean + standard deviation of at least three independent experiments.*significantly different from solvent control levels.

ATRA effect (LOEC = 12.5 μM) in comparison with the lowest 1 nM ATRA concentration (LOEC 100 μM; Fig. 1D).

3.2. PAHs and N-PAHs modulate Oct-4 protein levels

Our study confirmed that ATRA suppresses Oct-4 levels in P19 cells with more pronounced and dose-dependent effects after prolonged 24 h exposures (Fig. 2). As shown in Fig. 3A and B, 1,7-phenanthroline (a compound that inhibited ATRA-effects at both exposure times in the P19/A15 assay) suppressed levels of Oct-4 after 6 h when exposed alone (LOEC 100 μM). Only weak not significant suppression was observed in the co-exposure of 1,7-Pht with ATRA.

For the parent PAH compound phenanthrene, interesting recovery was observed after 6 h exposure. Levels of Oct-4 suppressed by ATRA 32 nM alone returned back to the solvent control levels when ATRA 32 nM was co-exposed for 6 h with the highest phenanthrene concentration (12.5 μM; Fig. 3C). In contrast, after 24 h, higher phenanthrene concentrations (3.1 μM) significantly suppressed Oct-4 levels when tested alone (Fig. 3D).

B[a]A and B[c]A (compounds that enhanced ATRA effects after 24 h in P19/A15 reporter gene assay) had generally weak effects on Oct-4 expression. Because these compounds had no or only weak effects were after 6 h in P19/A15 reporter gene assay, only experiments with 24 h exposures were performed. When tested alone, these two compounds did not show significant influence on Oct-4. Co-exposures (24 h) of B[a]A and B[c]A with ATRA 32 nM showed only weak effects (see Fig. 3E and F).

4. Discussion

Polycyclic aromatic hydrocarbons (PAHs) and their N-heterocyclic analogs (N-PAHs) are important contaminants with a number of negative effects in organisms including teratogenicity and embryotoxicity. N-PAHs were also found to act as teratogens in the frog embryo teratogenicity assay FETAX (Buryskova et al., 2006). Most of the studies linked their embryotoxicity with mechanisms related to the aryl hydrocarbon receptor and cytochrome P450 activation (Billiard et al., 2008) but exact mechanism is not known. Other mechanisms such as disruption of retinoid signaling were recently suggested to be of importance (Benisek et al., 2008). In the present study, PAHs and N-PAHs modulated both ATRA-mediated response and Oct-4 protein levels in P19 cells. Moreover, effects of studied compounds on ATRA-mediated response were confirmed for a range of ATRA concentrations in the reporter gene assay. Although tested PAHs were structurally related, variable effects were observed indicating multiple mechanisms of action.

Two 4-ring PAHs, B[a]A and B[c]A, compounds that enhanced retinoid-like response in the reporter gene assay, showed only slight modulating effects on Oct-4, and the effects were observed only in co-exposures with ATRA. Results from the reporter gene assay also revealed interesting concentration-dependent observations because lower concentrations of tested compounds were necessary for significant modulations when higher concentrations of ATRA were used in co-exposure (see Fig. 1). This might be related to several mechanisms such as interference of studied compounds with co-repressors or co-activators of retinoid signaling. As described in a study of Widerak et al. (2006), strong AhR ligand TCDD activates RARα through a silencing mediator of retinoid acid and antagonism of the thyroid hormone receptor (SMRT). This effect is further synergized when co-exposed with ATRA. As shown previously, those compounds which up-regulated ATRA-mediated effects are also strong AhR ligands (Buryskova et al., 2006; Santodonato, 1997; Sovadinova et al., 2006). It is also known that high concentrations of ATRA (>100 nM) are able to remove co-repressors and activate co-activators with higher efficiency than physiological concentrations of ATRA (Breems-de Ridder et al., 2000). Thus, co-repressors potentially destabilized by the studied PAHs and N-PAHs could be removed from the binding site only by higher ATRA concentrations, and lower ATRA concentrations were not sufficient to remove destabilized co-repressors.

Both B[a]A and B[c]A are also strong CYP activators (Jung et al., 2001), and CYPs could have an impact on the retinoic acid metabolism (McSorley and Daly, 2000). Compared to our results, effective concentrations of these compounds inducing CYP1A in PLHC-1 cells (measured as EROD activity; Jung et al., 2001) are in a similar range as effective concentrations in the present study. ATRA was also shown to be a weak CYP1A activator but also an inhibitor of strong EROD activities (Fallone et al., 2004). Therefore, CYPs induced during the exposures possibly metabolized original PAHs/N-PAHs, and the newly formed metabolites could also contribute to observed effects.

Another mechanism explaining stimulatory effects of PAHs/N-PAHs in the reporter-gene assay is the activation of RXR receptor. Several organic contaminants were shown to act as RXR activators in yeast two hybrid assay (Li et al., 2008), and it was also found that RXR protein levels are slightly enhanced in P19 cells treated with ATRA (Novak et al., 2007). It is thus possible that higher RXR levels (induced at higher ATRA concentrations) could be involved in the observed concentration-dependent stimulatory effects of PAHs. However, further experiments would be necessary to fully confirm this mechanism. Further, other mechanisms including complex interactions of RAR/RXR signaling have variable effects on both ATRA-mediated responses and Oct-4, and they could be involved in observed effects of PAHs as well (Minucci et al., 1997; Schoorlemmer et al., 1995).

Other factors controlled by retinoid receptors were also shown to modulate Oct-4 expression such as chicken ovalbumin upstream promoter-transcription factor I (COUP-TF I) (Schoorlemmer et al., 1995). Interestingly, activation of these factors as well as suppression of Oct-4 mRNA levels was shown to occur only at high ATRA doses (>100 nM) (Pikarsky et al., 1994; Schoorlemmer et al., 1995), and similar mechanisms could be involved also in the present study.

Other two structurally related PAHs had variable effects. While Phe showed biphasic effects only when co-exposed with 32 nM ATRA after shorter 6 h exposure, N-heterocyclic analog 1,7-Pht

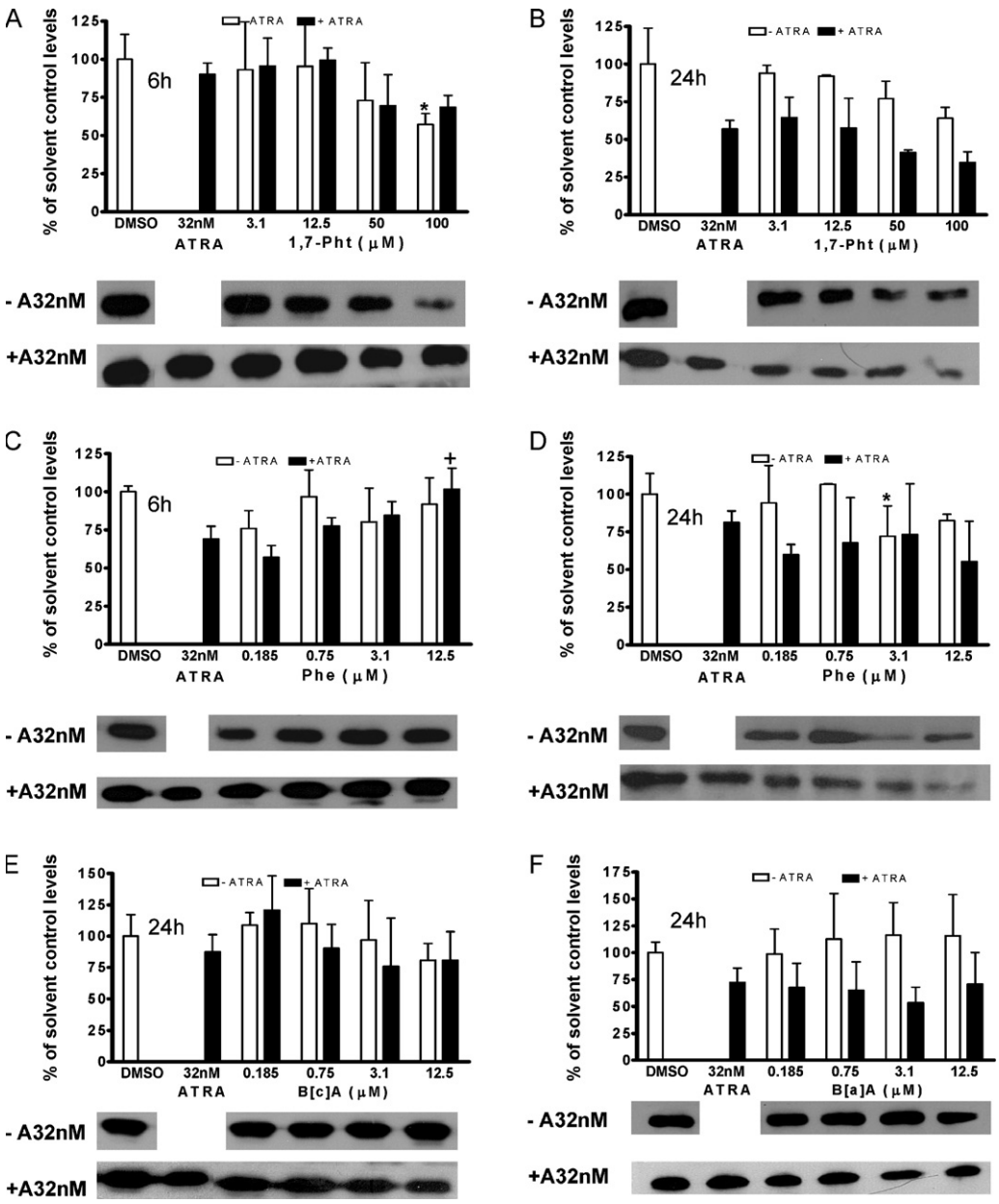

Fig. 3. Modulation of Oct-4 protein levels in P19 cells by selected PAHs and N-PAHs tested alone or in co-exposure with all-trans retinoic acid (ATRA 32 nM). Data in graphs are normalized to housekeeping protein Lamin B. Each column is the mean + standard deviation of at least three independent experiments. B[a]A – Benz[a]anthracene; B[c]A – Benz[c]acridine; 1,7-Pht – 1,7-phenanthroline; Phe – phenanthrene; * – significantly different from solvent control; + – significantly different from ATRA 32 nM.

systematically interacted with ATRA within a broad range of concentrations and exposure times. 1,7-Pht downregulated Oct-4 protein levels in a dose-dependent manner, while Phe showed mostly weak biphasic effect (Fig. 3). Although these findings might indicate that 1,7-Pht acts as a competitive antagonist of RAR, the interpretation could be more complicated. Study of Wilson et al. (2002) showed that competitive antagonists of nuclear receptors may cause inhibitions only when concentrations of natural ligands (ATRA in our case) are generally low. Much higher antagonist concentrations are necessary to modulate effects of natural ligands close to the plateau effect. Because suppressing effects of 1,7-Pht were similar within a whole range of concentrations of the natural ligand (ATRA), it seems that other mechanism than competitive inhibition are involved. Modulations of RAR/RXR, SF1 or COUP-TF1 could play a role as they were shown to control both ATRA-mediated transcription as well as Oct-4 levels (Barnea and Bergman, 2000; Benshushan et al., 1995; Pikarsky et al., 1994).

Another factor involved in Oct-4 regulation by ATRA is a testicular receptor TR2 (an orphan nuclear receptor). While non-modified TR2 activates Oct-4 gene, its SUMOylated form represses Oct-4. ATRA stimulates post-transcriptional modification (SUMOylation) of TR2 through the activation of extracellular signal-regulated kinases (ERKs) and subsequent TR2 phosphorylation (Gupta et al., 2008). Number of PAHs was also shown to activate ERKs (Rummel et al., 1999; Upham et al., 2008), and thus this mechanism could be at least partly responsible for the effects of PAHs/N-PaHs observed in the present study.

The importance of Oct-4 for pluripotency maintenance and its regulation during differentiation was confirmed both *in vitro* (Hough et al., 2006; Niwa et al., 2000) and *in vivo* (Pesce and Scholer, 2001). Interestingly, there are not many studies addressing modulation of Oct-4 by environmental pollutants or xenobiotics. For example, nicotine suppressed Oct-4 in human embryonic stem cells (Zdravkovic et al., 2008) but stimulated Oct-4 mRNA lev-

els in murine embryonic stem cells (Zhang et al., 2005). Nicotine was also found to inhibit ATRA-mediated RARβ expression in lung cancer cells via orphan receptor TR3 and COUP-TF, mechanisms discussed above (Chen et al., 2002). Another chemical that strongly downregulated Oct-4 levels was antidepressant drug fluoxetine, a suspected teratogen, which modulate multiple differentiation cellular processes (Kusakawa et al., 2008). *In vitro* effects of PAHs observed in the present study (e.g. suppression of Oct-4 by 1,7-Pht by almost 50%) may have direct effects on embryonal processes, because similar Oct-4 suppressions were shown to inhibit differentiation of pluripotent cells into the trophectoderm (Hough et al., 2006).

In conclusion, studied PAHs and N-PAHs interfered with the action of ATRA in the co-exposure experiments using P19/A15 cell reporter gene assay. Interestingly, when using higher concentrations of the natural ligand ATRA, lower concentrations of tested PAHs were sufficient to suppress the ATRA-induced effects. B[a]A and B[c]A, which stimulated ATRA-mediated effects in the reporter gene test, had weak effect on the Oct-4 protein levels in co-exposures with ATRA. In contrast, 1,7-Pht significantly downregulated Oct-4 and it also inhibited ATRA-mediated response in the P19/A15 reporter gene assay. Phenanthrene, a parental PAH of 1,7-Pht had biphasic and less pronounced effects. Observed *in vitro* effects of PAHs and N-PAHs may have deleterious effects on the differentiation and embryonic development, and further research is needed to fully explore underlying toxicity mechanisms.

Conflicts of interest

Authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.toxlet.2010.11.011.

References

Abbott, B.D., Birnbaum, L.S., 1989. Cellular alterations and enhanced induction of cleft-palate after coadministration of retinoic acid and TCDD. Toxicology and Applied Pharmacology 99, 287–301.

Barnea, E., Bergman, Y., 2000. Synergy of SF1 and RAR in activation of Oct-3/4 promoter. Journal of Biological Chemistry 275, 6608–6619.

Bastien, J., Rochette-Egly, C., 2004. Nuclear retinoid receptors and the transcription of retinoid-target genes. Gene 328, 1–16.

Benisek, M., Blaha, L., Hilscherova, K., 2008. Interference of PAHs and their N-heterocyclic analogs with signaling of retinoids in vitro. Toxicology in Vitro 22, 1909–1917.

Benshushan, E., Sharir, H., Pikarsky, E., Bergman, Y., 1995. A dynamic balance between ARP-1/COUP-TFII, EAR-3/COUP-TFI, and retinoic acid receptor retinoid-X-receptor heterodimers regulates Oct-3/4 expression in embryonal carcinoma-cells. Molecular and Cellular Biology 15, 1034–1048.

Billiard, S.M., Meyer, J.N., Wassenberg, D.M., Hodson, P.V., Di Giulio, R.T., 2008. Nonadditive effects of PAHs on early vertebrate development: mechanisms and implications for risk assessment. Toxicological Sciences 105, 5–23.

Blomhoff, R., Blomhoff, H.K., 2006. Overview of retinoid metabolism and function. Journal of Neurobiology 66, 606–630.

Breems-de Ridder, M.C., Lowenberg, B., Jansen, J.H., 2000. Retinoic acid receptor fusion proteins: friend or foe. Molecular and Cellular Endocrinology 165, 1–6.

Brehm, A., Ovitt, C.E., Scholer, H.R., 1998. Oct-4: more than just a POUerful marker of the mammalian germline? Apmis 106, 114–124.

Buryskova, B., Hilscherova, K., Blaha, L., Marsalek, B., Holoubek, I., 2006. Toxicity and modulations of biomarkers in *Xenopus laevis* embryos exposed to polycyclic aromatic hydrocarbons and their N-heterocyclic derivatives. Environmental Toxicology 21, 590–598.

Chen, G.Q., Lin, B.Z., Dawson, M.I., Zhang, X.K., 2002. Nicotine modulates the effects of retinoids on growth inhibition and RAR beta expression in lung cancer cells. International Journal of Cancer 99, 171–178.

Eifert, C., Sangster-Guity, N., Yu, L.M., Chittur, S.V., Perez, A.V., Tine, J.A., McCormick, P.J., 2006. Global gene expression profiles associated with retinoic acid-induced differentiation of embryonal carcinoma cells. Molecular Reproduction and Development 73, 796–824.

Faherty, S., Kane, M.T., Quinlan, L.R., 2005. Self-renewal and differentiation of mouse embryonic stem cells as measured by Oct4 gene expression: effects of LIF, serum-free medium, retinoic acid, and dbcAMP. In Vitro Cellular & Developmental Biology-Animal 41, 356–363.

Fallone, F., Villard, P.H., Seree, E., Rimet, O., Nguyen, Q.B., Bourgarel-Rey, W., Fouchier, F., Barra, Y., Durand, A., Lacarelle, B., 2004. Retinoids repress Ah receptor CYP1A1 induction pathway through the SMRT corepressor. Biochemical and Biophysical Research Communications 322, 551–556.

Feng, Y.C., Shi, G.L., Wu, J.H., Wang, Y.Q., Zhu, T., Dai, S.G., Pei, Y.Q., 2007. Source analysis of particulate-phase polycyclic aromatic hydrocarbons in an urban atmosphere of a northern city in China. Journal of the Air & Waste Management Association 57, 164–171.

Fields, A.L., Soprano, D.R., Soprano, K.J., 2007. Retinoids in biological control and cancer. Journal of Cellular Biochemistry 102, 886–898.

Freyberger, A., Schmuck, G., 2005. Screening for estrogenicity and anti-estrogenicity: a critical evaluation of an MVLN cell-based transactivation assay. Toxicology Letters 155, 1–13.

Gupta, P., Ho, P.C., Huq, M., Ha, S.G., Park, S.W., Khan, A.A., Tsai, N.P., Wei, L.N., 2008. Retinoic acid-stimulated sequential phosphorylation, PML recruitment, and SUMOylation of nuclear receptor TR2 to suppress Oct4 expression. Proceedings of the National Academy of Sciences of the United States of America 105, 11424–11429.

Hough, S.R., Clements, I., Welch, P.J., Wiederholt, K.A., 2006. Differentiation of mouse embryonic stem cells after RNA interference-mediated silencing of OCT4 and Nanog. Stem Cells 24, 1467–1475.

Jung, D.K.J., Klaus, T., Fent, K., 2001. Cytochrome P450 induction by nitrated polycyclic aromatic hydrocarbons, azaarenes, and binary mixtures in fish hepatoma cell line PLHC-1. Environmental Toxicology and Chemistry 20, 149–159.

Kaji, K., Norrby, K., Paca, A., Mileikovsky, M., Mohseni, P., Woltjen, K., 2009. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature.

Kusakawa, S., Yamauchi, J., Miyamoto, Y., Sanbe, A., Tanoue, A., 2008. Estimation of embryotoxic effect of fluoxetine using embryonic stem cell differentiation system. Life Sciences 83, 871–877.

Li, J., Ma, M., Wang, Z.J., 2008. A two-hybrid yeast assay to quantify the effects of xenobiotics on retinoid X receptor-mediated gene expression. Toxicology Letters 176, 198–206.

Lindenau, A., Fischer, B., 1996. Embryotoxicity of polychlorinated biphenyls (PCBs) for preimplantation embryos. Reproductive Toxicology 10, 227–230.

Love, J.M., Gudas, L.J., 1994. Vitamin-A, differentiation and cancer. Current Opinion in Cell Biology 6, 825–831.

Maden, M., 1999. The role of retinoic acid in embryonic: and post-embryonic development. In: Summer Meeting of the Nutrition-Society, Glasgow, Scotland, pp. 65–73.

McBurney, M.W., Jonesvilleneuve, E.M.V., Edwards, M.K.S., Anderson, P.J., 1982. Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell-line. Nature 299, 165–167.

McSorley, L.C., Daly, A.K., 2000. Identification of human cytochrome P450 isoforms that contribute to all-trans-Retinoic Acid 4-hydroxylation. Biochemical Pharmacology 60, 517–526.

Minucci, S., Leid, M., Toyama, R., SaintJeannet, J.P., Peterson, V.J., Horn, V., Ishmael, J.E., Bhattacharyya, N., Dey, A., Dawid, I.B., Ozato, K., 1997. Retinoid X receptor (RXR) within the RXR-retinoic acid receptor heterodimer binds its ligand and enhances retinoid-dependent gene expression. Molecular and Cellular Biology 17, 644–655.

Niwa, H., Miyazaki, J., Smith, A.G., 2000. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nature Genetics 24, 372–376.

Novak, J., Benisek, M., Hilscherova, K., 2008. Disruption of retinoid transport, metabolism and signaling by environmental pollutants. Environment International 34, 898–913.

Novak, J., Benisek, M., Pachernik, J., Janosek, J., Sidlova, T., Kiviranta, H., Verta, M., Giesy, J.P., Blaha, L., Hilscherova, K., 2007. Interference of contaminated sediment extracts and environmental pollutants with retinoid signaling. Environmental Toxicology and Chemistry 26, 1591–1599.

Pachernik, J., Bryja, V., Esner, M., Kubala, L., Dvorak, P., Hampl, A., 2005. Neural differentiation of pluripotent mouse embryonal carcinoma cells by retinoic acid: inhibitory effect of serum. Physiological Research 54, 115–122.

Pesce, M., Scholer, H.R., 2001. Oct-4: gatekeeper in the beginnings of mammalian development. Stem Cells 19, 271–278.

Pikarsky, E., Sharir, H., Benshushan, E., Bergman, Y., 1994. Retinoic acid represses Oct-3/4 gene-expression through several retinoic acid-responsive elements located in the promoter-enhancer region. Molecular and Cellular Biology 14, 1026–1038.

Rummel, A.M., Trosko, J.E., Wilson, M.R., Upham, B.L., 1999. Polycyclic aromatic hydrocarbons with bay-like regions inhibited gap junctional intercellular com-

munication and stimulated MAPK activity. Toxicological Sciences 49, 232–240.
Santodonato, J., 1997. Review of the estrogenic and antiestrogenic activity of polycyclic aromatic hydrocarbons: relationship to carcinogenicity. Chemosphere 34, 835–848.
Schoorlemmer, J., Jonk, L., Shen, S.B., vanPuijenbroek, A., Feijen, A., Kruijer, W., 1995. Regulation of Oct-4 gene expression during differentiation of EC cells. Molecular Biology Reports 21, 129–140.
Sovadinova, I., Blaha, L., Janosek, J., Hilscherova, K., Giesy, J.P., Jones, P.D., Holoubek, I., 2006. Cytotoxicity and aryl hydrocarbon receptor-mediated activity of N-heterocyclic polycyclic aromatic hydrocarbons: Structure-activity relationships. Environmental Toxicology and Chemistry 25, 1291–1297.
Takahashi, K., Yamanaka, S., 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663–676.
Thier, M., Roeb, E., Breuer, B., Bayer, T.A., Halfter, H., Weis, J., 2000. Expression of matrix metalloproteinase-2 in glial and neuronal tumor cell lines: inverse correlation with proliferation rate. Cancer Letters 149, 163–170.
Tzimas, G., Nau, H., 2001. The role of metabolism and toxicokinetics in retinoid teratogenesis. Current Pharmaceutical Design 7, 803–831.
Upham, B.L., Blaha, L., Babica, P., Park, J.S., Sovadinova, I., Pudrith, C., Rummel, A.M., Weis, L.M., Sai, K., Tithof, P.K., Guzvic, M., Vondracek, J., Machala, M., Trosko, J.E., 2008. Tumor promoting properties of a cigarette smoke prevalent polycyclic aromatic hydrocarbon as indicated by the inhibition of gap junctional intercellular communication via phosphatidylcholine-specific phospholipase C. Cancer Science 99, 696–705.
Widerak, M., Ghoneim, C., Dumontier, M.F., Quesne, M., Corvol, M.T., Savouret, J.F., 2006. The aryl hydrocarbon receptor activates the retinoic acid receptor alpha through SMRT antagonism. Biochimie 88, 387–397.
Wilson, V.S., Bobseine, K., Lambright, C.R., Gray, L.E., 2002. A novel cell line, MDA-kb2, that stably expresses an androgen- and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists. Toxicological Sciences 66, 69–81.
Zdravkovic, T., Genbacev, O., LaRocque, N., McMaster, M., Fisher, S., 2008. Human embryonic stem cells as a model system for studying the effects of smoke exposure on the embryo. Reproductive Toxicology 26, 86–93.
Zhang, H.P., Guo, D.X., Wang, L.Y., Zhao, Y.X., Cheng, Y., Qiao, Z.D., 2005. Effect of nicotine on Oct-4 and Rex-1 expression of mouse embryonic stem cells. Reproductive Toxicology 19, 473–478.

Článek VIII:

Hilscherova, K., Jones, P.D., Gracia, T., Newsted, J.L., Zhang, X., Sanderson, J.T., Yu, R., Wu, R., Giesy, J.P., 2004. Assessment of the effects of chemicals on the expression of ten steroidogenic genes in the H295R cell line using real-time PCR. Toxicological Sciences 81 (1), 78-89.

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Assessment of the Effects of Chemicals on the Expression of Ten Steroidogenic Genes in the H295R Cell Line Using Real-Time PCR

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The potential for a variety of environmental contaminants to disturb endocrine function in wildlife and humans has been of recent concern. While much effort is being focused on the assessment of effects mediated through steroid hormone receptor–based mechanisms, there are potentially several other mechanisms that could lead to endocrine disruption. Recent studies have demonstrated that a variety of xenobiotics can alter the gene expression or activity of enzymes involved in steroidogenesis. By altering the production or catalytic activity of steroidogenic or steroid-catabolizing enzymes, these chemicals have the potential to alter the steroid balance in organisms. To assess the potential of chemicals to alter steroidogenesis, an assay system was developed using a human adrenocortical carcinoma cell line, the H295R cell line, which retains the ability to synthesize most of the important steroidogenic enzymes. Methods were developed, optimized, and validated to measure the expression of 10 genes involved in steroidogenesis by the use of real-time quantitative reverse transcriptase PCR. The effects of several model chemicals known to alter steroid metabolism, both inducers and inhibitors, were assessed. Similar expression patterns were observed for chemicals acting through common mechanisms of action. Time-course studies demonstrated distinct time-dependent expression profiles for chemicals able to modulate steroid metabolism. The assay, which allows simultaneous analysis of the expression of numerous steroidogenic enzymes, would be useful as a sensitive and integrative screen for the many effects of chemicals on steroidogenesis.

Key Words: **steroidogenesis; bioassay; xenoestrogens; screening.**

Recently there has been much interest in the effects of endocrine disrupters on wildlife (Ankley *et al*., 1998) and humans (Kavlock *et al*., 1996). The Safe Drinking Water Act Amendments of 1995 and the Food Quality Protection Act of 1996 mandate screening for endocrine-disrupting properties of chemicals in drinking water or pesticides used in food production. In response to this legislation, the federal Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) recommended that chemicals be screened as agonists or antagonists of estrogen (ER), androgen (AR), and thyroid (ThR) hormone receptors (EDSTAC Final Report, 1998).

One type of endocrine disruption takes place when xenobiotics mimic steroid hormones. Of particular concern have been those compounds that mimic endogenous estrogens, sometimes called xenoestrogens. While some reports indicated that endocrine disruption functioned through this mechanism of action, subsequent studies have found that some compounds have more complex mechanisms of action. It has been observed that some compounds can bind to the androgen receptor and function as either androgen agonists or antagonists. Although the effects of endocrine-disrupting chemicals (EDCs) and methods to screen for them have focused on direct interactions with steroid hormone receptors such as ER, AR, and ThR, EDCs can operate several different ways. Firstly, there are several other receptor-mediated processes that control sexual development and homeostasis. Secondly, there are also some nonreceptor-mediated mechanisms. Finally, there are compounds that can modulate steroid hormone production or breakdown and cause endocrine disruption without acting as hormone mimics. These effects are often exerted indirectly via various effects on common signal transduction pathways or by acting on steroid metabolism pathways.

One such example is the effect of the herbicide atrazine. Atrazine has been observed to cause estrogenic effects both *in vitro* and *in vivo* but does not bind to the estrogen receptor (Connor *et al*., 1996; Sanderson *et al*., 1999, 2000, 2001). While the effects observed *in vitro* occurred at relatively great concentrations, these results serve as an example of the types of effects that can be observed with *in vitro* tests. The family of 2-chloro-*s*-triazine herbicides had a common ability to induce the catalytic activity and mRNA levels of *CYP19* using the H295R cell line as a steroidogenic model system (Sanderson *et al*., 2000, 2001). The H295R (a subpopulation of H295 that forms a monolayer in culture) human adrenocortical carcinoma cell line has been

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characterized in detail and shown to express most of the key enzymes involved in steroidogenesis (Gazdar *et al*., 1990; Rainey *et al*., 1993; Staels *et al*., 1993). Sanderson and coworkers suggested that the effects they observed in the H295R cells occurred by the inhibition of phosphodiesterase with a concomitant increase in cyclic-AMP. The model compound 8-bromo-c-AMP also resulted in the upregulation of *CYP19* (aromatase) mRNA.

While this mechanism may not be operating *in vivo* at all times in all tissues of all species or at relevant environmental concentrations, it is a plausible explanation for the observation that atrazine induced luciferase activity under the control of the ER in MVLN cells (MCF-7-*luc*, MVLN; Villeneuve *et al*., 1998). However, experiments demonstrating the expression of aromatase in this cell line have yielded equivocal results. Thus, in addition to other indirect mechanisms of action, it is possible that natural and synthetic chemicals can modulate the endocrine system by acting as direct or indirect stimulators or inhibitors of the enzymes involved in the production, transformation, and or elimination of steroid hormones. Here we present a procedure for screening for the effects of chemicals on the profile of expression of steroidogenic genes. Specifically, we report methods to simultaneously measure mRNA concentrations for 10 steroidogenic enzymes and two housekeeping genes in cultured H295R cells.

The key genes measured in the current study include *CYP11A* (cholesterol side-chain cleavage); *CYP11B1* (steroid 11β-hydroxylase); *CYP11B2* (aldosterone synthetase); *CYP17* (steroid 17α-hydroxylase and/or 17,20 lyase); *CYP19* (aromatase); *17βHSD1*, *17βHSD4*, *CYP21B2* (steroid 21-hydroxylase), and *3βHSD2* (3β-hydroxysteroid dehydrogenase); *HMGR* (hydroxymethylgutaryl CoA reductase); and the cholesterol transfer protein *StAR* (steroid acute regulatory protein). The H295R cells used have the physiological characteristics of zonally undifferentiated human fetal adrenal cells, with the ability to produce the steroid hormones of each of the three phenotypically distinct zones found in the adult adrenal cortex (Fig. 1; Gazdar *et al*., 1990; Staels *et al*., 1993). Since the cells maintain the ability to express these genes and produce these enzymes, which might otherwise only be expressed in certain tissues or periods of ontogeny, they are a useful model system for potential effects on steroidogenesis.

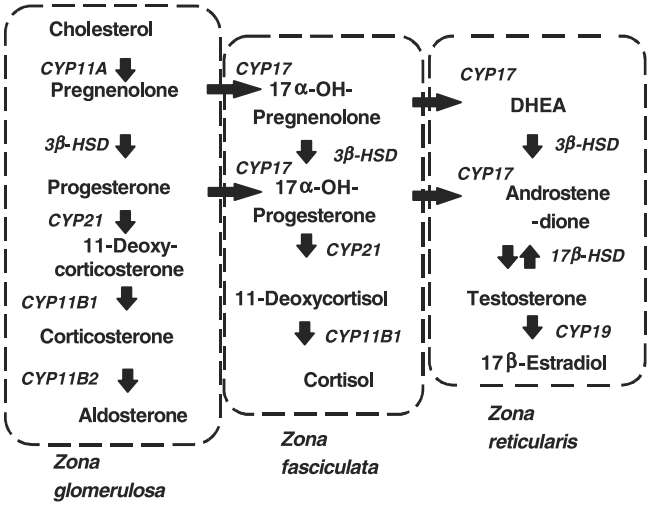

FIG. 1. Schematic representation of the steps involved in steroid hormone synthesis and the tissue localization of the reactions within the adrenal gland.

MATERIALS AND METHODS

Forskolin, 8BrcAMP, Phorbol-12-myristate-13-acetate (PMA), lovastatin, ketoconazole, aminoglutethimide, androstenedione, and spironolactone were obtained from Sigma Chemical Co. (St. Louis, MO). Metyrapone was from Aldrich (St. Louis, MO), and daidzein was from ICN Biochemicals Inc. (Aurora, OH). The chemicals used in this study were chosen based on their variety of known effects on steroid metabolism. That is, aminoglutethimide is an aromatase inhibitor; lovastatin is metabolized to produce a specific hydroxymethylglutaryl-CoA reductase (HMGR) inhibitor; 8BrcAMP and forskolin increase cellular cAMP concentrations; PMA is a diacylglycerol analogue that activates protein kinase C; ketoconazole works principally by the inhibition of cytochrome P450 14a-demethylase (*P45014DM*); and daidzein is a weak estrogen receptor agonist.

The H295R human adrenocortical carcinoma cell lines were obtained from the American Type Culture Collection (ATCC CRL-2128; ATCC, Manassas,VA) and were grown in 75 cm^2 flasks with 12.5 ml of supplemented medium at 37°C with a 5% CO_2 atmosphere. Supplemented medium was a 1:1 mixture of Dulbecco's modified Eagle's medium with Ham's F-12 Nutrient mixture with 15 mM HEPES buffer. The medium was supplemented with 1.2 g/l Na_2CO_3, ITS + Premix (1 ml Premix/100 ml medium), and 12.5 ml/500 ml NuSerum (BD Bioscience, San Jose, CA). Final component concentrations in the medium were as follows: 15 mM HEPES, 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenium, 1.25 mg/ml bovine serum albumin, 5.35 μg/ml linoleic acid, and 2.5% NuSerum. The medium was changed two to three times per week and cells were detached from flasks for subculturing by use of trypsin/EDTA (Sterile 1× Trypsin-EDTA; Life Technologies Inc., Grand Island, NY). Cells were exposed to chemicals of interest in 6-well Tissue Culture Plates (Nalgene Nunc Inc., Rochester, NY). Cells were dosed with chemicals dissolved in DMSO for 48–72 h after plating.

RNA isolation. Before nucleic acid isolation and analysis, cell viability was determined. Cells were visually inspected under a microscope to evaluate viability and cell numbers. Also, cell viability was determined with the Live/Dead cell viability kit (Molecular Probes, Eugene, OR). Cell death was only observed for 17α-Ethynylestradiol and lovastatin at concentrations greater than 30 μM; ketoconazole and cyproterone acetate inhibited cell growth at concentrations greater than 30 μM. No adverse effects on cell growth or viability were observed for any of the tested chemicals at maximum concentrations ranging from 30 to 100 μM. Exposures in which either cell death or decreased viability was observed were not used for gene expression analysis.

After removal of the medium, cells were lysed in the culture plate by the addition of 580 μl/well of Lysis Buffer-β-ME mixture (Stratagene, La Jolla, CA). Cells were mixed and collected by repeated pipetting and transferred to a microcentrifuge tube that was mixed to homogenize and ensure low viscosity of the lysate. After mixing, the homogenate was transferred to a prefilter spin cup seated in a 2-ml tube and was centrifuged in a microcentrifuge for 5 min. The spin cup was removed from the receptacle tube and discarded. For RNA isolation, 700 μl of 70% ethanol was added to the filtrate and the tube was vortexed to mix thoroughly. Half of the mixture was transferred to an RNA binding spin cup seated in a fresh 2-ml tube and this was then centrifuged for 1 min. The spin cup was removed and retained and the filtrate was discarded. This procedure was repeated with the same spin cup using the second half of the sample.

To remove residual DNA prior to reverse transcription, DNase treatment was used; 600 µl of 1× low-salt wash buffer were added to the spin cup containing the RNA, this was centrifuged for 1 min, and the filtrate was discarded. Next, 55 µl of RNase Free-DNase I solution (Stratagene) were added to the fiber matrix inside the spin cup. The sample was incubated at 37°C for 15 min. The sample was then washed with 600 µl of 1× high-salt wash buffer and 600 µl of 1× low-salt wash buffer, centrifuged at maximum speed for 30–60 s and discarding the filtrate after each wash. A final wash was done by adding 300 µl of 1× low-salt wash buffer to the spin cup, and the tube was centrifuged for 2 min to dry the fiber matrix. The spin cup was transferred to a fresh 1.5-ml microcentrifuge tube and 80 µl of nuclease-free water was added directly onto the center of the fiber matrix inside the spin cup. The tube was incubated for 2 min at room temperature before centrifugation for 1 min. This elution step was repeated to maximize the yield of RNA. The purified RNA was used immediately for RT-PCR or was stored at −80°C until analysis.

An appropriate dilution of the RNA sample (1:50) was prepared for RNA quantitation. The absorbance of the RNA solution was measured at 260 and 280 nm and the 260/280 ratio was calculated. The concentration of total RNA was estimated using the A_{260} value and a standard with an A_{260} of 1 that was equivalent to 40 µg RNA/ml.

cDNA preparation. Total RNA (1–5 µg) was combined with 50 µM oligo-$(dT)_{20}$ and 10 mM dNTPs diethylpyrocarbamate- (DEPC-) treated water to a final volume of 12 µl. RNA and primers were denatured at 65°C for 5 min and then incubated on ice for 5 min. Reverse transcription was performed using 8 µl of a master mix containing the following: 5× cDNA synthesis buffer, 0.1 M DTT, RNase OUT 40 U/µl, Cloned AMV Reverse Transcriptase (Invitrogen, Carlsbad, CA), and DEPC-treated water. Reactions were incubated at 50°C for 45 min and were terminated by incubation at 85°C for 5 min. Samples were either used directly for PCR or were stored at −20°C until analysis.

Real-time PCR. Real-time PCR (quantitative PCR) was performed by using a Smart Cycler System (Cepheid, Sunnyvale, CA) in 25-µl sterile tubes using a master mix containing the following: 25 mM $MgCl_2$, 1 U/µl AmpErase (Applied Biosystems, Foster City, CA), 5 U/µl Taq DNA polymerase AmpliTaq Gold, 10X SYBR Green (PE Biosystems, Warrington, UK), nuclease-free water, and between 10 pg and 1 µg of cDNA. The Thermal Cycling program was 94°C for 10 min as follows: 50–60°C for 30 s to 1 min; 68–72°C for 1 min/kb followed by 35–40 cycles of 94°C for 15–40 s; 50–60°C for 30 s to 1 min; 68–72°C for 1 min/kb; and a final cycle of 94°C for 15–40 s, 50–60°C for 30 s to 1 min, and 72°C for 5–10 min. Melting curve analyses were performed immediately following the final PCR cycle to differentiate between the desired amplicons and any primer-dimers or DNA contaminants.

For quantification of PCR results, C_t (the cycle at which the fluorescence signal is first significantly different from background) was determined for each reaction. C_t values for each gene of interest were normalized by division by the C_t for the endogenous control gene to produce ΔC_t. Therefore, the difference between ΔC_t values for a control and a chemically exposed culture (designated $\Delta\Delta C_t$) represent the degree of induction or inhibition of the gene of interest. Moreover, the degree of induction or inhibition can be calculated as a fold difference using the following relationship:

$$X_{exp}/X_{con} = 2^{-\Delta\Delta Ct}$$

where X_{exp} and X_{con} represent the degree of expression in the exposed and control samples, respectively, and X_{exp}/X_{con}, therefore, represents the fold induction. All data are reported and were statistically analyzed as fold induction between exposed and control cultures. Gene expression was measured at least in triplicate for each control or exposed cell culture and each exposure was repeated at least three times.

Statistical analysis. Statistical analyses of gene expression profiles were conducted using SYSTAT 10 (SPSS Inc., Chicago, IL). Differences in gene expression were evaluated by ANOVA followed by Tukey's test. Differences with $p \leq 0.05$ were considered significant. Statistical analysis of sequence homologies between amplicons and the GenBank database were conducted using the BLAST algorithm on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/).

RESULTS

PCR Assay Procedures

Quantitative PCR (Q-RT-PCR) conditions, including sense and antisense primers, temperatures, times, and reagent concentrations were optimized for all the steroidogenic genes (Table 1). Each amplicon yielded a single peak when the melting temperature curve was analyzed at the conclusion of the PCR reaction (Fig. 2). To further confirm the identities of the amplified sequences, the PCR products were analyzed by agarose gel electrophoresis (Fig. 3). After optimization, each PCR reaction produced a single amplicon of the expected size. No additional bands or excessive levels of primer-dimer products were

TABLE 1
Optimal Conditions for Quantitative Reverse-Transcriptase Polymerase Chain Reaction

Gene	Product length	Annealing °C (s)*	Primer concentration (µM)	Sense primer	Antisense primer
18S rRNA	124	62 (60)	0.4	CGTCTGCCCTATCAACTTTCG	TGCCTTCCTTGGATGTGGTAG
β-*actin*	100	64 (60)	0.2	CACTCTTCCAGCCTTCCTTCC	AGGTCTTTGCGGATGTCCAC
CYP11A	137	62 (50)	0.4	GAGATGGCACGCAACCTGAAG	CTTAGTGTCTCCTTGATGCTGGC
CYP11B2	146	62 (50)	0.2	TCCAGGTGTGTTCAGTAGTTCC	GAAGCCATCTCTGAGGTCTGTG
CYP17	134	64 (60)	0.6	AGCCGCACACCAACTATCAG	TCACCGATGCTGGAGTCAAC
CYP19	128	64 (50)	0.4	AGGTGCTATTGGTCATCTGCTC	TGGTGGAATCGGGTCTTTATGG
CYP21	108	64 (50)	0.4	CGTGGTGCTGACCCGACTG	GGCTGCATCTTGAGGATGACAC
3βHSD2	95	60 (50)	0.4	TGCCAGTCTTCATCTACACCAG	TTCCAGAGGCTCTTCTTCGTG
17βHSD1	136	64 (60)	0.4	CTCCCTCTGACCAGCAACC	TGTGTCTCCCACGCAATCTC
17βHSD4	121	62 (50)	0.4	TGCGGGATCACGGATGACTC	GCCACCATTCTCCTCACAACTC
StAR	168	64 (40)	0.4	GTCCCACCCTGCCTCTGAAG	CATACTCTAAACACGAACCCCACC
HMGR	152	60 (50)	0.4	TGCTTGCCGAGCCTAATGAAAG	AGAGCGTTCGTGGGTCCATC

*All PCR reactions were extended at 72°C for 30 s and denatured at 95°C for 15 s.

detected in any of the amplified DNA samples. To definitively confirm the identity of the amplicons, the DNA sequence of each band was determined (Table 2). During amplicon sequencing, the initial sequence determination (i.e., the first 20–30 base pairs) can be unclear and this low-quality sequence was identified by the sequencing facility (Michigan State University, Macromolecular Structure Facility, personal communication). Thus, only the middle portion of the sequence is of sufficient quality to match. This is why the sequences determined are not the full length of the amplicon.

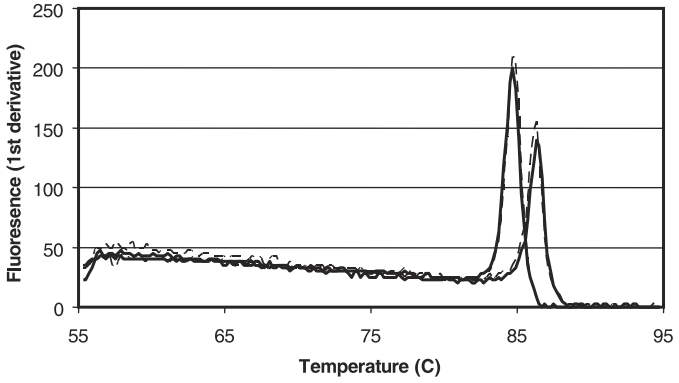

FIG. 2. Representative PCR product melting curves for *CYP17* and *StAR*. The lines represent the first derivative of fluorescence with varying temperature. The two curves with the melting temperature of 84.7°C are for *CYP17*. The two curves with a melting temperature of 86°C are for *StAR*.

The sequence of the amplicons showed a minimum of 89% homology to the desired target sequence and a minimum significance value (E-value) of 9×10^{-8}. The Expect value (E) is a parameter that describes the number of hits one can expect to observe by chance when searching a database of a particular size. The value decreases exponentially with the Score (S) that is assigned to a match between two sequences. The E value describes the random background noise that exists for matches between sequences. For example, an E value of 1 assigned to a ''hit'' can be interpreted as meaning that, in a database of the current size, one might expect to see one match with a similar score simply by chance. Hence, the smaller the E-value or the closer it is to 0, the more significant the match. The BLAST programs report E values rather than *p* values because it is easier to interpret the difference between, for example, E values of 5 and 10 than *p* values of 0.993 and 0.99995. However, when $E < 0.01$, *p* values and E values are nearly identical.

Due to the relatively short length of the amplified DNA, some bands could not be sequenced. While some differences were detected from the published sequences, these differences are not likely to be significant given that only a single sequence determination was conducted and the possibility that genetic variants different from the published sequences could occur.

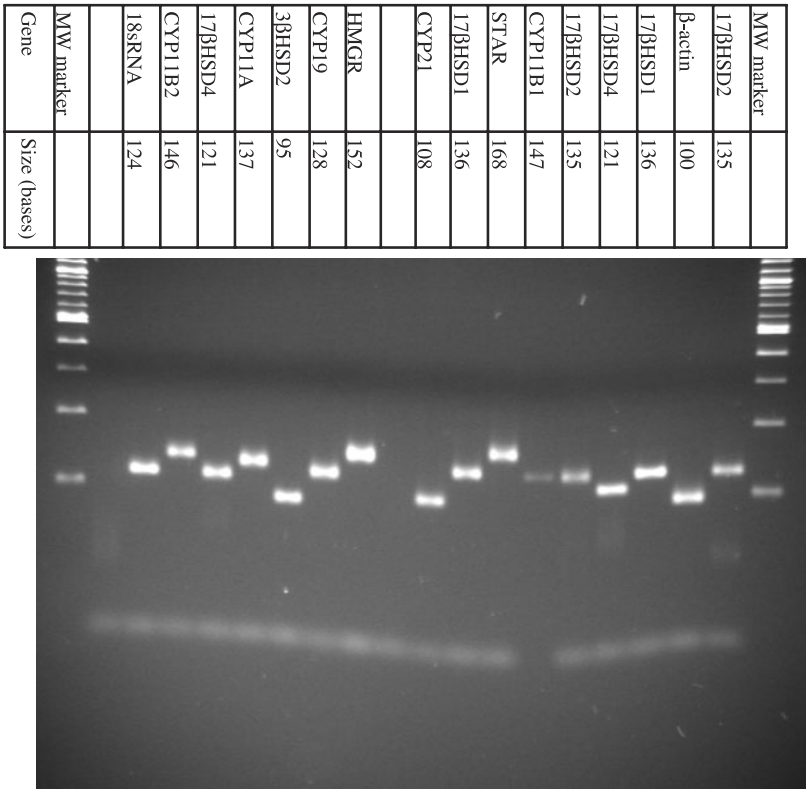

FIG. 3. Agarose gel electrophoresis of Q-RT-PCR products for the steroidogenic and housekeeping genes.

TABLE 2
Sequences of Amplicons for Steroidogenic Enzymes from H295R Cells Amplified by Q-RT-PCR

Target	Amplicon sequence*	Identities	E value
18S	CGGGGAATCAGGGTTCGATTCCGGATCGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGG	61/63	2e-22
β-*actin*	TCACTCCATCATGAAGTGTGACGTGTACATCCGCAAAGA	36/37	3e-9
CYP11A	CCGATGCTACAGCTGGTCCCCCTCCTCAAAGCCAGCATCAAGGAGACACTA	50/50	3e-19
CYP11B2	GCAGTGCAGCATGGGAAAGGAATAAGGGGGCAACAAGGTGCACAGACCTCAGAGATGGCT	60/60	4e-25
CYP17	CACAAGGCCAACGTTGACTCCAGCATCGGT	30/30	9e-8
CYP19	AGAGTTTGAGGGAGATCCAGTCGGTGAAGAAACCGTATCCATAAAGACCCGATTCCA	54/57	4e-16
CYP21	CGCCCTCCCTGCAGCCCCTGCCCCACTGCCGTGTCATCCTC	39/40	5e-11
17βHSD1	AAAGGAAGGCTTATCCTTGAGATTGCGTGGGAGACACAA	37/37	1e-11
17βHSD4	AGCCAGAGTATGTGGCACCTCTTGTCCTCTGGCTTTGTCACGAGAGTTGTGAGGAGAATGGTG	62/63	2e-24
HMGR	TCCTGTGGCCAGGAGGTTTGACTGAAACATTCACACAGGGCTCTTTGATGGACCCACGAACGC	63/65	2e-21
StAR	CCAGGAGAATCCCTACTGGAAGCCTGCAAGTCTAAGATCTCCATCTGGTGACAGTGGCATGGGT-GGGGTTCGTGTT	74/75	2e-31
CYP11B1	CTTGTCCCCAGCCCTACCTGGCCACTTTCTCCAGCAAGCACTGTCCTCTGGGCAGTTTGCACCCA-TCCCTCCCAGT	73/76	6e-25
3βHSD2	TGCTTTGTGCAGTATCTGGATGCGNTGGGCTTGATGTATTTGCCGGAGTCTTGAATGAAAAGGG-ACCAGGAGCTGAGGAATTGCNAANAACCTGCTCTCCGC	93/104	1e-21

Note. See text for discussion of the statistical significance of the E value. *All sequences are listed s 5′–3′.

The PCR methods were also optimized to ensure optimum efficiency (100%) over a range of tested RNA concentrations. Relative efficiencies were also determined to ensure that quantification of sequences of interest relative to housekeeping genes would remain constant even at a wide range of relative message concentrations (Fig. 4). The determination of all sequences of interest could be achieved quantitatively with 100% efficiency over a range of at least four orders of magnitude.

Chemical Exposure Results

In Exposure 1, H295R cells were exposed to several model inducers for 24 h. At the end of 24 h, relative responses of 10 genes involved in the steroidogenic pathway were evaluated and compared to negative controls that were analyzed along with the treated cells (Fig. 5). The levels of gene expression in blank and solvent control cell cultures were remarkably consistent when normalized to the housekeeping genes β-actin (Table 3) or 18S ribosomal RNA (Table 4). However, an evaluation of the coefficients of variation (CV) for blanks indicated that the variability in gene expression associated with 18S RNA–normalized data were greater than those associated with data normalized to β-actin. To evaluate the amount of variability associated with the housekeeping genes, separate from that associated with the measurement steroidogenic genes, a comparison of C_t values for 18S RNA and β-actin among all treatments was conducted with blank data from Exposure 1. Results of the data analysis indicated that the variability associated with 18S RNA (average CV of 26%) was greater than the variability associated with β-actin (average CV of 2.1%). Also, the coefficients of variation for 18S RNA ranged from 0.52 to 115% while for β-actin the range was 0.95 to 3.94%. This result demonstrates that gene expression data normalized to 18S RNA would incorporate additional sources of variability not associated with the measurement of specific genes.

Treatment of H295R cells with model inducers resulted in significant changes in gene expression (Fig. 5). Treatment of H295R cells with forskolin and 8BrcAMP resulted in significant increases in expression of *CYP17*, *CYP21*, *CYP11A*, *3*β-*HSD2*, *StAR*, and *CYP11B2* as normalized by β-actin. Also, treatment with 8BrcAMP resulted in a significant increase in *CYP19* gene expression. Of the genes that were significantly altered, *CYP11B2* was induced to the greatest extent (>15-fold increase in cells treated with forskolin or 8BrcAMP). PMA treatment of H295R cells resulted in statistically significant increases in *CYP21* and *CYP19* gene expression, while lovastatin did not significantly alter the expression of any steroidogenic genes. However, while *CYP11B2* expression was altered by PMA, the alteration in gene expression was not significantly different from that of the solvent control. Treatment of the cells with PMA also resulted in a decrease in the expression of *CYP11A* (3.3-fold), *CYP17* (10.9-fold), and *HMGR* (2.9-fold), but none of these reductions were statistically significant. In contrast to the differences in gene expression observed with data normalized to β-actin, no statistically significant differences were noted for treatments where gene expression was normalized to 18S RNA. The relatively great variability in measured 18S RNA activity in both the controls and treated cells masked any alterations in gene expression due to chemical treatment (Table 4). Thus, while there was a 47-fold increase in *3*β*HSD2* in 8BrcAMP-treated cells, compared to a 12-fold increase noted in β-actin–normalized data, normalization of expression to 18S RNA introduced variability into the data and resulted in no significant differences.

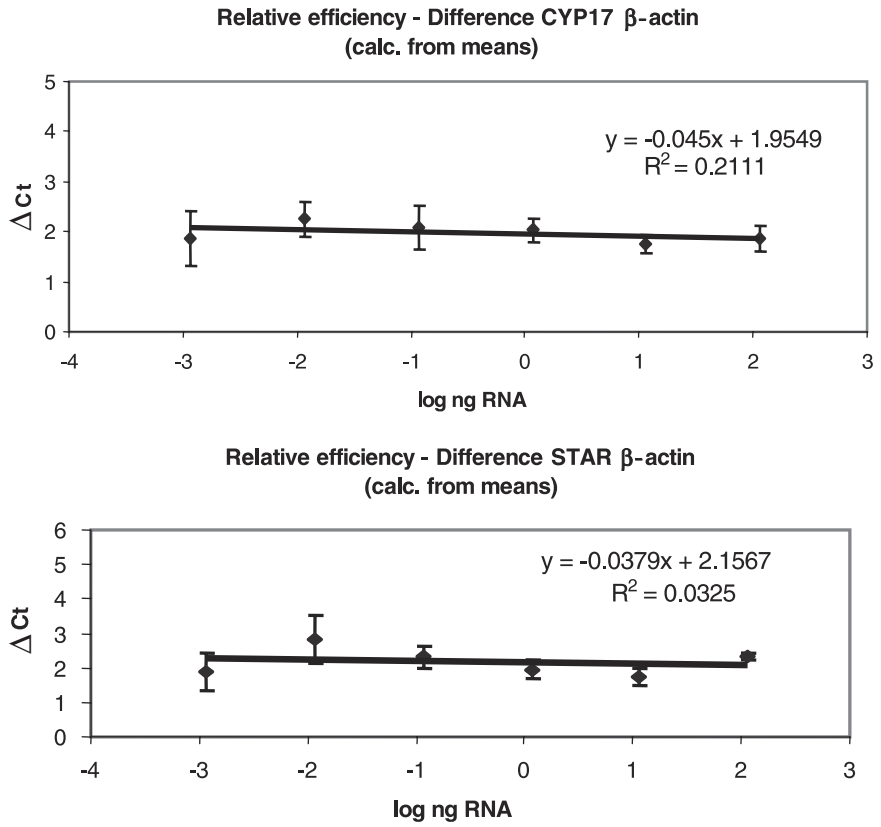

FIG. 4. Representative PCR efficiency diagrams for two of the steroidogenic genes, *CYP17* (upper) and *StAR* (lower). See text for methods of calculation.

To further elucidate the effects of forskolin and PMA on gene expression, cells were exposed to different concentrations of these compounds over time periods up to 48 h (Fig. 6). In general, treatment with PMA resulted in greater alteration in gene expression at 12 than at 24 h for both 10 and 40 nM PMA. As was observed in Exposure 1, PMA reduced the expression of *CYP11A* and *CYP17*; the inhibition of *CYP17* was not apparent until 24 h, whereas the reduction of *CYP11A* was initially evident at 12 h and continued on to 24 h. In cells treated with 10 nM PMA, the expression of *CYP11A* was somewhat greater at 24 than at 12 h. Furthermore, when *CYP11A* levels at 24 h in the 10 nM-PMA group were compared to levels at 12 and 24 h in the 40-nM PMA treatment group, no significant differences were observed. These results suggest some recovery for this gene may have occurred, but the exact mechanism of this recovery is unknown at this time. The most significant effect of exposure to PMA was the large increase in *CYP19* and *3βHSD2* gene expression at 12 h for both the tested concentrations. The concentration of *CYP19* mRNA was increased 240- and 274-fold by 10 and 40 nM PMA, respectively. Also, *13βHSD2* gene expression was increased 43.2- and 23-fold by 10 and 40 μM PMA, respectively. The expression of these genes was approximately 10-fold less at 24 h than it was at 12 h, with the expression levels of both genes being less than 1.5-fold different between concentrations. This general pattern of greater gene expression at 12 compared to 24 h occurred for most of the genes analyzed. Furthermore, at 24 h there was little difference in gene expression between cells treated with 10 or 40 nM PMA for genes monitored in the exposure. The consistency of this result among genes and between concentrations as well as to the results of the previous exposures adds to the validity of the great levels of mRNA induction observed.

Time- and concentration-dependent changes in gene expression were observed in cells treated with forskolin (Fig. 6). While gene expression at both doses tended to be greater at 12 h than at 24 h, like that observed with PMA, expression at 48 h for some of the genes returned to the levels measured at 12 h. Thus, for genes such as *CYP17*, *CYP11A*, and *StAR*, this resulted in measured gene expression that resembled an inverted time-response curve.

As was observed in Exposure 1, *3βHSD2*, *CYP11B2*, and *CYP19* were the genes for which expression was increased to the greatest extent over the three time periods. However, several specific time- and concentration-related differences in gene expression were noted among these three genes. For instance, a 40-fold induction in *CYP19* gene expression was observed at 12 h in cells exposed to 10 or 50 μM forskolin. This was followed by a reduction in gene expression to approximately 20-fold induction at 24 and 48 h sampling time in both treatment groups. For *CYP11B2* at 12 h, there was a 68- or 34-fold increase in gene expression in cell treated with 10 or 50 μM,

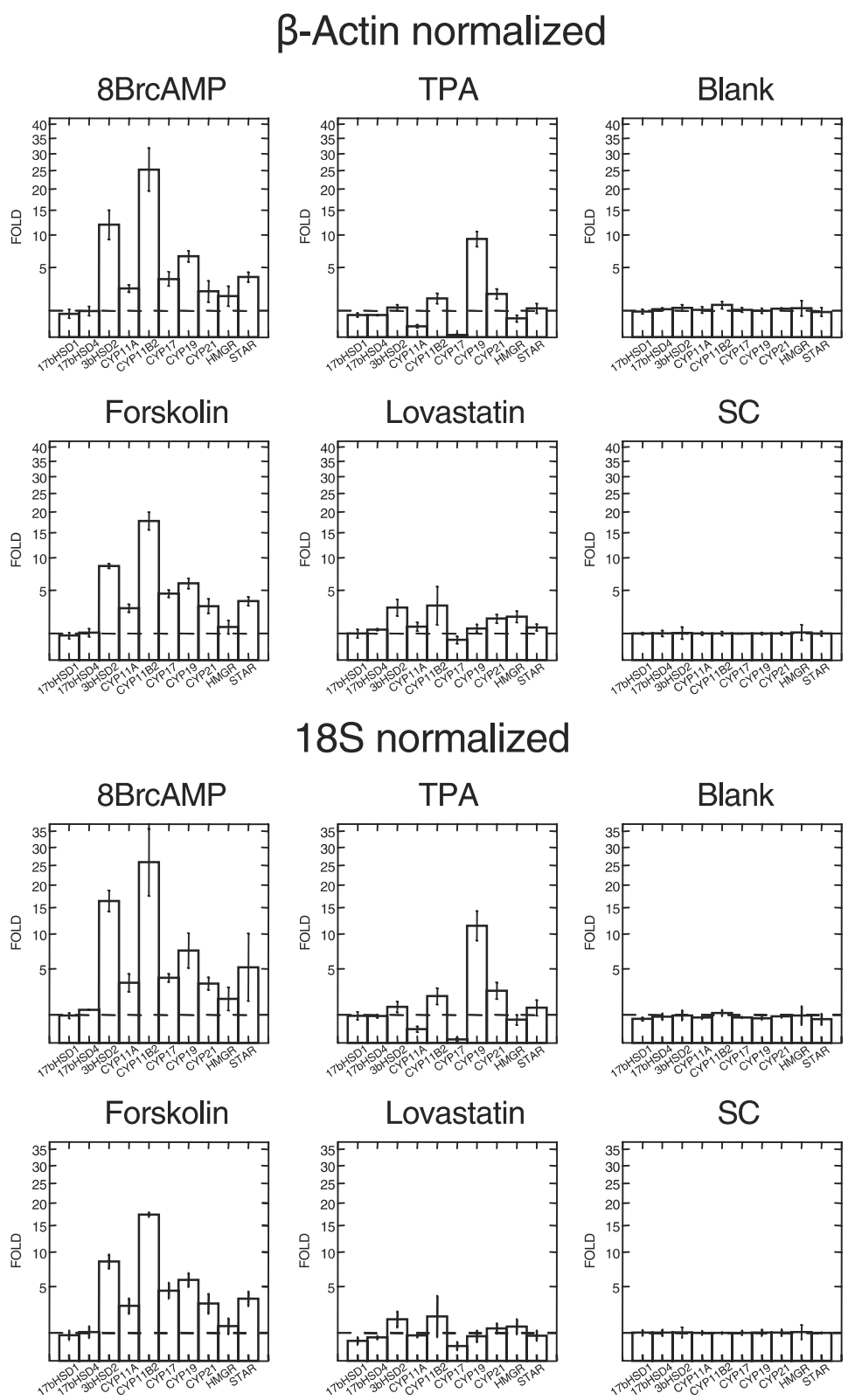

FIG. 5. The effects of different chemicals on expression of steroidogenic enzyme genes in H2195R cells in culture. Expression of steroidogenic genes was normalized to the expression of either β-actin or 18S ribosomal RNA as indicated. Fold induction represents the increase in expression compared to the relevant solvent control. Values presented are the means of three determinations on each of three replicate exposures. PMA, phorbol-12-myristate 13-acetate; sc, solvent control; blank, unexposed cells.

TABLE 3
Expression of Steroidogenic Genes in H295R Cells Exposed to Model Inducer, Responses Normalized to β-Actin

	Treatment					
Gene	Blank	Solvent	Forskolin	8BrcAMP	PMA	Lovastatin
CYP17	1.06 ± 0.18	1.05 ± 0.021	4.58 ± 0.59*	3.68 ± 0.98*	0.34 ± 0.40	0.44 ± 0.37
CYP21	1.11 ± 0.08	1.00 ± 0.11	3.22 ± 0.93*	2.51 ± 1.32*	2.40 ± 0.23*	1.87 ± 0.48
CYP11A	1.06 ± 0.24	1.00 ± 0.10	2.98 ± 0.50*	2.69 ± 0.47*	0.57 ± 0.40	1.18 ± 0.82
CYP19	1.01 ± 0.19	1.00 ± 0.08	5.93 ± 0.94	6.53 ± 1.05*	7.42 ± 4.94*	3.23 ± 3.64
StAR	0.94 ± 0.31	1.01 ± 0.17	3.71 ± 0.62*	3.86 ± 0.71*	1.45 ± 0.30	1.10 ± 0.31
3βHSD2	1.17 ± 0.26	1.07 ± 0.43	8.57 ± 0.49*	12.1 ± 3.92*	1.92 ± 1.02	2.36 ± 1.63
HMGR	1.19 ± 0.57	1.12 ± 0.55	1.45 ± 0.58	2.09 ± 0.97	1.41 ± 1.21	1.42 ± 0.94
17βHSD1	0.96 ± 0.17	1.00 ± 0.08	0.98 ± 0.20	0.85 ± 0.29	0.88 ± 0.06	0.92 ± 0.41
17βHSD4	1.09 ± 0.10	1.02 ± 0.23	1.06 ± 0.28	1.00 ± 0.35	0.89 ± 0.24	1.11 ± 0.30
CYP11B2	1.35 ± 0.29	1.08 ± 0.15	17.8 ± 2.76*	25.7 ± 8.34*	3.68 ± 2.78	1.72 ± 0.34

Note. Cell exposures were for 24 h; relative gene activity expressed as means and standard deviations.
*Statistically different from solvent control ($p < 0.05$).

TABLE 4
Expression of Steroidogenic Genes in H295R Cells Exposed to Model Inducers, Responses Normalized to 18S RNA

	Treatment					
Gene	Blank	Solvent	Forskolin	8BrcAMP	PMA	Lovastatin
CYP17	0.86 ± 0.03	1.01 ± 0.15	4.58 ± 1.20*	20.5 ± 28.6	0.29 ± 0.27	0.25 ± 0.18
CYP21	0.92 ± 0.07	1.02 ± 0.23	3.24 ± 1.18*	8.82 ± 9.46	2.74 ± 0.96	1.30 ± 0.43
CYP11A	0.86 ± 0.10	1.01 ± 0.13	2.99 ± 0.91*	12.0 ± 14.8	0.56 ± 0.19	0.69 ± 0.38
CYP19	0.83 ± 0.10	1.02 ± 0.22	5.87 ± 1.14*	35.3 ± 48.2	9.45 ± 7.13	3.03 ± 4.17
StAR	0.80 ± 0.33	1.00 ± 0.04	3.70 ± 0.96*	17.5 ± 21.1	1.59 ± 0.47	0.74 ± 0.13
3βHSD2	0.99 ± 0.32	1.05 ± 0.37	8.51 ± 1.47*	47.3 ± 53.4	1.93 ± 0.33	1.46 ± 0.73
HMGR	1.03 ± 0.59	1.08 ± 0.48	1.44 ± 0.60	11.6 ± 16.5	1.32 ± 0.68	0.85 ± 0.40
17βHSD1	0.79 ± 0.09	1.01 ± 0.19	0.89 ± 0.28	4.20 ± 5.61	0.97 ± 0.24	0.61 ± 0.19
17βHSD4	0.91 ± 0.20	1.01 ± 0.21	1.07 ± 0.37	4.17 ± 5.00	0.95 ± 0.07	0.74 ± 0.06
CYP11B2	1.11 ± 0.21	1.00 ± 0.06	17.4 ± 0.63*	159 ± 230	3.54 ± 1.42	1.17 ± 0.21

Note. Cell exposures were for 24 h; relative gene activity expressed as means and standard deviations.
*Statistically different from solvent control ($p < 0.05$).

respectively. Thereafter, there was a decrease in *CYP11B2* expression that resulted in expression levels that were similar among dose groups for the 24 and 48 h time points. In contrast to *CYP11B2*, there was no concentration-related difference in expression of *3βHSD2* with time but there was a general trend of reduced gene expression (11-fold reduction) over the experimental time period. Interestingly, while all previous exposures caused little effect on *HMGR* expression at 12 or 24 h of exposure to either 10 or 50 μM, an exposure to forskolin for 48 h resulted in a considerable and similar increase in the expression of this gene.

The reproducibility of the gene expression in the H295R bioassay was evaluated with forskolin- and PMA-treated cells from Exposures 1 and 3 (Table 5). In cells exposed to forskolin, the only significant differences in gene expression between the two experiments were for *CYP21*, *CYP19*, *StAR*, and *CYP11B2*. Of these genes, only *CYP21* and *CYP19* had interassay differences that were greater than 2-fold. In general, the expression of these genes was greater in Exposure 3 than in Exposure 1, and overall significances of these gene activities relative to solvent control values were consistent between assays. That is, if there was a significant alteration in gene expression in one assay it was also significant in the second assay. In the PMA exposures, only *CYP17*, *CYP21*, *CYP19*, *3βHSD2*, and *CYP11B2* significantly differed between assays. As was observed with forskolin, the level of gene expression in Exposure 3 was generally greater than that measured in Exposure 1, with all fold differences being greater than 2.5. Again, while the magnitude of gene expression activity differed between the two assays, the significances of gene expression as a consequence of PMA exposure were similar, indicating that the gene expression profile remained the same between assays. Overall, this analysis indicates that while there

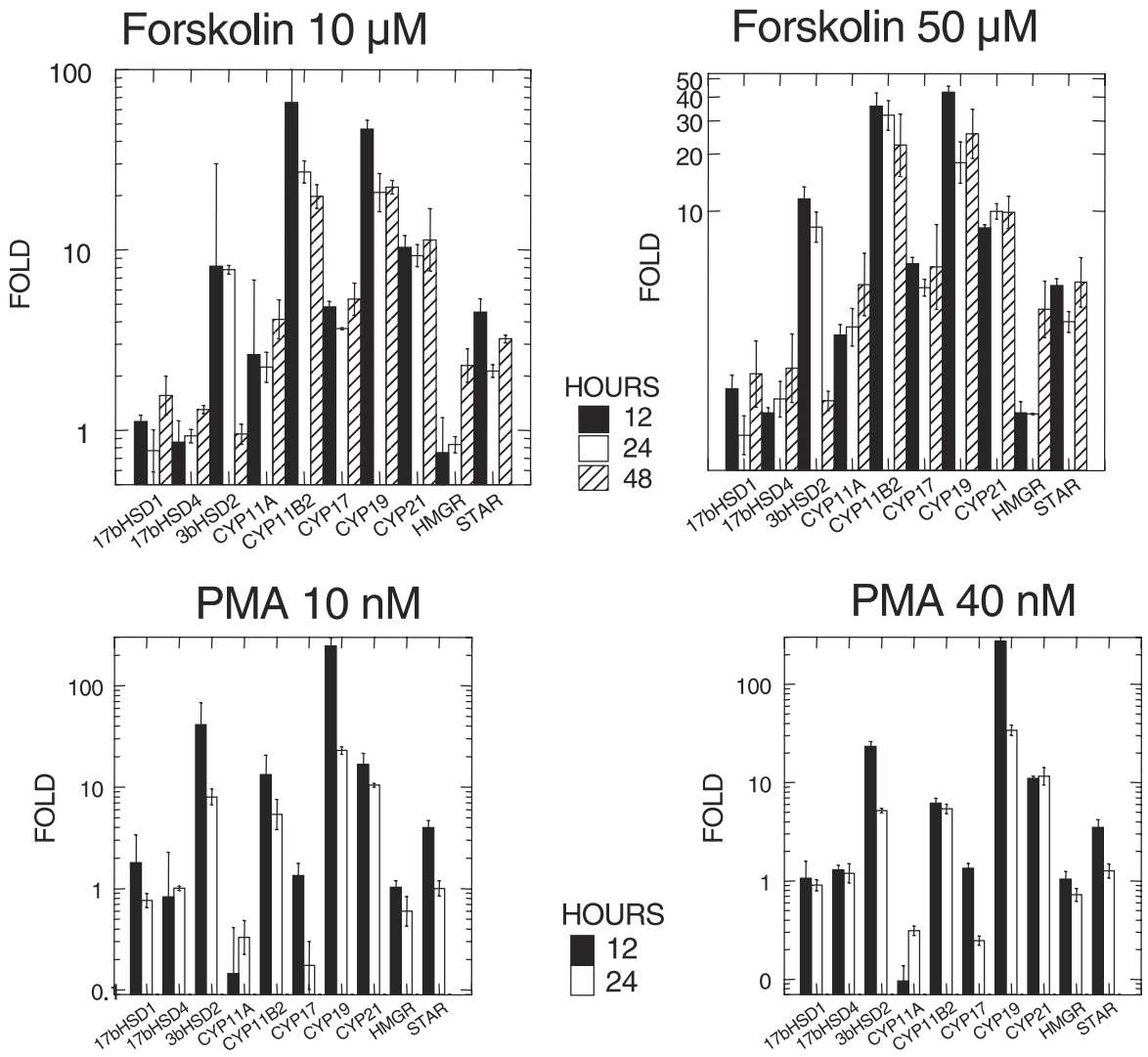

FIG. 6. Time course for the effects of forskolin and PMA on steroidogenic gene expression in H295R cells in culture. Expression of steroidogenic genes was normalized to the expression of β-actin. Fold induction represents the increase in expression compared to the relevant solvent control. Values presented are the means of three determinations on each of three replicate exposures. PMA, phorbol-12-myristate 13-acetate.

is some interassay variability in absolute expression, the overall conclusion that can be drawn relative to gene expression profiles is consistent between assays.

In another set of exposures (Exposure 2), the effects of a variety of inhibitors of steroidogenic genes were examined (Table 6). The most wide-ranging effects were observed after exposure to ketoconazole and spironolactone. Spironolactone decreased the expression of *StAR* by greater than 90% yet caused a 7-fold induction of *CYP19*. Spironolactone also significantly decreased the expression of *17βHSD4* (but not *17βHSD1*), *3βHSD2*, and *CYP11A*. Ketoconazole decreased the expression of *CYP11A* and *3βHSD2*, while it increased the expression of *CYP21*, *CYP19*, *HMGR*, *17βHSD1*, and *CYP11B2*. Of particular interest is the fact that ketoconazole was the only inhibitor tested that resulted in a significant induction of *CYP21* and *CYP11B2*. No other inhibitor tested significantly altered the expression of these two genes. None of the model inhibitors significantly induced the expression of *StAR*, but expression of this gene was significantly decreased by exposure to spirolactone, aminoglutethimide, or daidzein. However, the reduction in *StAR* was not correlated to any general decrease in the expression of the other steroidogenic genes monitored in the study. In contrast to the other genes monitored in this experiment, the expression of *CYP17* was not affected by any of the inhibitor chemicals.

DISCUSSION

An analytical procedure was developed that is capable of measuring gene expression of a range of steroidogenic enzymes as a result of exposure to chemicals. The Q-RT-PCR procedure was chosen over traditional enzyme assay techniques because it

offers the opportunity to screen expression of a wide range of genes using a single technique and a limited amount of sample. This latter criterion was essential to the development of a cell culture–based bioassay approach.

The production of steroids is a complex process with multiple sensitive control points. Given the complexity of the system and the number of enzymes and substrates involved, the potential for xenobiotic chemicals to interfere with this process is relatively great. Indeed the presence of genetic deficiencies in these steroidogenic enzymes leads to a condition known as congenital adrenal hyperplasia (CAH), which is often fatal (Richmond *et al*., 2001). While this condition is most frequently caused by a deficiency in *CYP21B* (Chiou *et al*., 1990), deficiencies in *StAR* and other steroidogenic enzymes are also capable of causing CAH (Richmond *et al*., 2001).

Mobilization of cholesterol to *CYP11A*, also known as $CYP450_{SCC}$, and its conversion to pregnenolone are the first and rate-limiting steps in the conversion of cholesterol to steroid hormones and is a point of both acute and chronic control (Hu *et al*., 2001; 2002). In our study, only 8BrcAMP and forskolin resulted in significant increases in *CYP11A1* expression; PMA significantly decreased *CYP11A* expression, while lovastatin appeared to have little effect on this enzyme. Alterations in *CYP11A* are also noteworthy since some studies indicate that the expression of other steroidogenic enzymes is coordinated with *CYP11A*. For example, it has been demonstrated that *CYP11A* activity may be coordinated with *CYP11B1* activity by the physical proximity of the two enzymes (Cauet *et al*., 2001). Such physical interrelationships between enzymes may be of greater significance *in vivo* than *in vitro* due to the tissue-specific expression of some enzymes. In fact, some tissues, particularly the adrenal gland, exhibit differential enzyme expression within different regions of the tissue (Gazdar *et al*., 1990; Sanderson *et al*., 2000; Staels *et al*., 1993).

Some of the chemicals tested resulted in some increase in *CYP21* gene expression. The *CYP21* gene product is required for the synthesis of both aldosterone and corticosteroids. Deficiency of this enzyme in CAH results in deficiencies in both cortisol and aldosterone that are also accompanied by overproduction of androgens (Chiou *et al*., 1990, Richmond *et al*., 2001). The overproduction of androgens is due to a combination of the general adrenal hyperplasia and substrate accumulation related to inhibition of the gluco- and mineralocorticoid pathways. In our experiments, increases in *CYP21* would be expected to lead to increased synthesis of cortisol and aldosterone and may result in decreased substrate availability for androgen and estrogen production.

CYP17 catalyzes the conversion of aldosterone to corticosteroid substrates and ultimately to sex steroid substrates.

TABLE 5
Comparison of Gene Expression Results of H295R Cells Exposed to Forskolin and PMA in Exposures 1 and 3, Responses Normalized to β-Actin

	Forskolin (50 μM)		PMA (40 nM)	
Gene	Fold change	*p* value	Fold change	*p* value
CYP17	−1.2	0.138	+2.51*	0.001
CYP21	+3.1*	0.001	+5.30*	0.009
CYP11A	−1.2	0.230	−1.01	0.953
CYP19	+3.1*	0.018	+3.6*	0.001
StAR	−1.05*	0.05	+1.09	0.707
3βHSD2	−1.05	0.634	+4.3*	0.001
HMGR	−1.73	0.216	+1.18	0.430
17βHSD1	−1.37	0.176	+1.17	0.231
17βHSD4	−1.10	0.667	+1.50	0.075
CYP11B2	+1.81*	0.011	+2.95*	0.001

Note. Fold change indicates direction and difference between Exposures 1 and 3; *p* value is based on the results of *t*-test between Exposures 1 and 3.
*Statistically significant difference between the two experiments.

TABLE 6
Expression of Steroidogenic Genes in H295R Cells Exposed to Model Inhibitors, Responses Normalized to β-Actin

	Treatment							
Gene	Blank	Solvent	Metyrapone	Daidzein	Ketoconazole	AMG	Androstedione	Spironolactone
CYP17	1.16 (0.26)	1.00 (0.06)	0.81 (0.01)	0.75 (0.05)	0.93 (0.15)	0.78 (0.08)	1.29 (0.03)	0.77 (0.46)
CYP21	1.17 (0.15)	1.02 (0.21)	1.11 (0.35)	1.14 (0.14)	2.18* (0.27)	0.89 (0.19)	0.68 (0.26)	1.36 (0.32)
CYP11A	1.06 (0.07)	1.00 (0.04)	0.83* (0.03)	0.88 (0.07)	0.73* (0.06)	0.84* (0.10)	1.07 (0.14)	0.37* (0.07)
CYP19	1.11 (0.41)	1.00 (0.10)	1.62 (0.35)	1.30 (0.18)	4.45* (0.95)	1.21 (0.12)	1.41 (0.20)	7.13* (2.72)
StAR	1.07 (0.31)	1.01 (0.18)	1.05 (0.42)	0.31* (0.18)	1.61 (0.65)	0.29* (0.07)	0.74* (0.45)	0.13* (0.13)
3βHSD2	1.15 (0.08)	1.03 (0.31)	0.48* (0.18)	0.50* (0.12)	0.43* (0.09)	0.92 (0.30)	0.76 (0.38)	0.26* (0.12)
HMGR	0.98 (0.27)	1.01 (0.48)	1.01 (0.06)	0.89 (0.14)	1.66* (0.24)	0.97 (0.26)	1.22* (0.29)	0.73 (0.29)
17βHSD1	1.18 (0.29)	1.01 (0.12)	1.70 (0.12)	1.60 (0.42)	2.00* (0.79)	1.41 (0.23)	1.70 (0.31)	1.38 (0.65)
17βHSD4	1.00 (0.13)	1.04 (0.35)	1.15 (0.10)	1.16 (0.19)	0.99 (0.69)	0.41* (0.44)	0.21* (0.03)	0.34* (0.28)
CYP11B2	0.92 (0.16)	1.02 (0.24)	1.65 (0.42)	1.13 (0.03)	5.89* (1.82)	0.87 (0.19)	1.25 (0.120	0.76 (0.16)

Note. Cell exposures were for 24 h; relative gene activity expressed as means with standard deviations in parentheses.
*Statistically different from solvent control ($p < 0.05$).

TABLE 7
Fold Differences in Gene Expression for H295R Cell Lines Exposed to Model Chemicals

Chemical	*CYP11A*	*CYP11B2*	*CYP17*	*CYP19*	*CYP21*	*17βHSD1*	*17βHSD4*	*3βHSD2*	*HMGR*	*StAR*
Inducers										
8BrcAMP	↑	↑↑↑↑	↑	↑↑	↑	–	–	↑↑↑	–	↑
PMA	↓	–	↓↓↓	↑↑	↑	–	–	–	↓	–
Forskolin	↑	↑↑↑↑	↑	↑↑	↑	–	–	↑↑	–	↑
Lovastatin	–	↑	–	–	↑	–	–	↑	↑	–
Inhibitors										
Aminogluteth-imide	–	–	–	–	–	–	↓↓	–	–	↓
Androstedione	–	–	–		–	–	↓↓	–	–	–
Spironolactone	↓	–	–	↑↑	–	–	↓↓	↓↓	–	↓↓↓
Daidzein	–	–	–	–	–	–	–	↓	–	↓
Ketoconazole	–	↓↓	–	↑	↑	↑	–	↓	–	–
Metyrapone	–	–	–	–	–	–	–	↓	–	–

Note. Symbols indicate difference relative to control. ↑, 2-fold or more; ↑↑, 5-fold or more; ↑↑↑, 10-fold or more; ↑↑↑↑, 15-fold or more. All other differences less than 2-fold.

Therefore, it is possible that this enzyme could redirect steroid output from mineralocorticoids to glucocorticoids or weak androgens. Inhibition of *CYP17* would have the opposite effect. Supporting this hypothesis is the observation that treatment with PMA, which results in an almost complete inhibition of *CYP17* expression, results in the greatest increase in *CYP19* expression ($p < 0.01$). *CYP19* is responsible for the final conversion of androgens to estrogens.

While only a limited number of chemicals were tested in this study, distinct gene expression profiles are apparent (Table 7). In particular, similar expression patterns were observed for 8BrcAMP and forskolin. These patterns included relatively great increases in the expression of *3βHSD2* and *CYP11B2* and moderate increases in expression of *CYP11A*, *CYP17*, *CYP19*, *CYP21*, *HMGR*, and *StAR*. In contrast, PMA resulted in decreases in *CYP11A* and *CYP17*, moderate increases in *CYP11B2* and *CYP21*, and a greater increase in *CYP19*. This variety of responses demonstrates the utility of the H295R cell line for the detection of both induction and down-regulation of gene expression for steroidogenic enzymes (Heneweer *et al*., 2004). Lovastatin resulted in only moderate increases of *3βHSD2*, *CYP11B2*, *CYP21*, and *HMGR* expression. We hypothesize that the expression profiles observed for forskolin and 8BrcAMP, which were similar, resulted from increased signaling through the cAMP pathway and that other chemicals causing a similar alteration would result in a similar expression profile, as reported previously (Sanderson *et al*., 2002). It has been shown that forskolin is able to increase cellular cAMP concentrations in H295R cell line (Sanderson *et al*., 2002). In contrast, the expression profiles observed for PMA and lovastatin appear to have been produced by a signaling pathway other than the cAMP pathway and were distinct from each other. PMA exerts effects on steroidogenesis primarily through the MAPKC pathway and so would be expected to have an expression profile distinct from the cAMP-dependent pathways. Lovastatin is known to specifically inhibit HMG-CoA reductase activity and, as expected, treatment with this chemical increased the expression of HMG-CoA reductase in H295R cells.

It has been hypothesized in recent studies that the ability of chemicals to alter activity of *CYP19* (aromatase) represents a potential mechanism of endocrine disruption (Hayes *et al*., 2002; Heneweer *et al*., 2004; Sanderson *et al*., 2002). While several of the chemicals tested in this study altered the expression of *CYP19*, this gene was in no case the only gene whose expression was altered. Indeed, in no case was the alteration in the expression of *CYP19* the most significant alteration in gene expression (Table 7). These observations clearly demonstrate the need to examine alterations in steroid metabolic processes in a far more holistic fashion, evaluating many different end points including but not limited to gene expression. While gene expression profiling offers detailed information on alterations in gene regulation, other procedures such as the measurement of enzymes activities and amounts of steroids produced offer more proximal measures of the effects of chemicals on steroidogenesis.

The ability to assess all of the key enzymes involved in steroidogenesis in a single assay procedure will clearly be of great interest to those studying the effects of xenobiotics on steroidogenesis. While initial work has focused on specific enzymes such as aromatase, the assay we have presented allows for more general assessment of steroidogenesis by evaluating both enzymes that determine the overall rate of steroidogenesis as well as those specific enzymes that can influence the overall fate or balance of steroid production. The H295R cell line has been previously used in such a bioassay approach, but the end points in those studies were either mRNA species and one or two specific enzymes (Sanderson *et al*., 2001, 2002) or were a variety of enzyme activities (Ohno *et al*., 2002).

Our findings demonstrate that the genes within the steroidogenesis pathway are not expressed to the same extent and that

different chemicals result in different relative changes in the expression of various genes. Chemical agents have the potential to alter gene expression profiles and, potentially, the steroids produced by this pathway. The changes in patterns of relative expression can be used to classify chemicals of unknown mechanisms of action on the steroidogenic pathways. In this way, chemicals can be grouped for further testing of a reduced set of model chemicals and for risk assessments.

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REFERENCES

Ankley, G., Mihaich, E., Stahl, R., Tillitt, D., Colborn, T., McMaster, S., Miller, R., Bantle, J., Campbell, P., Denslow, N., *et al*. (1998). Overview of a workshop on screening methods for detecting potential (anti-) estrogenic/androgenic chemicals in wildlife. *Environ. Toxicol. Chem.* **17,** 68–87.

Cauet, G., Balbuena, D., Achstetter, T., and Dumas, B. (2001). CYP11A1 stimulates the hydroxylase activity of CYP11B1 in mitochondria of recombinant yeast *in vivo* and *in vitro*. *Eur. J. Biochem.* **268,** 4054–4062.

Chiou, S. H., Hu, M. C., and Chung, B. C. (1990). A missense mutation at Ile172—Asn or Arg356—Trp causes steroid 21-hydroxylase deficiency. *J. Biol. Chem.* **265,** 3549–3552.

Connor, K., Howell, J., Chen, I., Liu, H., Berhane, K., Sciarretta, C., Safe, S., and Zacherewski, T. (1996). Failure of chloro-*s*-triazine–derived compounds to induce estrogen receptor–mediated responses *in vivo* and *in vitro*. *Fundam. Appl. Toxicol.* **30,** 93–101.

EDSTAC Final Report (1998). *Endocrine Disruptor Screening and Testing Advisory Committee Final Report.* U.S. Environmental Protection Agency. www.epa.gov/opptintr/opptendo/finalrpt.htm.

Gazdar, A. F., Oie, H. K., Shackleton, C. H., Chen, T. R., Triche, T. J., Myers, C. E., Chrousos, G. P., Brennan, M. F., Stein, C. A., and La Rocca, R. V. (1990). Establishment and characterization of a human adrenocortical carcinoma cell line that expresses multiple pathways of steroid biosynthesis. *Cancer Res.* **50,** 5488–5496.

Hayes, T. B. Collins, A., Lee, M., Mendoza, M., Noriega, N., Stuart, A. A., and Vonk, A. (2002). Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proc. Nat. Acad. Sci. USA* **99,** 5476–5480.

Heneweer, M., Van den Berg, M., and Sanderson, J. (2004). A comparison of human H295R and rat R2C cell lines as *in vitro* screening tools for effects on aromatase. *Toxicol. Lett.* **146,** 183–194.

Hu, M. C., Chiang, E. F.-L., Tong, S. K., Lai, W., Hsu, N. C., Wang, L. C.-K., and Chung, B. C. (2001). Regulation of steroidogenesis in transgenic mice and zebrafish. *Mol. Cell. Endocrinol.* **171,** 9–14.

Hu, M. C., Hsu, N. C., El Hadj, N. B., Pai, C. I., Chi, H. P. C, Wang, K. L., and Chung, B. C. (2002). Steroid deficiency syndromes in mice with targeted disruption of CYP11A1. *Mol. Endocrinol.* **16,** 1943–1950.

Kavlock, R. T., Daston, G. P., De Rosa, C., Fenner-Crisp, P., Gray, L. E., Kaattari, S., Lucier, G., Luster, M., Mac, M. J., Maczka, C., *et al*. (1996). Research needs for the risk assessment of health and environmental effects of endocrine disruptors: A report of the U.S. EPA sponsored workshop. *Environ. Health Perspect.* **104,** 715–740.

Ohno, S., Shinoda, S., Toyoshima, S., Nakazawa, H., Makino, T., and Nakajin, S. (2002). Effects of flavonoid phytochemicals on cortisol production and on activities of steroidogenic enzymes in human adrenocortical H295R cells. *J. Steroid Biochem. Mol. Biol.* **80,** 355–363.

Rainey, W. E., Bird, I. M., Sawetawan, C., Hanley, N. A., McCarthy, J. L., McGee, E. A., Wester, R., and Mason, J. I. (1993). Regulation of human adrenal carcinoma cell (NCI-H295) production of C19 steroids. *J. Clin. Endocrinol. Metab.* **77,** 731–737.

Richmond, E. J., Flickinger, C. J., McDonald, J. A., Lovell, M. A., and Rogol, A. D. (2001) Lipoid congenital adrenal hyperplasia (CAH): Patient report and a mini-review. *Clin. Pediatr. (Phila.)* **40,** 403–407.

Sanderson, J. T., Boerma, J., Lansbergen, G. W., and Van den Berg, M. (2002). Induction and inhibition of aromatase (CYP19) activity by various classes of pesticides in H295R human adrenocortical carcinoma cells. *Toxicol. Appl. Pharmacol.* **182,** 44–54.

Sanderson, J. T., Heneweer, M., Seinen, W., Giesy, J. P., and Van den Berg, M. (1999). Chloro-s-triazine herbicides and certain metabolites induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells. *Organohalogen Compounds* **42,** 5–8.

Sanderson, J. T., Seinen, W., Giesy, J. P., and Van den Berg, M. (2000). 2-Chloro-*S*-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: A novel mechanism for estrogenicity. *Toxicol. Sci.* **54,** 121–127.

Sanderson, J., Thomas, R. J., Letcher, M., Heneweer, Giesy, J. P., and Van den Berg, M. (2001). Effects of chloro-*S*-triazine herbicides and metabolites on aromatase (CYP19) activity in various human cell lines and on vitellogenin production in male carp hepatocytes. *Environ. Health Perspect.* **109,** 1027–1031.

Staels, B., Hum, D. W., and Miller, W. L. (1993). Regulation of steroidogenesis in NCI-H295R cells: A cellular model of the human fetal adrenal. *Mol. Endocrinol.* **7,** 423–433.

Villeneuve, D. L., Blankenship, A. L., and Giesy, J. P. (1998). Estrogen receptors environmental xenobiotics. In *Toxicant-Receptor Interactions and Modulation of Gene Expression*. (M. S. Denison and W. G. Helferich, Eds.), pp. 69–99. Lippincott-Raven Publishers, Philadelphia.

Článek IX:

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The H295R system for evaluation of endocrine-disrupting effects ☆

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Abstract

The present studies were undertaken to evaluate the utility of the H295R system as an in vitro assay to assess the potential of chemicals to modulate steroidogenesis. The effects of four model chemicals on the expression of ten steroidogenic genes and on the production of three steroid hormones were examined. Exposures with individual model chemicals as well as binary mixtures were conducted. Although the responses reflect the known mode of action of the various compounds, the results show that designating a chemical as "specific inducer or inhibitor" is unwise. Not all changes in the mixture exposures could be predicted based on results from individual chemical exposures. Hormone production was not always directly related to gene expression. The H295R system integrates the effects of direct-acting hormone agonists and antagonists as well as chemicals affecting signal transduction pathways for steroid production and provides data on both gene expression and hormone secretion which makes this cell line a valuable tool to examine effects of chemicals on steroidogenesis.

Keywords: Bioassay; Steroidogenesis; Screening; Endocrine disruptors; Mixtures

1. Introduction

Concern about the potential effects of chemicals on the endocrine systems of wildlife (Ankley et al., 1998) and humans (Kavlock et al., 1996) has increased over the past years. On October 26th, 2000 the European Parliament adopted a resolution on endocrine disrupters, emphasizing the application of the precautionary principle and calling on the Commission to identify substances for immediate action. In 2004, the Commission presented an update on the implementation of the strategy which among other recommendations includes an adaptation/amendment of current legislation to consider potential effects of Endocrine Disrupters. In particular, Regulation No 793/93 of the European Economic Community (EEC) on risk assessment and Directive 67/548/EEC on the classification of dangerous substances have been promulgated. In the United States, legislation such as the Safe Drinking Water Act Amendments of 1995 and the Food Quality Protection Act of 1996 have been promulgated. These legislative mandates require screening for endocrine-disrupting properties of chemicals used in commerce or resulting from processes that might occur in drinking water or food.

It has been difficult to develop the necessary screening tools because there are so many potential effects that could lead to endocrine disruption. In fact, any stressor, chemical or otherwise that forces any organisms out of its normal

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homeostatic range could be defined as an endocrine disruptor. The federal Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) recommended that chemicals be screened as agonists or antagonists of estrogen (ER), androgen (AR) and thyroid (ThR) hormone receptors (EDSTAC, 1998). Specifically, much of the early research focused on the effects of direct-acting effects such as chemicals that act as hormone mimics by acting as agonists for hormone receptors, in particular interest focused on the ER. Dodds and Lawson (1938) conducted perhaps the first published study to show the estrogenicity of bisphenol A and alkylphenols using ovariectomized rats. Although more evidence for the endocrine disruption effects of these compounds was found in the 70s (Mueller and Kim, 1978) health concerns were only raised when effects of bisphenol A and nonylphenol on cultured human breast cells were observed (Krishnan et al., 1993; Soto et al., 1991). A yeast screen containing a human AR receptor showed that bisphenol A can also act as an anti-AR (Sohoni and Sumpter, 1998). In the late 90s the list of endocrine disrupters grew when some PCBs metabolites were found to mimic estradiol (ER) (McKinney and Waller, 1994) and dioxins were shown to not only to alter hormone production but also to alter the immune system (Grassman et al., 1998). The pesticide *o,p*-DDT was also found to be a weak ER agonist (Colborn et al., 1997). The relevance of this finding is questionable since the primary form of the DDT metabolites found in the environment and animal tissues is actually *o,p*-DDE. Nevertheless, much of the initial research, public interest and legislation focused on hormone mimics. Furthermore, most of the initial work was on developing methods of predicting the ability of chemicals to serve as ER agonists. This included both structure activity models to predict ER binding (Kanno et al., 2001) as well as ER binding assays (Legler et al., 1999). More recently several eukaryotic cell-based expression assays have been developed where an endogenous or exogenous reporter gene is expressed under the control of the ER (Pons et al., 1990; Legler et al., 1999) or AR receptor (Sonneveld et al., 2004; Wilson et al., 2002). In addition, there have been some in vitro systems based on prokaryotic cells (Routledge and Sumpter, 1996). Together these systems have made possible the identification of many environmental contaminants which may act by binding directly to hormone receptors. The utility of in vitro assay systems for the identification of novel mechanisms of endocrine disruption was demonstrated by the observation that some chemicals are able to alter the production of enzymes involved in steroid production (Sanderson et al., 2000). While some chemicals have been shown to modulate the endocrine system as direct receptor agonists or antagonists (Villeneuve et al., 1998) other chemicals can cause effects by non-receptor-mediated mechanisms (Sanderson et al., 2000). In particular, chemicals that alter the expression of steroidogenic enzymes have the potential to alter rates, as well as absolute and relative concentrations of hormones in blood and tissues (Hilscherova et al., 2004).

The H295R assay system is now being developed and validated for use in a tiered screening approach by the US EPA (EDSTAC Final Report, 1998) and the results of preliminary work conducted in our laboratory have been presented to the OECD at their annual meeting in Paris (2005). At this time the US EPA is considering using the H295R system to replace two currently used assays, the Hershberger uterotropic assay for estrogenicity (Kanno et al., 2001) and the rat minced testis assay for determining effects on aromatase (CYP19). If the H295R assay is adopted, it is anticipated that it will result in more rapid, accurate and less expensive assays as well as obviating the need for the use of large numbers of live animals, which is required in the in vivo or ex vivo assays currently being utilized. Because of the great potential utility of the H295R assay as a screening tool to discern the mechanisms of action of specific endocrine modulating compounds, we present this demonstration and review the general characteristics and utility of the assay as a "frontiers" article.

The H295R cell line was derived from a human adrenal carcinoma and has all the enzymes necessary to produce steroid hormones (Gazdar et al., 1990; Rainey et al., 1993; Staels et al., 1993). H295R cells have physiological characteristics of zonally undifferentiated human fetal adrenal cells and as a result these cells have the ability to produce the steroid hormones of each of the three phenotypically distinct zones found in the adult adrenal cortex (Gazdar et al., 1990; Staels et al., 1993). Since the cells maintain the ability to express these genes and produce these enzymes, they are a useful model system for the study of potential effects on steroidogenesis. The genes measured in the current studies include *CYP11A* (cholesterol side-chain cleavage), *CYP11B2* (aldosterone synthetase), *CYP17* (steroid 17α-hydroxylase and/or 17,20 lyase), *CYP19* (aromatase), *17β-HSD1* and *17β-HSD4* (17β-hydroxysteroid dehydrogenase, type 1 and 4), *CYP21B2* (steroid 21-hydroxylase), *3β-HSD2* (3β-hydroxysteroid dehydrogenase), *HMGR* (hydroxymethylglutaryl CoA reductase) and the cholesterol transfer protein *StAR* (steroid acute regulatory protein). Treatment with a variety of agents has been shown to alter steroid production in H295R cells (Ohno et al., 2002). Previous studies have demonstrated that measurement of gene expression in the H295R system not only permits the evaluation of the potential of chemicals to interfere with the expression of steroidogenic enzymes, but also provides a means of profiling the modes of action of chemicals (Hilscherova et al., 2004; Zhang et al., 2005). Furthermore, the H295R cell line has also been shown to be useful for measuring the activity of the enzymes as observed in the studies of Sanderson co-workers (Sanderson et al., 2000, 2001) where it was demonstrated that commonly used 2-chloro-*s*-triazine herbicides dose-dependently induced aromatase (CYP19) activity in this cell line.

The H295R system therefore represents a unique bioassay system in that it allows the measurement of alterations in gene expression and at the same time permits

determination of alterations in steroid hormone production by the same cell cultures. In this paper we review the current status of the assay and report further on the development of the H295R assay system and for the first time present data demonstrating the relationship between gene expression and steroid production.

2. Materials and methods

2.1. Test chemicals

Forskolin, ketoconazole and aminoglutethimide (AMG), were obtained from Sigma (St. Louis, MO, USA), metyrapone was obtained from Aldrich (St. Louis, MO, USA); purity of all test chemicals exceeded 98%. The chemicals used in this study were chosen based on the known effects on steroid metabolism as well as their effects on steroidogenic gene expression (Hilscherova et al., 2004).

2.2. Experimental design

The H295R human adrenocortical carcinoma cell line was obtained from the American Type Culture Collection (ATCC # CRL-2128, ATCC, Manassas, VA, USA) and cells were grown in 75 cm^2 flasks with 12.5 ml of supplemented medium at 37 °C with a 5% CO_2 atmosphere. Supplemented medium was a 1:1 mixture of Dulbecco's modified Eagle's medium with Ham's F-12 Nutrient mixture with 15 mM HEPES buffer. The medium was supplemented with 1.2 g/L Na_2CO_3, ITS+ Premix (BD Bioscience, 1 ml Premix/100 ml medium), and 12.5 ml/500 ml NuSerum (BD Bioscience, San Jose, CA, USA). Final component concentrations in the medium were: 15 mM HEPES; 6.25 μg/ml insulin; 6.25 μg/ml transferrin; 6.25 ng/ml selenium; 1.25 mg/ml bovine serum albumin; 5.35 μg/ml linoleic acid; and 2.5% NuSerum. The medium was changed 2–3 times a week and cells were detached from flasks for sub-culturing using trypsin/EDTA (Sterile 1 × trypsin–EDTA (Life Technologies Inc.)). Cells were exposed to test chemicals dissolved in DMSO using 6-well tissue culture plates (Nalgene Nunc Inc., Rochester, NY, USA). Cells were detached from flasks with trypsin/EDTA (Sterile 1 × trypsin–EDTA (Life Technologies Inc.)) and were harvested into a final volume of 11 ml of medium. Cell density was determined using a hemocytometer. For dosing, 3 ml of cell suspension containing 1×10^6 cells/ml were placed in each well.

In the dose–response experiment, H295R cells were exposed to 0.03, 0.1, 1.0, 3.0, 10, or 50 μM forskolin for 24 h while only the 10 and 50 μM concentrations were measured at 48 h. The solvent used in these experiments was DMSO at a final concentration of 0.1%. Matching solvent controls were run concurrently and used to evaluate gene expression at each time interval.

To ascertain the effects of chemical mixtures on H295R cells, cells were treated with forskolin in combination with other chemicals previously shown to alter gene expression (Hilscherova et al., 2004). The chemicals used in this study were chosen based on their variety of known effects on steroid metabolism. Among other effects AMG is an aromatase inhibitor (Bastida et al., 2001) and has shown to block pregnenolone formation by inhibitory effects on CYP11A activity (Johansson et al., 2002); forskolin increases cellular cAMP concentrations (Thomson et al., 2001); ketoconazole works principally by inhibition of cytochrome P450 14 α-demethylase (*P45014DM*), however, it has been demonstrated that ketoconazole not only blocks the 11 β-hydroxylase conversion of deoxycortisone to corticosterone but also is responsible for the inhibition of cholesterol conversion to pregnenolone by mitochondrial fractions (Loose et al., 1983). Metyrapone is also considered an inhibitor of 11 β-hydroxylase (Parthasarathy et al., 2002). The data used in these analyses are a compilation of several different exposure studies where some data for individual compounds were run separately from those that evaluated chemical mixtures. All chemical mixtures contained 10 μM forskolin in combination with 300 μM metyrapone, 300 μM AMG or 20 μM ketoconazole (Table 1).

Table 1
Chemical mixtures exposure[a]

Chemical 1	Chemical 2
Solvent control	na
10 μM forskolin	300 μM Metyrapone[b]
10 μM forskolin	300 μM AMG[b]
10 μM forskolin	20 μM ketoconazole[b]

Na, Not applicable.
[a]H295R cells were exposed to individual or chemical mixtures for 24 h.
[b]Gene expression data for individual chemicals were previously reported in Hilscherova et al (2004).

2.3. Cell viability/cytotoxicity

Before nucleic acid isolation and hormone analysis, cell viability was determined. Cells were visually inspected under a microscope to evaluate viability and cell number. In addition, cell viability was determined with the Live/Dead cell viability kit (Molecular Probes, Eugene, OR, USA). While ketoconazole inhibited cell growth at concentrations greater than 30 μM, no adverse effects on cell growth or viability were observed for any of the tested chemicals at concentrations up to 300 μM. In instances where exposure to model compounds resulted in cell death or decreased viability the data were not used to evaluate gene expression or hormone production.

2.4. RNA isolation

For nucleic acid extraction, after removal of the medium, cells were lysed in the culture plate, by the addition of 580 μl/well of Lysis Buffer-β-ME mixture (Stratagene, La Jolla, CA, USA) and RNA was isolated as described in Hilscherova et al. (2004). Briefly, lysed cells were mixed and then centrifuged in a pre-filter spin cup and the mixture centrifuged. The filtrate was diluted with 70% ethanol and vortexed. The mixture was transferred to an RNA spin cup and centrifuged for 1 min. The filtrate was discarded and the spin cup was washed with a low-salt buffer and then centrifuged for 1 min. RNase-free DNase I solution (Strategene, La Jolla CA, USA) was added to the fiber matrix inside the spin cup and the sample was incubated at 37 °C for 15 min. The sample was then washed with high-salt followed by a low-salt buffer. After each wash cycle, the filtrate was discarded. After the final wash, the sample was centrifuged and nuclease-free water was added directly to the fiber matrix inside the spin cup. The tube was incubated for 2 min at room temperature and centrifuged. This elution step was repeated to maximize the yield of RNA. The purified RNA was used immediately or stored at −80 °C until needed. An appropriate dilution of the RNA sample (1:50) was prepared for RNA quantification. The absorbance of the RNA solution was measured at 260 and 280 nm and the 260/280 ratio was calculated. The concentration of total RNA was estimated using the A_{260} value and a standard with an A_{260} of 1 that was equivalent to 40 μg RNA/ml.

2.5. cDNA preparation

Total RNA (1–5 μg) was combined with 50 μM oligo-(dT)20, 10 mM dNTPs, and diethylpyrocarbamate (DEPC)-treated water to a final volume of 12 μl. RNA and primers were denatured at 65 °C for 5 min and then incubated on ice for 5 min. Reverse transcription was performed using 8 μl of a master mix containing; 5 × cDNA synthesis buffer (Carlsbad CA, USA) and DEPC-treated water. Reactions were incubated at 50 °C for 45 min and were terminated by incubation at 85 °C for 5 min. Samples were either used directly for PCR or were stored at −20 °C until analyzed.

2.6. Real-time PCR

Real-time PCR (quantitative PCR) was performed by using a Smart Cycler System (Cepheid, Sunnyvale, CA, USA) in 25 μl sterile tubes using a master mix containing 25 mM $MgCl_2$, 1 U/μl AmpErase (Applied Biosystems, Foster City, CA, USA), 5 U/μl *Taq* DNA polymerase AmpliTaq Gold, 10 × SYBR Green (PE Biosystems, Warrington, UK), nuclease-free water and between 10 pg and 1 μg of cDNA. The thermal cycling program included an initial denaturation step at 94 °C for 10 min, followed by 25–35 cycles of denaturation (95 °C for 15 s), primer annealing (at 60–64 °C for 40–60 s), and cDNA extension (72 °C for 30 s); a final extension step at 72 °C for 5–10 min was also included. Melting curve analyses were performed immediately following the final PCR cycle to differentiate between the desired amplicons and any primer–dimers or DNA contaminants. Specifics of the assay parameters such as primers used and annealing temperatures have been published previously (Hilscherova et al., 2004).

For quantification of PCR results C_t (the cycle at which the fluorescence signal is first significantly different from background) was determined for each reaction. C_t values for each gene of interest were normalized to the endogenous control gene, β-actin. Normalized values were used to calculate the degree of induction or inhibition expressed as a "fold difference" compared to normalized control values. Therefore, all data were statistically analyzed as "fold induction" between exposed and control cultures. Gene expression was measured in triplicate for each control or exposed cell culture and each exposure was repeated at least three times.

2.7. Hormone quantification

Hormone extraction and quantification by ELISA were conducted as previously described (Hecker et al., 2005). Briefly, frozen media samples were thawed on ice, and the hormones were extracted twice with diethyl ether (5 ml) in glass tubes. To determine extraction recoveries 10 μl of ^{3}H-testosterone 0.0002 μCi/μl was added to 500 μl of sample prior to extraction. The solvent extract was separated from the water phase by centrifugation at 2000 × *g* for 10 min and transferred into small glass vials. The solvent was evaporated under a stream of nitrogen, and the residue was dissolved in EIA buffer from Cayman Chemical Company and either immediately measured or frozen at −80 °C for later hormone determination. Concentrations of hormones in media were measured by competitive ELISA using Cayman Chemical® hormone EIA kits (Cayman Chemical Company, Ann Arbor, MI, USA; progesterone (P) [Cat # 582601], Testosterone (T) [Cat # 582701], E2 [Cat # 582251]). Because the antibody to progesterone exhibits cross-reactivity with *pregnenolone* of 61% and the method does not allow for the separation of these two hormones, P concentrations are expressed as P/*pregnenolone*. The working ranges of these assays for the determination of steroid hormones in H295R media were determined to be: P: 7.8–1000 pg/ml; T: 3.9–500 pg/ml; 17ß-E2: 7.8–1000 pg/ml. Media extracts were diluted 1:25 and 1:100 for T while for P and E2 dilutions were 1:50–1:100 and 1:2–1:10, respectively.

2.8. Statistical analysis

Statistical analyses of gene expression profiles and hormone quantification were conducted using SYSTAT (SYSTAT Software Inc., Point Richmond, CA, USA). Differences in gene expression were evaluated by ANOVA followed by Tukey's test. Differences with $P<0.05$ were considered significant.

3. Results

3.1. Chemical dose and time courses

Results from the time course study indicated that gene expression profiles could be grouped into three general categories. These categories were based on gene expression levels measured in H295R cells exposed to a range of forskolin concentrations for either 24 or 48 h (Fig. 1). The genes were grouped as follows:

3.1.1. Group I genes (CYP21, CYP19, 3β-HSD2, and CYP11β2)

At 24 h, the dose–response curve for Group I genes was characterized by a relatively great increase in gene expression from the solvent control levels to 10 μM that was followed by a "plateau" in expression from 10 to 50 μM. At 48 h, the pattern in gene expression was similar to that observed at 24 h except that gene expression was approximately 2–3-fold greater than that observed at similar concentrations at 24 h. Overall, the genes in this group showed the greatest levels of induction with gene expression levels commonly being 10-fold in excess of that observed in solvent controls.

3.1.2. Group II genes (CYP17 and CYP11A)

At 24 h, gene expression was characterized by an increase that reached a maximal level between 3 and 10 μM forskolin. At concentrations greater than 10 μM forskolin, gene expression increased but to a lesser degree indicating that maximal expression levels may have not yet been reached. In the 48 h exposure, genes were characterized by an increase in expression up to approximately 10 μM forskolin followed by a "plateau" up to 50 μM. However, unlike that observed at 24 h, gene expression did not significantly differ between 10 and 50 μM indicating that these genes may have reached a maximal expression level. Finally, while the shape of the gene expression profile for these genes was similar to that observed with Group I genes, the induction of these genes was not as pronounced and was generally in excess of 3 fold but no greater than 10-fold.

3.2. Group III genes (StAR, 17β-HSD1 and 17β-HSD4)

Unlike the profiles observed for Group I and II genes, the expression profiles at 24 and 48 h in the Group III genes differed considerably. At 24 h, the dose–response curve was characterized by relatively great increase in gene expression in cells exposed up to 3 μM forskolin, this was followed by a large decrease in expression at 10 μM. Levels of gene expression at 10 μM were similar to that observed in the solvent controls. However, this decrease was followed by a slight increase in activity at concentrations up to 50 μM. In contrast, in cells exposed for 48 h, gene expression increased sharply from control levels up to 10 μM forskolin that was followed by less than a 1.5-fold increase in activity in the 50 μM exposure. Overall, the level of gene expression observed at 10 and 50 μM at 48 h was similar to that observed at the 3 μM forskolin dose in cells exposed to 24 h.

Alterations in expression of HMGR did not appear to be similar to any of the above categories. The HMGR gene

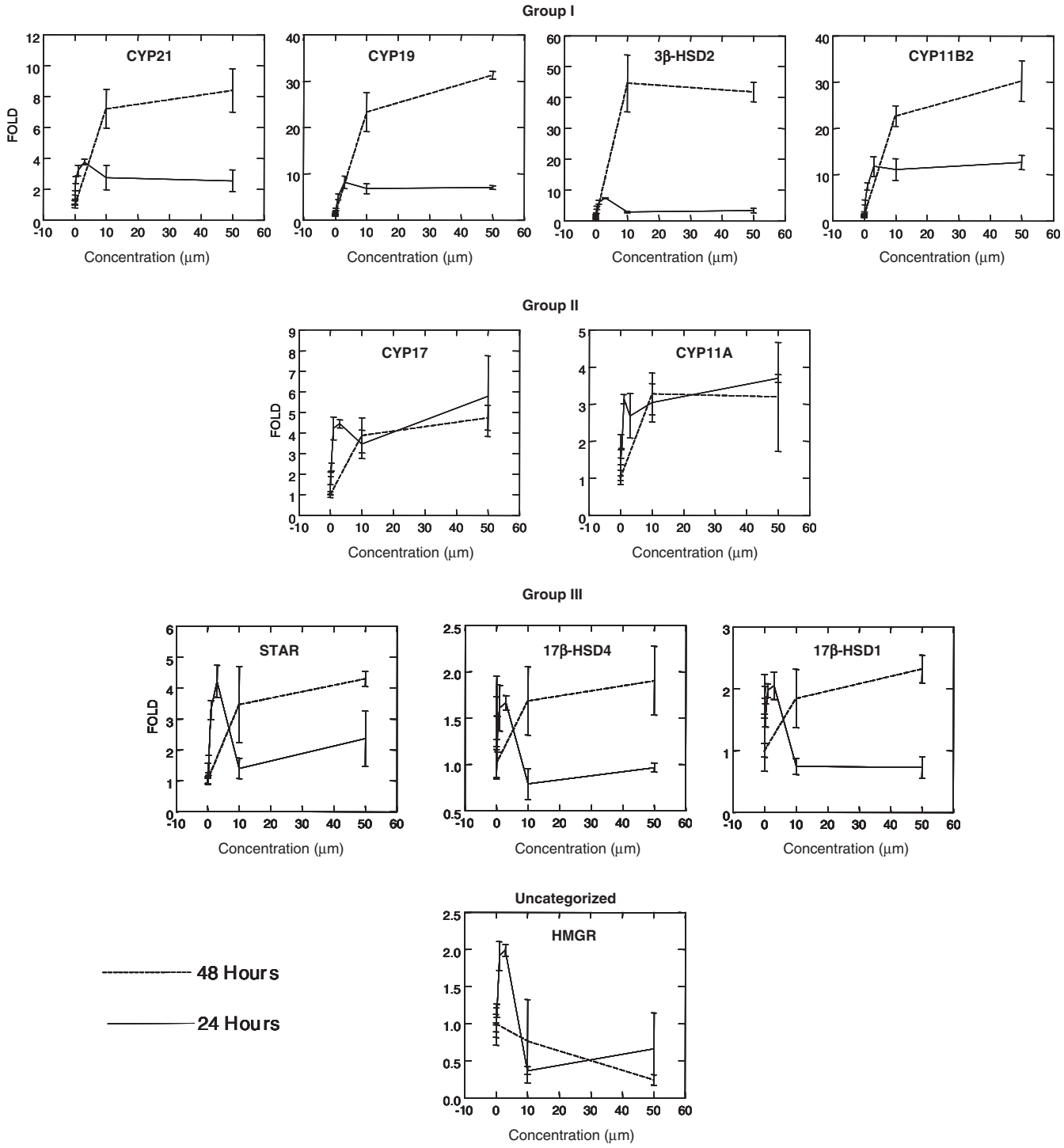

Fig. 1. Alterations in expression of steridogenic genes in H295R cells exposed to a range of concentrations of forskolin for 24 or 48 h.

expression profile at 24 h was characterized by a 2-fold increase in gene expression up to 3 μM that was followed by approximately a 4-fold decrease in expression at 10 μM and 50 μM. The gene expression profile at 48 h differed from that observed at 24 h in that gene expression was suppressed from control levels at all doses with the greatest suppression being observed in cells exposed to 50 μM.

These steroidogenic genes were also categorized by the time of induction with Group I genes being capable of further induction relatively 'late' (48 h) in the exposure. In contrast, HMGR along with the Group II and III genes appeared to be induced by low concentrations of forskolin to a greater degree at 24 h than higher doses at 48 h, unlike the response observed for the Group I genes. The observed results were in good agreement with those published previously (Hilscherova et al., 2004) demonstrating the general robustness of the assay procedure. While there were slight differences in gene expression alterations

between the two studies for Group II and Group III and, these differences were generally less than 2-fold between the two experiments. The most profound differences between the current study and Hilscherova's study were observed for Group I genes. In the current study, the expression levels of CYP11β2, CYP19, and CYP21 ranged from 3-fold less to 1.5-fold greater than the results observed by Hilscherova et al. for cells exposed under similar conditions. These results most likely represent differences in culture and exposure conditions.

The dose–response curves for forskolin at 24 h were trimodal for all of the genes studied (Fig. 1). This is particularly evident for HMGR, STAR, 17β-HSD1 and 17β-HSD4. For these genes, induction of expression was greatest at 1 and 3 μM forskolin and was decreased markedly at 10 μM. While there was a moderate increase in expression between 10 and 50 μM, the expression at these greater concentrations never reached the levels attained at 3 μM. In contrast, nearly all the gene expressions measured at 48 h were consistent, either increase or decrease, over the exposure range. The complex nature of the dose-response curve clearly demonstrates the complex, multiply regulated nature of the steroidogenesis pathway.

3.3. Patterns of gene response to chemical mixtures

Exposure of H295R cells to 10 μM forskolin for 24 h resulted in statistically significant (2-fold or greater) increases in the expression of the CYP17, CYP19, 17β-HSD4, CYP11A, StAR and CYP11β2 genes as compared to control levels (Table 2). No significant changes were observed in the expression of CYP21, 17β-HSD1, 3β-HSD2 or HMGR genes when compared to control gene expression levels. Furthermore exposure to forskolin was not associated with any decrease in the expression of the targeted genes indicating that this chemical was a general inducer of steroidogenic genes in H295R cells. As a result, all comparisons in the binary mixture studies were made relative to forskolin while changes in gene expression with single chemicals were evaluated relative to solvent controls.

Metyrapone did not alter the forskolin-induced expression of CYP19, 17β-HSD4, StAR or CYP11β2 in the mixture (Table 2). However, there was approximately a 2-fold decrease in forskolin-induced gene expression of CYP17 and CYP11A that was accompanied by an 11-fold decrease in 3β-HSD2 gene expression. The reduction of 3β-HSD2 is interesting in that forskolin alone induced 3β-HSD2 gene expression by about 1.5-fold whereas metyrapone decreased expression of this gene by approximately 2-fold when compared to control levels. However, the mixture resulted in 7.7-fold decrease in gene expression when compared to solvent control levels. All other metyrapone–forskolin mixture related changes in forskolin-altered gene expression were less than 1.5-fold.

AMG did not significantly alter the forskolin-induced gene expression of StAR, CYP17, or CYP11B2 in the mixture exposure (Table 2). However, there was approximately a 2-fold reduction in the forskolin-induced expression of the CYP11A and 17β-HSD1 genes in cells exposed to the mixture. The greatest effect in the mixture experiment was observed for CYP19, the expression of this gene was reduced approximately 13- fold less than that observed in cells treated with forskolin alone. Ketoconazole did not alter the forskolin-induced gene expression of CYP19, 17β- HSD1 or StAR while it reduced by approximately 2-fold the expression of CYP17 and CYP11A (Table 2). In addition, while forskolin itself did not alter the expression of HMGR, CYP21 and 3β-HSD2, the forskolin–ketoconazole mixture significantly decreased the expression levels of these genes when compared to controls. The reductions in the expression of these genes were similar to those observed in cells exposed to ketoconazole indicating there was no interaction but that the effects were being moderated only by ketoconazole. Forskolin and ketoconazole caused 6.6- and 4.8- fold increases in CYP11β2 expression, respectively. However, a binary mixture of these two chemicals resulted in a much

Table 2
Gene expression in H295R cells exposed to single chemicals and to binary mixtures[a]

Treatment[b]	CYP17	CYP19	CYP21	17βHSD1	17âHSD4	CYP11A	StAR	3βHSD2	HMGR	CYP11B2
Solvent control	1.03 (0.29)	1.12 (0.69)	1.01 (0.15)	1.34 (0.19)	1.05 (0.42)	1.00 (0.11)	1.01 (0.14)	1.01 (0.20)	1.00 (0.06)	1.09 (0.48)
Forskolin	3.10[c] (0.64)	4.64[c] (0.80)	1.36 (0.25)	1.00 (0.06)	4.03[c] (1.50)	3.53[c] (1.45)	1.98[c] (0.45)	1.48 (0.20)	1.34 (0.19)	6.60[c] (1.87)
Forskolin + metyrapone	1.59[c] (0.21)	4.56 (0.89)	1.13 (0.10)	0.77[c] (0.22)	4.96 (0.64)	1.77[c] (0.46)	1.63 (0.47)	0.13[c] (0.04)	1.32 (0.23)	6.45 (2.12)
Forskolin + aminoglutethimide	2.00 (0.51)	0.35[c] (0.02)	1.39 (0.59)	0.45[c] (0.16)	6.81[c] (0.52)	1.80[c] (0.25)	1.77 (0.36)	1.33 (0.30)	1.23 (0.21)	5.38 (1.16)
Forskolin + ketoconazole	1.24[c] (0.23)	4.32 (0.86)	0.61[c] (0.09)	1.24 (0.09)	6.53[c] (0.72)	1.62 (0.38)	2.59 (0.21)	0.38[c] (0.07)	0.41[c] (0.11)	37.0[c] (4.03)
Metyrapone	0.81 (0.01)	1.62 (0.35)	1.11 (0.35)	1.70[c] (0.12)	1.15 (0.100	0.83 (0.03)	1.05 (0.42)	0.48[c] (0.18)	1.01 (0.06)	1.65 (0.42)
Aminoglutethimide	0.78[c] (0.08)	1.21 (0.12)	0.89 (0.19)	1.41 (0.23)	0.41[c] (0.44)	0.84 (0.10)	0.29[c] (0.07)	0.92 (0.30)	0.97 (0.26)	0.87 (0.19)
Ketoconzole	0.33[c] (0.06)	1.00 (0.19)	0.75[c] (0.19)	1.04 (0.30)	5.62[c] (0.68)	0.81 (0.16)	1.18 (0.34)	0.56[c] (0.14)	0.45[c] (0.16)	4.79[c] (0.80)

[a]All exposures were conducted for 24 h under standard conditions. All gene expression values for fold change relative to control given as means and standard deviations.

[b]Concentrations of single chemicals and mixtures exposures were: forskolin (10 μM), metyrapone (300 μM), aminoglutethimide (300 μM), ketoconazole (20 μM).

[c]Indicates statistically significant differences at $P<0.05$. For individual treatments, comparisons made to solvent control. For mixtures, comparisons made to forskolin.

greater increase in expression (37-fold) than would have been predicted from exposures to the individual chemicals. This suggested "super-induction" was not observed for any of the other genes in that most other changes in gene expression were typically less than 3-fold. We hypothesize that this super-induction was due to the combined effects of increased CYP11A activity induced by forskolin and inhibition of CYP17 and CYP21 (Fig. 2). Increases in CYP11A activity caused by forskolin would increase the flux towards and production of pregnenolone (Cauet et al., 2001). At the same time the inhibition of CYP17 and CYP21 would prevent the conversion of pregnenolone to products other than P (Hu et al. 2001, 2002). This should lead to an increase in the flux of metabolites to P. The super-induced enzyme, CYP11β2 is responsible for the subsequent metabolism of P such that under these experimental conditions, the large increase in expression of this enzyme could be reasonably expected. Several other enzymes also metabolize P but their expression was not measured in this study. In addition at least one of these enzymes, 17-α-hydroxylase (EC 1.14.99.9) has previously been reported to be inhibited by ketoconazole (DiMattina et al., 1988). The interactive effects of the chemicals in this situation are also understandable since increased metabolism due to CYP11A induction by forskolin alone would not result in a great increase in CYP11β2 metabolism of P. This is because the increased flux would be dispersed to other parts of the synthetic pathway by CYP17 and CYP21 activities. Also in the absence of increased CYP11A activity the inhibition of CYP17 and CYP21 would not necessarily lead to accumulation of P and subsequent induction of CYP11B2.

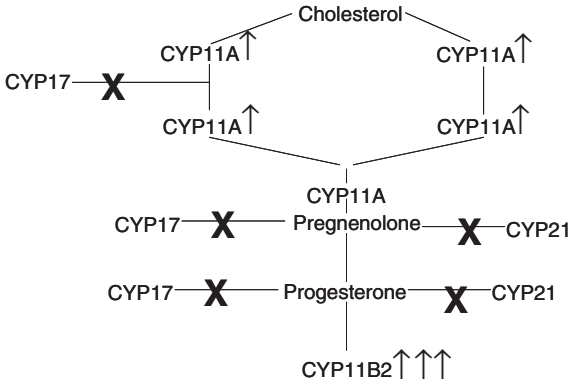

Fig. 2. Potential mechanism for super-induction of CYP11B2 by a mixture of forskolin and ketoconazole. Arrows indicate increased gene expression crosses represent decreased gene expression.

3.4. Hormone production

Medium from the solvent controls and most of the chemical treatments contained measurable concentrations of P, T and E2 (Table 3). The average concentrations of E2, T and P in the solvent controls were 14.2, 3845, and 13,948 pg/ml, respectively. Coefficients of variation for E2, T and P were 0.8%, 3.4% and 49%, respectively.

In H295R cells treated with forskolin, the production of P, T and E2 was increased from control levels by approximately 2.5-, 1.7- and 21-fold, respectively (Table 3). Thus, while forskolin increased all three hormone concentrations, the production of E2 preferentially increased when compared to the other two hormones. In contrast, treatment of H295R cells with AMG and ketoconazole resulted in decreased production of all three hormones compared to solvent controls. In cells treated with AMG, the production of E2 and P were decreased to below their assay detection limits (7.3 and 440 pg/ml, respectively) while T was reduced 3-fold when compared to the solvent control. Treatment with ketoconazole resulted in approximately a 7-fold reduction in P, a 10-fold reduction in T and a 1.1-fold reduction in E2 compared to control levels.

In the forskolin–AMG binary mixture, the concentration of P and E2 in the medium was reduced by approximately

Table 3
Hormone concentrations in media from H295R cells treated with single chemicals or in binary mixtures[a]

Treatment[b]	Progesterone (pg/ml)	Testosterone (pg/ml)	Estradiol (pg/ml)
Solvent control	13948 ± 6907	3845 ± 129	14.2 ± 0.109
Forskolin (FOR)	35542 ± 6006	6513 ± 151[d]	303 ± 4.55[d]
Aminoglutethimide (AMG)	<440[c]	440 ± 165[d]	<7.3[c]
Ketoconazole (KETO)	1949 ± 116[d]	374 ± 3.81[d]	12.6 ± 1.88[d]
Metyrapone (MET)	na	na	Na
Forskolin + AMG	693 ± 129[d]	1112 ± 190[d]	<7.3[c]
Forskolin + KETO	7429 ± 105[d]	149 ± 48.5[d]	207 ± 62.3
Forskolin + MET	5902 ± 892[d]	508 ± 105[d]	<7.3[c]

Na, No applicable.

[a]H295R cells exposed to either forskolin (10 μM); aminoglutethimide (300 μM); metyrapone (300 μM); ketoconazole (20 μM). Binary mixtures had the same chemical concentrations. All exposures were 24 h.

[b]Statistical comparisons for individual chemicals were to the solvent control. Binary mixtures were compared to forskolin alone.

[c]Indicates that hormone concentrations were less than the assay detection limit na is not analyzed.

[d]Indicates a Statistically significant difference ($P<0.05$; in a two-tailed test).

50-fold and greater than 40-fold, respectively, from that measured in cells exposed to forskolin alone. In contrast, T concentrations were only reduced approximately 6-fold from forskolin-induced levels. However, when compared to solvent control, there were 20- and 3.5-fold reductions in P and T concentrations while E2 concentrations were only 2-fold less than control. In the forskolin–ketoconazole mixture, there was approximately a 40-fold reduction of T concentrations from that observed in the forskolin alone experiment. The reductions in P and E2 concentrations were only 5- and 1.5-fold, respectively, compared to forskolin alone. A comparison of the mixture hormone data to the solvent control had a slightly different pattern in that P and T concentrations were reduced from control levels by 2- and 25-fold respectively. These reductions represent approximately a 50% change from that observed in the mixture. In contrast, E2 concentrations were more than 15-fold greater than observed in the solvent control confirming the observation that ketoconazole did not greatly affect E2 concentrations. In the forskolin-metyrapone exposure, concentrations of P and T were reduced approximately 6- and 13-fold from the forskolin alone while E2 was reduced by approximately 80-fold.

4. Discussion

Previous studies have demonstrated the utility of the H295R assay system as a rapid, sensitive and predictive in vitro system to assess the potential effects of chemicals on steroidogenesis (Hilscherova et al., 2004; Zhang et al., 2005). However, to more fully interpret the results obtained from this system, it was necessary to develop a more detailed understanding of the effects of exposure concentration and time on the results for model compounds. Additionally, to be of use in real-world scenarios the response of the system to chemical mixtures needs to be understood. Finally, the results of alterations on gene expression can now be related to alterations in actual steroidogenic function as determined by rates of synthesis and release of hormones to the culture medium.

4.1. Dose- and time-dependent patterns

The results of the time course experiments for the forskolin exposure demonstrated the coordinated expression of distinct groups of genes based on the shape and time dependence of the dose–response curve. The ability to group genes based on chemical- induced alterations in expression suggests a mechanistic linkage in the regulation of these genes. When a group of chemicals alter the same set of genes it is possible to establish the general mechanism by which they disrupt steroid production; furthermore, based on their chemical structures, response profiles may be established and used to predict the effects of other chemicals with similar chemical structure and unknown mechanism of action. Thus, the genes that exhibited the greatest change in transcription, those classified in Group I, are the genes coding for the enzymes involved directly in the production of the final steroid products such as aldosterone (CYP11β2) and E2 (CYP19). These downstream genes exhibited the greatest increase in expression when compared to the other genes studied in the 24 and 48 h exposure to forskolin. Group I also included genes involved in the production of key steroidogenic substrates such as 11-deoxycortisol, an important precursor in the production of glucocorticoids that is regulated by CYP21; and androstenedione, an indispensable substrate for the formation of sex steroids by 3β-HSD2; this gene, 3β-HSD2, is also involved in the production of P, a key steroid end-product as well as an important substrate in the formation of glucocorticoids. As observed in earlier studies, forskolin has relatively little impact on the expression of 17β-HSD1 and 17β-HSD4. These two genes code for two of the ten types of mammalian 17β-hydroxysteroid dehydrogenases (17β-HSDs) (Baker, 2001). These enzymes have the crucial role of controlling the last step in the formation of the essential active estrogens and androgens as well as the role of inactivating these potent sex steroids to produce compounds with little or no biological activity. Although the ten 17β-HSDs belong to the same protein super-family their amino acid sequences and structures demonstrate relatively low degrees of identity. In addition, each protein demonstrates distinct tissue-specific expression, substrate specificity, regulatory mechanisms and catalytic activity. In particular, human 17β-HSD type 1 and 4 have distinctly opposite functions. While 17β-HSD1 catalyzes the formation of 17β-E2 from estrone, 17β-HSD4 functions mainly in the conversion of 17β-E2 to estrone and androst-5-ene-3β, 17β diol (Labrie et al., 1997), in the current study the alteration of expression of these two genes by forskolin was minimal. The greatest induction observed relative to control was approximately 2-fold at 48 h for both, 17β-HSD1 and 17β-HSD4.

The relationship observed between HMGR and the other genes evaluated in this study was also of interest. At 24 h, the HMGR expression in cells exposed up to 3 μM forskolin increased to approximately 2-fold control levels but then decreased by approximately 2.5–3-fold from maximal levels at forskolin concentrations equal to or greater than 10 μM. This increase in HMGR gene expression was accompanied by increases in most other genes and was most evident for StAR and 17β-HSD4. In contrast, at 48 h there was a linear reduction in HMGR expression over the entire dose range (Fig. 1). This differed from that observed for the other genes evaluated in the study in that there was a general increase in their expression from control levels over the exposure range. The enzyme HMGR controls the biosynthesis of sterol and non-sterol isoprenoid biosynthesis pathway and catalyzes the synthesis of mevalonate, the precursor common to cholesterol, dolichol and coenzyme Q. In animal models, the reaction catalyzed by HMGR is the rate-limiting step in cholesterol biosynthesis and is subject to complex

regulatory controls (Goldstein and Brown, 1990). However, while it is the primary mechanism for controlling cholesterol biosynthesis, other mechanisms are also involved in these processes (Kojima et al., 2004). The interesting behavior of HMGR may be explained by the fact that the HMGR enzyme is controlled by several different mechanisms; among them are those conducted by cholesterol itself since cholesterol acts as a feed-back inhibitor of pre-existing HMGR as well as inducing rapid degradation of the enzyme. The sterol-mediate mechanisms include transcription of the reductase gene, translation of its mRNA and modulation of enzyme activity. Other mechanisms involve phosphorylation–dephosphorylation processes as result of cholesterol-induced polyubiquitnation of HMGR and its degradation in the proteosome (Panda and Devi, 2004).

When evaluating the forskolin dose–response analysis, it can be observed that most of the genes reach the maximum expression when exposed to a concentration of 10 μM for 48 h (Fig. 1). Based on these results, this concentration and time of exposure were chosen to evaluate the effects of other chemicals on the maximum effects observed by forskolin.

Overall, depending on the mode of action, time can be an important factor in evaluating the effect of chemicals or groups of chemicals on steroidogenic enzymes and genes.

4.2. Chemical treatments

The experiments with individual chemicals and simple binary mixtures in this study clearly indicate the existence of a variety of control mechanisms regulating the expression of these genes that result in responses that would not be easily predicted from the results of studies with the individual chemicals (Table 4). However, because of the limitations of the experiment design, the occurrence of antagonism, additivity and super-additivity can only be suggested. A complete study design, including a quantitative definition of summation and individual dose–effect relationships for model chemical 1, model chemical 2 and their mixture (at known ratios of 1 and 2) is required in order to definitively explain interactions between the chemicals. Here we can only conclude that for the binary mixtures cumulative effects and possible antagonist behavior were observed.

AMG, a drug also known as Cytraden, is used as an aromatase inhibitor in patients with breast cancer and adrenal anomalies. In our study, treatment of H295R cells with 300 μM AMG resulted in the inhibition of expression of CYP17, StAR and 17β-HSD4 while no effect on the expression of CYP19 was observed. While this would suggest that this dose of AMG would not adversely affect the production of E2 due to inhibition of degradation to estrone as a consequence of decreased expression of 17β-HSD4 (Fig. 3), it is important to note that this concentration is relatively high and that at lower concentrations E2 could be decreased due to an inhibition of aromatase (Hecker et al., 2005). On the other hand, AMG has also been found to reduce the production of pregnenolone, a P precursor, in rat neural tissues by inhibition of the CYP11A activity (Patte-Mensah et al., 2003). This effect on enzyme activity may explain the significant decrement in the production of P in our study. When H295R cells are exposed to a mixture of forskolin and AMG, CYP19 is inhibited but 17β-HSD4 is up-regulated possibly to compensate for the lack of E2 caused by aromatase

Table 4
Effects and interactions of single and mixture chemical exposures on steroidogenic genes in H295R cell line and hormone production[a]

Gene/Exposure	FORS[b]	METY[b]	FORS + METY[c]	AMG[b]	FORS + AMG[c]	KETO[b]	FORS + KETO[c]
CYP11A	↑	—	↑	—	↑	—	↓
CYP17	↑	—	↑	↓	↑	↓	↓
3βHSD2	—	↓	↓	—	—	↓	↓
CYP21	—	—	—	—	—	↓	↓
CYP11B2	↑↑	—	↑↑	—	↑↑	↑↑	↑↑↑↑[d]
CYP19	↑↑	—	↑[e]	—	↓↓[e]	↓	—[e]
17βHSD1	—	↑	↓[e]	—	↓[e]	—	—
17βHSD4	↑	—	↑↑	↓	↑	↑	↑
STAR	↑	—	—[e]	↓	—	—	—
HMGR	—	—	—	—	—	↓	↓
Hormone/exposure	FORS	METY	FORS + METY	AMG	FORS + AMG	KETO	FORS + KETO
Testosterone	↑	↓↓↓	na	↓↓	↓↓[e]	↓↓↓	↓↓↓↓+[e]
Progesterone	—	↓↓	na	<MDL	↓↓↓↓+[e]	↓↓	↓↓[e]
Estradiol	↑↑↑↑	↓↓↓	na	<MDL	↓↓↓↓+[e]	—	—[e]

Na, No applicable; MDL, minimum detection limit; +, More than 40-fold; ↑, Up-regulation, ↓, Down-regulation; ↑ or ↓, 2-fold or more/significant difference; ↑↑ or ↓↓, 5-fold or more, ↑↑↑ or ↓↓↓, 10-fold or more and ↑↑↑↑ or ↓↓↓↓, 15-fold or more.

[a]Chemicals were forskolin (FORS), metyrapone (METY), aminoglutethimide (AMG), and ketoconazole (KETO).

[b]Gene expression and hormone production comparisons for single chemicals made to solvent control.

[c]Gene expression and hormone production comparisons for mixtures made to forskolin alone.

[d]Suggested super-additivity.

[e]Suggested antagonism.

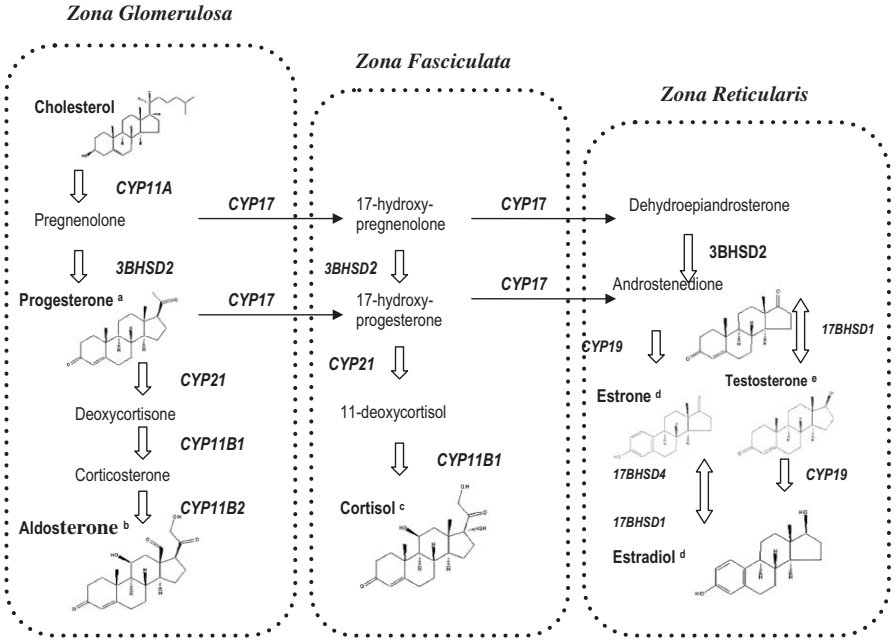

Fig. 3. Principal pathways in steroid biosynthesis where (a) major progestagen, (b) major mineralo-corticoid, (c) major gluco-corticoid, (d) major estrogen and (e) major androgen.

inhibition. The inhibition of aromatase is in agreement with the results of other studies (Bastida et al., 2001), in which the inhibitory action of AMG on protein kinase A was demonstrated. Recently, several aromatase promoter regions have been identified in H295R cells that were shown to be responsive to different stimuli and have implications relative to the regulation of aromatase activities (Heneweer et al., 2004). Thus, chemicals or chemical mixtures may have the potential to act on different promoter regions through alterations in second messenger systems such as PKA, PKC or Jak/STAT such that aromatase gene expression or activities are changed in a manner not predicted based on single chemical exposures. As a result, the mechanism of forskolin-mediated alterations in gene expression may be the action that makes AMG such a potent steroidogenic inhibitor rather than the blockade of the steroidogenesis pathway.

The forskolin-related induction of CYP17 and CYP11A gene expression was reduced to almost the same extent by all chemicals; only AMG did not significantly affect the forskolin-induced expression of 3β-HSD2 whereas the other chemicals reduced the expression of this enzyme to well below the level of expression seen in either the forskolin only treatment or that of the solvent control. Finally, while forskolin and ketoconazole both caused moderate increases (4–6-fold) in the expression of CYP11β2, a binary mixture of these two compounds resulted in a greater increase in expression (37-fold) than would have been predicted from exposures to the individual chemicals. It can be speculated that the reason for this notorious up-regulation may be explained by the interaction of the two known modes of action of the individual chemicals and the fact that these two modes of action come to a critical conjunction which forces the metabolic pathway toward specific products. In contrast, the other chemical mixtures tested had little to no effect on the forskolin-induced expression of CYP11β2 since they did not have modes of action which caused the specific alterations in the metabolic network.

Exposure of H295R cells to forskolin resulted in significant increases in T and E2 production. Although greater than those for E2 and T, differences in P concentrations were not statistically significant, which is likely due to the relatively high variability of control P concentrations. The super production of E2 was most likely directly related to an increase in the expression of the CYP19 gene, and a subsequent increase in aromatase enzyme concentrations. In addition, the up-regulation of the CYP17 gene expression by forskolin treatment may shift the steroidogenic process to the production of androgenic substrates leading to the formation of T and E2 (Cobb et al., 1996).

AMG treatment significantly decreased the production of all three hormones and in particular P and E2 where concentrations were reduced to less than their assay detection limit (Table 3). This result may be related to the decrease in CYP17 gene expression that could potentially result in a decrease in the production of androgenic substrates required for the formation of these hormones, but mostly, AMG is an endocrine antihormone that blocks adrenal steroidogenesis by inhibiting the enzymatic conversion of cholesterol to pregnenolone and also blocks the aromatization of androgenic precursors to estrogens by inhibiting aromatase activity. Therefore, it could be speculated that AMG decreased P and E2 production by a mechanism other than gene expression,

since 3β-HSD2 and CYP19 gene expression were not affected by this chemical treatment. Because the versatility of the H295R cell line for the evaluation of aromatase activity have been shown before (Sanderson et al., 2000, 2002), several experiments are being conducted in our laboratories to evaluate the effects of AMG in the activity of this enzyme in order to search for a more detailed explanation related to the E2 production. However, since all three hormones were greatly decreased by AMG the reduction was most likely due to general stress rather than a specific action of this chemical within the steroidogenic pathway. The powerful antagonism of AMG to forskolin is clearly observed when E2 concentrations dramatically decreased in the forskolin–AMG treatment compared to the greatest induction by forskolin. In addition, the ten-fold induction in T production by forskolin treatment matches the ten-fold decrement in T production observed in the forskolin–AMG exposure. The ketoconazole–forskolin treatment produced the same effects as those observed with either chemical singly on 17β-HSD1 gene expression resulting in no significant change in E2 concentrations. However, exposure to the mixture resulted in significant decreases in the production of T and P. The decrease in P may have been linked to the down-regulation of 3β-HSD2 since no effects were noted on the expression of CYP17 as compared to the solvent controls. Furthermore, the massive increase in CYP11B2 expression could have resulted in an increased synthesis of aldosterone, thus, resulting in the depletion of further upstream precursors including P. A possible explanation for the decrease in T could be a result of increased CYP19 expression in combination with the reduction of precursors such as P, with the lack of a concomitant increase of E2 due to the shift of the E2/estrone balance towards estrone due to increasing 17β-HSD4 gene expression. However, it is difficult to link changes in hormone production with changes in steroidogenic gene expression without the knowledge on how these translate into effects at the enzyme activity levels, and thus, the exact causes for the observed alterations in hormone concentrations remain unclear.

The results from this study underscore the utility of the H295R cell system to investigate the interactions of chemicals on steroidogenic gene expression and hormone production. However, to better understand these interactions it will be necessary to evaluate other endpoints such as steroidogenic enzyme activities, which link the alterations in gene expression to biologically important processes that are controlled by endocrine systems. Given that environmental exposure to chemical contaminants is almost always in the form of mixtures the use of a system such as the H295R assay is a powerful tool to investigate the effects of single compounds and complex mixtures of xenobiotics, and to investigate the molecular mechanisms of those effects and the molecular mechanisms of chemical interaction.

This study and our previous work (Hilscherova et al., 2004) have demonstrated the ability of the H295R assay system to investigate the effects of xenobiotics on steroidogenesis. By observing the effects of chemical exposure on 10 different steroidogenic endpoints (i.e., the expression of 10 different genes) the assay system has revealed that in general even "specific" inhibitors affect the expression of multiple genes. This is not surprising given the complex regulatory mechanisms controlling steroidogenesis, but clearly demonstrates that designating any chemical as a "specific inhibitor or inducer" is unwise. However, the responses observed clearly reflect the known mode of action of the various compounds. In the present study the complex non-additive responses observed as a result of exposure to some chemical mixtures can be explained by mechanistic interactions of the known modes of action of the specific chemicals. The additional complexity observed in many of the responses required considerably more effort to be put into interpretation of the gene expression profiles, thus hormone production was also evaluated to observe any correlation to gene expression. The H295R assay together with hormone quantification is a useful in vitro system to investigate regulatory and chemical interaction mechanisms as well as providing a system for screening chemicals for effects on steroidogenesis.

5. Conclusions

The H295R assay system is unique among bioassays in that it measures alterations in gene expression and hormone production at the same time. This dual response is particularly significant for chemicals that are able to alter the production of steroid hormones since the ultimate effects of these chemicals are expressed through the alterations in hormone concentrations in exposed organisms. The results of the H295R assay to date have demonstrated that chemicals maybe grouped alternatively by effects on gene expression or by effects on hormone production. These results clearly show that the chemicals tested have a range of modes of action. Significantly, there are clear examples of interaction between modes of action that may lead to supra-additive effects. This finding is clearly of significance given that environmental contaminants are most commonly found in complex mixtures. The H295R system is an effective tool for understanding potential mechanisms of action as well as a rapid, sensitive and cost-effective tool for high throughput screening of a range of potential effects of compounds. Furthermore, the H295R system is attractive because it minimizes the use of whole organisms.

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References

Ankley, G., Mihaich, E., Stahl, R., Tillitt, D., Colborn, T., McMaster, S., Miller, R., Bantle, J., Campbell, P., Denslow, N., Dickerson, R., Folmar, L., Fry, M., Giesy, J., Gray, L.E., Guiney, P., Hutchinson, T., Kennedy, S., Kramer, V., LeBlanc, G., Mayes, M., Nimrod, A., Patino, R., Peterson, R., Purdy, R., Ringer, R., Thomas, P., Touart, L., Van Der Kraak, G., Zachrewski, T., 1998. Overview of a workshop on screening methods for detecting potential (anti-) estrogenic/androgenic chemicals in wildlife. Environ. Toxicol. Chem. 17, 68–87.

Baker, M.E., 2001. Evolution of 17beta-hydroxysteroid dehydrogenases and their role in androgen, estrogen and retinoid action. Mol. Cell. Endocrinol. 171, 211–215.

Bastida, C.M., Tejada, F., Peñafiel, R., 2001. Aminoglutethimide, a steroidogenesis inhibitor abolishes hormonal induction of ornithine decarboxylase in steroidogenic tissues: evidence for its role as cAMP-dependent protein kinase inhibitor. Biochem. Biophys. Res. Comm. 281, 244–248.

Cauet, G., Balbuena, D., Achstetter, T., Dumas, B., 2001. CYP11A1 stimulates the hydroxylase activity of CYP11B1 in mitochondria of recombinant yeast in vivo and in vitro. Eur. J. Biochem. 268, 4054–4062.

Cobb, V.J., Williams, B.C., Mason, J.I., Walker, S.W., 1996. Forskolin treatment directs steroid production towards the androgen pathway in the NCI-H295R adrenocortical tumour cell line. Endocr. Res. 22 (4), 545–550.

Colborn, T., Dumanoski, D., Myers, J.P., 1997. Our Stolen Future: Are We Threatening our Fertility, Intelligence, and Survival? Penguin Group Publishers, New York, pp. 21–22.

DiMattina, M., Maronian, N., Ashby, H., Loriaux, D.L., Albertson, B.D., 1988. Ketoconazole inhibits multiple steroidogenic enzymes involved in androgen biosynthesis in the human ovary. Fertil.Steril. 49, 62–65.

Dodds, E.C., Lawson, W., 1938. Molecular structure in relation to oestrogenic activity. Compounds without a phenanthrene nucleus. Proc. Royal Soc. Lon. B. 125, 222–232.

EDSTAC Final Report, 1998. Endocrine Disruptor Screening and Testing Advisory Committee Final Report. US Environmental Protection Agency. Internet access at URL: 〈http://www.epa.gov/opptintr/opptendo/finalrpt.htm〉.

Gazdar, A.F., Oie, H.K., Shackleton, C.H., Chen, T.R., Triche, T.J., Myers, C.E., Chrousos, G.P., Brennan, M.F., Stein, C.A., La Rocca, R.V., 1990. Establishment and characterization of a human adrenocortical carcinoma cell line that expresses multiple pathways of steroid biosynthesis. Cancer Res. 50, 5488–5496.

Goldstein, J.L., Brown, M.S., 1990. Regulation of the mevalonate pathway. Nature 343, 425–430.

Grassman, J.A., Masten, S.A., Walker, N.J., Lucier, G.W., 1998. Animal models of human response to dioxins. Environ. Health Persp. 106 (Suppl. 2), 761–775.

Hecker, M., Newsted, J.L., Murphy, M.B., Higley, E.B., Tompsett, A.R., Jones, P.D., Giesy, J.P., 2005. Human adrenocarcinoma (H295R) cells for rapid in vitro determination of effects on steroidogenesis: hormone production. Tox. Appl. Pharm., in press.

Heneweer, M., van den Berg, M., Sanderson, J., 2004. A comparison of human H295R and rat R2C cell lines as in vitro screening tools for effects on aromatase. Toxicol. Lett. 146, 183–194.

Hilscherova, K., Jones, P.D., Gracia, T., Newsted, J.L., Zhang, X., Sanderson, J.T., Yu, R.M.K., Wu, R.S.S., Giesy, J.P., 2004. Assessment of the effects of chemicals on the expression of ten steroidogenic genes in the H295R cell line using real-time PCR. Toxicol. Sci. 81, 78–89.

Hu, M.C., Chiang, E.F.-L., Tong, S.K., Lai, W., Hsu, N.C., Wang, L.C.-K., Chung, B.C., 2001. Regulation of steroidogenesis in transgenic mice and zebrafish. Mol. Cell. Endocrinol. 171, 9–14.

Hu, M.C., Hsu, N.C., El Hadj, N.B., Pai, C.I., Chi, H.P.C., Wang, K.L., Chung, B.C., 2002. Steroid deficiency syndromes in mice with targeted disruption of Cyp11a1. Mol. Endocrinol. 16, 1943–1950.

Johansson, M.K., Sanderson, J.T., Lund, B.O., 2002. Effects of 3-MeSO2-DDE and some CYP inhibitors on glucocorticoid steroidogenesis in the H295R human adrenocortical carcinoma cell line. Toxicol In vitro 16 (2), 113–121.

Kanno, J., et al., 2001. The OECD program to validate the rat uterotrophic bioassay to screen compounds for in vivo estrogenic responses: Phase 1. Environ. Health Perspect. 109, 785–794.

Kavlock, R.T., Daston, G.P., De Rosa, C., Fenner-Crisp, P., Gray, L.E., Kaattari, S., Lucier, G., Luster, M., Mac, M.J., Maczka, C., Miller, R., Moore, J., Rolland, R., Scott, G., Sheehan, D.M., Sinks, T., Tilson, H.A., 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the US EPA sponsored workshop. Environ. Health Perspect. 104, 715–740.

Kojima, M., Masui, T., Nemoto, K., Degawa, M., 2004. Lead nitrate-induced development of hyperchlolesterolemia in rats: sterol-independent gene regulation of hepatic enzymes responsible for cholesterol homeostasis. Toxicol. Lett. 154, 35–44.

Krishnan, A.V., Starhis, P., Permuth, S.F., Tokes, L., Feldman, D., 1993. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 132, 2279–2286.

Labrie, F., Luu-The, V., Lin, S.X., Labrie, C., Simard, J., Breton, R., Belanger, A., 1997. The key role of 17β-hydroxysteroid dehydrogenase in sex steroid biology. Steroids 62, 148–158.

Legler, J., van den Brink, C.E., Brouwer, A., Murk, A.J., van der Saag, P.T., Vethaak, A.D., van der Burg, B., 1999. Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. Toxicol. Sci. 48.1, 55–66.

Loose, D.S., Kan, P.B., Hirst, M.A., Marcus, R.A., Feldman, D., 1983. Ketoconazole blocks adrenal steroidogenesis by inhibiting cytochrome P450-dependent enzymes. J. Clin. Invest. 71 (5), 1495–1499.

McKinney, J.D., Waller, C.L., 1994. Polychlorinated biphenyls as hormonally active structural analogues. Environ. Health Persp. 102, 290–297.

Mueller, G.C., Kim, U.-H., 1978. Displacement of estradiol from estrogen receptors by simple alkyl phenols. Endocrinology 102, 1429–1435.

Ohno, S., Shinoda, S., Toyoshima, S., Nakazawa, H., Makino, T., Nakajin, S., 2002. Effects of flavonoid phytochemicals on cortisol production and on activities of steroidogenic enzymes in human adrenocortical H295R cells. J. Steroid Biochem. Mol. Biol. 80, 355–363.

Panda, T., Devi, V., 2004. Regulation and degradation of HMGCo-A reductase. Appl. Microbiol. Biotechnol. 66, 143–152.

Parthasarathy, C., Yuvaraj, S., Sivakumar, R., Ravi, S.B., Balasubramanian, K., 2002. Metyrapone-induced corticosterone deficiency impairs glucose oxidation and steroidogenesis in Leydig cells of adult albino rats. Endocrinol. J. 49, 405–412.

Patte-Mensah, C., Kappes, V., Freund-Mercier, M., Tsutsui, K., Mensah-Nyagan, A., 2003. Cellular distribution and bioactivity of the key steroidogenic enzyme, cytochrome P450 side chain cleavage, in sensory neural pathways. J. Neurochem. 86 (5), 1233–1246.

Pons, M., et al., 1990. A new cellular model of response to estrogens: a bioluminescent test to characterize (anti) estrogen molecules. Biotechniques 9, 450–459.

Rainey, W.E., Bird, I.M., Sawetawan, C., Hanley, N.A., McCarthy, J.L., McGee, E.A., Wester, R., Mason, J.I., 1993. Regulation of human adrenal carcinoma cell (NCI-H295) production of C19 steroids. J. Clin. Endocrinol. Metab. 77, 731–737.

Routledge, E.J., Sumpter, J.P., 1996. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ. Toxicol. Chem. 15, 241–248.

Sanderson, J.T., Seinen, W., Giesy, J.P., van den Berg, M., 2000. 2-chloro-S-triazine herbicides induce aromatase (CYP-19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity. Toxicol. Sci. 54, 121–127.

Sanderson, J.T., Boerma, J., Lansbergen, W.A., van der Berg, M., 2002. Induction and inhibition of aromatase (CYP19) activity by various classes of pesticides in H295R human adrenocortical carcinoma cells. Tox. Appl. Pharm. 182, 44–54.

Sohoni, P., Sumpter, J.P., 1998. Several environmental oestrogens are also anti-androgens. J. Endocrinol. 158, 327–339.

Sonneveld, E., Jansen, H.J., Riteco, J.A., Brouwer, A., van der Burg, B., 2004. Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid responsive bioassays. Toxicol. Sci. 83, 136–148.

Soto, A.M., Justicia, H., Wray, J.W., Sonnenschein, C., 1991. *p*-Nonylphenol, an estrogenic xenobiotic released from 'modified' polystyrene. Environ. Health Persp. 92, 167–173.

Staels, B., Hum, D.W., Miller, W.L., 1993. Regulation of steroidogenesis in NCI-H295R cells: a cellular model of the human fetal adrenal. Mol. Endocrinol. 7, 23–433.

Thomson, L.M., Kapas, S., Carroll, M., Hinson, J.P., 2001. Autocrine role of adrenomedullin in the human adrenal cortex. J. Endocrinol. 170, 259–265.

Villeneuve, D.L., Blankenship, A.L., Giesy, J.P., 1998. Estrogen receptors—environmental xenobiotics. In: Denison, M.S., Helferich, W.G. (Eds.), Toxicant–Receptor Interactions and Modulation of Gene Expression. Lippincott-Raven Publishers, Philadelphia, pp. 69–99 (Chapter 4).

Wilson, V.S., Bobseine, K., Lambright, C.R., Gray Jr., L.E., 2002. A novel cell line, MDA-kb2, that stably expresses an androgen- and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists. Toxicol. Sci. 66.1, 69–81.

Zhang, X., Yu, R.M.K., Jones, P.D., Lam, G.K.W., Newsted, J.L., Gracia, T., Hecker, M., Hilscherova, K., Sanderson, J.T., Wu, R.S.S., Giesy, J.P., 2005. Quantitative RT-PCR methods for evaluating toxicant-induced effects on steroidogenesis using the H295R cell line. Environ. Sci. Technol. 39, 2777–2785.

Článek X:

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Modulation of steroidogenic gene expression and hormone production of H295R cells by pharmaceuticals and other environmentally active compounds

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Abstract

The H295R cell bioassay was used to evaluate the potential endocrine disrupting effects of 18 of the most commonly used pharmaceuticals in the United States. Exposures for 48 h with single pharmaceuticals and binary mixtures were conducted; the expression of five steroidogenic genes, 3βHSD2, CYP11β1, CYP11β2, CYP17 and CYP19, was quantified by Q-RT-PCR. Production of the steroid hormones estradiol (E2), testosterone (T) and progesterone (P) was also evaluated. Antibiotics were shown to modulate gene expression and hormone production. Amoxicillin up-regulated the expression of CYP11β2 and CYP19 by more than 2-fold and induced estradiol production up to almost 3-fold. Erythromycin significantly increased CYP11β2 expression and the production of P and E2 by 3.5- and 2.4-fold, respectively, while production of T was significantly decreased. The β-blocker salbutamol caused the greatest induction of CYP17, more than 13-fold, and significantly decreased E2 production. The binary mixture of cyproterone and salbutamol significantly down-regulated expression of CYP19, while a mixture of ethynylestradiol and trenbolone, increased E2 production 3.7-fold. Estradiol production was significantly affected by changes in concentrations of trenbolone, cyproterone, and ethynylestradiol. Exposures with individual pharmaceuticals showed the possible secondary effects that drugs may exert on steroid production. Results from binary mixture exposures suggested the possible type of interactions that may occur between drugs and the joint effects product of such interactions. Dose–response results indicated that although two chemicals may share a common mechanism of action the concentration effects observed may be significantly different.

Keywords: Bioassay; Steroidogenesis; Pharmaceuticals; Endocrine disruptors; Drug mixtures; Dose–response

Introduction

According to the U.S. Food and Drug Administration (USFDA), approximately 82,000 drugs are registered in the U.S. for human use, accounting for more than 3000 active ingredients. Adjuvants and, in some instances, pigments and dyes are also components of the formulated drug product. After administration to humans and animals, pharmaceuticals are excreted in waste products and many unused medications are disposed in drains or sewage systems. Sewage treatment facilities, depending on their technology and a chemical's physicochemical properties, are not always effective in removing active chemicals from wastewater. As a result, pharmaceuticals find their way into the environment, where they can directly affect terrestrial and aquatic organisms and can be incorporated into food chains (Díaz-Cruz et al., 2003; Cecchini and LoPresti, 2007).

Despite extensive and detailed reports about residues of pharmaceuticals in the environment have been published (Jorgensen

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and Halling-Sorensen, 2000; Hereber, 2002; Sanderson et al., 2004; Jones et al., 2004), the potential ecological effects associated with the presence of these compounds have been largely ignored. In the European Union, discharge of pharmaceutical products is regulated through mandatory submission of Environmental Risk Assessments (ERAs) that accompany Marketing Authorization Approval. Most of the methods used today for the identification and quantification of pharmaceuticals in the environment and the first attempts at eco-toxicity evaluations of these active compounds have been developed in European countries (Commission of the European Communities, 1992). Notably, the European Union has taken the lead on banning the use of the majority of growth-promoting antibiotics in livestock on the basis of the "Precautionary Principle" (Casewell et al., 2003).

Among the frequently detected substances in rivers are β-blockers such as metoprolol (at concentrations up to 1.5 μg/l) and β-sympathomimetics (Hirsch et al., 1996; Sedlak and Pinkston, 2001). Analgesic and anti-inflammatory drugs like diclofenac have been observed in several studies at concentrations up to 1.2 μg/l (Ternes, 1998; Stumpf et al., 1998; Buser et al., 1998); estrogens such as 17β-estradiol have been found at concentrations up to 13 ng/l (Kuch and Ballschmitter, 2000). In addition, antibiotics such as erythromycin have been reported to occur at concentrations as high as 1.7 μg/l (Hirsch et al., 1999; Lindsey et al., 2001). Estrogenic compounds have also been identified in rivers of southern and central Germany (Adler et al., 2001), as well as lipid-lowering agents such as clofibric acid at concentrations as great as 0.2 μg/l (Ollers et al., 2001), and anti-epileptic drugs such as carbamazepine at concentrations up to 2.1 μg/l (Mohle et al., 1999).

During 1999–2000, the U.S. Geological Survey conducted the first nationwide investigation of the occurrence of pharmaceuticals, hormones and other organic contaminants in 139 streams in 30 states (Kolpin et al., 2002). A total of 95 residues were targeted including antibiotics, prescription and nonprescription drugs, steroids and hormones, 82 of which were found in at least one sample. Although the authors cautioned that sites were chosen based on their increased susceptibility to contamination from urban or agricultural activities, a surprising 80% of streams sampled were positive for one or more of the targeted pharmaceuticals. Furthermore, 75% of the streams contained two or more of the targeted pharmaceuticals, 54% had more than five, while 34% had more than 10 and 13% tested positive for more than 20 targeted contaminants. Similar reconnaissance studies are ongoing all over the world to evaluate the presence of pharmaceuticals in groundwater and surface water sources of drinking water. Identification of the environmental exposure routes of these drugs is crucial for a realistic environmental assessment of pharmaceuticals because it is the prescribed drug dose and the duration of treatment that provides an estimate of environmental loading. The fact that the same drug may be used for several applications and that exposure routes may vary in different environmental matrices means that the fate of the drug may also vary, resulting in quite different environmental concentrations.

Pharmaceuticals are sometimes thought to be easily (bio) degraded in the environment, but it has been established that large proportions of many pharmaceuticals can be excreted from the body un-metabolized and enter wastewater as biologically active substances (Fent et al., 2006; Kummerer, 2001). Some drugs which have been metabolized can be converted back to the parent compound in the environment (Pickrell, 2002). This has been demonstrated for the glucoronide metabolite of chloramphenicol and the acetylated metabolite of sulphadimidine in samples of liquid manure (Berger et al., 1986). Thus, it is often not only the parent compound which should be the subject for a risk assessment but also the major metabolites. Additionally, drug residues found in the environment, especially in aquatic systems, usually occur as mixtures rather than as single contaminants, and their possible interactions should therefore be considered in risk assessments.

Since pharmaceuticals are specifically designed to be biologically active, they may have unintended effects on non-target organisms in the environment, even at low concentrations. However, there is a lack of information about effects other than the original innate function for which the chemical or pharmaceutical was designed and/or produced. Furthermore, the paucity of information concerning ecotoxicity of pharmaceuticals (van Wezel and Jager, 2002) also makes it difficult to characterize and assess the environmental risk of these compounds.

The objective of the present study was to evaluate the potential effects of 18 of the most used human and veterinary pharmaceuticals in the United States on steroidogenesis. Using the H295R cells as a study model, the effects of five antibiotics, four growth promoters (two of which are also used as antibiotics), one corticosteroid, one anti-cancer and one birth control drug, two analgesic and anti-inflammatory drugs, one anti-lipidic, one anti-depressive, one β-blocker and one insect repellent, on the expression of five steroidogenic genes encoding for the four rate-determining enzymes controlling the production of the three main hormones in the steroidogenic pathway was evaluated by use of Q-RT-PCR. The genes studied included 3βHSD2, CYP11β1, CYP11β2, CYP17 and CYP19. In addition, the production of the hormones estradiol (17β-estradiol, E2), testosterone (T), and progesterone (P) was quantified using ELISA methods and related to gene expression. Dose–response curves were also developed to evaluate the effects of chemical concentration on both gene expression and hormone production.

Methods

Test chemicals. The 18 pharmaceuticals used for this study are depicted in Table 1. All chemicals were obtained from Sigma (St. Louis, MO, USA), except for amoxicillin, cephalexin hydrate and erythromycin, which were obtained from BioChemika (St. Louis, MO, USA). Doxycycline hyclate was used in this study. Purity of all test chemicals from Sigma exceeded 98% while that of chemicals from BioChemika exceeded 97%. The chemicals used in this study were selected based on the list of the top 300 prescription drugs dispensed in the USA during 2005 (Rx List, www.rxlist.com) and also by their prevalence in surface waters. A set of high and low concentration exposures was run with the purpose of testing the range of response of the H295R cell system. High concentrations were established as higher than 3×10^3 1 μg/l and low concentrations were established to be less than 1 μg/l.

Experimental design. The H295R human adrenocortical carcinoma cell line was obtained from the American Type Culture Collection (ATCC # CRL-2128, ATCC, Manassas, VA, USA) and cells were grown in 75 cm^2 flasks with 12.5 ml of supplemented medium at 37 °C with a 5% CO_2 atmosphere. Supplemented

Table 1
Pharmaceuticals and environmentally active compounds used in H295R cell exposures[a,b]

Compound	Therapeutic use	Conc.[a] (μg/l)
Acetaminophen	Analgesic	3×10^5
Clofibrate	Lipid agent	3×10^3
Dexamethasone	Corticosteroid	2×10^3
Doxycycline	Antibiotic	1×10^4
DEET	Pesticide	3×10^3
Erythromycin	Antibiotic	3×10^3
Ibuprofen	NSAAID	25×10^4
Trimethoprim	Antibiotic	3×10^3
Tylosin	Antibiotic[c]	3×10^3

Compound	Therapeutic use	Conc.[b] (μg/l)
Amoxicillin	Antibiotic	71
Cephalexin	Antibiotic	73
Cyproterone	Cancer treatment	62
Ethynylestradiol	Oral contraceptive	1
Fluoxetine	Antidepressant	1
Oxytetracycline	Antibiotic[c]	81
Salbutamol	Asthma/β-agonist	5×10^{-2}
Trenbolone	Growth Promoter	25
α-Zearalanol	Hyperestrogen	2.8×10^{-3}

NSAAID: Non steroidal analgesic anti-inflammatory drug.
[a] High concentrations.
[b] Low concentrations.
[c] Used also as a growth promoter.

medium was a 1:1 mixture of Dulbecco's modified Eagle's medium with Ham's F-12 Nutrient mixture with 15 mM HEPES buffer. The medium was supplemented with 1.2 g/l Na_2CO_3, ITS+Premix (BD Bioscience, 1 ml Premix/100 ml medium), and 12.5 ml/500 ml NuSerum (BD Bioscience, San Jose, CA, USA). Final component concentrations in the medium were: 15 mM HEPES; 6.25 μg/ml insulin; 6.25 μg/ml transferrin; 6.25 ng/ml selenium; 1.25 mg/ml bovine serum albumin; 5.35 μg/ml linoleic acid; and 2.5% NuSerum. The medium was changed 2–3 times per week and cells were detached from flasks for sub-culturing using sterile 1× trypsin–EDTA (Life Technologies Inc.). For exposure, cells were harvested into a final volume of 10 ml of medium. Cell density was determined using a hemacytometer. For dosing, 3 ml of cell suspension containing approximately 10^6 cells/ml were placed in each well of 6-well tissue culture plates (Nalgene Nunc Inc., Rochester, NY, USA). Cells were exposed for 48 h to different groups of pharmaceuticals and several other compounds of relevant environmental importance dissolved in DMSO or methanol. The final concentration of both DMSO and methanol was 0.1%.

H295R cells were exposed for 48 h to individual antibiotics with different spectra of action (Table 1). Amoxicillin, cephalexin, erythromycin, tetracyclines, trimethoprim and tylosin were the antibiotics chosen. A group of drugs used for hormone therapies including cyproterone, dexamethasone and ethynylestradiol (EE2), and growth promoters such as trenbolone and α-zearalanol, were also tested. Over-the-counter drugs, such as the analgesics acetaminophen and ibuprofen were also included. The assessed pharmaceuticals included also other drugs from different therapeutic groups, such as the antidepressant fluoxetine, the antilipidic clofibrate, the β-agonist salbutamol, and the insect repellant DEET (*N*,*N*-diethyl-3-methylbenzamide), which are also frequently found in environmental samples. The effects of the target chemicals on gene expression were compared to the effects of exposures to solvent controls of DMSO or methanol where appropriate, at each time interval.

Since pharmaceuticals can occur in surface waters as mixtures, a set of four binary mixtures of pharmaceuticals were also used as exposure solutions for the H295R cells. These mixtures were EE2-trenbolone, EE2-cyproterone, EE2-tylosin and cyproterone-salbutamol. The chemicals used in mixture solutions were chosen based on the results of individual exposures. Moreover, dose–response curves were constructed for three of the pharmaceuticals used as hormone therapy drugs such as EE2, cyproterone and trenbolone to evaluate whether or not changes in gene expression and hormone production were directly related to changes in drug concentration.

All exposures, individual chemicals and binary mixtures, were run in triplicate.

Cell viability/cytotoxicity. Before nucleic acid isolation and hormone analysis, cell viability was determined. Cells were visually inspected under a microscope to evaluate viability and cell number. In addition, to establish the range of chemical concentrations that could be used without producing physical harm to the cells, a Live/Dead cell viability assay kit (Molecular Probes, Eugene, OR, USA) was used. In instances where exposure resulted in cell death or decreased viability (less than 85%) the data were not used to evaluate gene expression or hormone production.

RNA isolation. For nucleic acid extraction, cells were lysed in the culture plate after removal of the medium by the addition of 580 μl/well of lysis buffer–β-ME mixture (Stratagene, La Jolla, CA, USA) and RNA was isolated as previously described (Hilscherova et al., 2004). Briefly, lysed cells were mixed and then centrifuged in a pre-filter spin cup. The filtrate was diluted with 70% ethanol and vortexed. The mixture was transferred to an RNA spin cup and centrifuged for 1 min. The filtrate was discarded and the spin cup was washed with a low-salt buffer and then centrifuged for 1 min. RNase-Free DNase I solution (Stratagene, La Jolla CA, USA) was added to the fiber matrix inside the spin cup and the sample was incubated at 37 °C for 15 min. The sample was then washed with a high-salt followed by a low-salt buffer. After each wash cycle, the filtrate was discarded. After the final wash, the sample was centrifuged and nuclease-free water was added directly to the fiber matrix inside the spin cup. The tube was incubated for 2 min at room temperature and centrifuged. This elution step was repeated to maximize the yield of RNA. The purified RNA was used immediately or stored at −80 °C until needed. An appropriate dilution of the RNA sample (1:50) was prepared for RNA quantification. The absorbance of the RNA solution was measured at 260 nm and 280 nm and the 260/280 ratio was calculated. The concentration of total RNA was estimated using the A_{260} value and a standard with A_{260} of 1 that was equivalent to 40 μg RNA/ml.

cDNA preparation. Total RNA (1–5 μg) was combined with 50 μM oligo-(dT)20, 10 mM dNTPs, and diethylpyrocarbamate (DEPC)-treated water to a final volume of 12 μl. RNA and primers were denatured at 65 °C for 5 min and then incubated on ice for 5 min. Reverse transcription was performed using 8 μl of a master mix containing 5*X* cDNA synthesis buffer (Carlsbad CA, USA) and RNase/DNase free water. Reactions were incubated at 50 °C for 45 min and were terminated by incubation at 85 °C for 5 min. Samples were either used directly for PCR or were stored at −20 °C until analyzed.

Gene expression using real-time PCR. The studied genes include 3βHSD2 encoding for the enzyme catalyzing the production of the first biologically important steroid in the pathway, progesterone; CYP11β1 and CYP11β2, which work directly on cortisol production and aldosterone synthesis respectively; CYP17 required for androgen production and regulation of substrate supplies for aromatization, as well as for cortisol biosynthesis; and CYP19 gene encoding for the aromatase enzyme, which mediates the aromatization of C18 estrogenic steroids from C19 androgens. CYP11β1 was measured only for the analysis of dose responses of cyproterone and trenbolone exposures. The analysis of gene expression Real-time PCR (quantitative PCR) was performed by the Smart Cycler System (Cepheid, Sunnyvale, CA, USA) in 25 μl sterile tubes using a master mix containing 25 mM $MgCl_2$, 1U/μl AmpErase (Applied Biosystems, Foster City, CA, USA), 5 U/μl *Taq* DNA polymerase AmpliTaq Gold, 10× SYBR Green (PE Biosystems, Warrington, UK), nuclease free water and between 10 pg and 1 μg of cDNA. The thermal cycling program included an initial denaturing step at 94 °C for 10 min, followed by 25–35 cycles of denaturing (95 °C for 15 s), primer annealing (at 60–64 °C for 40–60 s), and cDNA extension (72 °C for 30 s); a final extension step at 72 °C for 5–10 min was also included. Melting curve analyses were performed immediately following the final PCR cycle to differentiate between the desired amplicons and any primer-dimers or DNA contaminants. Specifics of the assay parameters such as primers used and annealing temperatures have been published previously (Hilscherova et al., 2004).

For quantification of PCR results the threshold cycle C_t (the cycle at which the fluorescence signal is first significantly different from background) was determined for each reaction. C_t values for each gene of interest were normalized to the endogenous control gene, β-actin. Normalized values were used to calculate the degree of induction or inhibition expressed as a "fold difference" compared to normalized control values. Therefore, all data were statistically analyzed as "fold induction" between exposed and control cultures. Gene

expression was measured in triplicate for each control or exposed cell culture and each exposure was repeated at least three times.

Hormone quantification. Hormone extraction and quantification were conducted by ELISA were conducted as previously described (Hecker et al., 2006). Briefly, after exposure cell medium was collected from each well prior to cell lysis for RNA extraction and stored in 1 ml aliquots at −80 °C until needed. For analysis, frozen medium samples were thawed on ice, and the hormones were extracted twice with diethyl ether (5 ml) in glass tubes. To determine extraction recoveries a trace amount of ^{3}H-T was added to each sample prior to extraction. The solvent extract was separated from the water phase by centrifugation at 2000×*g* for 10 min and transferred into small glass vials. The solvent was evaporated under a stream of nitrogen, and the residue was dissolved in EIA buffer from Cayman Chemical Company and either immediately measured or frozen at −80 °C for later hormone determination. Concentrations of hormones in media were measured by competitive ELISA using Cayman Chemical® hormone EIA kits (Cayman Chemical Company, Ann Arbor, MI, USA; P [P; Cat # 582601], T [T; Cat # 582701], 17β-estradiol [E2; Cat # 582251]). Because the antibody to P exhibits 61% cross-reactivity with pregnenolone and the method does not allow for the separation of these two hormones, P concentrations are expressed as P/pregnenolone. Hormones in all media samples were measured in triplicate. The working ranges for the determination of steroid hormones in H295R media were, P: 7.8–1000 pg/ml; T: 3.9–500 pg/ml; E2 estradiol: 7.8–1000 pg/ml. Media extracts were diluted 1:25 and 1:100 for T, while dilutions for P and E2 were 1:50 to 1:100 and 1:2 to 1:10, respectively.

Statistical analysis. Statistical analyses of gene expression profiles were conducted using SYSTAT (SYSTAT Software Inc., Point Richmond, CA, USA). Differences in gene expression and hormone production were evaluated by ANOVA followed by Tukey's Test. Differences with $p<0.05$ were considered significant. Statistical correlations between gene expression and hormone production were established by Pearson correlation analysis followed by Bonferroni probability test. Correlations with $p<0.05$ were considered significant.

Results

Antibiotic exposure

Gene expression

Gene expression responses to the exposures conducted with seven of the most commonly used antibiotics in human medicine and for veterinary purposes are given in Table 2. The responses of gene expression for the blank and solvent control exposures were consistent. Treatment of the H295R cells with environmentally relevant or greater concentrations of the selected antibiotics resulted in significant changes in the expression of several genes. Exposure of H295R cells to environmentally relevant concentrations of amoxicillin, cephalexin, oxytetracycline and tylosin significantly altered the expression pattern of the four target genes relative to that of solvent-exposed cells. Because oxytetracycline was not soluble in DMSO methanol was used to dissolve this compound. A methanol control was also included among the exposures. Amoxicillin significantly increased the expression of CYP17 and CYP19 more than 4-fold compared to solvent controls, while cephalexin and oxytetracycline significantly increased expression of CYP19 more than 2-fold. Oxytetracycline was also the only antibiotic shown to affect the expression of the progesterogenic gene 3βHSD2. Tylosin increased expression of the aldosteronogenic gene CYP11β2 approximately 10-fold. Erythromycin, doxycycline and trimethoprim were used at non-relevant environmental concentrations of 3 to 10 μg/ml. Erythromycin increased the expression of CYP11β2 approximately 7-fold while doxycycline induced the expression of CYP19 almost 3-fold.

Hormone production

While the concentrations of all the hormones measured were very consistent in the blank, DMSO and methanol exposures (Table 2), some of the pharmaceuticals produced in changes in production of hormones. Erythromycin increased the production of P and E2 more than 2- and 3-fold respectively, and reduced the production of T by more than 50%. In contrast, tetracyclines did not significantly affect hormone production. Tylosin decreased the production of T and E2, while cephalexin only decreased T production and amoxicillin increased production of E2 more than 2-fold.

Table 2
Gene expression and hormone production in H295R cells exposed to single antibiotics

Treatment									
Gene	DMSO	MeOH	AMOXI	CEPHA	ERYT	OXYTC[a]	DOXYC	TRIME	TYLO
	0.1%	0.1%	71 μg/l	73 μg/l	3×10^{3} μg/l	81 μg/l	1×10^{4} μg/l	3×10^{3} μg/l	3×10^{3} μg/l
CYP11β2	1.00±0.33	1.00±0.19	1.95±0.24	0.77±0.33	6.91±0.97*	1.72±0.31	0.69±0.73	0.64±0.05	9.99±1.09*
CYP19	1.00±0.61	1.00±0.16	4.45±0.55*	2.87±1.13*	0.54±0.14	2.58±0.25*	2.87±0.40*	0.58±0.08	0.49±0.33
CYP17	1.00±0.10	1.00±0.16	4.48±0.35*	1.74±0.56	0.75±0.06	1.89±0.071	0.85±0.04	1.90±0.08	1.03±0.04
3βHSD2	1.00±0.13	1.00±0.16	1.42±0.75	0.72±0.52	0.92±0.25	2.51±0.17*	1.00±0.08	0.60±0.36	1.32±0.27

Hormone	DMSO	MeOH	AMOXI	CEPHA	ERYT	OXYTC	DOXYC	TYLO
	0.1%	0.1%	71 μg/l	73 μg/l	3×10^{3} μg/l	81 μg/l	1×10^{4} μg/l	3×10^{3} μg/l
Testosterone	1.00±0.27	1.00±0.34	0.71±0.02	0.11±0.03*	0.44±0.08*	1.23±0.007	1.55±0.085	0.42±0.09*
Progesterone	1.00±0.01	1.00±0.13	0.63±0.13	0.89±0.04	3.55±0.58*	1.32±0.55	1.26±0.20	1.30±0.28
Estradiol	1.00±0.32	1.00±0.60	2.5±0.61*	0.51±0.30	2.46±0.33*	0.15±0.05	2.04±0.60	0.12±0.08*

All exposures were conducted for 48 h under standard conditions. All gene expression and hormone production values for fold-change relative to DMSO the solvent control (=1.0), given as means and standard deviations. DMSO: Dimethylsulfoxide; MeOH: Methanol; AMOXI: Amoxicillin; ERYT: Erythromycin; OXYTC: Oxytetracycline; TRIME: Trimethoprim; TYLO: Tylosin.

[a] Compared to MeOH as solvent control.

* Indicates statistically significant differences at $p<0.05$.

Table 3
Gene expression and hormone production in H295R cells exposed to single hormone therapy drugs

Treatment						
Gene	DMSO	CYPROT	DEXAM	EE2	TRENB	ZEARA
	0.1%	62 μg/l	2×10^{3} μg/l	1 μg/l	25 μg/l	2.8×10^{-3} μg/l
CYP11β2	1.00±0.33	1.34±0.52	5.39±0.50*	4.88±0.97*	0.81±0.43	0.22±0.03
CYP19	1.00±0.61	4.62 ±1.51*	0.98±0.19	0.54±0.14	2.56±0.55*	0.32±0.10
CYP17	1.00±0.10	2.81±0.5*	0.71±0.04	0.75±0.06	1.79±0.41	0.20±0.04
3βHSD2	1.00±0.13	0.90±0.32	0.64±0.09	0.92±0.25	0.77±0.15	0.14±0.03*
Hormone	DMSO	CYPROT	DEXAM	EE2	TRENB	ZEARA
	0.1%	62 μg/l	2×10^{3} μg/l	1 μg/l	25 μg/l	2.8×10^{-3} μg/l
Testosterone	1.00±0.27	0.29±0.03*	0.25±0.05*	0.36±0.12*	0.48±0.09*	1.06±0.15
Progesterone	1.00±0.01	1.02±0.30	0.74±0.05	2.71±0.39*	0.72±0.20	1.02±0.21
Estradiol	1.00±0.32	1.25±0.47	1.4±0.26	2.33±0.27*	1.6±0.25	0.17±0.03

All exposures were conducted for 48 h under standard conditions. All gene expression and hormone production values are expressed as fold-change relative to the solvent control DMSO (=1.0), given as means and standard deviations. DMSO: Dimethylsulfoxide; CYPROT: Cyproterone; DEXAM: Dexamethasone; EE2: Ethynylestradiol; TRENB: Trenbolone Acetate; ZEARA: α-Zearalanol.
* Indicates statistically significant differences at $p<0.05$.

Hormone therapy drugs

Gene expression

None of the four hormone therapy drugs that are commonly used for cancer treatment, birth control, inflammatory processes and as growth promoters in animal production significantly affected the expression of 3βHSD2 (Table 3). Environmentally relevant concentrations of the cancer therapy drug cyproterone induced the expression of CYP19 more than 4-fold and the expression of the androgenic gene CYP17 3-fold. Dexamethasone and EE2 exposures induced expression of CY11β2 approximately 5-fold. The growth promoter trenbolone only increased the expression of CYP19 by about 3-fold.

Hormone production

Hormone therapy drugs also affected the hormone production (Table 3). EE2 significantly increased P and E2 production by more than 2-fold and at the same time significantly decreased T production by about 66%. Trenbolone and cyproterone decreased T production by approximately 50% and 66% respectively. However, no chemical except of EE2 affected E2 or P production.

Other pharmaceuticals and environmentally active compounds

Gene expression

A significant up-regulation of CYP17 was observed after the exposure to the β_2-agonist salbutamol, which increased the expression of this gene more than 10-fold (Table 4). None of the other genes studied were affected by this compound. Of the analgesics studied, only acetaminophen significantly affected the expression of CYP11β2 by increasing it approximately 4-fold. CYP11β2 was induced approximately 5-fold by the antilipidic clofibrate and the antidepressant fluoxetine, and ap-

Table 4
Gene expression and hormone production in H295R cells exposed to single pharmaceuticals

Treatment							
Gene	DMSO	ACETA	IBUPR	SALBU	CLOFI	DEET	FLUOX
	0.1%	3×10^{5} μg/l	25×10^{4} μg/l	5×10^{-2} μg/l	3×10^{3} μg/l	3×10^{3} μg/l	1 μg/l
CYP11β2	1.00±0.33	3.66±0.89*	2.6±0.77	2.00±0.38	4.67±1.24*	8.20±1.306*	5.69±0.77*
CYP19	1.00±0.61	0.88±0.10	0.72±0.01	1.88±0.62	1.30±0.15	0.5±0.04	1.41±0.09
CYP17	1.00±0.10	0.64±0.08	0.55±0.08	13.64±0.98*	1.04 ± 0.09	1.26±0.13	0.90±0.05
3βHSD2	1.00±0.13	0.45±0.20	0.37±0.23	1.05±0.19	0.66±0.03	1.04±0.44	0.69±0.07
Hormone	DMSO	ACETA	IBUPR	SALBU	CLOFI	DEET	FLUOX
	0.1%	3×10^{5} μg/l	25×10^{4} μg/l	5×10^{-2} μg/l	3×10^{3} μg/l	3×10^{3} μg/l	1 μg/l
Testosterone	1.00±0.27	1.12±0.04	0.88	0.51±0.17	0.68±0.13*	0.39±0.01*	0.75±0.01
Progesterone	1.00±0.01	2.3±0.15*	1.84	0.30±0.30	0.75±0.09	1.68±0.48	1.37±0.08
Estradiol	1.00±0.32	0.50±0.2	0.42	0.32±0.32*	1.55±0.05	0.17±0.10*	1.30±0.30

All exposures were conducted for 48 h under standard conditions. All gene expression and hormone production values are expressed as fold-change relative to the solvent control DMSO (=1.0), given as means and standard deviations. DMSO: Dimethylsulfoxide; ACETA: Acetaminophen; IBU: Ibuprofen; SALBU: Salbutamol; CLOFI: Clofibrate; DEET: *N*,*N*-diethyl-3-methylbenzamide; FLUOX: Fluoxetine.
* Indicates statistically significant differences at $p<0.05$.

proximately 4-fold by the analgesic acetaminophen. The insect repellent, DEET also increased the expression of this gene more than 8-fold. Although the natural phytoestrogen α-zearalanol decreased the expression of the four evaluated genes, only the decrease in 3βHSD2 was statistically significant. The non-steroidal analgesic anti-inflammatory drug (NSAAID) ibuprofen did not produce any significant changes in the expression of any of the steroidogenic genes studied.

Hormone production

Ibuprofen and fluoxetine did not cause significant changes in the production of any of the analyzed hormones compared to blank and solvent controls (Table 4). However, T production was decreased by clofibrate and DEET, while E2 production was significantly inhibited by salbutamol and DEET. Moreover, the analgesic acetaminophen produced a 2-fold increase in P concentrations.

Pattern of responses to chemical mixtures

Gene expression

When H295R cells were exposed to binary mixtures where one of the two components was EE2 the gene expression responses were very diverse (Table 5). In the response to trenbolone exposure CYP19 was up-regulated approximately 2.5-fold. Exposure to a mixture of trenbolone and EE2 caused a decrease in CYP19 expression of as much as 50% compared to solvent control. Individually cyproterone up-regulated expression of CYP19 more than 4-fold, but when cells were exposed to a mixture of cyproterone and EE2, expression of this gene was not significantly different from that of the control. Although tylosin reduced CYP19 expression this reduction was not statistically significant when compared to that of cells exposed to the solvent only. The tylosin–EE2 mixture did not produce changes in the expression of this gene compared to controls. Cyproterone significantly up-regulated the expression of the aromatase gene CYP19 up to 4.6-fold and salbutamol did not produce any effects on this gene, but when these two compounds were mixed together the expression of CYP19 was almost completely inhibited.

Salbutamol caused induction of CYP17 of more than 13-fold; while cyproterone also significantly induced this gene almost 3-fold. Neither tylosin nor EE2 affected the expression of CYP17, and moreover none of the binary mixture studied significantly affected the expression of this gene.

3βHSD2 was the least affected by any of the treatments. Individual exposures with the chosen chemicals did not produce any significant changes in expression of this gene. However, the cyproterone/salbutamol mixture significantly decreased the expression of 3βHSD2.

A variety of responses was also observed for CYP11β2. Expression of this gene was increased significantly by around 5-fold after exposure to EE2. Mixtures of cyproterone and trenbolone each with EE2 did not affect the expression of CYP11β2, and although tylosin treatment significantly induced (10-fold) the expression of this gene, the tylosin–EE2 mixture did not produce any significant change.

Hormone production

Responses in hormone production by cells exposed to the mixtures could not be predicted from the results for the individual chemical exposures. Although T production was reduced significantly by all of the five chemicals chosen for the mixture treatments, binary mixtures of these compounds with EE2 did not show significant changes in T production when compared to values from solvent controls.

Production of P was only significantly increased by treatment with EE2 when exposed to compounds individually. However, the cyproterone–EE2 mixture increased the production of P by more than 2-fold. E2 production, on the other hand, was significantly increased by more than 3-fold by the trenbolone–EE2 mixture. Exposure to the tylosin–EE2 did not cause significant changes in the production of any of the analyzed hormones.

Dose–response analysis

Gene expression

Dose–response curves were constructed after 48 h of exposure to trenbolone, EE2, and cyproterone in the ranges of 0–39 μg/l,

Table 5
Gene expression and hormone production in H295R cells exposed to single chemicals and to binary mixtures[a]

Treatment	CYP17	CYP19	3βHSD2	CYP11β2	PROG	TEST	ESTR
DMSO	1.04 (0.05)	0.99 (0.02)	0.94 (0.09)	1.00 (0.01)	1.00 (0.01)	1.00 (0.27)	1.00 (0.32)
Ethynylestradiol	1.05 (0.18)	1.05 (0.18)	0.76 (0.1)	4.88 (0.88)*	2.71 (0.39)*	0.36 (0.12)*	2.33 (0.27)*
Ethynylestradiol+Trenbolone	0.55 (0.50)	0.57 (0.50)	0.45 (0.25)	1.46 (1.16)	2.72 (1.67)	0.77 (0.02)	3.77 (1.13)*
Ethynylestradiol+Cyproterone	0.88 (0.01)	0.99 (0.03)	0.43 (0.33)	1.28 (0.39)	2.49 (0.42)	0.70 (0.14)	1.69 (0.19)
Ethynylestradiol+Tylosin	0.69 (0.43)	1.04 (0.44)	0.54 (0.47)	2.33 (0.27)	1.39 (0.95)	0.85 (0.17)	0.74 (0.09)
Cyproterone+Salbutamol	0.76 (0.27)	0.01 (0.00)*	0.33 (0.06)*	2.53 (1.34)	ND	ND	ND
Trenbolone	1.79 (0.41)	2.56 (0.55)*	0.77 (0.15)	0.81 (0.43)	0.72 (0.20)	0.48 (0.09)*	1.60 (0.25)
Cyproterone	2.81 (0.50)*	4.62 (1.51)*	0.90 (0.32)	1.34 (0.52)	1.02 (0.30)	0.29 (0.03)*	1.25 (0.47)
Tylosin	1.03 (0.04)	0.49 (0.03)	1.32 (0.27)	9.99 (1.09)*	1.03 (0.04)	0.42 (0.09)*	0.12 (0.08)*
Salbutamol	13.64 (0.5)*	1.00 (0.19)	1.88 (0.62)	2.00 (0.38)	0.30 (0.30)	0.51 (0.17)*	0.32 (0.32)*

Concentrations of single chemicals and mixtures exposures were: DMSO (0.1%), Ethynylestradiol (1 μg/l), Trenbolone (25 μg/l), Cyproterone (62 μg/l), Tylosin (3×10^{3} μg/l), Salbutamol (50×10^{-2} μg/l). ND: No Data.

[a] All exposures were conducted for 48 h under standard conditions. All gene expression and hormone production values are expressed as fold-change relative to the solvent control DMSO (=1.0), given as means and standard deviations in parenthesis. TEST: Testosterone, PROG: Progesterone, ESTR: Estradiol.

* Indicates statistically significant differences at $p<0.05$.

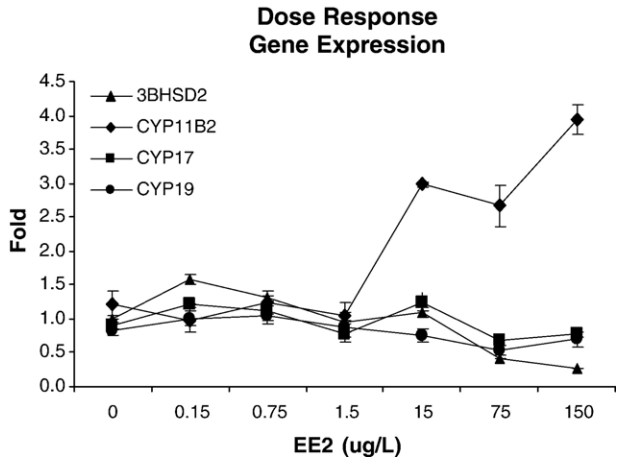

Fig. 1. Dose–response curve for the expression of steroidogenic genes and hormone production after 48 h exposure with different concentrations of ethynylestradiol.

0–150 μg/l and 0–45 μg/l respectively. Relative expression of 3βHSD2, CYP11β2, CYP17 and CYP19 values normalized to β-actin were compared to solvent controls. 17βHSD1 responses were also analyzed for the cyproterone and trenbolone only.

In the EE2 exposure (Fig. 1) 3βHSD2, CYP17 and CYP19 were not affected by the different concentrations of this chemical, however, CYP11β2 expression increased between 1.5 and 15 μg/l EE2 were constant between 15 and 75 μg/l EE2, and rose again between 75 and 150 μg/l EE2. For the cyproterone exposure (Fig. 2), 3βHSD2, CYP19 and 17BHSD1 expression remained at basal levels, while the androgenic gene CYP17 increased in expression by approximately 3-fold at a concentration of 0.9 μg/l. Exposure to trenbolone at concentrations ranging from 0 to 780 μg/l did not affect expression of any of the five genes studied in the dose–response exposures (Fig. 3). Most of the genes fluctuated positively around basal values of expression except for CY11β2, which showed a decrease in expression at 78 μg/l but returned to basal values at greater concentrations such as 780 μg/l.

Hormone production

For the EE2 exposure, the dose–response curve for the production of T was constant and did not change (Fig. 1).

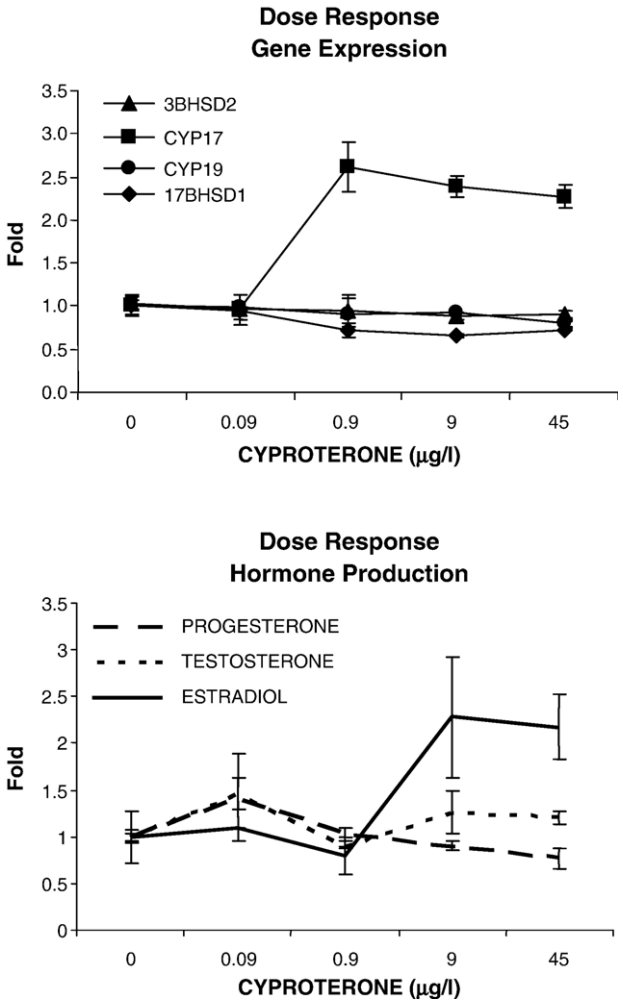

Fig. 2. Dose–response curve for the expression of steroidogenic genes and hormone production after 48 h exposure with different concentrations of cyproterone.

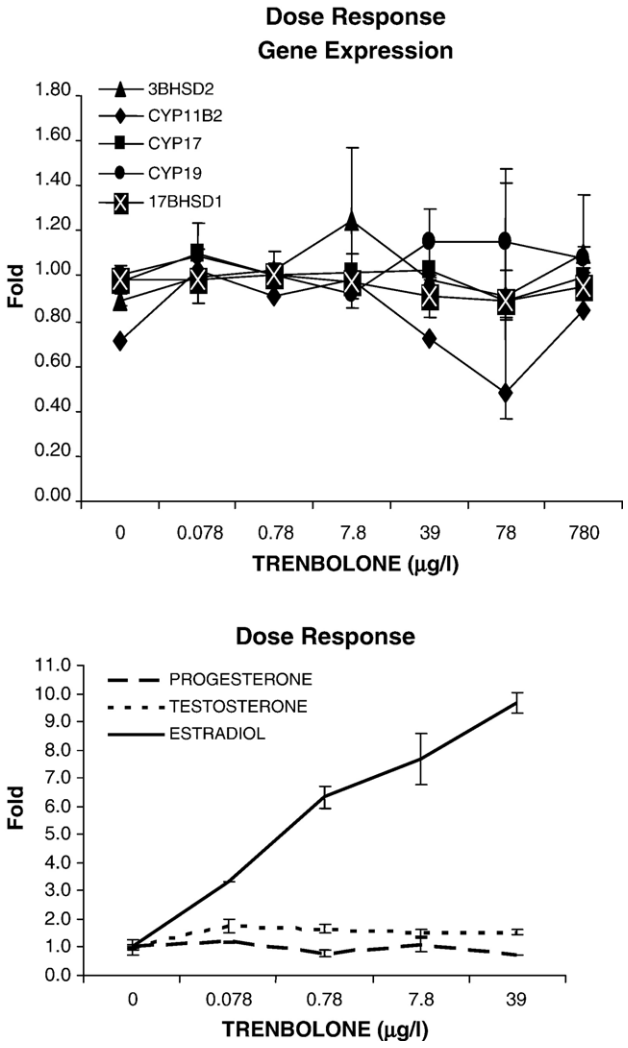

Fig. 3. Dose–response curve for the expression of steroidogenic genes and hormone production after 48 h exposure with different concentrations of trenbolone.

Table 6
Pearson correlation matrix for steroidogenic genes and steroid hormones for all chemical treatments

	CYP11β2	CYP17	CYP19	3βHSD2	TEST	PROG	ESTR
CYP11β2							
CYP17	−0.136						
CYP19	−0.395*	0.276					
3βHSD2	0.061	0.173	0.336				
TEST	−0.367	−0.148	−0.040	0.127			
PROG	0.254	−0.079	−0.285	−0.042	0.239		
ESTR	−0.065	0.193	0.191	−0.008	−0.207	0.088	

TEST: Testosterone, PROG: Progesterone, ESTR: Estradiol.
* Indicates statistically significant correlations at $p<0.05$.

Table 8
Pearson correlation matrix for steroidogenic genes and steroid hormones for the hormone therapy treatments

	CYP11β2	CYP17	CYP19	3βHSD2	TEST	PROG	ESTR
CYP11β2							
CYP17	−0.758						
CYP19	−0.677	0.913*					
3βHSD2	−0.255	0.567	0.728				
TEST	−0.504	0.123	0.032	−0.023			
PROG	0.430	−0.222	−0.254	0.111	0.062		
ESTR	0.385	0.369	−0.297	0.259	0.191	0.783	

TEST: Testosterone, PROG: Progesterone, ESTR: Estradiol.
* Indicates statistically significant correlations at $p<0.05$.

Moreover, P production started to increase at 0.15 μg/l EE2, reaching an 8-fold maximum induction at 1.5 μg/l EE2. P production then returned to basal levels at 15 μg/l EE2.

The dose–response curve for hormone production in response to cyproterone exposure was bimodal through the concentration range of 0 to 45 μg/l (Fig. 2). P and T production were slightly greater than control upon exposure to 0.09 μg/l cyproterone, then decreased at 0.9 μg/l and increased again at 9 μg/l, remaining constant up to 45 μg/l cyproterone. E2 production followed the same pattern as P and T but the fold-change was greater. Maximum E2 production was observed to be 2.5-fold when exposed to 9 μg/l of cyproterone, while for P and T the maximum was less than 1.5-fold. Production of P and T remained at control values for exposures of trenbolone concentrations ranging from 0 to 39 μg/l. In contrast, trenbolone exposures (Fig. 3) showed how E2 concentrations increased rapidly and significantly at concentrations greater than 7.8×10^{-2} μg/l of trenbolone reaching a maximum almost 10-fold compared to controls. On the other hand concentrations of P and T did not change at any concentration of the trenbolone exposure and remain at the levels of the solvent control. not changeA 6-fold induction was observed at 0.78 μg/l, and E2 production increased at 7.8 and 39 μg/l.

Relationship between gene expression and hormone production

The relationships between gene expression and hormone production were investigated by correlation analyses with all the chemicals and by group, that is, for the antibiotic group and the hormone therapy group separately (Tables 6–8).

A Pearson correlation matrix for all data from individual exposures showed low levels of positive and negative correlations between the four genes studied and between the three hormones analyzed. To ascertain the validity of these correlations Bonferroni probabilities were also calculated. The results of these analyses indicated that by pooling all treatments together only one correlation was statistically significant and it was the negative correlation between the responses of the aromatase gene CYP19 and the aldosterone gene CYP11β2. No statistically significant correlations between hormone production and gene expression were observed for the combined treatments.

Correlations for the group of antibiotics not only showed the negative correlation established before between CYP19 and CYP11β2 but also a positive correlation between CYP19 and CYP17; both correlations were statistically significant. Again, no significant correlations between gene expression and hormone production were observed. A single positive significant correlation was observed between expression of CYP19 and CYP17 for the group of chemicals used for hormone therapy, but the negative correlation between CYP11β2 and CYP19 was not observed.

Table 7
Pearson correlation matrix for steroidogenic genes and steroid hormones for the antibiotic treatments

	CYP11β2	CYP17	CYP19	3βHSD2	TEST	PROG
CYP17	−0.353					
CYP19	−0.803*	0.747*				
3βHSD2	−0.078	0.272	0.191			
TEST	−0.408	−0.052	0.299	0.404		
PROG	0.452	−0.511	−0.656	−0.159	−0.132	
ESTR	−0.107	0.121	0.133	−0.382	0.189	0.376

TEST: Testosterone, PROG: Progesterone, ESTR: Estradiol.
* Indicates statistically significant correlations at $p<0.05$.

Discussion

Previous studies have demonstrated the effectiveness of the H295R assay in identifying the potential effects that compounds may exert at different points in the steroidogenic pathway (Hilscherova et al., 2004; Hecker et al., 2006; Zhang et al., 2005; Gracia et al., 2006; Blaha et al., 2006). With the H295R cell culture system not only is it possible to analyze gene expression and hormone production, but also to evaluate enzyme activity. Moreover, this cell system has proven useful in identifying chemical mechanisms of action, in establishing patterns of gene and hormone responses, and also in the analysis of different interactions between chemicals when present in complex mixtures. Results from the experiments conducted in the present study confirm the effectiveness of the H295R screening system and its capacities when examining effects of environmentally relevant doses of pollutants on steroid production.

Pattern of responses by group of chemicals

Antibiotics

The present *in vitro* study demonstrates that environmentally relevant concentrations of pharmaceuticals have the

potential to interfere with the normal pathway of steroid production. In particular, antibiotics were shown to have a broad range of effects on steroidogenesis. Although the semisynthetic β-lactam antibiotics amoxicillin and cephalexin have a similar therapeutic mechanism of action, they affected steroidogenic gene expression and hormone production quite differently. In the case of the semi-synthetic macrolide antibiotics, erythromycin and tylosin, both caused the same gene expression profile, and yet they differed in their hormone production profile. Amoxicillin and cephalexin both have a β-lactam ring in their chemical structures (Saderm et al., 2007), while erythromycin and tylosin both have a macrolide ring, and these small differences in chemical structure may be responsible for the discrepancies observed in gene expression and hormone production profiles.

Since the effects of antibiotics on steroid production have not been previously studied, the mechanisms by which these compounds exert their effects on steroidogenesis are unknown. Given the extensive use of antibiotics and their loadings to the environment, endocrine disruption resulting from these pharmaceutical chemicals should be considered along with the promotion of antibiotic resistance and the potential of these compounds to influence growth in humans (Ternak, 2004) and other non-target organisms.

Hormone therapy group

Drugs employed as hormone therapy agents have a broad range of medical uses. Pharmaceuticals of this group are used in cancer treatment, birth control, in diagnostic procedures, and as growth promoters, among other uses. Cyproterone is a steroidal anti-androgen with weak progestagenic activity used in the treatment of prostate cancer (Wirth et al., 2007). This drug exerts its functions by suppressing androgen action both by binding directly to the androgen receptor and by inhibiting the positive feedback of androgens on the pituitary ultimately resulting in reduced production of sex steroids (Sharpe et al., 2004). The anti-androgenic properties of cyproterone were observed in the results for hormone analysis where concentrations of T were reduced by up to one-third. It is noteworthy that the expression of CYP19 and CYP17 were increased, probably in response to depletion of T in the medium. Induction of CYP17 would drive steroidogenesis towards the production of androgens while increase in CYP19 activity would ensure that E2 was produced despite small concentrations of substrate. The significant and strong negative correlation observed for these two genes for this group of pharmaceuticals supports the idea of a coordinated expression system.

EE2 is the most common and most potent estrogenic compound found in sewage effluents (Sarmah et al., 2006). This synthetic E2 analog is used in combination with other estrogenic substances in the manufacturing of contraceptive pills. Studies have demonstrated the effects of EE2 on the survival, sex ratio, gonadal growth, spawning and sexual differentiation of aquatic organisms, especially in fish (Scholz and Gutzeit, 2000). In H295R cells exposed to 1 μg/l EE2 the production of P and E2 in H295R cells doubled, while T production was greatly reduced. The observed decrease in T production may be a reaction to the increased production of E2 since T production may be substrate-limited.

Trenbolone acetate (TBA) is a synthetic steroid hormone commonly used to enhance growth in beef cattle. TBA is quickly metabolized to the potent androgen 17β-trenbolone (Durhan et al., 2006). Despite high affinity of 17β-trenbolone for the human androgen receptor, an affinity which is known to be similar to that of dihydrotestosterone (Bauer et al., 2001), decreases in T production in the H295R cells were observed. We speculate that these results may be an indication of the capability of the 17β-trenbolone metabolite for blocking other pathways directly or indirectly related to T production or for inducing pathways leading to T metabolism. At the same time the cell response to this lack of T is the induction in the expression of the aromatase gene CYP19 trying to keep E2 concentrations at normal levels.

The estrogenic equivalent of 17β-trenbolone is α-zearalanol; this chemical is the active metabolite of the mycotoxin zearalenone that is obtained from *Fusarium* spp. (Sheehan et al., 1984). This compound is also used in veterinary medicine as a growth promoter. T and P production were not affected by this chemical nor was the expression of the steroidogenic genes studied, except for 3βHSD2. Although both EE2 and α-zearalanol can strongly bind to estrogen receptor (ER) (Takemura et al., 2007) their observed effects on E2 production were very different. EE2 doubled E2 production whereas α-zearalanol reduced E2 production by almost 6-fold. As it was speculated before for the results of trenbolone exposure, we hypothesize that this reduction in E2 production may be the result of the activation or inhibition of other pathways not related to E2 receptors binding.

Together these results indicate that extensive attention must be directed to the use and fate of pharmaceuticals with hormonal properties since this is a group of chemicals that will surely produce significant effects when reaching non-target organisms. This is especially the case for compounds used for veterinary purposes which may be excreted in their active forms by treated animals and then reach aquatic ecosystems via runoff (Lange and Dietrich, 2002).

Other pharmaceuticals

Over-the-counter analgesics and anti-inflammatory drugs such as acetaminophen and ibuprofen did not produce significant changes in gene expression or hormone responses. No steroidogenic effects have been demonstrated for acetaminophen and even its exact mechanism of action as an analgesic is unknown. Antilipidic drugs such as clofibrate are commonly used to treat hyperlipidemia, a condition considered a major risk factor of cardio and cerebro-vascular diseases. Despite being withdrawn from the market in most countries in Western Europe, clofibrate concentrations in the ng/l to μg/l range have been reported in several sewage treatment plant effluents (Koutsouba et al., 2003). In H295R cells clofibrate concentrations of 3 μg/ml significantly reduced T production. Reduction of T levels by this drug has also been observed in rat where it was suggested that clofibrate may exert its action through direct effects on the microsomal

enzyme systems responsible for steroid metabolism (Xu et al., 2002).

The results from the exposure with the drug salbutamol, a short-acting, β_2-adrenergic receptor agonist used to treat broncho-spasm and in some cases used in obstetrics as a tocolytic to relax uterine smooth muscle and delay premature labor (Blanchard et al., 1993), were especially interesting. Salbutamol binds to β_2-adrenergic receptors with greater affinity than β_1-receptors; the activation of β_2-adrenergic receptors results in relaxation of smooth muscles. Salbutamol is also used in combination with other drugs as a growth promoter in livestock. This β_2-adrenergic drug enhances lipolysis and the rate at which fatty acids are oxidized producing leaner animals (Hernández-Carrasquilla, 2003). Thus, we hypothesized that the lipolytic effects of salbutamol could be responsible for the significant decreases of almost 50% in E2 production compared to solvent controls. The most obvious effect of this agonist compound was the increase in expression of the androgenic gene CYP17 by more than 10-fold. More specific studies need to be designed in order to reveal if this increase in the expression of CYP17 is in some way linked to the depletion of E2.

Effects of drug mixtures

From their first use, pharmaceuticals have been entering the environment and have been constantly detected at measurable concentrations; they are ordinarily found in mixtures of active ingredients with a variety of biological activities. Thus, non-target organisms are being exposed to compounds with different biological actions at the same time. Few toxicological studies have been conducted to address chronic toxicity upon exposure to mixtures of biologically active contaminants and the associated risks (Crane et al., 2006). Understanding of the effects of complex mixtures of compounds acting together must become a priority when evaluating the potential risks of pharmaceuticals in the environment. One of the major difficulties in analyzing effects of complex mixtures is the understanding of the different ways in which compounds in the mixture will interact to produce effects.

H295R cells were exposed to four binary mixtures of different pharmaceuticals. EE2, the most common component in birth control pills, was a common component for three of the four binary mixtures prepared. Because of the effects of individual compounds on the four genes studied, cyproterone, trenbolone and tylosin were chosen as the second components in the mixtures with EE2. In addition, due to the effects of cyproterone on gene expression and salbutamol on hormone production, H295R cells were also exposed to a mixture of these two compounds. Gene responses suggested that the chemicals present in these mixtures interact mostly by antagonistic mechanisms, although agonism was also observed in some cases. The dominant effects of EE2 were observed in mixtures with trenbolone and cyproterone. When expression values produced by individual exposures of EE2 were greater than those produced by the second component in the mixture the joint effects observed were similar to those caused by the second component, or in other terms, for the compound that produced lower fold-inductions.

In contrast to gene expression, several types of interactions were observed for the hormone production responses to the mixture treatments. For instance, in the case of P the binary mixtures produced the same effects as produced by EE2 alone, which was more than a 2-fold induction in the production of this hormone, an indication that the EE2 effects prevail in the mixture. On the other hand T production was down-regulated by all the individual treatments but the mixtures did not produce significant changes in the concentration of this hormone showing that these chemicals block each other's antagonistic effects with respect to T production. On the contrary, the results of E2 measurement showed that an additive effect was produced by the mixture of EE2 and trenbolone; such a response is likely due to the affinity of both these compounds for the ER. These results document that the steroidogenic effects exerted by the binary mixture exposures could not be predicted from the results of exposures of the individual chemicals in question. As an example, cyproterone significantly up-regulated the expression of the aromatase gene CYP19 while salbutamol did not produce significant changes in the expression of this gene, but a mixture of the two compounds resulted in complete suppression of CYP19 expression. These findings corroborate the premise that not only do compounds interact, but their effects are usually different from the responses of individual chemicals. The results show that pharmaceuticals and their mixtures act through additional unknown modes of toxic action that have to be understood in order to truly assess their potential effects as environmental contaminants.

Dose–response analysis

Three pharmaceuticals used in hormone therapy were selected to conduct dose–response studies. The results showed that the dose-dependent changes in gene expression behaved differently for each chemical. Exposure to EE2 affected only CYP11β2 expression. Of the hormones, only T production was not affected by exposure to EE2. E2 concentrations were proportional to the concentration of EE2. Changes were observed even at EE2 concentrations as small as 0.15 μg/l. The positive relationship between E2 production and EE2 in the medium is consistent with the great affinity of EE2 to for the ER. Despite the induction of CYP11β2 by EE2, P production was not affected in the same manner. Increased production of this hormone was only observed between 0.15 and 1.5 μg/l before returning to basal levels.

The anti-androgen cyproterone prevents dihydrotestosterone, the active form of T in mammals, from binding to receptors in carcinoma cells. Thus, induction of CYP17 may be a response to the presence of the anti-androgen that results in an increase in the production of active T to compete for the receptors. E2 was the only hormone to increase proportionally with cyproterone concentration. The mechanisms by which this process occurred are unknown.

Changes in trenbolone concentrations did not produce major effects on the expression of any of the steroidogenic genes, or hormone production except for its effects on E2. The production of E2 was greater than in the control at all trenbolone

concentrations tested, although the expression of the aromatase gene CYP19 was not increased and T production stayed within the basal concentration range. These results suggest the possibility that trenbolone induced the activity of the aromatase enzyme by the activation of other pathways.

Chemicals with the same mechanism of action may have different effects on the expression of steroidogenic genes and the production of steroid hormones. For instance, although cyproterone and trenbolone both interact with the androgen receptor, each caused different effects on gene expression and hormone production. Thus, it appears that each of these chemicals, in addition to its interaction with the androgen receptor, may induce or inhibit other points in the pathway resulting in the observed differences in effects.

The results from this study demonstrated that several pharmaceuticals, including compounds with unknown steroidogenic effects, have the potential to produce changes in steroidogenic gene expression and hormone production at different range of concentrations. Moreover, the steroidogenic effects of mixtures of pharmaceuticals may be different to the effects observed from individual compounds, which leads to conclude that interaction between pharmaceuticals occurs and such interaction has its own particular effects. Although compounds with the same therapeutic mechanism of action may show similar gene expression profiles their effects on hormone production may be different, perhaps due to differences in the particular effects that each compound can exert directly or indirectly into the steroidogenic pathway independently of their genomic effects. Since none statistical correlations were established between gene expression and hormone production it is suggested that not only other factors different to gene expression are influencing the production of steroid hormones, but also that alternatively chemicals may activate or desactivate pathways that may influence the production of steroid hormones. Despite no statistical correlations between gene expression and hormone production were observed, statistical correlations among some genes were shown to be significant, which may suggest a direct expression dependency between genes, dependency that may only be corroborated through functional genomic analysis.

Pharmaceuticals were just recently classified as environmental contaminants after years of being ignored in environmental studies; the presence of these bioactive compounds in the environment should be controlled and monitored due to the inherent potential biological effects that compounds such as these can exert on non-target organisms. The H295R cell bioassay is a very quick, practical, and sensitive pre-screening method by which the endocrine disruptive effects of environmentally relevant chemicals may be evaluated.

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References

Adler, P., Steger-Hartmann, T., Kalbfus, W., 2001. Distribution of natural and synthetic estrogenic steroid hormones in water samples from southern and middle Germany. Acta Hydrochim. Hydrobiol. 29, 227–241.

Bauer, E., Daxenberger, A., Petri, T., Sauerwein, H., Meyer, H., 2001. Characterization of the affinity of different anabolics and synthetic hormones to the androgen receptor, human sex hormone binding globulin and to the bovine progestin receptor. APMIS. Acta Pathol. Microbiol. Immunol. Scand., Suppl. 109, S452–S460.

Berger, K., Petersen, B., Büning-Pfaue, H., 1986. Persistence of drugs occurring in liquid manure in the food chain. Arch. Hyg. 37, 99–102.

Blanchard, P., Ellis, M., Maltin, C., Falkous, G., Harris, J., Mantle, D., 1993. Effect of growth promoters on pig muscle structural protein and proteolytic enzyme levels *in vivo* and *in vitro*. Biochimie 75, 839–847.

Blaha, L., Hilscherova, K., Mazurova, E., Hecker, M., Jones, P., Newsted, J., Bradley, P., Gracia, T., Duris, Z., Horka, I., Holoubek, I., Giesy, J.P., 2006. Alteration of steroidogenesis in H295R cells by organic sediment contaminants and relationships to other endocrine disrupting effects. Environ. Int. 32, 749–757.

Buser, H.R., Poiger, T., Müller, M.D., 1998. Occurrence and fate of the pharmaceutical drug Diclofenac in surface waters: rapid photodegradation in a lake. Environ. Sci. Technol. 33, 2529–2535.

Casewell, M., Friis, C., Marco, E., McMullin, P., Phillips, I., 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. J. Antimicrob. Chemother. 52, 159–161.

Cecchini, M., LoPresti, V., 2007. Drug residues store in the body following cessation of use: impacts on neuroendocrine balance and behavior. Med. Hypotheses 68 (4), 868–879.

Commission of the European Communities, 1992. Methods for determination of ecotoxicity. Off. J. Eur. Communities 383, 187–225.

Crane, M., Watts, C., Boucard, T., 2006. Chronic aquatic environmental risks from exposure to human pharmaceuticals. Sci. Total Environ. 367, 23–41.

Díaz-Cruz, M.S., López de Alda, M.J., Barceló, D., 2003. Environmental behavior and analysis of veterinary and human drugs in soils, sediments and sludge. Trends Anal. Chem. 22, 340–351.

Durhan, E.J., Lambright, C.S., Makynen, E.A., Lazorchak, J., Hartig, P.C., Wilson, V.S., Gray, L.E., Ankley, G.T., 2006. Identification of metabolites of trenbolone acetate in androgenic runoff from a beef feedlot. Environ. Health Perspect. 114 (Supl. 1), 65–68.

Fent, K., Weston, A., Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. Aquat. Toxicol. 76, 122–129.

Gracia, T., Hilscherova, K., Jones, P., Newsted, J., Zhang, X., Hecker, M., Higley, E., Sanderson, J., Yu, R.M.K., Wu, R.S.S., Giesy, J.P., 2006. The H295R system for evaluation of endocrine-disrupting effects. Ecotoxicol. Environ. Saf. 65, 293–305.

Hecker, M., Newsted, J.L., Murphy, M.B., Higley, E.B., Jones, P.D., Wu, R., Giesy, J.P., 2006. Human adrenocarcinoma (H295R) cells for rapid *in vitro* determination of effects on steroidogenesis: Hormone production. Toxicol. Appl. Pharmacol. 217, 114–124.

Hereber, T., 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol. Lett. 1331, 5–17.

Hernández-Carrasquilla, M., 2003. Gas chromatography-mass spectrometry analysis of β_2-agonists in bovine retina. Anal. Chim. Acta 408, 285–290.

Hilscherova, K., Jones, P.D., Gracia, T., Newsted, J.L., Zhang, X., Sanderson, J.T., Yu, R.M.K., Wu, R.S.S., Giesy, J.P., 2004. Assessment of the effects of chemicals on the expression of ten steroidogenic genes in the H295R cell line using real-time PCR. Toxicol. Sci. 81, 78–89.

Hirsch, R., Ternes, T.A., Haberer, K., Kratz, K.L., 1996. Nachweis von Betablockern und Bronchospasmolytika in der aquatischen Umwelt. Vom Wasser 87, 263–274.

Hirsch, R., Ternes, T.A., Haberer, K., Kratz, K.L., 1999. Occurrence of antibiotics in the aquatic environment. Sci. Total Environ. 225, 109–118.

Jones, O., Voulvoulis, N., Lester, J., 2004. Potential ecological and human health risks associated with the presence of pharmaceutically active compounds in the aquatic environment. Crit. Rev. Toxicol. 34, 335–350.

Jorgensen, S.E., Halling-Sorensen, B., 2000. Drugs in the environment. Editorial. Chemosphere 40, 691–699.

Kolpin, D., Furlong, E., Meyer, M., Thurman, E., Zaugg, S., Barber, L., Buxton, H., 2002. Pharmaceuticals, hormones and other organic wastewater contaminants in U.S. streams 1999–2000: a national reconnaissance. Environ. Sci. Technol. 36, 1202–1211.

Koutsouba, V., Heberer, T., Fuhrmann, B., Schmidt-Baumler, K., Tsipi, D., Hikskia, A., 2003. Determination of polar pharmaceuticals in sewage water of Greece by gas chromatography-mass spectrometry. Chemosphere 51, 69–75.

Kuch, H.M., Ballschmitter, K., 2000. Determination of endogenous and exogenous estrogens in effluents from sewage treatment plants at the ng/l-level. Fresenius' J. Anal. Chem. 366, 392–395.

Kummerer, K., 2001. Drugs in the environment: emission of drugs, diagnostic aids and disinfectants into wastewater by hospitals in relation to other sources — a review. Chemosphere 45, 957–969.

Lange, R., Dietrich, D., 2002. Environmental risk assessment of pharmaceutical drug substances—conceptual considerations. Toxicol. Lett. 131, 97–104.

Lindsey, M.E., Meyer, M., Thurman, E.M., 2001. Analysis of trace levels of sulfonamide and tetracycline antimicrobials in groundwater and surface water using solid-phase extraction and liquid chromatography/mass spectrometry. Anal. Chem. 73, 4640–4646.

Mohle, E., Horvath, S., Merz, W., Metzger, J.W., 1999. Determination of hardly degradable organic compounds in sewage water-identification of pharmaceutical residues. Vom Wasser 92, 207–223.

Ollers, S., Singer, H.P., Fässler, P., Müller, R.S., 2001. Simultaneous quantification of neutral and acidic pharmaceuticals and pesticides at the low-ng/l level in surface and waste water. J. Chromatogr. A 911, 225–234.

Pickrell, J., 2002. Killer cocktails. Sci. News 162 (7), 101.

Rx List. Top 200 Drugs Of 2003 By Prescriptions Dispensed. www.rxlist.com.

Saderm, H.S., Jacobs, M.R., Fritsche, T.R., 2007. Review of the spectrum and potency of orally administered cephalosporins and amoxicillin/clavulanate. Diagn. Microbiol. Infect. Dis. 57 (Suppl. 3), S5–S12.

Sanderson, H., Johnson, D.J., Reitsma, T., Brain, R.A., Wilson, C.J., Solomon, K.R., 2004. Ranking and prioritization of environmental risks of pharmaceuticals in surface waters. Regul. Toxicol. Pharmacol. 39, 158–183.

Sarmah, A.K., Northcott, G.L., Leusch, F.D., Tremblay, L.A., 2006. A survey of endocrine disrupting chemicals (EDCs) in municipal sewage and animal waste effluents in the Waikato region of New Zealand. Sci. Total Environ. 355, 135–144.

Scholz, S., Gutzeit, H., 2000. 17-α-ethynylestradiol affects reproduction, sexual differentiation and aromatase gene expression of the medaka (*Oryzias latipes*). Aquat. Toxicol. 50, 363–373.

Sedlak, D.L., Pinkston, K.E., 2001. Factors affecting the concentrations of pharmaceuticals released to the aquatic environment. Water Res. Update 120, 56–64.

Sharpe, R., MacLatchy, Deborah, L., Courtenay, S., Van der Draak, G., 2004. Effects of a model androgen (methyl T) and a model anti-androgen (cyproterone acetate) on reproductive endocrine endpoints in a short-term adult mummichog (*Fundulus heteroclitus*) bioassay. Aquat. Toxicol. 67, 203–215.

Sheehan, D., Branham, W., Medlock, K., Shanmugasundaram, E., 1984. Estrogenic activity of zearalenone and zearalanol in the neonatal rat uterus. Teratology 29, 383–392.

Stumpf, M., Ternes, T.A., Haberer, K., Baumann, W., 1998. Isolicrung von Ibuprofen-Metaboliten und deren Bedcutung als Kontaminanten der aquatischen Umwelt. Vom Wasser 91, 291–303.

Takemura, H., Shim, J.Y., Sayama, K., Tsubura, A., Zhu, B.T., Shimi, K., 2007. Characterization of the estrogenic activities of zearalenone and zeranol in vivo and in vitro. J. Steroid Biochem. Mol. Biol. 103 (2), 170–177.

Ternak, G., 2004. Antibiotics may act as growth/obesity promoters in humans as an inadvertent result of antibiotic pollution? Med. Hypotheses 64, 14–16.

Ternes, T., 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Res. 32, 3245–3260.

U.S. Food Drug Administration. Center for Drug and Evaluation. FDA Drug Register List. www.fda.gov.

van Wezel, A., Jager, T., 2002. Comparison of two screening level risk assessment approaches for six disinfectants and pharmaceuticals. Chemosphere 47, 113–1128.

Wirth, M., Hakenberg, O., Froehner, M., 2007. Antiandrogens in the treatment of prostate cancer. Eur. Urol. 51 (2), 306–313.

Xu, S., Zhu, B.T., Conney, A.H., 2002. Effect of clofibrate administration on the esterification and deesterification of steroid hormones by liver and extrahepatic tissues in rats. Biochem. Pharmacol. 63, 985–992.

Zhang, X., Yu, R.M.K., Jones, P.D., Lam, G.K.W., Newsted, J.L., Gracia, T., Hecker, M., Hilscherova, K., Sanderson, J.T., Wu, R.S.S., Giesy, J.P., 2005. Quantitative RT-PCR methods for evaluating toxicant-induced effects on steroidogenesis using the H295R cell line. Environ. Sci. Technol. 39, 2777–2785.

Článek XI:

Haeba, M.H., Hilscherová, K., Mazurová, E., Bláha, L., 2008. Selected endocrine disrupting compounds (vinclozolin, flutamide, ketoconazole and dicofol): Effects on survival, occurrence of males, growth, molting and reproduction of *Daphnia magna*. Environmental Science and Pollution Research 15, 222–227.

Area 6.4 • Monitoring and Fate of Persistent Chemicals

Research Article

Selected Endocrine Disrupting Compounds (Vinclozolin, Flutamide, Ketoconazole and Dicofol): Effects on Survival, Occurrence of Males, Growth, Molting and Reproduction of *Daphnia magna*

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Abstract

Background, Aim and Scope. Pollution–induced endocrine disruption in vertebrates and invertebrates is a worldwide environmental problem, but relatively little is known about effects of endocrine disrupting compounds (EDCs) in planktonic crustaceans (including *Daphnia magna*). Aims of the present study were to investigate acute 48 h toxicity and sub-chronic (4–6 days) and chronic (21 days) effects of selected EDCs in *D. magna*. We have investigated both traditional endpoints as well as other parameters such as sex determination, maturation, molting or embryogenesis in order to evaluate the sensitivity and possible use of these endpoints in ecological risk assessment.

Materials and Methods. We have studied effects of four model EDCs (vinclozolin, flutamide, ketoconazole and dicofol) on *D. magna* using (i) an acute 48 h immobilization assay, (ii) a sub-chronic, 4–6 day assay evaluating development and the sex ratio of neonates, and (iii) a chronic, 21 day assay studying number of neonates, sex of neonates, molting frequency, day of maturation and the growth of maternal organisms.

Results. Acute EC_{50} values in the 48 h immobilization test were as follows (mg/L): dicofol 0.2, ketoconazole 1.5, flutamide 2.7, vinclozolin >3. Short-term, 4–6 day assays with sublethal concentrations showed that the sex ratio in *Daphnia* was modulated by vinclozolin (decreased number of neonate males at 1 mg/L) and dicofol (increase in males at 0.1 mg/L). Flutamide (up to 1 mg/L) had no effect on the sex of neonates, but inhibited embryonic development at certain stages during chronic assay, resulting in abortions. Ketoconazole had no significant effects on the studied processes up to 1 mg/L.

Discussion. Sex ratio modulations by some chemicals (vinclozolin and dicofol) corresponded to the known action of these compounds in vertebrates (i.e. anti-androgenicity and anti-oestrogenicity, respectively). Our study revealed that some chemicals known to affect steroid-regulated processes in vertebrates can also affect sublethal endpoints (e.g. embryonic sex determination and/or reproduction) in invertebrates such as *D. magna*.

Conclusions. A series of model vertebrate endocrine disrupters affected various sub-chronic and chronic parameters in *D. magna* including several endpoints that have not been previously studied in detail (such as sex determination in neonates, embryogenesis, molting and maturation). Evaluations of traditional reproduction parameters (obtained from the 21 day chronic assay) as well as the results from a rapid, 4–6 day, sub-chronic assay provide complementary information on non-lethal effects of suspected organic endocrine disrupters.

Recommendations and Perspectives. It seems that there are analogies between vertebrates and invertebrates in toxicity mechanisms and in vivo effects of endocrine disruptors. However, general physiological status of organisms may also indirectly affect endpoints that are traditionally considered 'hormone regulated' (especially at higher effective concentrations as observed in this study) and these factors should be carefully considered. Further research of *D. magna* physiology and comparative studies with various EDCs will help to understand mechanisms of action as well as ecological risks of EDCs in the environment.

Keywords: *Daphnia magna*; dicofol; endocrine disruption; flutamide; ketoconazole; sex determination; vinclozolin

Introduction

Anthropogenic chemicals have been shown to cause endocrine disruption in numerous organisms. Endocrine disruptive chemicals (EDCs) have caused a wide range of effects in wildlife and possibly in humans (Tyler et al. 1998, StahlschmidtAllner et al. 1997, Basler & Lebsanft 1999, Keiter et al. 2006). There are also numerous EDC-induced effects documented in invertebrates, including planktonic crustaceans (Hense et al. 2005, LeBlanc 2007). The most often reported effects include an alteration in testosterone metabolism (Baldwin & Leblanc 1994, Baldwin et al. 1998), which could lead to imposex or intersex development, perturbations in the molt cycle (Zou & Fingerman 1997), growth retardation (LeBlanc & McLachlan 1999), developmental abnormalities (Olmstead & LeBlanc 2000) or modulations of fecundity (Bryan et al. 1986). In crustaceans, some ef-

fects of EDCs seem to be mediated by steroid or ecdysteroid regulated processes acting via intercellular receptors and transcription factors in a way similar to vertebrates (Chang 1993, LeBlanc & McLachlan 1999, Subramonian 2000).

Daphnia magna is one of the most often used organisms in ecotoxicology, and it has been evaluated as a model for studies of endocrine disruption (Kashian & Dodson 2004, Sanchez et al. 2005). Fecundity, growth rate and maturation are some of the parameters that might be affected by EDCs. Also the sex ratio (proportion of males in the population) has been shown to be a sensitive indicator of stress factors, including chemical pollutants (Dodson et al. 1999b). *Daphnia magna* reproduce mostly by parthenogenesis and females are usually dominant in daphnid populations. It has been found that juvenile hormone III and methyl farnesoate as well as their chemical analogs used as pesticides (such as pyriproxyfen and fenoxycarb) increase occurrence of male daphnids in the population (Olmstead and Leblanc 2002, Olmstead and LeBlanc 2003, Tatarazako et al. 2003, Wang et al. 2005). Similarly, a recent study reported that two insect juvenile hormones (JH I and JH II) and three juvenile hormone analogs (kinoprene, hydroprene and epofenonane) increased the proportion of males in the *Daphnia magna* population (Oda et al. 2005). The increase in the sex ratio has also been observed in *Daphnia* exposed to such chemicals as atrazine or acetone (Dodson et al. 1999). On the other hand, other compounds (e.g. methoprene and dieldrin) may decrease male production in crustaceans by mimicking or interfering with methyl farnesoate action (Peterson et al. 2001, Dodson et al. 1999).

Despite some previous studies, our understanding on the sublethal effects of possible EDCs in invertebrates is still limited. In the present study we have investigated effects of four chemicals that are suspected of interfering with normal reproduction and development in *Daphnia;* vinclozolin (dicarboximide fungicide known to act as an antagonist of androgen receptors in vertebrates; Sperry & Thomas 1999), flutamide (a drug clinically used in treatment of human prostate cancer acting as an anti-androgen; Kolvenbag et al. 2001), dicofol (an organochlorine acaricide manufactured from technical DDT known to be antioestrogenic in vertebrates; Vinggaard et al. 2000), and ketoconazole (an anti-fungal imidazole derivative that inhibits various CYP enzymes, acting also as an anti-androgen; Gray et al. 1999). The major goals of our study were to explore both acute toxicity and effects of sublethal doses in the sub-chronic (4–6 days) and chronic (21 days) assays with *D. magna*. We focused on several traditional endpoints as well as on less frequently employed parameters such as sex ratio, maturation, molting or embryogenesis, in order to evaluate the sensitivity of these parameters and their possible use in the ecological risk assessment.

1 Materials and Methods

1.1 Material

Daphnia magna (long-term laboratory culture originally collected from a freshwater reservoir in Brno, Czech Republic) have been permanently maintained for more than 3 years under controlled conditions: temperature 20 ± 2ºC, 16/8 hr light/dark cycle in the Elendt M4 medium (Samel et al. 1999, OECD 1996). Dimethylsulfoxide (DMSO) of analytical grade (99% purity) was used as a non-toxic solvent at 0.05% v/v. All tested chemicals (vinclozolin, flutamide, ketoconazole and dicofol) were purchased from Sigma-Aldrich.

1.2 Acute toxicity testing

In acute toxicity tests, neonates less than 24 h old (twenty animals for each treatment and control) were used. The exposure medium (8.88 g $CaCl_2$, 2.4 g $MgSO_4$, 2.59 g $NaHCO_3$ and 0.23 g KCl per litre of water) was not renewed during the test and organisms were not fed. Mortality (immobilization) was recorded after 24 and 48 hours.

1.3 Sub-chronic toxicity testing

Sub-chronic toxicity test was conducted with gravid (10–14-day-old adults) females with the first eggs in their brood chamber. Daphnids were examined microscopically for developmental stage of embryos and females having late embryonic maturation stages (Kast-Hutcheson et al. 2001) were used for experiments (exposed to sublethal doses estimated from the acute toxicity assays). The first batch of neonates (hatching within the first 24 h) was always discarded as these animals were not exposed to the tested chemicals during their entire developmental period. Neonates (from the second brood) spent their entire embryonic development under exposure to tested chemicals and they were used for toxicity evaluation. Ten replicate polypropylene jars (each with individual *D. magna* females in 50ml medium covered with saran wrap to prevent volatilization) were used per treatment. Exposure medium was renewed every 48h. The offspring was removed daily and counted. Development and the sex of neonates were assessed using a low magnification light microscope. Offspring males were identified by the presence of prominent first antennules and the sex ratio was calculated as the number of males divided by the total number of neonates (Dodson et al. 1999). Assay was terminated after all females released the second brood of neonates (typically 4–6 days of exposure).

1.4 Chronic reproduction assay

Chronic, 21 day, toxicity assays were started with neonate females younger than 24 h placed individually into beakers with 50 ml of M4 medium. Ten replicate jars (each with an individual *D. magna* female; covered with saran wrap to prevent volatilization) were used per treatment and the exposure medium was renewed every 48 h. The following parameters were evaluated: offspring counts and their sex, molting frequency, day of maturation (i.e. time to the first reproduction) and the lengths of maternal organisms (on days 0, 7, 15 and 21; LeBlanc & McLachlan 1999). The daphnids in the sub-chronic and chronic assays were fed every other day (at the time of medium exchange) with a *Selenastrum capricornutum* and *Chlorella kessleri* mixture (10^7 cells in 1 ml administered into 50 ml jar). Feeding habits during experiments were monitored and no differences between controls and exposed animals were recorded.

1.5 Stability of tested compounds

Stability of the tested compounds during 48 h (renewal period of the exposure media) was checked by monitoring changes in UV-VIS spectra (scan of 200–600 nm with Varian CARY cuvette spectrophotometer). For flutamide and vinclozolin, the results were confirmed by Ultra Performance Liqmilli-Q water was used. The compounds were detected by absorbance monitoring at a range of 200–300 nm. Analyses were performed using validated methods by the contract partner (pharmaceutical company Pliva-Lachema, a.s., Brno, Czech Republic). Less than 15% decrease in concentrations of all tested compounds was observed during a 48 h period of media exchange and nominal concentrations were used for calculations of toxicity values.

1.6 Statistics

The 48 h EC_{50} values were calculated by Probit analysis (Finney 1971). Statistical comparisons between exposure groups were performed by one way analysis of variance (ANOVA) followed by Dunnet's test to detect differences among treatment groups in comparison with controls. Non-parametric tests were employed when the data were heterogeneous. Statistics were calculated in Statistica for Windows 6.0. *P*-values less than 0.05 were considered statistically significant.

2 Results

Full dose-response curves for acute toxicity are in **Fig. 1** and estimated EC_{50} values for tested compounds are presented in **Table 1.** Dicofol had the highest toxicity with 48 h EC_{50} of 0.2 mg/L, while vinclozolin was the least toxic with no effect on immobilization up to its solubility (> 3 mg/L).

Concentrations causing no significant effects (No Observed Effects Concentrations – NOECs) in the acute tests were selected for further sub-chronic and chronic assays, whose results are summarized in **Table 2.** No mortalities were observed during sub-chronic and chronic exposures. Ketoconazole (up to 1.0 mg/L) had no significant effect on any of the investigated parameters.

Sex ratio in the sub-chronic assay was affected by dicofol and vinclozolin (Table 2, **Fig. 2**). Dicofol significantly increased the sex ratio in favour of males at the highest concentration tested, 0.1 mg/L (Fig. 2A), while vinclozolin (1 mg/L) decreased the number of neonate males (Fig. 2B).

Chronic, 21 day exposures to the highest concentration of flutamide (1.0 mg/L) significantly ($p<0.01$) suppressed total number of offspring (**Fig. 3A**). Flutamide also affected the growth of maternal daphnids during the initial 7 days, but growth-inhibitory effects were not apparent at the end

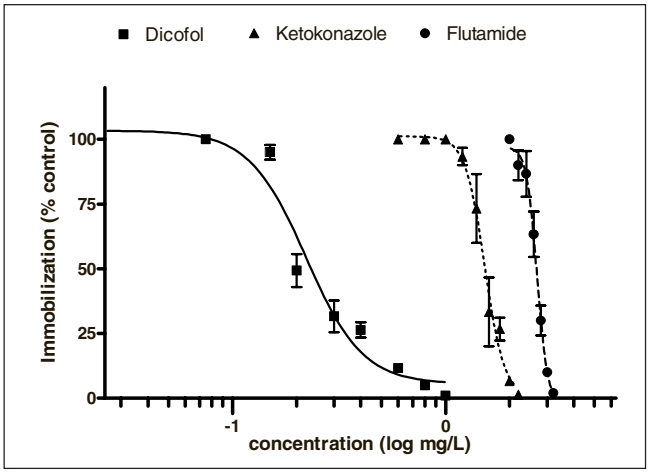

Fig. 1: Acute toxicity of the tested compounds to *D. magna* (48 h immobilization, concentration-response curves). Vinclozolin was not toxic up to its water solubility (3 mg/L)

Table 1: Acute effects (24 and 48 h EC50 values) of the tested compounds on immobilization in *D. magna* (EC_{50} in mg/L; 95% confidence limits for EC50 (in parentheses))

	Acute toxicity to *D. magna* (EC50; mg/L)	
	24 h	**48 h**
Dicofol	0.38 (0.32–0.46)	0.2 (0.17–0.24)
Ketoconazole	8.1 (4.6–10.8)	1.51 (1.16–1.91)
Flutamide	7.8 (5.9–28.4)	2.7 (2.15–3.41)
Vinclozolin	>3	>3

Table 2: Overview of the effects of tested compounds (at indicated concentrations) on the sublethal parameters in sub-chronic (4–6 day) and chronic (21 day) bioassays with *D. magna*

	Assay type	Dicofol (0.1 mg/L)	Ketoconazole (1 mg/L)	Flutamide (1 mg/L)	Vinclozolin (1 mg/L)
Sex ratio (males/total)	***sub-chronic***	increase (3-fold, p<0.05)	no	no	decrease (2-fold, p<0.05)
	chronic	no	no	no	No
Neonate numbers	***sub-chronic***	no	no	no	No
	chronic	no	no	decrease (2-fold, p<0.05)	no
Size of maternal organisms	***chronic***	no	no	suppression (64%, p<0.05)	no
Maturation	***chronic***	no	no	delayed (50%, p<0.05)	no
Molting	***chronic***	no	no	no	no

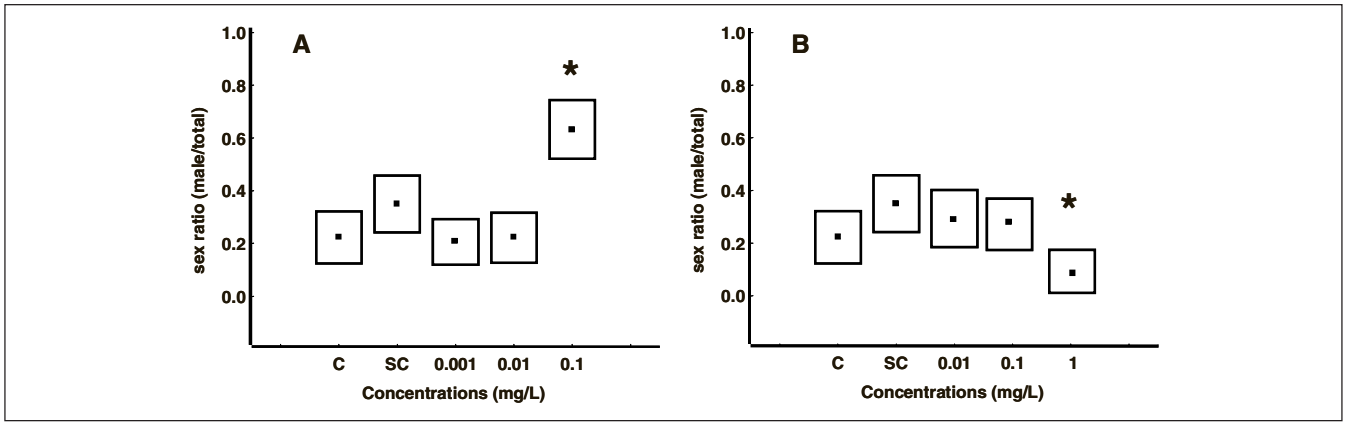

Fig. 2: Effects of dicofol (A) and vinclozolin (B) on the sex ratio in the neonates of *D. magna* during 4–6 day sub-chronic exposure (concentrations in mg/L). Data represent mean and the standard error. * Asterisks indicate significant ($P < 0.05$) difference from the control (ANOVA, followed by Dunnet's test); C – control/blank, SC – solvent control

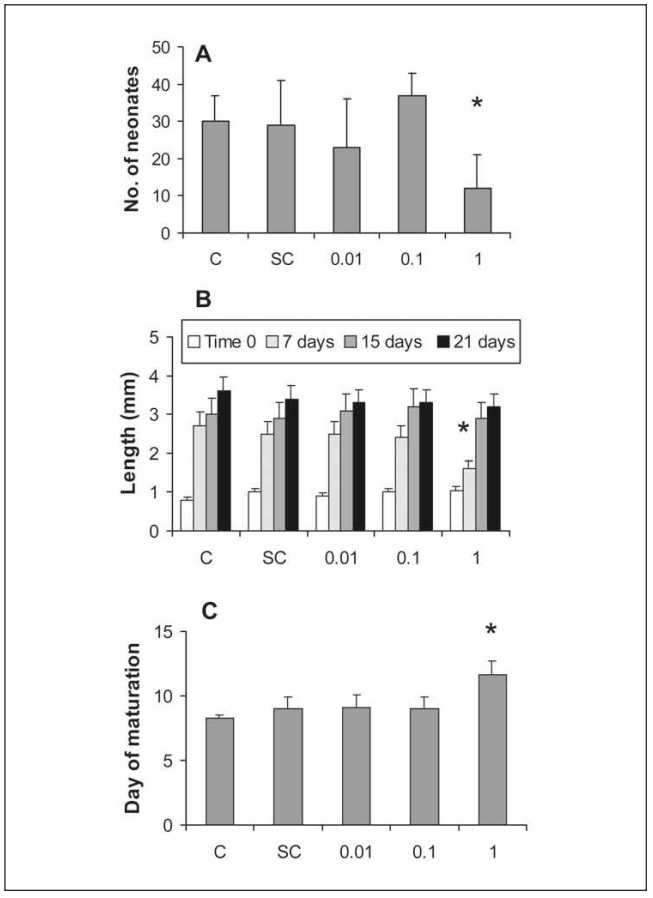

Fig. 3: Effects of flutamide (concentrations in mg/L) in the chronic assay on the number of neonates (A), the growth (length) of maternal organisms (B) and the maturation (C). Data are presented as the mean and the standard error of mean. Asterisks indicate a significant ($P<0.05$) difference from the control (ANOVA, followed by Dunnet's test). C – control, SC – solvent control

of 21 days of exposure (**Fig. 3B**). Further, flutamide delayed attainment of daphnid maturity by prolonging the time to the first reproduction at the greatest concentration tested (**Fig. 3C**). Flutamide had no effects during the 4–6 day, sub-chronic exposures and did not affect the sex ratio of exposed animals.

3 Discussion

Even though *Daphnia magna* is a commonly employed test organism in ecotoxicology, relatively little is known about its physiology and biochemical mechanisms involved in possibly endocrine regulated endpoints. Neonate sex ratio, maturation, growth rate, molting, and fecundity are some of the important life characteristics of this species that may be affected by environmental pollutants, including endocrine disruptors (LeBlanc 2007). In this study, we have assessed effects of selected model chemicals (acting as EDCs in vertebrates) on endpoints that were not previously evaluated in *D. magna*. We have compared results from both sub-chronic and chronic experiments to study the role of possible adaptation and/or detoxification that may occur during prolonged, 21 day experiments (Kashian 2004). From the practical point, it may be difficult to determine sex of neonates during 21 day experiments (labourous evaluation of a high number of neonates from three to five broods per single maternal organism). Further, increasing age of maternal daphnids during the 21 day experiments may affect the sex of the offsprings (Oda et al. 2006). Therefore, shorter sub-chronic experiments are of importance in the studies focusing on the sex ratio (Oda et al. 2006).

Of the compounds tested, vinclozoline had no effect on survival of *D. magna* in the acute 48 h assay up to its solubility (3 mg/L; see Table 1), a concentration several orders of magnitude higher than possible environmental levels that rarely exceed 0.5 µg/L (Tillmann et al. 2001). All other chemicals significantly affected viability of daphnids at concentrations comparable to those reported in previous studies with EC_{50} values ranging from 0.2 (dicofol) to 2.7 mg/L (flutamide; see Table 1; Andersen et al. 2001). Based on the acute assays, concentrations with no effects were selected for further sub-chronic and chronic experiments (up to 0.1 mg/L for dicofol and 1 mg/L for other chemicals).

Flutamide (1 mg/L; see Fig. 3) was the only tested compound that negatively affected several parameters in the chronic, 21 day assay (delayed the maturation and temporarily suppressed the growth of maternal organisms, and also reduced the total number of neonates). On the other hand, flutamide had no direct effects on the sex ratio in *D. magna* (see

Table 2). At the present time, there is only limited information on flutamide toxicity in aquatic invertebrates. Our observations with *D. magna* seem to correspond to the study with the freshwater rotifer *Brachionus calyciflorus*, where flutamide exhibited similar effects at concentrations around µg/L (Preston et al. 2000). As flutamide is a known anti-androgen in vertebrates including fish (Kunimatsu et al. 2004), potential interaction of this compound with analogs of steroid receptors in *Daphnia* could possibly explain our results (Köhler et al. 2007, LeBlanc 2007). This hypothesis can also be indirectly supported by similar results (i.e. delayed maturation and reduced growth of juvenile daphnids) reported previously for another anti-androgen – cyproterone acetate (LeBlanc & McLachlan 1999, Olmstead & LeBlanc 2001). However, it should also be emphasized that the growth suppression of maternal organisms and inhibition in offspring production (both observed in our study) could be inter-correlated (growth-inhibited, i.e. smaller daphnids could not carry enough eggs in their brood chambers). Further, effective concentrations of flutamide in our study were relatively high, and we cannot exclude possible indirect negative effects on general physiological status of daphnids that could lead to the lower reproduction. A study of Barata et al. (2000) with higher concentrations of fluoranthene reported anorexia in exposed daphnids,but this was not confirmed in our experiments (no differences in feeding of exposed and control organisms).

Sex ratio in *Daphnia magna* is a sensitive endpoint that may be affected by unfavourable environmental factors as well as chemical pollution, including EDCs (Dodson et al. 1999b, Peterson et al. 2001, Olmstead & LeBlanc 2003, Tatarazako et al. 2003, Kashian & Dodson 2004, Oda et al. 2005b). In our study, exposure to the organochlorine acaricide dicofol significantly increased the number of male neonates during the sub-chronic assay (see Fig. 2A). We have observed this effect in repeated 4–6 day experiments but it was not apparent during the prolonged 21-day assay (see Table 2). Acclimation and/or detoxification during longer periods could possibly play a role, but full elucidation of such differences would need further research. Dicofol-induced numbers of males could correspond to its action in vertebrates where it inhibits CYP19, an important steroidogenic enzyme catalyzing conversion of androgens to oestrogens, acting thus as an anti-oestrogen (Vinggaard et al. 2000). In spite of differences between vertebrates and crustaceans, some parallels in endocrine regulations were suggested in recent reviews (Köhler et al. 2007, LeBlanc 2007). Sex ratio in *D. magna* was also affected by another tested compound, vinclozolin, which caused a decrease in the number of newborn males (Fig. 2B). Interestingly, this finding is also consistent with the vinclozolin mechanism described in vertebrates, an antagonistic action brought about by binding to the androgen receptor (Kelce et al. 1994). Vinclozolin has also been reported to cause female virilization (imposex development) and reduction of accessory sex organ expression in the sensitive fresh water snail *Marisa cornuarietis* and two marine prosobranchs *Nucella lapillus* and *Nassarius reticulatus* (Tillmann et al. 2001).

4 Conclusions and Perspectives

Taken together, our study documents that some chemicals known to influence steroid-regulated processes in vertebrates can have an effect on embryonal sex determination and/or reproduction in *D. magna*. These effects may change the rate of sexual reproduction and genetic recombination affecting overall diversity of zooplankton populations (Dodson & Hanazato 1995). Our observations also seem to support the hypothesis that EDCs affecting steroid-mediated actions in vertebrates (such as dicofol or vinclozolin acting as anti-oestrogen and anti-androgen, respectively) can have similar sublethal effects in invertebrates including *Daphnia* (Zou & Fingerman 1997, Dodson et al. 1999). In spite of these analogies, general physiological status of organisms may also indirectly affect some endpoints that are traditionally considered as hormone regulated. For example, sex ratio may be affected by higher mortality of neonates of certain sensitive sex (e.g. males) or suppression in feeding or poor food quality may result in a slower growth, etc. Our studies did not suggest such effects (although effective concentrations were relatively high), but these factors should always be carefully considered. Further research of *D. magna* physiology and comparative studies with various EDCs may help to understand mechanisms of action as well as ecological risks of these compounds in the environment.

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References

Andersen H, Wollenberger L, Halling-Sorensen B, Kusk K (2001): Development of *Copepod nauplii* to copepodites – A parameter for chronic toxicity including endocrine disruption. Environ Toxicol & Chem 20, 2821–2829

Baldwin W, Graham S, Shea D, LeBlanc G (1998): Altered metabolic elimination of testosterone and associated toxicity following exposure of *Daphnia magna* to nonylphenol polyethoxylate. Ecotoxicol Environ Saf 39 (2) 104–111

Baldwin W, Leblanc G (1994): In-vivo biotransformation of testosterone by phase-I and phase-Ii detoxication enzymes and their modulation by 20-Hydroxyecdysone in *Daphni magna*. Aquat Toxicol 29 (1–2) 103–117

Barata C, Baird J (2000): Determining the ecotoxicological mode of action of chemicals from measurements made on individuals: Results from instar-based tests with *Daphnia magna* Straus. Aquat Toxicol 48, 195–209

Basler A, Lebsanft J (1999): Endocrine disrupters – Status and regulatory aspects. Env Sci Pollut Res 6, 44–48

Bayley M, Junge M, Baatrup E (2002): Exposure of juvenile guppies to three antiandrogens causes demasculinization and a reduced sperm count in adult males. Aquat Toxicol 56, 227–239

Bryan GW, Gibbs PE, Hummerstone (1986): The decline of the gastropod *Nucella lapillus* around south-west England: Evidence for the effect of tributyltin from antifouling paints. J Marine Biol Assoc UK 66, 611–640

Chang E (1993): Comparative endocrinology of molting and reproduction – Insects and crustaceans. Annu Rev Entomol 38, 161–180

Dodson S, Hanazato T (1995): Commentary on effects of anthropogenic and natural organic chemicals on the development, swimming behavior, and reproduction of *Daphnia*, a key member of aquatic ecosystem. Environ Health Perspect 103, 7–11

Dodson S, Merritt C, Shannahan J, Shults C (1999a): Low exposure concentrations of atrazine increase male production in *Daphnia pulicaria*. Environ Toxico Chem 18, 1568–1573

Finney D (1971): Probit analysis. Cambridge University, Cambridge, UK

Gray L, Lambright C, Mann P, Price M, Cooper R, Ostby J (1999): Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p '-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. Toxicol Ind Health 15, 94–118

Heckman W, Kane B, Pakyz R, Cosentino M (1992): The effect of ketoconazole on endocrine and reproductive parameters in malemice and rats. J Androl 13, 191–198

Heinz-RK, Werner K, Martin S, Iika L, Anna L, Reye, Rita T, Roland N, Gilbert S (2007): Sex steroid receptor evolution and signalling in aquatic invertebrates. Ecotoxicology 16 (1) 131–143

Hense B, Severin G, Pfister G, Welzl G, Jaser W, Schramm K (2005): Effects of anthropogenic estrogens nonylphenol and 17 alpha-ethinylestradiol in aquatic model ecosystems. Acta Hydrochim Hydrobiol 33 (1) 27–37

Kashian D (2004): Toxaphene detoxification and acclimation in *Daphnia magna*: do cytochrome P-450 enzymes play a role? Comp Biochem Phys C 137, 53–63

Kashian D, Dodson S (2004). Effects of vertebrate hormones on development and sex determination in *Daphnia magna*. Environ Toxicol Chem 23, 1282–1288

Kast-Hutcheson K, Rider C, LeBlanc G (2001): The fungicide propiconazole interferes with embryonic development of the crustacean *Daphnia magna*. Environ Toxicol Chem 20, 502–509

Keiter S, Rastall A, Kosmehl T, Wurm K, Erdinger L, Braunbeck T, Hollert H (2006): Ecotoxicological assessment of sediment, suspended matter and water samples in the upper Danube River – A pilot study in search for the causes for the decline of fish catches. Env Sci Pollut Res 13, 308–319

Kelce W, Monosson E, Gamcsik M, Laws S, Gray L (1994): Environmental Hormone Disruptors: Evidence That Vinclozolin Developmental Toxicity Is Mediated by Antiandrogenic Metabolites. Toxicol Appl Pharmacol 126, 276–285

Kolvenbag G, Iversen P, Newling D (2001): Antiandrogen monotherapy: A new form of treatment for patients with prostate cancer. Urol 58, 16–22

Kunimatsu T, Yamada T, Miyata K, Yabushita S, Seki T, Okuno Y, Matsuo M (2004): Evaluation for reliability and feasibility of the draft protocol for the enhanced rat 28-day subacute study (OECD Guideline 407) using androgen antagonist flutamide. Toxicol 200, 77–89

LeBlanc G, McLachlan J (1999): Molt-independent growth inhibition of *Daphnia magna* by a vertebrate antiandrogen. Environ Toxicol Chem 18, 1450–1455

LeBlanc G (2007): Crustacean endocrine toxicology: A review. Ecotoxicology 16 (1) 61–81

Oda S, Tatarazako N, Watanabe H, Morita M, Iguchi T (2005a): Production of male neonates in *Daphnia magna* (Cladocera, Crustacea) exposed to juvenile hormones and their analogs. Chemosphere 61, 1168–1174

Oda S, Tatarazako N, Watanabe H, Morita M, Iguchi T (2005b): Production of male neonates in four cladoceran species exposed to a juvenile hormone analog, fenoxycarb. Chemosphere 60, 74–78

Oda S, Tatarazako N, Watanabe H, Morita M, Iquchi T (2006): Genetic differences in the production of male neonates in *Daphnia magna* exposed to juvenile hormone analogs. Chemosphere 63 (9) 1477–1484

OECD (1996): Organization for Economic Cooperation and Development – Guideline 202, *Daphnia* sp., Acute Immobilisation Test and Reproduction Test

Olmstead A, LeBlanc GA (2000): Effects of endocrine-active chemicals on the development of sex characteristics of *Daphnia magna*. Environ Toxicol Chem 19, 2107–2113

Olmstead A, Leblanc G. (2002): Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia magna*. J Exp Zool 293, 736–739

Olmstead A, LeBlanc G (2003): Insecticidal juvenile hormone analogs stimulate the production of male offspring in the crustacean *Daphnia magna*. Environ Health Perspect 111, 919–924

Olmstead A, LeBlanc G (2001): Low exposure concentration effects of methoprene on endocrine-regulated processes in the crustacean *Daphnia magna*. Toxicol Sci 62, 268–273

Peterson J, Kashian D, Dodson S (2001): Methoprene and 20-OH-ecdysone affect male production in *Daphnia pulex*. Environ Toxicol Chem 20, 582–588

Preston B, Snell T, Robertson T, Dingmann B (2000): Use of freshwater rotifer brachionus calyciflorus in screening assay for potential endocrine disruptors. Environ Toxicol Chem 19, 2923–2928

Samel A, Ziegenfuss M, Goulden C, Banks S, Baer K (1999): Culturing and bioassay testing of *Daphnia magna* using Elendt M4, Elendt M7, and COMBO media. Ecotox Environ Safe 43, 103–110

Sanchez P, Alonso C, Fernandez C, Vega M, Garcia M, Tarazona J (2005): Evaluation of a multi-species test system for assessing acute and chronic toxicity of sediments and water to aquatic invertebrates – Effects of pentachlorophenol on *Daphnia magna* and *Chironomus prasinus*. J Soils Sediments 5, 53–58

Schurmeyer T, Nieschlag E (1984): Effect of ketoconazole and other imidazole fungicides on testosterone biosynthesis. Acta Endocrinologica 105, 275–280

Sperry T, Thomas P (1999): Identification of two nuclear androgen receptors in kelp bass (*Paralabrax clathratus*) and their binding affinities for xenobiotics: Comparison with Atlantic croaker (*Micropogonias undulatus*) androgen receptors. Biol Reprod 61, 1152–1161

StahlschmidtAllner P, Allner B, Rombke J, Knacker T (1997): Endocrine disruptors in the aquatic environment. Env Sci Pollut Res 4, 155–162

Subramonian T (2000): Crustacean ecdysteroids and embryogenesis. Comp Biochem Phys C 125, 135–156

Tatarazako N, Oda S, Watanabe H, Morita M, Iguchi T (2003): Juvenile hormone agonists affect the occurrence of male *Daphnia*. Chemosphere 53, 827–833

Tillmann M, Schulte-Oehlmann U, Duft M, Markert B, Oehlmann J (2001): Effects of endocrine disruptors on prosobranch snails (Mollusca : Gastropoda) in the laboratory. Part III: Cyproterone acetate and vinclozolin as antiandrogens. Ecotoxicology 10, 373–388

Tyler C, Jobling S, Sumpter J (1998): Endocrine disruption in wildlife: A critical review of the evidence. Crit Rev Toxicol 28 (4) 319–361

Vinggaard A, Hnida C, Breinholt V, Larsen J (2000): Screening of selected pesticides for inhibition of CYP19 aromatase activity in vitro. Toxicol in Vitro 14, 227–234

Wang H, Olmstead A, Li H, LeBlanc G (2005): The screening of chemicals for juvenoid-related endocrine activity using the water flea *Daphnia magna*. Aquat Toxicol 74, 193–204

Zou E, Fingerman M (1997): Effects of estrogenic xenobiotics on molting of the water flea, *Daphnia magna*. Ecotoxicol Environ Saf 38 (3) 281–285

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Článek XII:

Feldmannová, M., Hilscherová, K., Maršálek, B., Bláha, L., 2006. Effects of N-heterocyclic polyaromatic hydrocarbons on survival, reproduction, and biochemical parameters in *Daphnia magna*. Environmental Toxicology 21, 425–431.

Effects of N-Heterocyclic Polyaromatic Hydrocarbons on Survival, Reproduction, and Biochemical Parameters in *Daphnia magna*

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ABSTRACT: N-heterocyclic polycyclic aromatic hydrocarbons (N-PAHs) belong among newly identified classes of environmental pollutants with relatively high toxic potential. N-PAHs have been detected in air, soil, marine environments, and freshwater sediments. The N-PAHs are present at lower concentrations than their nonsubstituted analogues but their greater solubility would lead to greater bioavailibity and potential for toxic effects. Here we present results of acute and chronic toxicity in traditional aquatic invertebrate ecotoxicological model (*Daphnia magna*) along with assessment of biochemical responses. Studied biomarkers in *D. magna* exposed to N-heterocyclic derivatives included glutathione levels and activities of detoxication and antioxidative enzymes glutathione S-transferase and glutathione peroxidase. Phenanthrene and 1,10-phenathroline were the most toxic of all tested compounds ($EC_{50} < 6$ μM after 48 h exposure) and all tested N-PAHs suppressed reproduction of *Daphnia magna*. The data suggest that N-PAHs can induce oxidative stress in *D. magna*. The significant decline of glutathione content was found in animals treated with acridine, 1,10-phenanthroline, benzo(h)quinoline, phenantridine, and phenazine. Significant decrease of GPx activities relative to controls was found for all tested compounds except of phenanthrene and phenazine. Activities of GST increased after exposure to phenanthridine, phenazine, and benzo(h)quinoline, and declined in *D. magna* treated with phenanthrene (significant at one concentration) or anthracene (not significant). Our results confirmed significant acute as well as chronic toxicities of N-PAHs as well as potential of biochemical parameters to be used as early warning signals of toxicity in *Daphnia magna*. Environ Toxicol 21: 425–431, 2006.

Keywords: N-PAHs; *Daphnia magna*; biomarkers; glutathione; reproduction

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) represent a major class of organic contaminants in many industrial and urban regions worldwide. Besides the 16 traditionally monitored US EPA priority PAHs, there are many compounds that are currently overlooked in monitoring programs. These include for example high molecular weight mutagenic PAHs, nitroderivatives and oxygenated PAHs,

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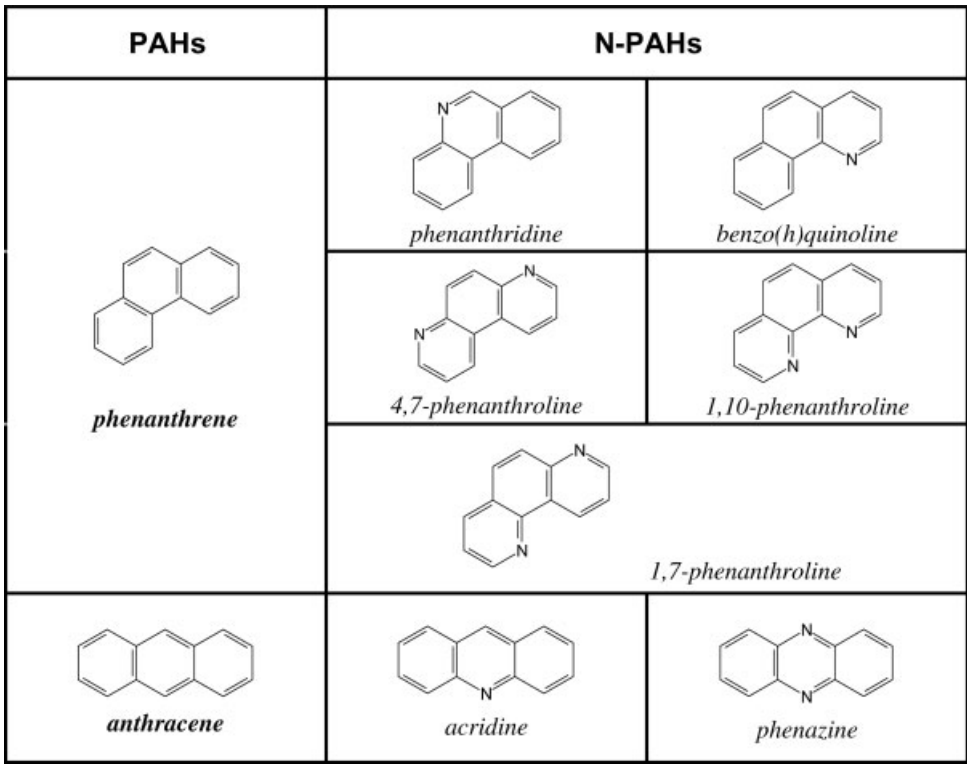

Fig. 1. Chemical structures of the studied compounds.

and also N-heterocyclic aromatic compounds (N-PAHs or aza-PAHs; Durant et al., 1998; Machala et al., 2001).

N-PAHs are a family of N-heterocyclic PAHs containing one or more in-ring nitrogen atoms. There are natural sources of aza-PAHs such as alkaloids, mycotoxins, nucleotides, or electron carriers, but significant amounts of these compounds are released into the environment by anthropogenic sources including incomplete combustion of fossil fuels, spills or industrial effluents, oil drilling, refining and storage, coal tar distillation, wood preservation, and also tobacco smoking (Chen and Preston, 2004). Aza-PAHs are widespread concomitantly with their parent analogues and have been detected in air (Durant et al., 1998), water and sediments (Machala et al., 2001), and also in soil (Brooks et al., 1998). However, our understanding of their occurrence, environmental fate, biological metabolism, and effects is still limited.

Although heterocyclic PAHs outnumber the unsubstituted homocycles, the environmental concentrations reported are lower (1–10%) than those of the parent analogous, unsubstituted PAHs (Benestad et al., 1987). However, the greater polarity and water solubility of heterocyclic PAHs may lead to increased bioavailability and thus potential toxic effects despite their lower environmental concentrations. For example, solubility of acridin is almost 3 orders of magnitude greater compared to anthracene (Kochany and Maguire, 1994).

The ecotoxicological effects of a few aza-PAHs (particularly low molecular weight compounds) on algae, invertebrates, and fish have been investigated (Parkhurst et al., 1981; van Vlaardingen et al., 1996; Kraak et al., 1997; Bleeker et al., 1999). Additionally, some N-heterocyclic aromatic compounds were found to have significant mutagenic, carcinogenic and teratogenic effects, and also non-genotoxic effects such as (anti)estrogenicity have been reported (Yamada et al., 2004).

Our study compared effects of two parent PAHs (anthracene and phenanthrene) and seven N-heterocyclic PAH derivatives (Fig. 1) in the aquatic invertebrate model *Daphnia magna*.

Although there are some toxicity data for few selected chemicals (particularly quinoline and acridine), little information is available on other N-PAHs under study.

In this study, we report effects of PAHs and their derivatives on both traditional parameters (survival and reproduction) in *Daphnia magna* but also sublethal biochemical markers playing important role in detoxification and

Abbreviations

PAH	Polycyclic aromatic hydrocarbons
GSH	Glutathione content
GST	Glutathione transferase
GPx	Glutathione peroxidase
DTNB	5,5′-Dithiobis-2-nitrobenzoic acid
CDNB	1-Chloro-2,4 dinitrobenzene
GR	Glutathione reductase
TTFR	Time of the first reproduction

protection against oxidative stress. The studied parameters included total glutathione content (GSH) and activities of detoxification enzyme glutathione transferase (GST) and antioxidant enzyme glutathione peroxidase (GPx).

MATERIALS AND METHODS

Acute Toxicity Bioassay

Juveniles of *Daphnia magna* (continuous laboratory breeding, juveniles less than 24 h old) were randomly transferred into separate polystyrene plate with standard exposure solution (containing basic inorganic salts according to CSN ISO 6341 (1997)). Neonates (twenty juveniles for each concentration) were exposed to N-PAHs (10 μL of dilutions in DMSO per 5 mL). Twenty neonates in standard solution served as control and twenty neonates exposed to DMSO were used as solvent control (final conc. 0.2% DMSO did not affect immobilization of *D. magna*). Each concentration and controls were tested in 4 replicates. Temperature was maintained at 20 $\pm$ 2°C during the exposure. Daphnias were inspected after 24 and 48 h exposure. Acute toxicity was expressed as the median effective concentration (EC_{50}) for immobilization. The concentrations used in acute tests were 2.5–40 μM for acridine, benzo[h]quinoline and phenazine, 0.3–6 μM for phenanthrene and anthracene, 2–150 μM for 1,7 and 4,7-phenanthroline and 0.4–110 μM for 1,10-phenanthroline and phenanthridine.

Chronic Bioassay

Juveniles of *Daphnia magna* less than 24 h old were randomly transferred into separate polystyrene jars (single animal per 50 mL jar) with standard Elendt's M4 solution (containing basic inorganic salts and vitamins prepared according to CSN ISO 10706 (2001)). *D. magna* neonates (ten jars, i.e., ten individual animals per treatment) were exposed to N-PAHs (5 μL of dilutions prepared in DMSO per 50 mL). Twenty neonates exposed to M4 solution served as control and twenty neonates as solvent control. During the 21 day exposure (16 h light: 8 h dark photoperiod), temperature was maintained at 20 $\pm$ 2°C. Exposure solutions were renewed three times per week (every Monday, Wednesday, and Friday, according to CSN ISO 10706 (2001)) and *D. magna* were fed with a viable green algae mixture (*Chlorella vulgaris, Scenedesmus subspicatus, Pseudokirchneriella subcapitata*). The concentration of algae was 10^5 cells per 1 daphnia in 50 mL jar. Neonates were counted daily and discarded. Mortality, time to the first reproduction, number of broods per female, and number of offspring per female were used to evaluate fecundity. The concentrations in chronic test were 0.25–15 μM for phenazine, phenanthridine and benzo[h]quinoline, 0.7–11 μM for acridine, 0.1–1.5 μM for 1,10-phenanthroline.

Assessment of Biomarkers

D. magna neonates (juveniles less than 24 h old; five jars with ten animals per treatment) were exposed to N-PAHs (5 μL of dilutions in DMSO per 50 mL). Control neonates were exposed to M4 solution and 5 μL of DMSO/50 mL served as solvent control. During the 96 h exposure (16 h light: 8 h dark photoperiod) temperature was maintained at 20 $\pm$ 2°C. Exposure solutions were renewed every 48 h and *D. magna* were fed daily. Experiments were repeated independently three times. At the end of the exposure period, 50 daphnias were harvested and homogenized in 1 mL of ice cold phosphate-buffered saline (PBS, pH 7.2). The homogenate was centrifuged at 4°C at 2500*g* and the supernatant was stored at -80°C until determination of biochemical parameters.

Assessments of biomarkers were optimized for measurement in microplates. Tecan GENios microplate reader (TECAN GmbH, Switzerland) was used for measurement of absorbance in all assays.

The determination of GSH content was based on the method of Ellman (1959). Sample was mixed in ratio 10:1 with 25% (w/v) trichloracetic acid, centrifuged at 6000*g* for 10 min. Supernatant was incubated in Tris/HCl buffer (0.8 M Tris/HCl, 0.02 M EDTA, pH 8.9) with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB 80 μM) for 5 min at room temperature. Absorbance of GSH-DTNB conjugate was determined at 420 nm. The total GSH in daphnia was expressed as nmol GSH/mg protein, using reduced GSH as a standard for calibration.

Glutathione S-transferase activities (GST) were determined spectrophotometrically at 340 nm by the procedure of Habig et al. (1974) in homogenate of *Daphnia magna* using 1-chloro-2,4 dinitrobenzene (CDNB), reduced glutathione (GSH), and phosphate buffer (pH 7.2). The final concentrations were 2 mM CDNB and 2 mM GSH. The units of GST activities are expressed as nmol/min/mg protein.

GPx activities were measured following NADPH oxidation at 340 nm in the presence of glutathione reductase (GR), reduced glutathione (GSH), and *tert*-butyl hydroperoxide as a substrate (Flohé and Gunzler, 1984). The method was performed with final concentrations of 3 mM GSH, 1 U GR, 0.15 mM NADPH, and 1.2 mM BHP in 0.1 M potassium phosphate/1 mM EDTA buffer (pH 7). The units of GPx activities are expressed as nmol NADPH oxidized/min/mg protein.

The protein concentrations were determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard. Absorbance was measured at 680 nm.

Data Analysis

Toxicity results from both acute and chronic tests were evaluated with probit model to estimate EC_{50} for immobilization in acute test and LC_{50} in the chronic test. The significance of differences in levels of biochemical parameters

TABLE I. Acute and chronic toxicity of PAHs and their derivatives to *D. magna*

	Immobilization		Survival	Reproduction
Compound	EC_{50} 24 h (μM)	EC_{50} 48 h (μM)	LC_{50} 21d (μM)	EC_{50} 21d (μM)
Phenanthrene	5.0 (4.5–5.5)	3.2 (2.9–3.5)	n/a[a]	n/a
Phenanthridine	28.8 (28.1–29.5)	15.0 (14.3–15.7)	6.6 (6.2–7.0)	<0.25
Benzo(h)quinoline	23.5 (22.9–24.1)	19.4 (18.1–20.7)	5 (4.9–5.1)	<0.25
4,7-phenanthroline	104.4 (104.1–104.7)	94.3 (93.5–95.1)	n/a	n/a
1,7-phenanthroline	111.0 (110.6–111.4)	98.7 (98.3–99.1)	n/a	n/a
1,10-phenanthroline	6.9 (6.1–7.7)	5.8 (5.2–6.4)	0.35 (0.33–0.36)	0.69 (0.68–0.7)
Anthracene	>solubility[b]	>solubility	n/a	n/a
Acridine	23.2 (22.8–23.6)	13.4 (12.9–13.9)	1.4 (0.9–1.9)	<0.7
Phenazine	28.1 (26.9–29.3)	16.3 (16.0–16.6)	>15	<0.25

Values indicate EC_{50} (for immobilization and reproduction), LC_{50} (chronic test), and 95% confidence intervals (in parentheses).
[a] Not analysed.
[b] Anthracene solubility limit 5 μM.

among treatment groups was examined by ANOVA with LSD and Dunett post hoc test. The homogeneity of variance was assessed by Levene's test. *P*-values less than 0.05 were considered statistically significant for all applied tests. All statistical analyses were performed with Statistica for Windows (StatSoft, Tulsa, OK, USA).

RESULTS

Our experiments showed significant effects of tested N-PAHs on survival, fecundity, and reproduction of *D. magna*. The effects of studied PAHs and N-PAHs on mobility of *Daphnia magna* after acute exposure (24 h/48 h) are summarized in Table I. Parent PAH phenanthrene was the most acutely toxic compound tested, while no toxicity was observed within water soluble doses of anthracene ($\leq$5 μM). The acute toxicity of tested N-heterocyclic derivatives increased in the following order: 1,7-phenanthroline $\leq$ 4,7-phenanthroline $<$ phenanthridine $\leq$ phenazine $\leq$ benzo(h)-quinoline $\leq$ acridine $<$ 1,10-phenanthroline.

Chronic experiments with *Daphnia magna* revealed significant lethal and reproduction-related effects of all tested compounds (Tables I and II).

The survival in control as well as solvent control groups was 100%. Low mortalities (10–20%) were observed in animals exposed to lower concentrations of most tested compounds and also at higher concentrations (15 μM) of phenazine (Table I). Higher concentrations of acridine (5.6 μM), benzo(h)quinoline, and phenanthridine (15 μM) caused immobilization (corresponding to death) of all animals within 6–11 days. The most toxic in chronic test was 1,10-phenanthroline (LC_{50} 0.35 μM, survival affected already at the lowest tested concentration 0.086 μM), and the toxicity decreased in the following order: 1,10-phenanthroline > acridine > benzo(h)quinoline > phenanthridine > phenazine.

The effects of the tested compounds on fecundity (reproduction) of *Daphnia magna* during 21 day experiments are summarized in Tables I and II. Significant effects of all studied N-PAHs on fecundity of *Daphnia magna* were observed already at the lowest tested concentrations (ranging from 0.086 μM for phenanthroline to 0.25 μM for other compounds). For the most toxic compound 1,10-phenanthroline, there were relatively high fecundities (more than 50% of control) at concentrations 0.086–0.35 μM.

On the other hand, sublethal concentration of other N-PAHs caused more pronounced effects. There was no repro-

TABLE II. Effects of N-PAHs in *Daphnia magna* after 21 days exposure

Concentration (μM)	Time of the First Reproduction (TTFR; days)	Number of Broods Per Female
Control/solvent control		
	8	5
Phenanthridine		
0.25	13	2–3
5	–	0
15	–	0
Benzo(h)quinoline		
0.25	11	2–3
5	20	1
15	–	0
1,10-phenanthroline		
0.086	8	4–5
0.69	8	4
1.4	8	3–4
Acridine		
0.7	8	3
5.6	11	1
11	–	0
Phenazine		
0.25	13	2
5	15	1
15	15	1

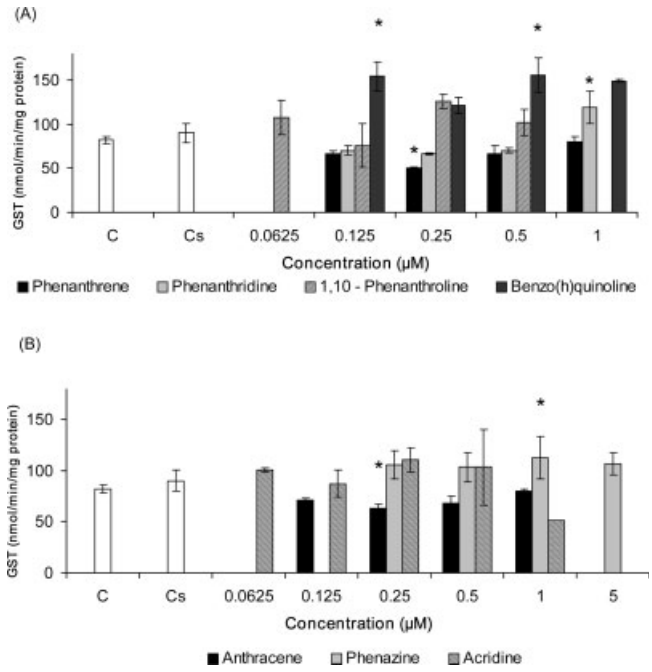

Fig. 2. Changes in GST activities in *D. magna* exposed to various concentrations of tested compounds (96 h exposure). (A) Effects of phenanthrene and its derivatives, (B) effects of anthracene and its derivatives, C-control, Cs-solvent control. Values represent the mean ± SD of triplicate determinations.

duction in the highest concentration of acridine (11 μM) and benzo(h)quinoline (15 μM) and two highest concentrations of phenanthridine (5 and 15 μM). Exposure to phenazine caused decrease of fecundity to 19% already at the lowest tested concentration (0.25 μM), although the effects of this compound on survival were the least pronounced of all tested chemicals.

Time of the first reproduction (TTFR) was 8 days in both controls (Table II). The same time to the first reproduction was found for animals exposed to 1,10-phenanthroline (all concentrations) and acridine (up to 2.7 μM). Significant prolongation of TTFR was observed for benzo(h)quinoline (11–20 days). Reproduction of daphnias exposed to phenathridine and phenazine was also delayed (13–15 days).

The results of assessment of biochemical parameters in daphnia after 96 h exposure to the tested compounds are displayed in Figures 2, 3, 4, and Table III.

Our study has revealed increase in GST activities after exposure to benzo(h)quinoline, phenanthridine, and phenazine (Fig. 2). Also the activities of GST for 1,10-phenanthroline and acridine were weakly elevated at some concentrations, but this effect was not significant. On the other hand, the unsubstituted PAHs did not cause any significant effect except of decrease of GST activity at 0.25 μM concentration of phenanthrene.

The effects on enzymatic activities of GPx are illustrated in Figure 3. All studied compounds except of phenanthrene and phenazine caused significant decrease in activities of GPx. The effects were most pronounced for 1,10-phenanthroline, where there was about 40% decrease of GPx activities already at the lowest tested concentration (0.0625 μM) and benzo(h)quinoline, which showed similar level of decrease at 0.25 μM and higher concentrations.

The next studied parameter was glutathione (GSH) content. The measurements have shown significant decline of GSH for all tested N-PAHs, while the parent compounds did not cause any effect at the same concentrations (Fig. 4). Acridine and 1,10-phenanthroline had the strongest effects. They have caused decrease in glutathione levels to about 30% of control already at the lowest tested concentration (0.0625 μM).

Biomarkers were generally affected at lower concentration than toxicity parameters (Tables I and III).

DISCUSSION

Although PAHs and their derivatives belong among major (dominant) contaminants in many areas worldwide, the characterization of their adverse effects is still incomplete. For practical reasons, most ecotoxicological studies of aza-

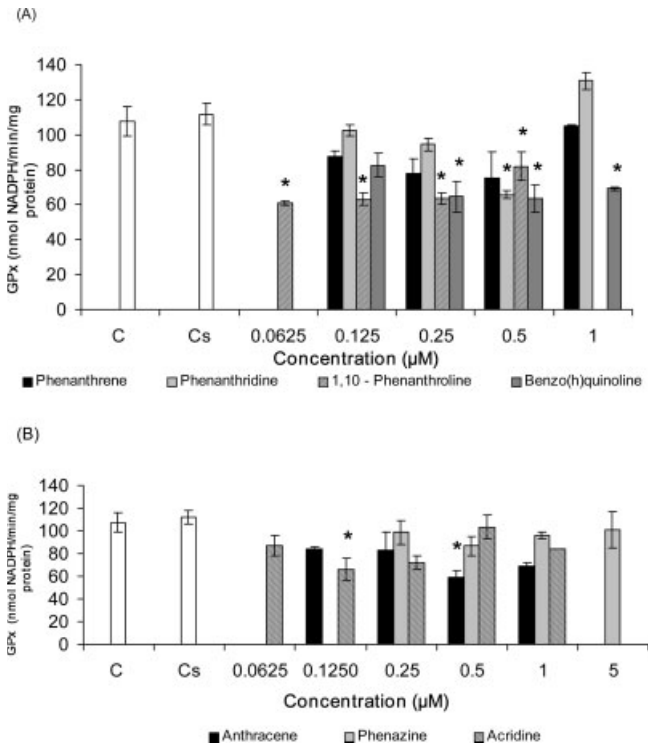

Fig. 3. Changes in GPx activities in *D. magna* exposed to various concentrations of tested compounds (96 h exposure). (A) Effects of phenanthrene and its derivatives, (B) effects of anthracene and its derivatives, C-control, Cs-solvent control. Values represent the mean ± SD of triplicate determinations.

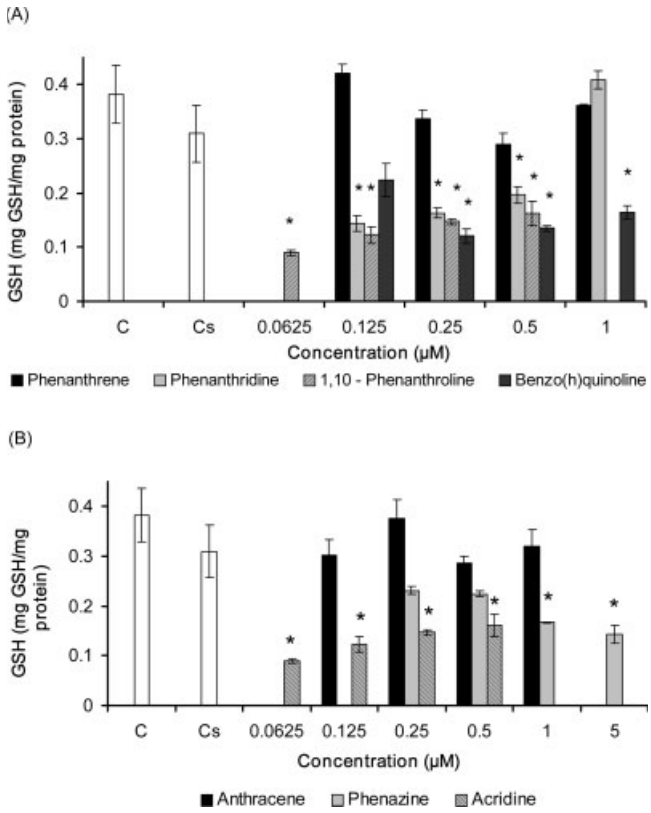

Fig. 4. Changes in levels of reduced GSH in *D. magna* exposed to various concentrations of tested compounds (96 h exposure). (A) Effects of phenanthrene and its derivatives, (B) effects of anthracene and its derivatives, C-control, Cs-solvent control. Values represent the mean ± SD of triplicate determinations.

PAHs have focused on lower molecular weight compounds such as quinolines, benzoquinolines, or acridine (van Vlaardingen et al., 1996; Kraak et al., 1997; Bleeker et al., 1999). Even though N-PAHs have been detected in the environment, there is limited information about their toxicity and to our knowledge, information about their sublethal effect to *Daphnia magna* does not exist with the exception of some prototypical compounds, such as acridine. Our study brings more complete information on toxicities of wider spectra of N-PAHs relative to their parent compounds.

The 48 h EC_{50} for acridine evaluated in our study 13.4 μM (2.4 mg/L), Table I corresponds to data published by Eastmond et al. (1984) and Parkhurst et al. (1981), who showed 48 h 50% effective concentration of 12.8 μM (2.3 mg/L). The EC_{50} for phenanthrene after 48 h exposure determined in our study 5 μM (0.89 mg/L) is also comparable with previously published EC_{50} value of 2.3–4.7 μM (0.38–0.84 mg/L) (Eastmond et al., 1984).

There are only few studies on N-PAH effects to pelagic crustaceans, but some toxicological data are available for other aquatic organisms. Previously published 48 h EC_{50} values for the zebra mussel (*Dreissena polymorpha*) exposed to acridine, phenanthridine, and benzo(h)quinoline were 2.8 μM (0.5 mg/L), 0.5 μM (0.09 mg/L), and 6.9 μM (1.25 mg/L), respectively (Kraak et al., 1997). N-PAH toxicity was also tested with *Chironomus riparius*, and LC_{50} values for acridine, phenanthridine, and benzo(h)quinoline were 0.4 μM (0.07 mg/L), 3.42 μM (0.61 mg/L), and 3.38 μM (0.6 mg/L), respectively (Bleeker et al., 1999). As these values are lower than those observed in our study (Table I), interspecific differences might be responsible for this observation.

In our study, we compared effects of both parent 3-ring PAHs (anthracene and phenanthrene), with the toxicities of their N-substituted derivatives. Interesting results were observed with the derivatives of phenathrene. Both the parent compound and 1,10-phenanthroline were the most toxic, while the other N-PAHs containing two nitrogen heterocyclic atoms (1,7-phenanthroline and 4,7-phenanthroline) were among the least toxic of the studied compounds.

The direct comparison of EC_{50} values for anthracene and its analogues is not possible, since the effective concentrations for acridine and phenazine are above the solubility of anthracene, which did not show any lethality within concentrations up to its solubility ($\leq$5 μM). However, the substituted compounds caused significant effects within their solubility range and thus are toxicologically more hazardous to *Daphnia magna* than anthracene.

Besides the acute toxicity, our study focused on characterization of chronic effects of N-PAHs including sublethal reproductive toxicity. Generally, all compounds significantly decreased fecundity at all tested concentrations. Number of broods was also affected by tested compounds, and there was significant delay in the time to the first brood (TTFR) for all compounds except of 1,10-phenanthroline. 1,10-Phenanthroline caused the greatest mortality but its effects on fecundity were comparable to that of the other studied compounds (see Table II). Opposite effect was observed for phenazine. This N-PAH caused relatively lower mortality but had the most pronounced effect on reproduction.

TABLE III. The effects of N-PAHs on biomarkers in *Daphnia magna* after 96 hours exposure

Compound	GPx	GST	GSH
Phenanthrene	n.s.	0.25 ↓	n.s.
Phenanthridine	0.5 ↓	1 ↑	0.125 ↓
Benzo(h)quinoline	0.25 ↓	0.125 ↑	0.25 ↓
1,10-phenanthroline	0.0625 ↓	n.s.	0.0625 ↓
Anthracene	0.5 ↓	0.25 ↓	n.s.
Acridine	0.125 ↓	n.s.	0.0625 ↓
Phenazine	n.s.	1 ↑	1 ↓

The lowest concentration of tested N-PAHs (LOEC, μM) that induced significant change in corresponding biochemical parameter in comparison with control (LOEC) (arrows indicate observed trends ↓ decrease, ↑ increase, n.s., no significant changes at all tested concentrations).

For structurally related acridine, previous studies demonstrated effects on number of broods and number of young per brood at concentrations 0.4–0.8 mg/L (2.2–4.5 μM) (Parkhurst et al., 1981). In our study, these concentrations caused strong effects and decreased the fecundity to 5.4–14% relative to control (Table II).

Several mechanisms of PAH-induced oxidative stress and overproduction of reactive oxygen species (ROS) have been recognized. They include photoreactions (Zafiriou et al., 1984), redox cycling of PAH derivatives (Cavalieri and Rogan, 1985), and also side release of ROS during oxidative PAH metabolism. In our study, we have observed significant inductions of oxidative stress in Daphnia exposed to PAHs and NPAHs. Biochemical responses such as changes in cellular antioxidant and detoxification status might be a suitable tool to trace the toxicity mechanisms. In our study, GSH was the most sensitive biochemical parameter. For all tested compounds, we found significant decline in GSH levels within sublethal concentrations (Table III and Fig. 4). The lower glutathione levels correspond with decreased activities of GPx for most compounds. The strongest effect on glutathione and GPx was caused by 1,10-phenanthroline, which was also the most toxic compound in both acute and chronic tests. It affected biochemical parameters at very low concentration (0.086 μM, Table III).

Modulations were found for GST activities, which is an important detoxication enzyme. While parental PAHs showed little effect, stimulations were observed after exposure to N-PAHs. Also Barata et al. (2005) suggested the glutathione enzymes and catalase as the most responsive biomarkers of oxidative stress in *Daphnia magna*.

In conclusion, our study brings new information on series of PAHs and their N-heterocyclic derivatives for which only limited ecotoxicological data exist so far. Our results revealed that GSH and GPx were generally sensitive and affected by most of the tested compounds. Consequently, these parameters could be used as potential early markers for long-term effects of N-PAHs in aquatic ecosystems and might serve as early warning parameters as significant changes were observed at concentrations in which *in vivo* effects were much less pronounced.

REFERENCES

Barata C, Navarro JC, Varo I, Riva MC, Arun S, Porte C. 2005. Changes in antioxidant enzyme activities, fatty acid composition and lipid peroxidation in *Daphnia magna* during the aging process. Comp Biochem Physiol B: Biochem Mol Biol 140:81–90.

Benestad C, Jebens A, Tveten G. 1987. Emission of organic micropollutants from waste incineration. Chemosphere 16:813–820.

Bleeker EAJ, Van der Geest HG, Klamer HJC, De Voogt P, Wind E, Kraak MHS. 1999. Toxic and genotoxic effects of azaarenes: Isomers and metabolites. Polycyclic Aromat Compd 13:191–203.

Brooks LR, Hughes TJ, Claxton LD, Austern B, Brenner R, Kremer F. 1998. Bioassay-directed fractionation and chemical identification of mutagens in bioremediated soils. Environ Health Perspect 106:1435–1440.

Cavalieri E, Rogan E. 1985. Role of radical cations in aromatic hydrocarbon carcinogenesis. Environ Health Perspect 64:69–84.

Chen H.-Y, Preston MR. 2004. Measurement of semi-volatile azaarenes in airborne particulate and vapor phase. Anal Chim Acta 501:71–78.

Durant JL, Lafleur AL, Plummer EF, Taghizadeh K, Busby WF, Thilly WG. 1998. Human lymphoblast mutagens in urban airborne particles. Environ Sci Technol 32:1894–1906.

Eastmond DA, Booth GM, Lee ML. 1984. Toxicity, accumulation, and elimination of polycyclic aromatic sulfur heterocycles in *Daphnia magna*. Arch Environ Contam Toxicol 13:105–111.

Ellman GL. 1959. Tissue sulfhydryl group. Arch Biochem Biophys 82:70–77.

Flohé L, Gunzler WA. 1984. Assays of glutathion peroxidase. Methods Enzymol 105:114–120.

Habig WH, Pabst MJ, Jakoby WB. 1974. Gluthatione S-transferase. The first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130–7139.

Kochany J, Maguire RJ. 1994. Abiotic transformations of polynuclear aromatic-hydrocarbons and polynuclear aromatic nitrogen-heterocycles in aquatic environments. Sci Total Environ 144: 17–31.

Kraak MHS, Ainscough C, Fernandez A, vanVlaardingen PLA, deVoogt P, Admiraal WA. 1997. Short-term and chronic exposure of the zebra mussel (*Dreissena polymorpha*) to acridine: Effects and metabolism. Aquat Toxicol 37:9–20.

Lowry OH, Rosebrough AL, Farr AL, Randall RJ. 1951. Protein measurements with Folin-phenol reagents. J Bio Chem 193: 265–275.

Machala M, Ciganek M, Blaha L, Minksova K, Vondrack J. 2001. Aryl hydrocarbon receptor-mediated and estrogenic activities of oxygenated polycyclic aromatic hydrocarbons and azaarenes originally identified in extracts of river sediments. Environ Toxicol Chem 20:2736–2743.

Parkhurst BR, Bradshaw AS, Forte JL, Wright GP. 1981. The chronic toxicity to *Daphnia magna* of acridine, a representative azaarene present in synthetic fossil fuel products and wastewaters. Environ Pollut Ser A: Ecol Biol 24:21–30.

van Vlaardingen PLA, Steinhoff WJ, deVoogt P, Admiraal WA. 1996. Property-toxicity relationships of azaarenes to the green alga *Scenedesmus acuminatus*. Environ Toxicol Chem 15:2035– 2042.

Water quality—Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) (Acute toxicity test). CSN ISO 6341, 1971.

Water quality—Determination of long term toxicity of substances to *Daphnia magna* Straus (Cladocera, Crustacea). CSN ISO 10706, 2001.

Yamada K, Suzuki T, Kohara A, Hayashi M, Mizutani T, Saeki K. 2004. In vivo mutagenicity of benzo[f]quinoline, benzo[h]quinoline, and 1,7-phenanthroline using the lacZ transgenic mice. Mutat Res 559:83–95.

Zafiriou OC, Joussot-Dubien J, Zepp RC, Zika RG. 1984. Photochemistry of natural waters. Environ Sci Technol 18:358–371.

Článek XIII:

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Changes in concentrations of hydrophilic organic contaminants and of endocrine-disrupting potential downstream of small communities located adjacent to headwaters

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ABSTRACT

Endocrine-disruptive potential and concentrations of polar organic contaminants were measured in seven headwaters flowing through relatively unpolluted areas of the Czech Republic. Towns with Wastewater Treatment Plant (WWTP) discharges were the first known sources of anthropogenic pollution in the areas. River water was sampled several kilometers upstream (US) and several tens of meters downstream (DS) of the WWTP discharges, by use of Pesticide and Pharmaceutical Polar Organic Integrative Samplers (POCIS-Pest, POCIS-Pharm). Extracts of passive samplers were tested by use of a battery of *in vitro* bioassays to determine overall non-specific cytotoxicity, endocrine-disruptive (ED) potential and dioxin-like toxicity. The extracts were also used for quantification of polar organics. There was little toxicity to cells caused by most extracts of POCIS. Estrogenicity was detected in all types of samples even though US locations are considered to be background. At US locations, concentrations of estrogen equivalents (EEq) ranged from less than the detection limits (LOD) to 0.5 ng EEq/POCIS. Downstream concentrations of EEqs ranged from less than LOD to 4.8 ng EEq/POCIS. Concentrations of EEq in POCIS extracts from all DS locations were 1 to 14 times greater than those at US locations. Concentrations of EEq measured in extracts of POCIS-Pest and POCIS-Pharm were in a good agreement. Neither antiestrogenic nor anti/androgenic activities were detected. Concentrations of 2,3,7,8-TCDD equivalents (TEq_{bio}) were detected in both types of POCIS at concentrations ranging from less than the LOD to 0.39 ng TEq_{bio}/POCIS. Nearly all extracts of POCIS-Pharm contained greater concentrations of TEq_{bio} activity than extracts of POCIS-Pest. Concentrations of pesticides and pharmaceuticals in extracts of POCIS were generally small at all sampling sites, but levels of some pharmaceuticals were significantly greater in both types of POCIS from DS locations. Chemical analyses along with the results of bioassays documented impacts of small towns with WWTPs on headwaters.

1. Introduction

Municipal and industrial waste waters can be sources of compounds that are able to cause acute toxicity as well as sublethal chronic abnormalities including disruption of hormonal balance in aquatic organisms (endocrine disruption, ED). Persistent and bioaccumulative organic chemicals have been conventionally monitored, but less persistent and less hydrophobic organic compounds are currently used as pesticides, prescription and non-prescription drugs and personal care products. Despite their lesser bioconcentration potential, relatively large fluxes of some of these compounds into aquatic systems might be acutely toxic and/or induce sublethal chronic abnormalities (Alvarez

Abbreviations: AEq, androgenic equivalent; AhR, Aryl hydrocarbon receptor; DS, downstream; E1, estrone; E2, 17β-estradiol; E3, Estriol; EC, effective concentration; ED, endocrine disruption; EDCs, endocrine disruptive compounds; EE2, 17α-ethynylestradiol; EEq, estrogenic equivalent; HpOCs, hydrophilic organic compounds; K_{ow}, octanol–water partition coefficient; LOD, limit of detection; LOQ, limit of quantification; NR, Neutral Red; PCBs, polychlorinated biphenyls; PCDDs, polychlorinated dibenzodioxins; PCDFs, polychlorinated dibenzofurans; PNEC, Predicted No Effects Concentration; POCIS, Polar Organic Chemical Integrative Sampler; POCIS-Pest, Polar Organic Chemical Integrative Sampler optimized for polar Pesticides; POCIS-Pharm, Polar Organic Chemical Integrative Sampler optimized for most Pharmaceuticals; R_s, sampling rate (L/day); TEq_{bio}, dioxin-like equivalent obtained in bioassay; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; US, upstream; WWTP, Waste Water Treatment Plant.

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et al., 2007). Furthermore, some of these chemicals (particularly pharmaceuticals) can be highly potent, such that even concentrations at or near analytical detection limits may have biological activity.

Concentrations and/or ecotoxicological effects of hydrophilic organic compounds (HpOCs, contain one or more polar functional groups or a significant molecular dipole moment) have been reported in discharges of Waste Water Treatment Plants (WWTP) and/or downstream receiving waters (Aguayo et al., 2004; Bolong et al., 2009; Caliman and Gavrilescu, 2009). Downstream reaches of rivers have been shown to be polluted by compounds of both industrial and communal origin (Bolong et al., 2009), and therefore it is difficult to evaluate contributions and effects of pollutants released by individual towns. There are fewer sources of HpOC pollution in the headwaters and their potential impacts are not easy to assess, since there is limited information on concentrations of pollutants in the background areas.

Although different groups of HpOCs can contribute to adverse effects, xenoestrogens and xenoandrogens have emerged as environmental issues due to their ability to mimic or otherwise adversely affect functions of natural reproductive hormones, which could result in impaired reproduction of aquatic organisms (Matthiessen and Johnson, 2007). Even though the efficiencies of conventional WWTPs with activated sludge systems to remove estrogenic and androgenic compounds seem to be relatively high (88–>99% for estrogens and 96–>99% for androgens (Korner et al., 2000; Leusch et al., 2010; Murk et al., 2002; Svenson and Allard, 2004), concentrations of these endocrine disruptive compounds (EDCs) in some effluents are sufficient to cause ED (Kirk et al., 2002). Since some EDCs can cause adverse effects at small concentrations (ng/L), it is difficult and expensive to detect them by instrumental analyses (Korner et al., 2000). Moreover, because they occur in mixtures, even if they can be quantified, it is difficult to predict the potential effects of these compounds (Leusch et al., 2005). Therefore, *in vitro* bioassays can serve as cheaper and more environmentally relevant alternative to screen for the combined effects of mixtures on specific biological endpoints (Kinnberg, 2003).

The most frequently reported effect connected with EDs in surface waters is feminization of male fish downstream of WWTPs (Jobling and Tyler, 2003). Among estrogenic EDCs, the steroidal estrogens estrone (E1), estradiol (E2), and synthetic estrogen analogue, ethinyl estradiol (EE2), are some of the most potent endocrine disruptors in sewage effluents, all having more than thousand times greater potency to cause ED, at least in fish, than most other xenobiotics (Young et al., 2004). Under environmental conditions, steroidal hormones have been identified to be primarily responsible for observed adverse estrogenic effects on fish downstream of WWTPs although other weakly estrogenic compounds, such as alkylphenols and bisphenol A, can contribute to the effects (Desbrow et al., 1998; Gross-Sorokin et al., 2006). Important is also the fact that effluents from WWTPs can contain antiandrogenic chemicals as well. Their presence has been suggested by previous studies as a potential complication in establishing the chemical causation of fish sexual disruption (Tyler and Jobling, 2008). Efforts to identify the contributing antiandrogens are now underway, using a targeted fractionation process combined with screening by recombinant yeast assay and high-quality analytical chemistry. It should also be mentioned that certain compounds may act as both estrogens and antiandrogens (*e.g.* Suzuki et al., 2005).

There are two different approaches of sampling water, either active or passive. We chose to use passive integrative sampling, rather than traditional grab or composite sampling, for two reasons: i) passive sampling permits determination of time-weighted average concentrations of HpOCs in water, which is especially important when concentrations of HpOCs fluctuate over time because of changes in weather or variable diurnal patterns of consumption of products which are primary sources of HpOCs and, ii) the most potent EDCs usually occur at small concentrations (ng/L) and passive integrative samplers serve as an effective alternative to collecting and handling large volumes of water (Alvarez et al., 2007).

One useful passive sampler for HpOCs is the Polar Organic Chemical Integrative Sampler (POCIS). Relatively good correlations have been observed between concentrations of estrogenic equivalent (EEq) determined in bioassays for POCIS and grab water samples (Arditsoglou and Voutsa, 2008; Vermeirssen et al., 2005). POCIS has been shown to sample a wide variety of polar as well as moderate hydrophobic organic compounds with log K_{ow} of less than 4. Two types of adsorbents are considered standard for deployment of POCIS in the field. One of the two standard configurations, POCIS-Pest, preferentially concentrates waterborne HpOCs such as polar pesticides, natural and synthetic hormones, and other wastewater-related contaminants. The other, POCIS-Pharm, incorporates a sorbent optimal for sequestering polar pharmaceuticals (Alvarez et al., 2007).

Both types of POCIS exhibited linear uptake of phenolic and steroid compounds during 28-day tests conducted in laboratory during which concentrations of analytes in water were held constant. The correlation coefficients of the linear regression with respect to timescale were greater than 0.995 for POCIS-Pest and 0.985 for POCIS-Pharm, which suggests that uptake was time-integrative and the rate of uptake was not time-dependent during the exposure period. Moreover, rates of sampling (R_s) were not affected by changes in concentrations of tested compounds (Arditsoglou and Voutsa, 2008; Matthiessen and Johnson, 2007).

In the present study, water quality in terms of HpOCs and EDCs was studied in several headwaters in the Czech Republic. A combination of instrumental analyses of individual chemicals and *in vitro* assays with extracts from POCIS-Pest and POCIS-Pharm was conducted to: i) determine background levels of anti/estrogenic, anti/androgenic and dioxin-like activities in headwater streams upstream of known sources of anthropogenic pollution, and ii) evaluate the impacts of small towns and their WWTP discharges on concentrations of mixtures of EDCs in rivers.

2. Methods

2.1. Collection of samples

One POCIS-Pest and one POCIS-Pharm (Exposmeter AB, Sweden) sampler were deployed at each location. Study locations were upstream and downstream of seven municipal WWTPs, which were situated on small rivers and streams in relatively unpolluted areas of the Czech Republic (Fig. 1). Upstream (US) POCIS were placed from 2 to 5 km upstream of WWTPs in highland forest areas with minimal anthropogenic impact, while downstream (DS) sites were within 150 to 250 m of WWTP effluents. The towns studied, Králíky, Jilemnice, Cvikov, Tachov, Volary, Vimperk and Prachatice, are the upstream-

Fig. 1. Location of the sampling sites on small rivers in the Czech Republic: 1 — River Tichá Orlice near town Králíky; 2 — Stream Roudnický potok (upstream) and Jizerka river (downstream) near town Jilemnice; 3 — Stream Boberský potok near town Cvikov; 4 — River Mže near town Tachov; 5 — River Volyňka near town Vimperk; 6 — Stream Volarský potok near town Volary; 7 — Stream Živný potok near town Prachatice.

most sources of anthropogenic pollution on the assessed rivers/streams. These rivers/streams have natural or seminatural habitats flowing mostly through woodlands but there are agricultural fields or pastures in close proximity (0.2–3 km) to most of the towns. All WWTPs applied mechanical–biological treatment with activated sludge and Cvikov WWTP had an additional stabilizing pond (1.4 ha). All locations were sampled in June 2008, except for Prachatice, which was sampled in January 2008. Duration of deployment of samplers was 2 to 3 weeks. Duration of deployment should be within the linear uptake period for most HpOCs. Characteristics of WWTPs and river/stream conditions are summarized (Table 1).

2.2. Extraction of POCIS

After collection of POCIS, all samples (entire POCIS) were stored at −18 °C until analysis. The exposed POCIS was disassembled; the sorbent was transferred to the glass gravity flow chromatographic column with glass wool plug and analytes were eluted by the appropriate solvent mixture. Methanol was used as the eluent for POCIS-Pharm and a mixture of dichlormethane: methanol: toluene (8:1:1) was used for POCIS-Pest. The eluate was then evaporated to a small volume, the solvent was changed to methanol and the sample volume was adjusted to 2 mL for chemical analyses. Hexane, dichloromethane, acetone, toluene (all in Suprasolv purity), water and methanol (Hypergrade for LC/MS) were purchased from Merck (Darmstadt, Germany). The aliquots of extracts were further concentrated four-fold under a gentle stream of nitrogen to decrease the LOD for *in vitro* assays. The process blank samples were prepared following sample preparation procedure of both POCIS types and they were analyzed together with the other samples.

2.3. Bioassays

Four individual bioassays were used to determine overall cytotoxicity, anti/estrogenicity, anti/androgenicity and dioxin-like potencies of extracts of POCIS-Pest and POCIS-Pharm samplers. The reporter gene assays employed mammalian cell lines MVLN and H4IIE-*luc* and two types of recombinant *Saccharomyces cerevisiae*. MVLN are human breast carcinoma cells stably transfected with luciferase gene under the control of estrogen receptor, which were used for the assessment of cytotoxicity and anti/estrogenicity. Cytotoxicity of the samples was also investigated by recombinant strain of *S. cerevisiae* which expresses genes for enzyme luciferase under standard conditions (Leskinen et al., 2005). The potency of POCIS extracts to modulate androgen receptor-mediated responses was examined by use of recombinant *S. cerevisiae* that were modified to express human androgen receptor along with firefly luciferase under transcriptional control of androgen-responsive element (Michelini et al., 2005). H4IIE-*luc* are rat hepato-carcinoma cells stably transfected with the luciferase gene under control of Aryl hydrocarbon receptor (AhR) and they were used for the assessment of dioxin-like activity (Sanderson et al., 1996). At least two independent experiments were conducted in each bioassay for each exposure variant. All dilutions of POCIS extracts or controls were tested at least in triplicate.

Cytotoxicity of the samples can bias the results of the bioassays, therefore viability of cells was assessed several ways: Viability of MVLN cells was determined by use of the Neutral Red (NR) test where the NR dye is incorporated in the lysosomes of living cells and the uptake of NR is proportional to the number of viable cells. For cytotoxicity testing by NR-test, MVLN cells were seeded at a density of 25 000 cells/well in 96-well microplate ViewPlates™ (Packard, Meriden, CT, USA) and incubated for 24 h at 37 °C under atmosphere enriched with 5% CO_2. During this period cells were grown in DMEM-F12 without phenol red (Sigma Aldrich, USA) containing 10% foetal calf serum previously treated with dextran-coated charcoal to reduce concentrations of natural steroids in the serum. After 24 h, cells were exposed to dilutions of extracts from POCIS and solvent control (methanol, 0.5% v/v). Cytotoxicity was determined after 24 h of exposure, when NR (Sigma-Aldrich, Czech Republic) was added to the exposure medium in microplates to make a final concentration of 0.5 mg/mL. Cells were then incubated for 1 h at 37 °C. Afterwards, the cells were washed twice with phosphate buffered saline and lysed in the presence of acetic acid–ethanol solution (25:25:0.5; ethanol:water:acetic acid) for 15 min on a shaker. Finally, NR uptake was determined spectrophotometrically (Power Wave, BioTek, USA) at 570 nm. Absorbance was related to the response of the solvent control and the percentage of cytotoxicity of each sample dilution (viability of the cells exposed to the sample dilution relative to viability of cells exposed to solvent control (considered as 100%)) was determined. For the other way of assessing the viability, the recombinant strain of *S. cerevisiae* which expresses genes for enzyme luciferase under standard conditions (Leskinen et al., 2005) was used. In the presence of cytotoxic substances in the medium, luminescent light, produced normally by interaction between luciferase and added substrate luciferin, is less. When reaching a linear phase of growth, yeast were seeded into 96-well culture ViewPlates™ (Packard, Meriden, CT, USA) and exposed to vehicle, dilutions of POCIS extracts or to medium alone. Yeast cells were incubated for 2.5 h at 30 °C and then the signal was detected after addition of D-luciferin substrate. Detected luminescence was used to express the percentage of cytotoxicity caused by each sample dilution, as determined by the viability of the cells exposed to sample dilution relative to viability of cells exposed to solvent control, which was assigned a value of 100%.

Exposure for the determination of the anti/estrogenic potency of extracts in MVLN cells was conducted the same way as for the NR cytotoxicity evaluation described above with the following difference: cells were exposed to dilutions of POCIS extracts, calibration of the reference estrogen E2 (dilution series 10^{-12}–0.5×10^{-9} M E2, Sigma-Aldrich, Czech Republic) and solvent control (methanol, 0.5% v/v). After 24 h of exposure, the intensity of luminescence was measured

Table 1
Description of sampling sites, river parameters and sampling dates and duration.

Site no.	Name of town	Inhabitants no.	Name of recipient river(stream)	Effluent %[a]	River Q355 [m^3/s]	River flow velocity [m/s]	Sampling duration [day]	Date of sampling[b]
1	Králíky	4800	Tichá Orlice	20%	0.07	0.23	16	26 May–11 June
2	Jilemnice	6000	Roudnický potok (US)/Jizerka (DS)[c]	5%	0.02	0.08 (US) 0.02 (DS)	16	26 May–11 June
3	Cvikov	1900	Boberský potok	10%	0.08	0.13	21	21 May–11 June
4	Tachov	13000	Mže	15%	0.40	0.17	22	21 May–12 June
5	Vimperk	7650	Volyňka	4%	0.11	0.06	21	22 May–12 June
6	Volary	4000	Volarský potok	5%	0.07	0.12	21	22 May–12 June
7	Prachatice	13000	Živný potok	30%	0.15	0.17	23/16[d]	7/14[d]–30 January

[a] Average contribution of WWTP effluent to the recipient.
[b] All samples were taken in 2008.
[c] US = upstream site, DS = downstream site.
[d] US POCIS-Pest and both DS POCISes have been exposed for 23 days while US POCIS-Pharm for 16 days.

using Promega Steady Glo Kit (Promega, Mannheim, Germany). After subtraction of the response of the solvent control, luminescence in the estrogenicity assay was related to the maximal response of standard ligand (E2max for estrogenicity) and converted to percentages of E2max. Maximal induction as well as the shape of the curve differed among samples, thus equal efficacy or parallelism of the dose–response curves could not be assumed (Villeneuve et al., 2000). To avoid any predictions beyond the measured responses with all samples and to estimate the estrogenic equivalents (EEq) in the samples (expressed in ng E2/POCIS) the EEq_{20} estimate based on the 20% E2max response was reported, since most of the active samples did not reach the 50% E2max. EEq_{20} values were based on relating the amount of E2 causing 20% of the E2max response (EC_{20}) to the amount of sample causing the same response determined from regression analysis (equivalent of amount of E2 per amount of sample). The EC values were calculated by nonlinear logarithmic regression of dose–response curve of calibration standard and samples in Graph Pad Prism (GraphPad Software, San Diego, USA). The anti/estrogenicity was assessed by simultaneous exposure of the sample extract and 17β-estradiol (33 pM E2).

Duration of sampling varied from 16 to 23 days at different locations. Based on the evidence from previous research that uptake of phenolic as well as steroidal estrogens is linear in terms of time and concentration up to at least 28 days (Alvarez et al., 2007; Arditsoglou and Voutsa, 2008), we present our results normalized to 20 days of deployment along with the primary data in Table 3. The normalization was performed to simplify the comparability of our results among different locations and also with other studies in discussion. The data are presented both these ways to demonstrate the possible influence of the somewhat different deployment periods of the samplers on the results and their interpretation.

Concentrations of EEq in water were estimated by use of the sampling rate of E2 (0.09 L/day) previously determined by Matthiessen and Johnson (2007). It is important to stress, that these recalculated values represent approximate estimates of EEq concentrations in water and the values should not be considered as definite concentrations. This estimation will be further discussed in detail.

Concentrations of EEq in water were calculated (Eq. (1)).

$$C_w = C_{POCIS}/R_s t \quad (1)$$

where: C_w is the estimated concentration of EEq in water (ng/L), C_{POCIS} are concentrations of EEq in extracts from POCIS (ng/POCIS; primary not normalized values), R_s is sampling rate (L/day) of E2 previously determined by Matthiessen and Johnson (2007) and t is the sampling period (days).

As it was mentioned, anti/androgenity of POCIS extracts was determined by use of recombinant strain of *S. cerevisiae*. Plating and dosing were the same as for determination cytotoxicity of sample extracts in another strain of *S. cerevisiae* described above, but in this case, yeast cells were exposed not only to POCIS extracts and controls of pure medium and vehicle but also to dilutions of standard (testosterone in a range from 10^{-11} to 10^{-6} M, Sigma-Aldrich, Czech Republic).

The H4IIE-*luc* model was used for analysis of dioxin-like activity of the samples (Sanderson et al., 1996). Cells were seeded at a density of 15 000 per well in 96-well microplate ViewPlates™ (Packard, Meriden, CT, USA) and incubated for 24 h under 5% CO_2 at 37 °C, in DMEM-F12 medium with phenol red (Sigma Aldrich, USA) containing 10% foetal calf serum. After 24 h, cells were exposed to the reference compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, with a dilution series of 10^{-12}–0.5×10^{-9} M, Ultra Scientific, USA), or dilutions of POCIS extracts and solvent control (methanol, 0.5% v/v). After 24 h of exposure, the intensity of luminescence was measured using Promega Steady Glo Kit (Promega, Mannheim, Germany). Results from the H4IIE-*luc in vitro* assay were analyzed by the same approach as described for the determination of the EEq above. Presented TEq_{bio} are expressed in ng of TCDD per POCIS. TEq_{bio} values were based on EC_{20} values because most samples did not reach greater EC responses.

For each bioassay the limit of detection was determined as the lowest observable effect concentration of standard chemical divided by the greatest non-cytotoxic extract concentration expressed as POCIS equivalent.

Table 2
List of pesticides and pharmaceuticals analyzed in extracts from both POCIS-Pest and POCIS-Pharm and list of perfluorinated organic compounds analyzed in extracts from POCIS-Pest.

Pharmaceuticals	Pesticides		Perfluorinated organics
Carbamazepine	2,4,5-T	MCPA	Perfluoro-1-hexanesulfonate
Cephalexin	2,4-D	MCPP_MECOPROP	2H-perfluoro-2-octenoic acid
Ciprofloxacin	Acetochlor	Metalaxyl	Perfluoro-1-octanesulfonamide
Diaveridine	Alachlor	Metamitron	N-methylperfluoro-1-octanesulfonamide
Diclofenac	Atrazine	Methabenzthiazuron	Perfluorooctanoic acid
Enrofloxacin	Atrazine desethyl	Methamidophos	Perfluorooctane sulfonic acid
Erythromycin	Azoxystrobin	Methidathion	Perfluorononanoic acid
Metronidazole	Bentazone	Metobromuron	
Norfloxacin	Bromacil	Metolachlor	
Ofloxacin	Carbofuran	Metoxuron	
Sulfachloropyridazine	Cyanazine	Metribuzin	
Sulfamethazine	Desmetryn	Monolinuron	
Sulfamethoxazole	Diazinon	Nicosulfuron	
Sulfamethoxypyridazine	Dichlobenil	Phorate	
Sulfapyridine	Dichlorprop	Phosalone	
Trimethoprim	Dimethoate	Phosphamidon	
	Diuron	Prometryn	
	Fenarimol	Propiconazole	
	Fenhexamid	Propyzamide	
	Fipronil	Pyridate	
	Fluazifop-p-butyl	Rimsulfuron	
	Hexazinone	Simazine	
	Chlorbromuron	Tebuconazole	
	Chlorotoluron	Terbuthylazine	
	Imazethapyr	Terbutryn	
	Isoproturon	Thifensulfuron-methyl	
	Kresoxim-methyl	Thiophanate-methyl	
	Linuron	Tri-allate	

2.4. LC/MS/MS analyses

Chemicals such as natrium sulfate, silicagel, methanol *etc.* were purchased from Merck (Darmstadt, Germany). ^{13}C labeled and native perfluorinated compounds were purchased from Wellington Laboratories. ^{13}C labeled Simazine, Sulfamethoxazol, 2.4D and Ciprofloxacin were purchased from Cambridge Isotope Laboratories. Native compounds were purchased from Dr. Ehrenstorfer, AccuStandards and Absolute Standards. All of the standards were purchased from Labicom ltd. (Olomouc, Czech Republic). A list of analyzed compounds is given in Table 2.

A cocktail of internal standards was spiked into each POCIS extract (100 μL of the standard mixture in water was added to 100 μL of POCIS extract). Chemicals were identified and quantified by use of LC/MS/MS. Analyses were performed using three different LC/MS/MS methods.

Chemicals in POCIS extracts were quantified by use of internal standards. A subsample (20 μL for pesticide and 10 μL for pharmaceuticals) was injected onto an analytical column (Phenomenex C18 Aqua, 2 mm × 50 mm, 5 μm particles). The HTS PAL (CTC) autosampler, Rheos2000 (Flux) quaternary pump and TSQ Quantum AccessTM (ThermoScientific, USA) triple quadrupole tandem mass spectrometer were used for analyses of polar pesticides, pharmaceuticals and perfluorinated compounds. Two MS/MS transitions were monitored (where possible) for native analytes to confirm identity. An agreement of results obtained from both transitions better than 30% was accepted as a confirmed result. Isotope dilution and internal standard methods were used for the quantification of target compounds. Quantification limits (LOQs) of analytes were calculated the same way as concentration but peak area corresponding to instrument LOQ was used instead of peak area found in sample. Thus, LOQs are adjusted to internal standards.

Most detected compounds have been shown to be in the linear uptake phase for at least 23 days (the maximal deployment period in our study) (Alvarez et al., 2007). Thus, we present concentrations of those compounds normalized to 20 days of deployment to enable more precise interpretation of our results across different locations and also better comparability with other studies in discussion.

2.5. Statistical analysis

Due to violations of the assumptions of parametric statistical testing, differences between results of the two applied cytotoxicity detection systems as well as between potencies of POCIS-Pest and POCIS-Pharm extracts to induce nonspecific cytotoxicity and act through specific modes of action were evaluated by nonparametric Wilcoxon Matched Pairs test. The same test was applied to assess differences between concentrations of pollutants detected in POCIS-Pest and Pharm extracts. The nonparametric Spearman rank correlation was used to assess the similarity of the potential of POCIS-Pest and Pharm extracts to act through specific modes of action. All statistical analyses were performed with Statistica for Windows® 9.0 (StatSoft, Tulsa, OK, USA), the tests were considered significant at $p<0.05$.

3. Results

There was no response above detection limits observed for blanks in any of the bioassays. The limits of detection in blanks were 0.06 ng EEq/POCIS for estrogenity, 1.29 ng AEq/POCIS for androgenity and 0.03 ng TEqbio/POCIS for dioxin-like activity.

3.1. Cytotoxicity

Most tested concentrations of POCIS extract equivalents (0.00125%–0.25% POCIS/mL) were not cytotoxic to yeast or to MVLN cells. At the greatest tested POCIS extract equivalent concentration 0.5% POCIS extract/mL samples from some locations caused cytotoxicity of as much as 50% (Fig. 2). For both types of POCIS the cytotoxic effects were comparable or greater at DS locations than at US locations with a single exception where the POCIS-Pharm extract at location 5 exhibited greater cytotoxicity at the US location (Fig. 2B). However, the greater cytotoxicity observed DS of WWTPs compared to US was statistically significant only for extracts of POCIS-Pest measured by yeast test. In all other cases, including all extracts of POCIS-Pharm in both bioassays and POCIS-Pest in MVLN cells, the magnitude of differences in cytotoxicity was not statistically significant between US and DS.

Although the yeast test was significantly more sensitive to cytotoxicity of POCIS-Pharm extracts ($p=0.009$) than the MVLN test, the results of the two tests were comparable among POCIS extracts, with no significant difference between the results of the two tests with extracts of POCIS-Pest ($p=0.79$). The yeast test was also significantly more sensitive to POCIS-Pharm extracts than POCIS-Pest extracts ($p=0.01$), whereas there was no statistically significant difference between cytotoxicity of extracts of the two types of samplers in the MVLN test.

3.2. Anti/estrogenicity

Estrogenicity was detected in extracts of both types of POCIS and differences were observed between US and DS locations. No extract showed significant antiestrogenic activity (data not shown). Although samples from DS locations were more estrogenic than those from US locations at all sites, some EEq was detected also in most US samples (Table 3).

Because uptake of the more potent and also some less potent estrogens has previously been demonstrated to be time integrative for more than 25 days (*e.g.* Arditsoglou and Voutsa, 2008), here estrogenic potentials detected in extracts of POCIS are reported also as normalized to 20 days of POCIS deployment. However, differences between data obtained before and after the normalization to 20 days of POCIS deployment were negligible (Table 3).

Concentrations of EEq greater than the LOD (0.1 to 0.6 ng/POCIS) were observed in four out of seven US locations in both types of POCIS. The variation among LOD is caused by slightly different cytotoxicity of extracts. Detected concentrations of EEq in US samples ranged from 0.3 to 0.5 ng/$POCIS_{20\ days}$ in POCIS-Pest as well as in POCIS-Pharm extracts. Since there were no known anthropogenic impacts near US sites, the detected EEq concentrations can be considered as background.

Estrogenic equivalents in extracts from DS samples were greater than the LOD at all sites with the single exception of the POCIS-Pest extract at site 2. Concentrations ranged from 0.7 to 4.0 ng/$POCIS_{20\ days}$ for POCIS-Pest and from 0.5 to 4.2 ng/$POCIS_{20\ days}$ for POCIS-Pharm extracts. The greatest concentrations of EEq were observed at DS locations at sites 3 and 7 (Table 3). At site 3 DS samples contained more than 10-fold greater concentration of EEq than the US sample in the case of POCIS-Pest and more than 14-fold greater concentration of EEq than the US POCIS-Pharm. At site 7 DS samples contained more than 7-fold greater concentrations of EEQ than the US sample from POCIS-Pest and more than 5-fold greater concentration than the US sample from POCIS-Pharm, respectively.

Estrogenic potential of water was estimated (Eq. (1)). For US localities sampled by both types of POCIS the calculated water EEq concentrations detected above LOD varied from 0.1 to 0.3 ng/L. Estimated estrogenic potential in water in DS locations sampled by POCIS-Pest ranged from less than 0.4 to 2.2 ng EEq/L and for those sampled by POCIS-Pharm from 0.3 to 2.3 ng EEq/L (Table 3).

There were statistically significant correlations between estrogenic potentials of the pesticide and pharmaceutical POCIS extracts (Spearman rank 0.79, $N=7$, LOD values were replaced by value of 1/2 LOD), despite the discrepancy at the DS location at site 6. At DS location at site 6, repeated evaluation of estrogenic potential confirmed the difference of estrogenicity in extract of POCIS-Pharm compared to POCIS-Pest. The likeness of estrogenicity in extracts of POCIS-Pest and Pharm was also confirmed by nonparametric Wilcoxon Matched Pairs test, which indicated no significant difference between POCIS-Pest and Pharm ($p=0.81$).

3.3. Anti/androgenicity

There was no significant androgenic activity in any extract in the test with recombinant yeast assay (data not shown). Detection limit was 1.29 ng AEq/POCIS. None of the extracts has shown antiandrogenic activity (data not shown).

3.4. Dioxin-like activity

Dioxin-like activity was detected in most extracts. At US locations sampled by POCIS-Pest, concentrations exceeded the detection limit of 0.03 ng TEq_{bio}/POCIS in only two cases whereas extracts from the POCIS-Pharm sampler deployed at the same locations had detectable concentrations at six out of seven sites (Fig. 3). Concentrations of TEq_{bio} at US locations ranged from less than the LOD to 0.08 and to 0.22 ng TEq_{bio}/POCIS for extracts of POCIS-Pest and POCIS-Pharm, respectively. DS sites mostly showed greater concentrations of TEq_{bio} in extracts from POCIS-Pharm than from POCIS-Pest. Extracts from DS POCIS-Pest contained concentrations of TEq_{bio} that ranged from less than LOD of 0.08 to 0.26 ng TEq_{bio}/POCIS and from 0.08 to 0.39 ng TEq_{bio}/POCIS in extracts of POCIS-Pharm.

When considering all samples together, significantly greater concentrations of TEq_{bio} were observed in extracts of POCIS-Pharm than extracts of POCIS-Pest (Wilcoxon Matched Pairs test; $P=0.0029$). Nevertheless, similar patterns of greater concentrations of TEq_{bio} at DS locations with similar orders of magnitudes were observed in extracts of both types of POCIS. At most sites, concentrations of TEq_{bio} were greater DS of WWTPs (Fig. 3). Concentrations TEq_{bio} in extracts of DS POCIS-Pest at sites 4 and 7 were greater than those in extracts of POCIS-Pest from US, by 1.4- and 4.9-fold, respectively. Concentrations of TEq_{bio} in extracts of POCIS-Pharm at sites 1, 2 and 5 were approximately equivalent

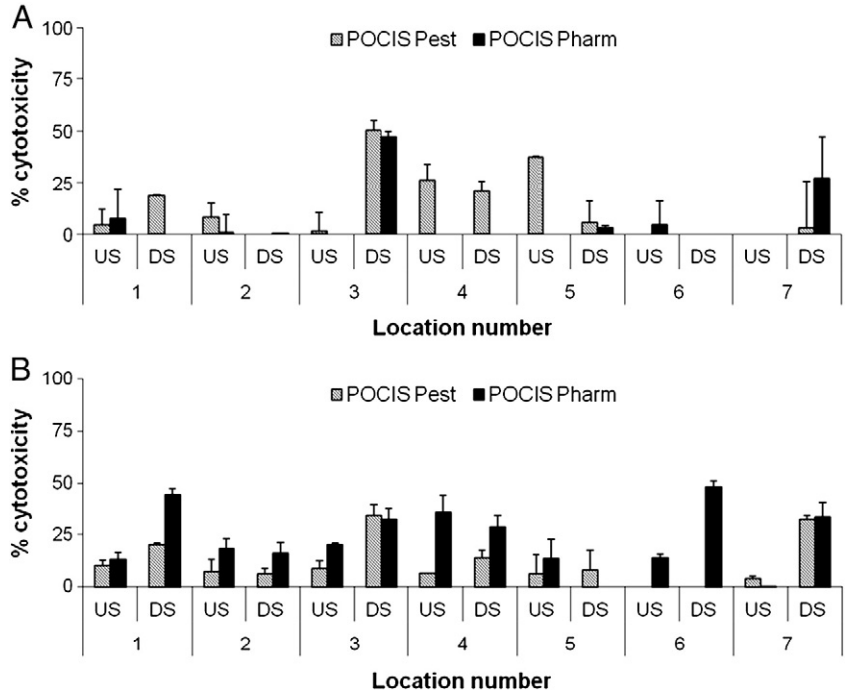

Fig. 2. Cytotoxicity of extracts (concentration of 0.5% POCIS/mL) from upstream (US) and downstream (DS) measured by the yeast screen (A) and by Neutral Red test with MVLN cells (B). Error bars show standard deviations. For samples without any cytotoxic effect, no values are presented.

for US and DS locations, whereas they were about 3-fold greater at the DS location of sites 3 and 4 and at least about 5-fold greater at the DS location at sites 6 and 7.

3.5. Chemical analyses

Although most of the selected chemicals that were monitored were not detected in extracts at concentrations greater than the LOQ (0.1 to 14 ng/POCIS), concentrations of several pharmaceuticals were greater at DS relative to US locations (Table 4). The greatest concentrations of pharmaceuticals were observed at the DS location of site 7. Pharmaceuticals found most frequently and also at the greatest concentrations were carbamazepine and diclofenac. Concentrations of carbamazepine ranged from less than the detection limit (2–8 ng/POCIS) to 9 ng/POCIS$_{20\ days}$ in extracts from US locations and from 13 to 339 ng/POCIS$_{20\ days}$ in extracts from DS locations. The concentrations of diclofenac ranged from less than the LOQ (2–8 ng/POCIS) to 31 ng/POCIS$_{20\ days}$ in extracts from US locations and from 18 to 409 ng/POCIS$_{20\ days}$ in extracts from DS locations. Concentrations in extracts of POCIS-Pest and POCIS-Pharm were comparable with a few exceptions, such as sulfapyridine at sites 3 and 4. Except pharmaceuticals presented in Table 4, a few other compounds — ofloxacin, norfloxacin, ciprofloxacin and erythromycin were detected above the detection limits (LOQ 0.6–14 ng/POCIS), all detected concentrations were lower than 100 ng/POCIS$_{20\ days}$.

Concentrations of most pesticides that were monitored were less than the LOQ (0.1–6.5 ng/POCIS). Most pesticides, which were quantifiable, were triazines, and their concentrations were generally small (<100 ng/POCIS$_{20\ days}$). Concentrations of all detected triazines, including atrazine, atrazine desethyl, hexazinone, simazine and terbuthylazine are summarized in Table 5. Besides triazines, acetochlor at a concentration of 1375 ng/POCIS$_{20\ days}$ was detected in one isolated POCIS-Pest sample from US location of site 2.

Beside the pharmaceuticals and pesticides, perfluorinated organic compounds (listed in Table 2) were also monitored in extracts of POCIS-Pest. However, concentrations greater than the LOQ of 0.21–1.15 ng/POCIS were observed only in a few cases

Table 3
Estrogenic activities in POCIS-Pest and POCIS-Pharm extracts measured by MVLN *in vitro* assay expressed as ng EEq/POCIS, normalized to sampling period of 20 days and recalculated (according to Eq. (1)) to approximate EEq water concentrations.

Site no.	US/DS[a]	POCIS depl.[b] (day)	EEq in POCIS extracts (ng/POCIS)		EEq in POCIS extracts normalized to 20 days of POCIS deployment (ng/POCIS$_{20\ days}$)		Estimated EEq in water derived from E2 R_s[c] and EEq of POCIS extract (ng/L)	
			POCIS Pest	POCIS Pharm	POCIS Pest	POCIS Pharm	POCIS Pest	POCIS Pharm
1	US	16	0.2 ± 0.01	<0.2	0.3	<0.3	0.1	<0.1
	DS		1.0 ± 0.1	0.7 ± 0.2	1.3	0.9	0.7	0.5
2	US	16	<0.3	<0.3	<0.4	<0.4	<0.2	<0.2
	DS		<0.3	0.7 ± 0.6	<0.4	0.8	<0.2	0.5
3	US	21	0.4 ± 0.3	0.3 ± 0.1	0.4	0.3	0.2	0.2
	DS		4.2 ± 1.5	4.3 ± 0.4	4.0	4.1	2.2	2.3
4	US	22	0.5 ± 0.2	0.3 ± 0.1	0.5	0.3	0.3	0.1
	DS		0.9 ± 0.2	0.5 ± 0.02	0.8	0.5	0.5	0.3
5	US	21	0.4 ± 0.1	0.5 ± 0.1	0.4	0.5	0.2	0.3
	DS		0.9 ± 0.6	1.0 ± 0.04	0.9	1.0	0.5	0.5
6	US	21	<0.3	<0.3	<0.3	<0.3	<0.2	<0.2
	DS		0.7 ± 0.7	2.3 ± 0.3	0.7	2.2	0.4	1.2
7	US	23/16[d]	<0.6	<0.6	<0.5	<0.8	<0.3	<0.4
	DS		4.5 ± 1.3	4.8 ± 1.0	3.9	4.2	2.2	2.3

[a] US = upstream site, DS = downstream site.
[b] Duration of POCIS deployment.
[c] R_s = sampling rate.
[d] US POCIS-Pest and both DS POCISes have been exposed for 23 days while US POCIS-Pharm for 16 days.

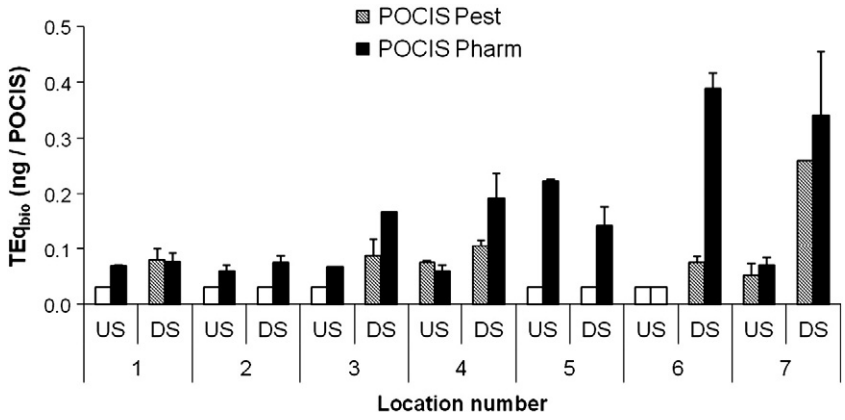

Fig. 3. Dioxin-like activity of upstream (US) and downstream (DS) POCIS-Pest and POCIS-Pharm extracts determined by H4IIE-*luc in vitro* assay. White columns indicate TEq_{bio} concentrations less than our detection limit (0.03 ng/POCIS); error bars show standard deviations.

and were less than 5 ng/POCIS with single exception of perfluorooctane sulfonic acid, which was detected at DS location 2 at concentration 36 ng/POCIS.

4. Discussion

Most previous studies assessing ED contamination of rivers focused on the influence of urbanized areas and larger WWTPs (Kinnberg, 2003), but there is less information on the impact of smaller sources on headwaters where better quality of water would be expected. Our study brings important information on the background levels of ED and HpOCs compounds and the influence of smaller towns without major industrial activities on headwaters pollution. Seven small rivers or streams were sampled by use of POCIS-Pest and POCIS-Pharm passive samplers US and DS of the most upstream sources of anthropogenic pollution, which were small towns with WWTP discharges.

Sampling rates for most compounds, which were investigated by use of POCIS in turbulent conditions, have been reported to range from 0.12 to 0.26 L/day (95%centile of published R_s; Alvarez et al., 2007; Arditsoglou and Voutsa, 2008; Harman et al., 2008; Macleod et al., 2007; Mazzella et al., 2007). This means that in 16 days, which is the minimal time of deployment of POCIS in the study, the results of which are reported here, the amount of the chemicals present in POCIS would be equivalent to 1.92–4.16 L of river water (0.12–0.26 L/day × 16 days). Thus, the least concentration causing cytotoxic effect — 0.5% POCIS/mL, would represent 9.6- to 20.8-fold concentrated river water. Therefore our results suggest little overall cytotoxicity of river water and weak impact of WWTPs onto this unspecific toxicity.

The results of the two systems used to detect cytotoxicity, yeast and mammalian cells, were similar with the exception of greater cytotoxicity of extracts of POCIS-Pharm in the yeast cells. This observation indicates greater sensitivity of the yeast model toward some chemicals that are more concentrated by POCIS-Pharm. Chemical analyses of POCIS-Pest and Pharm extracts did not reveal any significant differences in concentrations of monitored pollutants. However, it has been suggested that some pharmaceuticals have multiple functional groups, which have a tendency to strongly bind to the carbonaceous component of the triphasic adsorbent mixture contained in POCIS-Pest, which results in poor solvent extraction recoveries of some members of this class of compounds during sample processing (Alvarez et al., 2007). Our results demonstrating weak cytotoxicity correspond to another study of Alvarez et al. (2008), who used Microtox® assay to evaluate toxicity of POCIS from surface waters burdened by extensive agriculture. In that study, no extract from passive samplers (POCIS, SPMD) exposed for 29 to 65 days displayed acute toxicity.

Although the study, the results of which are reported here, was conducted in relatively unpolluted areas, some estrogenic activity was detected even at US locations (Table 3). Authors of some other studies had referred to detect concentrations of EEq in reference rivers. Nadzialek et al. (2010), who used active sampling and MCF-7 assay, found EEq concentrations at both tested reference sites in Belgium to be 0.01 and 0.03 ng/L. These concentrations are comparable with those estimated in our study (<0.1–0.3 ng EEq/L) especially if we consider our recalculated results as the worst case scenario. In contrast, Sellin et al. (2009), who used POCIS-Pharm and chemical analyses of their

Table 4
Results of the LC/MS/MS analyses — pharmaceuticals with greatest detected concentrations in extracts from POCIS-Pest and POCIS-Pharm ($ng/POCIS_{20\ days}$). Results are normalized to sampling period of 20 days.

Site no.	US/DS [a]	Sulfapyridine		Sulfamethoxazole		Trimethoprim		Carbamazepine		Diclofenac	
		POCIS Pest	POCIS Pharm	POCIS Pest	POCIS Pharm	POCIS Pest	POCIS Pharm	POCIS Pest	POCIS Pharm	POCIS Pest	POCIS Pharm
1	US	–	–	–	–	–	–	–	–	–	–
	DS	–	–	74	16	13	9	44	28	60	49
2	US	–	–	–	–	–	–	–	–	–	–
	DS	–	14	9	–	–	–	15	15	18	30
3	US	–	–	11	–	–	–	6	–	31	24
	DS	90	25	27	–	10	8	95	36	133	57
4	US	9	3	–	–	–	–	9	3	–	–
	DS	100	13	59	8	28	10	61	13	100	23
5	US	–	–	–	–	–	–	–	–	–	–
	DS	12	16	–	–	8	14	24	40	31	70
6	US	–	–	–	–	–	–	–	–	–	–
	DS	42	26	30	15	35	32	190	238	181	190
7	US	–	–	–	–	–	–	–	–	–	–
	DS	50	36	200	122	209	209	339	304	391	409

"–" less than LOQ (0.6–14 ng/POCIS).
[a] US = upstream site, DS = downstream site.

Table 5
Results of the LC/MS/MS analyses - concentrations of triazines (ng/POCIS$_{20\ days}$), which were the most frequently detected pesticides at tested sites. Results are normalized to sampling period of 20 days.

Site no.	US/DS[a]	Atrazine		Atrazine desethyl		Hexazinone		Simazine		Terbuthylazine	
		POCIS Pest	POCIS Pharm	POCIS Pest	POCIS Pharm	POCIS Pest	POCIS Pharm	POCIS Pest	POCIS Pharm	POCIS Pest	POCIS Pharm
1	US	–	–	–	–	5	7	–	–	15	21
	DS	14	14	8	6	–	–	–	–	2	3
2	US	8	12	18	19	1	–	4	5	1375	1875
	DS	4	7	5	5	4	3	1	1	475	713
3	US	7	7	8	3	32	19	5	4	2	1
	DS	24	11	17	5	49	20	8	4	3	1
4	US	2	3	8	5	6	5	–	–	2	2
	DS	5	2	11	3	8	3	1	–	4	3
5	US	8	7	13	7	18	12	–	–	2	2
	DS	5	11	7	9	12	16	–	1	2	3
6	US	–	–	–	–	1	–	–	–	1	1
	DS	21	31	25	22	20	18	–	1	6	6
7	US	2	2	16	13	9	9	1	2	–	–
	DS	14	11	25	18	10	9	2	1	2	1

"–" less than LOQ (0.1–6.5 ng/POCIS).
[a] US = upstream site, DS = downstream site.

extracts to monitor estrogens in rivers of Nebraska, reported calculated EEq concentrations above detection limit (1 ng/POCIS$_{7\ days}$) in 2 out of 3 reference sites and the concentrations (1.9 and 1.5 ng/POCIS$_{7\ days}$) were at least one order of magnitude greater than those found in our study. Matthiessen and Johnson (2007) evaluated, among others, estrogenic potential of 6 British headwaters with only few sources of estrogenic contamination (isolated houses with septic tanks). They used POCIS, which was previously calibrated in a laboratory study and yeast estrogen screen assay to evaluate estrogenic potential of the POCIS extracts. Their EEq concentrations ranged from less than the LOD (0.08 ng/L) to 1.4 ng/L with a median of 0.3 ng/L (except of 1 site with extremely great EEq value), which are slightly greater but comparable results to ours.

Greater estrogenic potential DS of WWTPs compared to US was detected at all sampled sites (Table 3). Comparable results were obtained by Vermeirssen et al. (2005), who monitored estrogens in POCIS Pest and Pharm extracts deployed US and DS of 5 municipal WWTPs in Switzerland. Four out of the five rivers were, according to earlier DS samples analyses, chosen as moderate to greatly estrogenic whereas one river as less estrogenic. The concentrations of EEq at the least burdened site were very similar to those obtained in our study (0.4 ng EEq/POCIS$_{22\ days}$ in extracts of both types of POCIS placed US and 1.9–2.0 ng EEq/POCIS$_{22\ days}$ in extract of POCIS-Pest and 1.7–1.9 ng EEq/POCIS$_{22\ days}$ of POCIS-Pharm situated DS of the WWTP). In contrast, the river with the greatest estrogenic pollution contained more than 20 ng EEq/POCIS$_{22\ days}$ in both POCIS extracts of US samples and comparable EEq concentrations in DS ones. Similar to our results most DS samples displayed increase of estrogenic activity compared to US ones. Greater concentrations of estrogens in all POCIS samplers deployed DS of municipal WWTPs of smaller towns compared to US sites were also found in Nebraska (Sellin et al., 2009). Those authors determined estrogenic equivalents analytically (based on known potential of steroidal estrogens to cause the effect) and the recalculated EEq concentrations were greater (up to 22.7 ng/POCIS$_{7\ days}$) than those detected by bioassays in our study. However, the greatest EEq concentrations were detected DS of WWTP with trickling filters technology which had been previously proved to be less effective in estrogens removal than activated sludge systems (Svenson et al., 2003) such as those in all WWTPs in our study.

Concentrations of EEq in POCIS extracts were converted to approximate concentrations of EEq in water by use of sampling rate of E2 because: i) in numerous studies steroidal estrogens have been identified to be responsible for most (often more than 90%) of estrogenic activity detected by *in vitro* assays in municipal waste waters effluents (*e.g.* Korner et al., 2001; Routledge et al., 1998) ii) compared to E1, Estriol (E3) and EE2, E2 has the least R_s (Arditsoglou and Voutsa, 2008), which enabled to estimate the worst case scenario (the greatest concentration) and iii) E2 is the standard reference compound used for EEq calculations. For estimating concentrations of EEq in water, R_s for E2 previously established for the same standardized POCIS configuration as used in our study was applied in calculation (0.09 L/day; Matthiessen and Johnson, 2007). From the rates of sampling for E2 given in the literature (Arditsoglou and Voutsa, 2008; Matthiessen and Johnson, 2007), the R_s calibrated at 10 °C was used because the temperature was similar to the conditions in the studied streams and rivers and the application of the lowest R_s value resulted in the worst case scenario estimate. Furthermore, application of the E2 sampling rate calibrated at 23.5 ± 0.5 °C by Arditsoglou and Voutsa (2008) would result in a range <0.1 to 1.8 ng/L EEq, which is similar to the currently presented results (Table 3). Rate of sampling can vary under different environmental conditions (*e.g.* diverse water flow rates, pH or temperature) but all the stations (with exception of location 7) were sampled at the same time eliminating thus at least partially variability. Moreover, the flow rates were always greater than 0.02 m/s and it has been demonstrated that under turbulent conditions sampling rates do not dramatically change as a function of flow velocity (Li et al., 2010). Another line of evidence, which supports the approach of EEq calculation applied in the study, is direct comparison of POCIS with grab samples as reported by Vermeirssen et al. (2005). Those authors measured estrogenic activity in both extracts of POCIS and grab samples and concentrations of EEq in extracts of POCIS were approximately 3-fold greater than the average concentrations of EEq in grab samples. These findings indicated the rate of sampling for estrogenic compounds is approximately 0.14 L/day. This experimentally established R_s is consistent with the results observed in this study where it was assumed that use of R_s for E2 could serve as an approximation to estimate concentrations of EEq in water and that these recalculated results represent a realistic estimate of the worst case scenario.

Even though the most estrogenic extracts came from POCIS exposed DS of Prachatice town (site 7), which has the most inhabitants and the largest proportion of WWTP effluent in relation to the recipient river (Table 1), these two parameters did not correlate with the estrogenic potentials in POCIS extracts from other sites. Other forces, for example different primary sources of estrogens or different WWTP capacity or technology, probably influenced the EEq concentrations in DS samples. Estrogenic activity detected in extracts of POCIS-Pest or POCIS-Pharm was similar, this observation is consistent with previous field as well as calibration studies (Arditsoglou and Voutsa, 2008; Vermeirssen et al., 2005).

Although dioxin-like compounds are usually investigated in less polar matrices such as SPMD or sediments, some recent studies (Dagnino et al., 2010; Reungoat et al., 2010) affirmed this activity also in water phase. In this study, dioxin-like activity was detected in both types of POCIS (0.05–0.39 ng TEq_{bio}/POCIS), even at several US locations. Sampling rates for known AhR active compounds and kinetic of their sampling has not been reported for POCIS yet. Therefore our results cannot be recalculated to water concentrations nor to unified number of days of their deployment. Dioxin-like activity has been traditionally connected with hydrophobic compounds such as polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) or polychlorinated biphenyls (PCBs). Since experimentally-determined values for log K_{ow} range from 6.1 to 8.2 for PCDD and PCDF congeners (Chrostowski and Foster, 1996) and from 4.66 up to 7.44 for PCB congeners, respectively (Zhou et al., 2005), these compounds are not expected to be sampled by POCIS. Our results suggest that less hydrophobic compounds like PAHs, which are also known to bind to AhR, or some unknown compounds might represent non-negligible part of dioxin-like activities in aquatic environment and this issue desires further research.

In this study concentrations of TEq_{bio} in extracts of POCIS-Pharm were approximately 2-fold greater than those in extracts of POCIS-Pest. Up to authors' knowledge, no other comparisons of concentrations of TEq_{bio} in extracts of POCIS-Pest and POCIS-Pharm have been published. However, since the same sorbent mass and membrane were used for both types of POCIS, it seems that different affinity of dioxin-like compounds to the POCIS-Pest *vs.* POCIS-Pharm sorbent might be responsible for the observed difference. Another reason could be the efficiency of extraction methods. However, the most potent and traditionally studied dioxin-like pollutants are hydrophobic substances and POCIS-Pest was extracted by less polar solvent than POCIS-Pharm.

Even though *in vitro* assays revealed some specific potencies of mixtures that might cause effects to the aquatic biota, chemical analyses of a wide range of compounds (Table 2) did not show significant contamination. The greatest effects were observed in estrogenic activity screening assay. However, steroidal estrogens, which have been shown to be responsible for most of the estrogen equivalents in waste waters (Desbrow et al., 1998), were not monitored in this study. Among detected chemicals, some triazines are known to be able to disturb endocrine system of organisms (Danzo, 1997; Vonier et al., 1996). In this study, triazines were detected at concentrations from less than 0.1 to 1875 ng/$POCIS_{20\ days}$ (Table 5) and their previously published sampling rates varied from 0.12 to 0.26 L/day (Alvarez et al., 2007; Mazzella et al., 2007). Estimated concentrations of triazines in water ranged from less than 0.02 ng/L to 781 ng/L, but these compounds are known to be effective at concentrations greater than mg/L (Danzo, 1997; Vonier et al., 1996) and thus their contribution to the responses detected by the *in vitro* systems can be considered negligible.

Concentrations of all monitored chemicals were small compared to the results of other studies (Arditsoglou and Voutsa, 2008; Soderstrom et al., 2009), which was in good agreement with our intention to sample relatively unpolluted areas. Despite the small concentrations of studied contaminants there were obviously increased concentrations of pharmaceuticals in DS samples. This was not so remarkable in case of pesticides. The reason of greater differences of pharmaceuticals concentrations in US and DS extract than pesticides might be the fact that pharmaceuticals are used only in human quarters or farms whereas pesticides are used also in areas distant from towns.

When considering the environmental significance of our results, some of the detected estrogenic equivalents concentrations had been reported to cause adverse effects. Authors of most studies, who observed estrogenic adverse effects on aquatic biota, reported EEq concentrations or corresponding concentrations of estrogens higher than those detected in our study (*e.g.* Sellin et al., 2009; Vermeirssen et al., 2005; Young et al., 2004). However, for example, Vethaak et al. (2005) found elevated levels of yolk protein vitellogenin in male bream (*Abramis brama*) in river with EEq levels determined by *in vitro* ER-CALUX assay as low as 0.17 ng/L. In that study, steroidal hormones were identified as the main contributors to the EEq (Vethaak et al., 2005). To authors' knowledge, the only estrogen, for which LOEC concentrations lower than 0.5 ng/L *in vivo* has been reported, was EE2 (Young et al., 2004). For example, Zha et al. (2008) demonstrated that the reproduction of the F-1 minnows was completely inhibited at EE2 concentration as low as 0.2 ng/L in a multigeneration study with Chinese rare minnows (*Gobiocypris rarus*). In our study, the upstream locations (with estimated EEqs <0.1–0.3 ng/L) were chosen as background sites without any grasslands or human settlements near the catchments and therefore we do not expect steroidal estrogens, particularly the synthetic EE2, to be responsible for the detected EEq. Contrariwise, at downstream locations with estimated EEq <0.2–2.3 ng/L, where municipal waste water effluents were considered as the main sources of estrogens, the presence of highly potent steroidal estrogens would be expected. The relative potency of any estrogens to E2 can differ for *in vitro* and *in vivo* studies (*e.g.* Johnson and Sumpter, 2001). The greatest difference has been reported for EE2. In the *in vitro* assay that we used (MVLN) the estrogenic potency of EE2 relative to E2 is 1.25 whereas in *in vivo* studies concerning production of yolk protein vitellogenin or alteration of ovarian somatic index in fish it has been reported to be approximately 25–30 (Gutendorf and Westendorf, 2001; Young et al., 2004). This indicates that the overall estrogenic equivalents for *in vivo* situation might be even greater that those derived from *in vitro* tests. As far as the authors know, there are no studies available on potential *in vivo* adverse effects in similar locations as examined in our study. Therefore it is not possible to reliably estimate the environmental significance of detected EEq yet.

The levels of vitellogenin in brown trout (*Salmo trutta fario* L.) from US and DS Prachatice (corresponding to our location 7) were investigated in September 2007 by researchers from Faculty of Fisheries and Protection of Waters, University of South Bohemia. There were significantly increased levels of vitellogenin in male brown trout captured downstream compared to the upstream site. The number of examined fish males was 6 at each US and DS location. The median plasma concentration were bellow detection limit of 10 ng/mL in male fish from upstream site and 3035 μg/mL in those from downstream site (Zlabek, personal communication). This corresponds with the results of our study, where the estrogenic activity was bellow detection limit in POCIS exposed upstream of Prachatice, while there were the greatest EEq among all sites in our study detected in POCIS from the Prachatice downstream site (2.3 ng/L). Thus, the increased EEq values from *in vitro* studies might indicate potential *in vivo* effects. Generally, the relevance of *in vitro* determined estrogenic equivalents for *in vivo* situation is a very important issue, which requires further research and which is also in focus of our further studies.

5. Conclusion

The study brought new information about concentrations of polar organic contaminants and endocrine-disruptive potential in relatively unpolluted rivers and about the influence of smaller towns on this type of contamination in affected headwaters. There was an obvious impact on all sites despite the fact that the towns are equipped with municipal WWTPs with advanced activated sludge systems of treatment. Increased exposure potential of estrogenic and dioxin-like compounds (determined by *in vitro* assays) downstream of the towns were demonstrated. Some of the detected estrogenic equivalents concentrations had been reported to cause adverse effects. The study also demonstrated the suitability of passive sampling combined with chemical analyses and *in vitro* bioassays to reveal these impacts.

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References

Aguayo S, Munoz MJ, de la Torre A, Roset J, de la Pena E, Carballo M. Identification of organic compounds and ecotoxicological assessment of sewage treatment plants (STP) effluents. Sci Total Environ 2004;328:69–81.

Alvarez DA, Huckins JN, Petty JD, Jones-Lepp T, Stuer-Lauridsen F, Getting DT, et al. Chapter 8 Tool for monitoring hydrophilic contaminants in water: polar organic chemical integrative sampler (POCIS). In: Greenwood R, Mills G, Vrana B, editors. Passive sampling techniques in environmental monitoring, vol. 48. Comprehensive analytical chemistry; 2007. p. 171–97.

Alvarez DA, Cranor WL, Perkins SD, Clark RC, Smith SB. Chemical and toxicologic assessment of organic contaminants in surface water using passive samplers. J Environ Qual 2008;37:1024–33.

Arditsoglou A, Voutsa D. Passive sampling of selected endocrine disrupting compounds using polar organic chemical integrative samplers. Environ Pollut 2008;156:316–24.

Bolong N, Ismail AF, Salim MR, Matsuura T. A review of the effects of emerging contaminants in wastewater and options for their removal. Desalination 2009;239:229–46.

Caliman FA, Gavrilescu M. Pharmaceuticals, personal care products and endocrine disrupting agents in the environment — a review. CLEAN—Soil Air Water 2009;37:277–303.

Chrostowski PC, Foster SA. A methodology for assessing congener-specific partitioning and plant uptake of dioxins and dioxin-like compounds. Chemosphere 1996;32:2285–304.

Dagnino S, Gomez E, Picot B, Cavailles V, Casellas C, Balaguer P, et al. Estrogenic and AhR activities in dissolved phase and suspended solids from wastewater treatment plants. Sci Total Environ 2010;408:2608–15.

Danzo BJ. Environmental xenobiotics may disrupt normal endocrine function by interfering with the binding of physiological ligands to steroid receptors and binding proteins. Environ Health Perspect 1997;105:294–301.

Desbrow C, Routledge EJ, Brighty GC, Sumpter JP, Waldock M. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and *in vitro* biological screening. Environ Sci Technol 1998;32:1549–58.

Gross-Sorokin MY, Roast SD, Brighty GC. Assessment of feminization of male fish in English rivers by the environment agency of England and Wales. Environ Health Perspect 2006;114:147–51.

Gutendorf B, Westendorf J. Comparison of an array of *in vitro* assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. Toxicology 2001;166:79–89.

Harman C, Tollefsen K-E, Bøyum O, Thomas K, Grung M. Uptake rates of alkylphenols, PAHs and carbazoles in semipermeable membrane devices (SPMDs) and polar organic chemical integrative samplers (POCIS). Chemosphere 2008;72:1510–6.

Jobling S, Tyler CR. Endocrine disruption in wild freshwater fish. Pure Appl Chem 2003;75:2219–34.

Johnson AC, Sumpter JP. Removal of endocrine-disrupting chemicals in activated sludge treatment works. Environ Sci Technol 2001;35:4697–703.

Kinnberg K. Evaluation of in vitro assays for determination of estrogenic activity in the environment. Copenhagen, Denmark: Danish Environmental Protection Agency; 2003.

Kirk LA, Tyler CR, Lye CM, Sumpter JP. Changes in estrogenic and androgenic activities at different stages of treatment in wastewater treatment works. Environ Toxicol Chem 2002;21:972–9.

Korner W, Bolz U, Sussmuth W, Hiller G, Schuller W, Hanf V, et al. Input/output balance of estrogenic active compounds in a major municipal sewage plant in Germany. Chemosphere 2000;40:1131–42.

Korner W, Spengler P, Bolz U, Schuller W, Hanf V, Metzger JW. Substances with estrogenic activity in effluents of sewage treatment plants in southwestern Germany. 2. Biological analysis. Environ Toxicol Chem 2001;20:2142–51.

Leskinen P, Michelini E, Picard D, Karp M, Virta M. Bioluminescent yeast assays for detecting estrogenic and androgenic activity in different matrices. Chemosphere 2005;61:259–66.

Leusch FDL, Chapman HF, Korner W, Gooneratne SR, Tremblay LA. Efficacy of an advanced sewage treatment plant in southeast Queensland, Australia, to remove estrogenic chemicals. Environ Sci Technol 2005;39:5781–6.

Leusch FDL, De Jager C, Levi Y, Lim R, Puijker L, Sacher F, et al. Comparison of five in vitro bioassays to measure estrogenic activity in environmental waters. Environ Sci Technol 2010;44:3853–60.

Li HX, Vermeirssen ELM, Helm PA, Metcalfe CD. Controlled field evaluation of water flow rate effects on sampling polar organic compounds using polar organic chemical integrative samplers. Environ Toxicol Chem 2010;29:2461–9.

Macleod SL, McClure EL, Wong CS. Laboratory calibration and field deployment of the polar organic chemical integrative sampler for pharmaceuticals and personal care products in wastewater and surface water. Environ Toxicol Chem 2007;26:2517–29.

Matthiessen P, Johnson I. Implications of research on endocrine disruption for the environmental risk assessment, regulation and monitoring of chemicals in the European Union. Environ Pollut 2007;146:9–18.

Mazzella N, Dubernet JF, Delmas F. Determination of kinetic and equilibrium regimes in the operation of polar organic chemical integrative samplers: application to the passive sampling of the polar herbicides in aquatic environments. J Chromatogr A 2007;1154:42–51.

Michelini E, Leskinen P, Virta M, Karp M, Roda A. A new recombinant cell-based bioluminescent assay for sensitive androgen-like compound detection. Biosens Bioelectron 2005;20:2261–7.

Murk AJ, Legler J, van Lipzig MMH, Meerman JHN, Belfroid AC, Spenkelink A, et al. Detection of estrogenic potency in wastewater and surface water with three *in vitro* bioassays. Environ Toxicol Chem 2002;21:16–23.

Nadzialek S, Vanparys C, Van der Heiden E, Michaux C, Brose F, Scippo M-L, et al. Understanding the gap between the estrogenicity of an effluent and its real impact into the wild. Sci Total Environ 2010;408:812–21.

Reungoat J, Macova M, Escher BI, Carswell S, Mueller JF, Keller J. Removal of micropollutants and reduction of biological activity in a full scale reclamation plant using ozonation and activated carbon filtration. Water Res 2010;44:625–37.

Routledge EJ, Sheahan D, Desbrow C, Brighty GC, Waldock M, Sumpter JP. Identification of estrogenic chemicals in STW effluent. 2. *In vivo* responses in trout and roach. Environ Sci Technol 1998;32:1559–65.

Sanderson JT, Aarts J, Brouwer A, Froese KL, Denison MS, Giesy JP. Comparison of Ah receptor-mediated luciferase and ethoxyresorufin-O-deethylase induction in H4IIE cells: implications for their use as bioanalytical tools for the detection of polyhalogenated aromatic hydrocarbons. Toxicol Appl Pharmacol 1996;137:316–25.

Sellin MK, Snow DD, Akerly DL, Kolok AS. Estrogenic compounds downstream from three small cities in eastern Nebraska: occurrence and biological effect. J Am Water Resour Assoc 2009;45:14–21.

Soderstrom H, Lindberg RH, Fick J. Strategies for monitoring the emerging polar organic contaminants in water with emphasis on integrative passive sampling. J Chromatogr A 2009;1216:623–30.

Suzuki T, Kitamura S, Khota R, Sugihara K, Fujimoto N, Ohta S. Estrogenic and antiandrogenic activities of 17 benzophenone derivatives used as UV stabilizers and sunscreens. Toxicol Appl Pharmacol 2005;203:9-17.

Svenson A, Allard AS. Occurrence and some properties of the androgenic activity in municipal sewage effluents. J Environ Sci Health A Tox Hazard Subst Environ Eng 2004;39:693–701.

Svenson A, Allard AS, Ek M. Removal of estrogenicity in Swedish municipal sewage treatment plants. Water Res 2003;37:4433–43.

Tyler CR, Jobling S. Roach, sex, and gender-bending chemicals: the feminization of wild fish in English rivers. Bioscience 2008;58:1051–9.

Vermeirssen ELM, Korner O, Schonenberger R, Suter MJF, Burkhardt-Holm P. Characterization of environmental estrogens in river water using a three pronged approach: active and passive water sampling and the analysis of accumulated estrogens in the bile of caged fish. Environ Sci Technol 2005;39:8191–8.

Vethaak AD, Lahr J, Schrap SM, Belfroid AC, Rijs GBJ, Gerritsen A, et al. An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands. Chemosphere 2005;59:511–24.

Villeneuve DL, Blankenship AL, Giesy JP. Derivation and application of relative potency estimates based on in vitro bioassay results. Environ Toxicol Chem 2000;19:2835–43.

Vonier PM, Crain DA, McLachlan JA, Guillette LJ, Arnold SF. Interaction of environmental chemicals with the estrogen and progesterone receptors from the oviduct of the American alligator. Environ Health Perspect 1996;104:1318–22.

Young WF, Whitehouse P, Johnson I, Sorokin N. Proposed predicted-no-effect-concentrations (PNECs) for natural and synthetic steroid oestrogens in surface waters. Technical Report P2-T04/1, Environment Agency, Bristol; 2004.

Zha JM, Sun LW, Zhou YQ, Spear PA, Ma M, Wang ZJ. Assessment of 17 alpha-ethinylestradiol effects and underlying mechanisms in a continuous, multigeneration exposure of the Chinese rare minnow (*Gobiocypris rarus*). Toxicol Appl Pharmacol 2008;226:298–308.

Zhou W, Zhai Z, Wang Z, Wang L. Estimation of n-octanol/water partition coefficients (K_{ow}) of all PCB congeners by density functional theory. J Mol Struct Thoechem 2005;755:137–45.

Článek XIV:

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Estrogen-, androgen- and aryl hydrocarbon receptor mediated activities in passive and composite samples from municipal waste and surface waters

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ABSTRACT

Passive and composite sampling in combination with *in vitro* bioassays and identification and quantification of individual chemicals were applied to characterize pollution by compounds with several specific modes of action in urban area in the basin of two rivers, with 400,000 inhabitants and a variety of industrial activities. Two types of passive samplers, semipermeable membrane devices (SPMD) for hydrophobic contaminants and polar organic chemical integrative samplers (POCIS) for polar compounds such as pesticides and pharmaceuticals, were used to sample wastewater treatment plant (WWTP) influent and effluent as well as rivers upstream and downstream of the urban complex and the WWTP. Compounds with endocrine disruptive potency were detected in river water and WWTP influent and effluent. Year-round, monthly assessment of waste waters by bioassays documented estrogenic, androgenic and dioxin-like potency as well as cytotoxicity in influent waters of the WWTP and allowed characterization of seasonal variability of these biological potentials in waste waters. The WWTP effectively removed cytotoxic compounds, xenoestrogens and xenoandrogens. There was significant variability in treatment efficiency of dioxin-like potency. The study indicates that the WWTP, despite its up-to-date technology, can contribute endocrine disrupting compounds to the river. Riverine samples exhibited dioxin-like, antiestrogenic and antiandrogenic potencies. The study design enabled characterization of effects of the urban complex and the WWTP on the river. Concentrations of PAHs and contaminants and specific biological potencies sampled by POCIS decreased as a function of distance from the city.

1. Introduction

There is increasing evidence that environmental contaminants have the potential to disrupt endocrine processes. This might result in adverse effects on reproduction, cause certain cancers, and other toxicities related to (sexual) differentiation, growth, and development (Giesy et al., 2000; Miles-Richardson et al., 1999; Sanderson and van den Berg, 2003; Snyder et al., 2000). A variety of pollutants that are found in surface and waste waters, such as organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), polychlorinated dioxins and furans (PCDD/Fs), polycyclic aromatic hydrocarbons (PAHs), alkylphenols, synthetic steroids, pesticides, pharmaceuticals and personal care products (PPCPs), but also natural products such as phytoestrogens, have been shown to elicit endocrine disruptive effects.

Sources of endocrine disrupting compounds (EDCs) are associated with larger urbanized and industrial areas. However, influences of smaller local sources can also be significant, especially where dilution is minimal (Jarosova et al., 2012). EDCs are also released to aquatic environments from both municipal and various industrial waste waters (Garcia-Reyero et al., 2004). Relative contributions of EDCs to surface waters depend on efficacies of sewage treatment systems, which is dependent on both capacity and technology of the wastewater treatment plant (WWTP). Potential risks of adverse effects of effluents from WWTPs to aquatic environments are influenced by volume of effluent, discharge of the receiving river, weather conditions and probably other factors that affect dissipation through dilution and/or degradation (Sumpter, 1995). Wastewater treatment plants receive mixtures of molecules from domestic, agricultural, and/or industrial wastes and

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thus waste waters can contain mixtures of many of the above listed pollutants and their degradation products (Alvarez et al., 2005). Despite intensive removal of xenobiotics by municipal WWTPs, which can range from 88 to >99% and 96 to >99% for xenoestrogens and xenoandrogens, respectively (Korner et al., 2000; Leusch et al., 2010; Murk et al., 2002; Svenson and Allard, 2004), they often do not remove all chemicals from the effluent. Moreover, during treatment some contaminants can be deconjugated to their more biologically active forms (Desbrow et al., 1998). Thus, most effluents still contain complex mixtures of molecules, including transformation products formed during treatment.

Adverse effects on endocrine function and/or reproductive health associated with exposure to effluents from WWTPs, which can persist several kilometers from the point of effluent entry (Harries et al., 1996), have been demonstrated in wild fish populations (Jobling et al., 1998) or fishes caged downstream from WWTPs (Snyder et al., 2004). Several studies combining the use of chemical analyses and *in vitro* assays have revealed steroid estrogens as the most potent endocrine disruptors in WWTP effluents with thresholds for adverse effects of a few ng/L (Korner et al., 2000; Matsui et al., 2000; Nakada et al., 2004; Routledge et al., 1998; Snyder et al., 2000). However, other EDCs can be effective in various landuse conditions (Sole et al., 2000) and special consideration should be paid to mixtures of pollutants. Also, more information is needed to assess the potential contribution from other sources than just the WWTPs.

Selection of an appropriate sampling approach is crucial to determining the presence of contaminants and assessment of their potential for effects on aquatic environment. Traditional grab samples represent the immediate situation, thus only those contaminants present at the time of sampling are characterized. Episodic events such as spills or stormwater runoff can be missed since contaminants can dissipate prior to the next sampling (Alvarez et al., 2005; Huckins et al., 1990, 1993). A more representative way to sample, that represents an integrated estimate of the time-averaged exposure is composite samples collected over time. But, even this type of extensive sampling represents isolated conditions over relatively short durations. This sort of intensive sampling program is resource-intensive, requiring sampling staff and/or special equipment, which cannot be easily employed at many sites, especially at locations where equipment might be at risk to vandalism.

An alternative protocol is passive sampling, which enables estimation of time-weighted concentrations of contaminants and sequesters residues from episodic events commonly not detected by use of intermitent grab sampling. Passive sampling requires minimal resources of both personnel and equipment. Passive samplers have no moving parts to fail and require no electricity to function. They can be placed out of sight to avoid vandalism. Passive sampling can be used in situations of variable water conditions and because they concentrate residues from water they can enable detection of ultra-trace, yet toxicologically relevant concentrations of contaminant mixtures over extended durations (Alvarez et al., 2004). Other advantages include relatively simple, single deployment as compared to collecting and processing multiple water samples, greater mass of chemical residues sequestered, and the ability to detect chemicals which dissipate quickly (Alvarez et al., 2005; Huckins et al., 1990). Passive sampling also eliminates the need for some tedious and time-consuming clean-up steps associated with other types of sample collection.

Semipermeable membrane devices (SPMDs) have been developed as *in situ*, integrating passive samplers for monitoring of trace-level, waterborne hydrophobic contaminants (Huckins et al., 1993) and have been used for effective sampling of multiple classes of chemicals, including PAHs, PCBs, OCPs, PCDD/Fs, alkylated phenols, moderately polar organophosphate insecticides, pyrethroid insecticides, neutral organometallic compounds, and certain heterocyclic aromatic compounds (Petty et al., 2000a). Since SPMDs can mimic accumulation by aquatic organisms that can bioconcentrate trace amounts of organic contaminants, SPMDs measure not only the presence, but also the bioavailability and bioconcentration potential of organic contaminants (Huckins et al., 1990; Petty et al., 2000b). Polar Organic Chemical Integrative Samplers (POCIS) sequester waterborne hydrophilic contaminants, such as polar pesticides, pharmaceuticals, ingredients from personal care and consumer products, natural and synthetic hormones (Alvarez et al., 2004, 2005; Petty et al., 2004). Depending on the sorbent used, POCIS can be modified for sampling of general hydrophilic contaminants or pharmaceuticals (Alvarez et al., 2005).

The aim of this study was characterization of the influence of the industrialized urban region of Brno, Czech Republic and its associated municipal WWTP on contamination of the Svratka and Svitava rivers by compounds with endocrine disruptive potency by joint use of bioassays, two types of passive samplers and identification and quantification of selected organic chemicals. One goal was to assess the year-round variability in endocrine disruptive potency of WWTP influent and effluent water and thus treatment efficiency for EDCs by collecting composite samples monthly. The second major goal was to determine the relative magnitude of contributions of the urban area and the WWTP on contamination of these two urban rivers by endocrine disruptive compounds that can modulate the arylhydrocarbon (AhR), estrogen (ER) and androgen (AR) receptors. A battery of *in vitro* bioassays was used to assess potencies of agonists of these three receptors. Two types of passive samplers, POCIS and SPMD, were used to collect integrated samples of hydrophobic and hydrophilic compounds and assess their potencies to interfere with the three receptors signalling.

2. Materials and methods

2.1. Sampling design

Samples were collected from the region around Brno, the second largest metropolitan district of the Czech Republic in Central Europe. The metropolitan region of Brno with more than 400,000 inhabitants is spread through the basin formed by the Svratka and Svitava Rivers. The city has a central wastewater treatment plant and a variety of industrial activities. The municipal WWTP treats wastewater conveyed by a system of sanitary sewers from the city of Brno and increasingly also by a system of pumping stations from its surroundings. The WWTP was recently reconstructed and enhanced to a capacity of 513,000 population equivalent with permissible volume of discharged wastewater of 4222 L/s. Waste water is subjected to primary (mechanical) treatment followed by biological stage of activation with pre-denitrification and anaerobic phosphorus removal (system of circulatory activation with change of anaerobic, anoxic and aerated zones). Excess activated sludge is then anaerobically stabilized (Brněnské vodárny a kanalizace, 2010; Ministry of the Environment, 2010).

The influent and effluent of the WWTP were sampled monthly from May 2007 until April 2008. In addition, SPMD and POCIS passive samplers were placed in the influent (*site 5*) and effluent (*site 6*) of the WWTP and at seven sites in the Svratka, Svitava and Bobrava Rivers at locations upstream and downstream of Brno and downstream of the WWTP effluent (Fig. 1). Passive samplers were deployed for 23 days and collected during October 2007. Sampling locations in the Svratka River were: *Knínicky* (*site 1*) upstream of the city of Brno (downstream of the dam of Brno reservoir) and a site downstream of Brno upstream of the confluence with the Svitava River (*Svratka before confluence*, *site 2*). Locations monitored in the Svitava River included *Bílovice* and *Svitavou* (*site 3*), a small town upstream of Brno, and another site downstream of Brno upstream of the confluence with the Svratka River (*Svitava before confluence*, *site 4*). Another sampling site was selected in the *Bobrava River* (*site 9*), which is a tributary affected mostly by agriculture that flows into the Svratka River downstream of the WWTP. Downstream of the WWTP and the confluence of the Bobrava and Svratka rivers samples were collected near a small town *Rajhradice (site 7)* and at *Zidlochovice* (*site 8*, approximately 20 km downstream from Brno).

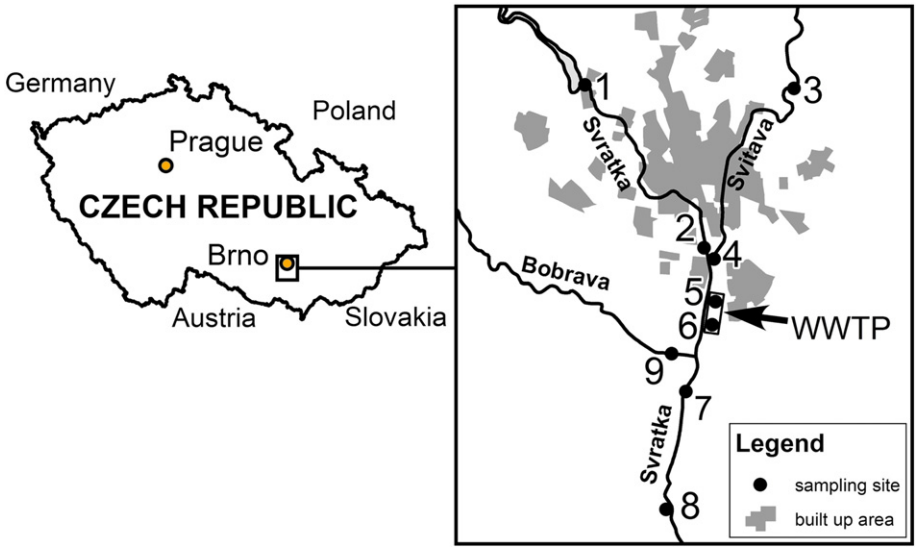

Fig. 1. Map of the Czech Republic showing locations of sampling sites in the vicinity of Brno. Sampling sites: 1—Svratka River, Knínicky, 2—Svratka River before confluence, 3—Svitava River, Bilovice nad Svitavou, 4—Svitava River before confluence, 5—WWTP Modrice, influent, 6—WWTP Modrice, effluent, 7—Svratka River, Rajhradice, 8—Svratka River, Zidlochovice, 9—Bobrava River.

2.2. Passsive water sampling and preparation of extracts

SPMD and POCIS disks were obtained from Exposmeter AB, Tavelsjo, Sweden. Prior to passive sampling, the sampling protocol was prepared with QA/QC. One POCIS was used for both chemical analysis and bioassay testing. Two SPMDs were used in duplicates for chemical analysis, one SPMD was used for toxicity assessment. SPMDs for chemical analysis contained performance reference compounds (PRC) used as onsite SPMDs calibration. Four deuterated PAHs ([$^2H_{10}$]acenaphthene, [$^2H_{10}$]fluorene, [$^2H_{10}$]phenanthrene, and [$^2H_{12}$]chrysene) and four $^{13}C_{12}$-labeled PCBs (PCB 3, 8, 37, and 54) were used as PRCs. Transport, field and laboratory blanks were used. A standard sampling arrangement was used as described in Grabic et al. (2010). It consists of a combination of POCIS and SPMDs mounted on commercially available stainless steel holders in protective deployment canisters made of perforated stainless steel plates. These samplers were suspended at 0.5–1 m depth of the water column in cryptic locations to minimize vandalism. After exposure for 23 days, samplers were recovered, cleaned and sealed in airtight, metal cans and placed on ice in a cooler for transport to the laboratory. Membranes were stored in sealed cans in a freezer at −18 °C until analysis. Before analysis SPMDs were cleaned and dialyzed with hexane in accordance with previously published methods (Ellis et al., 1995). Combined dialysates were adjusted to a volume of 10 mL. Chemical residues sampled by POCIS were recovered from the sorbent by organic solvent elution with a combination of methanol:toluene:dichloromethane (1:1:8, v/v/v). Volumes of all extracts were reduced by rotary evaporation and under a gentle stream of nitrogen, then solvent was exchanged to methanol (Alvarez et al., 2005). The final equivalent concentrations were 1 sampler/mL. A portion of each extract was transferred into DMSO for testing in bioassays.

2.3. Processing of waste water

Samples of influent and effluent were collected from the municipal WWTP on the Svratka River, downstream of Brno, once a month for 12 months. Water was collected every 2 h and composited over a 24-h period. Samples of influent were prefiltered through glass wool and 47 mm diameter glass fiber filter with 2.7 μm pores (Filap, Czech Republic) and both influent and effluent samples were filtered through glass fiber filters (1 μm pores, Whatman, Sigma-Aldrich, Czech Republic) to prevent solid phase extraction (SPE) cartridges from clogging during later extraction. Filters were extracted and tested separately to ensure that no compounds with significant potency in any of the assays were removed by filtration. Organic compounds in filtrates were extracted within 24 h by SPE by use of Oasis HLB cartridges (Waters, Czech Republic). Cartridges were activated by methanol and equilibrated by water according to producer instructions. After samples had passed through cartridges, they were dried by air for 10–15 min and eluted by use of 15 mL methanol. Extracts were rotary evaporated to reduce the volume to approximately 2 mL and then evaporated in a gentle stream of nitrogen to final volumes of 1 mL.

2.4. Instrumental analyses

Organic extracts of SPMD and POCIS samplers were analyzed for wide range of organic compounds. Samples were analyzed in accordance with standard EN ISO/IEC 17025. Detailed analytical procedures were described in Grabic et al. (2010). A set of internal standards was used in the analyses. These included carbon $^{13}C_{12}$-labeled PCBs (3, 15, 31, 52, 118, 153, 180, 194, 206, 209), TCS, PFOC (perfluorooctanesulfonic acid [PFOS], perfluoro-nonanoic acid [PFNA], perfluoro-octanoid acid [PFOA]), and native standards purchased from Wellington Laboratories (Canada). ^{13}C-labeled OCPs (γ-HCH and DDE), PAH ($^{13}C_{2-6}$-labeled PAHs U.S. Environmental Protection Agency [U.S. EPA] 16 PAH cocktail), and polar compounds (simazine, 2,4-D, sulfamethoxazol, ciprofloxacin) were purchased from Cambridge Isotope Laboratories (USA). The native ones were purchased from Dr. Ehrenstorfer, AccuStandards, and Absolute Standards via Labicom (Czech Republic). All solvents, including hexane, dichloromethane, acetone, toluene (SupraSolv purity), water, and methanol (hypergrade for LC/MS) were of the highest quality from Merck (Germany). Organic extracts of SPMDs were characterized by quantifying 16 US EPA polycyclic aromatic hydrocarbons (PAH): acenaphthene, acenaphthylene, anthracene, benzo[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*ghi*]perylene, benzo[*k*]fluoranthene, chrysene, dibenzo[*a*,*h*]anthracene, fluoranthene, fluorene, indeno(1,2,3-cd)pyrene, naphthalene, phenanthrene, and pyrene), polychlorinated biphenyls (PCBs): tri-, tetra-, penta-, hexa-, hepta-, octa-, nona-, and deca-congeners, organochlorine pesticides (OCPs): hexachlorbenzene, α-, β-, γ-, δ-stereoisomers of hexachlorohexane (HCH), two congeners of dichlorodiphenyltrichloroethane (DDT) and its degradation products,

dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD), triclosan (TCS) and its environmental transformation product methyl triclosan (MeTCS) and polybrominated diphenyl ethers (PBDEs), expressed as the sum of congeners. POCIS extracts were analyzed for polar pesticides, pharmaceuticals and perfluorinated compounds (PFCs), expressed as the sum of perfluoroorganic compounds (PFHxS, FHUEA, FOSA, N-MeFOSA, PFOA, PFOS, PFNA). A complete list of individual pesticides and pharmaceuticals analyzed in POCIS is attached in footnotes to Table 1. Gas chromatography/mass spectrometry (GC/MS) was used for identification and quantification of PAHs. PAHs with more rings that could not be analyzed by use of GC/MS were analyzed by use of high performance liquid chromatography with fluorescence detector (HPLC/FLD). Quantification of PCBs, OCPs, PBDEs, triclosan and its metabolite were performed by GC/MS-MS. Polar pesticides, pharmaceuticals and PFCs were identified and quantified by use of HPLC/MS-MS.

Limits of detection for identified groups of chemicals were as follows: PAHs 3 ng/SPMD, MeTCS/TCS 3 ng/SPMD, OCPs 0.2 ng/SPMD, PCBs 0.1 ng/SPMD, polar pesticides: 0.5–5 ng/POCIS, antibiotics: 1–2 ng/POCIS, other pharmaceuticals 5 ng/POCIS. Analytical procedure involved evaluation of recoveries of internal standards. Recoveries were within following ranges: PAHs: 80–100 %, MeTCS/TCS: 60–100 %, OCPs, PCBs: 60–100 %, polar pesticides, pharmaceuticals: 55–80 %. Both trip and analytical blanks were analyzed. Laboratory blanks were subtracted. Trip blanks contributed 0–5 % of the total exposure, therefore no subtraction was performed.

2.5. In vitro bioassays

Four transactivation reporter gene bioassays were used to assess receptor-mediated potencies of organic extracts of waters from the WWTP and passive samplers. All assays were conducted in 96 well microplates and included several dilutions of extracts in triplicate to provide a dose-response curve for each sample. All media and chemicals were purchased from Sigma-Aldrich (Czech Republic) unless otherwise specified.

2.5.1. AhR-mediated potency

AhR-mediated (dioxin-like) potency was determined by use of the H4IIE-*luc* bioassay, which is rat hepatoma cell line containing a luciferase reporter gene under control of dioxin-responsive enhancers (DRE) (Hilscherova et al., 2001; Sanderson et al., 1996; Villeneuve et al., 2002). H4IIE-*luc* cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (BioTech, Czech Republic) supplemented with 10% fetal calf serum Mycoplex (PAA, Austria). The H4IIE-*luc* cells were seeded in the culture medium at density of 15,000 cells/well and after 24 h exposed to samples, calibration reference or solvent control. Standard calibration was performed with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; Ultra Scientific, USA; dilution series 1–500 pM). After 24 h of exposure, intensity of luciferase luminescence corresponding to the receptor activation was measured by use of Promega Steady Glo Kit (Promega, USA).

2.5.2. ER-mediated potency

Estrogen receptor mediated potency was evaluated by use of the MVLN bioassay, a human breast carcinoma cell line transfected with the luciferase gene under control of estrogen receptor activation (Demirpence et al., 1993; Freyberger and Schmuck, 2005; Hilscherova et al., 2002). MVLN cells were cultured in medium DMEM/F12 supplemented with 10% fetal calf serum Mycoplex (PAA, Austria). MVLN cells were seeded at density of 20,000 cells/well in DMEM/F12 supplemented with 10% dialyzed fetal calf serum (PAA, Austria), which was additionally dextran/charcoal treated to further decrease background concentrations of hormones. Approximately 24 h after plating, cells were exposed to samples, calibration reference or solvent control in DMEM/F12. Standard calibration was performed with 17β-estradiol (E_2; dilution series 1–500 pM). Effects of extracts on MVLN were assessed either singly or in combination with competing

Table 1
The results of chemical analysis of passive samplers extracts. Ranges: the sum of detected compounds—the sum of detected compounds plus limit of detection for the nondetected compounds.

POCIS Sampling site	Pesticides[a]	Sulfonamides[b]	Other antibiotics[c]	Other pharmaceuticals[d]	PFCs
	ng/POCIS				
1	376–464	157–172	12–68	231–239	6–9
2	285–388	104–128	2–52	253–261	3–6
3	382–491	824–838	54–105	904–911	33–36
4	463–603	721–733	32–81	808–814	38–41
5	279–394	924–938	290–317	1242–1249	12–15
6	2726–2836	10,087–10,104	1534–1551	18,550–18,559	272–274
7	474–599	992–1004	120–157	1344–1350	29–32
8	342–441	889–903	98–138	1147–1154	21–24
9	613–723	926–938	51–108	1003–1009	10–12

SPMD Sampling site	PAHs	PCBs	OCPs	Triclosan	MeTriclosan	PBDEs
	ng/L	pg/L	pg/L	pg/L	pg/L	pg/L
1	40.8	408–438	809–825	431	168	16–27
2	52.9	724–734	831–845	190	155	8.8–14
3	38.2	2155–2168	737–747	360	812	21–28.2
4	40.8	1370–1373	718–720	247	642	13.7–16.8
5	2160	825–861	831–839	32,817	84.2	162
6	31.6	1440–1446	1183–1194	8747	24,365	136–140
7	36.2	1252–1259	775–782	1115	3197	27.6–30.2
8	28.6	1548–1567	1040–1044	1680	3344	30.3–37.4
9	51.2	507–526	684–701	554	867	10.3–19.2

[a] Pesticides: clopyralid, bentazone, bromoxynil, 2,4-D, MCPA, dichlorprop, mecoprop (MCPP), 2,4,5-T, imazethapyr, thifensulfuron-methyl, methamidophos, nicosulfuron, rimsulfuron, metamitron, dimethoat, atrazin_desethyl, metoxuron, phosphamidon, cyanazin, metribuzin, simazin, bromacil, carbofuran, hexazinon, thiophanate-methyl, monolinuron, chlorotoluron, isoproturon, metobromuron, atrazin, desmetryn, dichlobenil, methabenzthiazuron, diuron, methidathion, ethofumesat, azoxystrobin, linuron, terbuthylazine, chlorbromuron, propyzamide, prometryn, metolachlor, fenhexamid, fenarimol, acetochlor, terbutryn, fipronil, kresoxim-methyl, tebuconazole, diazinon, propiconazole, phorate, phosalone, fluazifop-p-butyl, tri-allate, pyridate, alachlor, metalaxyl.

[b] Sulfonamides: sulfapyridin, sulfamethazin, sulfamethoxypyridazin, sulfachloropyridazin, sulfamethoxazol.

[c] Other antibiotics: metronidazol, cefalexin, ofloxacin, norfloxacin, ciprofloxacin, enrofloxacin, erythromycin, trimetoprim.

[d] Other pharmaceuticals: diaveridin, carbamazepin, diclofenac.

endogenous ligand (33 pM 17β-estradiol)—given concentration is near its EC_{50} value. Exposure duration and final measurement was the same as in the case of H4IIE-*luc* bioassay described above.

2.5.3. AR-mediated potency

(Anti)androgenicity of passive samplers extracts was assessed in a bioassay with MDA-kb2 cells, a human breast carcinoma cell line stably transfected with luciferase reporter gene under control of functional endogenous androgen receptor (AR) and glucocorticoid receptor (GR) (Wilson et al., 2002). MDA-kb2 cells were cultured in L-15 Leibovitz medium supplemented with 10% fetal calf serum Mycoplex (PAA, Austria). MDA-kb2 were seeded at density of 50,000 cells/well and exposed after 24 h to samples, calibration reference or solvent control in L-15 Leibovitz medium supplemented with 10% dextran/charcoal treated dialyzed fetal calf serum. Standard calibration was performed with dihydrotestosterone (DHT; dilution series 1 pM–10 μM). In addition to androgenic effects, antiandrogenicity was assessed in combination with competing endogenous ligand (1 nM dihydrotestosterone). After 24 h of exposure, intensity of luciferase luminescence was measured with prepared luciferase reagent (Wilson et al., 2002).

Organic extracts of influent and effluent waters were assessed in a bioluminescent yeast assay based on recombinant *Saccharomyces cerevisiae* cells modified to express human androgen receptor along with firefly luciferase under transcriptional control of androgen-responsive element to detect compounds affecting AR-mediated hormonal signalling. The assay with the androgen-responsive yeast model was performed according to Leskinen et al. (2005). Yeast cells were seeded in 96-well microplates and exposed to reference testosterone (T; dilution series 1 pM–10 μM), the sample alone or in combination with testosterone (10 nM) to determine antiandrogenic effect. Yeast cells were incubated for 2.5 h and then the signal was detected after addition of D-luciferin substrate.

2.5.4. Cytotoxicity

Non-cytotoxic sample concentrations to be used in each bioassay with mammalian cell lines were determined by use of the neutral red uptake assay (Freyberger and Schmuck, 2005). Particular bioassays with individual cell lines were processed as previously described. At the end of the exposure period, neutral red solution (0.5 mg/mL of media) was added and cells were incubated for 1 h at 37 °C. Medium was removed and cells washed with PBS and lysed with 1% acetic acid in 50% ethanol. Absorbance was measured in a microplate spectrophotometer at 570 nm.

Yeast strain of recombinant *S. cerevisiae* constitutively expressing luciferase, which has shown greater sensitivity compared to the mammalian cells, was used for detailed cytotoxicity assessment (Leskinen et al., 2005; Michelini et al., 2005). Complete dose–responses relationships of cytotoxic effects for all samples were determined after 2.5 h exposure. The intensity of luciferase luminescence after addition of D-luciferin corresponded to the number of surviving cells (Leskinen et al., 2005).

2.6. Data analysis

Sample responses expressed as relative luminescence units were converted to percentage of maximum response of the standard curves (% TCDDmax/E_2max/DHTmax/Tmax). The response of the solvent control was substracted from both standard and sample responses prior to the conversion. EC values were calculated by nonlinear logarithmic regression of dose–response curves of calibration standards and samples (Graph Pad Prism, GraphPad® Software, San Diego, California, USA). Relative potencies expressed as TCDD equivalents (BIOTEQ)/E_2 equivalents (EEQ)/androgen equivalents (AEQ) were calculated by relating the EC_{50} value of standard calibration with the concentration of the tested sample inducing the same response (Villeneuve et al., 2000). Due to cytotoxicity, it was not possible to obtain complete dose–response curves in testing of waste water samples in the yeast assay. Thus, their AEQ values were calculated as point estimates because maximum detected luminescence induction at noncytotoxic concentrations did not exceed 15%.

Cytotoxicity, antiestrogenicity and antiandrogenicity corresponded to the decrease in detected luminescence/absorbance signal given by solvent control in case of cytotoxicity and specified amount of competing standard ligand for the other effects. IC_{50} values for antiestrogenicity and antiandrogenicity or IC_{20} values in cases that the effects did not cause 50% response, were calculated from dose–response curves expressed in percentage of signal of competitive concentration of added natural ligand (33 pM E_2, 1 nM DHT, 10 nM testosterone). For better clarity of the trends in graphs the values are expressed as an index of antiestrogenicity (AE) or antiandrogenicity (AA), which corresponds to reciprocal value of IC_{20} or IC_{50}. Similarly, the index of cytotoxicity was derived as the reciprocal value of IC_{20} or IC_{50} for the cytotoxic response.

2.7. Calculation of dissolved water concentrations from passive sampler data

Concentrations of target analytes in water were calculated from the mass absorbed by the SPMD, the *in situ* sampling rate of the compounds and their sampler–water partition coefficients using the kinetic uptake model by Huckins et al. (2006). Sampling rates of target compounds were estimated from dissipation of performance reference compounds (PRCs) from SPMDs during exposure using nonlinear least squares method by Booij and Smedes (2010), considering the fraction of individual PRCs that remain in the SPMD after the exposure as a continuous function of their partition coefficients, with sampling rate as an adjustable parameter. The necessary sampler–water partition coefficients values were estimated from the respective octanol/water partition coefficients according to Huckins et al. (2006).

For the purpose of comparison of toxic potencies of extracts from SPMDs from different sampling sites the measured toxic equivalent concentrations (TEQ) in extracts [ng/SPMD] were translated to water concentrations $C_{W\text{-}TEQ}$ [ng/L or pg/L] at the individual sites. Since physicochemical properties of the compounds that exhibit bioassay response in the extracts are not known, linear uptake was assumed (Eq. (1)).

$$C_{w-TEQ} = \frac{TEQ}{R_s t} \quad (1)$$

Where: R_S is the sampling rate and t is the exposure time. The necessary R_S values were obtained using the PRC model described above. Since R_S is only a weak function of hydrophobicity, values of R_S with a medium molecular mass (MW = 300) were applied in all calculations.

For POCIS data, no correction for the potential effect of environmental variables was performed and results were simply compared on the basis of toxic equivalent concentrations (TEQ) in sampler extracts [ng/POCIS]. It has been demostrated that water flow rate has a relatively minor influence on the accumulation of a number of pollutants including EDCs into POCIS (Li et al., 2010). Thus, it appears not necessary to adjust sampling rates for POCIS when they are deployed in areas where the water flows vary only slightly.

3. Results

3.1. Concentrations of individual residues

Greatest concentrations of polar pesticides, pharmaceuticals and perfluoroorganic compounds in POCIS were detected at site 6 (WWTP effluent) (Table 1). Concentrations of contaminants found in

POCIS from WWTP influent (site 5) were less than in POCIS at WWTP effluent and comparable or greater than in those from the other sites. The explanations of greater detected levels of some contaminants and biological potencies in passive samplers from WWTP effluent are elaborated in detail in the Discussion section. Concentrations of some pharmaceuticals in POCIS from the sites upstream of Brno were slightly greater than downstream, but concentrations in the Svratka River were generally approximately 4-fold less than in the Svitava River. Similarly, concentrations of PFCs were approximately 6-fold greater in Svitava than in Svratka, while concentrations of pesticides were comparable in both rivers. Greater concentrations of pesticides were found at site 9 on the tributary of the Svratka River. Concentrations of pharmaceuticals were greater bellow the WWTP effluent. There was a slight decrease of concentrations of contaminants in POCIS as a function of distance from the city and WWTP.

The greatest concentrations of most pollutants sampled by SPMD were observed in samples from the WWTP, with concentrations of PAHs and triclosan greatest in the influent (site 5), while concentrations of methyl triclosan were greatest in the effluent (site 6) (Table 1). Greater concentrations of PCBs and methyl triclosan were detected already upstream of Brno in the Svitava River (sites 3, 4). Concentrations of most pollutants did not increase much directly downstream of Brno on both rivers (sites 2, 4), except for PCBs in the Svratka River. Concentrations of PAHs were slightly lesser downstream of the WWTP (site 7) and further decreased at the longer distance from the city (site 8), while no such trend was observed for concentrations of PCBs and OCPs. Concentrations of PBDEs, triclosan and methyl triclosan were significantly greater downstream of the WWTP.

3.2. Cytotoxicity

Some samples of WWTP influent water caused 20% cytotoxicity even at 25-fold dilution, but effluent water samples caused cytotoxicity only at 100% water equivalents or were not cytotoxic (Fig. 2A). Removal efficiency for cytotoxicity in waste water was 83 to 98% throughout the year, except of one time point when toxicity of the influent was small and thus efficiency of removal was lower (46%). All POCIS extracts elicited cytotoxic effects, with the greatest cytotoxicity observed for samples from the WWTP effluent (site 6, Fig. 2B), which was about 50% greater than the effect of the WWTP influent sample (site 5). Cytotoxicity of POCIS exposed to river water was 4 to 10-fold lower, with greater toxicity in water from the Svitava River. It slightly increased downstream of the WWTP (site 7). A greater than 93% decrease in cytotoxicity after treatment of wastewater was observed in SPMD samples (Fig. 2C), where the WWTP influent sample (site 5) exhibited the greatest cytotoxicity. Cytotoxicity of compounds sampled by SPMD from upstream of Brno was greater in Svratka river, and it increased in river Svitava after flowing through the city and also downstream of WWTP (Fig. 2C).

3.3. AhR-mediated potency

Significant AhR-mediated (dioxin-like) potency expressed as bioassay-derived 2,3,7,8-TCDD equivalents (BIOTEQ) was detected in most samples. Samples of influent water from the WWTP generally elicited greater dioxin-like potency than did effluent water (Fig. 3A). Concentrations of BIOTEQ were between 0.1 and 3.4 ng TCDD/L for influent and 0.1 to 0.7 ng TCDD/L for effluent. Efficiency of treatment of the WWTP for compounds with dioxin-like potency varied during the year from 13 to 90%, except for two cases when the removal efficiency was even negative. In February and April effluent samples contained 8 and 27% greater levels of BIOTEQ than corresponding influent samples, respectively. Significant dioxin-like potency in POCIS samples was detected only for samples from the WWTP (sites 5, 6) and site 7 (sampling site directly downstream of the WWTP) (Fig. 3B, insert). Concentrations of BIOTEQs were between 0.3 and 2 ng TCDD/POCIS. Potency detected in the WWTP effluent (site 6) was 5-fold greater than that in the influent (site 5). All extracts of SPMD contained detectable AhR-mediated potency with the greatest response in the WWTP influent sample (site 5) and also in the Bobrava River which was affected by agriculture (site 9, Fig. 3B). Concentrations of BIOTEQ determined from SPMD ranged from 8.2 to 14.6 pg TCDD/L.

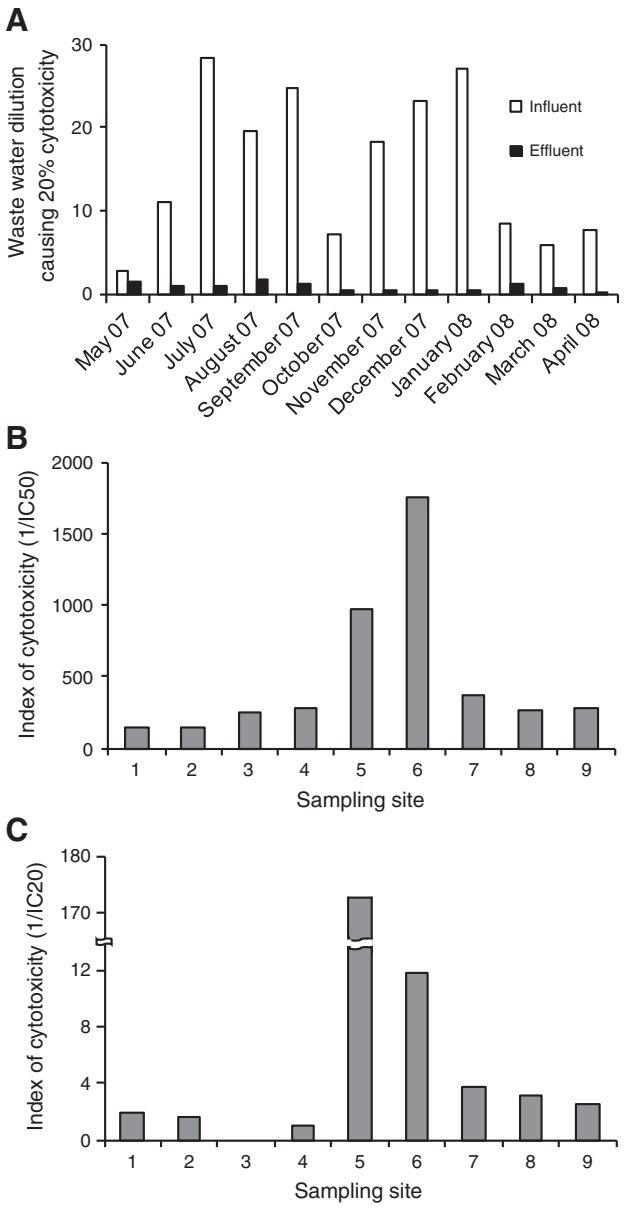

Fig. 2. Cytotoxicity of samples extracts detected in the bioluminescent yeast assay: (A) influent and effluent water samples from the WWTP; (B) POCIS (Index of cytotoxicity expressed as reciprocal value of IC_{50}, $[sampler/mL]^{-1}$); (C) SPMD (Index of cytotoxicity expressed as reciprocal value of IC_{20}, $[L/mL]^{-1}$); no column = no significant activity.

3.4. ER-mediated potency

Potency of ER agonists was detected in water from the WWTP during all samplings throughout the year (Fig. 4). Values of 17β-estradiol (E_2) equivalents (EEQ) varied from 5.4 to 124 ng E_2/L in influent and from 0.1 to 5.1 ng E_2/L in effluent. Efficiency of treatment to remove EEQ ranged from 80 to greater than 99 %. POCIS sample from the WWTP influent (site 5) had a concentration of EEQ of 7.3 ng E_2/

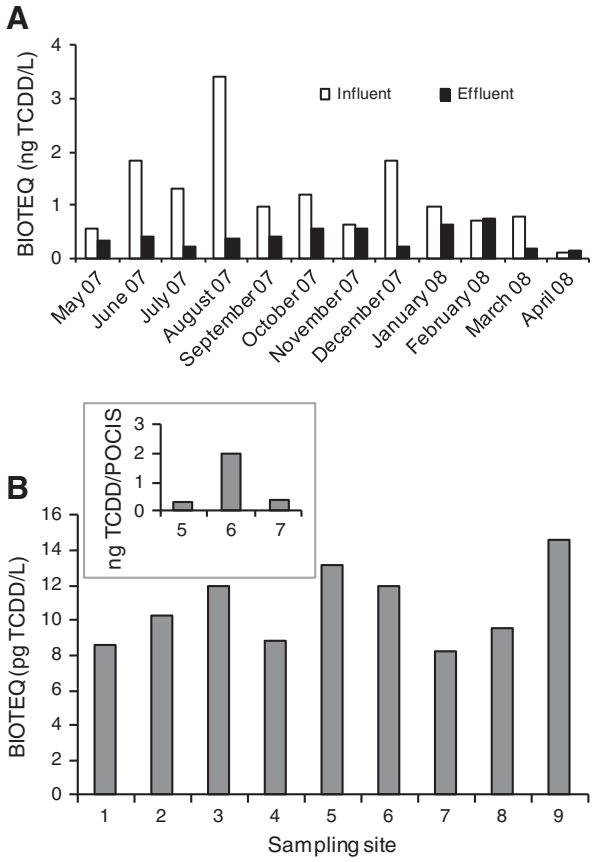

Fig. 3. AhR-Mediated (Dioxin-like) potency of samples extracts detected in H4IIE-*luc* assay expressed as BIOTEQ equivalents: (A) influent and effluent water from the WWTP; (B) SPMD and POCIS.

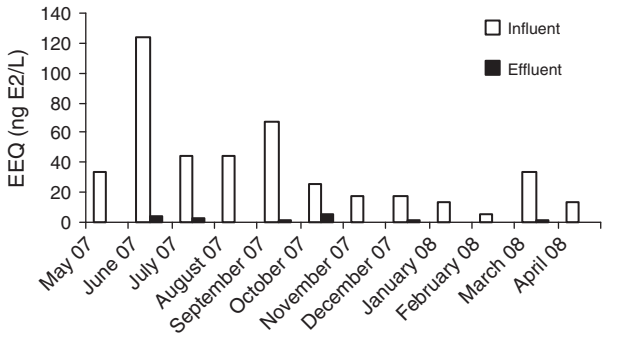

Fig. 4. Estrogenic potency, expressed as estradiol equivalents (EEQ) of extracts of WWTP influent and effluent water, detected in MVLN assay; no column = no significant activity.

sampler. The concentration of EEQ in the extract of POCIS exposed to effluent (site 6) was less than 0.6 ng E_2/sampler, which was the limit of detection. There were no EEQ detectable in POCIS from the rivers or in any SPMD samples.

Influent and effluent water samples from the WWTP showed no significant antiestrogenic potency when tested in the presence of E_2. Alternatively, antiestrogenic potency was detected in extracts of SPMD and POCIS from all sites. Data from SPMDs indicate greater antiestrogenicity in sites from river Svratka compared to Svitava already upstream of Brno. Greatest antiestrogenicity was observed in POCIS exposed to WWTP effluent while all samples from rivers and WWTP influent showed comparable potency (Fig. 5).

3.5. AR-mediated potency

Significant androgenic potencies were found mostly at the greatest non-cytotoxic concentrations of influent water samples and concentrations of androgen equivalents (AEQ) ranged from <23 to 193 ng testosterone/L (Table 2). Concentrations of AEQ determined for non-cytotoxic concentrations of effluent extracts were less than the limit of detection, which was 1–4 ng testosterone/L. Efficiency of treatment to remove androgenic compounds was greater than 96–99%. POCIS from WWTP influent and effluent were the only other samples to exhibit detectable AEQ with concentrations of 32.6 and 6.9 ng DHT/sampler, respectively. No antiandrogenic potency was observed in non-cytotoxic concentrations of samples from influent or effluent water from the WWTP. Antiandrogenic potency in competition with the added endogenous ligand DHT was detected in most extracts of SPMD and POCIS. The greatest antiandrogenic potency in extracts of POCIS was observed at site 4 in the Svitava River, directly downstream of Brno (Fig. 6A). The antiandrogenic potency of the extract of the POCIS exposed to WWTP influent (site 5) was comparable with the potency observed in samples from most sites on the rivers. There was no antiandrogenic potency observed in POCIS exposed to WWTP effluent (site 6). There was generally no antiandrogenic potency in extracts of SPMD exposed upstream of the WWTP, while there was antiandrogenic potency in samples from the WWTP (sites 5, 6) and from sites downstream of the WWTP. The antiandrogenic potency of compounds sampled by SPMD was approximately 60% greater in WWTP influent than that in effluent (Fig. 6B).

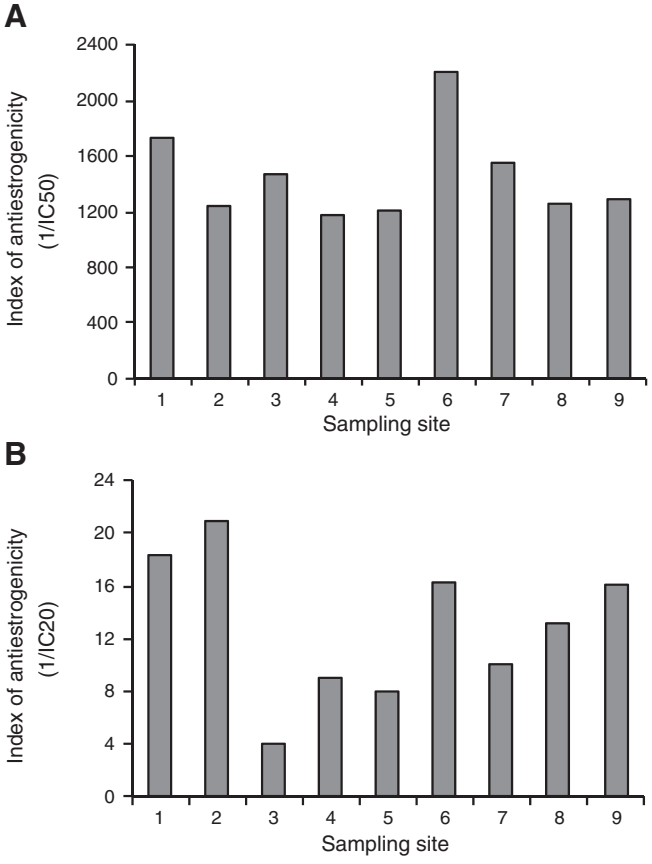

Fig. 5. Antiestrogenic potencies of samples extracts determined by use of the MVLN assay in the presence of 33 pM estradiol expressed as index of antiestrogenicity: (A) POCIS—reciprocal value of IC_{50} [sampler/mL]$^{-1}$), (B) SPMD—reciprocal value of IC_{20} [L/mL]$^{-1}$).

Table 2
Androgenic activity of influent and effluent water extracts from the WWTP detected in the yeast assay. (LOD ranged from 1.3 to 70 ng testosterone/L because of variable cytotoxicity of samples).

Sampling date	AEQ (ng testosterone/L)	
	Influent	Effluent
May 07	155	<3.7
June 07	97	<2.2
July 07	<70	<2.2
August 07	<70	<2.6
September 07	<23	<1.3
October 07	80	<1.3
November 07	193	<1.3
December 07	96	<1.3
January 08	107	<1.3
February 08	140	<1.3
March 08	47	<1.3
April 08	35	<1.3

4. Discussion

Rivers can be contaminated by many chemicals, some of which have the potential to affect normal reproduction, development and behavior of wildlife species and potentially also human health. Some of these compounds can be released to rivers from large city agglomerations via WWTP and other point-discharge or diffuse sources (Cargouet et al., 2004; Jobling et al., 1998; Sabaliunas et al., 2000; Snyder et al., 2000). In recent years, WWTP have been studied as potential sources of endocrine disruptive compounds to the aquatic environment (Harries et al., 1996; Murk et al., 2002; Tan et al., 2007). There are several studies that have investigated WWTPs by use of various approaches including passive sampling combined with instrumental analysis and/or bioassays (Tan et al., 2007; Vermeirssen et al., 2005). However, there has been less information on other possible sources. Moreover, the studies using bioassays were focused mainly on estrogenic potency and there is limited data on other specific biological potencies in mixtures extracted from surface or waste waters. In addition, mostly known endocrine disruptive compounds, such as estrogens, androgens, phthalates or alkylphenols are analyzed, but more data is needed for other pollutants, such as widely used compounds from the group of pharmaceuticals and personal care products.

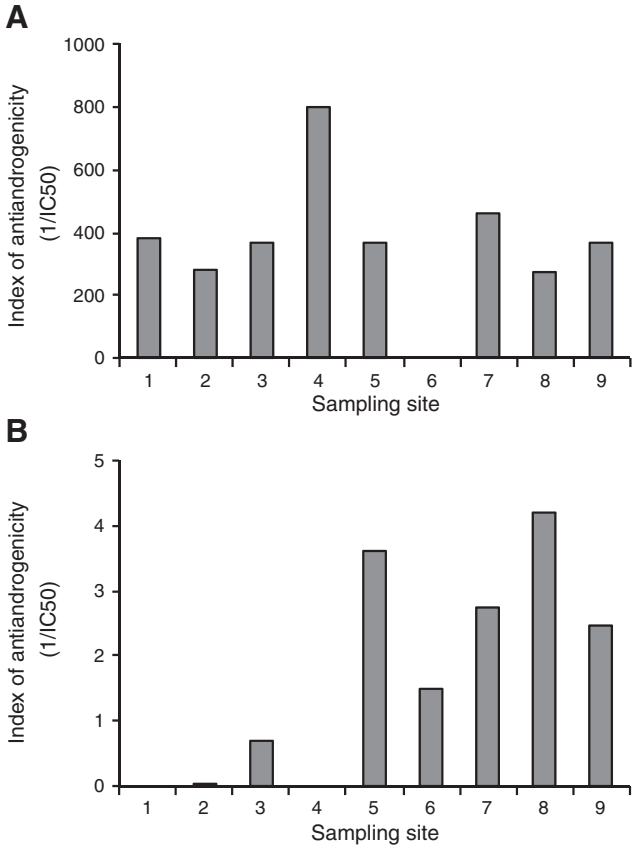

Fig. 6. Antiandrogenic potency of samples extracts determined by use of the MDA-kb2 assay in the presence of 1 nM dihydrotestosteron (DHT), expressed as an index of antiandrogenicity (reciprocal value of IC_{50}): (A) POCIS $[sampler/mL]^{-1}$, (B) SPMD $[L/mL]^{-1}$; no column = no significant activity.

In this study potencies for ligands in mixtures to interact with specific receptors as well as concentrations of several classes of pollutants were measured in waste waters and surface waters of two rivers in an urban metropolitan area in Central Europe with a variety of industries and modern recently renovated WWTP with advanced treatment capacity and efficiency. The sampling design and a complex approach using passive sampling along with chemical analysis and bioassays enabled to characterize the distribution and sources of pollutants in the model part of river basin. Based on measured residues, water of the Svitava River upstream of Brno seems to be more polluted than the Svratka River. Specifically, concentrations of pharmaceuticals, PFCs, PCBs and methyl triclosan were lower in the Svratka River. Furthermore, greater potencies for cytotoxicity of the hydrophilic fraction were observed in the Svitava River upstream of Brno. These data point to some pollution sources on river Svitava upstream of Brno agglomeration. There was no obvious influence of the city itself or WWTP on the concentrations of PAHs and organohalogenated compounds except of somewhat increased PCBs in Svratka downstream of Brno. Thus, neither runoff from the metropolitan region of Brno nor the effluent of the WWTP contributed significantly to the pollution with these compounds. Alternatively, concentrations of pharmaceuticals, antibiotics, triclosan and PBDEs were not affected by the city, but increased downstream of the WWTP, despite its up-to-date treatment technology. The data from passive samples document highly efficient removal of hydrophilic antiandrogenic and about 60% removal of hydrophobic antiandrogenic pollutants during WW treatment. Despite this removal, the concentrations of hydrophobic antiandrogenic pollutants in the river increased downstream of the WWTP similarly to the cytotoxic potency. Concentrations of triclosan and methyl triclosan were increased by the WWTP. For polar pesticides there was no influence of the city itself or WWTP. Concentrations of most of the polar compounds sampled by POCIS and associated biological potencies went down at the last study site about 20 km downstream of the city. There was no such decrease in levels of hydrophobic pollutants sampled by SPMD and their biological potencies, except of PAHs. The decrease of PAHs concentrations downstream of WWTP was not due to particle adsorption and sedimentation after flow out from WWTPs, since there was no increase of PAHs levels in river sediments (data not shown).

For all pollutants sampled by POCIS as well as some pollutants sampled by SPMD, the greatest concentrations were detected in WWTP effluent. Similarly, in the POCIS exposed to effluent there was also the greatest cytotoxicity, dioxin-like and antiestrogenic potency. All these concentrations and potencies were greater than for the WWTP influent. There are at least two explanations of the observed elevated concentrations and toxic potencies of compounds accumulated in passive samplers in the WWTP effluent in comparison to influent. Passive sampling methods measure the concentration of freely dissolved contaminants, which is directly related to the contaminants' chemical activity (Mayer et al., 2003). This also indicates the bioavailability or pressure (fugacity) of contaminants on organisms and consequently represents the exposure level for organisms. In the WWTP influent hydrophobic compounds are largely sorbed to the suspended particulate material so that their freely dissolved concentration is small (Lohmann et al., 2012). In the wastewater

treatment process the content of suspended material is efficiently reduced, which in turn results in a strong decrease of sorption capacity for hydrophobic compounds in WWTP effluent. However, some persistent compounds are not eliminated by the treatment process. As a result of the reduced uptake capacity of the particulate matter, free dissolved concentrations (chemical activity) in the effluent are higher than in the influent, which is in turn reflected in their levels found in passive samplers, especially in SPMDs.

Differences in uptake might be affected by different passive sampler exposure conditions in WWTP influent and effluent, respectively. Among potential factors that affect uptake kinetics into passive samplers, hydrodynamics and fouling are the most important ones. The visual observation of channels in WWTP influent and effluent indicates a similar turbulent water flow character in both cases. Thus, influent/effluent differences in hydrodynamics can hardly explain the observed up to ten-fold increase in accumulated amounts of some compounds in passive samplers (e.g. compounds in POCIS; Table 1). We hypothesize that fouling of samplers is the more important factor that affects the uptake of both hydrophobic as well as hydrophilic compounds into passive samplers. The raw waste water is a very complex mixture which contains debris, mud, various particles and even dispersed emulsions of liquids that are non-miscible with water (such as fats). Fouling and layers of dirt can reduce uptake of compounds into passive samplers (Stuer-Lauridsen, 2005) and lead to lower sampling rates by a) physical blockage of active surface of samplers by debris; b) thickening the diffusion barriers; c) reduction of the driving force for sampler uptake by shifting the partitioning equilibria between sampler and the surrounding environment. Our study indicates that passive sampling (especially for POCIS samples) may not be a reliable method in raw sewage water and could lead to significant underestimation of actual concentrations of dissolved pollutants. This problem is really specific to the raw sewage water and does not concern passive samples from any other site.

Most studies using *in vitro* assays include cytotoxicity tests, which determine the greatest possible sample concentration that is not cytotoxic for the cells to be used as the maximal tested concentration for the specific effects. In this study, dose–response curves and IC_{50} of extracts on yeast cells were determined. The efficient decrease of cytotoxicity in SPMD and waste water after waste water treatment might be due to activated sludge processes as well as flocculation, which have been shown to have the greatest efficiency of removal of cytotoxic compounds (Ma et al., 2005). Cytotoxicity of waste waters did not correlate with estrogenic or androgenic potencies of these waste waters. This observation is consistent with the results reported by Vega-Lopez et al. (2007), who found no correlation between estrogenic disruption and toxicity determined in MCF-7 cells for samples of water from two Mexican lakes, which receive domestic and industrial wastewaters after secondary treatment. These results support the theory that estrogenic potency in waste waters is caused primarily by steroidal estrogens, which are potent at ng/L concentrations and therefore does not correlate with the overall cytotoxicity. Cytotoxicity of extracts of all POCIS in the yeast assay can be related to sesquestered pollutants, especially antibiotics and other pharmaceuticals determined by chemical analysis.

There are few studies that have focused on effects of urban pollution on the overall toxicity of waters in municipal rivers. Toxicity determined by the Microtox assay was directly proportional to urban land cover in streams around six metropolitan areas in the USA (Bryant and Goodbred, 2009). Toxicity of river water sampled by SPMD in Microtox and *Daphnia pulex* test has been observed in the Neris River after flowing through the capital city of Lithuanina (Sabaliunas et al., 2000). This finding is consistent with the observation of greater toxicity of compounds sampled by SPMD from the Svitava River downstream of the metropolitan area compared to upstream of Brno observed in this study.

Detected AhR-mediated potency in both SPMD and POCIS indicated contribution of both hydrophobic and polar compounds to the overall dioxin-like potential of samples. Similarly in river sediments, mass-balance calculations based on fractionation with subsequent quantification have suggested that PAHs can account for a considerable portion of the dioxin-like potency together with unidentified more polar AhR-active compounds (Hilscherova et al., 2001). Dioxin-like potency found in all extracts of SPMDs was probably linked with the presence of known hydrophobic AhR ligands, such as PAHs or PCBs. Although dioxin-like compounds are usually investigated in less polar matrices such as SPMD or sediments, some recent studies (Dagnino et al., 2010; Reungoat et al., 2010) confirmed AhR potency in water phase. Results of another study (Jarosova et al., 2012) reported dioxin-like potency of 0.05 to 0.39 ng BIOTEQ/POCIS in headwaters with small local sources of pollution. In the current study, POCIS samples exhibited dioxin-like potency only at three sites, inside and downstream of the WWTP, which suggests that waste waters contain some hydrophilic dioxin-like compounds that are not completely removed during treatment. This result is in agreement with the dioxin-like potencies detected WWTPs influent and effluent waters. The data for waste water samples show dioxin-like potency specifically for the polar methanolic extracts and thus might not include influence of some hydrophobic pollutants. Efficiency of treatment by the WWTP determined from BIOTEQs of the waste water samples was not as great for chemicals with dioxin-like potency as in the case of elimination of cytotoxicity or hormone-like potencies. Efficiencies of treatment varied substantially throughout the year. Release of some particle-bound compounds during treatment and lesser efficiency of treatment related to greater persistence of some AhR-active compounds might have contributed to this difference. However, the absolute concentrations of BIOTEQ were less than those observed in other studies eventhough only a limited number of papers report dioxin-like potency in the dissolved phase. For example, Dagnino et al. (2010) detected AhR potency (by the same method as we used) in influent and effluent of French municipal WWTPs with an activated sludge system supplemented with biofilter to be as great as 37 to 112 ng TCDD/L, and 2.8 to 11.6 ng TCDD/L, respectively. Efficiency of removal was approximately 90% and the authors concluded that removal of AhR potency in this type of WWTPs depends primarily on removal of suspended solids with which they are associated. Alternatively, Ma et al. (2005) did not find concentrations of BIOTEQ that were greater than 14 pg TCDD/L in either influents or effluents from a pilot plant in a Beijing WWTP, China.

The observation that xenoestrogens and xenoandrogens were detected in waste water and POCIS samples from the WWTP, but not in SPMDs, implies that polar compounds accounted for the estrogenic and androgenic potencies. Since feminization of fish downstream from WWTPs has been observed in rivers worldwide, estrogenic potential of different types of waters has been evaluated in multiple studies. Examples of estrogenic potencies detected by various *in vitro* assays documenting the comparability of our findings to the situation in other parts of the world are compiled in Table 3.

Relatively great efficiency of removal of estrogenic potency in various WWTPs has been documented both by composite water sampling as well as POCIS sampling. The majority of municipal or domestic WWTPs have implemented at least physical and biological treatment techniques. Activated sludge processes, similar to those of WWTP investigated in this study, are the most widely used types of biological treatment processes worldwide. Most studies that have focused on WWTP of similar types to that studied here found the treatment efficiencies for estrogens ranging from >88 to >99% (Leusch et al., 2005; Murk et al., 2002), 90–95% (Korner et al., 2000; Murk et al., 2002) or greater than 95% (Tan et al., 2007), but other studies have reported lesser efficiencies (Cargouet et al., 2004). Efficiency of removal of estrogenic potency, as determined by the MVLN assay, in four mechanical–biological municipal or domestic WWTPs in Paris

Table 3
Examples of estrogenic activities in waste waters and surface waters as detected by various *in vitro* assays.

Matrix	EEQ ng/L	Country	In vitro assay[a]	Reference
Wastewater influent	51–70	Germany	E-Screen	Korner et al. (2000)
	17–23	Queensland, Australia	E-Screen	Leusch et al. (2005)
	1.1–120	The Netherlands	ER-CALUX, YES	Murk et al. (2002)
	35–72	Japan	YES	Onda et al. (2002)
	1–30	Sweden	YES	Svenson et al. (2003)
	108–356	Queensland, Australia	E-Screen	Tan et al. (2007)
	5.4–124	Czech Republic	MVLN	This study
Wastewater effluent	6	Germany	E-Screen	Korner et al. (2000)
	<0.75	Queensland, Australia	E-Screen	Leusch et al. (2005)
	0.03–16	The Netherlands	ER-CALUX, YES	Murk et al. (2002)
	4–25	Japan	YES	Onda et al. (2002)
	<0.1–15	Sweden	YES	Svenson et al. (2003)
	0.6–6.2	Japan	YES	Nakada et al. (2004)
	1.9–15	USA	MVLN	Snyder et al. (2001)
	<1–67.8	Queensland, Australia	E-Screen	Tan et al. (2007)
	0.1–5.1	Czech Republic	MVLN	This study
Surface water	0.07–0.5	The Netherlands	ER-CALUX	Murk et al. (2002)
	0.01–1.4	Belgium	E-Screen	Nadzialek et al. (2010)
	<0.18	Portugal	YES	Sousa et al. (2010)
	0.86–11	USA	MVLN	Snyder et al. (2001)
	<0.006–4.96	Sweden	YES	Svenson et al. (2003)
	0.025–0.68	Korea	E-Screen	Oh et al. (2009)

[a] E-Screen—cell proliferation assay, ER-CALUX—estrogen receptor chemical activated luciferase gene expression assay, YES—yeast estrogen screen, MVLN—luciferase reporter gene-based assay using the MVLN cell line.

ranged from 62 to 97% (Cargouet et al., 2004), which was similar to those reported for five WWTPs in the United Kingdom, which had reported efficiencies of 70 to 100% (Kirk et al., 2002). Efficiency of removal observed in this study was 80 to >99%, but in most tested samples it was greater than 96%.

In previous studies, concentrations of estrogen equivalents (EEQ) of river water upstream and downstream of several WWTPs, quantified by use of the yeast estrogen screen (YES), was significantly correlated with EEQ based on chemical analysis of steroidal estrogens for grab samples and POCIS (Vermeirssen et al., 2005). Also chemical and biological (E-Screen assay) analyses used to determine the concentrations of 15 endocrine disrupting compounds and estrogenicity in grab and passive samples from five municipal WWTPs showed good agreement (Tan et al., 2007). Alternatively, assessment of contamination of headwater streams from livestock farms documented that measured waterborne steroids accounted for some of the detected estrogenicity, but a considerable portion of estrogenicity could not be attributed to concentrations of identified estrogens (Matthiessen et al., 2006).

Androgenic potency of waste water in bioassays was shown to decrease during progression through the WWTP (Michelini et al., 2005). Concentrations of AEQ and efficiencies of removal observed in our study are similar to those reported for three Swedish municipal WWTPs that used activated sludge systems, and had androgenic potencies in yeast androgen screen (YAS) in influents ranging from 30 to 75 AEQ ng/L (and 0.8–3 AEQ ng/L in effluents) with efficiencies of removal of 96–98% (Svenson and Allard, 2004). However, some studies detected androgenic potencies in waste water influents that were greater than those observed in our study (Kirk et al., 2002; Leusch et al., 2006). Androgenic potencies in effluents of some WWTPs were as great as hundreds of ng AEQ/L, but in other WWTPs effluents they were less than the limits of quantification (Blankvoort et al., 2005; Kirk et al., 2002; Leusch et al., 2006; Sousa et al., 2010). Efficiencies of removal of androgens ranged from 82 to more than 99% when activated sludge was included in treatment processes, but significantly less when only primary treatment or for example biological trickling filters were employed (Kirk et al., 2002; Leusch et al., 2006). This observation is consistent with efficiencies of removal determined in this study which were greater than 96% in all cases. Also results obtained with POCIS samples confirmed significant removal of compounds with estrogenic and androgenic potency. Our results document that the efficiency of removal of both estrogenic and androgenic potency of the Brno WWTP can be ranked among the most efficient clarification WWTPs that do not implement advanced treatment. However, the results reported here also show that the efficiency of treatment can vary especially for dioxin-like and cytotoxic compounds, and thus one timepoint sampling might not be sufficient for its determination.

Results of this study provide unique information on the variability of cytotoxicity and specific potencies in waste waters during the whole year. Estrogenic potency seemed to be greater in the dryer summer season when there is less dilution than during winter when more precipitation results in greater runoff, but also greater dilution (Fig. 4). However, there was no clear trend for androgenic potencies. Lower temperatures in winter did not negatively influence removal of estrogenic potency by the WWTP, but it might have affected the breakdown of more persistent compounds causing the dioxin-like potency. The greatest cytotoxicity was observed during summer, which might be correlated with lesser dilution (Fig. 2), but with another peak in winter, when probably some other types of pollutants associated with more typical winter sources (such as combustion) might play more significant role. However, the dioxin-like potency did not vary as much as estrogenicity throughout the year, except for August when it was approximately 3-fold greater than during the rest of the year. This observation is probably due to less dilution in summer and possibly also some immediate pollution situation that can affect the samples collected during a single day. There is limited information on seasonal variability of specific potencies of contaminants in waste waters. A study conducted in the UK (Kirk et al., 2002) found that estrogenic and also androgenic potencies in influents and effluents were less in samples collected in months of rainy weather. The recombinant yeast assay was used to assess variability of estrogenic potencies in influent and effluent of Canadian municipal WWTP implementing an additional cleaning step of UV disinfection (Fernandez et al., 2008). Estrogenic potencies of composite samples of influent taken every week from September to December were not dependent on sampling season, while EEQ levels in final effluents were very high, exceeding 100 ng EEQ/L in September and ranging from about 50 to 80 ng EEQ/L from the end of October till the end of the campaign. Lower EEQ concentrations in effluent in autumn and winter compared to summer were seen also in our study, but the ranges of EEQ values were much lower than those reported by Fernandez et al. (2008).

Similar to the results of this study, small estrogenic potencies and/or concentrations of industrial estrogen mimics and natural estrogens were frequently detected in WWTP discharges, due to their incomplete removal by WWTPs (Table 3). However, even these concentrations have been shown to be effective in causing some biological effects. It has been demonstrated in a 7-year whole-lake experiment that long term exposure to estrogens (5-6 ng/L ethinyl estradiol) can affect sustainability of wild fish populations (Kidd et al., 2007). Moreover, a multigeneration study of Chinese rare minnows (*Gobiocypris rarus*) demonstrated that reproduction of the F_1 minnows was completely inhibited at the ethinyl estradiol concentration as low as 0.2 ng/L (Zha et al., 2008). These results suggest that even when efficiencies of removal of estrogen are as great as those observed in this study, risks to aquatic organisms can still occur due to the concentrations of estrogens that are constantly released from waste water effluents. The risk seems to be greatest in cases when the volume of effluent waters represents a greater proportion in relation to the receiving waters.

Next to the estrogenic and androgenic potencies detected in POCIS and water from WWTP, there were also some antiestrogenic and antiandrogenic pollutants in passive samples from WWTP, which however were not detected in the influent and effluent water samples. This difference indicates that antiestrogenic and antiandrogenic potency is related probably to less polar compounds, which were not in sufficient concentrations included in the methanolic extract of waste water. Moreover, the antiestrogenic/antiandrogenic potencies in waste waters could be masked by relatively great cytotoxicity of the methanolic extracts. Furthermore, passive samples enable higher preconcentration of the compounds compared to the composite water samples and thus the antiestrogenic/antiandrogenic activity detected in passive samples might have been bellow the limit of detection for the water samples. The passive samples from rivers exhibited neither estrogenic nor androgenic potency, but rather antiestrogenic and antiandrogenic potential. The antiestrogenic potency was detected in extracts from passive samplers exposed upstream of the city. In the study by Garcia-Reyero et al. (2001) (anti)estrogenicity was detected by recombinant yeast assay in waste waters and all samples of river water. The lack of estrogenic potency in POCIS and SPMD from river water in the study reported here could be caused by the presence of sufficient concentrations of chemicals that have been shown to have antiestrogenic potency, including pesticides, such as linuron or atrazine (Orton et al., 2009). Antiandrogenic potency was detected at most sampling sites. Hydrophilic antiandrogenic compounds were found in POCIS at sampling sites upstream of the city, whereas antiandrogenic potency in SPMD associated with the more hydrophobic pollutants was detected namely in the WWTP and downstream of the WWTP. Multiple contaminants are known to be associated with antiandrogenic potency (Orton et al., 2009; Sohoni and Sumpter, 1998), including some pesticides, which were detected by chemical analysis (e.g. p,p′-DDE, diuron).

5. Conclusion

This study revealed the presence of compounds with endocrine disruptive potency in both river water and WWTP influent and effluent. The results of year-round waste water assessment confirmed high treatment efficiency of the WWTP for cytotoxic compounds, xenoestrogens and xenoandrogens. There was significant seasonal variability of efficiency of treatment, especially of dioxin-like potencies. Despite its high efficiency WWTP had impact on the pollution with endocrine disruptive compounds. The approach employed enabled determination of contributions of the metropolitan urban area and the WWTP to contamination of the rivers. Concentrations of PAHs and most pollutants sampled by POCIS decreased as a function of distance downstream of the city. Passive sampling, along with *in vitro* bioassays and chemical analysis allowed determination of a broad spectrum of contaminants and specific biological potencies and revealed the pollution situation in this model region. More research should be performed in the future to better characterize passive sampler performance under complex exposure conditions in raw wastewaters.

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References

Alvarez DA, Petty JD, Huckins JN, Jones-Lepp TL, Getting DT, Goddard JP, et al. Development of a passive, in situ, integrative sampler for hydrophilic organic contaminants in aquatic environments. Environ Toxicol Chem 2004;23:1640–8.

Alvarez DA, Stackelberg PE, Petty JD, Huckins JN, Furlong ET, Zaugg SD, et al. Comparison of a novel passive sampler to standard water-column sampling for organic contaminants associated with wastewater effluents entering a New Jersey stream. Chemosphere 2005;61:610–22.

Blankvoort BMG, Rodenburg RJT, Murk AJ, Koeman JH, Schilt R, Aarts J. Androgenic activity in surface water samples detected using the AR-LUX assay: indications for mixture effects. Environ Toxicol Pharmacol 2005;19:263–72.

Booij K, Smedes F. An improved method for estimating in situ sampling rates of nonpolar passive samplers. Environ Sci Technol 2010;44:6789–94.

Brněnské vodárny a kanalizace. Sewage water treatment at WWTP Brno (Odvádění a čištění odpadních vod/ČOV Brno—Modřice). http://www.bvk.cz/o-spolecnosti/odvadeni-a-cisteni-odpadnich-vod/cov-brno-modrice/, 2010. [in Czech].

Bryant WL, Goodbred SL. The response of hydrophobic organics and potential toxicity in streams to urbanization of watersheds in six metropolitan areas of the United States. Environ Monit Assess 2009;157:419–47.

Cargouet M, Perdiz D, Mouatassim-Souali A, Tamisier-Karolak S, Levi Y. Assessment of river contamination by estrogenic compounds in Paris area (France). Sci Total Environ 2004;324:55–66.

Dagnino S, Gomez E, Picot B, Cavailles V, Casellas C, Balaguer P, et al. Estrogenic and AhR activities in dissolved phase and suspended solids from wastewater treatment plants. Sci Total Environ 2010;408:2608–15.

Demirpence E, Duchesne MJ, Badia E, Gagne D, Pons M. MVLN cells—a bioluminescent MCF-7-derived cell-line to study the modulation of estrogenic activity. J Steroid Biochem Mol Biol 1993;46:355–64.

Desbrow C, Routledge EJ, Brighty GC, Sumpter JP, Waldock M. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and *in vitro* biological screening. Environ Sci Technol 1998;32:1549–58.

Ellis GS, Huckins JN, Rostad CE, Schmitt CJ, Petty JD, Maccarthy P. Evaluation of lipid-containing semipermeable-membrane devices for monitoring organochlorine contaminants in the Upper Mississippi River. Environ Toxicol Chem 1995;14:1875–84.

Fernandez MP, Buchanan ID, Ikonomou MG. Seasonal variability of the reduction in estrogenic activity at a municipal WWTP. Water Res 2008;42:3075–81.

Freyberger A, Schmuck G. Screening for estrogenicity and anti-estrogenicity: a critical evaluation of an MVLN cell-based transactivation assay. Toxicol Lett 2005;155:1–13.

Garcia-Reyero N, Grau E, Castillo M, De Alda MJL, Barcelo D, Pina B. Monitoring of endocrine disruptors in surface waters by the yeast recombinant assay. Environ Toxicol Chem 2001;20:1152–8.

Garcia-Reyero N, Raldua D, Quiros L, Llaveria G, Cerda J, Barcelo D, et al. Use of vitellogenin mRNA as a biomarker for endocrine disruption in feral and cultured fish. Anal Bioanal Chem 2004;378:670–5.

Giesy JP, Pierens SL, Snyder EM, Miles-Richardson S, Kramer VJ, Snyder SA, et al. Effects of 4-nonylphenol on fecundity and biomarkers of estrogenicity in fathead minnows (*Pimephales promelas*). Environ Toxicol Chem 2000;19:1368–77.

Grabic R, Jurcikova J, Tomsejova S, Ocelka T, Halirova J, Hypr D, et al. Passive sampling methods for monitoring endocrine disruptors in the Svratka and Svitava Rivers in the Czech Republic. Environ Toxicol Chem 2010;29:550–5.

Harries JE, Sheahan DA, Jobling S, Matthiessen P, Neall P, Routledge EJ, et al. A survey of estrogenic activity in United Kingdom inland waters. Environ Toxicol Chem 1996;15:1993–2002.

Hilscherova K, Kannan K, Kang YS, Holoubek I, Machala M, Masunaga S, et al. Characterization of dioxin-like activity of sediments from a Czech river basin. Environ Toxicol Chem 2001;20:2768–77.

Hilscherova K, Kannan K, Holoubek I, Giesy JP. Characterization of estrogenic activity of riverine sediments from the Czech Republic. Arch Environ Contam Toxicol 2002;43:175–85.
Huckins JN, Tubergen MW, Manuweera GK. Semipermeable-membrane devices containing model lipid—a new approach to monitoring the bioavailability of lipophilic contaminants and estimating their bioconcentration potential. Chemosphere 1990;20:533–52.
Huckins JN, Manuweera GK, Petty JD, Mackay D, Lebo JA. Lipid-containing semipermeable-membrane devices for monitoring organic contaminants in water. Environ Sci Technol 1993;27:2489–96.
Huckins JN, Booij K, Petty JD. Theory and modeling. In: Huckins JN, Booij K, Petty JD, editors. Monitors of organic chemicals in the environment. Semipermeable membrane devices. New York: Springer; 2006. p. 45–85.
Jarosova B, Blaha L, Vrana B, Randak T, Grabic R, Giesy JP, et al. Changes in concentrations of hydrophilic organic contaminants and of endocrine-disrupting potential downstream of small communities located adjacent to headwaters. Environ Int 2012;45:22–31.
Jobling S, Nolan M, Tyler CR, Brighty G, Sumpter JP. Widespread sexual disruption in wild fish. Environ Sci Technol 1998;32:2498–506.
Kidd KA, Blanchfield PJ, Mills KH, Palace VP, Evans RE, Lazorchak JM, et al. Collapse of a fish population after exposure to a synthetic estrogen. Proc Natl Acad Sci U S A 2007;104:8897–901.
Kirk LA, Tyler CR, Lye CM, Sumpter JP. Changes in estrogenic and androgenic activities at different stages of treatment in wastewater treatment works. Environ Toxicol Chem 2002;21:972–9.
Korner W, Bolz U, Sussmuth W, Hiller G, Schuller W, Hanf V, et al. Input/output balance of estrogenic active compounds in a major municipal sewage plant in Germany. Chemosphere 2000;40:1131–42.
Leskinen P, Michelini E, Picard D, Karp M, Virta M. Bioluminescent yeast assays for detecting estrogenic and androgenic activity in different matrices. Chemosphere 2005;61:259–66.
Leusch FDL, Chapman HF, Korner W, Gooneratne SR, Tremblay LA. Efficacy of an advanced sewage treatment plant in southeast Queensland, Australia, to remove estrogenic chemicals. Environ Sci Technol 2005;39:5781–6.
Leusch FDL, Chapman HF, van den Heuvel MR, Tan BLL, Gooneratne SR, Tremblay LA. Bioassay-derived androgenic and estrogenic activity in municipal sewage in Australia and New Zealand. Ecotoxicol Environ Saf 2006;65:403–11.
Leusch FDL, De Jager C, Levi Y, Lim R, Puijker L, Sacher F, et al. Comparison of five *in vitro* bioassays to measure estrogenic activity in environmental waters. Environ Sci Technol 2010;44:3853–60.
Li H, Vermeirssen EL, Helm PA, Metcalfe CD. Controlled field evaluation of water flow rate effects on sampling polar organic compounds using polar organic chemical integrative samplers. Environ Toxicol Chem 2010;29:2461–9.
Lohmann R, Booij K, Smedes F, Vrana B. Use of passive sampling devices for monitoring and compliance checking of POP concentrations in water. Environ Sci Pollut Res 2012;19:1885–95.
Ma M, Li J, Wang ZJ. Assessing the detoxication efficiencies of wastewater treatment processes using a battery of bioassays/biomarkers. Arch Environ Contam Toxicol 2005;49:480–7.
Matsui S, Takigami H, Matsuda T, Taniguchi N, Adachi J, Kawami H, et al. Estrogen and estrogen mimics contamination in water and the role of sewage treatment. Water Sci Technol 2000;42:173–9.
Matthiessen P, Arnold D, Johnson AC, Pepper TJ, Pottinger TG, Pulman KGT. Contamination of headwater streams in the United Kingdom by oestrogenic hormones from livestock farms. Sci Total Environ 2006;367:616–30.
Mayer P, Tolls J, Hermens L, Mackay D. Equilibrium sampling devices. Environ Sci Technol 2003;37:184A–91A.
Michelini E, Leskinen P, Virta M, Karp M, Roda A. A new recombinant cell-based bioluminescent assay for sensitive androgen-like compound detection. Biosens Bioelectron 2005;20:2261–7.
Miles-Richardson SR, Kramer VJ, Fitzgerald SD, Render JA, Yamini B, Barbee SJ, et al. Effects of waterborne exposure of 17 beta-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). Aquat Toxicol 1999;47:129–45.
Ministry of the Environment of the Czech Republic, Czech Environmental Inspectorate. Report on the reconstruction and modernization of the WWTP in Brno. (in Czech) http://www.cizp.cz/(b1obdbr4uyzcvq454q1mucvb)/default.aspx?id=511&ido=362&sh=-711127208, 2010.
Murk AJ, Legler J, van Lipzig MMH, Meerman JHN, Belfroid AC, Spenkelink A, et al. Detection of estrogenic potency in wastewater and surface water with three *in vitro* bioassays. Environ Toxicol Chem 2002;21:16–23.
Nadzialek S, Vanparys C, Van der Heiden E, Michaux C, Brose F, Scippo ML, et al. Understanding the gap between the estrogenicity of an effluent and its real impact into the wild. Sci Total Environ 2010;408:812–21.
Nakada N, Nyunoya H, Nakamura M, Hara A, Iguchi T, Takada H. Identification of estrogenic compounds in wastewater effluent. Environ Toxicol Chem 2004;23:2807–15.
Oh SM, Kim HR, Park HK, Choi K, Ryu J, Shin HS, et al. Identification of estrogen-like effects and biologically active compounds in river water using bioassays and chemical analysis. Sci Total Environ 2009;407:5787–94.
Onda K, Yang SY, Miya A, Tanaka T. Evaluation of estrogen-like activity on sewage treatment processes using recombinant yeast. Water Sci Technol 2002;46:367–73.
Orton F, Lutz I, Kloas W, Routledge EJ. Endocrine disrupting effects of herbicides and pentachlorophenol: *In vitro* and *in vivo* evidence. Environ Sci Technol 2009;43:2144–50.
Petty JD, Orazio CE, Huckins JN, Gale RW, Lebo JA, Meadows JC, et al. Considerations involved with the use of semipermeable membrane devices for monitoring environmental contaminants. J Chromatogr A 2000a;879:83–95.
Petty JD, Jones SB, Huckins JN, Cranor WL, Parris JT, McTague TB, et al. An approach for assessment of water quality using semipermeable membrane devices (SPMDs) and bioindicator tests. Chemosphere 2000b;41:311–21.
Petty JD, Huckins JN, Alvarez DA, Brumbaugh WG, Cranor WL, Gale RW, et al. A holistic passive integrative sampling approach for assessing the presence and potential impacts of waterborne environmental contaminants. Chemosphere 2004;54:695–705.
Reungoat J, Macova M, Escher BI, Carswell S, Mueller JF, Keller J. Removal of micropollutants and reduction of biological activity in a full scale reclamation plant using ozonation and activated carbon filtration. Water Res 2010;44:625–37.
Routledge EJ, Sheahan D, Desbrow C, Brighty GC, Waldock M, Sumpter JP. Identification of estrogenic chemicals in STW effluent. 2. *In vivo* responses in trout and roach. Environ Sci Technol 1998;32:1559–65.
Sabaliunas D, Lazutka JR, Sabaliuniene I. Acute toxicity and genotoxicity of aquatic hydrophobic pollutants sampled with semipermeable membrane devices. Environ Pollut 2000;109:251–65.
Sanderson T, van den Berg M. Interactions of xenobiotics with the steroid hormone biosynthesis pathway. Pure Appl Chem 2003;75:1957–71.
Sanderson JT, Aarts J, Brouwer A, Froese KL, Denison MS, Giesy JP. Comparison of Ah receptor-mediated luciferase and ethoxyresorufin-O-deethylase induction in H4IIE cells: Implications for their use as bioanalytical tools for the detection of polyhalogenated aromatic hydrocarbons. Toxicol Appl Pharmacol 1996;137:316–25.
Snyder SA, Snyder E, Villeneuve D, Kurunthachalam K, Villalobos A, Blankenship A, Giesy J. Instrumental and bioanalytical measures of endocrine disruptors in water. Analysis of Environmental Endocrine Disruptors; 2000. p. 73–95.
Snyder SA, Villeneuve DL, Snyder EM, Giesy JP. Identification and quantification of estrogen receptor agonists in wastewater effluents. Environ Sci Technol 2001;35:3620–5.
Snyder EM, Snyder SA, Kelly KL, Gross TS, Villeneuve DL, Fitzgerald SD, et al. Reproductive responses of common carp (*Cyprinus carpio*) exposed in cages to influent of the Las Vegas Wash in Lake Mead, Nevada, from late winter to early spring. Environ Sci Technol 2004;38:6385–95.
Sohoni P, Sumpter JP. Several environmental oestrogens are also anti-androgens. J Endocrinol 1998;158:327–39.
Sole M, de Alda MJL, Castillo M, Porte C, Ladegaard-Pedersen K, Barcelo D. Estrogenicity determination in sewage treatment plants and surface waters from the Catalonian area (NE Spain). Environ Sci Technol 2000;34:5076–83.
Sousa A, Schonenberger R, Jonkers N, Suter MJF, Tanabe S, Barroso CM. Chemical and biological characterization of estrogenicity in effluents from WWTPs in Ria de Aveiro (NW Portugal). Arch Environ Contam Toxicol 2010;58:1–8.
Stuer-Lauridsen F. Review of passive accumulation devices for monitoring organic micropollutants in the aquatic environment. Environ Pollut 2005;136:503–24.
Sumpter JP. Feminized responses in fish to environmental estrogens. Toxicol Lett 1995;82–3:737–42.
Svenson A, Allard AS. Occurrence and some properties of the androgenic activity in municipal sewage effluents. J Environ Sci Health A Tox Hazard Subst Environ Eng 2004;39:693–701.
Svenson A, Allard AS, Ek M. Removal of estrogenicity in Swedish municipal sewage treatment plants. Water Res 2003;37:4433–43.
Tan BLL, Hawker DW, Muller JF, Leusch FDL, Tremblay LA, Chapman HF. Comprehensive study of endocrine disrupting compounds using grab and passive sampling at selected wastewater treatment plants in South East Queensland, Australia. Environ Int 2007;33:654–69.
Vega-Lopez A, Ramon-Gallegos E, Galar-Martinez M, Jimenez-Orozco FA, Garcia-Latorre E, Dominguez-Lopez ML. Estrogenic, anti-estrogenic and cytotoxic effects elicited by water from the type localities of the endangered goodeid fish *Girardinichthys viviparus*. Comp Biochem Physiol C Toxicol Pharmacol 2007;145:394–403.
Vermeirssen ELM, Korner O, Schonenberger R, Suter MJF, Burkhardt-Holm P. Characterization of environmental estrogens in river water using a three pronged approach: active and passive water sampling and the analysis of accumulated estrogens in the bile of caged fish. Environ Sci Technol 2005;39:8191–8.
Villeneuve DL, Blankenship AL, Giesy JP. Derivation and application of relative potency estimates based on *in vitro* bioassay results. Environ Toxicol Chem 2000;19:2835–43.
Villeneuve DL, Khim JS, Kannan K, Giesy JP. Relative potencies of individual polycyclic aromatic hydrocarbons to induce dioxinlike and estrogenic responses in three cell lines. Environ Toxicol 2002;17:128–37.
Wilson VS, Bobseine K, Lambright CR, Gray LE. A novel cell line, MDA-kb2, that stably expresses an androgen- and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists. Toxicol Sci 2002;66:69–81.
Zha JM, Sun LW, Zhou YQ, Spear PA, Ma M, Wang ZJ. Assessment of 17 alpha-ethinylestradiol effects and underlying mechanisms in a continuous, multigeneration exposure of the Chinese rare minnow (*Gobiocypris rarus*). Toxicol Appl Pharmacol 2008;226:298–308.

Článek XV:

Jarošová, B., Erseková, A., Hilscherová, K., Loos, R., Gawlik, B. M., Giesy, J. P., Bláha, L., 2014. Europe-wide survey of estrogenicity in wastewater treatment plant effluents: the need for the effect-based monitoring. Environmental Science and Pollution Research 21(18), 10970-10982.

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RESEARCH ARTICLE

Europe-wide survey of estrogenicity in wastewater treatment plant effluents: the need for the effect-based monitoring

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Abstract A pan-European monitoring campaign of the wastewater treatment plant (WWTP) effluents was conducted to obtain a concise picture on a broad range of pollutants including estrogenic compounds. Snapshot samples from 75 WWTP effluents were collected and analysed for concentrations of 150 polar organic and 20 inorganic compounds as well as estrogenicity using the MVLN reporter gene assay. The effect-based assessment determined estrogenicity in 27 of 75 samples tested with the concentrations ranging from 0.53 to 17.9 ng/L of 17-beta-estradiol equivalents (EEQ). Approximately one third of municipal WWTP effluents contained EEQ greater than 0.5 ng/L EEQ, which confirmed the importance of cities as the major contamination source. Beside municipal WWTPs, some treated industrial wastewaters also exhibited detectable EEQ, indicating the importance to investigate phytoestrogens released from plant processing factories. No steroid estrogens were detected in any of the samples by instrumental methods above their limits of quantification of 10 ng/L, and none of the other analysed classes of chemicals showed correlation with detected EEQs. The study demonstrates the need of effect-based monitoring to assess certain classes of contaminants such as estrogens, which are known to occur at low concentrations being of serious toxicological concern for aquatic biota.

Keywords In vitro bioassay · Monitoring · Sewage · Rivers · Hormones · EDCs · Endocrine disruptors

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Abbreviations

E1	Estrone
E2	17β-Estradiol
E2max	Maximal response of standard ligand - E2
EE2	17α-Ethynylestradiol
EEQ	17β-Estradiol equivalents
HDPE	High-density polyethylene
LOD	Limit of detection
LOQ	Limit of quantification
NP	Nonylphenol
OP	Octylphenol
PNECs	Predicted no-effect concentrations
PPCPs	Pharmaceuticals and personal care products
PFASs	Perfluoroalkyl substances
WWTP	Wastewater treatment plant
YES	Yeast estrogen screen

Introduction

Estrogenic compounds present in treated wastewaters have been shown to mainly contribute to adverse reproductive effects in aquatic biota. Feminisation of male fishes living downstream from wastewater treatment plants (WWTPs) has been observed worldwide (e.g. Sumpter and Johnson 2008; Wang et al. 2013). Steroid estrogens, particularly natural hormones such as estrone (E1) and 17β-estradiol (E2) and

the synthetic hormone 17α-ethynylestradiol (EE2) used in many contraceptives, have been identified as the major causative agents in treated, domestic wastewaters (Arditsoglou and Voutsa 2008; Jarosova et al. 2014). Feminisation of fishes has been also observed at several locations downstream from industrial WWTP discharges near places with textile and tannery industries, where greater concentrations of alkylphenols have been detected (Sumpter and Johnson 2008; Keith et al. 2001). The most potent estrogenic alkylphenols are 4-tertiary isomers of nonylphenol (NP) and to lesser extend also octylphenol (OP). NP and OP have been reported to be the primary cause of adverse effects downstream of the industrial WWTPs (Sumpter and Johnson 2008; Sole et al. 2000). Compared to steroid estrogens, alkylphenols are at least a thousand times less potent estrogens (Environment Agency 2004; Leusch et al. 2010), but their concentrations detected near textile industry may exceed 100 μg/L (e.g. Sole et al. 2000). That is approximately 100,000 times more than the common environmental concentrations of steroid estrogens (Runnalls et al. 2010).

The observation that steroid estrogens in surface waters can cause adverse effects on reproduction to sensitive organisms, such as fish at low nanogram per litre concentrations, stimulated efforts to improve analytical techniques for environmental samples (Sumpter and Johnson 2008). Despite these efforts, reliable quantification of steroid estrogens in environmental mixtures such as wastewaters remains a problem (Caldwell et al. 2012). In addition, even reliable detection of a few selected estrogens does not guarantee identification of actual estrogenic potential in environmental samples (Villeneuve et al. 1998). Some unexpected molecules or interactions increasing or inhibiting the overall estrogenicity have been observed in several studies (e.g. Cargouet et al. 2004; Pawlowski et al. 2003). Therefore, there was a need to complement the targeted chemical instrumental methods by biological approaches (Leusch et al. 2010). Naturally, in situ and in vivo bioassays would be the most relevant to detect adverse effects, but they are expensive and time and animal consuming which limits their application for broader monitoring of water quality. Alternatively, in vitro bioassays can serve as rapid and cost-effective screening methods to estimate total estrogenic activity of all compounds that act through the same mode of action (i.e. binding to estrogenic receptor) present in the mixtures, and they are currently being considered to be used in the tiered monitoring of estrogenicity of environmental waters (Leusch et al. 2010). The need for the effect-based monitoring and trigger values was recently highlighted also by other authors (Escher et al. 2013; Tang et al. 2013).

In past decades, several polar organic compound classes including estrogens were found to be discharged via WWTP effluents into receiving waters (Reemtsma et al. 2006). Majority of information about these so-called emerging chemicals and their eventual effects (such as estrogenic activity) in wastewaters is available from scattered national or local studies. Such studies are often narrow, focussing in detail on a specific chemical group and using different methodologies in the sample preparation and analysis. Therefore, it has been complicated to compare results across studies and to draw general estimates of probable concentrations or biological activities at a broader scale. In 2010, effluents from 90 WWTPs were collected within 16 European countries and analysed in order to obtain a large data set on many so far only locally investigated "emerging" compounds (Gawlik et al. 2012). The study was designed to provide the first concise overview of concentrations of emerging pollutants occurring in WWTP effluents across Europe including countries for which only limited information was publically available before such as Cyprus, Czech Republic or Lithuania. The study focussed on a range of pharmaceuticals and personal care products (PPCPs), veterinary (antibiotic) drugs, perfluoroalkyl substances (PFASs), organophosphate ester flame retardants, pesticides and their metabolites, industrial chemicals such as corrosion inhibitors benzotriazoles, polycyclic musk fragrances, X-ray contrast agents, gadolinium compounds, and siloxanes (Loos et al. 2012). Targeted chemical analyses were complemented by the effect-based monitoring approaches aiming at estrogenicity, dioxin-like activity, and yeast and diatom culture acute toxicity (Loos et al. 2013). In the present paper, we discuss in detail the results of estrogenicity detected using the reporter gene bioassay in the extracts of 75 WWTP effluents and compare the bioassay responses with the chemical analyses of emerging pollutants. Environmental risks of detected estrogenicity (expressed as nanograms per litre 17β-estradiol equivalents (EEQ)) are discussed by comparing the detected concentrations of EEQ with effective in vivo concentrations of major estrogens to aquatic biota such as fish.

Methods

Description of the campaign, WWTPs, sampling and sample storing

The selection of WWTP was not done by researchers. Instead, the selection of the WWTPs was done by voluntarily participating European Union Member States (and Switzerland), and no criteria were required by the coordinator of the project (Loos et al. 2013). Participants were, however, aware of the aims of the study and therefore wastewaters from WWTPs of different capacities and diverse sources (domestic with or without storm water; and also some with larger proportions of industrial effluents were collected from the participating countries). Table 1 gives a list of the 75 WWTPs from 16 different countries investigated in the present study. This table

Table 1 Characterisation of sampled wastewater treatment plants (WWTPs) and the detected estrogenic activity

Label in this article	Country	Location/WWTR name	Composition of wastewater	Plant capacity (thousands of m^3/d)	Capacity population equivalent (thousands)	Type of secondary (and tertiary if applied) treatment	Detected EEQ (ng/L)
WWTP A1	Italy	Roma nord ACEA	Dom. Ind. Rain	354	780	biological, not specified, final disinfection step	12.2
WWTP A2	Czech Rep.	Not displayed	Dom. Ind. Rain	>200	>500	AS, DN, N, CHP	2.1
WWTP A3	Czech Rep.	Not displayed	Dom. Ind. Rain	>100	>500	AS, DN, N, CHP	1.3
WWTP A4	Finland	Helsinki	Dom. Ind. probably Rain	30[a]	825[a]	AS, DN, N, CHP	<0.5
WWTP A5	Germany	Bremen	Dom. Ind. Rain	94	1 000	AS, D/N, CHP	<0.5
WWTP A6	Germany	Klärwerk Gut Marienhof	Dom. Ind. Rain	493	1 500	AS, DN, N, CHP	<0.5
WWTP A7	Ireland	Dublin		400	1 900	AS (sequencing batch reactor) with DN/N, UV Light Treatment	<0.5
WWTP A8	Netherlands	Harnaschpolder	Dom. Ind. Rain	150	1 400	AS, DN/N, BP	<0.5
WWTP A9	Netherlands	Rotterdam Dokhaven	Mainly Dom.	117	500	AS, D/N - SHARON® and ANAMMOX®, CHP	<0.5
WWTP A10	Switzerland	Zürich Werdhölzli	Dom. Ind. Rain		640	AS, DN, N, BP, CHP	<0.5
WWTP B1	Slovenia	Ljubljana	Dom. (62 %), Ind. (11 %), Rain (21 %)	103	360	AS not further specified	4.1
WWTP B2	Czech Rep.	Not displayed	Dom. Ind. Rain	52	170	AS, DN, N, CHP	1.7
WWTP B3	Lithuania	Kaunas		82	370	AS, DN/N, CHP	1.0
WWTP B4	Netherlands	Venlo		71[b]		AS, DN/N, BP	0.9
WWTP B5	Netherlands	Almere	Dom., Hospital, no Rain		330	not specified	0.6
WWTP B6	Austria	Wiener Neustadt - Sud	Dom. (90 %), Paper Ind.	37	260	AS, DN/N, P removal not specified,	0.5
WWTP B7	Austria	AWV Hall i. Tirol-Fritzens	Dom. Ind. (Rain was not further specified)	16	120	AS not further specified	<0.5
WWTP B8	Belgium	Deurne	Waste water from Antwerp	50[a]	325	AS not further specified	<0.5
WWTP B9	Finland	Espoo	Dom. Ind. Rain not specified	110	250	AS, DN, N, P removal not specified	<0.5
WWTP B10	Netherlands	Amstelveen	Dom.		125	AS not further specified	<0.5 ∇
WWTP B11	Netherlands	Nieuwgraaf	Dom. Ind. (30-40 %), Hospital		395	AS not further specified	<0.5 ∇
WWTP B12	Netherlands	Garmerwold (Noorderzijlvest)	Dom.		300	AS, DN/N - SHARON®, P removal not specified	<0.5
WWTP B13	Netherlands	Zaandam Oost	Dom. Urban runoff, Ind. Craft Industry		150	AS, DN/N, P removal not specified	<0.5
WWTP B14	Lithuania	Klaipedo vanduo	Dom. Ind. (Rain was not further specified)	95	200[a]	AS, DN/N, P removal not specified	<0.5
WWTP B15	Lithuania	Panevezys regional	Dom. Ind. Rain	70		not specified	<0.5
WWTP C1	Cyprus	Larnaka	Dom.	6	27.5	AS, no DN, N and P removal not specified, sand filtration, chlorination	3.6
WWTP C2	Spain	Ulldecona		1.6	13.5	not specified	3.3
WWTP C3	Czech Rep.	Not displayed	Dom. Rain	3	15	AS, N, DN, CHP	1.2
WWTP C4	Austria	Eisenstadt eisbachtal		12[b]	42[b]	AS, DN/N not specified, CHP	<0.5
WWTP C5	Austria	Feldkirchen		6.6	50	AS, N, DN, BP	<0.5
WWTP C6	Belgium	Hasselt	Dom.	12	65	AS, (DN/N and P removal not specified)	<0.5
WWTP C7	Cyprus	Limassol	Dom. Ind.	15	70	AS, N, DN, no BP (CHP not specified), sand filtration, chlorination	<0.5
WWTP C8	Czech Rep.	Not displayed	Dom. Rain	19	75	AS, N, DN, CHP	<0.5
WWTP C9	Ireland	Oberstown			80	cyclic AS, N, DN, CHP	<0.5
WWTP C10	Netherlands	Leek (Noorderzijlvest)	Dom.		34	not specified	<0.5 ∇
WWTP C11	Netherlands	Simpelveld	Dom., Health Care Unit		20.5	not specified	<0.5
WWTP C12	Netherlands	Winterswijk	Dom. Ind. (30-40 %). Hospital		83.5	not specified	<0.5
WWTP C13	Spain	Tortosa		10	46.8	not specified	<0.5
WWTP C14	Switzerland	Affoltern a.A.	Dom. Ind. Rain		14	AS, DN/N not specified, CHP	<0.5

Table 1 (continued)

Label in this article	Country	Location/WWTR name	Composition of wastewater	Plant capacity (thousands of m^3/d)	Capacity population equivalent (thousands)	Type of secondary (and tertiary if applied) treatment	Detected EEQ (ng/L)
WWTP D1	Czech Rep.	Not displayed	Dom. Ind. no Rain	0.3	2.5	AS, N, DN, CHP	1.9
WWTP D2	Germany	AZV Hungerbachtal			7[a]	AS not further specified	0.8
WWTP D3	Hungary	Alattyán	Mainly Dom.		0.25	not specified	0.8
WWTP D4	Switzerland	Wenslingen	Dom. Rain		0.7	AS (DN/N and P removal not specified)	0.6
WWTP D5	Czech Rep.	Not displayed	Dom. Ind. no Rain	0.7	5	AS, N, DN, CHP	<0.5
WWTP D6	Finland	Nummi-Pusula		1[b]	6[a]	Fe coag., As (no DN/N)	<0.5
WWTP D7	Spain	Godall		0.15	0.9	not specified	<0.5
WWTP D8	Switzerland	Konolfingen	Dom. Ind. Rain		7.9	AS, CHP (DN/N not specified)	<0.5
WWTP D9	Switzerland	Seuzach	Dom. Rain	4	6.5	AS, CHP (DN/N not specified)	<0.5 ∇
WWTP E1	Belgium	Agristo	Food industry (potato products)				3.4
WWTP E2	Belgium	TWZ Evergem	Tank cleaning and various ind. activities				1.8
WWTP E3	Belgium	Bayer Antwerpen	Chemical industry (e.g. pesticide production)				1.2
WWTP E4	Belgium	3M	Different industrial branches				0.8
WWTP E5	Belgium	Janssen Pharmaceuticals	Pharmaceutical industry				0.6
WWTP E6	Austria	WV Hofsteig	Dom. (25 %). Ind. (75 %) (Metal, food, textile)	138	216	AS not further specified	<0.5
WWTP E7	Belgium	Ajjinomoto Omnichem	Herbal extracts, polyphenols production				<0.5 ∇
WWTP E8	Belgium	Ardo	Food industry (frozen vegetable)				<0.5
WWTP E9	Belgium	Colortex	Textile industry (dyeing)				<0.5 ∇
WWTP E10	Belgium	EOC Oudenaarde	Chemical industry (e.g. adhesives, surfactants)				<0.5
WWTP E11	Belgium	Tack Oostrozebeke	Tank cleaning and various industrial activities				<0.5 ∇
WWTP E12	Belgium	Taminco	Chemical industry (Amine company)				<0.5 ∇
WWTP F1	Hungary	Martfű	Dom. or soya or brewery production?	1			17.9
WWTP F2	Portugal	Parada				AS, DN, N, no BP	6.0
WWTP F3	Austria	AWV Region Feldkirch		380		AS not further specified	1.2
WWTP F4	Portugal	Viana do Castelo			90[a]	AS not further specified	0.7
WWTP F5	Greece	Thessaloniki (EELTH)	Dom. Ind. probably Rain				0.7
WWTP F6	Italy	Depuratore 'Jugendwerk Brebbia'					0.6
WWTP F7	Belgium	Geel				trickling filter, AS (INVENT®), sand filtration	<0.5
WWTP F8	Belgium	Ronse					<0.5
WWTP F9	Belgium	Waregem	Region with textile industry				<0.5 ∇
WWTP F10	Finland	Lohja					<0.5
WWTP F11	Finland	Mäntsälä					<0.5
WWTP F12	Finland	Vihti					<0.5
WWTP F13	Greece	Thessaloniki (EEL AINEIA)	Waste water from Thermaikos city				<0.5
WWTP F14	Belgium	Claerebout					<0.5
WWTP F15	Belgium	Shanks lokeren					<0.5

Dom. domestic, *Ind*. industrial, *AS* reservoirs with activated sludge, *DN* denitrification, *N* nitrification, *DN/N* biological treatment of nitrogen (not specified if N, DN or both are used), *BP* biological removal of phosphorus, *CHP* chemical precipitation of phosphorus

[a] Approximate number

[b] Average daily discharge or currently connected equivalent citizens and not maximal capacity of WWTP

∇ ∇ Cytotoxic/antiestrogenic samples (open and full symbols indicate less and more pronounced effects, respectively)

contains (besides results of the estrogenicity of samples) information on the type of discharges treated in the plant (domestic or industrial), plant capacity (m^3/d), capacity in population equivalents, type of secondary treatment, and, if applicable, type of tertiary treatment applied. Unfortunately, not all participants of the campaign (owners of the WWTPs) provided all the information requested. Information was collected for 48 municipal and 12 industrial WWTPs, whereas no available metadata were available for 15 tested WWTPs (information not provided by the owners, neither found at other information sources nor on the internet). With the exception of a few small WWTPs (capacity of equivalent population, CEP<10,000) and possibly also some of the WWTPs for which there was no information, all investigated municipal WWTPs included activated sludge processes with nitrification and/or denitrification and chemical precipitation of phosphorus, which represent the most common WWTP technology in Europe. Only four municipal WWTPs reported use of biological phosphorus removal technology and other four municipal WWTPs utilised tertiary treatment step (filtration and chlorination or UV light). Some of the smaller WWTPs (CEP<10,000) utilised activated sludge and chemical precipitation of organics and phosphorus without denitrification/nitrification (Table 1).

With respect to the objective and broad character of the study, i.e. 'snapshot' screening of the European situation, both grab and 24-h composite samples were provided by WWTP owners. Eight 1-L aliquots of water were collected from each WWTP, stored in high-density polyethylene (HDPE) plastic bottles, then shipped to the coordinator (Joint Research Centre (JRC), Ispra, Italy) by fast courier in polystyrene boxes with cooling elements. Samples were stored at ~4 °C and further distributed as fast as possible to the other expert laboratories for analyses (Loos et al. 2013).

Due to high number of cooperating subjects, the time from sampling to extraction differed from days to 2 and occasionally even 3 months. Possible transformation of estrogenic compounds during shipping of samples was considered, and samples collected in the country where the bioassays were performed (Czech Republic) were divided into two aliquots and tested within 2 days after sampling as well as after the shipping procedure (45 days later). The samples originated from seven different WWTPs and represented municipal WWTPs with wide range of capacities from <10,000 to more than 1,000,000 equivalent citizens. Differences in the estrogenicity between the samples extracted immediately after sampling and with delay were studied to investigate stability of the samples during storage and shipping.

Sample preparation by solid-phase extraction

Water samples were extracted by solid-phase extraction with Oasis HLB cartridges (6 mL, 500 mg, Waters, CZ). Samples were filtered through glass fibre filters (2 μm, Fisher Scientific, CZ) prior to extraction. Each cartridge was activated by 6 mL of methanol (MeOH) and equilibrated by 8 mL of distilled water without vacuum. The water samples (500 mL) were passed through the wet cartridges at a flow rate of about 5 mL min^{-1}, then the columns were left to dry for 10 min, and consequently eluted by 6 mL of MeOH. Eluates were concentrated by nitrogen stream at laboratory temperature to final volumes which corresponded to 1,200 times of concentrated original effluents. This equivalent was selected as a maximal concentration which was not cytotoxic to the cells in our previous studies and enabled detecting estrogenic activity with the limits of detection (LOD) for estrogenicity of 0.5 ng EEQ per litre. Sample extracts were stored at −18 °C until analyses.

In vitro bioassays

To determine total estrogenicity of the sample extracts as well as specific potencies of individual estrogens (E1, E2, estriol (E3) and EE2), human breast carcinoma MVLN cells stably transfected under the control of estrogen receptor with firefly luciferase gene were used (Demirpence et al. 1993). Cells were grown in DMEM-F12 without phenol red (Sigma Aldrich, USA) containing 10 % foetal calf serum at 5 % CO_2 and 37 °C. Once the cells reached about 80 % confluence, they were trypsinised and seeded into a sterile 96-well plate at density 25,000 cells/well. For experiments, cells were grown in medium containing foetal calf serum treated with dextran-coated charcoal (strongly reduces concentrations of natural steroids in the calf serum). After 24 h, cells were exposed to the reference estrogen, 17β-estradiol (dilution series 1–500 pM E2), or the dilution series of other steroid estrogens (1–10,000 pM for E1 or E3; 0.1–500 pM for EE2), to the dilution series of the tested samples (at least five different concentrations), and blank and solvent controls (0.5 %*v*/*v* methanol). Exposures were conducted in three replicates for 24 h at 37 °C. After the exposure, intensity of luminescence was measured using Promega Steady Glo Kit (Promega, Mannheim, Germany). Analyses of the estrogenic potency of E1, E3 and EE2 were repeated and compared to E2 independently at least three times. Assessment of in vitro activity of the first 25 extracts of wastewaters was performed at least two times. The median standard deviation was 18 % (maximum 46 %) which was in a good agreement with our long-term results. The remaining 50 wastewaters were then analysed in a single experiment in three replicates.

Quantification of estrogenicity

Results of the estrogenicity bioassay were expressed as EEQ with respect to the standard estrogen, E2. After subtraction of the response in the solvent control, detected induction of

luminescence was related to the maximal response of standard ligand (E2max) and converted into percentages of E2max. Since most extracts did not reach 50 % of E2max (i.e. EC_{50}), the results were determined as EC_{25}. The EC_{25} values were based on relating the amount of E2 causing 25 % of the E2 response (EC_{25}) to the amount of sample causing the same level of response (Villeneuve et al. 2000). Values were determined from the nonlinear logarithmic regression of dose-response curve of calibration standard and samples using the GraphPad Prism Software (GraphPad Software, San Diego, USA).

Determination of the MVLN-cell-line-specific potencies of E1, E3 and EE2 relative to E2

After converting the results into percentages of E2max (as described in this section), EC_{50} values of dose-response curves of E2, E1, E3 and EE2 were determined from the nonlinear logarithmic regression in GraphPad Prism (GraphPad Software, San Diego, USA). The specific potencies were then determined as the ratio of EC_{50} of the model compound (E1, E3 or EE2) and EC_{50} of the reference E2. The EC_{50} of each of the model compound was always divided by EC_{50} of E2 which was obtained from measurements of cells exposed on the same microwell plate. The final specific potency relative to E2 was the mean of three independent experiments.

Statistical analyses

Nonparametric Wilcoxon match pairs test was used to assess the significance of differences of estrogenicity detected in samples extracted within 48 h and after delivery from the coordinator 45 d later. Differences in estrogenicity among the samples from six groups of WWTP effluents (four categories of municipal WWTP effluents divided according to the plant capacities, industrial WWTP effluents and 'unidentified' WWTP effluents) were tested by the nonparametric Kruskal-Wallis test. Spearman correlation was used to investigate the relationship between the results of chemical and biological analyses. All statistical analyses were performed with Statistica for Windows® 10.0 (StatSoft, Tulsa, OK, USA). For the statistical analyses, the concentrations below the LOD were replaced by one half of LOD.

Results and discussion

Verification of stability of selected samples during storage and shipping

Estrogenicities of the seven effluents extracted within 48 h after sampling were not significantly different from effects of the same samples extracted after delivery from the coordinator 45 d later (Wilcoxon match pairs test, $P=0.4$). Coefficients of variation between the freshly and later extracted samples were lower or comparable to the standard error of the used bioassay (Table 2). In two of the samples, greater concentrations of EEQs were detected in extracts prepared after longer storage (Table 2). These results demonstrate that, at least in the case of samples from the Czech Republic, there was no significant change in the estrogenic activity during prolonged storage and shipping.

Levels of detected estrogenic activity

The present study shows an overview of the pan-European situation regarding the estrogenic compounds, and for countries such as Cyprus, Lithuania or the Czech Republic, it brings some of the first publically available data on estrogenic potential in their WWTP effluents. Of the 75 WWTP effluents, 27 extracts showed estrogenic activity higher than the detection limit (>0.5 ng/L EEQ). Estrogenic activity in the 27 samples ranged from 0.53 to 17.9 ng/L EEQ with median and arithmetic mean being 1.2 and 2.7 ng/L EEQ, respectively (Fig. 1). Median and arithmetic mean of all 75 tested samples were <0.5 and 0.9 ng/L EEQ, respectively. The levels of detected EEQs are well comparable to the results of previous studies evaluating estrogenic activity of European WWTP

Table 2 Estrogenic activity in extracts of seven wastewater treatment plant (WWTP) effluents prepared directly after sampling (at 48 h) and after longer storage (45 d)

Number of WWTP	Extraction 48 h after sampling (ng/L EEQ)	Extraction 45 d after sampling (ng/L EEQ)	Coefficient of variation between samples extracted at 48 h and 45 d (%)
WWTP A2	2.0±0.4	2.1±0.5	2
WWTP A3	1.0±0.3	1.3±0.2	13
WWTP B2	0.7±0.2	1.7±0.7	40
WWTP C3	2.0±0.2	1.2±0.2	25
WWTP C8	0.8±0.2	<0.5	23[a]
WWTP D1	1.0±0.3	2.0±0.4	32
WWTP D5	<0.5	<0.5	0

Estrogenicity is expressed as 17β-estradiol equivalents (EEQ)

[a] Coefficient of variation was calculated as if the value <0.5 was 0.5

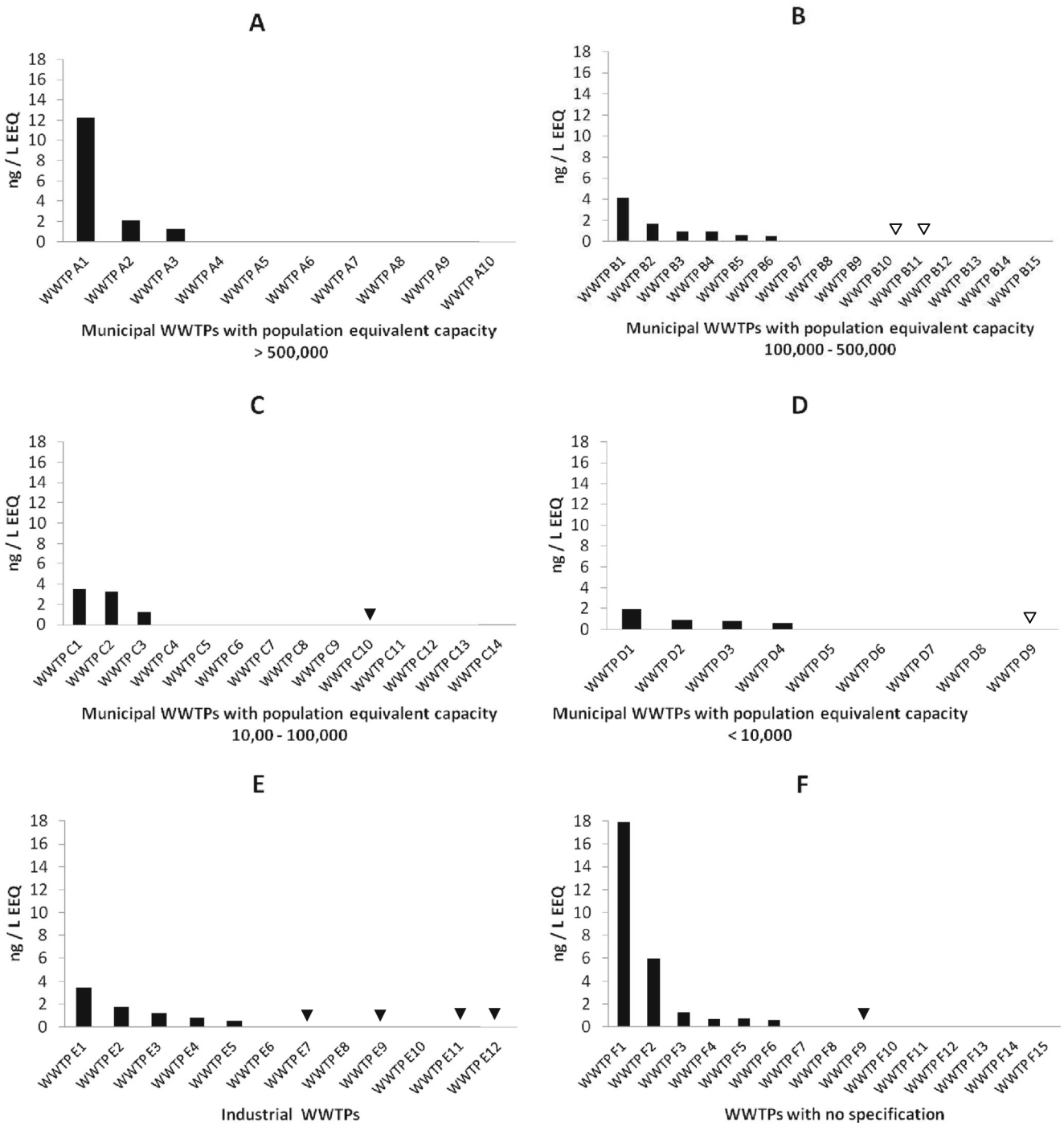

Fig. 1 Estrogenic activity expressed as 17β-estradiol equivalents (EEQ) of 75 extracts of European wastewater treatment plant (WWTP) effluents determined by MVLN in vitro assay. If no value is presented, the concentration of EEQ was less than the LOD (<0.5 ng/L). *Triangles* show cytotoxic/antiestrogenic samples (*open* and *full symbols* indicate less and more pronounced effects, respectively). **a**–**c** Municipal WWTPs, with domestic and some industrial wastewaters; **d** smaller WWTPs with most wastewaters of domestic origin; **e** industrial WWTPs; and **f** WWTPs for which no detailed information was available

effluents by different in vitro bioassays. For example, Svenson et al. (2003) used human estrogen receptor, hosted in a yeast strain, to quantify estrogenicity in samples of effluents from 20 Swedish municipal WWTPs. In this Swedish study, the treatment plants were selected to represent different treatment processes regarding chemical precipitation (coagulation and precipitation by Al or Fe to remove phosphorus and coagulate dissolved organic material) and microbial processes. The EEQs detected in Swedish WWTP effluents ranged from less than 0.1 to 15 ng/L. The other larger studies evaluating

estrogenicity of European WWTP effluents were for example those performed by Korner et al. (2001) in Germany, Vethaak et al. (2005) in the Netherlands, Aerni et al. (2004) in Switzerland or Cargouet et al. (2004) in France. After the exclusion of the one outlying value (53 ng/L EEQ) reported by Aerni et al. (2004), the levels of measured EEQ in all these studies varied from less than 0.03 to 24 ng/L, which is also in a good agreement with the results determined in the present study.

However, all the other studies reported higher frequencies of detection of positive samples in comparison to our survey. Several reasons could be considered. First, we have done no further concentrations of the initially negative samples. The detection limit of 0.5 ng/L EEQ in the present study was thus slightly higher in comparison to previous investigations (0.03–0.1 ng/L EEQ), which resulted in lower number of positive 'estrogenic' samples. Second, higher levels of EEQ in previously published studies were often detected at municipal WWTPs with other treatment technologies then activated sludge with nitrification, which was the most frequent in our study. For example, in the Dutch study by Vethaak et al. (2005) where most treatment plants consisted of an activated sludge system with nitrification step (similar to the present pan-European campaign), the frequency of positively estrogenic samples with EEQ>0.5 ng/L would be only 10 % (in contrast to reported 95 % with lower LOD of 0.1 ng/L EEQ). Second, nine samples showed decrease in estrogenic response compared to the solvent control, which may indicate either nonspecific cytotoxicity or antiestrogenicity. These two endpoints cannot be reliably distinguished by the used bioassay, and therefore, the samples were marked as antiestrogenic or cytotoxic. However, the cytotoxicity is much more common than the antiestrogenicicity in effluents, especially in municipal wastewaters. These usually contain highly potent estrogenic compounds like steroid estrogens having strong affinities to estrogenic receptors and overweighting thus the potencies of antiestrogenic compounds (e.g. Preuss et al. 2010; Johnson and Jurgens 2003). Many authors have reported estrogenic potential of WWTPs effluents (e.g. Vethaak et al. 2005; Aerni et al. 2004), but antiestrogenicity in this type of waters has only rarely been reported (Jalova et al. 2013). The nonspecific cytotoxicity can also mask the estrogenic potential, especially in highly contaminated samples. For example, Sole et al. (2000) reported fish feminisation downstream of a WWTP from textile industry where only cytotoxic (but not estrogenic) effects were detected using the in vitro system. In that case, high concentrations of contaminants (specifically the estrogenic alkylphenols) masked the actual estrogenic effect of these compounds (Sole et al. 2000). Therefore, samples found to be cytotoxic in the bioassay should be considered potentially estrogenic (or with lower probability potentially antiestrogenic).

Also, cytotoxicity in these nine specific samples could mask the effects of estrogens present in the complex mixtures. This would correspond to the study of Sole et al. (2000) who reported fish feminisation downstream of a WWTP from textile industry, where only cytotoxic (but not estrogenic) effects were detected using the in vitro system. The described arguments may explain the lower frequency of detection of estrogenic samples in the present study compared to other investigations. It should, however, be pointed out that although we have found good stability of estrogenic responses in seven of the studied samples, eventual degradation of the active compounds in other effluents cannot be fully excluded. Nevertheless, the detected ranges of EEQs provide a concise pan-European picture and correspond very well to the values reported in previous local studies from some of the countries.

Estrogenicity of different categories of WWTPs

Approximately one third of the 48 effluents that originated from municipal WWTPs displayed estrogenicity greater than the LOD of 0.5 ng/L EEQ, and 4 samples were cytotoxic/antiestrogenic (Fig. 1). The EEQ of positive extracts varied from 0.53 to 12.2 ng/L, and the greatest value was detected at a WWTP of one of the major cities with one of the highest capacities. However, statistical comparisons in estrogenicity among the groups of municipal WWTPs of different capacities showed no significant differences. Although the quantitative information on proportion of industrial and domestic wastewaters was available for a limited number of WWTPs (Table 1), the larger WWTPs with CEP of more than 100,000 typically contained not only domestic but also significant proportions of industrial wastewaters (about 11–40 %). The proportion of industrial wastewaters in effluents of municipal WWTPs had been reported to have little effect on observed rates of feminisation of fish (Jobling et al. 2006). There was no correlation between feminisation of fish and amounts of industrial wastewaters in rivers in the UK. In the same study, there was an association between the proportion of the municipal sewage effluent in the river and the incidence and magnitude of endocrine disruption in wild fishes (Jobling et al. 2006).

In the present study, there was no significant difference between estrogenicities of municipal and 'purely' industrial WWTP effluents where 9 out of 12 industrial WWTP effluents were either estrogenic (5 extracts with EEQ ranging from 0.6 to 3.4 ng/L) or cytotoxic/antiestrogenic (4 extracts). The sample with the greatest estrogenic activity among the industrial effluents originated from the WWTP of a factory that processes potatoes. The second most potent sample was from a WWTP treating wastewaters from tank vehicles, silo vehicles and cleaning of rail cars. Other samples exhibiting estrogenicity originated from a pharmaceutical factory and a company producing pesticides, whereas the cytotoxic/antiestrogenic extracts were treated wastewaters from companies processing plants in order to produce polyphenols, dyeing

textiles, cleaning tanks and vehicles (the company also accepts wastewaters from different industrial branches) or synthesizing amines. It should be pointed out that the majority of the industrial samples originated from a single country, Belgium (Table 1), so they cannot represent the Europe-wide situation for the industrial WWTP effluents.

Two of three treated wastewaters originating from factories processing plant materials were either antiestrogenic/estrogenic or cytotoxic. So far, only the phytoestrogen genistein (abundant in soya, flour and some vegetables) has been identified as the major contributor to detected estrogenicity in environmental waters (Kawanishi et al. 2004). Many other phytoestrogens (e.g. coumestrol, zearalenone, β-sitosterol, and enterolactone) have been identified in WWTP effluents or rivers, but their concentrations and/or estrogenic potencies were lower, relative to genistein or other anthropogenic estrogens (Pawlowski et al. 2003; Kawanishi et al. 2004; Lagana et al. 2004). Phytoestrogens in the environment can be significant contributors to estrogenicity of environmental waters, especially at locations close to factories processing plant materials (Liu et al. 2010).

Two out of three treated wastewaters originating from factories processing plant materials were found to be antiestrogenic or cytotoxic. As it was already discussed, this could mask the actual estrogenic effects in these samples. It is well known that many plants contain natural compounds structurally and/or functionally similar to estrogens and their active metabolites. These so-called phytoestrogens are widely present in, for example, soybeans, fruits, and cabbages. These are commonly consumed foods, and phytoestrogens thus occur in domestic wastewaters worldwide as reviewed by Liu et al. (2010). The in vitro potencies of phytoestrogens differ among individual bioassays (Liu et al. 2010), but the concentrations of phytoestrogens detected in the European municipal WWTP effluents seem to be too low to significantly contribute to detected EEQs. However, these patterns could be different in other than European municipal waters. For example, phytoestrogen genistein (abundant in soya, flour and some vegetables) has been identified as the major contributor for estrogenicity in a river water near a Japanese town where soya is a major food constituent (Kawanishi et al. 2004). Liu et al. (2010) also concluded that phytoestrogens may significantly contribute to adverse effects in organisms living downstream from WWTPs especially in countries with high consumption of phytoestrogen-rich plants (i.e. most Asian countries). Special attention should also be paid to the waters in vicinity of plant processing factories. Also, in the present study, two out of three tested effluents from WWTP serving to factories processing plants could be potentially estrogenic, and the risks associated with the phytoestrogens in these samples should be considered.

Of the 15 WWTPs for which no or limited data on collected waters or capacities were available (Table 1), six of the effluents contained detectable estrogenic activity and one sample was cytotoxic/antiestrogenic. One of the extracts contained the greatest concentration of EEQ observed in the present study, which was 17.9 ng/L, but the owner of this WWTP provided only the information on plant discharge capacity of 1,000 m^3/d, which is one of the smallest municipal WWTPs in the present study. This WWTP is situated near a town with about 7,000 citizens with light industry and agriculture including soya production and a brewery, which hypothetically could be sources of phytoestrogens that could contribute to estrogenicity. In the other five positive samples, concentrations ranged from 0.6 to 6.0 ng/L EEQ, a range that was not significantly different from estrogenicities detected in other groups of samples (Kruskal-Wallis, $P>0.05$).

Comparison of estrogenic activity with chemical analyses

Estrone, E2 and EE2, which are known to be the most potent estrogens in wastewater effluents (Gardner et al. 2012; Anderson et al. 2012), have been investigated but not detected at concentrations that were greater than the quantification limit (LOQ) of 10 ng/L in any of the samples (Loos et al. 2013). Future studies of WWTP effluents should therefore include further development of analytical methods for estrogens with detection limits in the sub nanogram per litre concentration range.

Some of the other chemicals detected have previously been reported to be estrogenic or antiestrogenic, but their actual concentrations were too low to induce observable effects in the in vitro assay. For example, effective estrogenic concentrations of triazines, hexazinon and diazinon are greater than miligrams per liter (Danzo 1997; Vonier et al. 1996), but the greatest sum of the detected concentrations of all measured triazines and triazols (atrazine, atrazine-desethyl, simazine, terbutylazine, terbutylazine-desethyl, propazine, hexazinon and diazinon) was 1.8 μg/L in the sample WWTP B12, which did not have estrogenic activity exceeding the LOD (Fig. 1).

Concentrations of target analytes in each sample that elicited estrogenic or antiestrogenic/cytotoxic effects in the bioassay were further searched for the presence of elevated (several times higher than median) concentrations of any detected chemical. A few samples contained elevated concentrations of, for example, perfluoroalkyl substances or the pharmaceutical fluconazole, but similar or even greater concentrations of these pollutants were always present also in extracts that did not elicit measurable responses in the in vitro assay. The only sample that was positive in the in vitro assay (strongly cytotoxic/antiestrogenic) and contained much greater concentrations of some selected chemicals than other samples was the industrial WWTP effluent coded WWTP E9. This sample contained high concentration of triclosan (more than 4 μg/L), and it was also the only sample where siloxanes were detected (Loos et al. 2012). This

WWTP is run by a company which uses textile dyes, and it is the only WWTP that processes effluents from the textile industry that was investigated in the present study (Table 1, WWTP E9). Triclosan is currently used as an antimicrobial agent in various household applications or cosmetics but also in functional clothing such as shoes and underwear. The maximum concentration observed was high compared to the other WWTP effluents reviewed in Dann and Hontela (2011), and its concentration might have been even greater because HDPE bottles used in the present study may affect sampling of this compound (Loos et al. 2013). Antiestrogenic or estrogenic effects of triclosan have been observed at concentrations of 20 to 100 μg/L, which are greater than those detected in this survey. However, triclosan has been shown to disrupt thyroid hormone homeostasis and possibly the reproductive axis of tadpoles (*Rana catesbeiana*) at concentrations greater than those detected in the present study (e.g. 0.15 μg/L), and the detected concentration might also be toxic to algae (Dann and Hontela 2011; Brausch and Rand 2011). Much less information is available on the toxicity of large production volume chemicals, such as siloxanes, polymeric ingredients in the synthesis of silicone products (Warner et al. 2010). The main concerns are the possibility of their accumulation in arctic organisms and their toxicity via inhalation (Warner et al. 2010; Siddiqui et al. 2007), but recent investigations suggested rather minor risk under current emission levels (Redman et al. 2012).

We also investigated possible relationships (nonparametric Spearman correlation) between the results of the in vitro assay and total concentrations of all analysed contaminants as well as with the levels of various analysed chemical classes (concentrations of pharmaceuticals, personal care products, veterinary drugs, perfluoroalkyl substances, organophosphate ester flame retardants, pesticides and their metabolites, benzotriazoles, polycyclic musk fragrances, X-ray contrast agents, gadolinium compounds, and siloxanes). None of the sums of concentrations of pollutants from each of the investigated groups was correlated with concentrations of EEQ. Some of the correlations among the chemical classes were significant (Supplementary Table SI 2). The greatest value of the correlation coefficient was found between the sums of concentrations of pharmaceuticals and sweeteners (R=0.56, Supplementary Table SI 2).

A weak correlation between concentrations of EEQ and concentrations of analytes is consistent with the fact that most investigated WWTP effluents were municipal, in which steroid hormones are most likely responsible for the estrogenicity. While most of their residues were less than the LOQ of 10 ng/L, estrogenicity was detected by the in vitro assay, demonstrating the need of complementing the chemical analyses with bioanalytical approaches. Many other studies showed the advantage of using different in vitro bioassays as monitoring tools especially in (but not limited to) wastewaters (e.g. Smital et al. 2013; Vasquez and Fatta-Kassinos 2013). The current efforts aim to utilise these bioassays within routine monitoring programmes, to harmonise and standardise the protocols and to set up proper effect-based trigger values (Escher et al. 2014; Leusch et al. 2010). Recently, bioassay-based target values for estrogenicity of municipal wastewaters were suggested by the authors of the present study (Jarosova et al. 2014; see also the next chapter). The target values for estrogenic, androgenic and other endocrine-disruptive potentials in drinking waters assessed by various bioassays were also suggested (Brand et al. 2013).

Environmental risks and specific sensitivities to E1, E3 and EE2 relative to E2

Concentrations of EEQs observed in this study (0.53–17.9 ng/L) are comparable or even greater than the lowest observable effective concentration of the most potent estrogens expected to occur in WWTP effluents. For example, complete inhibition of reproduction by Chinese rare minnows (*Gobiocypris rarus*) was caused by 0.2 ng/L of EE2 (Zha et al. 2008). Therefore, detected EEQ concentrations might be of toxicological concern even though some dilution of the effluents by recipients is considered. Unfortunately, information on the proportion of sewage effluent in the recipient river was not available for WWTPs in this study; thus, the only estimation of environmental risks could be done considering the undiluted effluents.

In other studies, estrogen-related adverse effects on aquatic organisms were observed at different concentrations of EEQ determined by use of various in vitro assays. For example, Vethaak et al. (2005) found elevated concentrations of the yolk phospholipoprotein vitellogenin in blood plasma of male bream (*Abramis brama*) in a river with 0.17 ng/L EEQ as quantified by use of the in vitro ER-CALUX assay. No in vivo response was observed in fish exposed to WWTP effluents containing 7 ng/L EEQ as quantified by use of the yeast estrogen screen, YES assay (Huggett et al. 2003). However, the same study (Huggett et al. 2003) showed elevated concentrations of vitellogenin in the blood plasma of male fish exposed to effluent from different WWTPs containing similar EEQ concentrations (around 7 ng/L EEQ) as measured by YES. These inconsistencies might be due either to different sensitivities among assays or to different compositions of specific mixtures and the fact that in vitro and in vivo sensitivities to individual compounds can be significantly different (Jarosova et al. 2014; Environment Agency 2004). Another reason might be interactions among molecules within the mixtures (Leusch et al. 2005). Nevertheless, the usefulness of in vitro assays for evaluating estrogenic activity in different types of waters has been recognised (Leusch et al. 2010; Murk et al. 2002), and bioassays are now being harmonised and standardised as a prospective tiered monitoring tool.

Although the concentration of EEQ that is of toxicological concern based on in vitro assays has not yet been determined (Leusch et al. 2010), some suggestions for municipal wastewaters have been developed recently (Jarosova et al. 2014). By combining data from the literature on the occurrence and bioassay-specific in vitro potencies of the most potent estrogens found in municipal WWTPs (i.e. E1, E2, E3 and EE2) and taking into account predicted no-effect concentrations (PNECs) for these compounds derived from fish studies (Caldwell et al. 2012; Supplementary Table SI 1), we have recently derived concentrations of EEQ less than which none of the PNECs of any of the major steroids would be exceeded (Jarosova et al. 2014). When estrogenicity of certain samples exceeds suggested PNEC for EEQ based on a specific in vitro bioassay, potential in vivo risk cannot be excluded. For the MVLN assay used in the present study, the derived estrogenic limits were 0.3 ng/L EEQ for longer-term exposures and 1.4 ng/L EEQ for shorter-term exposures (Jarosova et al. 2014). The longer-term limits were derived from PNECs of lifetime and multigeneration studies and therefore were meant to be generally used. In contrast, the shorter-term limits are relevant for events lasting only several days like sewage overflows. All samples in which estrogenicity was detected in this study (n=27) exceeded the longer-term limit, and nine of them exceeded also the shorter-term limit. This indicates that estrogens in the 'positively estrogenic samples' can cause risks to aquatic organisms unless the dilution of recipient is higher than factor of 2–60. For example, the longer-term limit in a recipient of effluent containing 17.9 ng/L EEQ would be met only if the contribution of the effluent was less than 2 % of total water volume in the recipient. For recipient of effluent with averaged estrogenicity (0.9 ng/L EEQ), the longer-term limit would be met in causes when the effluent accounts for less than about 30 % of water mass. Thus, according to the results of the bioassay, a considerable number of European WWTP effluents might pose risks to aquatic organisms living in their receiving waters.

Conclusions

This study of estrogenic potential in European WWTPs effluents clearly demonstrated how bioanalytical / bioassay tools complement the knowledge gained by traditional analytical techniques. Routine analyses of steroid estrogens were not sensitive enough to capture these compounds occurring in low concentrations, whereas bioassays revealed the overall estrogenic potential of the same samples. Furthermore, the bioanalytical results confirmed the hypothesis that a considerable number of wastewater effluents across Europe are estrogenic, and detected estrogenicity levels might be of serious toxicological concern. The study shows the importance of the effect-based monitoring approaches, which provide complementary information on potential toxicological and ecotoxicological risks of chemical mixtures.

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References

Aerni HR, Kobler B, Rutishauser BV, Wettstein FE, Fischer R, Giger W, Hungerbuhler A, Marazuela MD, Peter A, Schonenberger R, Vogeli AC, Suter MJF, Eggen RIL (2004) Combined biological and chemical assessment of estrogenic activities in wastewater treatment plant effluents. Anal Bioanal Chem 378:688–696

Anderson PD, Johnson AC, Pfeiffer D, Caldwell DJ, Hannah R, Mastrocco F, Sumpter JP, Williams RJ (2012) Endocrine disruption due to estrogens derived from humans predicted to be low in the majority of U.S. surface waters. Environ Toxicol Chem 31:1407–1415

Arditsoglou A, Voutsa D (2008) Determination of phenolic and steroid endocrine disrupting compounds in environmental matrices. Environ Sci Pollut Res 15:228–236

Brand W, de Jongh CM, van der Linden SC, Mennes W, Puijker LM, van Leeuwen CJ, van Wezel AP, Schriks M, Heringa MB (2013) Trigger values for investigation of hormonal activity in drinking water and its sources using CALUX bioassays. Environ Int 55:109–118

Brausch JM, Rand GM (2011) A review of personal care products in the aquatic environment: environmental concentrations and toxicity. Chemosphere 82:1518–1532

Caldwell DJ, Mastrocco F, Anderson PD, Lange R, Sumpter JP (2012) Predicted-no-effect concentrations for the steroid estrogens estrone, 17 beta-estradiol, estriol, and 17 alpha-ethinylestradiol. Environ Toxicol Chem 31:1396–1406

Cargouet M, Perdiz D, Mouatassim-Souali A, Tamisier-Karolak S, Levi Y (2004) Assessment of river contamination by estrogenic compounds in Paris area (France). Sci Total Environ 324:55–66

Dann AB, Hontela A (2011) Triclosan: environmental exposure, toxicity and mechanisms of action. J Appl Toxicol 31:285–311

Danzo BJ (1997) Environmental xenobiotics may disrupt normal endocrine function by interfering with the binding of physiological ligands to steroid receptors and binding proteins. Environ Health Perspect 105:294–301

Demirpence E, Duchesne MJ, Badia E, Gagne D, Pons M (1993) MVLN cells—a bioluminescent Mcf-7-derived cell-line to study the modulation of estrogenic activity. J Steroid Biochem Mol Biol 46:355–364

Environment Agency (2004) Proposed predicted-no-effect-concentrations (PNECs) for natural and synthetic steroid oestrogens in surface waters. P2-T04/1. R&D Technical Report. Bristol, UK

Escher BI, van Daele C, Dutt M, Tang JYM, Altenburger R (2013) Most oxidative stress response in water samples comes from unknown chemicals: the need for effect-based water quality trigger values. Environ Sci Technol 47(13):7002–7011

Escher BI, Allinson M, Altenburger R, Bain PA, Balaguer P, Busch W et al (2014) Benchmarking organic micropollutants in wastewater, recycled water and drinking water with in vitro bioassays. Environ Sci Technol 48(3):1940–1956

Gardner M, Comber S, Scrimshaw MD, Cartmell E, Lester J, Ellor B (2012) The significance of hazardous chemicals in wastewater treatment works effluents. Sci Total Environ 437:363–372

Gawlik BM, Loos R, Bidoglio G, Fauler G, Guo X, Lankmayr E, Linsinger T (2012) Testing sample stability in short-term isochronous stability studies for EU-wide monitoring surveys of polar organic contaminants in water. Trends Anal Chem 36:36–46

Huggett DB, Foran CM, Brooks BW, Weston J, Peterson B, Marsh KE, La Point TW, Schlenk D (2003) Comparison of in vitro and in vivo bioassays for estrogenicity in effluent from North American municipal wastewater facilities. Toxicol Sci 72:77–83

Jalova V, Jarosova B, Blaha L, Giesy JP, Ocelka T, Grabic R, Jurcikova J, Vrana B, Hilscherova K (2013) Estrogen-, androgen- and aryl hydrocarbon receptor mediated activities in passive and composite samples from municipal waste and surface waters. Environ Int 59:372–383

Jarosova B, Blaha L, Giesy JP, Hilscherova K (2014) What level of estrogenic activity determined by in vitro assays in municipal waste waters can be considered as safe? Environ Int 64:98–109

Jobling S, Williams R, Johnson A, Taylor A, Gross-Sorokin M, Nolan M, Tyler CR, van Aerle R, Santos E, Brighty G (2006) Predicted exposures to steroid estrogens in UK rivers correlate with widespread sexual disruption in wild fish populations. Environ Health Perspect 114:32–39

Johnson A, Jurgens M (2003) Endocrine active industrial chemicals: release and occurrence in the environment. Pure Appl Chem 75: 1895–1904

Kawanishi M, Takamura-Enya T, Ermawati R, Shimohara C, Sakamoto M, Matsukawa K, Matsuda T, Murahashi T, Matsui S, Wakabayashi K, Watanabe T, Tashiro Y, Yagi T (2004) Detection of genistein as an estrogenic contaminant of river water in Osaka. Environ Sci Technol 38:6424–6429

Keith TL, Snyder SA, Naylor CG, Staples CA, Summer C, Kannan K, Giesy JP (2001) Identification and quantification of nonylphenol ethoxylates and nonylphenol in fish tissues of Michigan, USA. Environ Sci Technol 10:10–13

Korner W, Spengler P, Bolz U, Schuller W, Hanf V, Metzger JW (2001) Substances with estrogenic activity in effluents of sewage treatment plants in southwestern Germany. 2. Biological analysis. Environ Toxicol Chem 20:2142–2151

Lagana A, Bacaloni A, De Leva I, Faberi A, Fago G, Marino A (2004) Analytical methodologies for determining the occurrence of endocrine disrupting chemicals in sewage treatment plants and natural waters. Anal Chim Acta 501:79–88

Leusch FDL, Chapman HF, Korner W, Gooneratne SR, Tremblay LA (2005) Efficacy of an advanced sewage treatment plant in southeast Queensland, Australia, to remove estrogenic chemicals. Environ Sci Technol 39:5781–5786

Leusch FDL, De Jager C, Levi Y, Lim R, Puijker L, Sacher F, Tremblay LA, Wilson VS, Chapman HF (2010) Comparison of five in vitro bioassays to measure estrogenic activity in environmental waters. Environ Sci Technol 44:3853–3860

Liu ZH, Kanjo Y, Mizutani S (2010) A review of phytoestrogens: their occurrence and fate in the environment. Water Res 44:567–577

Loos R, Carvalho R, Comero S, António DC, Ghiani M, Lettieri T, Locoro G, Paracchini B, Tavazzi S, Gawlik B, Blaha L, Jarosova B, Voorspoels S, Schwesig D, Haglund P, Fick J, Gans O (2012) EU wide monitoring survey on waste water treatment plant effluents. JRC scientific and policy report, JRC 76400, EUR 25563 EN, ISBN 978-92-79-26784-0. doi:10.2788/60663

Loos R, Carvalho R, Antonio DC, Comero S, Locoro G, Tavazzi S, Paracchini B, Ghiani M, Lettieri T, Blaha L, Jarosova B, Voorspoels S, Servaes K, Haglund P, Fick J, Lindberg RH, Schwesig D, Gawlik BM (2013) EU-wide monitoring survey on emerging polar organic contaminants in wastewater treatment plant effluents. Water Res 47:6475–6487

Murk AJ, Legler J, van Lipzig MMH, Meerman JHN, Belfroid AC, Spenkelink A, van der Burg B, Rijs GBJ, Vethaak D (2002) Detection of estrogenic potency in wastewater and surface water with three in vitro bioassays. Environ Toxicol Chem 21:16–23

Pawlowski S, Ternes T, Bonerz M, Kluczka T, van der Burg B, Nau H, Erdinger L, Braunbeck T (2003) Combined in situ and in vitro assessment of the estrogenic activity of sewage and surface water samples. Toxicol Sci 75:57–65

Preuss TG, Gurer-Orhan H, Meerman J, Ratte HT (2010) Some nonylphenol isomers show antiestrogenic potency in the MVLN cell assay. Toxicol in Vitro 24:129–134

Redman AD, Mihaich E, Woodburn K, Paquin P, Powell D, McGrath JA, Di Toro DM (2012) Tissue-based risk assessment of cyclic volatile methyl siloxanes. Environ Toxicol Chem 31:1911–1919

Reemtsma T, Weiss S, Mueller J, Petrovic M, Gonzalez S, Barcelo D, Ventura F, Knepper T (2006) Polar pollutants entry into the water cycle by municipal wastewater: a European perspective. Environ Sci Technol 40:5451–5458

Runnalls TJ, Margiotta-Casaluci L, Kugathas S, Sumpter JP (2010) Pharmaceuticals in the aquatic environment: steroids and anti-steroids as high priorities for research. Hum Ecol Risk Assess 16: 1318–1338

Siddiqui WH, Stump DG, Reynolds VL, Plotzke KP, Holson JF, Meeks RG (2007) A two-generation reproductive toxicity study of decamethylcyclopentasiloxane (D-5) in rats exposed by whole-body vapor inhalation. Reprod Toxicol 23:216–225

Smital T, Terzić S, Lončar J, Senta I, Žaja R, Popović M, Mikac I, Tollefsen KE, Thomas KV, Ahel M (2013) Prioritisation of organic contaminants in a river basin using chemical analyses and bioassays. Environ Sci Pollut Res Int 20(3):1384–1395

Sole M, de Alda MJL, Castillo M, Porte C, Ladegaard-Pedersen K, Barcelo D (2000) Estrogenicity determination in sewage treatment plants and surface waters from the Catalonian area (NE Spain). Environ Sci Technol 34:5076–5083

Sumpter JP, Johnson AC (2008) 10th anniversary perspective: reflections on endocrine disruption in the aquatic environment: from known knowns to unknown unknowns (and many things in between). J Environ Monit 10:1476–1485

Svenson A, Allard AS, Ek M (2003) Removal of estrogenicity in Swedish municipal sewage treatment plants. Water Res 37:4433–4443

Tang JYM, McCarty S, Glenn E, Neale PA, Warne MSJ, Escher BI (2013) Mixture effects of organic micropollutants present in water: towards the development of effect-based water quality trigger values for baseline toxicity. Water Res 47:3300–3314

Vasquez MI, Fatta-Kassinos D (2013) Is the evaluation of "traditional" physicochemical parameters sufficient to explain the potential toxicity of the treated wastewater at sewage treatment plants? Environ Sci Pollut Res 20(6):3516–3528

Vethaak AD, Lahr J, Schrap SM, Belfroid AC, Rijs GBJ, Gerritsen A, de Boer J, Bulder AS, Grinwis GCM, Kuiper RV, Legler J, Murk TAJ, Peijnenburg W, Verhaar HJM, de Voogt P (2005) An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands. Chemosphere 59: 511–524

Villeneuve DL, Blankenship AL, Giesy JP (1998) Estrogen receptors-environmental xenobiotics. In: Denison MS, Helferich WG (eds)

Toxicant-receptor interactions and modulation of gene expression. Lippincott-Raven Publishers, Philadelphia, pp 69–99

Villeneuve DL, Blankenship AL, Giesy JP (2000) Derivation and application of relative potency estimates based on in vitro bioassay results. Environ Toxicol Chem 19:2835–2843

Vonier PM, Crain DA, McLachlan JA, Guillette LJ, Arnold SF (1996) Interaction of environmental chemicals with the estrogen and progesterone receptors from the oviduct of the American alligator. Environ Health Perspect 104:1318–1322

Wang R, Liu J, Yang X, Lin C, Huang B, Jin W, Pa X (2013) Biological response of high-back crucian carp (*Carassius auratus*) during different life stages to wastewater treatment plant effluent. Environ Sci Pollut Res 20:8612–8620

Warner NA, Evenset A, Christensen G, Gabrielsen GW, Borga K, Leknes H (2010) Volatile siloxanes in the European arctic: assessment of sources and spatial distribution. Environ Sci Technol 44:7705–7710

Zha JM, Sun LW, Zhou YQ, Spear PA, Ma M, Wang ZJ (2008) Assessment of 17 alpha-ethinylestradiol effects and underlying mechanisms in a continuous, multigeneration exposure of the Chinese rare minnow (*Gobiocypris rarus*). Toxicol Appl Pharmacol 226:298–308

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Review

What level of estrogenic activity determined by *in vitro* assays in municipal waste waters can be considered as safe?

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ABSTRACT

In vitro assays are broadly used tools to evaluate the estrogenic activity in Waste Water Treatment Plant (WWTP) effluents and their receiving rivers. Since potencies of individual estrogens to induce *in vitro* and *in vivo* responses can differ it is not possible to directly evaluate risks based on *in vitro* measures of estrogenic activity. Estrone, 17beta-estradiol, 17alfa-ethinylestradiol and to some extent, estriol have been shown to be responsible for the majority of *in vitro* estrogenic activity of municipal WWTP effluents. Therefore, in the present study safe concentrations of Estrogenic Equivalents (EEQs-SSE) in municipal WWTP effluents were derived based on simplified assumption that the steroid estrogens are responsible for all estrogenicity determined with particular *in vitro* assays. EEQs-SSEs were derived using the bioassay and testing protocol-specific *in vitro* potencies of steroid estrogens, *in vivo* predicted no effect concentration (PNECs) of these compounds, and their relative contributions to the overall estrogenicity detected in municipal WWTP effluents. EEQs-SSEs for 15 individual bioassays varied from 0.1 to 0.4 ng EEQ/L. The EEQs-SSEs are supposed to be increased by use of location-specific dilution factors of WWTP effluents entering receiving rivers. They are applicable to municipal wastewater and rivers close to their discharges, but not to industrial waste waters.

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Abbreviations: cEEQ, calculated E2-Equivalents; E1, Estrone; E2, 17β-estradiol; E3, Estriol; EE2, 17α-ethinylestradiol; EEF, Estrogenic Equivalency Factor; EEQ, 17β-estradiol equivalent; EEQ-SSE, concentration of EEQ which is safe regarding major Steroid Estrogens; Ei, E1, E2, E3 or EE2; EQS, Environmental Quality Standard; ER, Estrogenic Receptor; NP, Nonylphenol; OP, Octylphenol; *P*, Percentage of total cEEQ; PNEC, Predicted No Effect Concentration; TIE, Toxicity Identification and Evaluation; VTG, Vitellogenin; WWTP, Waste Water Treatment Plant; YES, Yeast Estrogenicity Screening Assay.

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1. Introduction

Municipal waste waters are one of the main sources of estrogenic compounds in aquatic environments (*e.g.* Bolong et al., 2009). Feminization of fish downstream of Waste Water Treatment Plants (WWTPs) discharges has been observed worldwide (Sumpter and Johnson, 2008). Some estrogenic chemicals, particularly steroid estrogens, are known to cause disruption of the endocrine system of fishes and abnormalities of the reproductive tract (*e.g.* Bolong et al., 2009; Petrovic et al., 2004) in ng/L concentrations, which commonly occur in aquatic environment worldwide.

Several approaches exist to monitor the presence of estrogenic compounds in surface waters. Traditional assessment of water contamination has been based on identifying and quantifying individual chemicals, but this approach has some limitations. It is expensive because it requires sophisticated equipment and highly trained personnel (Caldwell et al., 2012). Furthermore, the individual constituents of complex mixtures occurring in the environment might not be known or there might not be methods or standards for them. In addition, the methods might not be sufficiently sensitive to measure the individual constituents or there might be matrix interferences affecting the quantification (Caldwell et al., 2012; Korner et al., 2000). Finally, chemical analyses of selected individual micropollutants cannot always identify total estrogenic potential present in environmental samples because some antagonistic or synergistic interactions can occur (Leusch et al., 2005). Therefore, biological monitoring approaches are needed. *In situ* and *in vivo* bioassays are the most relevant tools for the detection of adverse effects but they are also expensive and time and animals consuming.

In vitro bioassays can serve as a rapid, sensitive and relatively inexpensive integrative screening method to estimate total estrogenic activity of all compounds in the mixtures that act through the same mode of action (Hilscherova et al., 2000). The most frequently used *in vitro* assays for detection of estrogenicity are transactivation assays (Kinnberg, 2003) which evaluate the ability of samples/chemicals to stimulate estrogen receptor and upregulate subsequent expression of a reporter gene (hereinafter *in vitro* estrogenicity assays). Moreover, *in vitro* estrogenicity assays are currently being considered to be used in tiered monitoring of environmental waters (Leusch et al., 2010). Several studies comparing estrogenic activity detected in environmental samples by different *in vitro* assays have been conducted showing that the assays are useful for environmental monitoring (Leusch et al., 2010; Murk et al., 2002). However, the *in vitro* potency of individual estrogens can be significantly different from their *in vivo* potencies (Environmental Agency, 2004). This was demonstrated *e.g.* in a study by Wehmas et al. (2011) who observed *in vivo* responses in male fathead minnows (*Pimephales promelas*) such as elevated levels of hepatic vitellogenin (VTG) and estrogen receptor α subunit transcripts after exposure to WWTP effluent containing 1–2 ng/L EEQ determined by T47D-KBluc assay. In contrast, isolated E2 induced *in vivo* responses at much greater concentrations (10–100 ng/L) (Wehmas et al., 2011). Therefore more work is needed to better understand what can be learned from the results of these *in vitro* assays towards *in vivo* situation; and to identify trigger levels of estrogenic activity which would allow prioritization of samples for further investigation (Leusch et al., 2010).

Concentrations greater than 1 ng/L EEQ from *in vitro* assays are often considered to be associated with adverse effects on individuals *in vivo*. This could be based on observation that the standard reference compound E2 causes adverse *in vivo* effects at concentrations greater than 1 ng/L (Environmental Agency, 2004). However, such direct comparison is not relevant because other compounds also contribute to estrogenicity detected by *in vitro* assays. For example, in a study by Vethaak et al. (2005) elevated levels of VTG in male bream (*Abramis brama*) were found in a river with EEQ levels as low as 0.17 ng/L determined by *in vitro* ER-CALUX assay. Another reason why 1 ng/L EEQ might be considered is that UK Environmental Agency (2004) derived 1 ng/L E2 equivalent as a predicted-no-effect concentration (PNEC) for instrumental analyses of total steroid oestrogens. However, this instrumental PNEC accounted for concentrations of individual steroids and their *in vivo* potencies which are, as the authors of the derivation clearly stated, significantly different from their *in vitro* potencies (Environmental Agency, 2004). Therefore this PNEC of total steroid oestrogens should not be misinterpreted as a safe concentration for *in vitro* bioassays.

The goal of this paper was the derivation of safe concentrations of total EEQ measured by *in vitro* bioassays in municipal effluents that are expected to cause no adverse effects. The main purpose of their derivation was to improve the interpretation of *in vitro* results towards *in vivo* situation. The safe EEQ concentrations were derived by: i) comparing estrogenic potencies of major known estrogens among different *in vitro* assays; ii) considering *in vivo* potencies of major steroid estrogens; and iii) taking into account relative contributions of steroid estrogens to the overall *in vitro* estrogenic activities detected in municipal WWTP effluents. The applicability of derived safe EEQ concentrations is discussed in detail.

2. Methods

2.1. Selection of the most relevant compounds responsible for estrogenic activity in municipal waste waters

A variety of diverse chemicals present in the environment have been shown to interfere with regulation of endogenous estrogens. Despite their relatively great concentrations in the environment, their potency is mostly too small to significantly contribute to observed overall estrogenic activity in complex samples (Sumpter and Johnson, 2008). There is a strong evidence from both *in vivo* and *in vitro* studies that both endogenous and synthetic steroid estrogens, including estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), and for most *in vitro* assays also estriol (E3) are usually responsible for most of the estrogenic activity in municipal waste waters and their receiving waters (*e.g.* Aerni et al., 2004; Korner et al., 2001). The first researchers who described these compounds as the causative estrogens were Desborow et al. (1998) in UK WWTP effluents. They used a Toxicity Identification and Evaluation (TIE) approach combining fractionation procedures with biological screening to separate the active extract until a sample is clean enough for efficient chemical analyses. Purdom et al. (1994) and Routledge et al. (1998) demonstrated that concentrations of steroid estrogens present in the effluents (ng/L range) could cause the effects (such as elevated levels of plasma VTG) observed in wild fish living downstream of some WWTPs. Other studies (reviewed in Caldwell et al., 2012; Environmental Agency, 2004) demonstrated that environmentally relevant concentrations of steroid estrogens can cause effects like impaired reproduction, disrupted gonadal development or altered development of sexual characteristics. Another piece of evidence that human-excreted chemicals are most probably responsible for feminization of fish is that there was no correlation between feminization of fish and amounts of industrial waste waters in UK Rivers (Jobling et al., 2006). On the other hand, the same study demonstrated clear links between the degree of endocrine disruption in wild fish and the proportion of sewage effluent in the river, and showed that predicted exposures to steroid estrogens in UK rivers correlated well with widespread sexual disruption in wild fish populations (Jobling et al., 2006).

A similar situation was observed also in other countries. For example, Snyder et al. (2001) concluded by the use of a TIE approach that E2 and EE2 were the dominant estrogens (contributed 88–99% to the total EEQ) in water samples from 3 municipal WWTPs in Michigan and Nevada, USA. Also in vicinity of Paris, France and Tamagawa River in Tokyo, Japan, steroid estrogens were identified to cause most observed estrogenicity in WWTPs effluents and their receiving waters (Cargouet et al., 2004; Nakada et al., 2004). A bioassay-directed

fractionation method was also developed and applied on male fish bile, since estrogens are mainly excreted *via* bile into the intestine in fish (Houtman et al., 2004). The natural hormones E2, E1, and E3 accounted for the majority of estrogenic activity in male bream bile at all 3 tested locations in the Netherlands (Houtman et al., 2004).

Other studies which have focused on identifying and quantifying causative estrogens in municipal WWTP effluents used comparison of chemical analyses of known estrogenic compounds with *in vitro* assessment of estrogenicity. Concentrations of detected compounds were multiplied by their relative potencies compared to E2 (derived using the *in vitro* assay); and summed using concentration additivity. The calculated E2-equivalents (cEEQ) were compared to the overall estrogenic activity determined for the whole sample extract by the *in vitro* assay (EEQ). Authors of these studies mostly concluded that steroid estrogens contributed more than 90% of the measured estrogenic activity (*e.g.* Aerni et al., 2004; Korner et al., 2001; Rutishauser et al., 2004). However, at some locations concentrations of cEEQ were significantly different from the EEQs determined by the bioassays (*e.g.* Aerni et al., 2004; Thorpe et al., 2006; Vermeirssen et al., 2005). Authors of these studies often stated that it was not clear whether the difference was caused by the combination of uncertainties in the accuracy of analytical and bio-analytical methods or by unknown estrogenic compounds or their interactions (Aerni et al., 2004; Thorpe et al., 2006; Vermeirssen et al., 2005). To address the methodological uncertainties, Avbersek et al. (2011) developed a protocol for determining steroid estrogens in environmental samples which unified the sample preparation for chemical and biological analyses. The authors obtained strong correlations ($r^2 > 0.92$) between calculated concentrations of cEEQ based on steroid estrogens and EEQ measured *in vitro* for both spiked and environmental waste water samples. However, until now their approach had not been applied to a sufficient number of waste waters to make a general conclusion.

Beside steroid estrogens, alkylphenols particularly 4-tertiary isomers of nonylphenol (NP) and to lesser extend also octylphenol (OP) have been reported to be responsible for adverse effects on fish at several hot spots associated with certain industries (Sole et al., 2000; Sumpter and Johnson, 2008). In these rivers, concentrations of NP exceeded 100 μg/L whereas their common environmental concentrations occur in the low μg/L units or less (Johnson and Jurgens, 2003; Sole et al., 2000). NP and OP are transformation products of two of the most important alkylphenol polyethoxylates which have been economically important as nonionic surfactants for decades and used in a variety of industrial and household applications and therefore are ubiquitous (Johnson et al., 2005). Despite their ubiquity, their contributions to *in vitro* estrogenicity in rivers and municipal WWTPs effluents, contrary to WWTP effluents from textile industries, is usually small and corresponds with their small *in vitro* potencies in nearly all *in vitro* assays (Table 1). In the European Union (EU), in contrast to the USA, use of nonylphenol ethoxylates as surfactants has been restricted (Directive 2003/53/EC) and consequently, their concentrations in the environment and relative contributions to estrogenicity have been decreasing in the EU in recent years. Correspondingly, the reduction of adverse estrogenic effects to fish as a result of decrease in the concentration of alkylphenol polyethoxylates and NP has been described *e.g.* in Aire river, England (Sheahan et al., 2002). In the EU, NP and OP are considered priority pollutants and their concentrations in surface waters should be reduced to less than the Environmental Quality Standards (EQSs) which are 0.3 μg NP/L and 0.1 μg OP/L as annual averages of all detected concentrations (Directive 2008/105/EC). In a recent British study of more than 160 WWTP effluents, the median concentration of NP was 0.22 μg/L, while the median concentration in streams of the USA was reported to be 0.8 μg NP/L (Gardner et al., 2012; Kolpin et al., 2002). Although the median concentration of NP reported for the study of streams in the USA was influenced by a greater focus on more polluted locations (Kolpin et al., 2002), these results indicate that different legislative regulation could result in different environmental concentrations of estrogens in various countries.

In a few studies, natural estrogenic compounds, such as phytoestrogens, have been reported to contribute significant proportions of estrogenicity in municipal WWTP effluents or their receiving waters (Liu et al., 2010). In one river in Japan, genistein was identified as the compound responsible for most of the estrogenic activity (Kawanishi et al., 2004). Genistein is one of the most abundant phytoestrogens present in soya, flour and many vegetables and it was also identified in substantial concentrations (around 10 μg/L) in treated effluents from wood pulp mills (Kiparissis et al., 2001). Some other flavonoids have been identified in WWTP effluents or rivers but their concentrations and/or estrogenic potencies were much lower (Kawanishi et al., 2004; Lagana et al., 2004; Pawlowski et al., 2003). Compounds with relatively high estrogenic potency are also mycoestrogens, such as zearalenol, although few studies (Lagana et al., 2004; Pawlowski et al., 2003) document their occurrence. A few other studies have investigated estrogenicity in surface water at localities with minimal sources from human activities and detected some estrogenic activity which might have been caused by phytoestrogens (Jarosova et al., 2012; Nadzialek et al., 2010) but these studies were not designed to identify the responsible compounds. Overall, it seems that the wide variety of phytoestrogens present in WWTP effluents and/or in surface waters could contribute to measured estrogenic activity, even though the examples of their identification are rare. Phytoestrogens should be considered as possible significant contributors to estrogenicity detected in samples from places in the vicinity of plant–product manufactures or places with greater consumption of soya (Liu et al., 2010).

Although there is always the possibility that some unexpected compounds could contribute to estrogenicity of municipal WWTP effluents at specific places, the information in literature document that steroid estrogens, particularly E1, E2, EE2 and occasionally also E3 (when *in vitro* assays responsive to E3 are used) are usually responsible for majority of estrogenic activity of municipal WWTP effluents entering rivers (Sumpter and Johnson, 2008). Therefore, the present study further focused in detail on these compounds.

2.2. In vitro potency of model estrogens

Estrogenic potencies of various compounds relative to that of E2 in different *in vitro* assays, expressed as Estrogenic Equivalency Factors (EEF), have been reviewed and the results are summarized in Table 1. The EEFs were obtained by dividing EC50 of E2 as a reference by the EC50 of corresponding compound. According to the reviewed data, EEFs of estrogens can differ by orders of magnitude, not only among different *in vitro* models but also for the same model among laboratories using different testing protocols. For example, Gutendorf and Westendorf (2001) used 48 h exposure in the MVLN assay and reported EEF of E1 to be 0.01 whereas Van den Belt et al. (2004) used 20 h exposure in the same assay and reported the EEF of E1 to be 0.2. The largest differences in EEFs of steroid estrogens among different assays can be seen for E3 (Table 1). In the YES assay, the EEF of E3 was lower by a factor of 15–416 compared to other assays. Since there can be relatively large differences in EEFs even for the same models depending on the testing procedure, the most accurate determination of the safe EEQs would be with the EEFs for the major estrogens derived in the same *in vitro* model with the same testing protocol as used for the assessment of samples. In our approach, specific sets of EEFs reported for each model and testing approach in literature and also the set determined in the model used in our laboratory (MVLN) were used to derive the safe EEQs concentrations to see potential differences among assays with various potencies of the standard estrogens. Thus, further in the text when we write about bioassays it refers not only to the used model but also to the specific testing protocol used in each laboratory that derived the EEFs, which is described in detail in the references listed in Tables 1 and SD1–SD7.

2.3. Predicted-no-effect concentrations of steroid estrogens

Steroid estrogens are known to be the most potent estrogens in *in vivo* assays, all having potencies more than a thousand-fold greater in the most sensitive organism (fish) than other estrogenic xenobiotics (Caldwell et al., 2012; Environmental Agency, 2004). Data from studies of effects on reproduction of fishes were used to develop a species sensitivity distribution and PNECs of 0.1 and 2 ng/L for EE2 and E2, respectively, were derived (Caldwell et al., 2012). These PNECs were derived from long-term studies of reproduction used as the most sensitive endpoint in fishes, and should be sufficient for protection of reproductive health in fish exposed continuously for several life stages or multiple generations. PNECs for shorter-term exposure of less than 60 d, were also derived at 0.5 and 5 ng/L for EE2 and E2, respectively (Caldwell et al., 2012). Insufficient data were available to use the same methods to derive PNECs for E1 and E3, and therefore, PNECs were based on *in vivo* VTG induction studies and *in vitro* estrogenicity study accompanied with application of safety factors and the assumption that the relative ability to induce VTG by each of the steroid estrogens corresponds with the relative effects on reproductive endpoints (Caldwell et al., 2012). Resulting PNECs were 6 ng/L for E1 and 60 ng/L for E3 during longer-term exposures, and 20 and 200 ng/L for E1 and E3 in shorter-term exposures, respectively (Caldwell et al., 2012).

2.4. Derivation of safe concentrations of EEQ

Considering that E1, E2, E3 and EE2 are usually responsible for more than 90% of *in vitro* estrogenicity of treated municipal waste waters and that these compounds are highly potent *in vivo* (especially EE2), we derived safe concentrations of EEQ for municipal WWTP effluents based on the simplified assumption that steroid estrogens are responsible for all estrogenicity determined with the *in vitro* assays. The safe concentration of EEQ is hereinafter called EEQ Safe regarding Steroid Estrogens (EEQ-SSE) to reflect how they were derived. To determine EEQ-SSE knowledge of maximal contributions of the individual steroids to total estrogenic activity of municipal WWTP effluents was needed. Therefore the literature on occurrence of E1, E2, E3 and EE2 in municipal wastewaters was reviewed. Consequently, the maximal contributions of the individual steroids to total estrogenic activity were calculated.

2.4.1. Occurrence of steroid estrogens in municipal WWTP effluents

Concentrations of all four major estrogens were analyzed in 112 samples from 51 WWTP effluents (Table 2). In total about 150 papers investigating concentrations of estrogens in WWTP effluents were reviewed but most studies either reported only summarized results or did not investigate the presence of E3 because of its relatively small potencies to cause endocrine disruption compared to E1, E2 and EE2 (Caldwell et al., 2012). However, E3 can occur in significant amounts in WWTP effluents (Table 2) and it is quite potent estrogen in some *in vitro* systems (Table 1) and therefore it might be important for interpretation of the overall results. Forty seven out of the 51 WWTP listed in Table 2 included activated sludge treatment, which is the most common technology in municipal WWTPs. Most WWTPs also employed a nitrification step, which is known to enhance degradation of steroid estrogens (*e.g.* Khanal et al., 2006). Three WWTPs utilized nitrifying and denitrifying bacteria supported by solid filters and one WWTP was a system of lagoons without any artificial biological or chemical treatment. Authors of some studies reported concentrations of steroid estrogens as means of multiple samples collected at particular WWTP. Results of these studies were also included in the dataset (Table 2, samples with $N > 1$).

Estrone was the most frequently detected steroid estrogen with the greatest concentrations in most WWTP effluents (Table 2). There are two main reasons for this. First, E1 was the second most abundant steroid estrogen in WWTP influents (*e.g.* Anderson et al., 2012; Liu et al., 2009), but the most abundant one—E3 is known to be quickly degraded in the treatment processes (Anderson et al., 2012; Jin et al., 2008). Second, besides degradation of E1 during treatment, E1 can also be newly formed as a degradation product of E2 (Johnson and Sumpter, 2001). Based on the published reviewed studies (supplementary materials in Anderson et al., 2012), it can be generally concluded that conventional WWTPs, utilizing activated sludge systems without de/nitrification steps, are efficient at removal of E2 (median removal 85%) and E3 (median 97%), but removal of E1 is lower with median of 67%. Some studies found E2 to occur at the greatest concentrations in WWTP effluents (Table 2), which indicates the importance of operational conditions and technology of the specific WWTPs. Comparable or greater concentrations of E2 than E1 are typically detected at municipal WWTPs with solid supported bacteria or at conventional WWTPs with shorter retention time of solids, which does not support development of diverse microbial community, particularly nitrifiers (Kirk et al., 2002; Svenson et al., 2003). Due to its relatively lower potency, E3 is rarely investigated compared to E1, E2, and EE2. E3 has been reported to be rather rapidly degraded in conventional WWTPs (Anderson et al., 2012). However, in effluents of some municipal WWTPs E3 was detected at concentrations that were greater than E1, E2 or EE2. E3 which has been reported to be the most polar estrogen, might be lost during some procedures in analytical laboratories especially cleanup of samples by use of silica (Aerni et al., 2004; Fernandez et al., 2007). The lowest concentrations and frequency of detection were reported for synthetic steroid EE2 (Table 2). Since the primary route of entry of EE2 into the aquatic environment is through excretion by women using contraceptives, the initial load of this chemical is lower than E1, E2 or E3 (Environmental Agency, 2004). EE2 is the least abundant steroid estrogen in effluents of municipal WWTPs (Table 2 and 3), but its potency to cause ED, especially in fish, is high. Moreover, its limits of detection are mostly greater than concentrations considered to be biologically potent (Table 2, Environmental Agency, 2004).

To confirm the representativeness of concentrations of steroid estrogens included in this study, their median and maximal concentrations were compared with previously reported comprehensive data sets on occurrence of estrogens in treated waste waters (Gardner et al., 2012; Miege et al., 2009). Miege et al. (2009) compiled data about concentrations of emerging pollutants including E1, E2, E3 and EE2 in WWTP influents and effluents but this compilation was not limited to the studies where all four compounds were analyzed simultaneously as in our study. Gardner et al. (2012) reported recent results of a British national study of more than 160 different municipal WWTP effluents. The medians of all three investigations are similar (Table 3). The maximal observed concentration of E1 was greater in present study compared to study by Miege at al. (2009). However, the 95%ile of concentration of E1 in this study was much lower compared to the maximal value and the 95%ile was also comparable to 95%ile reported by Gardner et al. (2012). Similarly, the maximal observed concentration of E2 was 158 ng/L in the present study and 30 ng/L in a previous study by Miege et al. (2009). However, this difference was caused by one outlier value detected in the sample from a Canadian lagoon system and the 95%ile concentration of E2 was similar to that reported by others (Table 3). The 95%ile of EE2 in the British study by Gardner et al. (2012) was lower than those reported in the present study or by Miege et al. (2009). The data in the study by Gardner et al. (2012) were more consistent with predictions by Hannah et al. (2009) who calculated concentrations of EE2 based on estimates of per capita use of EE2, water use of 200 L/capita/day, loss of EE2 *via* metabolism, and loss *via* removal in secondary treatment step in Europe and the USA to range from 0.4 to 1.2 ng/L. However, higher concentrations of EE2 reported in the present study as well as in the database presented by Miege et al. (2009) largely originate from the study of 4 WWTPs around Paris, France, where greater concentrations could be explained by greater consumption of EE2 compared to other cities (Cargouet et al., 2004).

2.4.2. Determination of percentage contribution of steroid estrogens to total cEEQ

Based on known concentrations of E1, E2, E3 and EE2 ([E1], [E2], [E3] and [EE2]) in municipal WWTP effluents and *in vitro* potencies of individual compounds relative to E2 (EEF); the cEEQ for each WWTP effluent and each bioassay were calculated (Eq. (1)).

$$cEEQ = ([E1] \times EEF_{E1}) + ([E2] \times EEF_{E2}) + ([E3] \times EEF_{E3}) + ([EE2] \times EEF_{EE2}) \quad (1)$$

As demonstrated above, the relative potencies of these four major estrogens can vary widely among different bioassays (Table 1) and this can affect the detection power of the specific assay for each estrogen. Thus, the percentage contribution of each of these four estrogens to total EEQ was derived specifically for each set of relative potencies, this means for every bioassay. Fifteen sets of EEFs for all four major steroid estrogens in estrogenicity bioassays were available in literature. MVLN assay, used at laboratory where the authors mainly work, was chosen as an example (Table 2). Calculated EEQ for the other 14 assays are available in supplementary data (Table SD 1–7).

Consequently, the percentage of total cEEQ for each steroid estrogen and each *in vitro* bioassay was determined (Eq. (2)).

$$P_{Ei} = ([Ei] \times EEF_{Ei}/cEEQ) \times 100\% \quad (2)$$

Where: P_{Ei} is percentage of total cEEQ for Ei, where Ei is E1, E2, E3 or EE2, [Ei] is concentration of Ei.

Within the extensive dataset (Table 2) we had to deal with the important issue if and how to take into consideration the concentrations bellow limits of detection (LOD) to make sure that it would not lead to underestimation or overestimation of the actual proportions of contribution of each estrogen to total EEQ. Thus, to obtain the most realistic proportions we have compared two different approaches of calculations regarding LOD to assess how much the values below LOD influence the maximal P_{Ei} values ($P_{Ei\text{-}max}$). The first approach included all samples where at least two steroid estrogens were detected (N = 78) and 1/2 of LOD was taken into account when some estrogen was not detected at concentrations greater than LOD. The second approach included only those samples in which concentrations of all 4 steroids were detected above LODs (N = 32), thus there was no influence of LOD at all. The summary of these two approaches are listed in the bottom lines of Table 2 and Tables SD 1–7 in Supplementary materials. $P_{Ei\text{-}max}$

Table 1
Estrogenic potencies of model compounds relative to 17β-estradiol (Estrogenic Equivalency Factors—EEFs) determined in different *in vitro* assays.

Chemical	YES	ER-CALUX	MELN	T47D-KBluc	E-screen	MVLN
Estrone	0.19 [a]	0.06 [b]	0.03 [c]	1.4 [d]	0.01 [c]	0.01 [e]
	0.40 [f]	0.02 [g]	0.25 [c]	0.02 [c]	0.01 [c]	0.19 [h]
	0.38 [i]	0.15 [j]			0.13 [k]	0.2 [f]
	0.10 [c]	0.4 [l]			0.10 [m]	0.13 [n]
	0.25 [c]	0.12 [o]			0.04 [c]	
	0.10 [c]				0.01 [c]	
	0.50 [p]					
	0.33 [q]					
	0.10 [q]					
	0.68 [r]					
Estriol	3.50E−03 [a]	1.00 [c]	0.18 [c]	0.23 [d]	0.07 [c]	0.083 [e]
	6.31E−03 [c]	0.04 [g]	0.08 [c]	0.05 [c]	0.30 [k]	0.11 [n]
	2.40E−03 [i]	0.14 [l]			0.25 [c]	
	3.00E−03 [q]	0.13 [o]			0.09 [c]	
	3.70E−03 [q]					
17α-ethinylestradiol	2.20 [a]	1.20 [b]	2.45 [c]	7.23 [d]	1.26 [c]	1.25 [e]
	0.89 [f]	1.86 [g]	1.15 [c]	0.35 [c]	1.07 [c]	1.6[f]
	1.19 [s]	1.2 [j]			0.17 [c]	0.10 [t]
	2.29 [c]	1.68 [l]			1.35 [k]	1.09 [n]
	0.95 [c]	1.12 [o]			1.91 [c]	
	0.71 [c]				0.91 [m]	
	0.89 [c]				1.12 [c]	
	1.23 [c]				0.68 [c]	
	1.20 [c]					
	1.14 [p]					
	1.00 [q]					
	0.50 [q]					
	1.8 [r]					
4-Nonylphenol	2.19E−05 [c]	2.29E−05 [b]	1.58E−06 [c]	3.72E−05 [c]	1.29E−05 [c]	1.3E−05 [e]
	5.75E−04 [c]	2.29E−05 [c]	9.55E−06 [c]		2.88E−05 [c]	2.8E−06 [h]
	1.00E−04 [f]	1.20E−04 [c]			7.76E−05 [c]	1.3E−05 [t]
	2.51E−05 [s]	2.30E−05 [j]			2.34E−07 [c]	3.30E−05 [f]
	7.24E−07 [c]	3.70E−05 [o]			5.75E−05 [k]	
	2.69E−04 [c]				7.59E−05 [m]	
	1.10E−03 [c]				3.89E−05 [c]	
	4.7E−04 [r]				6.92E−05 [c]	
4-tert-Octylphenol	4.79E−04 [c]	Cytotoxic [uc]	4.79E−06 [c]	1.91E−05 [c]	6.46E−05 [v]	8.3E−05 [e]
	3.63E−06 [c]	7.30E−05 [o]			9.77E−05 [k]	6.7E−06 [h]
	2.14E−03 [c]				7.59E−05 [m]	1.90E−05 [t]
	1.70E−03 [c]				6.03E−04 [c]	
	7.80E−06 [s]				4.17E−04 [c]	
Genistein	2.45E−04 [c]	6.03E−05 [c]	6.46E−04 [c]	3.02E−05 [w]	1.29E−05 [e]	1.32E−04 [e]
	4.90E−04 [c]				2.82E−04 [m]	
	4.50E−05 [x]				1.41E−04 [c]	
	3.00E−03 [yz]				8.91E−05 [c]	

values calculated by both approaches were in very good agreement for E2 and E3, and the values from more conservative second approach were used for these two compounds for further calculations. There were greater differences in case of E1 and EE2. E1 was quite often the dominant steroid detected in WWTPs effluents at high concentrations many fold greater than the LODs of other compounds (see Table 2), the determination of its $P_{E1\text{-}max}$ was not affected by LOD. Therefore $P_{E1\text{-}max}$ calculated from the measurements including LOD (91% in case of MVLN assay, see bottom of Table 2) is more realistic and relevant. On the other hand, different situation can be seen for EE2. $P_{EE2\text{-}max}$ could be more influenced by use of 1/2 of LOD, since it was much more often bellow limit of detection (more than 60% of samples) and the limits of detection varied greatly among studies (Table 2). Hence for this compound, the way of LOD calculation could have stronger effect and lead to overestimation of the actual proportions of EE2. Thus, in case of EE2 the maximal relative contributions derived from the samples where all 4 estrogens are detected is more realistic and precise. These values were also in very good agreement with 95%ile of $P_{EE2\text{-}max}$ determined by the approach including 1/2 of LOD across all assays. In summary, derivation of the most realistic EEQ-SSE_{Ei} was thus based on $P_{E2\text{-}max}$, $P_{E3\text{-}max}$ and $P_{EE2\text{-}max}$ from measurements with all values above LOD and on $P_{E1\text{-}max}$ derived from all measurements where least two steroid estrogens were detected. When less than two steroids were detected at concentrations greater than the LOD in some WWTP effluents, the percentage of total cEEQs was not determined for any steroid in this effluent, because the values would rather be indicative of the LOD than the actual contribution of cEEQ.

Percentages of contributions to total cEEQ which were derived by use of EEFs specific for the MVLN *in vitro* assay are presented in Table 2 as an example. Percentages of contributions to total cEEQ calculated for the other 14 bioassays are presented in Supplementary data (Table SD 1–7). In case of the MVLN *in vitro* assay, the ranges of percentages of total cEEQ for E1 and E2 among individual WWTPs of total cEEQ were very wide (from <10 to >90%, Table 2). The maximal percentages of total cEEQ for E3 and EE2 were 40 and 39%, respectively. Similar patterns were obtained when other *in vitro* assays were used. The maximal contribution of E1 to total cEEQ was 97% in case of YES assays and also ER-CALUX assays, 95% in case of MELN assays and 91% in case of E-screen assays (Supplementary materials—Table SD 1–7). Maximal percentage of contribution to cEEQ for E2 was more than 90% in all assays. E3 was responsible maximally for 4% of the cEEQ in the assessment on YES assays but the maximal contribution to total cEEQ by E3 was 69% when assessed by other bioassays. EE2 was usually responsible for 8–34% of total cEEQ (medians of percentage of cEEQ), but the maximal value from all of the assays was 77% (Table SD 1–7).

2.4.3. Derivation of EEQ-SSE for municipal waste waters

After determination of maximal percentage of total cEEQ contributed by each considered estrogen by use of each bioassay, EEQ Safe regarding each Steroid Estrogen (EEQ-SSE_{Ei}) was derived (Eq. (3)). It is defined as the concentration of EEQ in every bioassay below which PNECs of the steroids would not be exceeded.

$$\text{EEQ-SSE}_{Ei} = \text{EEF}_{Ei} \times \text{PNEC}_{Ei} / (P_{Ei\text{-}max}/100\%) \quad (3)$$

Where: Ei is E1, E2, E3 or EE2, EEF_{Ei} is estrogenic potency of a compound (Ei) relative to 17β-estradiol determined in specific *in vitro* assay, $PNEC_{Ei}$ is *in vivo* derived PNEC for individual Ei, and $P_{Ei\text{-}max}$ is maximal percentage of total cEEQ for each Ei determined for specific bioassay.

Here a final EEQ-SSE *i.e.* concentration of total measured EEQ in municipal effluents that is expected to cause no adverse effects is derived and represents *in vitro* EEQ at which none of the PNECs for individual estrogens, E1, E2, E3 or EE2 is exceeded. When EEQ-SSE_{Ei} were calculated for all four of these compounds, the lowest concentration was reported as the proposed EEQ-SSE.

As it was mentioned in Section 2.4.2 EEQ-SSEs were derived specifically for the 15 bioassays for which the data on EEFs of all 4 estrogens were available. For nine of the 15 included bioassays (Table 4) the lowest EEQ-SSE_{Ei} was found for EE2 (EEQ-SSE_{EE2}) despite the fact that EE2 occurred at the lowest concentrations of the investigated compounds (Table SD 8). The reason for this is the greater *in vivo* estrogenic potency

Notes to Table 1:
YES—yeast estrogenicity screening assay (Routledge and Sumpter, 1996).
ER-CALUX—Estrogen Receptor mediated Chemical Activated LUciferase gene eXpression assay (Van der Burg et al., 2010).
MELN—MCF-7 cells stably transfected with the estrogen responsive gene ERE-betaGlob-Luc-SVNeo (Balaguer et al., 2000).
T47D-KBluc—T47D human breast cancer cells stably transfected with a triplet estrogen-responsive elements–promoter–luciferase reporter gene construct (Wilson et al., 2004).
E-SCREEN—the MCF7 cell proliferation assay (Soto et al., 1998).
MVLN—MCF-7 cells stably transfected with luciferase gene under the control of estrogen receptor (Demirpence et al., 1993).
[a] Svenson et al. (2003).
[b] Murk et al. (2002).
[c] Leusch et al. (2010).
[d] Bermudez et al. (2012).
[e] Gutendorf and Westendorf (2001).
[f] Van den Belt et al. (2004).
[g] Sonneveld et al. (2006).
[h] Furuichi et al. (2004).
[i] Aerni et al. (2004).
[j] Legler et al. (2002).
[k] Drewes et al. (2005).
[l] Avbersek et al. (2011).
[m] Korner et al. (2001).
[n] Original unpublished data—*in vitro* potencies determined by the authors of the present study by comparing the EC_{50} values from dose–response curves of E_2 and other estrogens.
[o] Houtman et al. (2004).
[p] Pawlowski et al. (2004).
[q] Caldwell et al. (2012).
[r] Thorpe et al. (2006).
[s] Rutishauser et al. (2004).
[t] Snyder et al. (2001).
[u] 4-tert-Octylphenol was cytotoxic to the cells at concentrations lower than EC_{50}.
[v] Leusch et al. (2006).
[w] Wilson et al. (2004).
[x] Breinholt and Larsen (1998).
[y] Value based on EC_{10}, not EC_{50}.
[z] Nishihara et al. (2000).

Table 2
Concentrations of four main steroid estrogens (E1, E2, E3 and EE2) and their relative percentage contribution (*P*) to total calculated estrogenic equivalents (cEEQ) if assessed by MVLN assay in municipal WWTP effluents.

Country	WWTP name or code	Equiv. citizens (thousands)	N	Concentration (ng/L)				cEEQ MVLN[a]	*P*-Percentage of total cEEQ for MVLN assay[a]			
				E1	E2	E3	EE2	(ng/L)	E1	E2	E3	EE2
Austria (Clara et al., 2005)	WWTP 1	2 500	1	72	30.0	275	5.0	73.6	12	41	40	7
	WWTP 2	167	1	8.0	<5	17.0	3.0	8.6	12	29[b]	21	38
	WWTP 3	135	1	<1	<5	<1	<1	–	–	–	–	–
	WWTP 4	6	1	4.0	<5	<1	4.0	7.4	7	34[b]	1[b]	59
			1	<1	8.0	1.0	<1	8.7	1[b]	92	1	6[b]
			1	2.0	4.0	<1	<1	4.8	5	83	1[b]	11[b]
California (Drewes et al., 2005)	WWTP 1	>100	1	0.6	<1	<2	<0.7	–	–	–	–	–
	WWTP 2	>100	1	<1	<1	<1	<0.7	–	–	–	–	–
	WWTP 3	>100	1	17.7	4.4	4.0	4.1	11.5	19	38	4	39
	WWTP 4	>500	1	50.4	1.5	<4.7	<0.7	8.4	75	18	3[b]	5[b]
	WWTP 5	>100	1	11.1	6.0	4.9	<0.7	8.3	17	72	6	5
	WWTP 6	>100	1	27.5	<0.6	<3.3	<0.7	–	–	–	–	–
	WWTP 7	>500	1	16.4	1.8	<3.3	<0.7	4.4	47	41	3[b]	9[b]
Canada (Fernandez et al., 2007)	WWTP B[TF]	740	1	69.0	5.0	8.0	1.0	15.6	55	32	5	7
			1	147.0	2.0	<1.5	<7.1	24.3	76	8	0[b]	16[b]
			1	<7.6	10.0	<1.5	1.0	11.6	4[b]	86	1[b]	9
			1	<7.6	1.0	<1.5	<7.1	–	–	–	–	–
			1	<7.6	3.0	<1.5	1.0	4.6	10[b]	65	2[b]	23
			1	25.0	6.0	<1.5	<7.1	13.1	24	46	1[b]	30[b]
			1[c]	85.0	6.0	1.0	<7.1	20.6	52	29	1	19[b]
	WWTP C	195	1	10.0	<7.1	<1.5	<7.1	–	–	–	–	–
	WWTP D	720	1	18.0	<7.1	<1.5	<7.1	–	–	–	–	–
	WWTP E[W]	20	1	28.0	57.0	<1.5	<7.1	64.4	5	88	0[b]	6[b]
			1	39.0	72.0	4.0	<7.1	81.2	6	89	1	5[b]
			1	56.0	158	23.0	5.0	172.9	4	91	1	3
China, Chongqing (Ye et al., 2012)	WWTP A	117	1[d]	4.7	<1.5	<2.5	<2.5	–	–	–	–	–
	WWTP B	214	1[d]	30.4	1.9	<2.5	<2.5	7.2	53	26	2[b]	19[b]
	WWTP C	330	1[d]	4.9	<1.5	<2.5	<2.5	–	–	–	–	–
	WWTP D	59	1[d]	8.6	<1.5	8.4	<2.5	4.1	26	18[b]	22	33[b]
	WWTP E	144	1[d]	3.8	<1.5	7.7	<2.5	3.4	14	22[b]	24	40[b]
	WWTP F	150	1[d]	4.0	<1.5	<2.5	<2.5	–	–	–	–	–
	WWTP G	160	1[d]	10.6	<1.5	<2.5	<2.5	–	–	–	–	–
	WWTP H	88	1[d]	8.1	<1.5	11.0	<2.5	4.3	24	17[b]	27	32[b]
	WWTP I	n.a.	1[d]	8.4	<1.5	<2.5	<2.5	–	–	–	–	–
	WWTP J	170	1[d]	4.0	<1.5	<2.5	<2.5	–	–	–	–	–
Finland (Bjorkblom et al., 2008)	Turku	160	1[d]	65.5	0.7	<0.6	<0.2	9.0	91	8	0[b]	1[b]
France, Boredeaux (Labadie and Budzinski, 2005a)	Eysines	50	1	71.4	<2	<1	<4	–	–	–	–	–
			1[d]	57.8	4.4	2.9	<2	13.0	56	34	2	8[b]
			1[d]	17.2	<1.0	<1.0	<1.0	–	–	–	–	–
France, Saine (Labadie and Budzinski, 2005b)	Elbeuf	110	1	<2.0	<1.9	<4.5	<3.0	–	–	–	–	–
			1	4.3	<3.8	<8.0	<5.3	–	–	–	–	–
			1	<3.5	<0.6	<4.9	<0.8	–	–	–	–	–
			1	<0.5	<0.4	<0.8	<0.8	–	–	–	–	–
			1	<4.3	<2.4	<5.6	<1.1	–	–	–	–	–
	Rouen	450	1	<1.8	<1.9	<4.0	<2.9	–	–	–	–	–
			1	<3.0	<3.8	<8.0	<5.3	–	–	–	–	–
			1[d]	<3.3	<0.5	3.5	<1.1	–	–	–	–	–
			1	<0.5	<0.4	<2.1	<1.0	–	–	–	–	–
			1	<3.4	<2.5	<7.3	<1.2	–	–	–	–	–
	Tancarville	n.a.	1	<2.8	<2.5	<3.0	<2.5	–	–	–	–	–
			1[d]	4.2	<0.8	<1.8	<0.7	–	–	–	–	–
			1[d]	1.8	<0.3	<3.6	<1.0	–	–	–	–	–
			1[d]	8.3	<0.3	<1.9	<0.7	–	–	–	–	–
			1[d]	4.9	<1.4	<5.0	<1.0	–	–	–	–	–
Italy, Roma (Baronti et al., 2000), (Johnson et al., 2000)	Cobis	40	1	<0.5	<0.5	0.7	<0.5	–	–	–	–	–
			1	13.0	2.9	3.3	1.0	6.0	27	49	6	18
			1	17.0	2.2	7.3	<0.3	5.3	40	42	15	3[b]
			1	6.9	0.7	5.7	0.5	2.7	32	27	22	19
			1	5.8	0.6	1.3	<0.3	1.6	46	35	9	10[b]
			1	5.4	1.0	1.1	0.4	2.3	30	44	5	21
	Fregene	120	1	2.0	4.0	4.0	<0.5	4.9	5	81	9	5[b]
			1	3.0	7.0	5.0	2.2	10.3	4	68	5	23
			1	6.5	2.1	1.6	1.7	4.9	17	43	3	37
			1	2.5	0.6	2.2	<0.3	1.3	25	44	18	13[b]
			1	3.7	0.4	0.6	0.3	1.2	39	29	5	27
			1	4.3	0.4	0.4	0.3	1.3	40	31	3	25
			1	3.3	1.2	0.9	0.4	2.2	19	55	5	21
	Ostia	350	1	31.0	3.0	<0.5	0.6	7.6	51	40	0[b]	9
			1	54.0	6.0	18.0	<0.5	14.9	45	40	13	2[b]
			1	82.1	3.3	1.4	1.1	14.9	69	22	1	8
			1	13.0	0.7	0.6	<0.3	2.6	63	28	3	6[b]
			1	46.0	3.0	1.5	0.5	9.4	61	32	2	5

Table 2 (*continued*)

Country	WWTP name or code	Equiv. citizens (thousands)	N	Concentration (ng/L)				cEEQ MVLN[a]	*P*-Percentage of total cEEQ for MVLN assay[a]			
				E1	E2	E3	EE2	(ng/L)	E1	E2	E3	EE2
			1	35.0	1.7	0.7	0.8	7.0	62	24	1	12
Italy, Roma (Baronti et al., 2000),			1	47.0	3.5	1.1	<0.3	9.7	61	36	1	2[b]
(Johnson et al., 2000)	Roma Sud	1200	1	20.0	3.0	7.0	<0.5	6.5	38	46	11	4[b]
			1	52.0	4.0	20.0	<0.5	12.9	50	31	16	2[b]
			1	51.0	3.1	11.0	1.2	12.0	53	26	10	11
			1	30.0	1.9	6.7	<0.3	6.5	58	29	11	2[b]
			1	22.0	1.6	5.8	0.5	5.5	50	29	11	10
			1	8.7	0.5	1.8	0.5	2.4	46	22	8	24
			1	4.0	2.3	18.0	0.4	5.1	10	45	37	8
	Roma Est	800	1	9.7	0.8	0.6	0.4	2.5	49	33	3	16
			1	8.0	0.7	0.4	<0.3	1.9	52	37	2	8[b]
			1	3.7	0.6	0.8	0.4	1.6	30	40	6	25
			1	6.9	0.8	0.8	0.7	2.6	34	32	3	31
			1	10.0	0.8	1.4	0.3	2.5	49	32	6	13
	Roma Nord	800	1	11.0	3.0	11.0	<0.5	5.8	24	52	20	5[b]
			1	19.0	2.0	28.0	<0.5	7.6	31	26	39	4[b]
			1	10.0	0.9	1.1	0.3	2.7	47	35	4	14
			1	6.4	0.4	0.7	<0.3	1.5	54	30	5	11[b]
			1	6.4	0.9	1.7	0.6	2.5	32	36	7	24
			1	6.6	0.7	1.0	0.5	2.2	37	33	5	26
			1	40.0	1.9	8.4	0.5	8.3	60	23	11	7
Slovenia (Avbersek et al., 2011)	WWTP 1	50	1	4.0	1.5	12.5	<2.0	4.4	11	34	30	25[b]
			1	1.7	2.9	18.4	<2.0	6.1	3	47	32	18[b]
	WWTP 2	360	1	16.5	2.1	<1.4	<2.0	5.3	39	39	1[b]	20[b]
	WWTP 3	100	1	61.8	8.1	<1.4	<2.0	17.0	46	48	0[b]	6[b]
			1	51.1	9.0	45.7	<2.0	21.3	30	42	23	5[b]
			1	5.2	<0.4	<1.4	<2.0	–	–	–	–	–
France (Cargouet et al., 2004)	Evry	250	6	7.2	4.5	7.3	3.1	9.5	9	47	8	35
	Valenton	1200	6	6.5	7.2	5.0	4.4	13.3	6	54	4	36
	Colombes [TF]	800	6	4.3	6.6	5.7	2.7	10.7	5	62	6	28
	Aheres	8000	6	6.2	8.6	6.8	4.5	15.0	5	57	5	33
France (Muller et al., 2008)	WWTP 1	120	3[d]	5.0	1.0	<1.0	2.0	3.9	16	26	1[b]	56
			3[d]	2.0	3.0	<1.25	<2.5	4.7	5	64	1[b]	29[b]
Grees (Pothitou and Voutsa, 2008)	WWTP 1	n.a.	5	<3	<2	<3	<2.0	–	–	–	–	–
Norway (Thomas et al., 2007)	Oslo	610	6	4.0	<3	<3	<0.3	–	–	–	–	–
Switzerland (Aerni et al., 2004)[e]	Glatt	88	7	11.9	0.7	7.2	<(0.7–1)	3.4	44	20	22	14[b]
	Rontal	27	4	27.3	3.4	9.9	1.6	9.6	35	36	11	18
	Surental	38	5	4.0	2.0	<(1–1.5)	<(0.7–1)	3.0	16	66	2[b]	15[b]
France (Aerni et al., 2004)[e]	Fr. 1	30	5	5.3	2.7	<(1–1.5)	<(0.7–1)	3.8	17	69	2[b]	12[b]
	Fr. 2	28	4	4.2	6.5	<(1–1.5)	<(0.7–1)	7.6	7	86	1[b]	6[b]
Values below LOD included as ½ LOD (n = 78)	Average			17.6	5.1	6.6	1.2	11.9	32	42	8	17
	Median			6.8	1.7	1.4	0.6	6.3	31	37	5	13
	95%ile			67.1	8.8	18.2	3.8	30.4	64	86	30	38
	Max			147	158	275	5.0	173	91	92	40	59
Measurements with all values above LOD (n = 32)	Average			20.7	7.1	11.0	1.5	13.9	33	40	8	20
	Median			8.7	2.5	4.0	0.9	5.7	33	35	5	20
	95%ile			69.8	17.0	23.0	4.6	41.7	62	65	29	37
	Max			147	158	275	5.0	173	69	91	40	39

cEEQ—calculated Estrogenic Equivalent (Eq. (1)).
N—number of samples. If N > 1, only the averaged concentrations for N samples were available.
n—number of causes (measurements) included in this calculations.
n.a.—not available.
[TF]—trickling filter technology.
[W]—wetland lagoons without any other treatment steps (17d hydraulic retention time).
[a] EEF_{E1} was 0.13; EEF_{E2} was 1; EEF_{E3} was 0.11; and EEF_{EE2} was 1.09 as determined by the authors of the present study by comparing the EC50 values from dose–response curves of E2 and other estrogens in MVLN assay.
[b] ½ of LOD was taken into account.
[c] one measurement was excluded from displayed data as outlier value.
[d] N samples were measured in triplicates. Mean concentrations from repeated measurements are displayed.
[e] Only minimal and maximal values were reported in this study, therefore the averages were calculated from these values.

of EE2. The PNEC of EE2 was lower than PNECs of E1, E2 or E3 by factors ranging 10–600. For six of the 15 bioassays the EEQ-SSE_{E1} was the lowest EEQ-$SSE_{Ei.}$ These 6 bioassays had EEF_{E1} values ranging from 0.01 to 0.03, which is approximately an order of magnitude less than the EEF_{E1} derived by use of most bioassays (Table 1). In all investigated bioassays EEQ-SSE_{E2} and especially EEQ-SSE_{E3} were much greater (by factors 3–15 in the case of EEQ-SSE_{E2} and 20–95 in the case of EEQ-SSE_{E3}), than the final EEQ-SSEs, which is indicative of the lower risks posed by E3 and to a lesser extent E2 compared to E1 and EE2. This result is consistent with previous assumptions as discussed *e.g.* by Johnson and Sumpter (2001).

3. Results and discussion

3.1. Derived concentrations of EEQ-SSEs

Since *in vivo* PNECs for steroids have been determined for longer-term exposures (multi-generation studies, more than 60 d) and shorter-term

Table 3
Comparison of medians and maximal concentrations of steroid estrogens in municipal waste water treatment plant effluents among different data sets.

	E1 (ng/L)				E2 (ng/L)				E3 (ng/L)				EE2 (ng/L)			
	N	Med	Max	95%ile	N	Med	Max	95%ile	N	Med	Max	95%ile	N	Med	Max	95%ile
This study[a]	112	7	147	67	112	1.7	158	8.8	112	1.4	275	18	112	0.6	5	3.8
Miege et al. (2009)	79	10	95	n.a.	63	1.5	30	n.a.	33	1.4	275	n.a.	33	0.5	5	n.a.
Gardner et al. (2012)	162	12	n.a.	80	162	1.3	n.a.	9.5	0	–	–	–	162	0.47	n.a.	1.36

med—median.
n.a.—not available.
N—number of investigated WWTP effluents.
[a] Values below LOD included as ½ LOD.

situations (less than 60 d), EEQ-SSEs were also calculated for both exposure scenarios. Calculated *in vitro* EEQ-SSEs for longer-term exposures ranged among individual bioassays from 0.1 to 0.4 ng/L EEQ with a median of 0.3 ng/L EEQ, while EEQ-SSEs for shorter-term exposures ranged from 0.5 to 2 ng/L EEQ with a median of 1.4 ng/L EEQ (Table 4). The smaller values for the EEQ-SSEs are near LOD of most bioassays (Leusch et al., 2010). However, it is important to emphasize that WWTP effluents are usually diluted by recipients so EEQ-SSEs should further be divided by appropriate dilution factor. For example if the contribution of WWTP effluent to the river flow was 10%, the EEQ-SSEs would vary from 1 to 4 ng EEQ/L and 5 to 20 ng/L EEQ for longer-term and shorter-term exposures, respectively. Use of EEFs for individual steroid hormones and knowledge of dilution factors for specific points in space and time enable comparison of LODs of the bioassays with the EEQ-SSEs. This allows qualified decisions *e.g.* whether less expensive assays (with greater LODs) can be used for specific WWTP.

Under environmental conditions concentrations of the steroids in rivers receiving WWTP effluents vary depending on EEQ concentrations in the effluents, on amounts of waste waters discharged and on river flow, hence the dilution factor of the effluent in the river (Anderson et al., 2012). EEQ-SSEs derived for longer-term exposure scenarios are more protective and should be generally used. The EEQ-SSEs for shorter-term exposures can be used in specific cases when the samples are collected during short periods of highest concentrations of EEQ (*e.g.* during sewage over-flows or during short periods of low flows of rivers receiving concentrated WWTP effluents). In some rivers, river flow can be much lower during rainless days and/or dryer seasons and since there is less dilution, concentrations of estrogens in rivers can be greater. Increasing concentrations will increase the risk to fish health especially if this occurs during critical windows of development. However, such conditions can be of relatively short duration, lasting only several days (Anderson et al., 2012). Therefore if samples of WWTP effluents are collected during these short periods of greatest EEQ concentrations, shorter-term derived EEQ-SSE might be more accurate limit than the longer-term EEQ-SSE.

The EEQ-SSEs recalculated for the dilution factor are more relevant than the previously suggested 1 ng/L. The Table 4 demonstrates that EEQ of 1 ng/L would be protective for shorter-term exposures in 67% of the bioassays. However, for longer term exposure it would not be protective enough for any of the bioassays. As demonstrated in Section 2.2 there can be relatively great differences in the potencies of the individual estrogens among bioassays and thus the same sample can cause different levels of responses in various bioassays. The differences in EEFs among laboratories using the same model actually demonstrate the need of standardized protocols (including media, serum, cell density, exposure time etc.) for each model to be able to apply the specific set of EEFs in calculations relative to environmental samples. Certainly, the most precise EEQ-SSE derivation is based on EEFs for the major estrogens determined in the same model with the same procedure as used for the samples. On the other hand, there are at maximum 4fold differences in the overall EEQ-SSE among assays (Table 4). If some general EEQ-SSE should be derived, it should be based on the bioassays with the lowest EEFs.

3.2. EEQ-SSEs for untreated waste waters and rivers receiving municipal WWTP effluents

When untreated waste waters are considered as a possible source of estrogenic contamination, the percentage of total cEEQ for EE2 would be lower due to the presence of greater concentrations of natural estrogens (Anderson et al., 2012; Liu et al., 2009; Miege et al., 2009; Muller et al., 2008). Therefore, EEQ-SSEs derived for municipal WWTP effluents are likely to be protective enough also for untreated municipal waste waters.

EEQ-SSEs developed to assess municipal WWTP effluents might be directly applicable for the reaches of rivers that are influenced primarily by municipal WWTP effluents. The values presented in Table 4 are protective regarding all 4 considered estrogens. With increasing distance from discharges, proportions of total cEEQ might change due to differential weathering in rivers. For E1 and E2 similar ranges of half-lives at 20 °C in river water were reported to be 5 and 3 d, respectively, whereas EE2 was more persistent (Jurgens et al., 2002). Photodegradation is the primary mechanism of transformation of EE2 with a half-life in water of approximately 17 d (Jurgens et al., 2002; Sumpter et al., 2006). Greater proportions of EE2 to cEEQ were observed in river water compared to WWTP discharge (Cargouet et al., 2004). Information about compounds responsible for estrogenicity as well as for other specific modes of actions in rivers is limited compared to what is available for WWTP effluents or rivers close to their discharges. Therefore, more research is needed to enable derivation of safe concentrations of EEQ for parts of rivers which are not in close vicinity of WWTP discharges.

3.3. Applicability of derived EEQ-SSEs and future research

The derived *in vitro* EEQ-SSEs are applicable for municipal WWTP effluents and parts of rivers close to their discharges where E1, E2, E3 and EE2 are expected to be responsible for the majority of the estrogenicity. Most information on the occurrence of steroid estrogens in waste waters presented here originate from European countries, therefore the best applicability of the EEQ-SSEs should be for the situation in Europe. Different patterns might occur in other regions of the world which could change the proportion of occurrence of estrogenic compounds in waters. For instance, in Japan, there is little use of the contraceptives and therefore the contribution of EE2 to the estrogenicity would be expected to be less than in EU countries (Sumpter and Johnson, 2008). This demonstrates the possibility of different $P_{EE2\text{-}max}$ compared to those reported in dataset used in this study. Most WWTP effluents investigated in this study employed primary treatment followed by activated sludge treatment, which represent the most common type of municipal WWTPs. However, different types of treatment could also result in different ratios of steroid estrogens. Once the proposed EEQ-SSE approach is applied, the datasets used for $P_{Ei\text{-}max}$ derivation can be enlarged or modified according to relevant available information *e.g.* from national reports.

It is also necessary to point out the limited ability of *in vitro* estrogenicity assays to detect some compounds with lower *in vitro*

Table 4
Safe estrogenic equivalents regarding steroid estrogens (EEQ-SSE) as calculated for *in vitro* bioassays and municipal waste water treatment plant effluents and/or rivers close to their discharges. The EEQs-SSEs are supposed to be increased by use of location-specific dilution factors of WWTP effluents entering receiving rivers.

Assay	EEQ-SSE (ng/L EEQ)	
	Longer-term exposures	Shorter-term exposures
YES (Aerni et al., 2004), (Rutishauser et al., 2004)	0.3	1.7
YES (Svenson et al., 2003)	0.4	2.0
YES (Caldwell et al., 2012)	0.3	1.6
YES (Leusch et al., 2010)	0.2	1.2
ER-CALUX (Sonneveld et al., 2006)	0.2	0.6
ER-CALUX (Avbersek et al., 2011)	0.4	2.0
ER-CALUX (Houtman et al., 2004)	0.3	1.4
MELN (Leusch et al., 2010)	0.2	0.8
MELN (Leusch et al., 2010)	0.3	1.6
E-screen (Gutendorf and Westendorf, 2001)	0.1	0.5
E-screen (Drewes et al., 2005)	0.3	1.6
E-screen (Leusch et al., 2010)	0.3	1.1
E-screen (Leusch et al., 2010)	0.1	0.5
MVLN[a]	0.3	1.4
MVLN (Gutendorf and Westendorf, 2001)	0.1	0.5
Min	0.1	0.5
Max	0.4	2.0
Median	0.3	1.4

YES—yeast estrogenicity screening assay (Routledge and Sumpter, 1996).
ER-CALUX—Estrogen Receptor mediated Chemical Activated LUciferase gene eXpression assay (Van der Burg et al., 2010).
MELN—MCF-7 cells stably transfected with the estrogen responsive gene ERE-betaGlob-Luc-SVNeo (Balaguer et al., 2000).
E-SCREEN—the MCF7 cell proliferation assay (Soto et al., 1998).
MVLN—MCF-7 cells stably transfected with luciferase gene under the control of estrogen receptor (Demirpence et al., 1993).
[a] Unpublished data—*in vitro* potencies were determined by the authors of the present study by comparing the EC50 values from dose–response curves of E2 and other estrogens.

potencies such as NP and OP, which might lead to underestimation of their potential estrogenic effects *in vivo*. *In vivo* PNECs have not been determined yet for many estrogenic compounds and therefore more research is needed to evaluate the applicability for the samples where the steroid estrogens cannot be expected as the dominant estrogens. It should be always kept in mind that all mentioned *in vitro* estrogenicity assays evaluate one specific mechanism of action (activation of estrogen receptor, ER) and that there are usually compounds with different modes of actions in environmental matrices which might induce similar effects (*i.e.* reproduction disorders) *in vivo*.

With respect to the issue of direct modulation of ER, one should also consider potential interference of anti-estrogenic compounds, which could be present in the sample along with the steroid estrogens (Johnson and Jurgens, 2003; Preuss et al., 2010). However, several lines of evidence indicate that antiestrogens are not a major issue in common municipal waste waters. First, steroid estrogens addressed in the present study are strong activators of ER, and their presence in the complex mixture is likely to overweigh potential effect of, generally weaker, antiestrogens. There is little information on antiestrogenic potency of effluents of municipal WWTPs, whereas numerous studies have found estrogenicity (*e.g.* Aerni et al., 2004; Vethaak et al., 2005). Nevertheless, in the samples containing eventual antiestrogens, the effect of the whole mixture determined in the *in vitro* assay would probably underestimate the actual content of estrogens. Antiestrogens could partially mask the effect of estrogenic compounds. Further research is needed to quantify the possible influence of antiestrogens.

The main purpose of derivation of EEQ-SSEs was not to derive any guideline value but to better understand what can be learned from the results of *in vitro* bioassays towards *in vivo* situation. According to our opinion, adoption of such limits into legislation needs further consideration. Traditional guideline limits are derived from PNECs of particular compounds and multiplied by factors of uncertainties. When such limits for E2 and EE2 were proposed for consideration under EU Water Framework Directive, the suggested EQSs for surface waters were as low as 0.4 ng/L for E2 and 0.035 ng/L for EE2, respectively (European Commission, 2012). Correspondingly, values of EEQ-SSEs are relatively low (yet higher than mentioned EQSs), since they are derived from the low PNEC values. EEQ-SSEs based on PNEC were however derived to protect individuals not populations, which will be most probably affected at higher concentrations of estrogens (Harris et al., 2011).

4. Conclusions

Safe levels of estrogenic equivalents (EEQ-SSE) in municipal WWTP effluents were derived considering bioassay specific *in vitro* potencies of major steroidal estrogens, *in vivo* derived PNECs of these compounds, and their relative contributions to the overall estrogenic activity detected in common municipal WWTP effluents. Since the *in vivo* PNECs for the steroids have been determined for longer-term (more than 60 d) and shorter-term (less than 60 d) exposures, also the EEQ-SSEs have been calculated for shorter-term and longer-term exposure scenarios. The derived EEQ-SSEs for 15 individual bioassays varied from 0.1 to 0.4 ng/L EEQ for longer-term exposures and from 0.5 to 2 ng/L EEQ for shorter-term exposures, respectively. The EEQs-SSEs are supposed to be increased by dilution factors of WWTP effluents in receiving rivers. The best applicability of the derived EEQ-SSEs is for areas, where steroidal estrogens have been confirmed or suspected as being responsible for fish feminization downstream municipal WWTPs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.envint.2013.12.009.

References

Aerni HR, Kobler B, Rutishauser BV, Wettstein FE, Fischer R, Giger W, et al. Combined biological and chemical assessment of estrogenic activities in wastewater treatment plant effluents. Anal Bioanal Chem 2004;378(3):688–96.
Anderson PD, Johnson AC, Pfeiffer D, Caldwell DJ, Hannah R, Mastrocco F, et al. Endocrine disruption due to estrogens derived from humans predicted to be low in the majority of U.S. surface waters. Environ Toxicol Chem 2012;31(6):1407–15.
Avbersek M, Zegura B, Filipic M, Heath E. Integration of GC-MSD and ER-Calux (R) assay into a single protocol for determining steroid estrogens in environmental samples. Sci Total Environ 2011;409(23):5069–75.
Balaguer P, Fenet H, Georget V, Comunale F, Terouanne B, Gilbin R, et al. Reporter cell lines to monitor steroid and antisteroid potential of environmental samples. Ecotoxicology 2000;9(1–2):105–14.
Baronti C, Curini R, D'Ascenzo G, Di Corcia A, Gentili A, Samperi R. Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. Environ Sci Technol 2000;34(24):5059–66.
Bermudez DS, Gray LE, Wilson VS. Modelling defined mixtures of environmental oestrogens found in domestic animal and sewage treatment effluents using an in vitro oestrogen-mediated transcriptional activation assay (T47D-KBluc). Int J Androl 2012;35(3):397–406.
Bjorkblom C, Salste L, Katsiadaki I, Wiklund T, Kronberg L. Detection of estrogenic activity in municipal wastewater effluent using primary cell cultures from three-spined stickleback and chemical analysis. Chemosphere 2008;73(7):1064–70.
Bolong N, Ismail AF, Salim MR, Matsuura T. A review of the effects of emerging contaminants in wastewater and options for their removal. Desalination 2009;239(1–3):229–46.

Breinholt V, Larsen JC. Detection of weak estrogenic flavonoids using a recombinant yeast strain and a modified MCF7 cell proliferation assay. Chem Res Toxicol 1998;11(6):622–9.
Caldwell DJ, Mastrocco F, Anderson PD, Lange R, Sumpter JP. Predicted-no-effect concentrations for the steroid estrogens estrone, 17 beta-estradiol, estriol, and 17 alpha-ethinylestradiol. Environ Toxicol Chem 2012;31(6):1396–406.
Cargouet M, Perdiz D, Mouatassim-Souali A, Tamisier-Karolak S, Levi Y. Assessment of river contamination by estrogenic compounds in Paris area (France). Sci Total Environ 2004;324(1–3):55–66.
Clara M, Kreuzinger N, Strenn B, Gans O, Kroiss H. The solids retention time—a suitable design parameter to evaluate the capacity of wastewater treatment plants to remove micropollutants. Water Res 2005;39(1):97–106.
Demirpence E, Duchesne MJ, Badia E, Gagne D, Pons M. Mvln cells—a bioluminescent Mcf-7-derived cell-line to study the modulation of estrogenic activity. J Steroid Biochem Mol Biol 1993;46(3):355–64.
Desbrow C, Routledge EJ, Brighty GC, Sumpter JP, Waldock M. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. Environ Sci Technol 1998;32:1549–58.
Drewes JE, Hemming J, Ladenburger SJ, Schauer J, Sonzogni W. An assessment of endocrine disrupting activity changes during wastewater treatment through the use of bioassays and chemical measurements. Water Environ Res 2005;77(1):12–23.
Environment Agency. Proposed Predicted-No-Effect-Concentrations (PNECs) for Natural and Synthetic Steroid Oestrogens in Surface Waters. P2–T04/1. Bristol, UK: R&D Technical Report; 2004.
European Commission. Analytical methods relevant to the European Commission's 2012 proposal on Priority Substances under the Water Framework Directive. Ispra, Italy: JRC Scientific and Policy Report; 2012. http://dx.doi.org/10.2788/51497.
Fernandez MP, Ikonomou MG, Buchanan I. An assessment of estrogenic organic contaminants in Canadian wastewaters. Sci Total Environ 2007;373(1):250–69.
Furuichi T, Kannan K, Giesy JP, Masunaga S. Contribution of known endocrine disrupting substances to the estrogenic activity in Tama River water samples from Japan using instrumental analysis and in vitro reporter gene assay. Water Res 2004;38(20):4491–501.
Gardner M, Comber S, Scrimshaw MD, Cartmell E, Lester J, Ellor B. The significance of hazardous chemicals in wastewater treatment works effluents. Sci Total Environ 2012;437:363–72.
Gutendorf B, Westendorf J. Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. Toxicology 2001;166(1–2):79–89.
Hannah R, D'Aco VJ, Anderson PD, Buzby ME, Caldwell DJ, Cunningham VL, et al. Exposure assessment of 17 alpha-ethinylestradiol in surface waters of the United States and Europe. Environ Toxicol Chem 2009;28(12):2725–32.
Harris CA, Hamilton PB, Runnalls TJ, Vinciotti V, Henshaw A, Hodgson D, et al. The consequences of feminization in breeding groups of wild fish. Environ Health Perspect 2011;119:306–11.
Hilscherova K, Machala M, Kannan K, Blankenship AL, Giesy JP. Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. Environ Sci Pollut Res 2000;7(3):159–71.
Houtman CJ, Van Oostveen AM, Brouwer A, Lamoree MH, Legler J. Identification of estrogenic compounds in fish bile using bioassay-directed fractionation. Environ Sci Technol 2004;38(23):6415–23.
Jarosova B, Blaha L, Vrana B, Randak T, Grabic R, Giesy JP, et al. Changes in concentrations of hydrophilic organic contaminants and of endocrine-disrupting potential downstream of small communities located adjacent to headwaters. Environ Int 2012;45:22–31.
Jin S, Yang F, Liao T, Hui Y, Xu Y. Seasonal variations of estrogenic compounds and their estrogenicities in influent and effluent from a municipal sewage treatment plant in China. Environ Toxicol Chem 2008;27(1):146–53.
Jobling S, Williams R, Johnson A, Taylor A, Gross-Sorokin M, Nolan M, et al. Predicted exposures to steroid estrogens in UK rivers correlate with widespread sexual disruption in wild fish populations. Environ Health Perspect 2006;114:32–9.
Johnson A, Jurgens M. Endocrine active industrial chemicals: release and occurrence in the environment. Pure Appl Chem 2003;75(11–12):1895–904.
Johnson AC, Sumpter JP. Removal of endocrine-disrupting chemicals in activated sludge treatment works. Environ Sci Technol 2001;35(24):4697–703.
Johnson AC, Belfroid A, Di Corcia A. Estimating steroid oestrogen inputs into activated sludge treatment works and observations on their removal from the effluent. Sci Total Environ 2000;256(2–3):163–73.
Johnson AC, Aerni HR, Gerritsen A, Gibert M, Giger W, Hylland K, et al. Comparing steroid estrogen, and nonylphenol content across a range of European sewage plants with different treatment and management practices. Water Res 2005;39(1):47–58.
Jurgens MD, Holthaus KIE, Johnson AC, Smith JJL, Hetheridge M, Williams RJ. The potential for estradiol and ethinylestradiol degradation in English rivers. Environ Toxicol Chem 2002;21(3):480–8.
Kawanishi M, Takamura-Enya T, Ermawati R, Shimohara C, Sakamoto M, Matsukawa K, et al. Detection of genistein as an estrogenic contaminant of river water in Osaka. Environ Sci Technol 2004;38(23):6424–9.
Khanal SK, Xie B, Thompson ML, Sung SW, Ong SK, Van Leeuwen J. Fate, transport, and biodegradation of natural estrogens in the environment and engineered systems. Environ Sci Technol 2006;40(21):6537–46.
Kinnberg K. Evaluation of in vitro assays for determination of estrogenic activity in the environment. Danish Environmental Protection Agency, Danish Ministry of the Environment; 2003.
Kiparissis Y, Hughes R, Metcalfe C, Ternes T. Identification of the isoflavonoid genistein in bleached kraft mill effluent. Environ Sci Technol 2001;35(12):2423–7.
Kirk LA, Tyler CR, Lye CM, Sumpter JP. Changes in estrogenic and androgenic activities at different stages of treatment in wastewater treatment works. Environ Toxicol Chem 2002;21(5):972–9.
Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, et al. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999–2000: a national reconnaissance. Environ Sci Technol 2002;36(6):1202–11.
Korner W, Bolz U, Sussmuth W, Hiller G, Schuller W, Hanf V, et al. Input/output balance of estrogenic active compounds in a major municipal sewage plant in Germany. Chemosphere 2000;40(9–11):1131–42.
Korner W, Spengler P, Bolz U, Schuller W, Hanf V, Metzger JW. Substances with estrogenic activity in effluents of sewage treatment plants in southwestern Germany. 2. Biological analysis. Environ Toxicol Chem 2001;20(10):2142–51.
Labadie P, Budzinski H. Determination of steroidal hormone profiles along the Jalle d'Eysines River (near Bordeaux, France). Environ Sci Technol 2005a;39(14):5113–20.
Labadie P, Budzinski H. Development of an analytical procedure for determination of selected estrogens and progestagens in water samples. Anal Bioanal Chem 2005b;381(6):1199–205.
Lagana A, Bacaloni A, De Leva I, Faberi A, Fago G, Marino A. Analytical methodologies for determining the occurrence of endocrine disrupting chemicals in sewage treatment plants and natural waters. Anal Chim Acta 2004;501(1):79–88.
Legler J, Zeinstra LM, Schuitemaker F, Lanser PH, Bogerd J, Brouwer A, et al. Comparison of in vivo and in vitro reporter gene assays for short-term screening of estrogenic activity. Environ Sci Technol 2002;36(20):4410–5.
Leusch FDL, Chapman HF, Korner W, Gooneratne SR, Tremblay LA. Efficacy of an advanced sewage treatment plant in southeast Queensland, Australia, to remove estrogenic chemicals. Environ Sci Technol 2005;39(15):5781–6.
Leusch FDL, van den Heuvel MR, Chapman HF, Gooneratne SR, Eriksson AME, Tremblay LA. Development of methods for extraction and in vitro quantification of estrogenic and androgenic activity of wastewater samples. Comp Biochem Physiol C Toxicol Pharmacol 2006;143(1):117–26.
Leusch FDL, De Jager C, Levi Y, Lim R, Puijker L, Sacher F, et al. Comparison of five in vitro bioassays to measure estrogenic activity in environmental waters. Environ Sci Technol 2010;44(10):3853–60.
Liu ZH, Kanjo Y, Mizutani S. Removal mechanisms for endocrine disrupting compounds (EDCs) in wastewater treatment—physical means, biodegradation, and chemical advanced oxidation: a review. Sci Total Environ 2009;407(2):731–48.
Liu ZH, Kanjo Y, Mizutani S. A review of phytoestrogens: their occurrence and fate in the environment. Water Res 2010;44(2):567–77.
Miege C, Choubert JM, Ribeiro L, Eusebe M, Coquery M. Fate of pharmaceuticals and personal care products in wastewater treatment plants—conception of a database and first results. Environ Pollut 2009;157(5):1721–6.
Muller M, Rabenoelina F, Balaguer P, Patureau D, Lemenach K, Budzinski H, et al. Chemical and biological analysis of endocrine-disrupting hormones and estrogenic activity in an advanced sewage treatment plant. Environ Toxicol Chem 2008;27(8):1649–58.
Murk AJ, Legler J, van Lipzig MMH, Meerman JHN, Belfroid AC, Spenkelink A, et al. Detection of estrogenic potency in wastewater and surface water with three in vitro bioassays. Environ Toxicol Chem 2002;21(1):16–23.
Nadzialek S, Vanparys C, Van der Heiden E, Michaux C, Brose F, Scippo M-L, et al. Understanding the gap between the estrogenicity of an effluent and its real impact into the wild. Sci Total Environ 2010;408(4):812–21.
Nakada N, Nyunoya H, Nakamura M, Hara A, Iguchi T, Takada H. Identification of estrogenic compounds in wastewater effluent. Environ Toxicol Chem 2004;23(12):2807–15.
Nishihara T, Nishikawa J, Kanayama T, Dakeyama F, Saito K, Imagawa M, et al. Estrogenic activities of 517 chemicals by yeast two-hybrid assay. J Health Sci 2000;46(4):282–98.
Pawlowski S, Ternes T, Bonerz M, Kluczka T, Van der Burg B, Nau H, et al. Combined in situ and in vitro assessment of the estrogenic activity of sewage and surface water samples. Toxicol Sci 2003;75(1):57–65.
Pawlowski S, Ternes TA, Bonerz M, Rastall AC, Erdinger L, Braunbeck T. Estrogenicity of solid phase-extracted water samples from two municipal sewage treatment plant effluents and river Rhine water using the yeast estrogen screen. Toxicol in Vitro 2004;18(1):129–38.
Petrovic M, Eljarrat E, de Alda MJL, Barcelo D. Endocrine disrupting compounds and other emerging contaminants in the environment: a survey on new monitoring strategies and occurrence data. Anal Bioanal Chem 2004;378(3):549–62.
Pothitou P, Voutsa D. Endocrine disrupting compounds in municipal and industrial wastewater treatment plants in Northern Greece. Chemosphere 2008;73(11):1716–23.
Preuss TG, Gurer-Orhan H, Meerman J, Ratte HT. Some nonylphenol isomers show antiestrogenic potency in the MVLN cell assay. Toxicol in Vitro 2010;24:129–34.
Purdom CE, Hardiman PA, Bye VVJ, Eno NC, Tyler CR, Sumpter JP. Estrogenic effects of effluents from sewage treatment works. Chem Ecol 1994;8(4):275–85.
Routledge EJ, Sumpter JP. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ Toxicol Chem 1996;15(3):241–8.
Routledge EJ, Sheahan D, Desbrow C, Brighty GC, Waldock M, Sumpter JP. Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach. Environ Sci Technol 1998;32(11):1559–65.
Rutishauser BV, Pesonen M, Escher BI, Ackermann GE, Aerni HR, Suter MJF, et al. Comparative analysis of estrogenic activity in sewage treatment plant effluents involving three in vitro assays and chemical analysis of steroids. Environ Toxicol Chem 2004;23(4):857–64.
Sheahan DA, Brighty GC, Daniel M, Jobling S, Harries JE, Hurst MR, et al. Reduction in the estrogenic activity of a treated sewage effluent discharge to an English river as a result of a decrease in the concentration of industrially derived surfactants. Environ Toxicol Chem 2002;21(3):515–9.

Snyder SA, Villeneuve DL, Snyder EM, Giesy JP. Identification and quantification of estrogen receptor agonists in wastewater effluents. Environ Sci Technol 2001;35(18):3620–5.
Sole M, de Alda MJL, Castillo M, Porte C, Ladegaard-Pedersen K, Barcelo D. Estrogenicity determination in sewage treatment plants and surface waters from the Catalonian area (NE Spain). Environ Sci Technol 2000;34(24):5076–83.
Sonneveld E, Riteco JAC, Jansen HJ, Pieterse B, Brouwer A, Schoonen WG, et al. Comparison of in vitro and in vivo screening models for androgenic and estrogenic activities. Toxicol Sci 2006;89(1):173–87.
Soto AM, Lin TM, Justicia H, Silvia RM, Sonnenschein C. An "in culture" bioassay to assess the estrogenicity of xenobiotics (E-SCREEN). J Clean Technol Environ Toxicol Occup Med 1998;7(3):331–43.
Sumpter JP, Johnson AC. 10th Anniversary Perspective: reflections on endocrine disruption in the aquatic environment: from known knowns to unknown unknowns (and many things in between). J Environ Monit 2008;10(12):1476–85.
Sumpter JP, Johnson AC, Williams RJ, Kortenkamp A, Scholze M. Modeling effects of mixtures of endocrine disrupting chemicals at the river catchment scale. Environ Sci Technol 2006;40(17):5478–89.
Svenson A, Allard AS, Ek M. Removal of estrogenicity in Swedish municipal sewage treatment plants. Water Res 2003;37(18):4433–43.
Thomas KV, Dye C, Schlabach M, Langford KH. Source to sink tracking of selected human pharmaceuticals from two Oslo city hospitals and a wastewater treatment works. J Environ Monit 2007;9(12):1410–8.
Thorpe KL, Gross-Sorokin M, Johnson I, Brighty G, Tyler CR. An assessment of the model of concentration addition for predicting the estrogenic activity of chemical mixtures in wastewater treatment works effluents. Environ Health Perspect 2006;114:90–7.
Van den Belt K, Berckmans P, Vangenechten C, Verheyen R, Witters H. Comparative study on the in vitro in vivo estrogenic potencies of 17 beta-estradiol, estrone, 17 alpha-ethynylestradiol and nonylphenol. Aquat Toxicol 2004;66(2):183–95.
Van der Burg B, Winter R, Weimer M, Berckmans P, Suzuki G, Gijsbers L, et al. Optimization and prevalidation of the in vitro ER alpha CALUX method to test estrogenic and antiestrogenic activity of compounds. Reprod Toxicol 2010;30(1):73–80.
Vermeirssen ELM, Korner O, Schonenberger R, Suter MJF, Burkhardt-Holm P. Characterization of environmental estrogens in river water using a three pronged approach: active and passive water sampling and the analysis of accumulated estrogens in the bile of caged fish. Environ Sci Technol 2005;39(21):8191–8.
Vethaak AD, Lahr J, Schrap SM, Belfroid AC, Rijs GBJ, Gerritsen A, et al. An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands. Chemosphere 2005;59:511–24.
Wehmas LC, Cavallin JE, Durhan EJ, Kahl MD, Martinovic D, Mayasich J, et al. Screening Complex Effluents for Estrogenic Activity with the T47d-Kbluc Cell Bioassay: Assay Optimization and Comparison with in Vivo Responses in Fish. Environ Toxicol Chem 2011;30(2):439–45.
Wilson VS, Bobseine K, Gray LE. Development and characterization of a cell line that stably expresses an estrogen-responsive luciferase reporter for the detection of estrogen receptor agonist and antagonists. Toxicol Sci 2004;81(1):69–77.
Ye X, Guo XS, Cui X, Zhang X, Zhang H, Wang MK, et al. Occurrence and removal of endocrine-disrupting chemicals in wastewater treatment plants in the Three Gorges Reservoir area, Chongqing, China. J Environ Monit 2012;14(8):2204–11.

Článek XVII:

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CHARACTERIZATION OF DIOXIN-LIKE ACTIVITY OF SEDIMENTS FROM A CZECH RIVER BASIN

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Abstract—Synthetic organic chemicals are present in environmental compartments as complex mixtures and therefore their potential effects are difficult to predict. In this study, in vitro bioassays using wild-type fish and rat hepatoma cell lines and their corresponding recombinant cell systems were used to evaluate 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-like activity in extracts of sediments collected from rivers of the Czech Republic. All the sediment extracts elicited statistically significant responses in all the cell lines tested. For most sediment extracts, a complete dose–response relationship was obtained. The maximal efficacy of the samples was between 57 and 143% of the maximal induction elicited by TCDD. Greater responsiveness, sensitivity, and reproducibility were observed for recombinant than wild-type cells. Cell line-specific differences in the sensitivity to compounds present in the complex sediment extracts were observed. The TCDD equivalents (TCDD-EQs) determined from the different cell bioassays were correlated. Greater concentrations of TCDD-EQs were obtained with fish cell lines. The TCDD-EQs calculated from the results of chemical analysis of toxic equivalents (TEQs) were in good agreement with those determined by bioassays; the arly hydrocaron receptor (AhR)-effects of the identified chemicals appear to be generally additive. This indicates that most of the TCDD-like activity was accounted for by the compounds identified and quantified by instrumental analysis. Fractionation along with mass-balance calculations allowed identification of the active fractions and classes of compounds. Polycyclic aromatic hydrocarbons (PAHs) were found to be responsible for most of the AhR-mediated activity in sediments.

Keywords—In vitro bioassays 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Polycyclic aromatic hydrocarbons Organochlorines Dioxin-like activity

INTRODUCTION

Sediments serve both as a sink and a source for a number of environmental pollutants, especially hydrophobic organic contaminants [1]. Some hydrophobic organic contaminants have slow rates of degradation and can persist in the environment for long periods of time and tend to bioaccumulate and biomagnify in the food chain [2,3]. In rivers, during certain times of year due to floods or human activities, residues associated with sediments can be resuspended and become bioavailable. Classical chemical analysis of complex mixtures of organic residues present in sediments can be both resource and time intensive. Instrumental quantification methods are available for some compounds, whereas other compounds for which neither methods nor standards are available may not be identified or quantified. Chemical analyses provide little information on the biological effects of complex mixtures, and they do not account for possible interactions between or among individual chemicals. In vitro bioassays can be used as a specific chemical detector for complex mixtures. They serve as simple, rapid, and sensitive screening systems for presence of and mutual interactions of chemicals with specific modes of action [4]. The application of instrumental analyses to quantify specific compounds in combination with bioassays to quantify the total activity along with specific fractionation techniques can be applied to assess the potential effects of complex mixtures and determine putative causative agents [5].

The critical mechanism of toxicity for some hydrophobic organic contaminants is their dioxin-like activity. Dioxin-like compounds elicit a variety of toxic effects in animals, including lethality, teratogenicity, embryotoxicity, carcinogenesis, tumor promotion, and others [6,7]. These chemicals bind to the arly hydrocarbon receptor (AhR) present in the cytosol; their binding affinity has been related to the incidence and intensity of toxic effects [8,9]. Binding of a ligand to the AhR initiates a cascade of actions leading to enhanced transcription of AhR-regulated genes and increases in activities of AhR-responsive enzymes [10]. In addition to the responses of AhR-responsive enzyme activities, expression of specific reporter genes under control of AhR-mediated transcription can be measured [11]. The use of the AhR reporter gene construct often increases sensitivity of the bioassay and eliminates potential interferences and other limitations of endogenous reporters [5].

Organic compounds known to bind to the AhR include, among others, polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/DFs), polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs). These compounds are often found together in environmental matrices including sediments. The relative potencies of complex mixtures can be expressed as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) equivalent (EQ) units, determined either by bioassays or cal-

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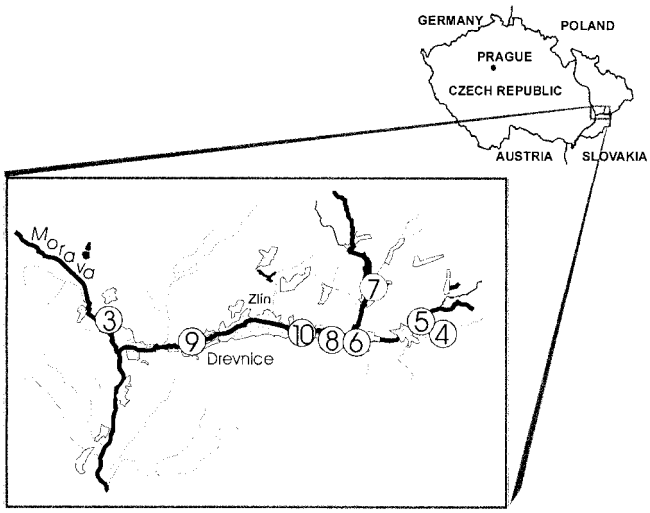

Fig. 1. Map of the Czech Republic, showing sampling locations on rivers.

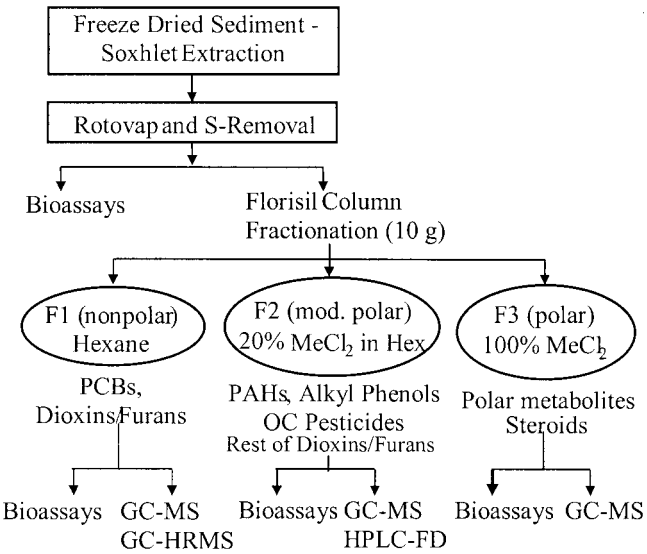

Fig. 2. Sediment extract fractionation and analysis scheme. PCB = polychlorinated biphenyls; PAH = polycyclic aromatic hydrocarbon; GC-MS = gas chromatography-mass spectrometry; GC-HRMS = gas chromatography-high resolution mass spectrometry; HPLC-FD = high-performance liquid chromatography-florescence.

culated from the concentrations of individual compounds and their relative potency factors (TEFs) [12,13]. For calculations, additivity of the effects of individual chemicals is usually assumed, while bioassays take into account nonadditive interactions such as synergism or antagonism [14].

There are no facilities in the Czech Republic to measure concentrations of dioxins and similar compounds; however, there is a great need to assess contaminated sites and prioritize remediation efforts. The objectives of this study were to test the applicability of in vitro cell bioassays for assessment of dioxin-like activity of complex mixture extracts from river sediments. Furthermore, the responsiveness of different cell lines, mammalian versus fish cell lines, and wild-type versus genetically engineered was assessed. After validation of the bioassays by comparison with the results of instrumental analyses, the less expensive but rapid and sensitive bioassay methods could be used to assess sediments for dioxin-like activity. To validate the use of the bioassay, mass-balance calculations were performed by comparison of calculated toxic equivalents based on instrumental analysis and TCDD-EQs based on bioassay results. The TCDD-EQ concentration represents the total AhR-mediated activity as determined in the bioassay. This includes, among other classes of compounds, PCDD/DFs, certain PCB congeners, and PAHs. The assay was used in conjunction with the fractionation to determine the classes of compounds causing the activity, to determine the proportion caused by each fraction, and to account for potential interactions in the complex mixture. The assay was not applied to predict the dioxin-like activity that would be likely to be accumulated into biota and to cause in vivo dioxin-like effects. In addition, to evaluate the potential for mobilization of contaminants in sediments, the results of analyses of river sediments collected before and after floods that occurred in the summer of 1997 were compared.

MATERIALS AND METHODS

Sample collection

Surface sediments were collected in the Czech Republic from the Morava River and the Drevnice River and its tributaries (Fig. 1). These rivers are in a narrow valley that contains three industrial cities. Sediments were collected after the floods in October 1997 (denoted by AF). For comparison, sediments collected in October 1996 prior to floods (denoted by BF) were also analyzed. Sediments were collected from the top 5-cm layer by use of a trowel. Large pieces of wood, leaves, and stones, greater than approximately 1 cm, were removed by hand and sediments were freeze dried. Dry sediments were homogenized, ground with a pestle and mortar, and sieved using a 2-mm sieve.

Extraction and fractionation

Twenty grams of sediment were extracted with 400 ml high-purity dichloromethane (DCM; Burdick and Jackson, Muskegon, MI, USA) in a Soxhlet apparatus for 16 h [15]. Sulfur was removed by treatment with acid-activated copper. The extracts were concentrated to approximately 5 ml in a rotary evaporator and then to 1 ml under a gentle stream of nitrogen. The whole extracts were fractionated (Fig. 2) and interferences removed by use of 10 g of activated florisil (60–100 mesh size; Sigma, St. Louis, MO, USA) packed into a glass column (10-mm i.d.). The first fraction (F1), which was eluted with 90 ml of high-purity hexane (Burdick and Jackson), contained PCBs and a portion of PCDD/DFs. Organochlorine (OC) pesticides, PAHs, and a number of their derivatives and the remaining PCDD/DFs and alkylphenolethoxylates were eluted in the second fraction (F2) with 100 ml 20% DCM in hexane. The third fraction (F3) eluted with 100 ml of 100% DCM contained the most polar compounds, including breakdown products of steroids.

Instrumental analysis

The PAHs were quantified by injecting the samples into a Hewlett-Packard 5890 series II gas chromatograph equipped with a 5972 series mass spectrometer detector (GC-MSD) (Avondale, PA, USA). Further details of PAH analysis and quantification are given elsewhere [15]. The PAH standard consisted of 16 components listed by the U.S. Environmental Protection Agency (U.S. EPA, method 8310), including acenaphthene, acenaphthylene, anthracene, benzo[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*ghi*]perylene,

benzo[*k*]fluoranthene, chrysene, dibenzo[*a*,*h*]anthracene, fluoranthene, fluorene, indeno(1,2,3-cd)pyrene, naphthalene, phenanthrene, and pyrene. Calibration standards of 0.2 to 5 μg/ml of each PAH were used.

Concentrations of noncoplanar PCBs were determined using a Hewlett-Packard 5890 series II gas chromatograph equipped with a capillary column HP-5 (Hewlett-Packard; 50-m length × 0.2-mm i.d.), with a film thickness of 0.33 μm and with an electron capture detector (GC-ECD) operated in splitless mode. The temperature program began with an 80°C hold for 1 min, followed by an increase of 20°C/min to 180°C, 3°C/min to 280°C, 20°C/min to 300°C, with a 10-min hold at 300°C. Injector and detector temperatures were 280 and 310°C, respectively. Seven indicator congeners of PCBs (28, 52, 101, 118, 153, 138, 180) were quantified by the internal standard method using calibration standard solutions at concentrations ranging from 0.01 to 4 μg/ml. The recoveries of individual PAHs and PCBs were highly consistent among sediment samples from each location.

Concentrations of 2,3,7,8-substituted PCDDs and PCDFs and non-*ortho* coplanar PCBs were measured in F1. The ^{13}C-labeled congeners of TCDD and OCDD were spiked into F1 fractions and treated with concentrated sulfuric acid. The extracts were then passed through 1 g of silica gel impregnated activated carbon to remove interfering compounds. The PCDDs/DFs and non-*ortho* coplanar PCBs were eluted with toluene and analyzed using a high-resolution gas chromatograph and a high-resolution mass spectrometer (HRGC-HRMS). A Hewlett-Packard 6890 series HRGC interfaced with an Autospec Ultima (Vg) HRMS was used. Injection was splitless. A DB-5 (60 m × 0.25-mm i.d.), at 0.25-μm film thickness, capillary column was used to separate PCDD/DF congeners. The column oven temperature was programmed from 160°C (3 min) to 200°C at a rate of 40°C/min and then to 306°C at a rate of 2°C/min. Injector and ion source temperatures were held at 280 and 250°C, respectively. The mass resolution of the mass spectrometer was greater than 10,000 MU. The mass spectrometer was operated at an electron impact energy of 40 eV. The PCDD/DF congeners and coplanar PCBs were determined by selected ion monitoring (SIM) at the two most intensive ions of the molecular ion cluster. Recoveries of internal standards varied from 55 to 96%. The estimated concentrations were not corrected for recovery. The method detection limit for PCDD/DF congeners was 0.02 pg/g dry weight. A portion of the PCDDs/DFs occurred in F2 of the florisil column fractionation but was not quantified.

Cell lines and cell culture conditions

Exposure to compounds exhibiting aromatic hydrocarbon receptor (AhR)-mediated activity was measured in two ways, i.e., as an increase in 7-ethoxyresorufin-*O*-deethylase (EROD) activity in the wild-type cells [16]; and as an increase in luciferase activity in the recombinant cells. In the wild-type cells, EROD was assessed as a specific measure of the activity of cytochrome P4501A1 that is regulated via the AhR [5]. In the recombinant cell lines, the firefly luciferase gene is under the control of a dioxin-responsive DNA enhancer element. Stimulation of the expression of this gene by AhR agonists was detected by luminiscence. Wild types of the rat hepatoma cells (H4IIE) [16,17] and dessert top minnow (*Poeciliopsis lucida*) hepatoma cells (PHLC-1) [18] were used. Recombinant cell lines derived from the wild-type rat (H4IIE-luc) [17] and rainbow trout hepatoma cell line (RLT2.0) [19,20] by stable transfection with the luciferase gene plasmid under the transcriptional regulation of dioxin-specific enhancers were also used. Cells were cultured by standard procedures developed at the Michigan State University Aquatic Toxicology Laboratory (East Lansing, MI, USA) [16,20]. Cells were cultivated in appropriate media with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) at specific temperature in a humidified CO_2 incubator, 5/95% CO_2/air, >90% humidity. The H4IIE-wt and H4IIE-luc cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma D-2902, St. Louis, MO, USA) at 37°C. The PHLC-1 cells were grown in minimal essential medium (α-MEM, Sigma M-3024) and 2 mM L-glutamine (Sigma G-5763) at 30°C. The RLT2.0 cells were grown in Basal Medium Eagle (BME; Life Technologies, Grand Island, NY, USA) with phenol red and 2 mM L-glutamine at 21°C.

For quantification of TCDD-EQ activity, cells were plated in 96-well microplates. RLT2.0 cells were plated at a density of 50,000 cells/well. All other cells were plated at 15,000 cells/well. Cells were preincubated overnight to attach and treated 24 h after plating with standards or extracts in DCM. The final concentration of solvent (dichloromethane) was 0.5%. To determine a dose–response relationship, cells were exposed to six different concentrations of the whole extract (each dilution threefold). Three to four replicates were dosed for each dilution. With every experiment, three separate TCDD calibration standards in DCM (each in three replicates) were measured. Full dose–responses were achieved for standards with final TCDD concentrations between 1.25 and 1,250 pM. After 72 h of exposure, the endpoints measured were EROD activity in H4IIE-wt and PHLC-1 cells and luciferase activity in H4IIE-luc and RLT 2.0 cells. Before measurement, confluent cells were examined microscopically to check for possible cytotoxicity or microbial/fungal contamination. The cell condition was also checked by use of a viability index [19].

Luciferase assay. Culture medium was removed, cells were washed with phosphate buffer saline, and incubated for 20 min with LucLite™ reagent (Packard Instruments, Meriden, CT, USA) at room temperature. Luciferase activity was measured as luminiscence produced at 30°C with a microplate-scanning Dynatech ML 3000 luminometer (Dynatech Laboratories, Chantilly, VA, USA) [16].

EROD assay. CYP1A activity (EROD) was measured by determining the rate of cleavage of 7-ethoxyresorufin to resorufin [16]. Briefly, medium was removed, cells were washed with PBS and lysed by freezing with nanopure water. Cells were then incubated with buffer and 4 μM 7-ethoxyresorufin at 30°C; after 20 min, nicotinamide adenine dinucleotide phosphate (NADPH) was added. After incubating for 1 h at 30°C, the reaction was stopped by addition of fluorescamine in acetonitrile for concurrent determination of protein content [21,22]. The fluorescences of resorufin and fluorescamine-protein adduct were measured simultaneously using a microplate Cytofluor 2300 (Millipore, Bedford, MA, USA) spectrofluorometer at excitation/emission wavelengths of 530/590 nm for resorufin and 400/460 nm for fluorescamine-protein adduct. Bovine serum albumin was used as a standard for quantification of protein.

Data analysis

The EROD activity in wild-type cells was recalculated for the produced resorufin, normalized to protein content, and expressed as mean pmol resorufin/min/mg protein. For recom-

Table 1. Comparison of TCDD-induction of AhR-mediated response in fish and mammalian cell lines (n = 9 for every cell line)[a]

Cell line	EC50 (pM)	Standard error	CV (%)	Linear working range (pg TCDD/well)	Variability = CV (%) within experiment: TCDD concentration/well (pg) 0.3	3	30	Maximal induction factor (max/control)
H4IIE-wt	34.5	1.96	9.9	0.1–10	10	7.3	15	4
H4IIE-luc	23.7	1.4	10	0.1–10	11	10	7	15
PHLC-1	52.6	0.95	3.1	0.3–30	7	25	22	5
RLT2.0	74.9	6.3	14.7	1–100	8	24	9	8

[a] H4IIE-wt and PHLC-1, measured EROD activity; H4IIE-luc and RLT2.0, measured luciferase activity; CV = coefficient of variation = standard deviation divided by mean, studied at three different TCDD concentrations; TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR = aryl hydrocarbon receptor.

binant cell lines, normalization to protein was found to be unnecessary. Wells were inspected to verify that they had approximately the same numbers of cells. Sample responses were expressed as relative luminiscence units (RLU) for the recombinant cell lines. Sensitivities of the cell lines to TCDD were compared by use of TCDD standard dose–response curves; EC50 concentrations were calculated by probit analysis. Due to unequal slopes and efficacies (maximal induction) of the responses, probit analysis could not be used for dose–responses of the sediment extracts. Calculation of multiple point estimates of the TCDD-EQs [23] was used to characterize these responses when slopes and efficacies were not equal. Sample responses were converted to a percentage of the mean maximum response observed for the TCDD standard ($TCDD_{max}$) and plotted as a function of log μl sample. Regression equations were derived for the linear portion of each dose–response curve. 2,3,7,8-TCDD equivalents, based on bioassay results (TCDD-EQs), were then calculated from the amount of sample producing a response equivalent to 50% of the maximal response (EC50) produced by the standard (TCDD). To account for the nonparallel slopes, the range of TCDD-EQs based on the level of response produced by EC20 and EC80 of TCDD are presented [23].

For fractions, single point estimates of TCDD-EQs were calculated by projecting the magnitude of induction caused by 1:3 dilutions of the extract fractions on the TCDD standard dose–response curve.

Total concentrations of TEQs based on instrumental analysis were calculated as the sum of TEQs from individual compounds, assuming additive responses to chemicals in the mixture [5]. The TEQs were obtained by multiplying the concentration by appropriate toxic equivalent factor (TEF) or relative potency (RP) values. The human/mammalian World Health Organization (Paris, France) (WHO) toxic equivalency factors (TEF) values were used for PCDD/DFs and PCBs [13]. For PAHs, relative potencies derived from the in vitro H4IIE-wt cell line assays were applied [24].

Because sample sizes were small and in some cases results were not normally distributed, the relationships and/or differences in the EC50 and TCDD-EQ values determined in bioassays were analyzed by nonparametric methods. The statistical significance of differences among EC50 values for TCDD were evaluated by use of the Mann–Whitney test. Relationships among TCDD-EQs derived from different cell lines and TEQs were evaluated by correlation analysis. All statistical analyses were performed with Statgraphics (Rockville, MD, USA).

RESULTS AND DISCUSSION

Comparison of cell lines

Testing conditions. The choice of DCM as the extract vehicle was directed by the extraction and analytical procedure. This solvent was equally as efficient for delivering analytes to cells as other solvents (i.e., isooctane, toluene, hexane) and it did not cause any background response in any of the tested cell lines. During incubation, DCM evaporates quickly, leaving the extracted compounds in the medium. However, due to its relatively great volatility, caution must be taken to keep dosing reproducible. Furthermore, sample dilutions must be stored at low temperature (4°C or less).

The longer exposure time (72 h) was chosen based on our previous studies and for potential metabolization of labile compounds. Time-course studies determined that maximum induction of EROD in H4IIE-wt and of luciferase activity in H4IIE-luc occurred at 72 and 24 h, respectively [16,25]. Induction of luciferase in recombinant fish cells (RLT2.0) is significantly slower than in mammalian cells, with maximal response of RLT2.0 cells to inducing compounds observed after 6 d [19]. The absolute values of EROD induction have been reported to be greater at time periods shorter than 72 h in PHLC-1 cells, with the activity least variable at 72 h [26].

Standard dose–response. The responsiveness of wild-type cells and stably transfected fish or rat hepatoma cells to TCDD standard were compared. The EC50 values in the studied cell lines were calculated from nine replicate measurements and ranged from 23 to 75 pM TCDD as determined by probit analysis (Table 1), with coefficients of variation of up to 15%. Statistically significant differences ($p < 0.05$, Mann–Whitney test) were observed between the EC50 for mammalian and fish cell lines. The EC50 values were greater for fish than for mammalian cell lines. The EC50 value for H4IIE wild-type cells was significantly greater than that for the recombinant H4IIE-luc cells. Other parameters describing the performance of different cell lines are summarized in Table 1. Variability in three different parts of the curve (lower, middle, upper) was less than 25%. The maximal induction factor, defined as the magnitude of induction relative to solvent control, was 1.5-fold to threefold greater for the recombinant cell lines. The linear working range was about 100-fold for all studied cell lines. Earlier studies have reported a threefold greater maximum induction and greater linear working range for recombinant mammalian cells relative to wild-type cells [16,27]. The inhibition of EROD activities by TCDD [11,26] was observed

Table 2. TCDD-like activity of sediment samples 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TCDD-EQs) determined in *in vitro* bioassays; presented are TCDD-EQs determined by the response equivalency approach at the level of response equivalent to 50% median effective concentration (EC50) of the maximal response produced by the standard $TCDD_{max}$ and the range of TCDD-EQs calculated from responses equivalent to 20% (EC20) and 80% (EC80) of $TCDD_{max}$ (range in parentheses)

	TCDD-EQs in studied cell lines (ng TCDD equivalents/g dry wt)			
Sample	H4IIE-wt	H4IIE-luc	PHLC-1	RLT2.0
3A	2.3 (1.8–3.0[a])	4.4 (3.5–5.7)	12.8 (9.3–17)	18.9 (18.5–19.3)
4A	9.1 (7.2–11.4)	8.6 (7.2–10.4)	27.8 (20.6–30.6)	34.6 (31–38)
5A	2.6 (2.4–2.8[a])	7.2 (8.2–6.4)	27 (20.4–30.8)	13.4 (29.7–6.2[a])
8A	1.0 (0.9–1.0[a])	1.9 (2.2–1.7[a])	6.4 (7.9–5.3[a])	2 (1.8–2.3)
9A	5.2 (4.1–6.8[a])	6.7 (5.3–8.3)	24.6 (19–31)	14.7 (14.3–15)
10A	1.9 (1.6–2.2[a])	4.6 (4.1–5.1)	19.2 (14.6–24.7)	7.1 (9.0–5.7)
5B	8.7 (6.6–11.5)	16.3 (11.8–22)	35.3 (28.6–35.3)	230[b]
7B	17.4 (11.6–26)	23 (15–35.7)	80 (54.6–113.8)	293[b]

[a] Relative potency estimates generated at magnitudes greater than the observed efficacy of sample.
[b] Response was over 50% $TCDD_{max}$ at all tested concentrations; the single point estimate is based on 90% $TCDD_{max}$ (EC90).

only at the greatest tested concentration (100 pg/well = 1,250 pM).

Dose–responses for complex mixtures. The bioassays were used as a chemical detector to report TCDD-EQ values for complex mixtures extracted from sediments. The responsiveness of different cell lines to sediment extracts was compared for six sediments sampled after floods in October 1997 (samples AF) and two sediments sampled before floods in October 1996 (samples 5BF, 7BF), which contained greater concentrations of AhR-active residues. All cell types were sufficiently sensitive to determine TCDD-EQs in sediment extracts. Depending on the cell line and sampling site, whole extracts equivalent to as little as 0.03 to 1 mg sediment were sufficient to cause significant induction relative to the solvent control. No significant cytotoxicity was observed except at the greatest dosed concentration (dose equivalent to 25 mg sediment). The wells that exhibited cytotoxicity were excluded from calculations.

Complete dose–response curves were obtained with most whole extracts in all cell lines. However, the efficacy (maximal level of induction) varied among cell lines. Some whole extracts reached efficacies greater than the maximal induction caused by the TCDD standard ($TCDD_{max}$), whereas other samples did not reach the maximal standard efficacy. In H4IIE-luc cells, all the whole extracts caused approximately the same maximal induction as TCDD. The greatest differences were observed for H4IIE-wt cells, where efficacies ranged from 53% (sample 8AF) to 154% (sample 7BF). This violates the assumption of equal efficacy required for the use of linearized functions to calculate TCDD-EQs [23]. Thus, the values calculated for the samples with variable efficacies are considered to be semiquantitative approximations [28].

Variations of the replicate measurements for samples were relatively small for both recombinant cell lines' coefficients of variation ([CV] generally <20%), but greater variations and some outlier values occurred for wild-type cells. The results from H4IIE-luc cells were most reproducible with least variability. Greater concentrations of some extracts caused inhibition of EROD activity in both mammalian and fish wild-type cells, suggesting that some compounds in the mixture can act as competitive inhibitors for the induced enzyme [26,11]. Estimation of TCDD-EQs was based on the calculation of the amount of sample needed to produce a response equivalent to EC20 to EC80 of TCDD. The estimate of the range is more appropriate than a single point estimate due to the nonparallelism of the dose–response curves (the lower the range, the more parallel the curves are) [23]. The values based on the response equivalent to EC50 were used for statistical comparison among cell lines and samples.

Caution must be exercised when calculating the TEQs for complex environmental mixtures. The effective concentration (EC) value used as a reference must elicit a response within the linear portion of the log-transformed sample dose–response curve; otherwise, the calculation would lead to significant under- or overestimation. The responses of RLT2.0 cells to sample extracts 5B and 7B were 66 to 120% of $TCDD_{max}$. Thus, the EC50 for TCDD could not be used for TCDD-EQ calculations. In these cases, the calculation was based on EC90 values, which were within the linear portion of the sample dose–response curves. However, due to the fact that these curves did not reach the same maximum, application of a different basis for calculations may have resulted in overestimation when compared with the other samples.

The dioxin-like activity expressed as ng TEQ/g sediment (TCDD-EQ) was cell-line specific (Table 2). However, while the absolute TCDD-EQ varied among cell lines, there was good correlation among TCDD-EQs of the whole extracts estimated by different cell lines (Table 3). The rank order of potency agreed well among different cell lines, with samples 5BF, 7BF, and 4AF exhibiting the greatest activity and sample 8AF the least. The results from the H4IIE-wt and H4IIE-luc were highly correlated ($R^2 > 0.85$, $p < 0.01$); the TCDD-EQs derived from

Table 3. Coefficients of determination (R^2) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TCDD-EQs) calculated on median effective concentration EC50 values evaluated by the different cell lines and toxic equivalents (TEQs) calculated from the results of chemical analysis (ANALYT)[a]

	Cell line			
	H4IIE-WT	H4IIE-LUC	PHLC-1	RLT2.0
H4IIE-LUC	0.88			
PHLC-1	0.88	0.90		
RLT2.0	0.75	0.93	0.75	
ANALYT.	0.88	0.88	0.96	0.79

[a] All correlations are significant at $p < 0.05$ ($n = 8$).

the recombinant cells were consistently greater by as much as 2.5-fold. Greater relative potencies of some polyhalogenated aromatic hydrocarbons in the H4IIE-luc cells compared with the wild-type cells have been previously reported [16].

Concentrations of TCDD-EQ determined by fish cell lines were generally greater than those determined by mammalian cells (Mann–Whitney test, $p < 0.05$). As suggested by studies developing species-specific TEFs for organochlorine compounds [20,25], some PCDD and PCDF congeners can have greater relative potency in fish (specifically rainbow trout) cell lines, whereas other compounds, such as mono- and di-*ortho*-substituted PCBs, elicit greater relative potencies in mammalian cells. Relative potencies for hexachloro-dibenzo-*p*-dioxin and dibenzofuran congeners in RLT2.0 cells were 4- to 45-fold greater relative to mammalian cell lines [20]. Other classes of chemicals may also have contributed to the differences. Differences in ligand-binding affinity and ligand specificity among species and tissues have been previously reported [29]. The reasons for cell-line-specific differences in induction include different structure, quantity, and physicochemical properties of the AhR, transcription factors, and other associated proteins [11,25]. Another potentially important factor could be metabolism of the compounds in the mixture. Studies with single compounds have documented faster metabolism of some organochlorines in rat cells compared with fish cells [30].

Concentrations of residues in sediments

Relatively great concentrations of PAHs were measured in all sediments (Table 4). The sum concentrations of 16 PAHs ranged from 1,132 to 40,000 ng/g dry weight. Pyrene, fluoranthene, and benzo[*b*]fluoranthene occurred at the greatest concentrations. No risk limits for concentrations of PAHs in sediments have been promulgated in the Czech Republic. However, in comparison with maximal permissible concentrations (MPCs, concentrations above which the risk of adverse effects is considered unacceptable) used as risk limits in The Netherlands [31], at least two of the PAHs in each sample, except for sample 8AF, exceeded the limits. In some samples collected before the floods (7BF, 5BF, 4BF), concentrations of most PAHs were greater than the MPCs for sediments.

Concentrations of organochlorine compounds in sediments were relatively low. This is the first study to report concentrations of PCDD/DFs and coplanar PCBs in the study area. Concentrations of the dominant congeners along with the sums for PCDD/DFs and coplanar PCBs are given (Table 4). Concentrations of both PCDDs and PCDFs were generally near the limit of detection of 0.02 pg/g dry weight in F1, with the total concentration less than 2.2 pg/g dry weight. However, PCDD/DFs that eluted in F2 were not analyzed in this study. The concentration of PCDD/Fs in this fraction were generally low (<50% of the concentration in F1). Coplanar PCB concentrations were less than 90 pg/g dry weight, with major contributions from congeners 77 and 126. The sum of other PCBs ranged from 14 to 114 ng/g dry weight. There was no significant difference in concentrations of PCDD/Fs or coplanar PCBs among locations.

Concentrations of TCDD-EQs in sediments

Because the H4IIE-luc bioassay was found to have the greatest sensitivity, least variability, and greater tolerance to cytotoxic effects, this cell line was chosen for detailed studies of all the samples taken before (BF) and after floods (AF). Extracts equivalent to as little as 0.1 mg dry weight sediment were sufficient to cause significant induction in this assay. Complete dose–response curves were obtained for all samples with maximal efficacies between 80 and 150% of the maximal induction observed for TCDD. The TCDD-EQs based on the EC50 response equivalent were used for comparison with analytical results.

To estimate the relative contribution of each analyte identified in the sediment extract to the whole sediment TCDD-EQs, the toxic equivalency quotients for each analyte were

Table 4. Levels of polycyclic aromatic hydrocarbons (PAHs) [μg/g dry wt], polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) [pg/g dry wt], and non-*ortho*-substituted polyclorinated biphenyls (PCBs) [pg/g dry wt] in sediments; for dioxins/furans, sum concentrations and dominant compound are listed; total PAHs = sum of the 16 PAHs listed in the methods

Sample	Total PAHs	OCDD	Total PCDDs	23478-PeCDF[a]	Total PCDFs	Non-*ortho*-substituted PCBs				
						#81	#77	#126	#169	Total
3B	3.5	0.88	0.88	0.25	0.25	0.45	4.7	9.6	0.5	15
4B	16.5	0.75	0.95	0.2	0.2	1.1	13	9.2	1.15	25
5B	12.4	0.62	0.7	0.12	0.12	1	11	3.6	<0.02	16
6B	11.9	1.2	1.5	0.18	0.38	1.6	21	29	0.95	52
7B	39.9	0.7	0.7	0.16	0.56	0.96	11	3.6	<0.02	15
8B	20.4	0.8	1.1	0.2	0.2	1.9	31	12	0.93	46
9B	10.5	0.95	0.95	0.58	0.58	4.4	56	24	<0.02	85
3A	61.7	0.62	0.76	0.12	0.2	<0.02	8.9	3.8	<0.02	13
4A	20	1.6	1.8	0.24	0.24	0.72	8.6	3	0.58	13
5A	16.7	0.52	0.56	0.1	0.14	1.2	13	3.7	<0.02	18
8A	11.3	0.62	0.7	0.1	0.1	0.46	4	2.7	1.6	9
9A	8.4	0.54	0.58	0.08	1.16	2.1	27.9	4.5	<0.02	34
10A	8.8	0.52	0.52	0.12	0.16	<0.02	6.3	3.1	0.02	10

[a] 23478-PeCDF = 2,3,4,7,8-Pentachlorodibenzofuran.

Table 5. Contribution of the identified analytes to the total concentrations of toxic equivalents (TEQs) of whole sediment extracts[a]

	TEQs (pg TCDD-equivalent/g dry wt sediment)					
Sample	PAHs	PCDDs	PCDFs	non-*ortho* PCBs	mono-*ortho* and di-*ortho*PCBs	Sum of TEQs
3B	2,803	0.00009	0.12	0.96	0.94	2,805
4B	8,812	0.02	0.1	0.94	1.1	8,814
5B	22,294	0.008	0.06	0.36	0.25	22,295
6B	6,565	0.003	0.1	2.9	0.24	6,570
7B	21,808	0.023	0	0.98	0.7	21,810
8B	12,627	0.23	0.1	1.2	1.2	12,630
9B	5,469	0.0001	0.29	2.4	1.5	5,473
3A	3,752	0.068	0.06	0.38	0.48	3,753
4A	15,072	0.003	0.12	0.31	0.6	15,073
5A	9,039	0.04	0.05	0.38	0.35	9,040
8A	781	0.0009	0.05	0.28	0.19	782
9A	6,382	0.04005	0.051	0.45	0.5	6,383
10A	5,911	0.00005	0.064	0.32	0.37	5,912

[a] TEQ = toxic equivalents; TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PAH = polycyclic aromatic hydrocarbons; PCDD = polychlorinated dibenzo-*p*-dioxin; PCDF = polychlorinated dibenzofuran; PCB = polychlorinated biphenyl.

calculated by multiplying the analyte's concentration by its toxic equivalence factor or specific relative potency. Contributions of PCDD/DFs, PCBs, and PAHs to the total TEQs are presented (Table 5). The TEFs used for dioxins, furans, and PCBs were WHO I-TEFs for humans/mammals, which are consensus values based on many different endpoints [13]. They represent overestimates of the actual potency because they are meant to be protective. They were used because this is the most complete source of TEFs available and is widely accepted. None of the organochlorine compounds, including PCBs, PCDDs, and PCDFs, contributed significantly to the total TEQs. Despite the great TEF values of organochlorines relative to RPs determined in the respective bioassays, their contribution was less than 1% of the total TEQs in all sediments. There are no consensus TEF values for PAHs. In this study, we have applied relative potencies determined from in vitro bioassays with H4IIE-wt cells [24]. Because TEFs or RPs can vary among cell types, calculated TEQs were interpreted in a semiquantitative manner. The greatest contributions to PAH-TEQs were by benzo[*b*]fluoranthene and benzo[*k*]fluoranthene. Polycyclic aromatic hydrocarbons contributed the greatest proportion of the TEQs due to their relative great concentrations, representing over 99% of the total TEQs. Significant induction of TCDD-like activities by PAHs has been documented in studies testing effects of extracts of air particulate material [32], fly ash samples [33], or semipermeable membrane devices exposed to stream water [34]. Possible concern for comparison of the bioassay and analytical results could be losses of some compounds, namely PAHs, due to metabolization in longer exposure studies. However, recent time-series studies of induction of the cell lines with PAH indicate that there is no loss of potency of PAHs with time. This is probably because the interactions with the AhR, which initiates the responses of the cells, occur very rapidly at the beginning of the incubation (Villeneuve et al., unpublished data).

The TCDD-EQs derived by H4IIE-luc bioassay were significantly correlated with TEQs ($R^2 > 0.8$, $p < 0.01$; see Fig. 3). The differences between TCDD-EQs and TEQs were mostly insignificant, considering the variability in bioassay results, the uncertainty of TEFs, and analytical errors within the assays. Most of the compounds responsible for the AhR-mediated activity have been accounted for. Only in some samples (3BF, 8AF) did unknown compounds contribute significantly to the total TEQ. Cases where concentrations of TCDD-EQ were significantly less than those of TEQ suggest possible less-than-additive (antagonistic) interactions among compounds in the mixture. Previous studies have reported that some compounds, especially di-*ortho*-PCBs, can act as antagonists or partial ag-

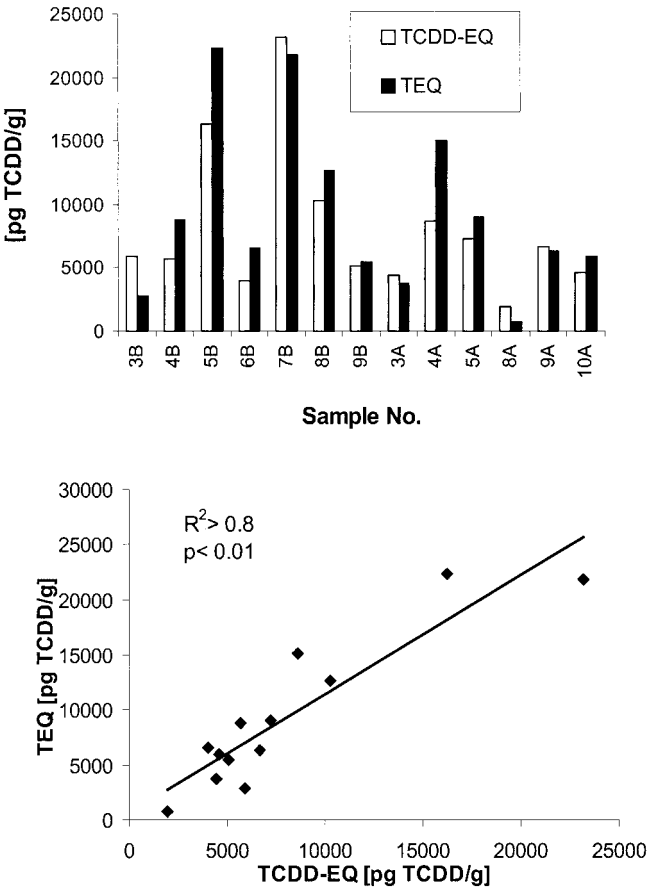

Fig. 3. Relationship between toxic equivalents determined from H4IIE-luc bioassays (2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents [TCDD-EQ] based on response equivalent to 50% [EC50] of the maximal response produced by the standard [$TCDD_{max}$]) and those calculated from analytical toxic equivalents (TEQ).

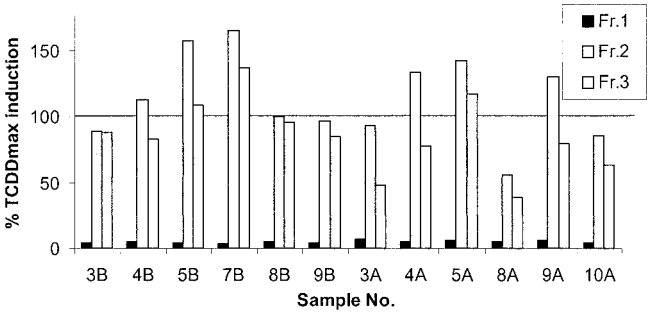

Fig. 4. Luciferase induction in the H4IIE-luc cell bioassay elicited by sediment fractions separated from the whole extract (nondiluted, 1:1). Response magnitude is expressed as percentage of maximal induction caused by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) standard ($TCDD_{max}$).

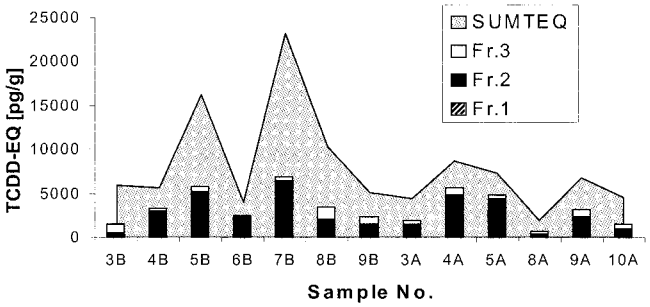

Fig. 5. Comparison of toxic equivalents determined as the sum of the toxic equivalent estimates from the three individual fractions and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TCDD-EQ) of the whole extract (SUMTEQ).

onists for the AhR, thus reducing the total potency of the mixture [12,13,16].

Fractionation

Compounds in the sediment extracts were separated, based on their polarity, into three different fractions by use of florisil column chromatography (Fig. 2). Two concentrations of each fraction (1:1 and 1:3 dilutions) were then tested with H4IIE-luc cells (Fig. 4). Little activity was observed in the first fraction (F1), with significant responses elicited in 3AF-F1 and most of the BF-F1 samples. The greatest induction was only about 1.5-fold greater than that of the solvent control. The nonpolar compounds eluted in the first fraction include PCBs and some of the PCDD/DFs, compounds with relatively great AhR-mediated activity [7,22,35]. Since the concentrations of these compounds were small, their small contribution to the total TEQs was expected (Tables 4 and 5).

The greatest induction of luciferase activity was caused by compounds in the second fraction (F2) for all samples with magnitudes of induction ranging between 8- and 26-fold above that of the solvent control. Most of the BF-F2 samples and also some AF-F2 samples (5, 4, 9) elicited responses greater than $TCDD_{max}$. This fraction contained PAHs, their derivatives, organochlorine pesticides, and a portion of the PCDD/DFs. These results confirm the conclusion of the mass-balance calculation, which suggested that PAHs were the major source of TEQs. Also in studies measuring EROD activity induced by Swedish sediment extracts in livers of cultured or in ovo injected chicken embryos, the greatest induction was caused by the sediment fraction containing PAHs [36,37]. However, since there may be additional compounds in F2 that could be AhR-active, caution must be exercised in assigning the causality to PAHs as the major contributors to toxicity.

The third fraction (F3) also caused significant induction (5- to 23-fold greater than the solvent control), but the maximal induction was less than that caused by F2. For some samples (5AF, 5BF, 7BF), the magnitude of induction was greater than $TCDD_{max}$. The compounds responsible for dioxin-like activity in F3 are unknown. They may include polar compounds that are relatively weak AhR agonists, which may be of either natural or synthetic origin. Recently, some studies have suggested additional types of AhR ligands and inducers with a wide structural variety, some of which could have been present in our sediment samples at concentrations sufficient to cause the observed induction [38]. Also, studies with marine sediments have documented the presence of unknown AhR-active compounds in the most polar fractions of sediment extracts [39].

Our results demonstrate that the individual fractions can elicit induction as great as the total extract and greater than maximal induction caused by TCDD. Presented results suggest that the interactions within complex mixtures can lead to induction greater than the maximal induction caused by TCDD, standard reference AhR agonist. Maximal responses greater than those caused by TCDD have been reported for complex mixtures [23,39] as well as for single compounds [32]. Some PAHs tested in recombinant mouse cells, benzo[*a*]pyrene in particular, induced a response significantly greater than that caused by TCDD even though their AhR-inducing potency was less than that for TCDD [32]. Benzo[*a*]pyrene was present in all the sediment samples studied.

Semiquantitative estimates of toxic equivalents of individual fractions were determined based on the magnitude of induction calculated from the log-transformed TCDD calibration (Fig. 5). The sum of the toxic equivalents from the three fractions were significantly correlated with TCDD-EQs from the total extract ($R^2 = 0.72$, $p < 0.05$). But sums of the TCDD-EQs from the fractions were significantly less than the TCDD-EQs estimated for total extract. This may suggest synergistic interactions among compounds in different fractions. However, the estimates for the fractions were based on single induction values. The shape of the sample dose–response curve was unknown and the magnitude of induction was directly projected to the standard dose–response curve. Furthermore, the florisil column could have adsorbed some of the compounds that could elicit dioxin-like activity.

Effect of floods on dioxin-like activity in sediments

Comparison of the results for sediment samples collected both in the fall 1996 and of 1997 provided information on the potential effects of great floods that occurred in the studied rivers in the summer of 1997. The results of the comparison of dioxin-like activity between the two sampling periods indicate that there was little change (sites 3, 4, 9) or the TCDD-EQs were significantly less after floods (sites 5, 8). Concentrations of PCDD/DFs were small and did not change significantly. Concentrations of coplanar PCBs were less after the floods. There was no clear trend for concentrations of PAHs, but the apparent decrease that occurred at sites 5 and 8 confirmed the results of the bioassays. Thus, the influence of floods on contaminant concentration in sediments was site specific. Decreases in concentrations at sites 5 and 8 suggest resuspension, transport, and redistribution of contaminated sedi-

ments during floods. Slight increases in concentrations at sites 4 and 9 may be due to increased runoff from the denudation area of the rivers during floods.

CONCLUSIONS

In vitro bioassays proved their applicability for assessment of the dioxin-like activity of complex environmental mixtures. All analyzed sediment samples elicited significant dioxin-like activity in in vitro bioassays. The results correlated well among the cell lines, with greater toxic equivalents for fish than mammalian cell lines. The H4IIE-luc cells were the least variable and most sensitive cell system. Great caution must be taken when calculating the toxic potencies from nonideal dose–response curves obtained from complex mixtures. The mass-balance calculations based on chemical analyses suggested that PAHs can account for a considerable portion of the dioxin-like activity. These results were confirmed by a fractionation approach, where little activity was observed in the first fraction, which contained relatively small concentrations of organochlorine pollutants, and the greatest activity was observed in the second fraction containing PAHs. Detection of significant dioxin-like activity in the third fraction suggests the presence of unidentified polar AhR-active compounds in the sediments. The effect of floods on dioxin-like activity in the sediment is site-specific, with no obvious trend.

Caution must be applied when assessing the risk posed by TCDD-EQs in sediments. The approach presented here is meant to be a screening tool to allow for prioritizing of contaminated sediments for subsequent study including instrumental analyses. For instance, since the fractionation demonstrated that PCDD/DFs and PCBs contributed little to the TEQ, they would not be considered contaminants of concern and there would be no requirement for subsequent analyses of these compounds by use of resource-intensive high-resolution mass spectrometry.

While PAHs can be toxic to benthic invertebrates, they would not be expected to be biomagnified by vertebrates like PCDD/DFs and PCBs are. The results of this study indicate that the use of the H4IIE-luc bioassay in combination with fractionation was effective and accurate and allowed most of the conclusions to be made that would have been made based on extensive instrumental analyses. In this study, the results of the bioassays were verified by instrumental analyses. Therefore, it can be concluded that the approach could be a cost-effective alternative to the more resource-intensive and time-consuming instrumental analyses in initial screening of river sediments.

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REFERENCES

1. Jaffe R. 1991. Fate of hydrophobic organic pollutants in the aquatic environment: A Review. *Environ Pollut* 69:237–257.
2. Duursma EK, Niewenhuize J, Van Liere JM, Hillebrand MTJ. 1986. Partitioning of organochlorines between water, particulate matter and some organisms in estuarine and marine systems of The Netherlands. *Neth J Sea Res* 20:239–251.
3. Fortner AR, Sick LV. 1985. Simultaneous accumulation of naphthalene, a PCB mixture and benzo[*a*]pyrene by the oyster. *Bull Environ Contam Toxicol* 34:256–264.
4. Bols N, Whyte J, Clemons J, Tom D, van den Heuvel M, Dixon M. 1997. Use of liver cell lines to develop TCDD equivalency factors and to derive TCDD equivalent concentrations in environmental samples. In Zelikoff JT, ed, *Ecotoxicology: Responses, Biomarkers and Risk Assessment.* SOS Publications, Fair Haven, NJ, USA, pp 329–350.
5. Hilscherova K, Machala M, Kannan K, Blankenship AL, Giesy JP. 2000. Cell bioassays for detection of dioxin-like and estrogen receptor mediated activity. *Environ Sci Pollut Res* 7:159–171.
6. Poland A, Knutson JC. 1982. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. *Annu Rev Pharmacol Toxicol* 22:517–554.
7. Nebert DW, Puga A, Vasiliou V. 1993. Role of the Ah receptor and the dioxin-inducible [Ah] gene battery in toxicity, cancer, and signal transduction. *Ann NY Acad Sci* 685:624–640.
8. Lucier GW, Portier CJ, Gallo MA. 1993. Receptor mechanism and dose–response models for the effects of dioxins. *Environ Health Perspect* 1:36–44.
9. Hankinson O. 1995. The aryl hydrocarbon receptor complex. *Annu Rev Pharmacol Toxicol* 35:307–340.
10. Zacharewski TR, Berhane K, Gillesby BE, Burnison BK. 1995. Detection of estrogen- and dioxin-like activity in pulp and paper mill black liquor and effluent using in vitro recombinant receptor/reporter gene assays. *Environ Sci Technol* 29:2140–2146.
11. Garrison PM, Tullis K, Aarts JMMJG, Brouwer A, Giesy JP, Denison MS. 1996. Species-specific recombinant cell lines as bioassay systems for the detection of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-like chemicals. *Fundam Appl Toxicol* 30:194–203.
12. Sanderson JT, Van den Berg M. 1999. Toxic equivalency factors (TEFs) and their use in ecological risk assessment: A successful method when used appropriately. *Hum Ecol Risk Assess* 5:43–52.
13. Van den Berg M, et al. 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environ Health Perspect* 106:775–790.
14. Balaguer P, Joyeux A, Denison MS, Vincent R, Gillesby BE, Zacharewski TR. 1996. Assessing the estrogenic and dioxin-like activities of chemicals and complex mixtures using in vitro recombinant receptor-reporter gene assay. *Can J Physiol Pharmacol* 74:216–222.
15. Khim JS, Kannan K, Villeneuve CH, Koh DL, Giesy JP. 1999. Characterization and distribution of trace organic contaminants in sediment from Masan Bay, Korea: 1. Instrumental analyses. *Environ Sci Technol* 33:4199–4205.
16. Tillit DE, Giesy JP, Ankley DA. 1989. Characterization of the H4IIE rat hepatoma cell bioassay as a tool for assessing toxic potency of planar halogenated hydrocarbons in environmental samples. *Environ Sci Technol* 25:87–92.
17. Sanderson JT, Aarts JMMJG, Brouwer A, Froese KL, Denison MS, Giesy JP. 1996. Comparison of Ah receptor-mediated luciferase and ethoxyresorufin-*O*-deethylase induction in H4IIE cells: Implications for their use as bioanalytical tools for detection of polyhalogenated aromatic hydrocarbons. *Toxicol Appl Pharmacol* 137:316–325.
18. Hightower LE, Renfro JL. 1988. Recent applications of fish cell culture to biomedical research. *J Exp Zool* 248:290–302.
19. Richter CA, Tieber VL, Denison MS, Giesy JP. 1997. An in vitro rainbow trout cell bioassay for aryl hydrocarbon receptor-mediated toxins. *Environ Toxicol Chem* 16:543–550.
20. Villeneuve D, Richter CA, Giesy JP. 1999. Rainbow trout cell bioassay derived TEFs for halogenated aromatic hydrocarbons: A comparison and sensitivity analysis. *Environ Toxicol Chem* 18: 879–888.
21. Lorenzen A, Kennedy SW. 1993. A fluorescence-based protein assay for use with a microplate reader. *Anal Biochem* 214:346–348.
22. Sanderson JT, Giesy JP. 1998. Wildlife toxicology, functional response assays. In Meyers RA, ed, *Encyclopedia of Environmental Analysis and Remediation.* John Wiley, New York, NY, USA, pp 5272–5297.
23. Villeneuve DL, Blankenship AL, Giesy JP. 2000. Derivation and application of relative potency estimates based on in vitro bioassay results. *Environ Toxicol Chem* 19:2835–2843.
24. Willett KL, Gardinali PR, Sericano JL, Wade TL, Safe SH. 1997.

Characterization of the H4IIE rat hepatoma cell bioassay for evaluation of environmental samples containing polynuclear aromatic hydrocarbons (PAHs). *Arch Environ Contam Toxicol* 32:442–448.
25. Clemons JH, van den Heuvel MR, Stegeman JJ, Dixon DG, Bols NC. 1994. Comparison of toxic equivalent factors for selected dioxin and furan congeners derived using fish and mammalian liver cell lines. *Can J Fish Aquat Sci* 51:1577–1584.
26. Hahn ME, Woodward BL, Stegeman JJ, Kennedy SW. 1996. Rapid assessment of induced cytochrome P4501A protein and catalytic activity in fish hepatoma cells grown in multiwell plates: Response to TCDD, TCDF, and two planar PCBs. *Environ Toxicol Chem* 15:582–591.
27. Murk AJ, Legler J, Denison MS, Giesy JP, Van De Guchte C, Brouwer A. 1996. Chemical-activated luciferase gene expression (CALUX): A novel in vitro bioassay for Ah receptor active compounds in sediments and pore water. *Fundam Appl Toxicol* 33: 149–60.
28. Koistinen J, Soimasuo M, Tukia K, Oikari A, Blankenship A, Giesy JP. 1998. Induction of EROD activity in Hepa-1 mouse hepatoma cells and estrogenicity in MCF-7 human breast cancer cells by extracts of pulp mill effluents, sludge, and sediments exposed to effluents. *Environ Toxicol Chem* 17:1499–1507.
29. El-Fouly MH, Richter CA, Giesy JP, Denison MS. 1994. Production of a novel recombinant cell line for use as a bioassay system for detection of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-like chemicals. *Environ Toxicol Chem* 10:1581–1588.
30. Clemons JH, Dixon DJ, Bols NC. 1997. Derivation of 2,3,7,8-TCDD toxic equivalent factors (TEFs) for selected dioxins, furans and PCBs with rainbow trout and rat liver cell lines and the influence of exposure time. *Chemosphere* 34:1105–1119.
31. Kalf DF, Crommentuijn T, van de Plassche EJ. 1997. Environmental quality objectives for 10 polycyclic aromatic hydrocarbons (PAHs). *Ecotoxicol Environ Saf* 36:89–97.
32. Clemons JH, Allan LM, Marvin CH, Wu Z, McCarry BE, Bryant DW, Zacharewski TR. 1998. Evidence of estrogen- and TCDD-like activities in crude and fractionated extracts of PM_{10} air particulate material using in vitro gene expression assay. *Environ Sci Technol* 32:1853–1860.
33. Till M, Behnisch P, Hagenmaier H, Bock KW, Schrenk D. 1997. Dioxin-like components in incinerator fly ash: A comparison between chemical analysis data and results from a cell culture bioassay. *Environ Health Perspect* 105:1326–1332.
34. Villeneuve D, Crunkilton RL, DeVita WM. 1997. Aryl hydrocarbon receptor-mediated toxic potency of dissolved lipophilic organic contaminants collected from Lincoln creek, Milwaukee, Wisconsin, USA, to PLHC-1 (*Poeciliopsis lucida*) fish hepatoma cells. *Environ Toxicol Chem* 16:977–984.
35. Safe SH. 1986. Comparative toxicology and mechanism of action of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. *Annu Rev Pharmacol Toxicol* 26:371–399.
36. Engwall M, Broman D, Dencker L, Naf C, Zebuhr Y, Brunstrom B. 1997. Toxic potencies of extracts from sediments and settling particulate matter collected in the recipient of a bleached pulp mill effluent before and after abandoning chlorine bleaching. *Environ Toxicol Chem* 16:1187–1194.
37. Brunstrom B, Broman D, Dencker L, Naf C, Vejlens E, Zebuhr Y. 1992. Extracts from settling particulate matter collected in the Stockholm archipelago waters: Embryolethality, immunotoxicity and EROD, inducing potency of fractions containing aliphatics/monoaromatics, diaromatics or polyaromatics. *Environ Toxicol Chem* 11:1441–1449.
38. Denison MS, Heath-Pagliuso. 1998. The Ah receptor: A regulator of the biochemical and toxicological actions of structurally diverse chemicals. *Bull Environ Contam Toxicol* 61:557–568.
39. Khim JS, Villeneuve D, Kannan K, Koh CH, Giesy JP. 1999. Characterization and distribution of trace contaminants in sediment from Masan Bay, Korea. 2. In vitro gene expression assay. *Environ Sci Technol* 33:4206–4211.

Článek XVIII:

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Characterization of Estrogenic Activity of Riverine Sediments from the Czech Republic

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Abstract. Extracts of sediments from rivers in an industrialized area in the Czech Republic were used to evaluate suitability of a simple *in vitro* bioassay system to detect estrogen receptor (ER)-mediated activity in the complex mixture. Total estrogenic activity was detected by measuring luciferase activity in a stably transfected cell line containing an estrogen-responsive element linked to a luciferase reporter gene. For appropriate interpretation of ER-mediated activity, the effect of sediment extracts on the cell cytotoxicity was assessed at the same time. All sediment samples elicited considerable estrogenic activity. Fractionation of the extracts along with bioassay testing and subsequent instrumental analysis allowed the estrogenic fractions to be identified. The Florisil fraction, which was intermediate in polarity, was the most estrogenic. Instrumental analysis documented that the concentration of the degradation products of alkylphenol ethoxylates did not occur at sufficient concentrations to account for the estrogenic activity. Mass-balance calculations and testing of fractions confirmed that certain polycyclic aromatic hydrocarbons (PAHs) or their metabolites were the most likely compounds contributing to estrogenicity. Some other compounds, such as PCNs and PAH derivatives, that were present in the first and second fraction were tested for their potential estrogenic activity. Their ER-mediated activity and contribution to the overall responses of the complex extracts were very low. The concentrations of 17β-estradiol present in the bioassay media was an important factor for the evaluation of (anti)estrogenicity of single compound(s) or complex mixtures.

A number of compounds present in the environment have been reported to elicit disrupting effects on normal physiological function of the endocrine system of mammals, fish, birds, reptiles, as well as invertebrates (Ankley *et al.* 1998). Most studies of such effects have focused on individual chemicals at relatively high concentrations. However, in environmental matrices, these chemicals are present as complex mixtures with other compounds, often in low concentrations. Thus, humans and wildlife are exposed to complex mixtures of both artificial and natural chemicals, which may interact to produce additive, greater than additive, or antagonistic effect (Safe *et al.* 1997). Effects of endocrine-disrupting chemicals on animals in the aquatic environment, especially river ecosystems, have been documented (Sumpter and Jobling 1995; Bortone and Davis 1994).

Aquatic sediments serve as a sink for a number of contaminants and thus as an integrative measure of exposure of the aquatic ecosystem. Sediment can contain mixtures of biologically active compounds with different mechanism of action. The bottom-dwelling animals are directly exposed to these chemicals and through them the pollutants can enter aquatic food chains. In addition, contaminants in sediments can directly affect micro- and meiobenthic communities.

Endocrine disruptors (EDs) present in sediments can have a variety of structures, and thus, their analytical determination would be daunting. Moreover, for a number of compounds, the endocrine-disrupting potency is unknown. Thus, the analytical determination of total ED activity of the complex mixture is not possible at this point. Some integrative measures of exposure are needed to determine endocrine-disrupting potential of complex mixtures. Several basic mechanisms exist for endocrine disruption, including receptor-mediated mechanism (ligand agonists and antagonists), inhibition of synthesis, inhibition or acceleration of metabolism of endogenous hormones. Despite their various structures, a number of chemicals can elicit effects via a mode of action similar to estrogen. *In vitro* recombinant cell bioassays, in which a reporter gene is under the control of receptor binding, enable estimation of the total receptor-mediated activity of samples and also account for possible interactions between compounds in the mixture (Joyeux *et al.* 1997). In this way estrogenic compounds, which are defined as compounds producing effects that are mediated through the estrogen receptor, can be characterized (Gillesby and Zacharewski 1998; Zacharewski 1997). The complex mixture of contaminants present in environmental matrices includes both estrogenic and antiestrogenic components. Effects of such mixtures can be determined by the relative contribution

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of each type of estrogen receptor (ER)-active compound and the nature of their interactions (Kramer and Giesy 1995).

Estrogenic activity has been previously detected in complex extracts from environmental samples, such as pulp and paper mill sludge and effluents (Koistinen *et al.* 1998) or particulate matter in air (Clemons *et al.* 1998). In most studies, the active agents have not been identified. Identification of causative agents is complicated due to complex composition of the samples. A useful strategy for determining the causative agents is the toxicant identification and evaluation (TIE) approach, including fractionation of the active extract (Hilscherova *et al.* 2000). Fractionation enables to separate groups of compounds with different characteristics. In the active fractions the causative agents can be identified by more specific chemical analysis.

There are no previous records of the endocrine-disrupting potential of contaminants present as complex mixtures in sediments of Czech rivers. The objectives of this study were (1) to determine potential estrogenic chemicals in sediments from an industrial area, (2) to examine the utility of *in vitro* recombinant cell line system for screening sediments, and (3) to estimate estrogenic or antiestrogenic potency of sediments. Other goals include comparison of the responses of the whole extracts at different concentrations of 17β-estradiol in the medium and assessment of the effects of sediment extracts on cytotoxicity and protein content of the cells. Sediment extracts were fractionated based on the polarity and tested on bioassays and instrumental analysis to determine the classes of compounds responsible for the (anti)estrogenic activity. Limited mass-balance calculations were performed to determine the proportion of the estrogenicity accounted for by the analyzed compounds of known potency. Relative potencies of some of the chemicals present in the active fractions were determined.

Materials and Methods

Complete details of the sample collection, processing, extraction, and fractionation procedure have been described in a previous study (Hilscherova *et al.* 2001). Surface sediments (top 5 cm layer) were collected in the Czech Republic from Rivers Morava, Drevnice, and Drevnice's tributaries in an industrial region of the Czech Republic (Figure 1). Seven sediments were collected in October 1996 (samples B = before floods), and six were collected in October 1997 (samples A = after floods). Dry sediments were homogenized, and 20 g of the sediment fraction < 2 mm were Soxhlet extracted for 16 h with dichlormethane (DCM; Burdick & Jackson, Muskegon, MI), and the extracts were fractionated into three fractions of different polarity by use of a Florisil column (Khim *et al.* 1999). The first fraction (F1), eluted with 90 ml high-purity hexane (Burdick & Jackson), contained polychlorinated biphenyls (PCBs), a portion of polychlorinated dioxins/furans (PCDD/DFs), and n-alkanes. The second fraction (F2) containing polycyclic aromatic hydrocarbons (PAHs), organochlorine (OC) pesticides, alkylphenols (APs), and rest of PCDD/DFs was eluted with 100 ml 20% DCM in hexane. Polychlorinated naphthalenes (PCNs) eluted in both F1 and F2. The third fraction eluted with 100 ml 100% DCM contained polar metabolites and sterols.

Instrumental Analysis

Analysis of PCDD/DFs, PCBs, and PAHs has been described in detail previously (Hilscherova *et al.* 2001). The concentrations of 16 U.S.EPA priority pollutant PAHs were determined. Alkylphenols and OC pesticides in F2 were determined following the method described (Khim *et al.* 1999). Reverse-phase high-performance liquid chromatography (HPLC) with fluorescence detection was used to quantify nonylphenol (NP) and octylphenol (OP). Samples and standards were injected (10 μl) by a Perkin Elmer Series 200 autosampler (Perkin Elmer, Norwalk, CT) onto an analytical column, Prodigy™ ODS (3), 250 × 4.6 mm column (Phenomenex, Torrance, CA), which was connected to a guard column Prodigy ODS (3), 30 × 4.6 mm and eluted with a flow of acetonitril (ACN) and water at a gradient from 50% ACN in water to 98% ACN in water delivered by Perkin Elmer Series 200 pump for 20 min. Detection was accomplished using a Hewlett Packard 1046A fluorescence detector (Hewlett-Packard, Wilmington, DE) with an excitation wavelength of 229 nm and an emission wavelength of 310 nm. NP and OP detection limits for the analytical method were 1 ng/g on a dry weight basis (DW).

Concentrations of OC pesticides were determined using a Hewlett Packard 5890 series II gas chromatograph equipped with a capillary column HP-5 (Hewlett Packard; 50 m length × 0.2 mm ID) coated at a film thickness of 0.33 μm and with an electron capture detector (GC/ECD). Hydrogen was used as the carrier gas with a constant flow (1.3 ml/min). Injection volume of 1 μl was made splitless. Injector and detector temperatures were set at 280°C and 310°C. The column oven temperature was programmed as described previously (Khim *et al.* 1999). Detection limits were 0.02 ng/g DW for HCB and HCH congeners and 0.1 ng/g DW for other compounds.

Cell Line and Cell Culture Conditions

A bioassay based on a human breast cancer cell line MCF-7 stably transfected with a reporter gene, allowing expression of the firefly luciferase enzyme under control of the estrogen-regulatory element was used (Pons *et al.* 1990). The cells were obtained from Dr. Michel Pons, Institut National de la Sante et la Recherche Medicale, Montpelier, France. MCF-7-luc cells (MVLN) were grown in Dulbecco's modified Eagle medium with Hams F-12 nutrient mixture (Sigma D-2906) supplemented with $NaHCO_3$, 1 mM sodium pyruvate (Sigma), 1 μg/ml insulin (Sigma I-1882). For culturing the cells on 100-mm plates 10% of defined fetal bovine serum (FBS; Hyclone, Logan, UT) was added to media. For bioassays in 96-well plates 5% charcoal-stripped FBS (Hyclone) with lesser background for 17β-estradiol ($E_2 < 5$ pg/ml) was used. The cells were cultivated until almost confluent with 10 ml media at 37°C in humidified CO_2 incubator, 5/95% CO_2/air, $> 90\%$ humidity. For bioassays cells were plated in 96-well culture ViewPlates (Packard Instruments, Meriden, CT) at a density of 15,000 cells in 250 μl media. Cells were dosed 24 h after plating in triplicate with 1.25 μl extract solution; the final concentration of solvent (DCM) was 0.5%. At least three separate standard calibrations with concentrations of 0.15 to 500 pM 17β-estradiol (E_2) were used. There were always at least three replicates of blank without any treatment and solvent control on every plate. The exposure time for all bioassays was 72 h. Each sample was dosed in six serial dilutions (1:3 diluting step) with three or four replicates per dilution. Two concentrations of the separated fractions (1:1 and 1:3 dilution) were tested on the bioassay with charcoal-stripped media and also in the media with addition of competing endogenous substrate. To examine the antiestrogenic potency of the extracts, 10 pM of E_2 (EC_{20} concentration) was added as a competitive inhibitor of ER binding. The responses were compared to solvent plus 10 pM E_2 as positive control that was run in parallel with the samples. Luciferase activity was determined by measurement of substrate-induced luminescence as described in previous studies (Koistinen *et al.* 1998; Hilscherova *et al.* 2001).

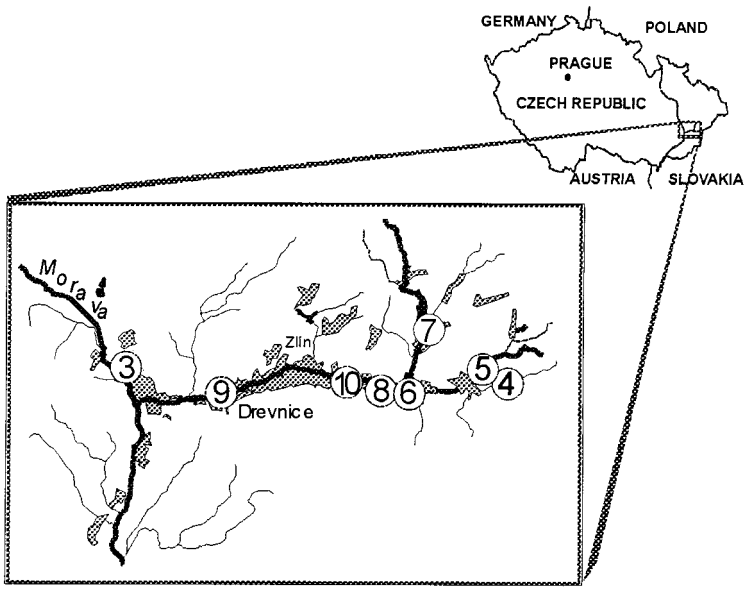

Fig. 1. Location of sampling sites on rivers in the Czech Republic.

Cell Viability Assay

A cell viability index was calculated as a ratio of fluorescence of viable and nonviable cells (Kramer and Giesy 1995; Richter *et al.* 1997). In viable cells the substrate calcein-AM (Molecular Probes, Eugene, OR) was hydrolyzed by esterases to a green fluorescent product, which was detected by fluorescence with excitation and emission wavelengths of 485 and 530 nm, respectively. In dead cells, ethidium bromide (Sigma) can enter cells with damaged membranes and forms a fluorescent product by binding to DNA (excitation 530 nm, emission 645 nm). Ethidium bromide and calcein-AM were added to the incubation media at final concentration of 0.5 μM, plates were incubated at room temperature for 15 min. Fluorescence was measured with a microplate scanning fluorometer, Cytofluor 2300 (Millipore, Bedford, MA).

The protein content was determined by Fluorescamine assay as described previously (Lorenzen and Kennedy 1993; Sanderson *et al.* 1996). The amount of protein per well was calculated based on calibration with standard bovine serum albumin.

Standard Compounds

In addition to the target compounds analyzed in sediments in this study, derivatives of PAHs (methyl and hydroxy PAHs) and PCNs were expected to occur in sediment extracts. Although these compounds were not quantified in sediments, their estrogenic potency was tested in the bioassay to predict their possible contribution to estrogenicity observed in sediments. Because estrogenic potential of PAH derivatives and PCNs have not been reported earlier, this study provided additional information by testing these compounds. The following derivatives of PAHs were tested for their (anti)estrogenic activity with the MCF-7-luc cells and AhR-mediated with the H4IIE-luc cells: 1-methyl-naphthalene (1 CH_3-NAPT), 1,2-dimethyl-napthalene (1,2 CH_3-NAPT), 3,6-dimethyl-phenanthrene (3,6 CH_3-PHE), 9-methyl-anthracene (9 CH_3-ANT), 9,10-dimethylanthracene (9,10 CH_3-ANT), 3,9-dimethyl-benzo(a)anthracene (3,9 CH_3-BaA), 1-methyl-benzo(c-)phenanthrene (1 CH_3-BcPHE), 6-hydroxy-chrysene (6 OH-CHR), 1-hydroxy-pyrene (1 OH-PYR). The standards were obtained from AccuStandard (New Haven, CT) and were greater than 99% purity. All compounds were tested at six different dilutions, with a range of concentrations of 2.5–500 μg/L for hydroxylated PAHs and 0.75 to 250 μg/L for methylated PAHs. The role of competing E_2 in ER-mediated activity of PAHs derivatives was examined by testing at three different levels of E_2: in charcoal-stripped media in which E_2 had been reduced ($E_2 < 0.9$ pM), at the ED_{20} concentration of 10 pM and at the ED_{90} concentration of 170 pM of E_2 concentration. Dilutions of PAH derivatives as well as appropriate E_2 calibrations were prepared in toluene.

Twenty PCNs and six technical mixtures of PCNs (Halowaxes) were screened in MCF-7-luc cells to determine ER-mediated activity (Table 1). For screening purposes two different concentrations were used, the maximum concentration as reported in Table 1 and one-third of this concentration. PCN standards were all high purity (> 93% up to > 99% purity), obtained from different sources (Blankenship *et al.* 2000). Both PCN congeners and E_2 standards were prepared in isooctane and the response was evaluated in charcoal-stripped medium as well as in the presence of 10 pM of E_2.

Data Analysis

Luciferase activity responses in samples were expressed as relative luminescence units (RLU). The viability index, protein content, and microscopic examination were used to evaluate cell condition. When cytotoxicity was observed, those data points were assessed by analyzing the data two ways. First, these values were excluded from the calculations of E_2-EQs. Also, in an attempt to make use of these values where cytotoxicity was observed and extend the linear working range of the data set, the response (estrogenicity) was normalized to the viability index. Nonnormalized data were compared with data normalized to the viability index. Protein normalization was not used for ER-mediated activity, because response induction is correlated with estrogen-induced protein synthesis (Villeneuve *et al.* 1998). The mean solvent control response was subtracted from both standard and sample responses. The significance of response relative to solvent control was evaluated by Student's *t*-test and nonparametric Mann-Whitney test ($\alpha = 0.05$). The EC_{20}, EC_{30}, and EC_{50} concentrations from standard (E_2) dose-response curves were calculated by probit analysis. The dose-

Table 1. ER-mediated activities of polychlorinated napthalenes (PCNs) and Hallowax mixtures tested at two different concentrations of E_2: in charcoal-stripped medium deprived of E_2 (< 0.9 pM) and in medium with addition of 10 pM E_2 (= EC_{20})

PCN Substitution	PCN Congener No.	Highest Tested Dose (ng/well)	Effect	% Solvent Control[a]	
				Stripped Media	10 pM E_2
2,3	10	625	A	80*	89*
1,2,5,6	36	1.25	A	82*	105
2,3,6,7	48	12.5	E	94	116*
1,2,3,5,8	53	12.5	E	113*	117*
1,2,3,4,6,7	66	12.5	A	79*	78*
1,2,3,5,6,8	68	12.5	A	74*	89*
1,2,3,6,7,8	70	12.5	E	94	114*
1,2,3,4,5,6,7	73	1.25	A	85*	63.5*
1,2,3,4,5,6,8	74	12.5	E	105	120*
Halowax 1013		1250	A	80*	78*
Halowax 1014		1250	A	69*	92*
Halowax 1051		1250	A	78*	82*
Halowax 1099		12.5	E	97	128*
Halowax 1001		12.5	A	88*	107

[a] Solvent control = 100%.

Effect: A = antiestrogenic, E = estrogenic. Listed percents of solvent control at the highest tested concentration (*marks significant effects, Mann-Whitney, *t* test, $p < 0.05$).

The following PCN congeners were also tested but did not elicit significant (anti)estrogenic activity: 2-CN; 1,4-DiCN; 1,5-DiCN; 1,2,7-TriCN; 1,2,3,4-TetraCN; 1,2,4,6-TetraCN; 1,2,6,8-TetraCN; 1,2,3,6,7-PentaCN; 1,2,3,5,6,7-HexaCN; 1,2,4,5,6,8-HexaCN; 1,2,3,4,5,6,7,8-OctaCN and Hallowax 1000.

response curves of sediment extracts did not meet the criteria for applying probit analysis, which are equal slope and equal efficacy (maximal induction). Thus, the multiple point estimates method (Villeneueve *et al.* 2000), which enables to account for the nonparallel slopes of the samples dose-response curves, was used for calculations of the estrogenic equivalents per g (ng E_2-EQ/g) sample. Sample responses were converted to a percentage of the mean maximum response observed for the E_2 standard and plotted as a function of log μl sample. Linear regression was applied to the linear part of the log-transformed dose response curve. The concentration producing a response equivalent to 20% (EC_{20}), 30% (EC_{30}), and 50% (EC_{50}) of the maximal response of the E_2 standard was calculated and used to determine relative potency.

Simple mass-balance calculation was conducted based on a limited number of compounds to compare the estradiol equivalents (E_2-EQ) from bioassay and analytical results (EEq). An equivalency factor approach was applied where the measured concentrations of individual compounds were multiplied by the appropriate E_2-relative potency values (ERPs) to calculate the analytical estrogenic equivalent (EEq) (Safe 1995). ERPs were previously determined with MCF-7-luc cells for some alkylphenols (Villeneueve *et al.* 1998) and PAHs (Clemons *et al.* 1998). Nonparametric (Spearman) correlation analysis was performed to characterize relationship between E_2-EQ determined from bioassays and those calculated from analytical results. Statistical calculations were conducted by use of the STATISTICA/w 5.0 program (StatSoft, Tulsa, OK).

Results and Discussion

Estrogenic Activity of Sediment Extracts

Complete dose-response relationship was obtained with 0.15–500 pM of 17β-estradiol (E_2) standard. Reproducibility of the replicate standard calibrations exhibited a coefficient of variation (CV) of between 5 and 25%. Overall, the EC_{50} for E_2 was 44.3 ± 11.9 pM (n = 26, CV = 0.27).

Significant induction of luciferase activity was observed with total extracts of all sediments. The DCM extracts represent total extractable organic contaminants present in sediments. As little as 0.1 mg of sediment was sufficient in some samples (5A, 9A, 3B, 6B, 9B) to elicit a significant response. The maximal induction (% E_2-max) caused by extracts was between 30% and 126% of the maximal induction elicited by E_2. Most samples did not reach the maximal efficacy (Figure 2A). Cytotoxicity, as determined by the viability test, was observed at the two greatest concentrations of some sediment extracts tested. Other studies testing environmental extracts, such as air particulate or black liquor from pulp mills also reported dose-dependent induction in ER-mediated activity with maximal induction less than the E_2 maximum (Clemons *et al.* 1998; Balaguer *et al.* 1996). Even though they did not evaluate cytotoxicity, they reported apparent distress of the cells (*i.e.*, spherical morphology) at the greatest concentrations (Balaguer *et al.* 1996).

Multiple point estimates of E_2-EQs were calculated to account for the low level of induction and nonparallel slopes observed for some sample. ER-mediated potency was expressed as the amount of sample causing the same level of response as the EC_{20}, EC_{30}, and EC_{50} of E_2. Due to the unequal slopes and efficacies, point estimates based on different levels of response can vary (Figure 3; Villeneuve *et al.* 1998). However, the shapes of the dose-response curves were similar and the E_2-EQ values based on the point estimates were correlated (Table 2). The E_2-EQs of the whole extracts varied significantly among sites ranging from 10 to 1,200 pg E_2/g sample.

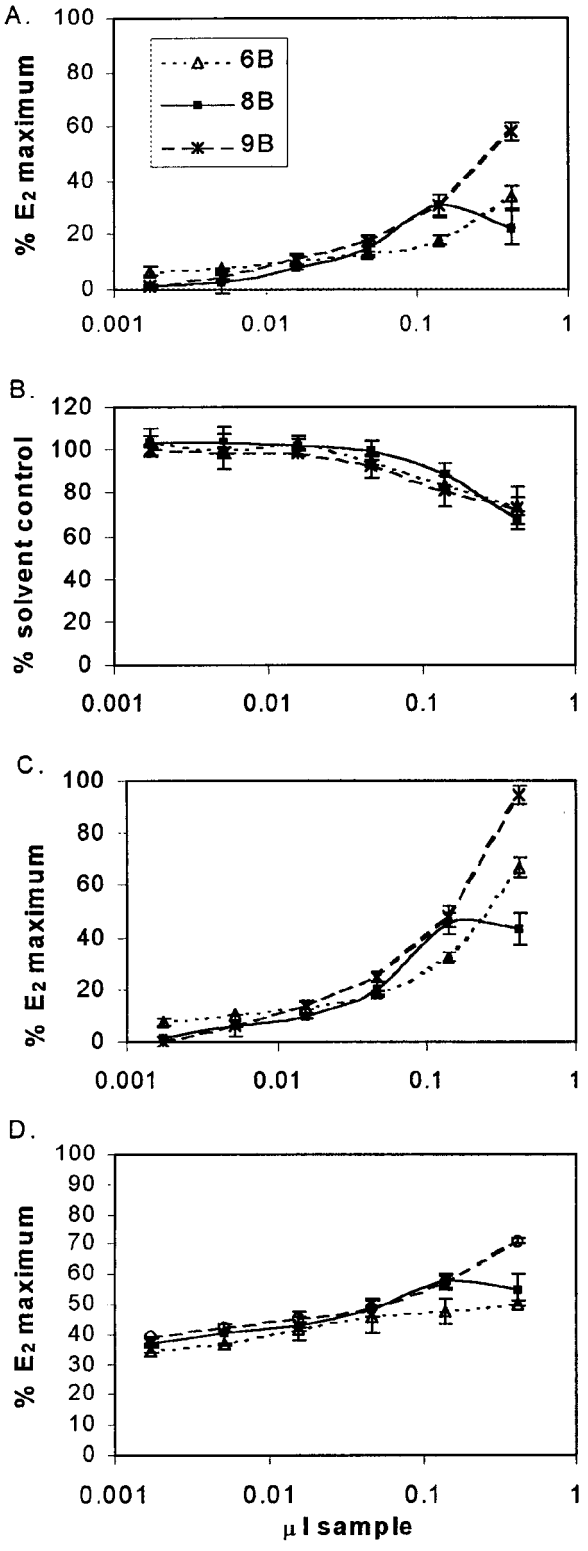

Fig. 2. Examples of dose-response curves for A: luciferase activity, B: viability index, C: luciferase activity normalized to viability index, D: luciferase activity after addition of 10 pM in the media for complex organic sediment extracts. The responses are expressed as % E_2 maximum = percent of induction caused by sample extract relative to maximal induction obtained with E_2 calibration (A, C, D) and as percent of solvent control (for B, solvent control = 100%)

Addition of E_2

A common practice in *in vitro* estrogenicity testing is to perform the assay in media deprived of available steroid hormones. This can maximize the sensitivity for detection of weak estrogens and evaluate the maximal ability of the sample to bind to the ER (Kramer *et al.* 1997). However, under natural body conditions, a certain level of E_2 is always present in exposed animals. To determine the estrogenic potential of the sediment extracts in the presence of competing E_2, the dose-response study was conducted in a charcoal-stripped media (deprived of $E_2 < 0.9$ pM) and also with E_2 addition. In our study, E_2 at a concentration of 10 pM, which was equivalent to the EC_{20}, was added to all samples. The addition of natural ligand did not change the character of response of the complex sediment extracts, all samples still elicited pronounced estrogenic effects even in the presence of E_2 (Figure 2D). The responses at lower sample concentrations were increased by addition of E_2 (Figure 2D), however the maximal fold induction was similar to that without E_2, reaching a maximum between 50% (sample 6B) and 134% of E_2-max (sample 9A). These result document additive effect of the samples and E_2 added at the 10 pM level. The role of E_2 concentrations in the testing media for complex mixtures was documented in a study where treatment of the cells with black liquor from pulp and paper production plus E_2 caused significantly higher induction than any component alone or even higher than the maximal induction caused by E_2 (E_2-max) (Zacharewski *et al.* 1995). The authors suggested that black liquor can potentiate the inducing activity of E_2. Alternatively, as in our results, neither synergistic nor antagonistic interactions were observed in studies of estrogenic activity of organic extract from air particulate matter or methanol-extracted pulp and paper mill effluent fraction after cotreatment with E_2 (Clemons *et al.* 1998; Zacharewski *et al.* 1995).

Cytotoxicity

As mentioned, cytotoxicity could be a significant confounding factor when evaluating (anti)estrogenic effects of complex environmental mixtures. In our study, cytotoxicity was assessed as a viability index, which significantly decreased at the greatest or two greatest concentrations of all extracts (Figure 2B). This observation confirmed the morphological damages to cells observed by microscopic examination. Despite the decrease in the viability index, there was a significant dose-dependent increase in ER-mediated activity in all samples (Figure 2A). To account for cytotoxicity, the results were normalized to the viability index. In samples collected after floods (A), the cytotoxicity was measured separately from the luciferase assay. Averaging of both luciferase activity and viability index and calculation of the ratio of the averages resulted in great variation and did not enable reasonable calculation. Further optimization of the assay enabled sensitive simultaneous measurement of viability index, protein content and luciferase activity in each of the 96 wells. This approach was applied for the B-type (before flood) samples and for these the luciferase responses from each well could be normalized separately for the specific viability index (Figure 2C). E_2-EQs

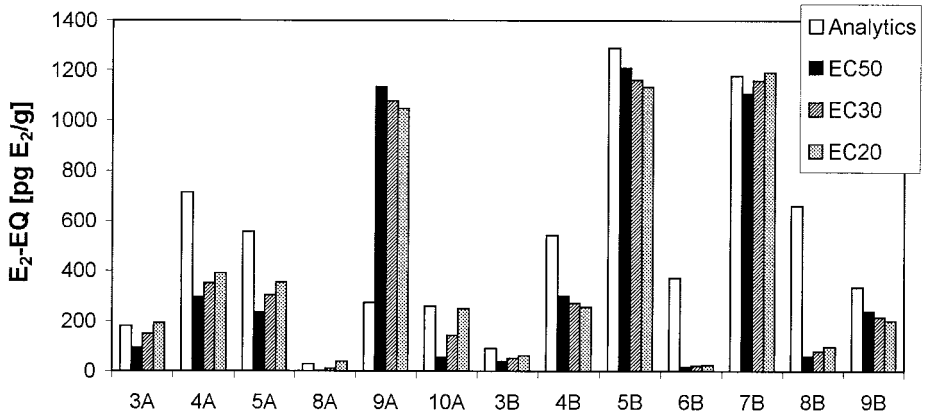

Fig. 3. Estrogenic equivalents (E_2-EQs) in DCM extracts of sediment samples (pg E_2 equivalents/g dry weight) were calculated from the analytical results and from the bioassays as described in the data analysis section. For the bioassays the estrogenic equivalents values were determined as multiple point estimates at levels of response equivalent to 20% (EC_{20}), 30% (EC_{30}), and 50% (EC_{50}) of the maximal response produced by the standard (E_2max). For samples 6B and 8B the values based on EC_{50} are approximations because maximal response for these sample did not reach 50% E_2max. The analytical EEq was calculated on limited number of compounds for which E_2-relative potency values (ERPs) are known (some PAHs, alkylphenols) by multiplying the ERP by concentration of the compound. A = sediments sampled after floods (October 1997), B = sediments sampled before floods (October 1996). Numbers refer to sample site

Table 2. Spearman rank order correlations of estrogenic equivalents determined from analytical results and calculated from bioassays (all samples, not normalized for viability index) at levels of response equivalent to 20% (EC_{20}), 30% (EC_{30}), and 50% (EC_{50}) of the maximal response produced by the standard (E_2); *p*-level in parentheses

		Bioassays		
		EC_{20}	EC_{30}	EC_{50}
Analytic	EQ	0.654 (0.015)	0.698 (0.008)	0.654 (0.015)
Bioassays	EC_{20}		0.972 (< 0.001)	0.912 (< 0.001)
	EC_{30}			0.962 (< 0.001)

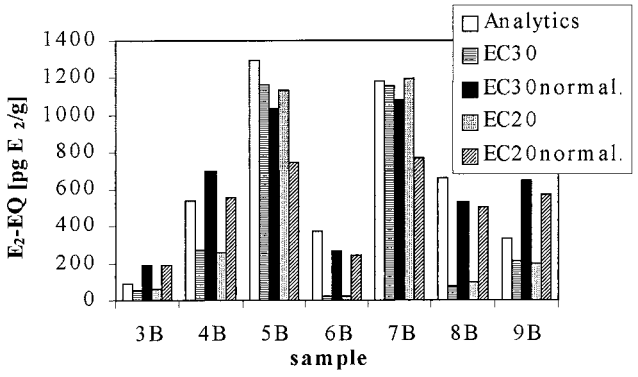

Fig. 4. Effect of normalization of luciferase induction value to viability index. Comparison of E_2-EQs determined from the bioassays raw data and the same data normalized to viability index. The results for two levels of response are compared: 20% (EC_{20}) and 30% (EC_{30}) of the maximal response produced by the standard (E_2max). Sample labels as in Figure 1

for the B samples were compared before and after normalization of the response to viability index (Figure 4). After normalization, the efficacy ranged from 50% to 115% of E_2-max. The E_2-EQs estimated from the normalized data are generally greater than those that were estimated before normalization. Protein content was determined in cells after 72 h of treatment with the extracts (data not shown) as another measure of cell condition. Protein content was correlated with viability index. Decreases of both were observed at the greatest concentrations of extracts. But protein content is a less sensitive measure and more variable than the viability index.

Fractions

Sample extracts were separated on Florisil column into three fractions based on polarity. The effect of concentration of competing E_2 was examined by testing the fractions at two different levels, at 10 pM of E_2 and in charcoal stripped medium (E_2 < 0.9 pM) (Figure 5).

Some of the compounds separated to the first fraction, including PCDDs, PCDFs, and PCBs, are known to elicit effects mediated by the aryl hydrocarbon receptor (AhR). Modulation of endocrine pathways by AhR agonists, such as AhR-mediated antiestrogenicity, has been reported along with complex interactions between ER and AhR signal transduction (Safe 1995; Navas and Segner 1998; Kharat and Saatcioglu 1996). No significant antiestrogenic activity was detected in the first fraction when tested in the E_2 stripped media. However, after addition of E_2 (10 pM) to the media, antiestrogenic activity was observed in the first fraction of some samples (3A, 4A, 9A, 5B). The small effect reflects the low concentrations of OC compounds in samples (Hilscherova *et al.* 2001). However,

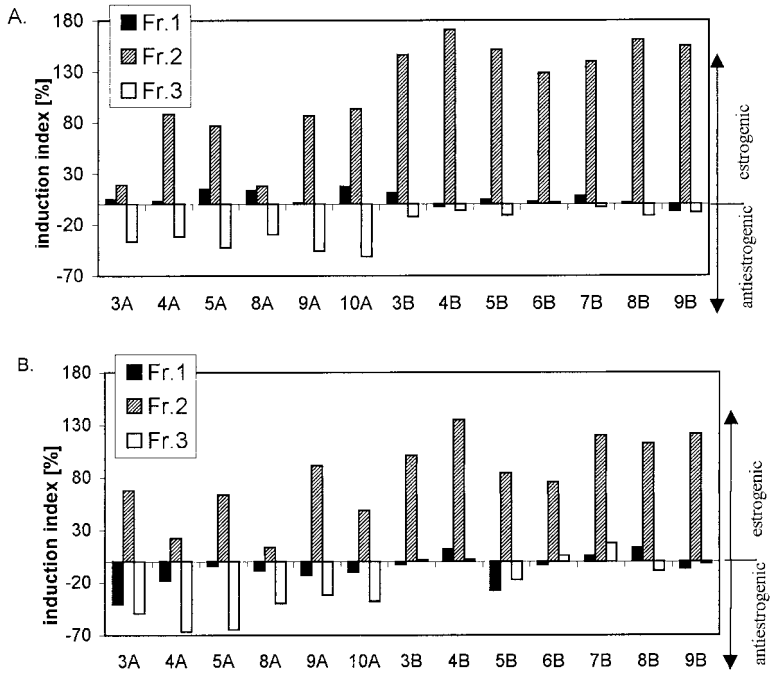

Fig. 5. (Anti)estrogenic activity in the sediment extract fractions. Induction index is defined as the % of sample induction over solvent control minus 100%. Thus, zero value corresponds to the solvent control level, positive values show estrogenic effects, and negative values antiestrogenic effects. A: tested in charcoal-stripped medium deprived of E_2. B: tested in addition of 10 pM E_2 (compared to solvent control with 10 pM E_2). Sample labels as in Figures 1 and 3

some PCBs and their hydroxylated metabolites may act as weak estrogens (Gierty *et al.* 1997; Soto *et al.* 1995; Waller *et al.* 1995). They can contribute slightly to the estrogenic activity in the first fraction and partly compensate the antiestrogenic effects of PCDD/DFs and coplanar PCBs. Mono-*ortho* PCB congeners such as PCB28 or PCB118 and di-*ortho*-congeners PCB52, 101, 138, 153, and 180 were analyzed in the sediment samples (Hilscherova *et al.* 2001). Concentrations of these compounds were relatively low (sum between 14 and 114 ng/g DW).

Significant estrogenic activity was observed in fraction 2, both before and after the addition of E_2. Pesticides that have been shown to elicit weak estrogenic activity, such as *o,p'*-DDT, endosulfan, toxaphene, and chlordecone, elute in this fraction (Khim *et al.* 1999). Another major group of compounds found in this fraction was PAHs. The studies of (anti)estrogenicity of PAHs are equivocal (Navas and Segner 1998). Affinity of certain PAHs for the ER and estrogenic activity has been reported in a study with MCF-7-luc cells (Clemons *et al.* 1998). Other studies documented only antiestrogenic effects of PAHs (Arcaro *et al.* 1999). Alkylphenols, such as NP, which elicit weak estrogenic activity, are also eluted in this fraction.

Antiestrogenicity was apparent in the third fraction, especially in the sediments sampled after floods. The antiestrogenicity was confirmed after the addition of 10 pM of E_2, when some samples caused a decrease of induction to about 35% of the solvent control. The contributors to antiestrogenic effects in the third fraction of A-type samples are probably some polar compounds, which remain to be identified. The antiestrogenic effects may be related to high AhR-mediated (dioxin-like) activity observed in this fraction (Hilscherova *et al.* 2001). An important confounding factor in the detection of antiestrogenicity could be potential cytotoxicity at greater extract concentrations, because reductions in luciferase expression could have been caused by decreased cell viability and inhibition of ER-mediated activities (Kramer *et al.* 1997). However, no obvious decrease in the viability of cells was detected in the third fraction.

To confirm the estrogenic effect of the second fraction and antiestrogenic effects of third fraction, six dilutions of these fractions were tested on bioassay (Figure 6). Both estrogenic activity in fraction 2 and antiestrogenic activity in fraction 3 were observed to be dose-dependent.

Mass-Balance Calculations

Estrogenic equivalents in the mixture are calculated as the sum of the products of the concentrations of individual compounds multiplied by their potency relative to E_2 (ERPs) (Safe 1995). Because not all of the compounds in the mixture could be quantified and ERPs were not available for all of the compounds that were quantified, only limited mass-balance calculations could be performed to determine the relative contribution of the estrogenic compounds analyzed to the total E_2 equivalents. The second fraction that elicited the greatest activity contained, among other compounds, alkylphenols, PAHs, and OC pesticides. For some of these compounds, specific ERPs were available from previous *in vitro* studies with MCF-7-luc cells (Villeneuve *et al.* 1998; Clemons *et al.* 1998).

Alkylphenols, such as NP and OP, have been reported to be estrogenic in both *in vitro* and *in vivo* laboratory studies (Nimrod and Benson 1996; Routledge and Sumpter 1997; Servos 1999). In the river sediments were detected OP and NP, which are degradation products of their corresponding ethoxylates. This is the first report of concentrations of alkylphenols in Czech sediments. The concentrations ranged from 1.7 to 154 ng/g DW (Table 3). All three PAHs for which ERPs are known

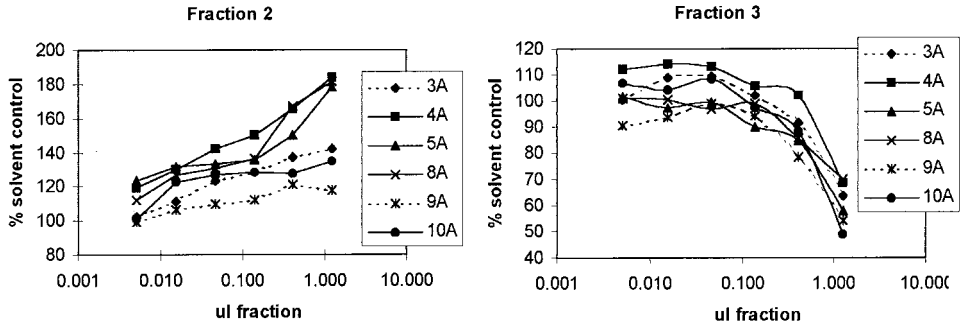

Fig. 6. Dose-response curves of the luciferase activity of the second and third fraction of the sediment extract tested in the stripped media ($E_2 < 0.9$ pM). Sample labels as in Figure 1

Table 3. Alkylphenol and PAH concentrations (ng/g DW) in river sediments and estrogenic equivalents (E_2-EQ, expressed as pg E_2/g DW) contributed by these classes of chemicals (OP = octyphenol, NP = nonylphenol).

	Alkylphenols			PAHs	
Sample	OP	NP	E_2-EQ (pg E_2/g)	Σ PAHs	E_2-EQ (pg E_2/g)
3A	7	43.2	1.12	6,172	177.7
4A	6.8	22.5	0.64	20,060	712.2
5A	8.8	82.9	2.07	16,725	556
8A	1.8	6.4	0.18	1,132	27.4
9A	5.2	137.1	3.27	8,396	269.5
10A	2.2	23.6	0.58	8,778	258.2
3B	4.9	61.6	1.51	3,530	87.9
4B	2	26.5	0.65	16,463	541.3
5B	1.8	9.4	0.25	33,998	1,291.1
6B	2.8	7.1	0.21	11,885	371.7
7B	2.1	23.9	0.59	39,951	1,177.4
8B	5.3	94	2.27	20,395	658.2
9B	3	154.1	3.63	10,530	329.4

(benzo(a)pyrene, benzo(a)anthracene, chrysene) were relatively abundant in the sediment samples. Their concentrations ranged from 21 to 4,260 ng/g DW. ERPs for PAHs were one order of magnitude greater than those for alkylphenols. The concentrations of PAHs were also greater. Therefore, the contribution of PAHs to the calculated EEq was about 98% compared to 2% contributed by alkylphenols (Table 3). F2 also contained small concentrations of some OC pesticides. Studies using multiple assays for assessing estrogenic activity have identified estrogenic potential of some DDT metabolites (*o,p′*-DDT, *o,p′*-DDD, *o,p′*-DDE, *p,p′*-DDE, *p,p′*-DDT) (Soto *et al.* 1995; Klotz *et al.* 1996; Shelby *et al.* 1996; Gaido *et al.* 1997), with ER-affinity approximately 1,000-fold less than 17β-estradiol. The concentration of *p,p*'-DDT was less than the detection limit of 0.1 ng/g in most sediment samples. Concentrations of other OC pesticides were less than 5 ng/gDW. Previous studies have documented the estrogenic activity of some of these OC pesticides at concentrations greater than 1 μg/g (Soto *et al.* 1994). Thus, the relatively small concentrations of these pesticides present in the sediments suggest their contribution to estrogenic potency is insignificant. The mass-balance calculations suggest that PAHs are the primary source of estrogenic activity in the sediments. However, recent studies document that not the parent PAH compounds but their hydroxylated metabolites that are formed during incubation of the cells are probably responsible for big part of the observed estrogenicity (Charles *et al.* 2000). Also, other compounds with unknown estrogenic potency could be present in the complex environmental mixture and contribute to the overall (anti)estrogenic activity.

E_2-EQs calculated from bioassays based on the EC_{20}, EC_{30}, and EC_{50} from the E_2 dose-response and EEqs from analytical results (based on PAHs and alkylphenols) are shown (Figure 3). Concentrations of EEqs were between 28 and 1,178 pg E_2/g DW. E_2-EQs were in good agreement with calculated EEq ($r_{Sp} > 0.65$, $p < 0.05$, see Table 2). In some cases (6B, 8B) the E_2-EQs were significantly less than EEq. This could have been the result of overestimating the relative potencies or due to the antagonistic interactions of other constituents of the mixture. Strong antiestrogenic effects were observed especially in fraction 3 of A samples.

The E_2-EQs for the B samples were also calculated for the data normalized to the viability index. The differences in analytical and bioassay-derived E_2-EQs are much less after normalization to viability index (Figure 4). The absolute values of E_2-EQs were more similar to the EEqs, and the correlation between the estrogenic equivalents derived from bioassays and those that were analytically determined was greater after normalizing the data for viability index. These results suggest that data from bioassays need to be interpreted with caution and cytotoxicity measurement should always be included.

Table 4. Estrogen receptor (ER-)mediated activities of PAHs derivatives

Compound	Abbreviation	ER-mediated activity		
		Stripped Media	10 pM E_2	E_2max
1-methyl-naphthalene	1 CH_3-NAPT	—	E 2.5–75	E 2.5–250
1,2-dimethyl-napthalene	1,2 CH_3-NAPT	A 75–250	—	E 7.5–250
3,6-dimethyl-phenanthrene	3,6 CH_3-PHE	—	E 250	E 7.5–250
9-methyl-anthracene	9 CH_3-ANT	A 25–250	A 250	E 25–250
9,10-dimethylanthracene	9,10 CH_3-ANT	—	E 2.5–75	E 7.5–75
3,9-dimethyl-benzo(a)anthracene	3,9 CH_3-BaA	A 2.5–250	A 75–250	A 25
1-methyl-benzo(c)phenanthrene	1 CH_3-BcPHE	E 7.5–250	E 2.5–75	E 2.5–75
6-hydroxy-chrysene	6 OH-CHR	A 500	—	E2.5–75
1-hydroxy-pyrene	1 OH-PYR	A 500	—	E 7.5–250

ER-mediated activity was tested at three different levels of endogenous substrate E_2: in stripped media deprived of E_2, at 10 pM, and at 170 pM (E_2 max). Observed effects: A = antiestrogenic, E = estrogenic. Listed are only those concentrations (μg/L) where the effects were significant (Mann-Whitney, *t* test, $p < 0.05$)

Model Compounds Tested

As reported, PAHs were the dominant residues in the most active fraction. The results concerning (anti)estrogenic activity of PAHs are ambiguous. There are structural similarities between PAHs and some steroids. It has been documented that, depending on dose and employed assay system, the same chemical may elicit both estrogenic and antiestrogenic effects (Santodonato 1997). In most studies only carcinogenic PAH congeners with four or more rings (four-plus congeners) have been assessed. These PAHs have been reported to be either weakly estrogenic or antiestrogenic. Some studies have reported antiestrogenicity of some PAHs with AhR-mediated activity (Arcaro *et al.* 1999; Chaloupka *et al.* 1992; Tran *et al.* 1996). A study of PAHs with MCF-7-luc cells demonstrated that some PAHs, namely benzo(a)pyrene, benzo(a)anthracene, and chrysene, are capable of interacting *in vitro* with the ER and inducing ER-mediated response (Clemons *et al.* 1998). Detailed studies suggested that estrogen-like activity exhibited by benzo(a)pyrene is predominantly produced by its hydroxylated metabolites (Charles *et al.* 2000) and this could possibly apply for other PAHs as well.

In our study, we tested some PAH derivatives with four or fewer rings and with methyl or hydroxyl substitution. These compounds were found in the second fraction of sediment extracts (qualitative data, not shown). Two hydroxylated and seven methylated PAH-derivatives that were examined for their potential (anti)estrogenic activity are listed (Table 4). The results of these studies demonstrated that the concentration of E_2 in the medium is important parameter in the assay (Table 4). All (anti)estrogenic effects caused by studied compounds were small and close to the limit of significance. These effects were not always completely dose-dependent. Significant dose-dependent antiestrogenicity was found only for 3,9 CH_3-BaA at all three levels of E_2. However, the antiestrogenicity was more pronounced without E_2 addition and decreased at greater E_2 concentrations.

Antiestrogenicity was not significant for most dilutions in the presence of 170 pM E_2. A similar trend was observed for other compounds where estrogenic effects were observed at the greater E_2 concentration, even though the compounds were slightly antiestrogenic in the absence of E_2. The (anti)estrogenic potency of the studied compounds is dependent on the compound concentration and concentrations of ER ligands, if any are present. Probably not only E_2 but also other ER ligands within the complex mixtures can influence the (anti)estrogenic potential of PAH derivatives in environmental samples. Previous findings indicated that the endocrine effect of PAHs may be dependent on the concentration ratio of exo- and endoestrogens (Navas and Segner 1998). Also for hydroxylated PCBs tested on MCF-7-luc cells (Kramer *et al.* 1997) the effect depended on the concentration of E_2 in the media, and the antiestrogenicity decreased at higher E_2 concentrations. *In vitro* studies with MCF-7 cells have documented that only those PAHs that bind to the AhR are antiestrogenic (Chaloupka *et al.* 1992). This observation agrees with our results because only 3,9-CH_3-BaA caused greater AhR-mediated effect (results not shown) as well as significant antiestrogenicity.

Other compounds tested for potential (anti)estrogenicity were PCNs (Table 1). PCNs would have eluted in F1 and F2 of the Florisil column fraction. However, we did not quantify these compounds by instrumental techniques. The primary goal in this study is to test for their potential (anti)estrogenicity. The results for active congeners and technical PCN mixtures that elicited significant response on the bioassays are reported (Table 1). The results are expressed as percentage induction relative to solvent control. Slight estrogenic effect was observed for PCN congeners 48, 53, and 74. Compounds that elicited significant antiestrogenic effect were mostly AhR agonists (congeners 66, 68, 73) (Blankenship *et al.* 2000). However, PCN congener 10 elicited response in MCF-7-luc cells but did not elicit AhR activity. All the other tested congeners elicited no significant ER-mediated activity at tested concentrations. Three of the technical PCN preparations (Halowaxes 1013, 1014, 1051) were found to be antiestrogenic. These mixtures also elicited significant AhR-mediated activity (Blankenship *et al.* 2000). Halowax 1001 also exhibited some antiestrogenicity in media from which most of the E_2 had been removed, but it was not confirmed in the addition of E_2. Halowax 1099 caused induction of luciferase only in presence of E_2. Halowaxes 1000, 1001, and 1099 with no or very little activity contained primarily mono-through tetra-CNs. Alternatively, the active preparations consist primarily of higher chlorinated PCNs (tetra-through octa-CNs). The ER-mediated effects of studied

PAH derivatives and PCNs are relatively weak, and because their concentrations in tested sediments are expected to be much lower that those of other active compounds (such as not substituted PAHs), they would not be expected to contribute significantly to the overall estrogenic responses of the complex sediment extracts.

Conclusions

In vitro cell bioassays can serve as sensitive, specific, and rapid bioanalytical tools to characterize receptor-mediated responses in complex environmental mixtures. While examining receptor mediated responses for the environmental samples, it is important to test the effect of extracts on cell condition to avoid misrepresentation of the results due to cytotoxicity. All studied river sediment extracts elicited estrogenic activity. Bioassays of the total extract coupled with the results of assays on individual fractions can account for interactions within complex mixtures that are not possible to consider in conventional chemical residue analysis and also to account for compounds for which the ER-mediated activity is not known. Fractionation of extracts enables to separate classes of compounds based on their different polarities and thus different characteristics. Thus, fractionation assists in characterization of the complex mixtures while assisting in determining the most active classes of compounds. Fractionation along with limited mass-balance calculations suggested an important contribution of PAHs and/or their metabolites to the overall estrogenic activity. The contribution of alkylphenolic compounds was relatively small. The polar compounds causing antiestrogenic activity in F3 have not yet been identified.

The concentrations of E_2 in the assay medium is an important factor in determining (anti)estrogenicity of single compounds and complex mixtures. Substituted PAHs and some PCNs were relatively less potent to ER-mediated effects, and the effects were dependent on the E_2 concentration in the media.

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References

Ankley G, Mihaich E, Stahl R, Tillit D, Colborn T, McMaster S, Miller R, Bantle J, Campbell P, Denslow N, Dickerson R, Folmar L, Fry M, Giesy J, Gray LE, Guiney P, Hutchinson T, Kennedy S, Kramer V, Leblanc G, Mayes M, Nimrod A, Patino R, Peterson R, Purdy R, Ringer R, Thomas P, Touart L, Van Der Kraak G, Zacharewski T (1998) Overview of a workshop on screening methods for detecting potential (anti-)estrogenic/androgenic chemicals in wildlife. Environ Toxicol Chem 17:68–87

Arcaro KF, O'Keefe PW, Yang Y, Clayton W, Gierthy JF (1999) Antiestrogenicity of environmental polycyclic aromatic hydrocarbons in human breast cancer cells. Toxicology 133:115–127

Balaguer P, Joyeux A, Denison MS, Vincent R, Gillesby BE, Zacharewski TR (1996) Assessing the estrogenic and dioxin-like activities of chemicals and complex mixtures using in vitro recombinant receptor-reporter gene assay. Can J Physiol Pharmacol 74:216–222

Blankenship A, Kannan K, Villalobos S, Villeneuve D, Falandysz J, Imagawa T, Jakobsson E, Giesy JP (2000) Relative potencies of individual polychlorinated napthalenes and Halowax mixtures to induce Ah receptor-mediated responses. Environ Sci Technol 34: 3153–3158

Bortone SA, Davis WB (1994) Fish intersexuality as an indicator of environmental stress. Bioscience 44:165–172

Chaloupka K, Krishnan V, Safe S (1992) Polynuclear aromatic hydrocarbon carcinogens as antiestrogens in MCF-7 human breast cancer cells: role of the Ah receptor. Carcinogenesis 12:2233–2239

Charles GD, Bartels MJ, Zacharewski TR, Gollapudi BB, Freshour NL, Carney EW (2000) Activity of benzo[a]pyrene and its hydroxylated metabolites in an estrogen receptor-α reporter gene assay. Toxicol Sci 55:320–326

Clemons JH, Allan LM, Marvin CH, Wu Z, McCarry BE, Bryant DW, Zacharewski TR (1998) Evidence of estrogen- and TCDD-like activities in crude and fractionated extracts of PM_{10} air particulate material using in vitro gene expression assay. Environ Sci Technol 32:1853–1860

Gaido KW, Leonard LS, Lovell S, Gould JC, Babai D, Portier CJ, McDonnel DP (1997) Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. Toxicol Appl Pharmacol 143:205–212

Gierty JF, Arcaro KF, Floyd M (1997) Assessment of PCB estrogenicity in a human breast cancer cell line. Chemosphere 34:1495–1505

Gillesby BE, Zacharewski TR (1998) Exoestrogens: mechanisms of action and strategies for identification and assessment. Environ Toxicol Chem 17:3–14

Hilscherova K, Machala M, Kannan K, Blankenship AL, Giesy JP. 2000. Cell bioassays for detection of dioxin-like and estrogen receptor mediated activity. Environ Sci Poll Res 7:159–171

Hilscherova K, Kannan K, Kang Y-S, Holoubek I, Machala M, Masunaga S, Nakanishi J, Giesy JP (2001) Characterization of dioxin-like activity of riverine sediments from the Czech Republic. Environ Toxicol Chem 20:2768–2777

Joyeux A, Balaguer P, Germain P, Boussioux AM, Pons M, Nicolas JC (1997) Engineered cell lines as a tool for monitoring biological activity of hormone analogs. Anal Biochem 249:119–130

Kharat I, Saatcioglu F (1996) Antiestrogenic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin are mediated by direct transcriptional interference with the liganded estrogen receptor. J Biol Chem 271: 10533–10537

Khim JS, Kannan K, Villeneuve D, Koh CH, Giesy JP (1999) Characterization and distribution of trace contaminants in sediment from Masan Bay, Korea. 1. Instrumental analysis. Environ Sci Technol 33:4199–4205

Klotz DM, Beckman BS, Hill SM, McLachlan JA, Walters MR, Arnold SF (1996) Identification of environmental chemicals with estrogenic activity using a combination of in vitro assays. Environ Health Perspect 104:1084–1089

Koistinen J, Soimasuo M, Tukia K, Oikari A, Blankenship A, Giesy JP (1998). Induction of EROD activity in Hepa-1 mouse hepatoma cells and estrogenicity in MCF-7 human breast cancer cells by extracts of pulp mill effluents, sludge, and sediments exposed to effluents. Environ Toxicol Chem 17:1499–1507

Kramer VJ, Giesy JP (1995) Environmental estrogens: a significant risk? Human Ecol Risk Assess 1:37–42

Kramer VJ, Helferich WG, Bergman A, Klasson-Wehler E, Giesy JP (1997) Hydroxylated polychlorinated biphenyl metabolites are

anti-estrogenic in a stably transfected human breast adenocarcinoma (MCF7) cell line. Toxicol Appl Pharmacol 144:363–376

Lorenzen A, Kennedy SW (1993) A fluorescence-based protein assay for use with a microplate reader. Anal Biochem 214:346–348

Navas JM, Segner H (1998) Antiestrogenic activity of anthropogenic and natural chemicals. Environ Sci Pollut Res 5:75–82

Nimrod AC, Benson WH (1996) Environmental estrogenic effects of alkylphenol ethoxylates. Crit Rev Toxicol 26:335–364

Pons M, Gagne D, Nicolas JC, Mchtali M (1990) A new cellular model of response to estrogens: a bioluminiscent test to characterize (anti)estrogen molecules. Biotechniques 9:450–459

Richter CA, Tieber VL, Denison MS, Giesy JP (1997) An *in vitro* rainbow trout cell bioassay for aryl hydrocarbon receptor-mediated toxins. Environ Toxicol Chem 16:543–550

Routledge EJ, Sumpter JP (1997) Structural features of alkylphenolic chemicals associated with estrogenic activity. J Biol Chem 272: 3280–3288

Safe S (1995) Environmental and dietary estrogens and human health: is there a problem? Environ Health Perspect 103:346–351

Safe S, Connor K, Ramamoorthy K, Gaido K, Maness S (1997) Human exposure to endocrine-active chemicals: hazard assessment problems. Reg Toxicol Pharmacol 26:52–58

Sanderson JT, Aarts JMMJG, Brouwer A, Froese KL, Denison MS, Giesy JP (1996) Comparison of Ah receptor-mediated luciferase and ethoxyresorufin-o-deethylase induction in H4IIE cells: implications for their use as bioanalytical tools for detection of polyhalogenated aromatic hydrocarbons. Toxicol Appl Pharmacol 137:316–325

Santodonato J (1997) Review of the estrogenic and antiestrogenic activity of polycyclic aromatic hydrocarbons: relationship to carcinogenicity. Chemosphere 34:835–848

Servos MR (1999) Review of the aquatic toxicity, estrogenic responses and bioaccumulation of alkylphenols and alkylphenol polyethoxylates. Water Qual Res J Canada 34:123–177

Shelby MD, Newbold RR, Tully DB, Chae K, Davis VL (1996) Assessing environmental chemicals for estrogenicity using a combination of *in vitro* and *in vivo* assays. Environ Health Perspect 104:1296–1300

Soto AM, Chung KL, Sonnenschein C (1994) The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. Environ Health Perspect 102:380–383

Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO (1995) The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ Health Perspect 103 (suppl 7):113–122

Sumpter JP, Jobling S (1995) Vitellogenesis as biomarker for estrogenic contamination of the aquatic environment. Environ Health Perspect 103:173–178

Tran DQ, Ide CF, McLachlan JA, Arnold SF (1996) The anti-estrogenic activity of selected polynuclear aromatic hydrocarbons in yeast expressing human estrogen receptor. Biochem Biophys Res Comm 229:102–108

Villeneuve D, Blankenship AL, Giesy JP (1998) Interactions between environmental xenobiotics and estrogen receptor-mediated responses. In: Denison MS, Helferich WG (eds) Toxicant–receptor interactions. Taylor and Francis, Philadelphia, PA, 69–99.

Villeneuve DL, Blankenship AL, Giesy JP (2000) Derivation and application of relative potency estimates based on in vitro bioassay results. Environ Toxicol Chem 19:2835–2843.

Waller CL, Minor D, McKinney JD (1995) Using three-dimensional quantitative structure-activity relationships to examine estrogen receptor binding affinities of polychlorinated hydroxybiphenyls. Environ Health Perspect 103:702–707

Zacharewski TR (1997) *In vitro* bioassays for assessing estrogenic substances. Environ Sci Technol 31:613–623

Zacharewski TR, Berhane K, Gillesby BE, Burnison BK (1995) Detection of estrogen- and dioxin-like activity in pulp and paper mill black liquor and effluent using *in vitro* recombinant receptor/reporter gene assays. Environ Sci Technol 29:2140–2146

Článek XIX:

Hilscherová, K., Dušek, L., Šídlová T., Jálová V., Čupr P., Giesy J.P., Nehyba S., Jarkovský J., Klánová J., Holoubek I., 2010. Seasonally and regionally determined indication potential of bioassays in contaminated river sediments. Environmental Toxicology and Chemistry 29 (3), 522-534.

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SEASONALLY AND REGIONALLY DETERMINED INDICATION POTENTIAL OF BIOASSAYS IN CONTAMINATED RIVER SEDIMENTS

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Abstract—River sediments are a dynamic system, especially in areas where floods occur frequently. In the present study, an integrative approach is used to investigate the seasonal and spatial dynamics of contamination of sediments from a regularly flooded industrial area in the Czech Republic, which presents a suitable model ecosystem for pollutant distribution research at a regional level. Surface sediments were sampled repeatedly to represent two different hydrological situations: spring (after the peak of high flow) and autumn (after longer period of low flow). Samples were characterized for abiotic parameters and concentrations of priority organic pollutants. Toxicity was assessed by Microtox test; genotoxicity by SOS-chromotest and green fluorescent protein (GFP)-yeast test; and the presence of compounds with specific mode of action by in vitro bioassays for dioxin-like activity, anti-/androgenicity, and anti-/estrogenicity. Distribution of organic contaminants varied among regions and seasonally. Although the results of Microtox and genotoxicity tests were relatively inconclusive, all other specific bioassays led to statistically significant regional and seasonal differences in profiles and allowed clear separation of upstream and downstream regions. The outcomes of these bioassays indicated an association with concentrations of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) as master variables. There were significant interrelations among dioxin-like activity, antiandrogenicity and content of organic carbon, clay, and concentration of PAHs and PCBs, which documents the significance of abiotic factors in accumulation of pollutants. The study demonstrates the strength of the specific bioassays in indicating the changes in contamination and emphasizes the crucial role of a well-designed sampling plan, in which both spatial and temporal dynamics should be taken into account, for the correct interpretations of information in risk assessments. Environ. Toxicol. Chem. 2010;29:522–534. © 2009 SETAC

Keywords—Sediment Polycyclic aromatic hydrocarbons Polychlorinated biphenyls Organic carbon In vitro bioassays

INTRODUCTION

The dynamics of sediment contamination are an important issue, i.e., in areas with historical and existing pollution sources also in the practical pursuit of the European Water Framework Directive [1]. The effort of governmental environmental organizations is to set concentration limits for the most widespread pollutants in river sediments and thus control the contamination [2]. However, insofar as river sediments represent a dynamic system in areas with occurrence of floods, both spatial and temporal dynamics should be taken into account in risk assessment.

Aquatic sediments serve as a sink for various environmental pollutants. They are considered an important stocking place for contaminants and thus can integrate potential exposure in aquatic ecosystems [3]. Sediments can contain complex mixtures of biologically active compounds with different mechanisms of action, often in relatively small concentrations, which may interact to produce additive, supra-additive, or infra-additive effects.

Association with sediments and particulate matter is of major importance for fate and effects of trace contaminants in aquatic systems. Among the important pollutants accumulating in sediments are the hydrophobic organic contaminants (HOCs), such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and organochlorine pesticides (OCPs), which can persist in this matrix for long periods and bioaccumulate and biomagnify in the food chain. However, it has been well documented that classes of chemicals other than HOCs, such as polyphenolic plasticizers, xenohormones, various pesticides, personal care products, and pharmaceuticals [4–6], can enter sediments and possibly affect aquatic life. It has been also recognized that riverine sediments can be a major sink for and a potential source of estrogenic contaminants [7,8]. Many of these compounds are not routinely monitored, and their toxic effects are not yet fully described. Moreover, it is possible that other chemicals of artificial as well as of natural origin

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whose toxicities have not yet been determined may also be present in sediments [9].

Chemical analyses of complex mixtures of organic residues in sediments provide little information on the biological effects of complex mixtures, and they do not take into account possible interactions among individual chemicals. Moreover, instrumental quantification methods and standards are available only for some compounds, whereas others may not be identified or quantified. Bioassays for general toxicity and genotoxicity as well as biotests based on specific cellular responses have been applied to evaluate biological potencies of various types of environmental samples, including sediments [10–12]. Responses mediated through specific receptors such as estrogen receptor (ER), androgen receptor (AR), and aryl hydrocarbon receptor (AhR) are known to be involved in some endocrine-disruptive and other adverse effects of xenobiotics [13]. Aryl hydrocarbon receptor-mediated effects are considered a valuable marker of contamination by dioxin-like compounds [14] that can negatively affect liver functions as well as immunity or the endocrine and nervous systems. Sediments polluted with persistent organic pollutants (POPs), many of which are ligands of the AhR, are a cause of concern around the industrialized world [6,15,16]. Bioassays that can integrate the effects and determine the potencies of mixtures in environmental samples offer relatively rapid and cost effective means of prioritizing samples before more elaborate chemical analyses [17]. These bioanalytical techniques serve as simple, sensitive screening systems for the presence of chemicals and also account for their possible interactions without the need to identify and quantify individual compounds [18]. The application of instrumental analyses to quantify specific compounds in combination with bioassays to quantify the total activity can help in assessing the potential effects of complex mixtures and determining the probability of the presence of unidentified active compounds.

Polluted sediments in rivers can be subject to remobilization, transport, and redistribution during certain times of year because of periods of high flow, floods, or human activities [19]. In remobilization processes, the long-term role of river sediments as sinks changes into a secondary contamination source, and the residues associated with sediments can be resuspended [20,21] and redistributed throughout the aquatic ecosystem, posing potential risk to the downstream sites [3,22]. An important role in interaction of organic pollutants with sediments and their release under certain conditions is played by the abiotic and biotic sediment characteristics; the most crucial ones are the amount and character of the particulate organic matter as well as the size and specific surface of the sediment particles [23–25].

Risk assessment of river sediments as a complex and dynamic environmental matrix requires an integrative approach of several environmental disciplines, including sedimentology, geochemistry, environmental chemistry, and ecotoxicology. In the present study, we report the results from a large-scale assessment of river sediments in a prototypical industrial area in the Czech Republic, which represents a suitable model ecosystem for pollutant distribution research at a regional level [26]. Results of related investigations focused on contact sediment toxicity in relation to contamination, and sediment characteristics are reported in another study by Bláha et al. [27] in this issue. Parts of the selected model study area (Zlín region of the Czech Republic) are regularly flooded, and the water and sediment quality has been impacted by historical industrial and agricultural activities. Thus there is potential for contamination by various types of pollutants and also a risk of redistribution of the contamination during floods, which has been documented in our previous study [26]. The present study investigates the seasonal and spatial dynamic of contamination of the sediments from the Morava River and the Drevnice River and its tributaries in this regularly flooded model area. A two-year study of river sediments has been performed to reveal temporal and spatial variations in contamination with traditionally studied pollutants, compounds with specific mode of action (dioxin-like and endocrine disruptive potencies), as well as toxic or genotoxic potencies. The principal aims of the study were to examine associations among outcomes of chemical and biological activity-based analyses in regionally and seasonally designed biomonitoring study, to quantify main sources of variability in the environmental data and their influence on bioindication potential of applied bioassays, to perform multivariate pattern analysis and extract clusters of abiotic and biotic measures with environmentally relevant interpretation, and to assess seasonally driven changes in profiles of environmental parameters.

MATERIALS AND METHODS

Sediments were sampled at 14 localities of the Morava River (one of the major tributaries of the Danube) as well as the Drevnice River, which flows into the Morava, and its tributaries, in the Zlín region, Czech Republic, where diverse sources of anthropogenic contamination (rural, industrial, diffuse) have existed for centuries (Fig. 1). Samples were collected during four campaigns in spring and autumn of 2005 and 2006, with a total of 56 sediment samples. The campaigns represent two different hydrological situations: spring (after the peak of early spring high flow) and autumn (after longer period of low flow). The sampling sites represent five regions (labeled I–V) within this area (Fig. 1A), which include rural as well as urban and industrialized areas. The characteristics of each region and potential sources of contamination are provided in Table 1. The sites were classified into these regions according to their location and contamination characteristics based on longer-term pollution data presented in our previous study [26]. The division into the regions has been validated by cluster analysis (Fig. 1B). Regions I and II represent the source streams of the river Drevnice, region III corresponds to the downstream part of this river in urban industrial area, region IV covers the larger river Morava above the confluence with Drevnice, and region V covers the rivers below the confluence and the outflow of the whole area. Surface sediments (from top 10 cm layer) were collected by use of a trowel from the sedimentation basis of the riverbed in zones of calm flow close to the riverbank. Representative samples were prepared by mixing five to eight subsamples from an area of approximately 4 m^2. Pieces of material greater than 1 cm were removed, and sediments were freeze dried. Dry sediments were homogenized, ground with a pestle and mortar, and sieved using a 2-mm sieve.

Total organic carbon content (TOC) was determined by use of a high-temperature TOC/TNb Analyzer LiquiTOC II (Elementar Analysensysteme). Detailed grain size distribution was determined with combined sieving and laser diffraction

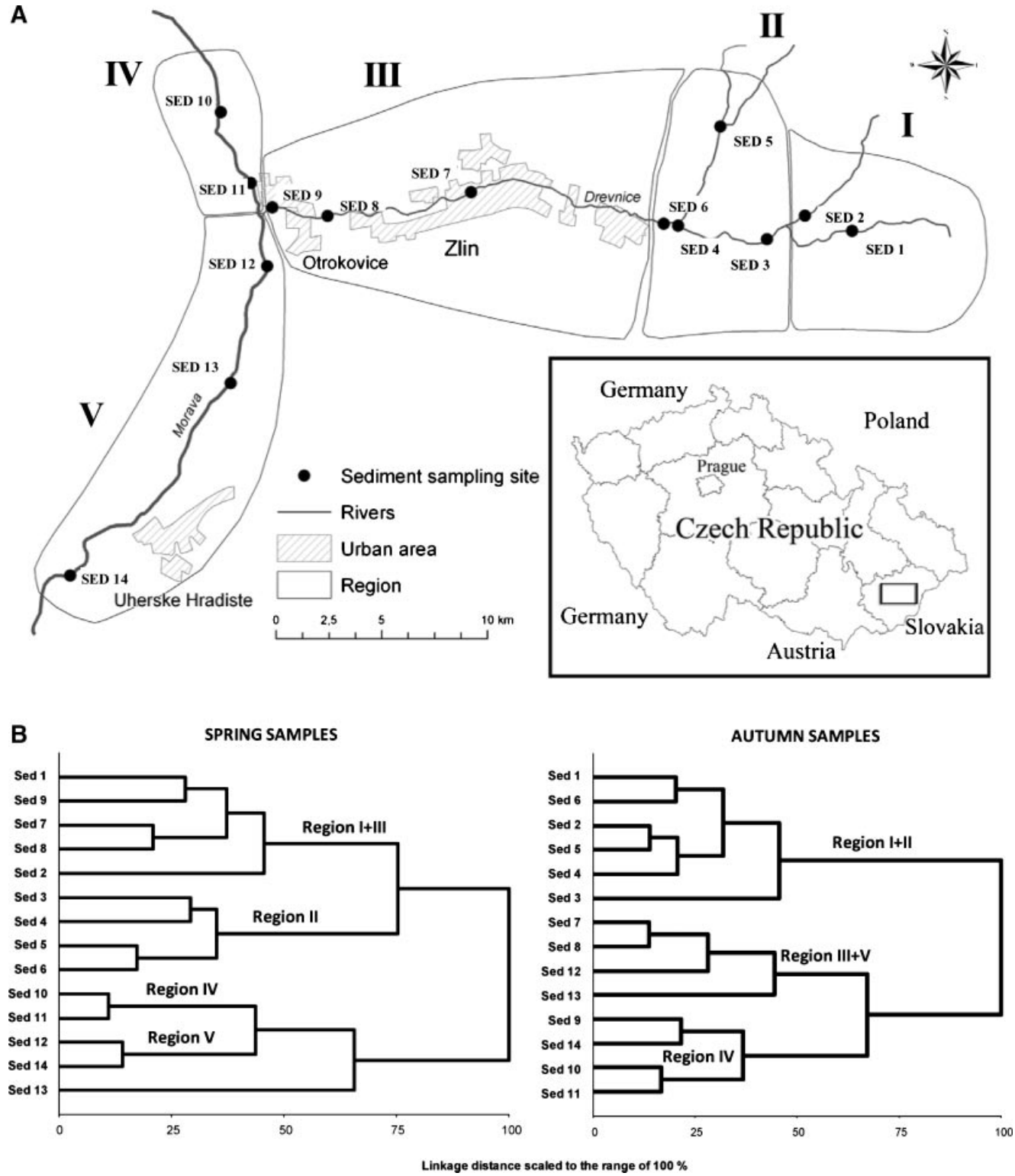

Fig. 1. Locations of sediment sampling sites (SED) and regions I to V in the area of interest (**A**) and multivariate clustering of sediment sites on the basis of concentration of organic pollutants (**B**).

methods to cover the wide size spectra. The Retsch AS 200 sieving machine covers the coarser grain fractions (0.063–4 mm), whereas the Cilas 1064 laser diffraction granulometer was used for finer grained sediments (0.0004–0.5 mm). Ultrasonic dispersion and distilled water were used prior to analyses in order to avoid flocculation of particles. Particles of less than 4 μm diameter were described as clay.

Cation exchange capacity (CEC) of sediments was calculated as the sum of chemical equivalents of H^+ (from pH), Ca^{2+}, Mg^{2+} (determined by flame atomic absorption

Table 1. Characteristics of sampled sediments (based on sediment dry wt) and sampling sites (Czech Republic)[a]

Site code	Site name	Texture (prevailing type)	TOC (%) median (min–max)	CEC (meq/kg) median (min–max)	Clay (%) median (min–max)	Anthropogenic matrices (%) median (min–max)	Contamination sources
Region I							Local sources (SFC)
SED1	Bratrejovka—Bratrejov	Silty sand	3.1 (1.6–4.6)	382 (356–431)	5.6 (4–7.5)	6.0 (3–7)	
SED2	Lutoninka upstream	Silty sand	1.8 (1.5–3.9)	478 (387–671)	5.9 (4.4–7.9)	12.5 (3–45)	
Region II							AG, SFC, OEL, SW, TF,
SED3	Lutoninka downstream	Sandy gravel	0.6 (0.4–0.9)	255 (238–306)	1.5 (0.9–2.1)	11.5 (8.8–14)	industry: chemistry
SED4	Lutoninka above the confluence with Drevnice	Silty sand	2.2 (0.6–5.2)	341 (206–614)	4.9 (3.8–6.6)	6.0 (4–8.1)	
SED5	Drevnice (below the confluence with Trnavka)—Slusovice	Silty sand	1.0 (1.0–1.2)	273 (174–394)	3.4 (1.6–4.0)	10.0 (9–12)	
SED6	Drevnice (below the confluence with Lutoninka)—Lipa	Silty sand	1.7 (1.2–2.9)	288 (239–695)	3.4 (3.3–4.4)	3.5 (1–49)	
Region III							AG, OEL, SFC, SW,
SED7	Drevnice (Zlin—Prstne)	Silty sand	4.3 (3.3–4.5)	598 (554–634)	4.7 (4–7.1)	50.0	TF industry: chemistry,
SED8	Drevnice (below Malenovice)	Sandy silt	3.3 (2.6–4.1)	549 (350–565)	8.7 (6.9–17)	20.5 (1–40)	energy, processing
SED9	Drevnice—Otrokovice	Silty sand	2.8 (1.4–3.5)	393 (193–495)	5.0 (0.6–9.8)	48.5 (37–51)	of metals
Region IV							AG, OEL, SW industry:
SED10	Morava—Kvasice	Silty sand	1.5 (0.8–2.0)	266 (123–282)	7.2 (2–12.7)	9.0 (4–14)	chemistry and other
SED11	Morava (above the confluence with Drevnice)	Sandy silt	1.3 (0.7–2.4)	321 (124–344)	7.9 (1.4–8.4)	15.0 (45–34)	
Region V							AG, SW, SFC, TF, OEL,
SED12	Morava—Napajedla	Silty sand	1.4 (0.1–3.6)	212 (32–388)	3.4 (0.3–9.7)	14.5 (4–31)	industry: chemistry,
SED13	Morava—Spytihnev	Sandy silt	2.9 (1.3–3.1)	285 (164–629)	12.0 (2.8–12.4)	14.0 (13–36)	food, energy
SED14	Morava—Kostelany	Silty sand	1.4 (0.6–3.0)	193 (162–715)	4.5 (1.4–6.8)	6.3 (5.6–7)	

[a] TOC = total organic carbon content; CEC = cation exchange capacity; AG = agriculture; OEL = old ecological loads; SFC = solid fuels combustion; SW = sewage waters; TF = traffic emissions.

spectrometry), and K^+ (determined by flame atomic emission spectrometry) in Mehlichs II extractants. For the analyses of organic pollutants and assessment of the extracts in bioassays, 10 g of lyophilized sediments was extracted with dichloromethane using an automatic Büchi extractor (Büchi Labortechnik AG). Laboratory blank and reference material were analyzed with each set of samples, and surrogate recovery standards were used for quality assurance/quality control samples prior to extraction. Terphenyl and PCB 121 were used as internal standards for PAHs and PCBs analyses, respectively. Activated Cu was used for S removal. Fractionation was achieved on a silica gel column; a sulfuric acid-modified silica gel column was used for PCBs/OCPs analysis. Samples were analyzed using a GC-MS instrument (HP 6890, HP 5973; Agilent) supplied with a J&W Scientific fused-silica column DB-5MS 5% Ph for PCBs (PCB 28, PCB 52, PCB 101, PCB 118, PCB 153, PCB 138, PCB 180), OCPs (isomers of hexachlorocyclohexane: α-HCH, β-HCH, γ-HCH, δ-HCH; *p,p′*-dichlorodiphenyldichloroethylene DDE, *p,p′*-dichlorodiphenyldichloroethane DDD, *p,p′*-dichlorodiphenyltrichloroethane DDT; hexachlorobenzene HCB; pentachlorbenzene PeCB), and 16 U.S. Environmental Protection Agency polycyclic aromatic hydrocarbons. Concentrations of contaminants were quantified using Pesticide Mix 13 (Dr. Ehrenstorfer, Augsburg, Germany) and PAH Mix 27 (Promochem) standard mixtures.

Bioassays

Toxicities of extracts were quantified by use of the bacterial Microtox test and genotoxicity with SOS-chromotest and green fluorescent protein (GFP)-yeast test. Sediment contamination by compounds with specific modes of action was assessed by in vitro bioassays for the dioxin-like activity, anti-/androgenicity, and anti-/estrogenicity.

The Microtox assay uses freeze-dried luminescent bacteria (*Vibrio fischeri*) as test organisms [28]. Toxicity of the sample was determined by measuring the decrease in bioluminescence of bacteria and expressed as the concentration causing 50% light reduction compared with negative control (EC50). To compare the toxicity of individual samples, EC50 was transformed into toxic units (TU = 100/EC50).

Genotoxicity was quantified spectrophotometrically from the response of reporter gene directly linked to the DNA damage of *Escherichia coli* tester strain in the SOS-chromotest [29]. In the GFP assay, the *Saccharomyces cerevisiae* strain contains a multiple-copy plasmid bearing the RAD54 gene fused to the GFP gene [30]. Induction of the RAD54 promoter as a result of DNA damage results in production of the GFP, which was quantified by using a fluorescence polarization detector. In both assays for genotoxicity, the general toxicity was assessed at the same time. Cytotoxicity values as a result of more general macromolecular damage (SOS-T, GFP-T) are based on absorbance data (SOS, alkaline phosphatase activity; GFP, cell density) that give an indication of reduction in proliferative potential, and these data were normalized to the untreated control. Genotoxicity data (SOS-G, GFP-G) are based on induction of reporter gene (absorbance or fluorescence detection) in the cells of test strains normalized to the untreated control (=1). The samples for which the induction factor was greater than 1.5 (SOS) or 1.3 (GFP) for any tested concentration were determined to be significantly genotoxic.

The potencies of the sediment extracts to elicit dioxin-like effects via AhR signaling were determined with the H4IIE-*luc* bioassay (rat hepatocarcinoma cells stably transfected with luciferase gene under control of AhR) [11]. The ER-mediated activity of sediment extracts was evaluated in bioassay with human breast carcinoma cell line MVLN transfected with estrogen receptor-linked luciferase gene. Procedural details for both assays have been described previously [10,11]. The H4IIE-*luc* cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and MVLN cells in DMEM/F12 medium (Sigma-Aldrich), both supplemented with 10% fetal calf serum Mycoplex (PAA). All cell culture bioassays were performed in 96-well microplates. The H4IIE-*luc* cells were exposed in the cultivation medium, and the MVLN cell line was exposed in DMEM/F12 supplemented with 5% dialyzed fetal calf serum (PAA), which was additionally dextran/charcoal treated to decrease background levels of estradiol further. After plating, cells were exposed to dilutions of sediment extracts or standards for 24 h in triplicate. The standard calibration was performed with reference compounds: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 0–500 pM) in case of H4IIE-*luc* or 17β-estradiol (E2; 0–500 pM) for MVLN. Effects of sediment extracts on MVLN cells were assessed either singly or in combination with 33 pM E2 as the competing endogenous ligand. After 24 h of exposure, the intensity of luciferase luminescence was measured using the Promega Steady Glo Kit. Cytotoxicity of tested dilutions of the samples was measured using neutral red uptake assay [31], and data from cytotoxic sample dilutions were excluded from calculations. The effects elicited by sediment extracts were related to the luciferase induction caused by the reference compounds and expressed as TCDD or estradiol equivalents (BIOTEQ and EEQ, respectively).

The bioluminescent yeast assay was used for detecting anti-/androgenic activity of the sediment extracts. The assay is based on the *S. cerevisiae* strain stably transfected with human AR along with firefly luciferase under transcriptional control of androgen-responsive element [32,33]. The yeast cells were incubated in 96-well culture plates with the sample alone or in combination with testosterone (10^{-8} M) for 3 h, and then the signal was detected after adding D-luciferin substrate. The results from the AR-specific yeast strain have been normalized to the results from the constitutively luminescent strain to cover the effects of the samples on yeast propagation [32]. The results from sample dilutions that were considered cytotoxic were discarded from the data analyses. Antiestrogenicity and antiandrogenicity correspond to the decrease in activity of the signal given by a specified amount of competing estradiol or testosterone. The responses are shown either as IC50 (concentration that reduces the effect by 50%) values or, for better clarity of the trends in graphs, expressed as an index of antiestrogenicity (AE) or antiandrogenicity (AA), which correspond to reciprocal values of IC50.

Statistical analysis

Sample frequency distributions were examined prior to statistical analyses, and standard, robust summary statistics were used to describe distribution patterns in the primary data. Nonparametric tests were applied for mutual comparisons of two or more variants (Mann–Whitney test, Kruskal–Wallis test, mean ranks post hoc test). Two-way ANOVA was applied to

examine relative contribution of regions and seasons to the overall experimental variability. Log transformation $\mathrm{Ln}(X+1)$ of both chemical and biological parameters was verified as effective in reaching the normality (goodness-of-fit test and Shapiro–Wilk's test). For toxicity tests with possible outcome in negative values, the transformation function $\mathrm{Ln}(X+100)$ was applied. The transformation also sufficiently stabilized the variability of parameters (Levene's test), which further facilitated usage of the ANOVA model. Both Spearman rank correlation coefficient (r_s) and Pearson's product-limit correlation (with log-transformed variables) were applied as measures of association among the compared parameters. The log transformation allowed multivariate clustering of sediment sites according to concentration profile of organic pollutants (based on Euclidean distances and the farthest neighbor algorithm).

Multivariate variation of the bioassays performed, as well as chemical parameters, was further summarized by use of principal component analysis (PCA). Component loading vectors explained the relationships among the biotests and chemical contamination. Component score vectors were second key outcomes of PCA as pairwise uncorrelated variables that were used for the final exploratory survey of the data from sites and regions. The most informative bilinear projections showing the associations between objects (examined sediment sites) and variables (bioassays and chemical parameters) were reached if logarithmically transformed variables directly entered the PCA, i.e., analyses based on covariance matrix. Component weight vectors were scaled to the length one. Biplot was used as a common graphical tool representing not only projections on extracted principal components but also the 2-D loadings of original variables by lines. The analyses were performed in Statistica® for Windows® 8.0 (StatSoft) and SPSS 12.0.

RESULTS AND DISCUSSION

Because sediments are a dynamic system, which has been shown to function as a secondary source of contamination in areas with existing pollution sources and occurrence of floods [34], a range of sites was selected to cover all locally important regions and types of areas in the river basin and to detect probable active sources of pollution (Table 1 and Fig. 1). The selection of site and region sampling design was based on our previous study, which has documented the potential for redistribution of the pollutants in the studied model area during floods as well as regional differences in contamination with HOCs [26]. The seasonal sampling strategy effectively extended the range of detectable environmental concentrations of all key pollutants and thus allowed relevant correlation with responses of applied bioassays. In both years, the spring sampling characterized the period after the greatest annual discharge, whereas both autumn samplings characterized the conditions of least discharge. The repeated successive sampling revealed substantial heterogeneity in many sediment characteristics, with the following regional profile (Table 1 and Fig. 2). The more upstream sediments in the Drevnice watershed (sed 1–6) had typically low concentration of TOC, low content of clay, and low proportion of anthropogenic materials during both seasons. The same was observed for the most downstream sampling points (sed 10–14), but mostly during spring. The downstream sites accumulated both clay and TOC from spring to autumn; the seasonal changes are responsible for greater variability of abiotic characteristics in the most downstream locations. On the other hand, the sites located in the middle of the area of interest (sed 7–9) were easily and significantly distinguished from the others by substantially greater TOC and clay content, greater proportion of anthropogenic materials, and greater CEC (Table 1 and Fig. 2).

No sediment quality criteria (SQC) have been promulgated in the Czech Republic, but the concentrations of PAHs at some sites exceeded the maximal permissible limit for sediments and also the effect range median values suggested in the literature [2,35]. However, the PAHs concentrations were comparable to those in other contaminated sediments from the Czech Republic [11] and worldwide [25,36–38]. Concentrations of PCBs and OCPs in sediments were generally less than SQC suggested in the literature, with few exceptions [37].

Results from some of the sites demonstrated large regional differences among the concentrations of residues and the character of the sediments and the potencies of the extracts in the bioassays. Therefore, clustering of searching for homogeneous groups of sites should improve interpretation of results, both from ecotoxicological and from regional perspectives. Distribution of all main organic contaminants was found to be strongly regionally and seasonally determined. Because of the significant seasonal differences, the sites were clustered according to the concentration of the primary organic pollutants of concern (PAHs, PCBs, HCHs, HCB, PeCB) separately for spring and autumn. The clustering classified sediments into five regions (Figs. 1B and 2). Regions I and II, Drevnice upstream areas, were characterized by local sources of contamination predominated by PAHs, in both spring and autumn. A seasonally changeable central area was defined as region III. In spring, contamination in this region resembled that in the more upstream region, but, in autumn, this region was clustered with the downstream sites. These changes hypothetically reflected interseasonal transport of sediment material. Region III was distinct from the others in having greater concentrations of PAHs, HCHs, HCB, and DDTs. Region IV expectedly groups the sites located upstream in the Morava River, which has sources of contamination different from those of the Drevnice River and is classified differently from the other areas both in spring and autumn (Fig. 1B). Region V can be regarded as the efflux of the whole area, exposed to waters from the Drevnice River and from upstream areas of the Morava River. It inevitably resulted in increased heterogeneity among samples. All sites clustered in this region exhibited the greatest seasonal changes in contamination. The clustering (Fig. 1B) and concentrations of residues measured (Fig. 2) indicate that both regional and seasonal differences are important. Such a structured area cannot be reliably represented by a single sampling campaign. However, the regional and seasonal differences are not necessarily coupled. Polycyclic aromatic hydrocarbons are evidently the predominant pollutants of the region, in spring with increased upstream concentrations and in autumn as a ubiquitous group of contaminants. Polychlorinated biphenyls are also an important but dynamic group, in spring with higher concentrations upstream; however, in autumn, the profile was reversed, with greater concentrations observed downstream than upstream. The other chlorinated POPs were found to be less regionally dynamic, with profiles similar to the profile of

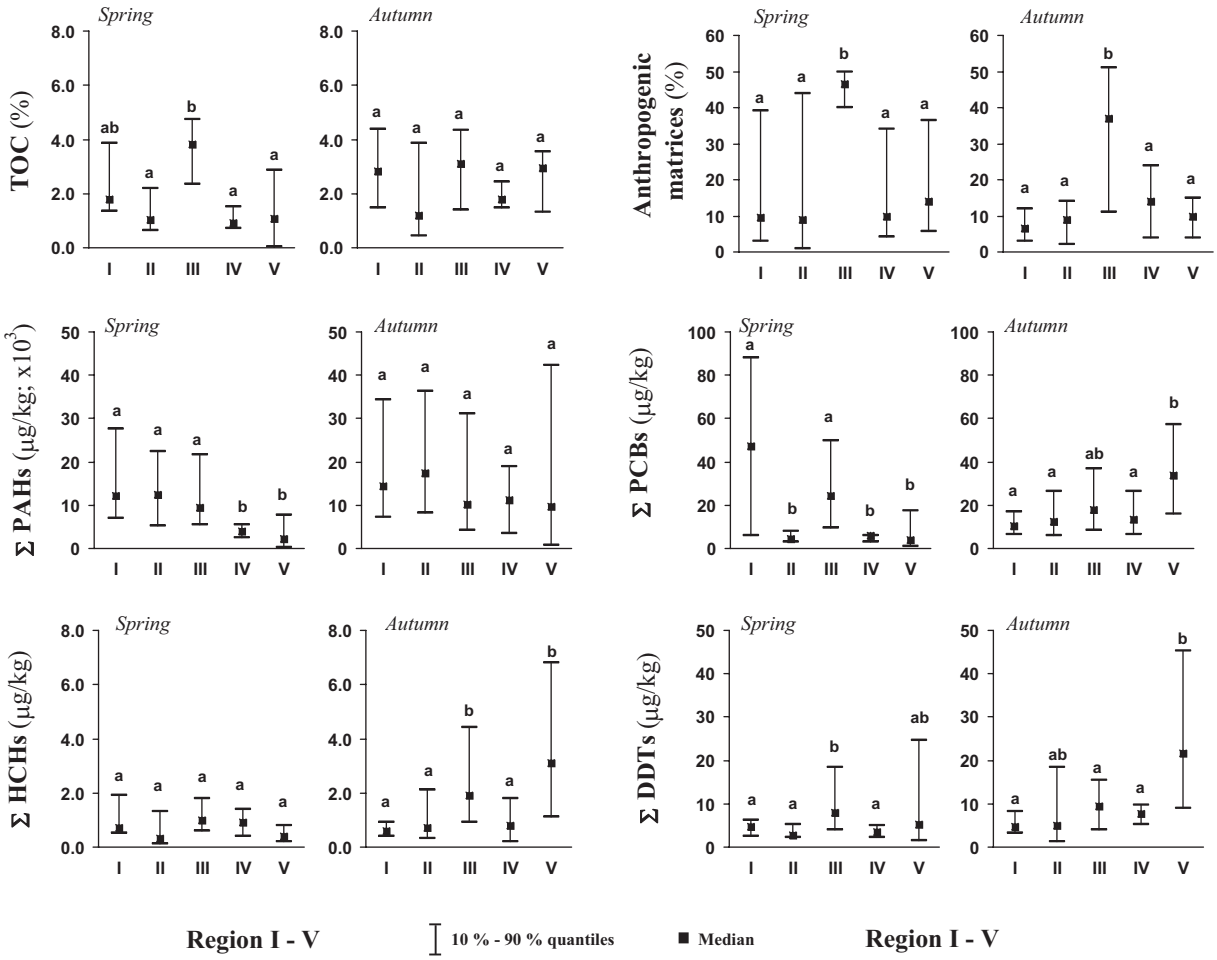

Fig. 2. Regional levels of selected sediment characteristics and concentrations of organic pollutants in spring and autumn. Values marked by the same lowercase letter were not significantly different ($p < 0.05$; Kruskal–Wallis test and mean ranks post hoc test). TOC = total organic carbon; PAHs = polycyclic aromatic hydrocarbons; PCBs = polychlorinated biphenyls; HCHs = hexachlorocyclohexanes.

Table 2. Range of abiotic characteristics (based on sediment dry wt) and contamination of examined sites in a two-way ANOVA model[a]

Soil characteristics/ pollutant	Range of regional median values[b]			Two-way ANOVA model[d]		
	Spring samples	Autumn samples	Range of seasonal median values[c] (spring–autumn)	Regions (%)	Seasons (%)	Interaction regions × seasons (%)
TOC (%)	0.81–3.8	1.6–2.9	1.54–2.42	41*	9.2	NS
CEC (meq/kg)	162–554	307–481	303–404	50*	9.2	15*
Clay (%)	2.9–6.9	3.1–9.4	4.7–5.7	32*	3.5	21*
Anthropogenic mixture (%)	9.7–48	7 – 32.5	8.5–21.3	23*	11	NS
∑PAHs (μg/kg)	1 569–14 757	11,170–18,728	9,444–16,061	38.5*	21*	NS
3-ring PAHs (μg/kg)	184–1,745	1,151–3,158	1,006–1,820	24*	17*	NS
4–5-ring PAHs (μg/kg)	788–7,895	6 630–12,856	5,105–8,496	43*	18*	NS
>5-ring PAHs (μg/kg)	598–5,117	3 389–7,289	3,433–4651	43*	19*	NS
∑PCBs (μg/kg)	2.6–47	11.0–29.4	5.9–14.6	26*	8.5	48*
3–4-Cl PCB (μg/kg)	0.4–1.8	0.7–3.1	0.8–1.1	28*	6.1	NS
5-Cl PCB (μg/kg)	0.4–7.8	1.1–3.3	1.1–1.8	22*	2.3	50*
6–7-Cl PCB (μg/kg)	2.0–38	7.6–19.9	4.0–11.7	25*	9.1	41*
∑HCHs (μg/kg)	0.4–1.1	0.6–2.5	0.9–1.1	8.5	9.2	25*
∑DDTs (μg/kg)	1.9–5.8	5.2–23.8	4.2–9.1	11.9	6.5	18*
HCB (μg/kg)	1.1–8.4	1.0–4.9	2.5–2.0	62*	3.1	NS
PeCB (μg/kg)	0.04–0.47	0.08–0.52	0.08–0.15	22*	0.2	NS

[a] TOC = total organic carbon content; CEC = cation exchange capacity; PAHs = polycyclic aromatic hydrocarbons; PCBs = polychlorinated biphenyls; HCHs = hexachlorocyclohexanes; HCB = hexachlorobenzene; PeCB = pentachlorbenzene; NS = nonsignificant.
[b] Minimum and maximum of regionally calculated median values (regions I–V; see also Table 1).
[c] Spring–autumn median values calculated over all examined sites.
[d] Components of overall variability that belong to the differences among regions, seasons and their interaction (if significant). Calculated as ratios of relevant sum of squares (two-way ANOVA model; log-transformed primary data.
* Statistically significant effect of a given component: $p < 0.05$.

Table 3. Response of bioassays (based on sediment dry wt) in examined sites in two-way ANOVA model[a]

Toxicological parameters	Range of regional median values[b]		Range of seasonal median values[c] (spring–autumn)	Two-way ANOVA model[d]		
	Spring samples	Autumn samples		Regions (%)	Seasons (%)	Interaction: regions × seasons
Microtox (TU)	39–218	181–925	83–329	3.3	66*	NS
SOS-T 15 mg/ml (%)[e]	(−)10.8–(+)13.8	9.7–31.7	7.9–22.9	3.3	20*	NS
SOS-G 15 mg/ml[e]	0.88–1.03	0.85–0.99	0.93–0.90	8.5	1.3	NS
GFP-T 15 mg/ml (%)[e]	(−)5.1–(+)53.0	40.7–60.3	35.3–49.7	11	27*	16*
GFP-G 15 mg/ml[e]	0.9–1.3	0.8–1.5	1.1–1.2	12	0.7	NS
BIOTEQ (ng/g)	1.0–8.7	2.6–7.6	4.3–4.4	43*	1.9	NS
EEQ (ng/g)	0.01–0.12	0.11–0.34	0.05–0.18	19*	53*	NS
AE IC50 (mg)	1.31–5.76	0.20–0.81	1.55–0.35	16*	37*	NS
AA IC50 (mg)	0.11–0.72	0.15–0.49	0.23–0.28	30*	0.1	NS

[a] TU = toxic unit (TU = 100/EC50), SOS-T and SOS-G = toxicity and genotoxicity from SOS chromotest, GFP-T and GFP-G = toxicity and genotoxicity from GFP test, BIOTEQ = TCDD-equivalent, EEQ = estradiol-equivalent, AE IC50/AA IC50 = sediment equivalent that reduces the effect by 50% for antiestrogenic/antiandrogenic effect; NS = nonsignificant.
[b] Minimum and maximum of regionally calculated median values (regions I–V; see also Table 1).
[c] Spring–autumn median values calculated over all examined sites.
[d] Components of overall variability that belong to the differences among regions, seasons and their interaction (if significant). Calculated as ratios of relevant sum of squares (two-way ANOVA model; log-transformed primary data.
[e] Applied dose: 15 mg sediment in ml assay reaction mixture.
* Statistically significant effect of a given component: $p < 0.05$.

PCBs in autumn, when concentrations were greater in regions IV and V than in regions I and II. Concentrations of HCHs and DDTs were greater in autumn than in spring, whereas the opposite trend was observed for HCB.

The results are confirmed also in Table 2, in which both regional and seasonal components of variability are investigated by use of the two-way ANOVA model. There were statistically significant contributions of most of the pollutants to discrimination among regions (sites), but only PAHs exhibited systematic seasonal changes (remarkably increased concentration from spring to autumn, in median values from 9.4 to 16 mg/kg, dry wt). Polycyclic aromatic hydrocarbons comprising more than three rings contributed to the discrimination among regions more substantially than the dynamic group with three rings, which corresponds to their higher K_{OW} values and increased association with bottom sediments. The seasonality of PAH concentrations along with TOC has also been shown in a recent study from Chinese rivers [25]. Different seasonal distribution patterns among regions were found for PCBs, HCHs, and DDTs and statistically justified by the region–season interaction component of the model. Polychlorinated biphenyls containing more than four chlorine atoms were the most variable contaminant group in the area of interest, with the most significant interaction regions × seasons in the ANOVA model (Table 2).

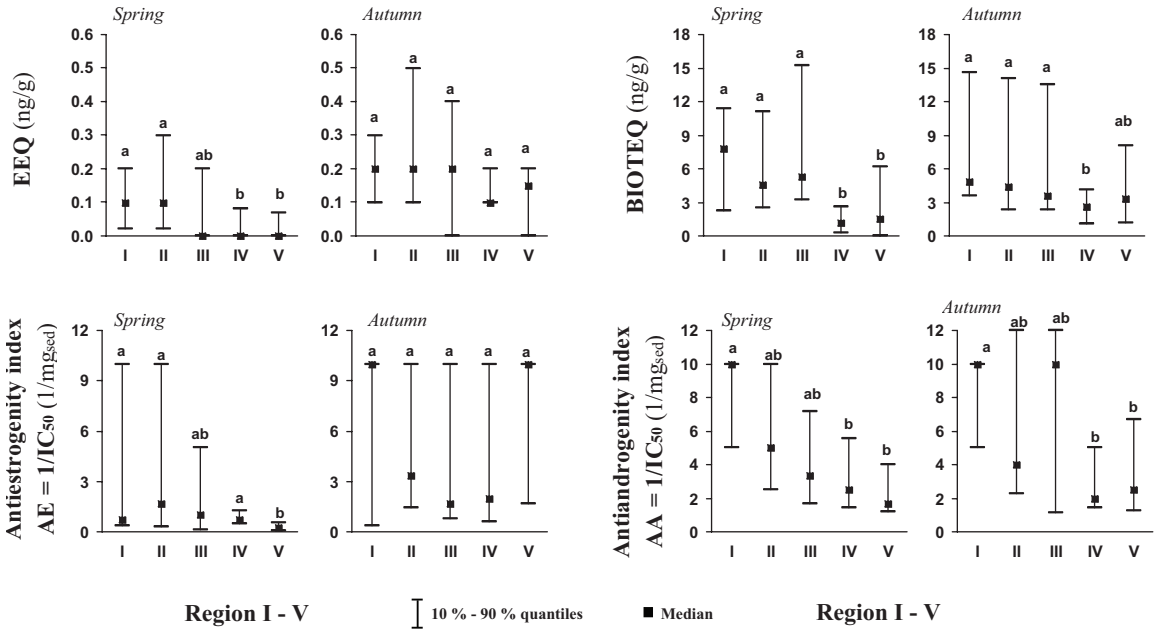

Fig. 3. Regional values of the performed bioassays analyzed separately for spring and autumn seasons. Values marked by the same lowercase letter were not significantly different ($p < 0.05$; Kruskal–Wallis test). EEQ = estradiol-equivalent; BIOTEQ = TCDD-equivalent; AE/AA = antiestrogenic/antiandrogenic index; IC50 (mg) = sediment equivalent that reduces the effect by 50%.

All the other main groups of pollutants showed only locally specific differences.

In comparison with the health risk assessment, which is usually based on chemical analyses of only a small part of the present contaminants, the bioassays allowed us to evaluate the potential effects of the mixture. Bioassays, both specific and nonspecific, were employed to characterize the sediments and to augment the information provided by the concentrations in the measurement of traditional contaminants (Table 3). Generally, the Microtox toxicity and genotoxicity tests were relatively inconclusive and showed high variability of the repeated measurements, so they were not able to separate regions. The results of the Microtox test were the most variable of all of the bioassays, but there were still statistically significant differences between seasons. The results of the genotoxicity tests (SOS-G and GFP-G) were not able to separate regions and

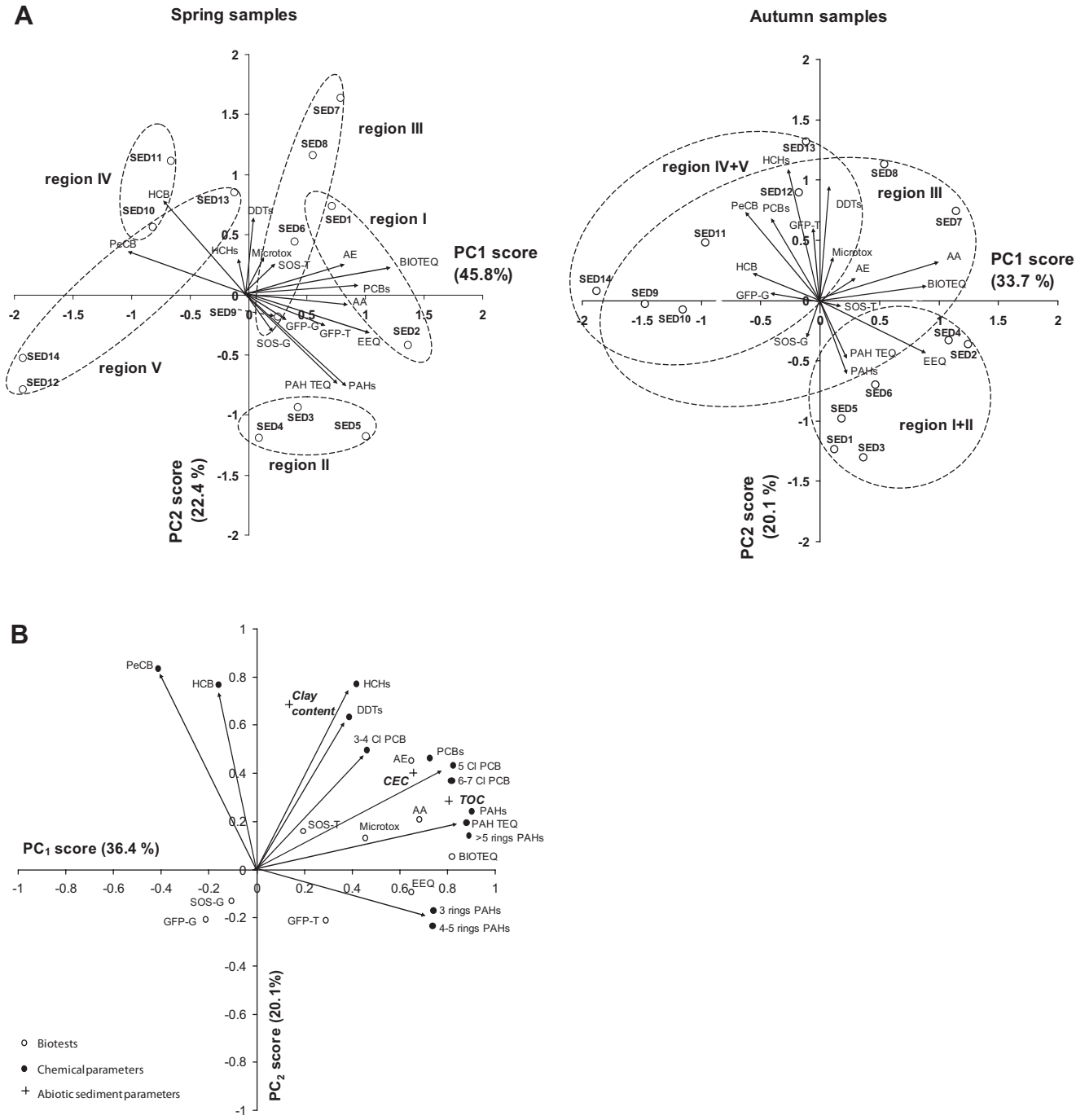

Fig. 4. Biplot presentation of regionally specific associations between main groups of contaminants and performed biotests in season-specific factor analysis (**A**) and multivariate pattern of association among main groups of contaminants, abiotic parameters, and bioassays calculated with aggregated regional and seasonal data (**B**). EEQ = estradiol-equivalent; BIOTEQ = TCDD-equivalent; AE/AA = antiestrogenic/antiandrogenic index; SOS-T and SOS-G = toxicity and genotoxicity from SOS-chromotest; GFP-T and GFP-G = toxicity and genotoxicity from GFP test; PAH TEQ = dioxin equivalent calculated from chemical analysis; CEC = cation exchange capacity; TOC = total organic carbon; PAHs = polycyclic aromatic hydrocarbons; PCBs = polychlorinated biphenyls HCHs = hexachlorocyclohexanes; HCB = hexachlorobenzene; PeCB = pentachlorbenzene; SED = sediment.

provided slight differences between seasons. As for the toxicity responses, the results of SOS-T test were also inconclusive, and only seasons could be discriminated on the basis of this assay; similar results were also found for GFP-T, with which the influence of season and region × season interaction was found to be as statistically significant. There was a lack of correlation of the toxicity/genotoxicity with the studied groups of pollutants, which indicates that the toxic/genotoxic potency was probably more related to other pollutants/stressors than measured in our study.

In contrast to the toxicity and genotoxicity tests, all the other specific bioassays led to statistically detectable differences among regions and between seasons profiles (Fig. 3). The primary conclusion that corresponded to the findings in the chemical part of the monitoring was the clear distinction of the upstream (I–III) from downstream (IV–V) regions, especially in spring. The multivariate PCA analyses combining biological and chemical measures confirmed that mutual association of the parameters clearly separated upstream and downstream sites (Fig. 4), with additional separation of Morava River sites in region IV. Results of sediments from the central area (region III) were clustered differently in spring and autumn.

The pattern corresponded to the profile of PAHs and was typical for EEQ and BIOTEQ. The results of both assays were correlated with PAHs and partially with PCBs as master variables in the season-specific factor analysis. Aggregating spring and autumn samples in the PCA revealed internal separation of different PAHs and PCBs as well (Fig. 4B). The concentrations of EEQ were more closely correlated with PAHs containing fewer than five rings, whereas concentrations of BIOTEQ were more associated with PAHs containing more than five rings. Polychlorinated biphenyls could not be analyzed as a thoroughly homogeneous group; the three- or four-Cl group differed in position from the others. The summed three- or four-Cl PCBs were less significantly correlated with EEQ and with BIOTEQ than the other PCBs.

Apart from the internal diversity, the associations of concentrations of PAHs with EEQ or BIOTEQ were statistically significant and formed the primary trajectory, which explained the greatest proportion of the variability and was associated with the first principal component in all analyses (Fig. 4). Antiandrogenic potency was correlated with concentrations of both PAHs and PCBs, similarly to the relationships with concentrations of both BIOTEQ and EEQ. All these measures were able to discriminate significantly regions I and II from IV and V, especially in spring (Figs. 3 and 4). Although PAHs were the primary discriminating factor in the analysis, other chlorinated compounds, such as HCHs, HCB, DDTs, and PeCB, followed the interseasonal behavior of PCBs. Therefore, all the bioassay results correlated with PCBs partially associated also with these POPs. However, none of the other POPs could be regarded as master independent variables for these associations. Increased concentrations of HCHs and DDTs were partially correlated with Microtox and SOS-T assay, but without statistical significance (Fig. 4).

The correlation of the outcomes of specific bioassays with PAH and PCB concentrations suggests their potential contribution to the observed activities, namely, to the dioxin-like and partially to the estrogenic activity, as was shown in our previous studies [10,11]. However, the greater presence of these studied pollutants also indicates the overall higher level of pollution in some regions. Namely, for the estrogenic and antiandrogenic

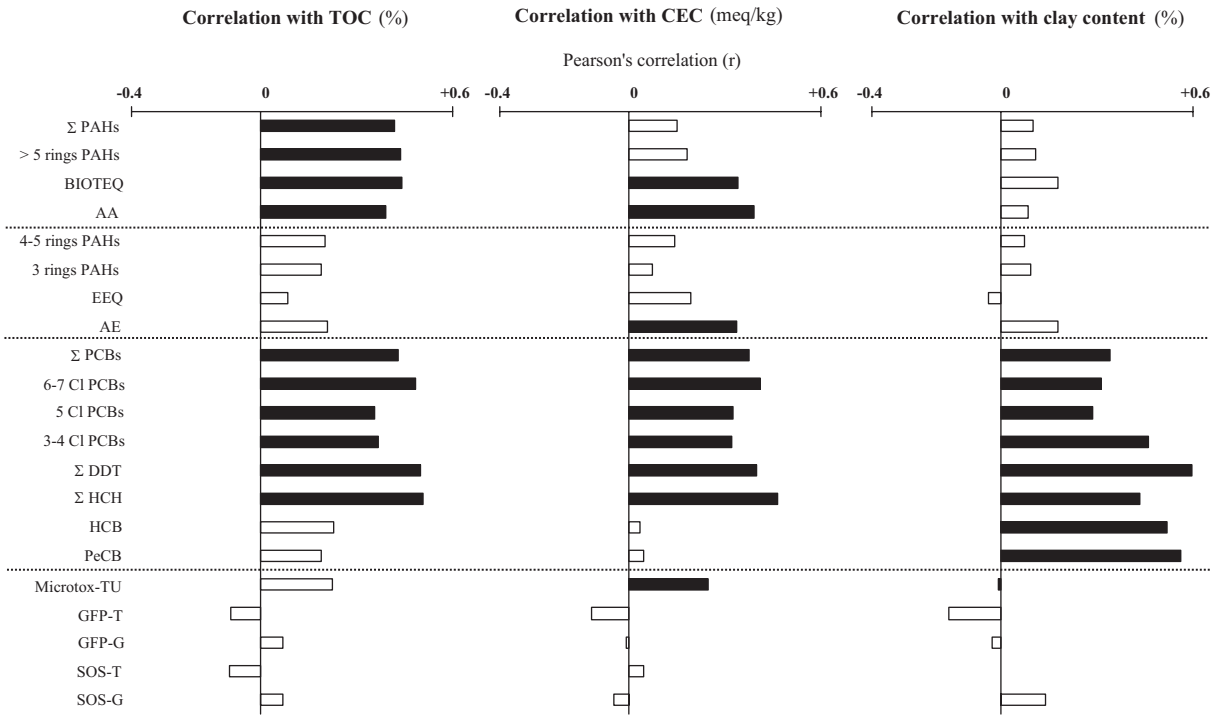

Fig. 5. Rank plots of Pearson correlation coefficients among concentrations of persistent organic pollutants, biotest measures, and abiotic parameters of sediments. Chemical and biotest parameters are grouped according to their mutual correlation; black: statistically significant correlation; $p < 0.05$. Abbreviations as for Figure 4.

potencies, there is a probable significant contribution of other pollutants with specific mode of action.

The primary abiotic characteristics of sediment that were significantly associated with the profiles of bioassay potencies and concentrations of residues were TOC, CEC, and clay content. There was significant interrelation among the concentration of BIOTEQ, antiandrogenic potency and TOC, clay and silt content, and concentrations of PAHs and PCBs. This result demonstrates the significance of abiotic factors in accumulation of pollutants with specific modes of action (Fig. 5). However, these relationships were partially distorted by seasonal fluctuations. All abiotic attributes varied within region and season but still exhibited discernible profiles (Tables 1 and 2, Fig. 2). Although samples taken in spring exhibited maximal TOC, CEC, and clay content, the average values of all these characteristics were greater in autumn than in spring at most locations.

All three characteristics significantly contributed to the multivariate association of environmental parameters. Correlation with abiotic factors improved the separation of clusters of locations that had been previously identified by use of concentrations of residues and potencies predicted by bioassays (Fig. 4B). The correlation profiles are displayed as univariate relationships (Fig. 5). The concentration of TOC contributed

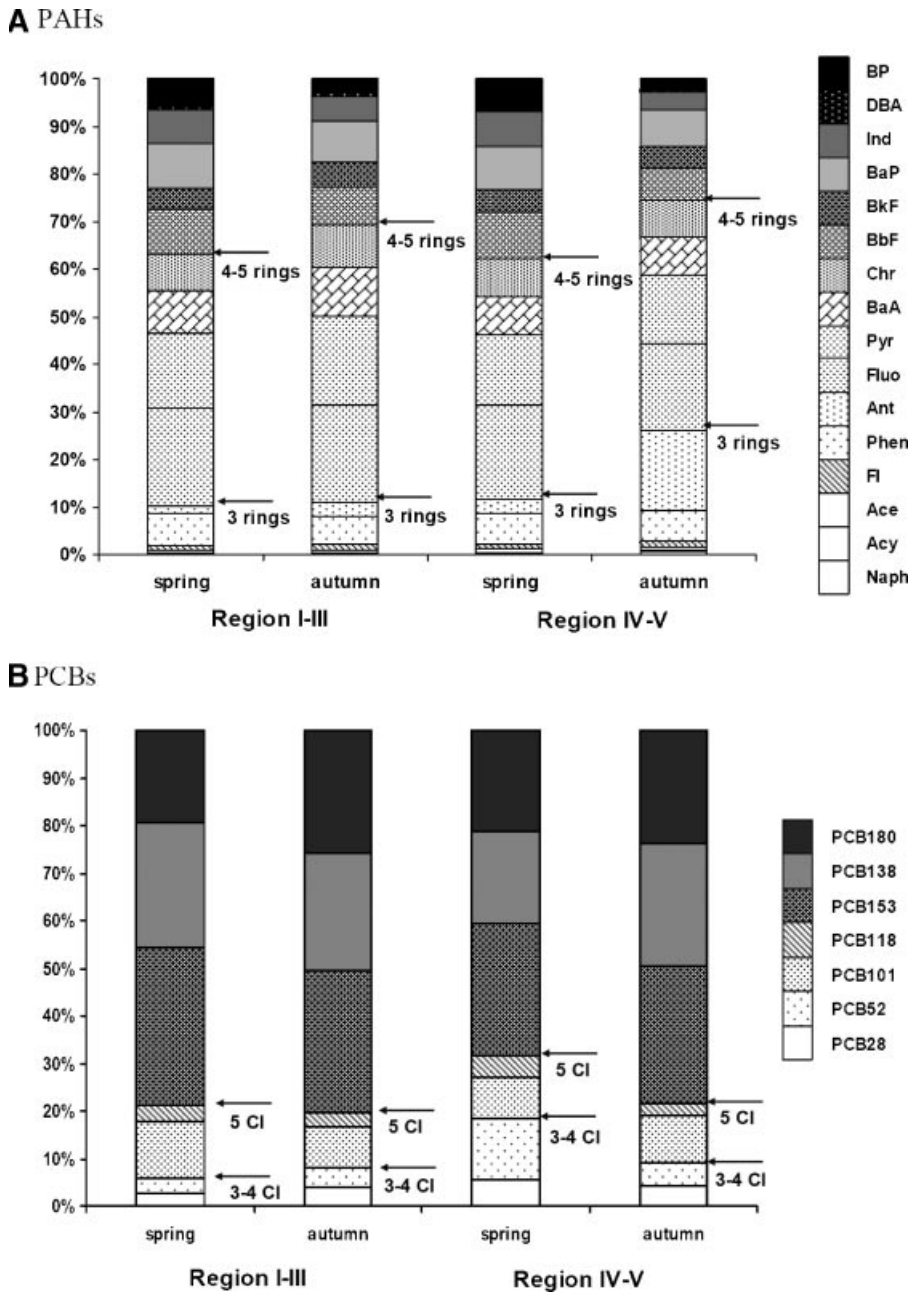

Fig. 6. Structure of polycyclic aromatic hydrocarbon (PAH; **A**) and polychlorinated biphenyl (PCB; **B**) mixtures according to sampled region and season. BP = benzo[*ghi*]perylene; DBA = dibenzo[*a,h*]anthracene; Ind = indeno(1,2,3,*cd*)pyrene; B*a*P = benzo[*a*]pyrene; B*k*F = benzo[*k*]fluoranthene; Chr = chrysene; B*a*A = benzo[*a*]anthracene; Pyr = pyrene; Fluo = fluoranthene; Ant = anthracene; Phen = phenanthrene; Fl = fluorene; Ace = acenaphthene; Acy = acenaphthylene; Naph = naphthalene.

preferentially to the first principal component, which also correlated with PAHs and particularly with concentrations of PAHs with more than five rings, and the concentration of BIOTEQ and antiandrogenic potency. Total organic carbon also mastered correlation with most of the Cl POPs, except for HCHs and PeCB, which do not tend to partition to sediment organic matter because of their lower K_{OW} values. Concentrations of all chlorinated POPs were significantly correlated with clay content, but no such correlation was observed for PAHs or the potencies determined with any of the biotests. Cation exchange capacity was most correlated with Cl POPs, Microtox, BIOTEQ, AE, and AA potency.

The relationships indicated by the observed correlations between concentrations of residues and potencies in bioassays can help to explain the transport and exposure pathways of the various compounds. Chlorinated POPs seem to move among the regions in a downstream direction, probably in association with solid sediment material represented by clay content. Alternatively, the ubiquitous distribution of PAHs appeared to be independent of clay content or seasonal transport of material. The PAHs were more preferentially associated with TOC, and concentrations of both increased significantly from spring to autumn.

The importance of physical and chemical properties of river sediments, including the particle size distribution and organic carbon in total sorption capacity for organic compounds and toxic potential of sediments, have been documented in previous studies [24,39]. In some field studies, strong correlations have been found between the heavier PAHs and TOC content [25,40], but there have also been several field studies in which the relationship between concentrations of PAH and TOC has been less strong. The absence of correlation is sometimes considered an indicator of anthropogenic pollution; it has been documented that results from sites with high concentrations of contaminants can distort and mask the correlation relationship with TOC [40].

Concentrations of some compounds, such as PAHs, DDTs, HCHs, increased from spring to autumn, whereas the regional distributions of others, such as PCBs and HCB, changed completely. The relative distribution of concentrations of individual PAHs and PCB congeners was similar between seasons in the Drevnice regions (I–III). They were more variable in the Morava part of the study area (Fig. 6), where the proportion of individual congeners visibly changed from spring to autumn. In connection with the changes in concentrations, these results suggest that PCBs are transported seasonally from upstream sites in spring to downstream sites in autumn (Fig. 6B). The hypothesis is indirectly supported by significant correlation of Cl POPs with clay sediment content.

CONCLUSIONS

The contamination situation of surface river sediments is seasonally changeable, and just one sampling period cannot be accepted as sufficient for conclusive results; both regional and seasonal component of variability have to be taken into account. Repeated sampling under different hydrological conditions allows us to reveal the significant determining relations that affect the fate of contaminants in a dynamic river ecosystem. The area of interest was clearly separated into regions that clustered sediment sites according to similar ecological, environmental, and contamination situations. This regional component of the variability was reflected in all influential measures and was accompanied by seasonally determined changes. This study documents the strength of specific bioassays in indicating the contamination changes and providing results that clearly separated both seasons and regions. The results emphasize the crucial role of a well-designed sampling plan in environmental biomonitoring for correct risk assessment interpretation and the complementarity of the bioassay results with chemical analysis data.

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REFERENCES

1. Hollert H, Duerr M, Haag I, Wölz J, Hilscherova K, Blaha L, Gerbersdorf S. 2007. Influence of hydrodynamics on sediment ecotoxicity. In Förstner U, Westrich B, eds, *Sediment Dynamics and Pollutant Mobility in Rivers—An Interdisciplinary Approach*. Springer-Verlag, New York, NY, USA. pp 401–416.
2. Crommentuijn T, Sijm D, de Bruijn J, van Leeuwen K, van de Plassche E. 2000. Maximum permissible and negligible concentrations for some organic substances and pesticides. *J Environ Manag* 58:297–312.
3. Forstner U, Heise S, Schwartz R, Westrich B, Ahlf W. 2004. Historical contaminated sediments and soils at the river basin scale—Examples from the Elbe catchment area. *J Soils Sediments* 4:247–260.
4. Jobling S, Tyler CR. 2003. Endocrine disruption in wild freshwater fish. *Pure Appl Chem* 75:2219–2234.
5. Ricking M, Schwarzbauer J, Franke S. 2003. Molecular markers of anthropogenic activity in sediments of the Havel and Spree Rivers (Germany). *Water Res* 37:2607–2617.
6. Brack W, Klamer HJC, de Ada ML, Barcelo D. 2007. Effect-directed analysis of key toxicants in European river basins—A review. *Environ Sci Pollut Res* 14:30–38.
7. Peck M, Gibson RW, Kortenkamp A, Hill EM. 2004. Sediments are major sinks of steroidal estrogens in two United Kingdom rivers. *Environ Toxicol Chem* 23:945–952.
8. Stachel B, Ehrhorn U, Heemken OP, Lepom P, Reincke H, Sawal G, Theobald N. 2003. Xenoestrogens in the River Elbe and its tributaries. *Environ Pollut* 124:497–507.
9. Petrovic M, Eljarrat E, de Alda MJL, Barcelo D. 2004. Endocrine disrupting compounds and other emerging contaminants in the environment: A survey on new monitoring strategies and occurrence data. *Anal Bioanal Chem* 378:549–562.
10. Hilscherova K, Kannan K, Holoubek I, Giesy JP. 2002. Characterization of estrogenic activity of riverine sediments from the Czech Republic. *Arch Environ Contam Toxicol* 43:175–185.
11. Hilscherova K, Kannan K, Kang YS, Holoubek I, Machala M, Masunaga S, Nakanishi J, Giesy JP. 2001. Characterization of dioxin-like activity of sediments from a Czech river basin. *Environ Toxicol Chem* 20:2768–2777.
12. Houtman CJ, Booij P, Jover E, del Rio DP, Swart K, van Velzen M, Vreuls R, Legler J, Brouwer A, Lamoree MH. 2006. Estrogemnic and dioxin-like compounds in sediment from Zierikzee Harbour identified with CALUX assay-directed fractionation combined with one and two dimensional gas chromatography analyses. *Chemosphere* 65:2244–2252.
13. Janosek J, Hilscherova K, Blaha L, Holmoubek I. 2006. Environmental xenobiotics and nuclear receptors—Interactions, effects and in vitro assessment. *Toxicol in Vitro* 20:18–37.
14. Whyte JJ, Schmitt CJ, Tillitt DE. 2004. The H4IIE cell bioassay as an indicator of dioxin-like chemicals in wildlife and the environment. *Crit Rev Toxicol* 34:1–83.
15. Kannan K, Yun SH, Ostaszewski A, McCabe JM, Mackenzie-Taylor D, Taylor AB. 2008. Dioxin-like toxicity in the Saginaw River watershed: Polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls in sediments and floodplain soils from the Saginaw and Shiawassee Rivers and Saginaw Bay, Michigan, USA. *Arch Environ Contam Toxicol* 54:9–19.

16. Umlauf G, Bidoglio G, Christoph EH, Kampheus J, Kruger F, Landmann D, Schulz AJ, Schwartz R, Severin K, Stachel B, Stehr D. 2005. The situation of PCDD/Fs and dioxin-like PCBs after the flooding of river Elbe and Mulde in 2002. *Acta Hydrochim Hydrobiol* 33:543–554.
17. Hilscherova K, Machala M, Kannan K, Blankenship AL, Giesy JP. 2000. Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. *Environ Sci Pollut Res* 7:159–171.
18. Khim JS, Lee KT, Villeneueve DL, Kannan K, Giesy JP, Koh CH. 2001. *In vitro* bioassay determination of dioxin-like and estrogenic activity in environmental samples from Ulsan Bay and its vicinity, Korea. *Arch Environ Contam Toxicol* 40:151–160.
19. Koethe F. 2003. Existing sediment management guidelines: An overview. What will happen with the sediment/dredged material? *J Soils Sediments* 3:139–143.
20. Ahlf W, Hollert H, Neumann-Hensel H, Ricking M. 2002. A guidance for the assessment and evaluation of sediment quality: A German approach based on ecotoxicological and chemical measurements. *J Soils Sediments* 2:37–42.
21. Hollert H, Durr M, Erdinger L, Braunbeck T. 2000. Cytotoxicity of settling particulate matter and sediments of the Neckar River (Germany) during a winter flood. *Environ Toxicol Chem* 19:528–534.
22. Schwartz R, Gerth J, Neumann-Hensel H, Bley S, Forstner U. 2006. Assessment of highly polluted fluvisol in the Spittelwasser floodplain—Based on national guideline values and MNA criteria. *J Soils Sediments* 6:145–155.
23. Jaffe R. 1991. Fate of hydrophobic organic pollutants in the aquatic environment—A review. *Environ Pollut* 69:237–257.
24. Vigano L, Arillo A, Buffagni A, Camusso M, Ciannarella R, Crosa G, Falugi C, Galassi S, Guzzella L, Lopez A, Mingazzini M, Pagnotta R, Patrolecco L, Tartari G, Valsecchi S. 2003. Quality assessment of bed sediments of the Po River (Italy). *Water Res* 37:501–518.
25. Guo W, He MC, Yang ZF, Lin CY, Quan XC, Wang HZ. 2007. Comparison of polycyclic aromatic hydrocarbons in sediments from the Songhuajiang River (China) during different sampling seasons. *J Environ Sci Health Part A* 42:119–127.
26. Hilscherova K, Dusek L, Kubik V, Cupr P, Hofman J, Klanova J, Holoubek I. 2007. Redistribution of organic pollutants in river sediments and alluvial soils related to major floods. *J Soils Sediments* 7:167–177.
27. Bláha L, Hilscherová K, Čáp T, Klánová J, Machát J, Zeman J, Holoubek I. 2009. Kinetic bacterial bioluminescence assay for contact sediment toxicity testing—Relationships with the matrix composition and contamination. *Environ Toxicol Chem* 29: (this issue).
28. International Organization for Standardization. 2007. Water quality—Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (luminescent bacteria test)—Part 3: Method using freeze-dried bacteria. ISO Standard 11348-3, Geneva, Switzerland.
29. Quillardet P, Hofnung M. 1993. The SOS chromotest—A review. *Mutat Res* 297:235–279.
30. Afanassiev V, Sefton M, Anantachaiyong T, Barker G, Walmsley R, Wolfl S. 2000. Application of yeast cells transformed with GFP expression constructs containing the RAD54 or RNR2 promoter as a test for the genotoxic potential of chemical substances. *Mutat Res* 464:297–308.
31. Freyberger A, Schmuck G. 2005. Screening for estrogenicity and anti-estrogenicity: a critical evaluation of an MVLN cell-based transactivation assay. *Toxicol Lett* 155:1–13.
32. Leskinen P, Michelini E, Picard D, Karp M, Virta M. 2005. Bioluminescent yeast assays for detecting estrogenic and androgenic activity in different matrices. *Chemosphere* 61:259–266.
33. Michelini E, Leskinen P, Virta M, Karp M, Roda A. 2005. A new recombinant cell-based bioluminescent assay for sensitive androgen-like compound detection. *Biosens Bioelectron* 20:2261–2267.
34. Westrich B, Forstner U. 2005. Sediment dynamics and pollutant mobility in rivers (SEDYMO) Assessing catchment-wide emission–immission relationships from sediment studies—BMBF Coordinated Research Project SEDYM (2002–2006). *J Soils Sediments* 5:197–200.
35. Long ER, Macdonald DD, Smith SL, Calder FD. 1995. Incidence of adverse biological effects within ranges of chemical concentrations in marine and estuarine sediments. *Environ Manag* 19:81–97.
36. Giacalone A, Gianguzza A, Mannino MR, Orecchio S, Piazzese D. 2004. Polycyclic aromatic hydrocarbons in sediments of marine coastal lagoons in Messina, Italy. Extraction and GC/MS analysis, distribution and sources. *Polycyclic Aromatic Compounds* 24:135–149.
37. Muller A, Heininger P, Wessels M, Pelzer J, Grunwald K, Pfitzner S, Berger M. 2003. Contaminant levels and ecotoxicological effects in sediments of the river Odra. *Acta Hydrochim Hydrobiol* 30:244–255.
38. Stachel B, Jantzen E, Knoth W, Kruger F, Lepom P, Oetken M, Reincke H, Sawal G, Schwartz R, Uhlig S. 2005. The Elbe flood in August 2002—Organic contaminants in sediment samples taken after the flood event. *J Environ Sci Health Part A* 40:265–287.
39. Chiou CT, McGroddy SE, Kile DE. 1998. Partition characteristics of polycyclic aromatic hydrocarbons on soils and sediments. *Environ Sci Technol* 32:264–269.
40. Evans HE, Evans RD, Lingard SM. 1989. Factors affecting the variation in the average molecular-weight of dissolved organic-carbon in freshwaters. *Sci Total Environ* 81/82:297–306.

Článek XX:

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RESEARCH ARTICLE

Longer-term and short-term variability in pollution of fluvial sediments by dioxin-like and endocrine disruptive compounds

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Abstract Changes in pollutant loads in relatively dynamic river sediments, which contain very complex mixtures of compounds, can play a crucial role in the fate and effects of pollutants in fluvial ecosystems. The contamination of sediments by bioactive substances can be sensitively assessed by in vitro bioassays. This is the first study that characterizes detailed short- and long-term changes in concentrations of contaminants with several modes of action in river sediments. One-year long monthly study described seasonal and spatial variability of contamination of sediments in a representative industrialized area by dioxin-like and endocrine disruptive chemicals. There were significant seasonal changes in both antiandrogenic and androgenic as well as dioxin-like potential of river sediments, while there were no general seasonal trends in estrogenicity. Aryl hydrocarbon receptor-dependent potency (dioxin-like potency) expressed as biological TCDD-equivalents (BIOTEQ) was in the range of 0.5–17.7 ng/g, dry mass (dm). The greatest BIOTEQ levels in sediments were observed during winter, particularly at locations downstream of the industrial area. Estrogenicity expressed as estradiol equivalents (EEQ) was in the range of 0.02–3.8 ng/g, dm. Antiandrogenicity was detected in all samples, while androgenic potency in the range of 0.7–16.8 ng/g, dm dihydrotestosterone equivalents (DHT-EQ) was found in only 30 % of samples, most often during autumn, when antiandrogenicity was the least. PAHs were predominant contaminants among analyzed pollutants, responsible, on average, for 13–21 % of BIOTEQ. Longer-term changes in concentrations of BIOTEQ corresponded to seasonal fluctuations, whereas for EEQ, the inter-annual changes at some locations were greater than seasonal variability during 1 year. The inter- as well as intra-annual variability in concentrations of both BIOTEQ and EEQ at individual sites was greater in spring than in autumn which was related to hydrological conditions in the river. This study stresses the importance of river hydrology and its seasonal variations in the design of effective sampling campaigns, as well as in the interpretation of any monitoring results.

Keywords Sediments · Seasonality · Monitoring · Dioxin-like potency · Estrogenicity · Antiandrogenicity

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Introduction

Sediments are considered as an important compartment of aquatic ecosystems that provide substratum for benthic organisms and represent a deposit of nutrients that can be returned to the biocycles during natural flooding (Forstner and Salomons 2010). Association with sediments and particulate matter also plays a crucial role in the fate and effects of contaminants in aquatic systems. Sediments serve as a sink for various hazardous chemicals, especially hydrophobic organic contaminants (HOCs) due to their hydrophobic nature and low-water solubility. Important parameters for the binding of organic pollutants to sediments are the specific surface of particles as well as quantity and quality of organic carbon (Jaffe 1991). Sediments contain a wide spectrum of compounds, of both natural and anthropogenic origin, that can affect organisms through different modes of action to cause additive, supra-additive, or infra-additive effects. Among HOCs, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) or polychlorinated dibenzo-*p*-dioxins (PCDDs), and dibenzofurans (PCDFs) have been detected in sediments worldwide (Colombo et al. 2006; Hilscherova et al. 2010; Kannan et al. 2008; Koh et al. 2004). Apart from the traditionally monitored hydrophobic pollutants, other classes of compounds such as pharmaceuticals and personal care products, polyphenolic compounds, phthalates, or various pesticides may be present in sediments (Brack et al. 2007; Jobling and Tyler 2003; Vigano et al. 2008). It has also been shown that sediments can serve as a sink of xenohormones and other endocrine disrupting compounds (Higley et al. 2012; Peck et al. 2004; Urbatzka et al. 2007).

To achieve good water quality within the European Union (EU), the Water Framework Directive (Directive 2000/60/EC) has been introduced into the EU legislation and limits for concentration of several hazardous priority substances in surface waters, so-called Environmental Quality Standards (EQS), have been defined (Directive 2008/105/EC). Recently, the list of priority substances has been revisited and EQS for more compounds in surface waters as well as EQS for some compounds in biota have been proposed (European Commission 2012). Contamination of sediments plays a crucial role in the pollution of aquatic environment. The Water Framework Directive recommends the monitoring of sediments at an adequate frequency to provide sufficient data for reliable determination of long-term status and trends and to establish limits for contaminants in sediments according to the local situation in each country. Specific approaches for sediment quality assessment along with EQS for sediments are under development, which is one of the remaining challenges for better protection of aquatic ecosystems. Sediment quality guidelines (SQGs) developed on the base of ecological and ecotoxicological information for several HOCs as well as metals have been introduced in Flanders, Belgium and incorporated into Flemish legislation in 2010 (de Deckere et al. 2011). Another approach was previously used in the Netherlands, where limits for some organic substances and pesticides were derived by use of the equilibrium partitioning method (Crommentuijn et al. 2000). An SQG for PCBs corresponding to the regulatory fish consumption limit based on biota-to-sediment accumulation factor has been derived for the Rhone River basin, France (Babut et al. 2012). No SQG have been promulgated by the Czech Republic yet.

Implementation of EQS for priority substances in sediments is a crucial step in better protection of aquatic environments. However, priority pollutants remain to be identified. Recently, more attention has been driven to "emerging contaminants" in addition to HOCs since they can elicit various biological responses (Brack et al. 2007; Kaplan 2013). While quantification of individual contaminants by instrumental analysis is an important tool to investigate the fate and distribution of known pollutants in the environment, possible biological effects of complex mixture are difficult to predict solely from chemical analysis. Instrumental analysis of individual, known contaminants does not account for possible interactions among chemicals or for those compounds that are not identified or not quantified. Thus, various in vitro bioassays have been applied to characterize contamination by bioactive substances in various environmental compartments, such as surface water, sediments, soil, air, or biota (e.g., Higley et al. 2012; Martinez-Gomez et al. 2013; Novak et al. 2009; Urbatzka et al. 2007; Wolz et al. 2011). In vitro bioassays are relatively rapid, cost-effective, and useful, especially in screening and long-term monitoring of contamination. Some of these assays are applied to estimate the potency of individual compounds as well as of complex mixtures to elicit biological responses mediated through specific nuclear receptors, such as the aryl hydrocarbon receptor (AhR), estrogen receptor (ER), or androgen receptor (AR). Activation of the AhR is considered critical in mediating effects of dioxin-like compounds that have been shown to cause hepatotoxicity, teratogenicity, carcinogenesis, immunotoxicity, and other adverse effects (Janosek et al. 2006). Estrogens and androgens are endogenous steroid sex hormones that control reproduction, development, differentiation, and growth. Functions of these hormones are mainly mediated by ER and AR, and many compounds have been shown to disrupt their signaling (Janosek et al. 2006). Reproductive disorders, such as feminization or masculinization of aquatic vertebrates and invertebrates were observed in the environment (as reviewed in Sumpter 2005). Exposure to synthetic estrogens can even lead to collapse of whole fish populations (Kidd et al. 2007).

River sediments represent a dynamic system and their potential risks are connected primarily with transport and deposition of contaminated solids in downstream regions

(Forstner et al. 2004; Hilscherova et al. 2003). Rivers can exhibit large differences in hydrodynamic characteristics during an annual cycle. In remobilization processes, pollutants associated with particles can be resuspended, thus, sediments can serve as a secondary source of contamination (Brinkmann et al. 2013; Hilscherova et al. 2007). Strong fluctuations in concentrations of contaminants can occur upon stronger floods that have been discussed in recent years in possible relation to the global climate change (Hunt 2002). Further, seasonal variability of contamination was observed at some places (Hilscherova et al. 2010; Zhao et al. 2011). Both temporal and spatial dynamics should be considered when assessing contamination of river ecosystems as was documented in a previous study (Hilscherova et al. 2010). Even though pollution of sediments by compounds with the above-listed modes of action has been reported from rivers in many parts of the world, there is a lack of information regarding long- as well as short-term variations or trends in their concentrations and/or potencies.

The present year-long study was focused on temporal (both seasonal and long-term) and spatial variability of contaminants in river sediments of a typical industrial area in the south-eastern part of the Czech Republic (Central Europe) that represents a suitable model ecosystem for research on the accumulation and distribution of pollutants on a local and regional scale. The studied region is a part of Danube River basin, situated near the city of Zlin. It includes two rivers, the Morava and its tributary, the Drevnice (Fig. 1). This area has been affected for many years by industrial and agricultural activities as well as effluents from wastewater treatment facilities and runoff from urban landscapes. Chemical, boot-and-shoe, plastics-and-rubber, food-stuff industry, agricultural crops and livestock production, as well as transport are among the most important sources of contamination (Hilscherova et al. 2007). The goal of this year-long study with monthly sampling was to characterize seasonal and spatial variability of contamination of fluvial sediments by compounds with dioxin-like and endocrine disruptive modes of action. Sediments were sampled monthly at five locations throughout a whole year. Extracts of sediments were assessed for AhR-, ER-, and AR-dependent potencies. Another goal was to address longer-term trends/variability through comparison of current and previous results from the region (Hilscherova et al. 2010, 2002). Thus, a comparison of seasonal as well as inter-annual trends in contamination by bioactive compounds could be conducted.

Materials and methods

Sampling and locations

Sediments were collected, monthly, from July 2007 to July 2008 at five locations in the south-eastern part of the Czech Republic in the Morava River and its tributary Drevnice River (Fig. 1). The Malenovice (MA) location is situated on the Drevnice River and is affected mainly by contamination from the city of Zlin and its surroundings. The Belov (BE) location is situated on the Morava River upstream from the confluence with the Drevnice River, whereas the Spytihnev (SP) location is downstream on Morava River and integrates contamination from both rivers. The Certak oxbow lake (CR) is a unique location that was separated from the active Morava River channel in the 1930s, but water communication with the river is provided via underground piping that enables the lake to act as a trap for suspended sediments from the river (Babek et al. 2008). The Certak (CE) location is situated on the Morava River near the oxbow lake to better assess differences between the active and abandoned channel. Samples were taken from each location in a period of 28 days, in 15 sampling campaigns. A total of 73 samples were collected. Two samples could not be obtained because of weather conditions. Samples were clustered according to four hydrologically defined seasons (Table S1): spring (March–May), summer (June–August), autumn (September–November), and winter (December–February). Data on river discharge and temperature were obtained from gauging stations in Zlin (representative for location MA), Kromeriz (representative for location BE), and Spytihnev (representative for locations SP, CE). The following parameters were used: Q=average discharge over the 28 days prior to each sampling campaign, T_{actual}=temperature on the day of sampling, $T_{average}$=time-weighted, average temperature over the 28 days prior to each sampling campaign. Composite samples of surface sediments were collected from the top 10-cm layer by use of pre-cleaned trowels. Large pieces of wood, leaves, and stones were removed manually and sediments were homogenized and freeze-dried. Dry sediments were sieved (2 mm). Total organic carbon content (TOC) was determined by use of high-temperature TOC/TNb Analyzer liquiTOC II (Elementar Analysensysteme, Hanau, Germany).

Chemical analysis

For quantification of organic pollutants, 10 g of freeze-dried sediments were extracted with dichloromethane by use of automated warm Soxhlet extraction (1 h, min. 15 cycles; Büchi B-811, Büchi, Switzerland). Laboratory blanks and reference material were analyzed with each set of samples. Surrogate recovery standards (final amount in each sample 10 ng PCB30, 10 ng PCB185, 333 ng D8-naphthalene, 333 ng D10-phenanthrene, and 333 ng D12-perylene) and 13C labeled PCDD/Fs standards (800 pg tetra-hexa PCDD/Fs, 1,600 pg hepta-octa PCDD/Fs) were used prior to extraction. Extracts were cleaned-up on silica column (for PAHs analysis), sulfuric acid-modified silica column was used for analysis of organohalogens. Copper powder was used to remove

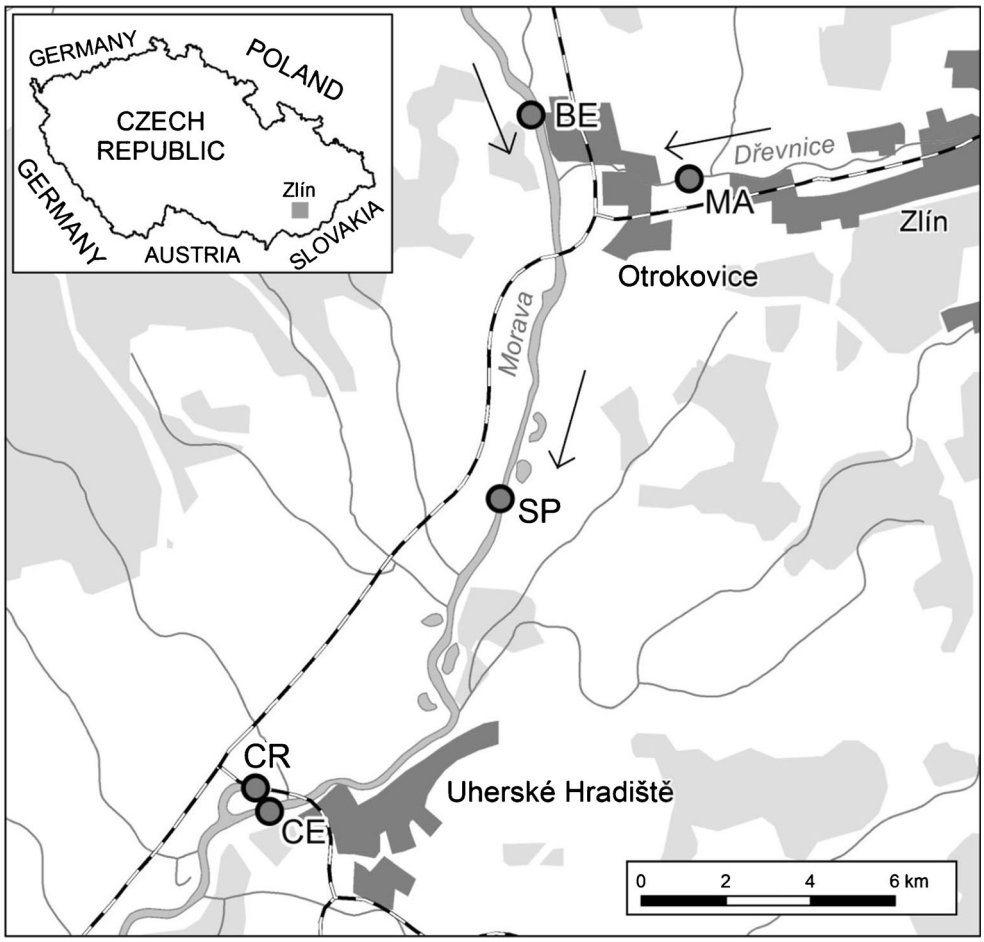

Fig. 1 Sampling localities on the Morava and Drevnice Rivers. *MA* Malenovice, *BE* Belov, *SP* Spytihnev, *CE* Certak Morava river, *CR* Certak oxbow lake; *arrows* indicate the river flow direction

sulfur. Further fractionation step was needed to analyze dioxin-like PCBs (dl-PCBs) and PCDD/Fs. Samples were applied on columns containing charcoal/silica mixture and eluted with DCM/cyclohexane in fraction 1 (mono-*ortho* dl-PCBs) and with toluene in fraction 2 (PCDD/Fs, non-*ortho* dl-PCBs). Terphenyl (200 ng/mL), PCB 121 (200 ng/mL) and 13C-labeled PCDD/Fs (16 ng/mL) were used as injection standards for quantification of PAHs, PCBs, and PCDD/Fs, respectively. Samples were analyzed using GC-MS instrument (Agilent 6890N GC–Agilent 5973N MS, Agilent, USA), separation of individual compounds was achieved on a DB-5MS (J&W Agilent, USA) for indicator PCBs (congeners 28, 52, 101, 118, 138, 153, 180), OCPs (dichlorodiphenyltrichloroethane *p*, *p*′-DDT and its metabolites *p*, *p*′-DDE, *p*, *p*′-DDD; hexachlorocyclohexane isomers α-, β-, γ-, δ-HCH; hexachlorobenzene), and 16 US EPA PAHs. Concentrations of contaminants were quantified using Pesticide Mix 13 (Dr. Ehrenstorfer GmbH, Germany) and PAH Mix 27 (Promochem, Germany) standard mixtures. HRGC/HRMS instrumental analysis for PCDD/Fs and dl-PCBs (congeners 77, 81, 105, 114, 123, 126, 156, 157, 167, 169, 189) was performed on Agilent 7890A GC (Agilent, USA) coupled to AutoSpec Premier MS (Waters, Micromass, UK). The GC was fitted with a capillary column DB5-MS, 60 m×0.25 mm i.d., 0.25-μm film. MS was operated in EI+ mode at $R>10$ k (Kukucka et al. 2010).

Total potency of samples to cause AhR-mediated effects, expressed as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-equivalents (TEQ), were calculated as the sum of the product of concentrations of individual AhR-active compounds multiplied by their relative potency (REP) to activate AhR-mediated responses in H4IIE-*luc* cells (Eq. 1).

$$\mathrm{TEQ} = \sum c_X * \mathrm{REP}_X \tag{1}$$

TEQ for individual groups of pollutants were calculated (Eqs. 2 and 3).

$$\mathrm{PAHs{-}TEQ} = \sum c_{\mathrm{PAHs}} * \mathrm{REP}_{\mathrm{PAHs}} \tag{2}$$

$$\mathrm{nonPAHs{-}TEQ} = \left(\sum c_{\mathrm{PCBs}} * \mathrm{REP}_{\mathrm{PCBs}}\right) + \left(\sum c_{\mathrm{PCDDs}} * \mathrm{REP}_{\mathrm{PCDDs}}\right) + \left(\sum c_{\mathrm{PCDFs}} * \mathrm{REP}_{\mathrm{PCDFs}}\right) \tag{3}$$

REPs derived by Machala et al. (2001) were used for PAHs, REPs derived by Behnisch et al. (2003) were used for PCBs and PCDD/Fs (Table S2).

Bioassays

For in vitro testing, 20 g of freeze-dried sediments without any surrogate standards were extracted as described above (Section Chemical Analysis). Extracts were treated with copper powder to remove sulfur, enriched under a gentle stream of nitrogen, and aliquots were transferred to ethanol (EtOH) and dimethyl sulfoxide (DMSO). Final concentration of sediment equivalents (SEQ) in the extracts was 20 g/mL. Three different mammalian cell lines transfected with the luciferase gene under control of several intracellular receptors were used to determine potencies of extracts of sediments to interfere with receptor-mediated responses. The potency to elicit dioxin-like effects via activation of AhR was quantified by use of the H4IIE-*luc* rat hepatocarcinoma cells (Hilscherova et al. 2001). ER-mediated response was evaluated by use of MVLN human breast carcinoma cells (Demirpence et al. 1993). MDA-kb2 human breast cancer cell line was used to assess AR-dependent response (Wilson et al. 2002).

H4IIE-*luc* cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % (*v*/*v*) fetal calf serum (FCS; both PAA laboratories, Austria) and exposed in the same medium supplemented with 1 % (*v*/*v*) gentamicin to prevent bacterial contamination. MVLN cells were cultured in DMEM/F12 medium (Sigma-Aldrich, Czech Republic) supplemented with 10 % (*v*/*v*) FCS and exposed in DMEM/F12 supplemented with 5 % (*v*/*v*) stripped (dextran/charcoal treated) FCS and 1 % (*v*/*v*) gentamicin. MDA-kb2 cells were cultured in Leibowitz L-15 medium (Sigma-Aldrich, Czech Republic) supplemented with 10 % (*v*/*v*) FCS and exposed in Leibowitz L-15 medium supplemented with 5 % (*v*/*v*) stripped FCS and 1 % (*v*/*v*) gentamicin. H4IIE-*luc* and MVLN cells were incubated and exposed at 5 % CO_2 and 37 °C. MDA-kb2 cells were incubated and exposed at 37 °C without addition of CO_2.

In the first step, test of cytotoxicity of the sediment extracts was conducted to determine the non-cytotoxic concentrations for testing of receptor-mediated effects. Upon testing, cells were seeded into sterile 96-well microplates in exposure medium. After 24-h incubation, cells were exposed to extracts of sediment samples in several dilutions. The greatest tested concentration for cytotoxicity assessment was 100 mg SEQ/mL. Cytotoxicity of the samples was measured using colorimetric Neutral Red (NR) uptake assay (Babich and Borenfreund 1990). Fifty microliters of NR dissolved in DMEM (0.5 mg/mL) were added into each well with cells and exposure medium after 24-h exposure. The mixture was incubated with cells for 1 h and then the medium with NR was removed. An aliquot of 150 μL of lysis solution (water, ethanol, acetic acid) was added and cells were shaken for 15 min (Orbital Shaker OS-20, BIOSAN, at 150 rpm). Absorbance was measured using a spectrophotometer (Tecan-Genios, $\lambda = 570$ nm). Data from the cytotoxic sample dilutions were excluded from calculations.

The interference with the receptor signaling was tested at several dilutions that did not significantly affect the viability of the cells, in three independent experiments. Cells were seeded into 96-well microplates and after 24-h incubation, exposed to extracts of sediment samples and appropriate standard calibration for agonistic potency along with blanks and solvent controls (0.5 % *v*/*v*). Reference compounds used for calibration were TCDD (Ultra Scientific, USA; concentration range 0.4–500 pM in EtOH) for H4IIE-*luc*, 17β-estradiol (E2; Sigma-Aldrich, Czech Republic; 1.23–100 pM in DMSO) for MVLN, and dihydrotestosterone (DHT; Sigma-Aldrich, Czech Republic; 10 pM–10 μM in DMSO) for MDA-kb2, respectively. For ER- and AR-antagonistic potency assessment, the exposure medium was supplemented with the reference compound (competing ligand) at approximately EC50 level, e.g., 33.3 pM E2 (MVLN) and 1 nM DHT (MDA-kb2), thus solvent concentration was 1 % (*v*/*v*). After 24-h exposure, cells were lysed, Promega Steady Glo Kit (Promega, USA) was added and the intensity of luminescence was measured by Luminoskan Ascent Microplate Luminometer (Thermo Scientific).

Data analysis

After subtraction of solvent control response, effects elicited by extracts of sediments were related to the luminescence caused by the reference compounds in the transactivation assay. The dose–response curves were fitted using non-linear logarithmic regression in GraphPad Prism (GraphPad Software, USA). AhR-mediated potency was expressed as TCDD-equivalents (BIOTEQ) calculated as $EC50_{TCDD}/EC50_{sample}$. Since many of the active samples did not reach 50 % of $E2_{max}$ induction, to avoid any predictions beyond the measured responses, estrogenicity was expressed as estradiol equivalents (EEQ) calculated as $EC25_{E2}/EC25_{sample}$. Antiestrogenicity of samples was expressed as the concentration (in sediment equivalents) that caused 25 % inhibition of luminescence in the presence of the competing ligand E2 (IC25). Androgenicity was expressed as DHT-equivalents (DHT-EQ) calculated as a point estimate based on the percentage of the luminescence induction caused by the greatest non-cytotoxic sample concentration because the dose–response curve for most samples did not exceed 20 % induction. DHT-EQ was calculated as ECX_{DHT}/ECX_{sample}, where X represents percentage induction of the greatest non-cytotoxic sample concentration. Antiandrogenicity was expressed as

percent inhibition of luminescence in the presence of the competing ligand DHT caused by the greatest concentration that was non-cytotoxic (as described for DHT-EQ).

The limit of detection (LOD) for each bioassay used in this study was derived as the ratio of the lowest amount of standard that elicits statistically significant response per the greatest tested non-cytotoxic concentration of SEQ. To calculate the LOD, the lowest concentration of reference compound significantly affecting the receptor-mediated response (lowest observed effect concentration for the receptor-mediated effect; LOEC), and the greatest non-cytotoxic sample concentration (no observable effects concentration for cytotoxicity; NOEC) were determined. Responses obtained for reference compounds and sample extracts were compared with solvent control response using ANOVA followed by Dunnet's test to determine significant effects ($p < 0.05$). Nonparametric Kruskal-Wallis test was used in case of non-homogenous variances (as tested by Levene's test). The LOD was then calculated as follows: LOD (pg/g, dry mass (dm) of sediment) = $LOEC_{standard\ ligand}$ (pg/mL)/$NOEC_{sample}$ (g SEQ/mL). Spatial and seasonal variability of dioxin-like toxicity (TEQ, BIOTEQ), estrogenic potency (EEQ), and antiandrogenic potency (AA) was tested by nonparametric Kruskal-Wallis test and visualized using boxplots.

Multivariate variation of bioassays results as well as chemical and environmental parameters was further summarized in the principal component analysis (PCA) as an effective technique simplifying the correlation structure through linear transformation of the original variables. PCA based on the correlation matrix was performed to provide component loading vectors explaining the relationships among the bioassays, pollutants, and other parameters and component score vectors as pair-wise uncorrelated variables that were used for the final exploratory survey of the data from the examined locations. Only variables with less than 10 % values below LOD were used for multivariate analysis. Values < LOD were replaced by ½ LOD. The variables with non-normal distribution were transformed by logarithmic transformation before use in PCA and parametric correlation analyses. The most important variables (estimates by eigenvalues) were selected for creating PCA (active variables), some other variables were visualized in the same ordination space as supplementary variables. Biplot was used as a common graphical tool representing not only projections on extracted principal components but also the 2-D loadings of original variables by lines. Additionally, Pearson's correlation analysis was used to quantify relationships between variables. All statistical analyses were performed with the software STATISTICA for Windows 10 (StatSoft, Inc. USA).

Results and discussion

This study documents variability of pollution in surface sediments of the rivers during the year. Sediments contained all chemically analyzed classes of pollutants (Table 1) at each location. SP was the most polluted location with the greatest concentrations of most contaminants and also with the greatest median of TOC content, whereas CR (oxbow lake) contained, overall, the least levels of contaminants. Detailed information about temporal and spatial distribution of HOCs will be described elsewhere (Prokes et al., in preparation). Comparing the contamination in assessed areas with SQGs derived from ecological and ecotoxicological data by de Deckere et al. (2011) (Table S3), all of the investigated locations are polluted. The SQGs proposed to be achieved in a long-term objective (so-called consensus 1 values) were exceeded by concentrations of PAHs, PCBs, and DDE in all samples up to 6-, 7-, and 30-fold, respectively, especially in winter and spring (PAHs), and in autumn (PCBs, DDE). In the case of DDD, the SQGs were exceeded even more than 200-fold in winter samples from location SP. According to these results, the studied locations are not in a good ecological sediment status as it was defined in de Deckere et al. (2011) during the year. The SQGs proposed to be achieved as a short-term objective (consensus 2 values) were only slightly exceeded by concentrations of some PAHs and DDE (1.3-fold), while the concentration of DDD was up to 5-fold greater in winter samples from location SP than the proposed limits. Consensus 2 values are described as values above which toxic and in situ effects are most likely to occur (de Deckere et al. 2011). From this point of view, all investigated sediments are likely to negatively affect biota. Desorption of contaminants from sediments might enhance their bioavailability, which plays a crucial role in manifestation of toxic effects on organisms. Results of previous studies indicated that sediments from the studied area represent a potential source of PAHs into the water column (Prokes et al. 2012).

Comparison of chemical analysis results to SQGs documents pollution by HOCs. However, compounds other than those HOCs that were quantified were present in the mixtures in sediments; therefore, specific biological activities were assessed in order to estimate the potential effects on organisms. Three transactivation cell lines were used to investigate specific biological potential of sediment samples. Mixtures extracted from sediments were cytotoxic; therefore, extracts were first treated with copper to remove sulfur, which is a frequent cause of cytotoxicity. Cytotoxicity of treated extracts was measured by use of the NR assay for each cell line in order to avoid any interference with specific endpoints measured in this study. Only concentrations of extracts that did not cause cytotoxicity were included in the evaluation of specific potencies. MDA-kb2 cells were more sensitive to effects on viability than were H4IIE-*luc* and MVLN cells. For MDA-

Table 1 Median and range (in brackets) of concentrations of pollutants, organic carbon, and biological potencies in extracts of sediments from studied localities from all sampling campaigns over a year (based on sediment dry mass)

Sampling site	PAHs (μg/g)	Ind. PCBs (ng/g)	Dl-PCBs (pg/g)	PCDDs (pg/g)	PCDFs (pg/g)	OCPs (ng/g)	TOC (%)	TEQ (ng/g)	TEQ/BIOTEQ (%)	BIOTEQ (ng/g)	EEQ (pg/g)[a]	DHT-EQ (ng/g)[a]	AA (% inhibition)
MA	4.4 (0.6–14.3)	11.6 (3.5–21.4)	399 (179–494)	38 (8–94)	15 (2–23)	9.2 (1.6–19)	3.3 (0.6–7.7)	0.6 (0.1–1.4)	14 (5–52)	3.7 (0.9–14.7)	99 (20–954) (n=13)	3.1 (1.8–3.9) (n=3)	72 (51–84)
BE	7.5 (0.5–10.7)	7.5 (1.7–10.5)	238 (105–340)	90 (11–190)	34 (4–49)	16.7 (0.9–53.1)	3.1 (0.2–5.1)	1.0 (0.1–1.2)	15 (6–39)	4.9 (1.0–12.6)	76 (40–3,753)	2.8 (1.4–8.3) (n=4)	68 (17–91)
SP	8.5 (5.3–13.8)	12.0 (6.5–33.4)	300 (127–612)	354 (152–818)	37 (24–109)	25.3 (8.9–58.1)	4.1 (2.9–5.3)	0.9 (0.6–1.9)	17 (8–109)	6.4 (1.5–17.7)	143 (45–895)	4.1 (0.7-5.9) (n=7)	72 (32–98)
CE	5.2 (3.0–9.0)	7.1 (4.5–20.6)	317 (142–643)	106 (57–268)	17 (11–24)	5.4 (2.9–39.5)	1.8 (1.2–2.6)	0.7 (0.4–1.1)	13 (6–49)	3.7 (1.3–15.4)	110 (62–229)	5.4 (0.8-16.8) (n=7)	56 (17–84)
CR	2.6 (1.5–8.8)	6.2 (0.7–13.7)	290 (188–431)	52 (36–94)	13 (11–28)	5.3 (1.2–11.6)	2.9 (2.1–5.9)	0.3 (0.2–0.9)	21 (10–44)	1.9 (0.5–5.3)	75 (39–198) (n=12)	1.9 (n=1)	74 (52–98)

Abbreviations of sites as in Fig. 1

PAHs polycyclic aromatic hydrocarbons, *ind. PCBs* indicator polychlorinated biphenyls, *dl-PCBs* dioxin-like PCBs, *PCDDs* polychlorinated dibenzo-*p*-dioxins, *PCDFs* polychlorinated dibenzofurans, *OCPs* organochlorine pesticides, *TOC* total organic carbon, *TEQ* TCDD-equivalent (derived based on chemical analysis), *BIOTEQ* TCDD-equivalent (bioassay-derived), *EEQ* estradiol-equivalent (bioassay derived), *DHT-EQ* dihydrotestosterone-equivalent (bioassay-derived), *AA* antiandrogenic activity (bioassay-derived)

[a] Total number of samples (n) with observed activity is noted in brackets in case when significant activity was not detected in all 15 samplings over the year

kb2 cells, the greatest NOEC corresponded to 50 mg SEQ/mL for most samples, four sediment extracts from location MA showed a greater cytotoxicity with NOEC of 15 mg SEQ/mL. For H4IIE-*luc* and MVLN cells, the cytotoxicity NOEC was 100 mg SEQ/mL for all samples.

AhR-mediated potency

AhR-mediated potency, expressed as BIOTEQ, was found in extracts of all sediments and was in the range of 0.5–17.7 ng/g, dm of sediment (LOD=1.3 pg/g, dm). Seasonal changes in BIOTEQ were obvious at all locations except CR (Figs. 2a and 3). The greatest dioxin-like potency was detected in sediments collected during winter. Concentrations of BIOTEQ in sediments collected during winter were significantly greater than in those collected during summer, which contained the least concentration of BIOTEQ ($p<0.05$). The same trend was observed for content of TOC (Fig. S1), which is an important parameter in accumulation of hydrophobic pollutants (Jaffe 1991). The trend of greatest concentrations in winter was most pronounced in the Morava River below the confluence with the Drevnice River (locations SP, CE). There was a trend of increasing concentration of BIOTEQ at SP compared to upstream locations (MA, BE) in samples collected during the summer and winter (Fig. 3 and S2). However, this trend was not obvious in spring and autumn, which indicates that spatial differences can be more pronounced during some seasons. SP is an integrating location for contamination from both rivers and additional nearby sources of pollution. Location CR (oxbow lake) was the least contaminated location, with concentrations of BIOTEQ significantly lesser ($p<0.05$) than those in sediments from locations BE, SP, and CE (Fig. 2a, Table 1). CR also exhibited lesser variability among seasons with AhR-mediated potency only slightly greater in sediments collected during winter (Fig. 3). This finding demonstrates the function of the oxbow lake as a more stable deposit of various HOCs without greater fluctuations in pollution that are obvious in the active channel. Concentrations of BIOTEQ in sediments from all riverine locations exhibited least variability during summer (Fig. 3), which was probably related to the least fluctuations in river water discharge during this season (Fig. S3). Variability in concentrations of BIOTEQ was greater among all riverine locations during winter and spring (Fig. 3).

Results of this study confirmed the role of PAHs as the predominant contributors to the overall AhR-mediated potency observed in previous studies from the region (Hilscherova et al. 2001; Vondracek et al. 2001). Total TEQ calculated from concentrations of individual AhR-active compounds (0.1–1.9 ng/g; Table 1) exhibited similar seasonal patterns as did concentrations of BIOTEQ (Fig. S4). However, concentrations of BIOTEQ were greater than concentrations of TEQ in extracts of all sediments

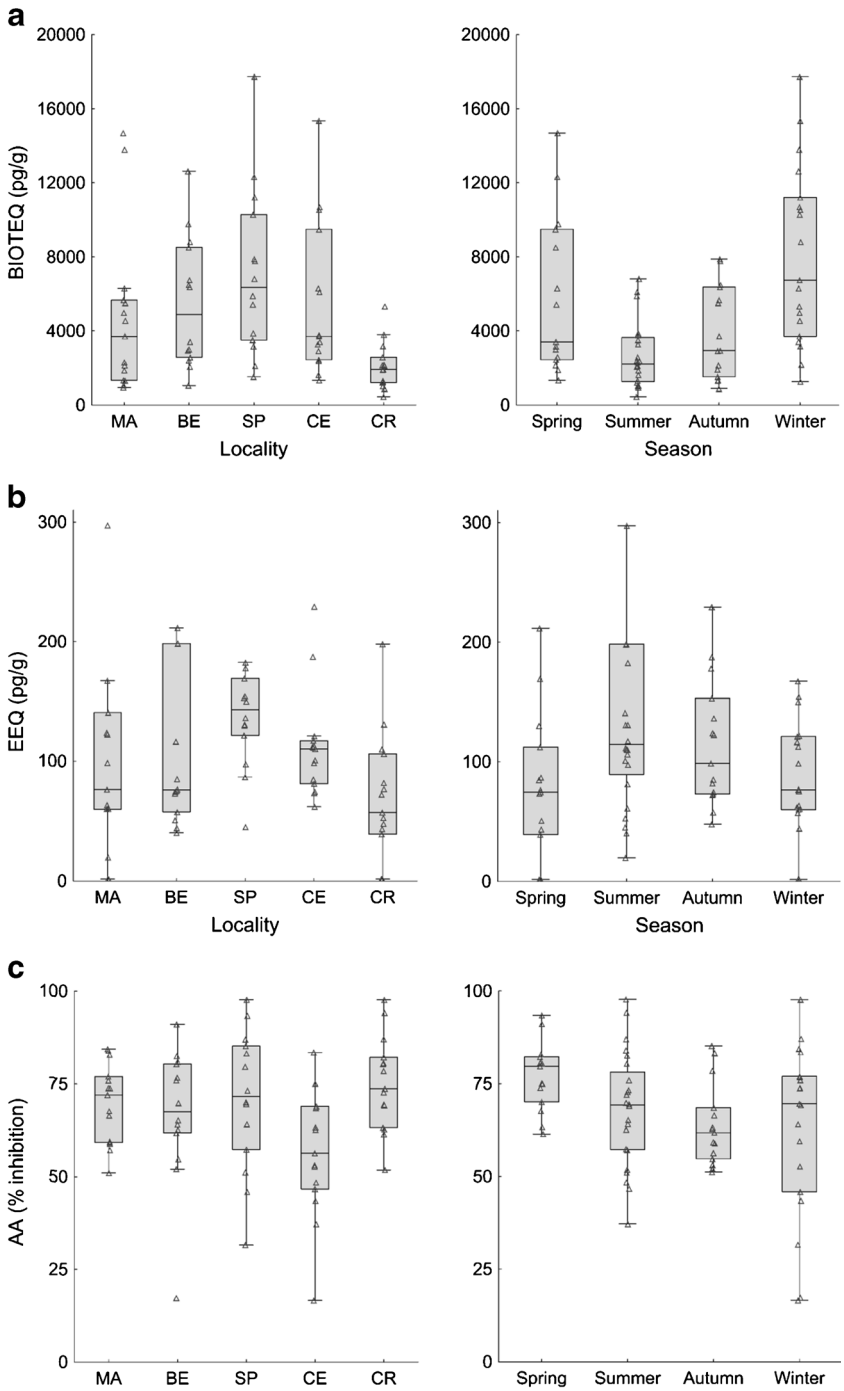

Fig. 2 Spatial and seasonal variability of bioassay-derived: **a** dioxin-like potency (BIOTEQ, pg/g, dm of sediment), **b** estrogenic potency (EEQ, pg/g, dm), **c** antiandrogenic potency (AA, % luminescence inhibition in competition with DHT caused by the highest non-cytotoxic sample concentration) in sediment samples from the 15 sampling campaigns in July 2007–July 2008 ($n=73$). *Middle line* is median, *box* means quartile range (25–75 %), *whisker* is non-outlier range and *triangles* are measured values

with a single exception. Generally, only 13–21 % BIOTEQ (median values "TEQ/BIOTEQ (%)" among locations; Table 1) could be explained by the presence of known AhR ligands, namely PAHs. PAHs accounted on average for 99.4 % of the total TEQ, which is consistent with the results of previous studies conducted in this area (Hilscherova et al. 2001; Vondracek et al. 2001). The main contributors among PAHs were benzo[k]fluoranthene and indeno[123-cd]perylene. Other source of dioxin-like toxicity might be azaarenes and oxygenated PAH derivatives (oxy-PAHs) that were previously detected in sediments from the studied area (Machala et al. 2001). Comparable levels of pollution with dioxin-like compounds were found in sediments from rivers affected by municipal and industrial activities from other areas, such as sediments from two Chinese rivers (BIOTEQ 0.3–13.9 ng/g, dm), where greatest potencies were observed in fractions containing PAHs, OCPs, a portion of PCDD/Fs and unknown compounds (Song et al. 2006). In sediments from the

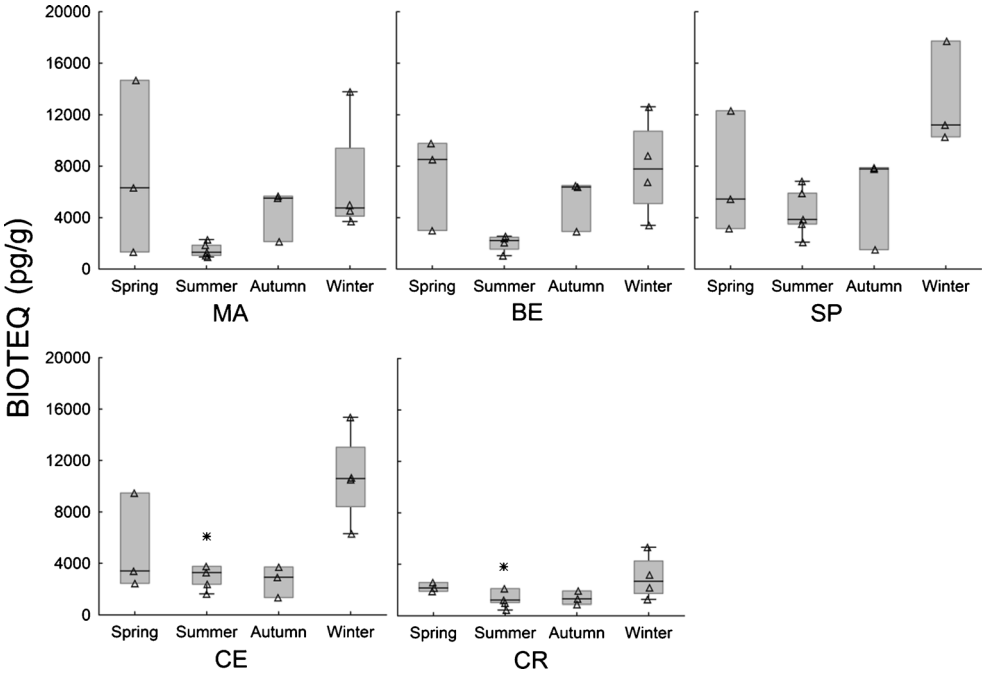

Fig. 3 Seasonal variability of bioassay derived dioxin-like activity (BIOTEQ, pg/g, dm) at each sampling site during the 15 sampling campaigns in July 2007–July 2008 ($n=73$). *Middle line* is median, *box* means quartile range (25–75 %), *whisker* is non-outlier range and *triangles* are measured values

Netherlands, most AhR-mediated potency was caused by acid-labile compounds, such as PAHs (Houtman et al. 2004). Alternatively, only 6 % of AhR-mediated potency was attributed to PAHs in sediments from Germany (Brack et al. 2008). Furthermore, PCDD/Fs were a major source of dioxin-like potency observed in the sediments in the USA where concentrations were as great as 19.9 and 17.7 ng/g, dm PCDDs and PCDFs, respectively (Hilscherova et al. 2003), and 46.5 ng/g, dm for the sum of PCDD/Fs (Kannan et al. 2008). However, in the study, the results of which are reported here, dl-PCBs and PCDD/Fs, due to their relatively small concentrations relative to PAHs, contributed little of the total concentrations of TEQ (Table 1). Despite their greater potency, the average contribution of PCDDs, PCDFs, and dl-PCBs to the total TEQ was only 0.2, 0.3, and 0.1 %, respectively.

ER-mediated potency

Results of the bioassay documented the presence of estrogenic compounds in almost all sediments. Estrogenic potency expressed as EEQ was detected in 93 % of samples in the range of 20–3,753 pg/g, dm, but most samples (88 %) contained 20–300 pg/g, dm (Fig. 2b). Four samples taken in summer 2007 at locations MA ($n=1$), BE ($n=2$), and SP ($n=1$) were noted for great values of EEQ that reached 895–3,753 pg/g, dm. These extreme concentrations are not included in Fig. 2b. Estrogenic potency of 7 % of the sediment samples ($n=5$) was less than the limit of detection (LOD= 3.25 pg/g). Median concentration of EEQ among seasons was greatest in sediments from location SP, similarly to median concentration of BIOTEQ. Samples from BE and MA were noted for a very variable estrogenicity among sampling campaigns including extreme EEQ values. When seasonal variability throughout the year was taken into account, statistically significant difference in estrogenic potency was observed only between locations SP and CR. But there were more pronounced differences in separate seasons. For example, EEQ were always greater in SP compared to BE in autumn and winter samples, while there was no such trend in the other two seasons. The greatest median estrogenic potency across locations was observed in summer (Fig. 2b), even without the extreme concentrations of EEQ observed in a few samples. These extremes could indicate exceptional inputs of (xeno-)estrogens of unknown origin that occurred during early summer 2007 at the above mentioned localities. No general significant seasonal trends in estrogenicity were observed in this study (Fig. 2b). However, sediments sampled at locations MA and CE tended to have lesser concentrations of EEQ in spring compared to autumn. A similar trend was observed previously in this region (Hilscherova et al. 2010; Table 2). Alternatively, greater estrogenicity was observed in sediments collected in spring than in those collected in autumn in a study where a smaller sample set was compared (Creusot et al. 2013).

A weak antiestrogenic potency in the presence of competing E2 was detected only in two sediment samples taken from CR in November and December 2007. Concentration of extract causing 25 % inhibition of luminescence (IC25) in competition with E2 was 25.6 and 16.6 mg SEQ/mL, respectively. These two samples exhibited none and little estrogenic potency, respectively, but the presence of estrogenic pollutants might be masked by antiestrogenic compounds present in

Table 2 Concentrations of AhR-mediated potency (BIOTEQ, pg TCDD/g, dm) and estrogenic potency (EEQ, pg E2/g, dm) of sediments at Malenovice (MA), Belov (BE) and Spytihnev (SP) determined by bioassays

	BIOTEQ (pg TCDD/g)			EEQ (pg E2/g)		
	MA	BE	SP	MA	BE	SP
October 1996[a]	6,542	4,223	NA	239	39	NA
October 1997[a]	6,675	4,449	NA	1,134	93	NA
May 2005[b]	15,368	8,123	9,867	95	29	186
October 2005[b]	7,868	1,442	4,611	231	107	175
May 2006[b]	7,768	914	14,356	<1	<1	90
October 2006[b]	1,660	764	5,573	442	66	127
October 2007	5,506	6,488	7,779	124	85	178
May 2008	1,333	3,001	3,166	<3	51	130
Summer 2007	1,058–2,302	1,042–2,566	2,098–5,893	141–954	198–3,753	45–895
Autumn 2007	2,139–5,663	2,927–6,488	1,515–7,884	99–124	58–85	136–178
Winter 2007/08	3,708–13,797	3,413–12,624	10,276–17,722	60–167	44–117	122–154
Spring 2008	1,333–14,690	3,001–9,768	3,166–12,317	<3–76	51–212	87–130
Summer 2008	940–1,867	2,062	3,870–6,824	20–61	40	97–131

NA data not available

[a] Hilscherova et al. (2001) (2002)

[b] Hilscherova et al. (2010)

these samples. There were four samples that elicited neither estrogenic nor antiestrogenic potency. Antiestrogenic effects might play an important role in some regions. For example, 81 % of sediments from the Pearl River, China, exhibited estrogenicity but at the same time, 61 % of all samples were antiestrogenic meaning that both estrogenic and antiestrogenic compounds were present (Zhao et al. 2011). Sediments from the Svratka and Svitava Rivers that flows into the Morava River downstream from the studied area of Zlin vicinity, elicited only antiestrogenic potencies (Jalova, personal communication) despite the fact that the region is relatively densely populated. The estrogenicity detected in sediments from the region around the city of Zlin indicates that there might be greater inputs of estrogenic compounds due to less effective wastewater treatment plants (WWTPs) and/or more intensive agriculture.

ER-dependent potency was previously assessed in samples from the studied area. Concentrations of EEQ were in the range of 5–23 (Vondracek et al. 2001) and 10–1,200 pg/g, dm in extracts of sediments (Hilscherova et al. 2002). After major floods in 1997, antiestrogenic potencies became more apparent in sediments compared to the situation before floods (Hilscherova et al. 2002). Approximately 10 years after the floods, regional median concentrations of EEQ in sediments from the studied area were in the range of 10–340 pg/g, dm (Hilscherova et al. 2010). Estrogenic compounds (EEQ 21.3–29.9 pg/g, dm) were found in sediments from both upstream and downstream of WWTPs that are considered to be an important source of estrogenic compounds in UK; estrone (E1) and E2 were determined as major estrogenic pollutants (Peck et al. 2004). On the other hand, EEQ in the range of 3.3–10.6 pg/g, dm was detected in sediments from downstream locations from WWTPs in Korea, whereas no potency was observed in upstream locations (Oh et al. 2000). High contamination by estrogenic compounds was observed in sediment from a river in Italy, where E1, estriol (E3), and nonylphenol contributed to the observed estrogenicity; phthalates and octylphenol isomers were suggested as potential contributors (Vigano et al. 2008). In the area around the city of Zlin, rivers receive treated effluents from a number of WWTPs as well as untreated sewage effluents from smaller villages and farms. Effects of large as well as smaller towns as sources of estrogenic compounds have been documented (Jarosova et al. 2012; Vermeirssen et al. 2005). Natural and synthetic estrogens, such as E1, E2, E3, and ethinyl estradiol, were not analyzed in our study but they can enter the rivers and are likely to accumulate in sediments (Luo et al. 2011; Peck et al. 2004; Streck 2009). Therefore, they could be important contributors to the estrogenic potency of extracts of sediments. In addition, PAHs have been found to be a source of estrogenicity in sediments (Hilscherova et al. 2002, 2010; Houtman et al. 2004; Luo et al. 2011). In this study, concentrations of EEQ in sediments were not correlated with concentrations of measured PAHs. However, some of their metabolites produced in sediments by microbial degradation such as hydroxylated PAHs could play a role in the estrogenic effects (e.g., Hayakawa et al. 2007; Luan et al. 2006).

AR-mediated potency

AA was more profound in extracts of sediments than androgenic potency. All extracts at non-cytotoxic concentrations inhibited luminescence in competition with natural ligand DHT with median inhibition during the year at 56–74 % at all sites (Table 1). Antiandrogenicity was greater in extracts of sediments from CR than that from CE (Fig. 2c), namely in summer and winter. These results suggest that antiandrogenic compounds could accumulate better in relatively stable sediments of the oxbow lake. This could be also affected by lower TOC content at CE compared to the other sites, since AA was shown to correlate with organic carbon level (Fig. 4). The AA was least in autumn and significantly greater concentrations were observed in spring.

Androgenic potency expressed as DHT-EQ greater than LOD (580 pg/g) was detected in 30 % of extracts of sediments. Concentrations of DHT-EQ were 0.7–16.8 ng/g, dm. Androgenic potency was detected in at least one sampling period at all locations, but in more than half of samples from the individual locations there was no detectable androgenicity (Table 1). Androgenic potency was detected most frequently in samples from locations SP and CE, whereas only one sample from CR exhibited androgenic potency. Androgenicity was detected most often in sediments collected during autumn (73 % of autumn samples), followed by summer (29 % of summer samples), while only three samples collected during winter and one during spring were androgenic.

To our knowledge, this is the first study that documents the significant seasonal changes in both antiandrogenic and androgenic potential of organic extracts of sediments from a river. Seasonal changes in both androgenic and antiandrogenic potencies were in good agreement. The least antiandrogenic potency, which was observed during autumn, corresponded to the frequent detection of androgenicity in extracts of sediments collected during autumn. Alternatively, AA was greatest in spring when only one sample was androgenic. Previously, AA potency of few sediment samples was shown to be greater in dry season compared to wet season (Zhao et al. 2011). Furthermore, androgenic potency was observed in 34 of 50 extracts of sediments collected in Germany, but seasonal trends were not investigated (Galluba and Oehlmann 2012).

Antiandrogenic potency has been frequently detected in studies of unfractionated extracts of sediments (Hilscherova et al. 2010; Zhao et al. 2011). In some studies, AA was a predominant effect in extracts of sediments, whereas androgenic potency was found in only some fractions (Urbatzka et al. 2007; Weiss et al. 2009). Effect-directed analysis was previously applied to reveal both AA and androgenic compounds in sediments. PAHs, such as fluoranthene, benz[a]anthracene, pyrene and phenanthrene, nonylphenol

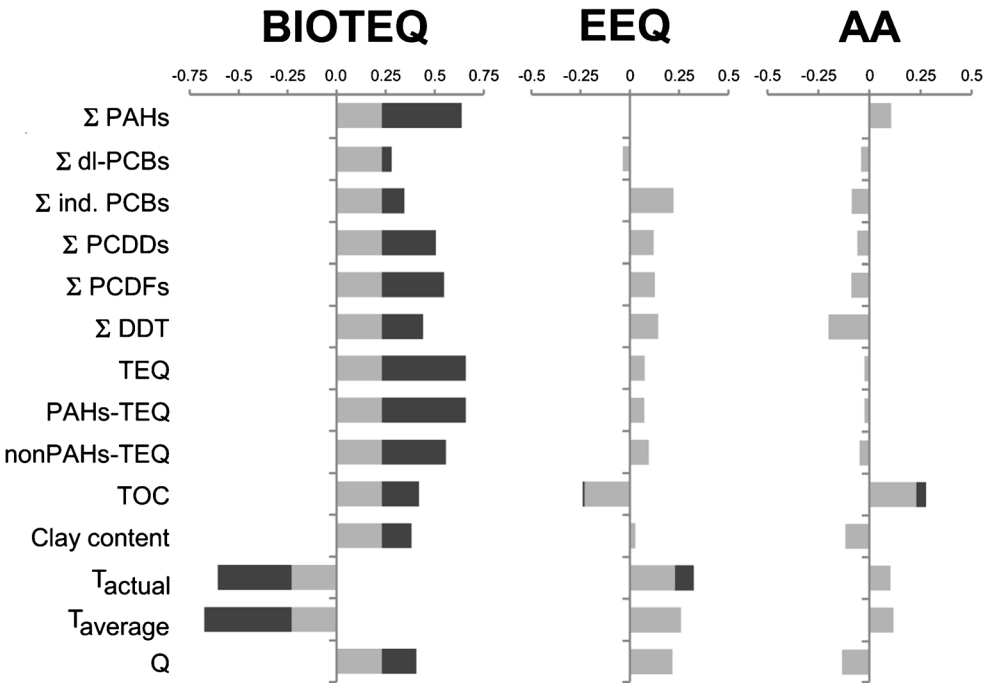

Fig. 4 Pearson's correlation coefficient of bioassay-derived dioxin-like potency (*BIOTEQ*), estrogenic potency (*EEQ*), and antiandrogenic potency (*AA*) with other parameters. *Dark bands* indicate a significant correlation ($p<0.05$). Abbreviations as in Table 1; Σ DDT = sum of concentrations of dichlorodiphenyltrichloroethane (p, p'-DDT) and its metabolites p, p'-DDE, p, p'-DDD; *PAHs-TEQ* TCDD-equivalent calculated based on PAHs concentration, *nonPAHs-TEQ* TCDD-equivalent calculated based on dl-PCBs and PCDD/Fs concentration, T_{actual} river water temperature on the day of sampling, $T_{average}$ time-weighted, average temperature over the 28 days prior to each sampling campaign, Q average discharge over the 28 days prior to each sampling campaign

(Weiss et al. 2009), and the metabolite of DDT, *p*, *p*′-DDE (Urbatzka et al. 2007) were found in antiandrogenic fractions. Various compounds, including oxygenated PAHs, organophosphates, musks, and steroids, were detected in androgenic fractions (Weiss et al. 2011). A number of contaminants analyzed in this study, including some PAHs, PCBs, PCDD/Fs, and OCPs, have also been reported to be antiandrogenic (Vinggaard et al. 2008).

Correlation and multivariate analysis

The correlation profiles of bioassay results with environmental parameters and concentrations of measured residues are displayed as bivariate relationships (Fig. 4). Concentrations of BIOTEQ were significantly positively correlated with TOC, clay content, and flow and negatively with temperature even when the seasonal variability was taken into account. These correlations document a significant role of abiotic parameters in accumulation of dioxin-like compounds, which was demonstrated for TOC and clay also in a previous study (Hilscherova et al. 2010). The fine-grained fraction of sediment particles plays an important role in the accumulation of HOCs in sediments (Jaffe 1991). BIOTEQ was also correlated with concentrations of all studied classes of HOCs. The most significant correlation has been found with PAHs and TEQ derived from PAHs, which documents their important contribution to BIOTEQ. However, from the comparison of TEQ and BIOTEQ it was calculated that only a negligible portion of dioxin-like activity was attributed to dl-PCBs and PCDD/Fs. The correlation does not imply causal relationship but rather indicates that compounds with similar properties like measured HOCs were responsible for the observed AhR-potency of sediments.

There was a significant negative correlation of concentrations of BIOTEQ with actual and average monthly temperature (Fig. 4). This corresponds with the greater concentrations of BIOTEQ observed during winter, which is probably related to slower rates of degradation of chemicals as well as greater PAHs inputs from local combustion during colder periods. Furthermore, concentrations of EEQ were significantly correlated with actual temperature (Fig. 4). Concentrations of PAHs might be partially reduced by microbial degradation that is greater during warmer months. Consequently, this could result in an increased estrogenic potency of sediments due to the formation of estrogenic metabolites, such as hydroxylated PAHs (Hayakawa et al. 2007; Luan et al. 2006; Wang et al. 2012). The opposite trend is observed during winter, because microbial degradation is lower at lower temperatures. Further, lesser dilution of (xeno)estrogens can be expected during warmer months due to the lesser discharge (Sumpter 2005; Figs. S3 and S5). However, no significant correlation between concentrations of EEQ and discharge was observed.

Antiandrogenic potency was significantly correlated with TOC (Fig. 4), which was also shown in a previous study (Hilscherova et al. 2010). Thus, relatively hydrophobic compounds are likely to contribute to the AA potency of extracts of sediments. However, no significant correlation was found between AA and concentrations of studied HOCs among locations and seasons (Fig. 4). The only correlations with AA were found with concentrations of *p*, *p*′-DDE at location MA and with both *p*, *p*′-DDE and *p*, *p*′-DDD in sediments from CE, respectively. These DDT metabolites are considered as antiandrogenic compounds (Vinggaard et al. 2008).

The data were further analyzed using multivariate PCA. Firstly, data from all localities and time points were included in the PCA. The first and second principal components (PC) accounted for 54 % of the total variance (40 and 14 %, respectively), and simplified the multivariate pattern which allowed the variables and samples to be projected onto a two-dimensional space (Fig. 5). Variables with the main influence were TEQ, BIOTEQ and concentrations of measured HOCs in the direction of first PC, and EEQ and AA in the direction of second PC. Secondly, only locations from the active river channel were assessed and temperature (T_{actual}) and discharge (Q) of the river were included as active variables in PCA (Fig. 6).[1] The first and second PC accounted for 52 % of the total variance (39 and 13 %, respectively). Variables with the main influence were concentrations of most classes of HOCs (excluding dl-PCBs) in one direction and T_{actual} and Q in the other direction (Fig. 6a). The influence of EEQ and AA was not apparent anymore in this two-dimensional projection. AA was the dominant parameter associated with PC3, which explained 10 % of the total variance.

AhR-mediated potency determined in bioassay (expressed as BIOTEQ) was clearly associated with concentrations of analyzed HOCs in the first PCA (Fig. 5a). However, if only locations from the active river channel were included, BIOTEQ was projected in the very same direction as HOCs along PC1 but somewhat separated by the direction along PC2 (Fig. 6a). This observation further supported the interpretation that the observed AhR-mediated potency of sediments cannot be fully explained by analyzed HOCs and there were other contaminants with similar properties contributing to the potency. In contrast, analyzed HOCs cannot explain concentrations of EEQ and AA that were projected in a different direction from concentrations of HOCs in both multivariate analyses (Figs. 5a and 6a).

When all locations and time points were included in the analysis, the outcomes of specific bioassays used together with concentrations of the measured pollutants as active variables did not separate the sediments from different locations

[1] Locality CR (oxbow lake) has no water discharge (lentic locality) and temperature was not measured, therefore, these two variables could not have been included in Fig. 5.

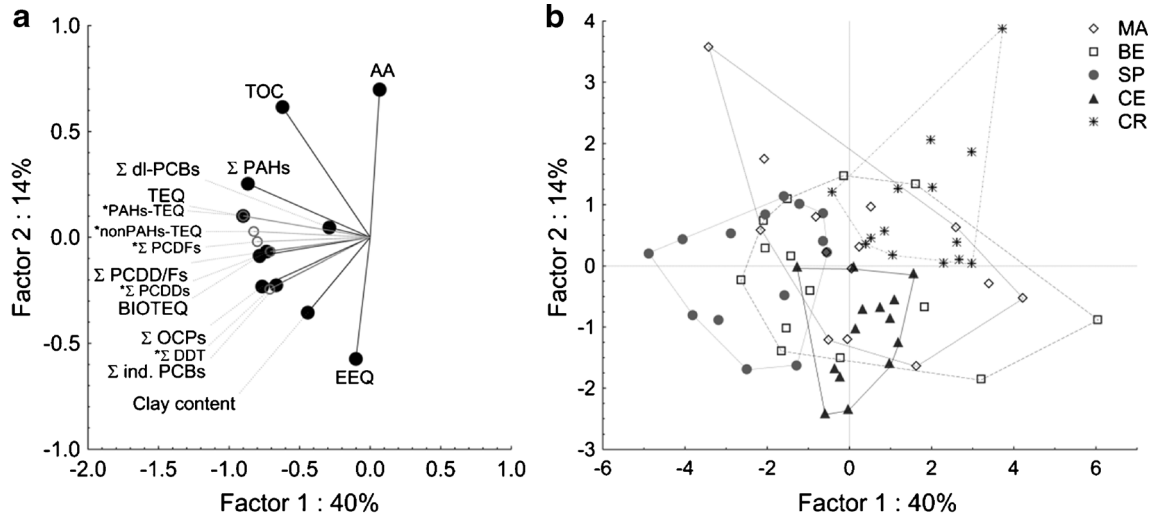

Fig. 5 Principal component analysis (PCA) based on the data from all sampling sites. The ordination diagrams show the relationship among variables (**a**) and distribution of samples according to localities (**b**). Variables marked by *full circles* were used for creating PCA (active variables). Variables marked by *empty circles* are displayed in the same ordination space but they were not used for creating PCA (supplementary variables). Abbreviations as in Table 1 and Fig. 4

(Fig. 5b). Seasonal variability of contamination had a stronger influence on the distribution of variables and samples in PCA than the differences among locations. Results of different samplings from all locations were relatively overlapping and only individual samples from various locations were outliers. Only if T_{actual} and Q were included as active variables in PCA, SP was obviously separated from the other locations in the direction of greater pollutant concentrations (Fig. 6a, b). In conclusion, seasonal changes play a dominant role and can be more important in the studied locations than spatial differences. This finding is consistent with the results of a previous study, which demonstrated no good separation of samples from several study regions in autumn compared to spring (Hilscherova et al. 2010).

Long-term trend analysis

Concentrations of dioxin-like and estrogenic potencies measured in fluvial sediments from the three locations (MA, BE, and SP) (Table 2) during this study were compared to those of several previous studies (Hilscherova et al. 2010, 2002). Data from autumn (October) were available from 5 years between

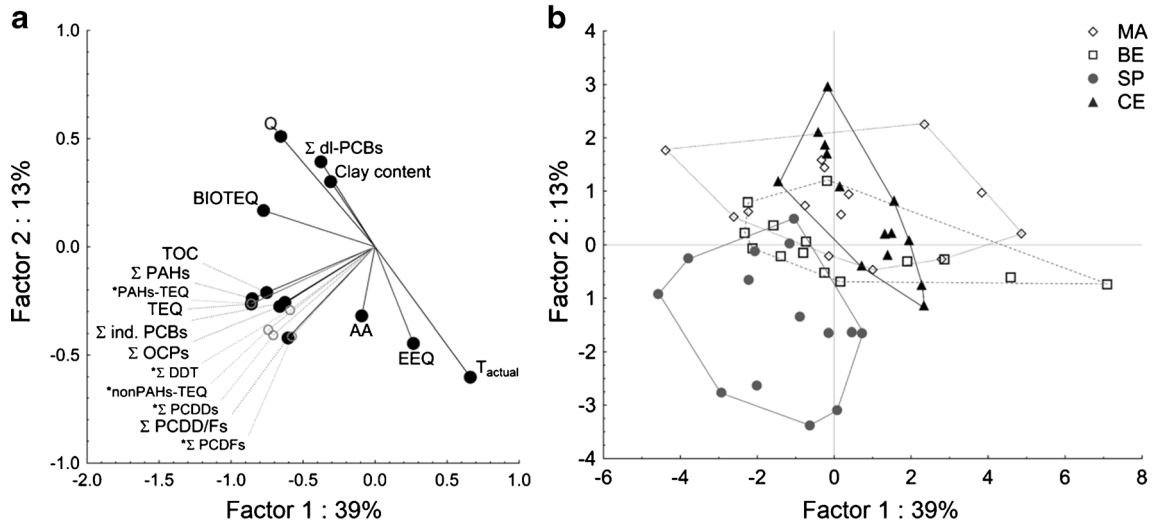

Fig. 6 Principal component analysis (PCA) based on data from sites in the active river channel (i.e., except CR) including also flow and temperature. The ordination diagrams show the relationship among variables (**a**) and distribution of samples according to localities (**b**). Variables marked by *full circles* were used for creating PCA (active variables). Variables marked by *empty circles* are displayed in the same ordination space but they were not used for creating PCA (supplementary variables). Abbreviations as in Table 1 and Fig. 4

1996 and 2008, while for spring (May) from three different years (2005–2008), respectively. There was no continuous trend of changes in concentrations of BIOTEQ or EEQ that would indicate the decrease or increase of contamination in time. Rather, the long-term (inter-annual) differences corresponded well with seasonal fluctuations documented in the current study. Greater differences in potencies measured in the bioassays were observed among spring samples from different years while concentrations were more stable during autumn. Inter-annual as well as seasonal fluctuations were the least at location SP; maximally 4- and 2-fold differences were observed in case of concentrations of BIOTEQ and EEQ, respectively. This was probably related to the greater overall discharge and long-term greater contamination at this location. On the other hand, the greatest differences were found for location MA on river Drevnice (up to 11-fold for BIOTEQ), where discharge was relatively small and thus, fluctuations in discharge could have had larger effects. Both short- and long-term variability in contamination by estrogenic compounds were substantially greater than in the case of dioxin-like compounds. Inter-annual variation in concentrations of EEQ was greater than variation among seasons. As much as 95- and 51-fold difference in EEQ was observed at location MA and BE, respectively, when comparing situations between May 2005, 2006, and 2008, while 25- and 4-fold difference was observed within estrogenic potency of sediments from these two locations in spring 2008, respectively (Table 2). The greater differences on locations MA and BE are associated mainly with a strong decrease of EEQ (below limit of detection) in spring 2006, which is a result of local floods that occurred in the region (Hilscherova et al. 2010). Alternatively, differences in concentrations of EEQs in extracts of sediments from location SP were only 2-fold among spring and autumn samples across the studied years. The results of this 1-year study also show that concentrations of both BIOTEQ and EEQ were more variable in spring compared to autumn. This is probably related to the hydrology of the studied rivers. The discharge of the river was relatively less and stable in autumn 2007 (except for one major rainfall), whereas greater discharge with stronger fluctuations occurred in spring 2008 which can be linked to a greater resuspension of sediments (Fig. S3). A similar comparison of a smaller data set from sediments in a French river showed 3.6- and 5-fold inter-annual differences in dioxin-like and estrogenic potency, respectively (Creusot et al. 2013). Unlike in this study, lesser fluctuation was found in spring than in autumn. However, spring was described as dry season, whereas autumn as wet season in the French study, which differs from the hydrology situation in our study region (Fig. S3). This supports the conclusion that hydrology of the river is a very important parameter that needs to be taken into account in evaluation of river sediments contamination.

Conclusions

The characterization of toxic potencies of environmental mixtures of pollutants might be an important step in the risk assessment of contaminated ecosystems allowing the assessment of potential risks connected with the exposure of organisms, next to comparing concentrations of selected contaminants with quality criteria or EQS. This study documents that the endocrine disruptive and dioxin-like potencies observed in sediments were not, respectively only to a minor extent, associated with routinely monitored hydrophobic organic pollutants. The contribution of PAHs, which were the predominant contaminants in the studied region, to the dioxin-like potency was 13–21 % across locations (median values). Despite the correlation between concentrations of dl-PCBs and PCDD/Fs with BIOTEQ, contribution of these contaminants to the dioxin-like potency was negligible as calculated based on their concentrations and relative potencies in the bioassay. Analyzed HOCs could not explain the observed estrogenic and antiandrogenic activities. The bioassays used in this study provided important information indicating the presence of yet unknown pollutants with dioxin-like and endocrine disruptive potencies in sediments.

This 1-year long study of fluvial sediments also revealed seasonal differences in contamination with dioxin-like AA and androgenic compounds. Further, a long-term comparison of the unique data set originating from three locations point to a greater inter-annual fluctuations in estrogenic than dioxin-like potency. Both short-term and long-term data documents greater fluctuations in biological potencies as well as in river water discharge at the individual locations during spring season. Hence, hydrology of the river and its seasonal differences should be taken into account both in design and interpretation of any monitoring studies. Locations and time points need to be chosen carefully to make sure that the variability of contamination is not overlooked. In addition, to be able to monitor long-term trends in a region, it is necessary to sample in the same period of the year and under comparable hydrological situation. If this is not possible, the interpretation of results from long-term monitoring should be corrected to these factors.

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References

Babek O, Hilscherova K, Nehyba S, Zeman J, Famera M, Francu J, Holoubek I, Machat J, Klanova J (2008) Contamination history of suspended river sediments accumulated in oxbow lakes over the last 25 years. J Soils Sediments 8:165–176

Babich H, Borenfreund E (1990) Cytotoxic effects of food-additives and pharmaceuticals on cells in culture as determined with the Neutral Red Assay. J Pharm Sci 79:592–594

Babut M, Lopes C, Pradelle S, Persat H, Badot PM (2012) BSAFs for freshwater fish and derivation of a sediment quality guideline for PCBs in the Rhone Basin, France. J Soils Sediments 12:241–251

Behnisch PA, Hosoe K, Sakai S (2003) Brominated dioxin-like compounds: in vitro assessment in comparison to classical dioxin-like compounds and other polyaromatic compounds. Environ Int 29: 861–877

Brack W, Klamer HJC, de Ada ML, Barcelo D (2007) Effect-directed analysis of key toxicants in European river basins—a review. Environ Sci Pollut Res 14:30–38

Brack W, Blaha L, Giesy JP, Grote M, Moeder M, Schrader S, Hecker M (2008) Polychlorinated naphthalenes and other dioxin-like compounds in Elbe River sediments. Environ Toxicol Chem 27:519–528

Brinkmann M, Hudjetz S, Kammann U, Hennig M, Kuckelkorn J, Chinoraks M, Cofalla C, Wiseman S, Giesy JP, Schaffer A, Hecker M, Wolz J, Schuttrumpf H, Hollert H (2013) How flood events affect rainbow trout: evidence of a biomarker cascade in rainbow trout after exposure to PAH contaminated sediment suspensions. Aquat Toxicol 128:13–24

Colombo JC, Cappelletti N, Lasci J, Migoya MC, Speranza E, Skorupka CN (2006) Sources, vertical fluxes, and equivalent toxicity of aromatic hydrocarbons in coastal sediments of the Rio de la Plata Estuary, Argentina. Environ Sci Technol 40:734–740

Creusot N, Tapie N, Piccini B, Balaguer P, Porcher JM, Budzinski H, Ait-Aissa S (2013) Distribution of steroid- and dioxin-like activities between sediments, POCIS and SPMD in a French river subject to mixed pressures. Environ Sci Pollut Res 20:2784–2794

Crommentuijn T, Sijm D, de Bruijn J, van den Hoop M, van Leeuwen K, van de Plassche E (2000) Maximum permissible and negligible concentrations for metals and metalloids in the Netherlands, taking into account background concentrations. J Environ Manage 60:121–143

de Deckere E, De Cooman W, Leloup V, Meire P, Schmitt C, von der Ohe PC (2011) Development of sediment quality guidelines for freshwater ecosystems. J Soils Sediments 11:504–517

Demirpence E, Duchesne MJ, Badia E, Gagne D, Pons M (1993) Mvln cells—a bioluminescent Mcf-7-derived cell-line to study the modulation of estrogenic activity. J Steroid Biochem Mol Biol 46:355–364

Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy, Brussels, p 72

Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008 on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council, Brussels, p 14

European Commission (2012) Proposal for a Directive of the European Parliament and of the Council amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy 2011/0429 (COD), Brussels, p 35

Forstner U, Salomons W (2010) Sediment research, management and policy. J Soils Sediments 10:1440–1452

Forstner U, Heise S, Schwartz R, Westrich B, Ahlf W (2004) Historical contaminated sediments and soils at the river basin scale. J Soils Sediments 4:247–260

Galluba S, Oehlmann J (2012) Widespread endocrine activity in river sediments in Hesse, Germany, assessed by a combination of in vitro and in vivo bioassays. J Soils Sediments 12:252–264

Hayakawa K, Onoda Y, Tachikawa C, Hosoi S, Yoshita M, Chung SW, Kizu R, Toriba A, Kameda T, Tang N (2007) Estrogenic/antiestrogenic activities of polycyclic aromatic hydrocarbons and their monohydroxylated derivatives by yeast two-hybrid assay. J Health Sci 53:562–570

Higley E, Grund S, Jones PD, Schulze T, Seiler TB, Lubcke-von Varel U, Brack W, Wolz J, Zielke H, Giesy JP, Hollert H, Hecker M (2012) Endocrine disrupting, mutagenic, and teratogenic effects of upper Danube River sediments using effect-directed analysis. Environ Toxicol Chem 31:1053–1062

Hilscherova K, Kannan K, Kang YS, Holoubek I, Machala M, Masunaga S, Nakanishi J, Giesy JP (2001) Characterization of dioxin-like activity of sediments from a Czech river basin. Environ Toxicol Chem 20:2768–2777

Hilscherova K, Kannan K, Holoubek I, Giesy JP (2002) Characterization of estrogenic activity of riverine sediments from the Czech Republic. Arch Environ Contam Toxicol 43:175–185

Hilscherova K, Kannan K, Nakata H, Hanari N, Yamashita N, Bradley PW, McCabe JM, Taylor AB, Giesy JP (2003) Polychlorinated dibenzo-p-dioxin and dibenzofuran concentration profiles in sediments and flood-plain soils of the Tittabawassee River, Michigan. Environ Sci Technol 37:468–474

Hilscherova K, Dusek L, Kubik V, Cupr P, Hofman J, Klanova J, Holoubek I (2007) Redistribution of organic pollutants in river sediments and alluvial soils related to major floods. J Soils Sediments 7:167–177

Hilscherova K, Dusek L, Sidlova T, Jalova V, Cupr P, Giesy JP, Nehyba S, Jarkovsky J, Klanova J, Holoubek I (2010) Seasonally and regionally determined indication potential of bioassays in contaminated river sediments. Environ Toxicol Chem 29:522–534

Houtman CJ, Cenijn PH, Hamers T, Lamoree MH, Legler J, Murk AJ, Brouwer A (2004) Toxicological profiling of sediments using in vitro bioassays, with emphasis on endocrine disruption. Environ Toxicol Chem 23:32–40

Hunt JCR (2002) Floods in a changing climate: a review. Phil Trans R Soc A Math Phys Eng Sci 360:1531–1543

Jaffe R (1991) Fate of hydrophobic organic pollutants in the aquatic environment—a review. Environ Pollut 69:237–257

Janosek J, Hilscherova K, Blaha L, Holoubek I (2006) Environmental xenobiotics and nuclear receptors—interactions, effects and in vitro assessment. Toxicol in Vitro 20:18–37

Jarosova B, Blaha L, Vrana B, Randak T, Grabic R, Giesy JP, Hilscherova K (2012) Changes in concentrations of hydrophilic organic contaminants and of endocrine-disrupting potential downstream of small communities located adjacent to headwaters. Environ Int 45:22–31

Jobling S, Tyler CR (2003) Endocrine disruption in wild freshwater fish. Pure Appl Chem 75:2219–2234

Kannan K, Yun SH, Ostaszewski A, McCabe JM, Mackenzie-Taylor D, Taylor AB (2008) Dioxin-like toxicity in the Saginaw river watershed: polychlorinated dibenzo-p-dioxins, dibenzofurans, and biphenyls in sediments and floodplain soils from the Saginaw and Shiawassee rivers and Saginaw bay, Michigan, USA. Arch Environ Contam Toxicol 54:9–19

Kaplan S (2013) Review: pharmacological pollution in water. Crit Rev Environ Sci Technol 43:1074–1116

Kidd KA, Blanchfield PJ, Mills KH, Palace VP, Evans RE, Lazorchak JM, Flick RW (2007) Collapse of a fish population after exposure to a synthetic estrogen. Proc Natl Acad Sci U S A 104:8897–8901

Koh CH, Khim JS, Kannan K, Villeneuve DL, Senthilkumar K, Giesy JP (2004) Polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs) and 2,3,7,8-TCDD equivalents (TEQs) in sediment from the Hyeongsan River, Korea. Environ Pollut 132:489–501

Kukucka P, Audy O, Prokes R, Komprdova K, Klanova J (2010) Temporal and spatial trends of selected POPs in riverine sediments: What can we learn for assessment of risks associated with frequent flood events? Organohalogen Compd 72:134–137

Luan TG, Yu KSH, Zhong Y, Zhou HW, Lan CY, Tam NFY (2006) Study of metabolites from the degradation of polycyclic aromatic hydrocarbons (PAHs) by bacterial consortium enriched from mangrove sediments. Chemosphere 65:2289–2296

Luo JP, Lei BL, Ma M, Zha JM, Wang ZJ (2011) Identification of estrogen receptor agonists in sediments from Wenyu River, Beijing, China. Water Res 45:3908–3914

Machala M, Vondracek J, Blaha L, Ciganek M, Neca J (2001) Aryl hydrocarbon receptor-mediated activity of mutagenic polycyclic aromatic hydrocarbons determined using in vitro reporter gene assay. Mutat Res Genet Toxicol Environ Mutagen 497:49–62

Martinez-Gomez C, Lamoree M, Hamers T, van Velzen M, Kamstra JH, Fernandez B, Benedicto J, Leon VM, Vethaak AD (2013) Integrated chemical and biological analysis to explain estrogenic potency in bile extracts of red mullet (*Mullus barbatus*). Aquat Toxicol 134:1–10

Novak J, Jalova V, Giesy JP, Hilscherova K (2009) Pollutants in particulate and gaseous fractions of ambient air interfere with multiple signaling pathways in vitro. Environ Int 35:43–49

Oh SM, Choung SY, Sheen YY, Chung KH (2000) Quantitative assessment of estrogenic activity in the water environment of Korea by the E-SCREEN assay. Sci Total Environ 263:161–169

Peck M, Gibson RW, Kortenkamp A, Hill EM (2004) Sediments are major sinks of steroidal estrogens in two United Kingdom rivers. Environ Toxicol Chem 23:945–952

Prokes R, Vrana B, Klanova J (2012) Levels and distribution of dissolved hydrophobic organic contaminants in the Morava river in Zlin district, Czech Republic as derived from their accumulation in silicone rubber passive samplers. Environ Pollut 166:157–166

Song MY, Jiang QT, Xu Y, Liu HX, Lam PKS, O'Toole DK, Zhang QH, Giesy JP, Jiang GB (2006) AhR-active compounds in sediments of the Haihe and Dagu Rivers, China. Chemosphere 63:1222–1230

Streck G (2009) Chemical and biological analysis of estrogenic, progestagenic and androgenic steroids in the environment. Trac Trends Anal Chem 28:635–652

Sumpter JP (2005) Endocrine disrupters in the aquatic environment: an overview. Acta Hydrochim Hydrobiol 33:9–16

Urbatzka R, van Cauwenberge A, Maggioni S, Vigano L, Mandich A, Benfenati E, Lutz I, Kloas W (2007) Androgenic and antiandrogenic activities in water and sediment samples from the river Lambro, Italy, detected by yeast androgen screen and chemical analyses. Chemosphere 67:1080–1087

Vermeirssen ELM, Korner O, Schonenberger R, Suter MJF, Burkhardt-Holm P (2005) Characterization of environmental estrogens in river water using a three pronged approach: active and passive water sampling and the analysis of accumulated estrogens in the bile of caged fish. Environ Sci Technol 39:8191–8198

Vigano L, Benfenati E, van Cauwenberge A, Eidem JK, Erratico C, Goksoyr A, Kloas W, Maggioni S, Mandich A, Urbatzka R (2008) Estrogenicity profile and estrogenic compounds determined in river sediments by chemical analysis, ELISA and yeast assays. Chemosphere 73:1078–1089

Vinggaard AM, Niemela J, Wedebye EB, Jensen GE (2008) Screening of 397 chemicals and development of a quantitative structure-activity relationship model for androgen receptor antagonism. Chem Res Toxicol 21:813–823

Vondracek J, Machala M, Minksova K, Blaha L, Murk AJ, Kozubik A, Hofmanova J, Hilscherova K, Ulrich R, Ciganek M, Neca J, Svrckova D, Holoubek I (2001) Monitoring river sediments contaminated predominantly with polyaromatic hydrocarbons by chemical and in vitro bioassay techniques. Environ Toxicol Chem 20: 1499–1506

Wang X, Lin L, Luan T, Yang L, Tam NFY (2012) Determination of hydroxylated metabolites of polycyclic aromatic hydrocarbons in sediment samples by combining subcritical water extraction and dispersive liquid-liquid microextraction with derivatization. Anal Chim Acta 753:57–63

Weiss JM, Hamers T, Thomas KV, van der Linden S, Leonards PEG, Lamoree MH (2009) Masking effect of anti-androgens on androgenic activity in European river sediment unveiled by effect-directed analysis. Anal Bioanal Chem 394:1385–1397

Weiss JM, Simon E, Stroomberg GJ, de Boer R, de Boer J, van der Linden SC, Leonards PEG, Lamoree MH (2011) Identification strategy for unknown pollutants using high-resolution mass spectrometry: androgen-disrupting compounds identified through effect-directed analysis. Anal Bioanal Chem 400:3141–3149

Wilson VS, Bobseine K, Lambright CR, Gray LE (2002) A novel cell line, MDA-kb2, that stably expresses an androgen- and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists. Toxicol Sci 66:69–81

Wolz J, Schulze T, Lubcke-von Varel U, Fleig M, Reifferscheid G, Brack W, Kuhlers D, Braunbeck T, Hollert H (2011) Investigation on soil contamination at recently inundated and non-inundated sites. J Soils Sediments 11:82–92

Zhao JL, Ying GG, Yang B, Liu S, Zhou LJ, Chen ZF, Lai HJ (2011) Screening of multiple hormonal activities in surface water and sediment from the pearl river system, South China, using effect-directed in vitro bioassays. Environ Toxicol Chem 30:2208–2215

Článek XXI:

Novák, J., Beníšek, M., Pacherník, J., Janošek, J., Šídlová, T., Kiviranta, H., Verta, M., Giesy, J.P., Bláha L., Hilscherová K., 2007. Interference of contaminated sediment extracts and environmental pollutants with retinoid signaling. Environmental Toxicology and Chemistry 26(8), 1591-1599.

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INTERFERENCE OF CONTAMINATED SEDIMENT EXTRACTS AND ENVIRONMENTAL POLLUTANTS WITH RETINOID SIGNALING

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Abstract—Retinoids are known to regulate important processes such as differentiation, development, and embryogenesis. Some effects, such as malformations in frogs or changes in metabolism of birds, could be related to disruption of the retinoid signaling pathway by exposure to organic contaminants. A new reporter gene assay has been established for evaluation of the modulation of retinoid signaling by individual chemicals or environmental samples. The bioassay is based on the pluripotent embryonic carcinoma cell line P19 stably transfected with the firefly luciferase gene under the control of a retinoic acid–responsive element (clone P19/A15). The cell line was used to characterize the effects of individual chemicals and sediments extracts on retinoid signaling pathways. The extracts of sediments from the River Kymi, Finland, which contained polychlorinated dioxins and furans and polycyclic aromatic hydrocarbons (PAHs), significantly increased the potency of all-*trans* retinoic acid (ATRA), while no effect was observed with the extract of the sediment from reference locality. Considerable part of the effect was caused by the labile fraction of the sediment extracts. Also, several individual PAHs potentiated the effect of ATRA; on the other hand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and several phthalates showed slightly inhibiting effect. These results suggest that PAHs could be able to modulate the retinoid signaling pathway and that they could be responsible for a part of the proretinoid activity observed in the sediment extracts. However, the effects of PAHs on the retinoic acid signaling pathways do not seem to be mediated directly by crosstalk with aryl hydrocarbon receptor.

Keywords—Retinoid Polycyclic aromatic hydrocarbons 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Sediments Retinoic acid receptor

INTRODUCTION

Retinoids, such as vitamin A, retinol, and their derivatives, have an essential role in regulation of development and homeostasis of all vertebrate tissues through regulation of cell differentiation, proliferation, and apoptosis [1]. Furthermore, retinoids can act as anticarcinogenic substances because of their antioxidant properties and control of differentiation [2,3]. Studies on retinoic acid deficiency or excess support the view that tissue distribution of retinoic acid is finely controlled. Vitamin A deficiency results in a spectrum of malformations that include abnormal development of the eye, brain, heart, somite, and limb [1]. Conversely, excessive retinoic acid intake during pregnancy can lead to developmental defects, such as limb malformations and craniofacial and heart defects, the type and degree of which depend on the magnitude, duration, and timing of the exposure [4]. Various studies have found that negative effects of environmental pollutants such as frog malformations [2] or impaired metabolism of retinoids in birds [5] could be mediated by modulation of the retinoid-signaling pathway [3]. For example, it has been reported that some fish species exposed to pulp mill effluents exhibited reduced hepatic levels of natural retinoids, while vitamin E levels were unaffected [6]. This was confirmed by other studies that showed that some constituents of pulp mill effluents could bind to both retinoic acid receptor (RAR) and retinoic X receptor (RXR) and displace the natural ligands in vitro [7]. While still controversial and not yet definitively proven, it has been suggested that the occurrence of deformed frogs in North America and Japan may be at least partly mediated by persistent organic pollutants that are present in surface waters and that interfere with retinoid signaling pathway [8,9]. However, the mechanism by which retinoids can cause these deformities is not well understood. Retinoid signaling has been reported to be affected by some pesticides [4], and several pesticides have been reported to activate RARs [9]. Plasma retinoid profiles have been reported to be different in bullfrogs from areas of intensive agriculture than from areas less affected by agriculture [9]. Frog deformities have been observed to be related to the proximity of pollution sources [3].

The complex retinoid signaling pathway contains numerous potential targets for disruption by environmental pollutants. The retinoid signal is transduced by two families of nuclear receptors, the RARs and the RXRs, which function as RXR/RAR heterodimers or RXR/RXR homodimers [10]. Each family consists of three isoforms (α, β, and γ) encoded by separate genes [11]. The RARs are activated by all-*trans* retinoic acid (ATRA) and its 9-cis isomer, while RXRs are activated only by 9-cis RA [11]. The potential interactions are made more complex by the fact that the retinoid signaling pathway seems

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to be able to crosstalk with other signaling pathways, such as those connected with the aryl hydrocarbon receptor (AhR), thyroid receptor [12,13], MAP kinases [10], or peroxisome proliferator activated receptors [14].

Nilsson and Hakansson [15] have shown that ligands of the AhR cause severe changes in metabolism of retinoids. The AhR binds with high affinity to planar, aromatic substances, including, among others, congeners of polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs). The primary known biochemical response to AhR activation is induction of drug-metabolizing enzymes such as cytochromes P450 (CYPs), glutathione-*S*-transferase, and uridine diphosphate-glucuronyltransferase. However, CYP enzymes do not participate only in detoxification of xenobiotics but they may also greatly enhance their toxic and/or mutagenic potency [16]. Furthermore, up-regulation of the various CYP mono-oxygenase enzymes can cause adverse effects through modulation of endogenous processes, such as modulation of specific cellular signaling pathways [17]. Numerous chronic adverse health effects of many xenobiotics, such as neurotoxicity, embryotoxicity, immunotoxicity, changes in cell proliferation, and carcinogenicity, have been reported to be AhR-dependent events [16].

Mobilization of retinol storage forms in liver and increase of retinoic acid levels in serum of rats are typical effects of exposure to the prototypal AhR activators such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [15]. Similar in vivo effects have been observed in lake trout after exposure to non-*ortho* PCB 126 [18]. In vitro exposure to TCDD has been shown to cause a significant decrease of ATRA action in human keratinocytes [19].

Retinoid signaling may also be affected by compounds with molecular structures similar to the natural ligands of retinoid receptors. That could be the case for phthalates, which belong to peroxisome proliferators. This group of chemicals is known to activate peroxisome proliferator activated receptors and cause peroxisome proliferation in the liver and other tissues [14]. Phthalates, which are widely used plasticizers and important contaminants of the environment [20], have been shown to cause hepatocarcinogenesis and damage to the testis, and their toxic effect in testes was assigned to change of RARα signaling [21].

Here we introduce a novel in vitro bioassay for evaluation of the potential of several model compounds and extracts of environmental matrices to affect the retinoic acid signaling system. The model is based on embryonic carcinoma P19 cell line [22]. This cell line retains the responsiveness to retinoid signals and pluripotent characteristics so that the cells are able to differentiate into cells of all three germ layers [23]. It is thus possible to differentiate them into neurons [24], cardiomyocytes [23], or primitive endoderm [25]. To determine if the tested samples were able to activate retinoid-signaling pathway and/or modulate the effect of ATRA, assessments were conducted with or without concurrent ATRA exposure. Extracts of contaminated river sediments and sediment from a reference locality were tested to determine if they contained compounds capable of affecting the retinoic acid signaling system. The organic extracts were applied as either raw or sulfuric acid–treated extracts to distinguish between the effects of persistent and more acid-labile compounds to the observed effects. The individual model compounds were selected to reflect the nature of contamination of the tested sediments. The model activator of AhR, TCDD, was used as standard because it is the most effective ligand among the PCDDs, PCDFs, and PCBs that were measured in persistent fraction of the sediment extracts. Several representatives of polycyclic aromatic hydrocarbons (PAHs) that were present in the extracts represented the nonpersistent fraction together with phthalate esters that possess the retinoid-like structure and might be therefore modulating the activity of RAR.

MATERIALS AND METHODS

Preparation of the sediment samples

Sediments were collected in 2000 from regions of the Kymi River in southeastern Finland, which is known to be polluted by organochlorinated compounds and mercury from production of chloralkali and wood preservatives and from pulp bleaching [26]. Sediment cores were collected from soft sediment sites with different degree of pollution. A reference sediment sample was collected from Steinbach Creek near Talheim south of Tübingen, Baden-Wüttenberg, Germany, an area that is known to be relatively free of significant concentrations of pollutants.

Sediment samples were freeze-dried and extracted with dichloromethane in a Büchi System B-811 automatic extractor. Extracts were used to determine residues of PCBs, PAHs, and other organic chlorinated pollutants (OCPs) or in the in vitro cell culture assays. Polychlorinated dioxins and furans were extracted with toluene in a Soxhlet apparatus. The volume of the dichloromethane extracts was reduced after extraction under a gentle nitrogen stream at ambient temperature. Half the extract for bioassays was evaporated under nitrogen until dryness and dissolved in 100 μl of dimethyl sulfoxide (DMSO), and the second half of the extract was vigorously mixed with 3 ml of concentrated sulfuric acid for 30 min to degrade the less persistent AhR ligands such as PAHs. The layers were separated by centrifugation at 1,000 *g* for 10 min, after which the top dichloromethane layer was transferred into a clean tube and the mixing repeated after adding 4 ml of dichloromethane to the tube containing the sulfuric acid layer. Finally, the top dichloromethane layer was combined with the first fraction, and the samples were concentrated under nitrogen until dryness and dissolved in 100 μl DMSO.

Chemical analyses

Concentrations of PCBs, PAHs, and OCPs were determined at RECETOX, Masaryk University Brno, Czech Republic and the polychlorinated dioxins and furans (PCDD/Fs) analyses were conducted in the Laboratory of Chemistry of the Department of Environmental Health in the Finnish National Public Health Institute. Polychlorinated dioxins and furans were determined in the purified extract with a high-resolution mass spectrometry equipped with a fused silica capillary column DB-DIOXIN (Krackeler Scientific, Albany, NY, USA) and a VG 70 SE mass spectrometer (resolution 10,000). Sixteen ^{13}C-labeled PCDD/F congeners were used as internal standards. A more thorough description of the PCDD/F method is given by Isosaari et al. [27]. Sample 1 was not analyzed for PCDD/Fs because there was no dioxin-like activity in the sulfuric acid–treated extract according to H4IIE-*luc* assay.

For PCBs, PAHs, and OCPs analysis, the laboratory blank and the reference material were analyzed with the set of sediment samples, and surrogate recovery standards were used for quality assurance and quality control samples prior to extraction. Volume was reduced after extraction under a gentle

nitrogen stream at ambient temperature and fractionation achieved on silica gel column; sulfuric acid–modified silica gel column was used for PCB/OCP samples. Sulfur was removed by activated copper treatment. Samples were analyzed using gas chromatography with electron capture detector HP 5890 supplied with a Quadrex fused silica column 5% Ph for PCBs and OCPs. Sixteen U.S. Environmental Protection Agency (U.S. EPA) polycyclic aromatic hydrocarbons were determined in all samples using gas chromatography with mass spectrometry (HP 6890, HP 5973) supplied with a J&W Scientific (Folsom, CA, USA) fused silica column DB-5MS. Samples were quantified using Pesticide Mix 13 (Dr. Ehrenstorfer GmbH, Augsburg, Germany) and PAH Mix 27 (Promochem, Teddington, UK) standard mixtures. Terfenyl and PCB 121 were used as internal standards for PAHs and PCBs analyses, respectively.

Chemicals

The reference TCDD was from Ultra Scientific (North Kingstown, RI, USA), and ATRA, phthalates, and polycyclic aromatic hydrocarbons were purchased from Sigma-Aldrich (Prague, Czech Republic). All chemicals were of the highest purity commercially available.

Cell cultures

The murine embryonal carcinoma cell line P19 was purchased from the European Collection of Cell Cultures (Wiltshire, UK). Stable transfectants of P19 cells were prepared by electroporation as described previously [24]. Cells were transfected with the mixture of 10 μg luciferase reporter pRAREβ2-TK-luc plasmid (provided by Christopher Glass, University of California, San Diego, La Jolla, CA, USA) and 2 μg selection vector pSV2Neo (Clontech, Saint-Germain-en-Laye, France). Transfected cells were then selected in medium containing 400 μg/ml of G418 (Sigma Aldrich), cloned, and screened for the response to ATRA by determining the amount of luciferase expression by luminometry. Positive clones that retained the phenotype and in vitro differentiation potential of maternal cells were used for further tests. The resulting clone P19/A15 cells were cultured in tissue culture flasks (Techno Plastic Products AG, Trasadingen, Switzerland), in Dubelco's modified Eagle medium containing 10% fetal calf serum Mycoplex (PAA Laboratories GmbH, Pasching, Austria). For differentiation, the cells were seeded on sterile cell-culture dishes at a density of 5,000 cells/cm^2 in DMEM medium with 125 nM ATRA (Sigma Aldrich). After 48 h of incubation, the medium was replaced by fresh medium without ATRA and cultivated for another 72 h before experimentation.

The H4IIE-*luc* (rat hepatocarcinoma) cells stably transfected with the luciferase gene under control of the AhR were used for analysis of receptor activation. This bioassay is a well-established model for evaluation of AhR-mediated activities of pure substances as well as environmental samples [28]. The cells were grown under the same conditions as P19/A15 cells.

Experiments

To describe the responsiveness and its possible changes during differentiation, a standard dose–response curve was developed for the standard ligand, ATRA, with both differentiated and nondifferentiated P19/A15 cells. Differentiation was induced by ATRA.

Effects of sediment extracts and model compounds (PAHs, TCDD, phthalates) alone or in combination with ATRA on induction of RAR-dependent luciferase were assessed. Both raw (containing persistent and labile compounds) and sulfuric acid–treated extracts (only persistent fraction) of contaminated sediment were tested. Effects of sediments were also correlated with AhR-mediated effects determined with H4IIE-luc cells.

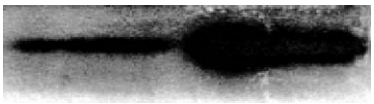
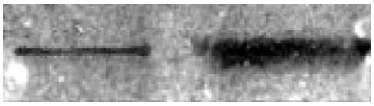
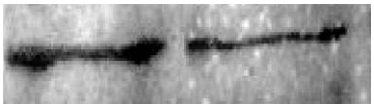
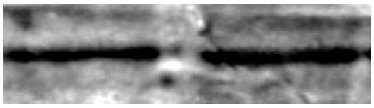
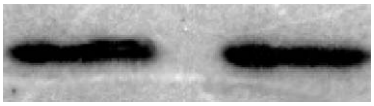
The cells were exposed to individual compounds that represented several classes of pollutants known to be present in the sediment extracts. In the cases where there was no response, such as TCDD and phthalates, the compounds were also tested on the differentiated cells. The level of differentiation in each experiment was evaluated by Western blotting.

Experiments with P19/A15 cells were performed in 96-well microplates. For the assay, either undifferentiated or differentiated P19/A15 cells were seeded at a density of 10,000 or 15,000 cells/well, respectively. After plating, the cells were exposed in triplicates to ATRA (dilution series 1–10,000 nM ATRA) and tested extracts or model compounds for 24 h at 37°C. All samples were dissolved in DMSO. The final concentration of the solvent was less than 0.5% v/v in the exposure media, and appropriate solvent controls were tested. The sediment extracts and model compounds were used for the exposure either alone or in combination with 32 nM ATRA (concentration within normal physiological range). Intensity of luciferase luminescence was measured using the Promega Steady Glo Kit (Promega, Madison, WI, USA). The H4IIE-*luc* cells were seeded on 96-well culture plates at a density 15,000 cells/well. The TCDD dissolved in DMSO was used as a reference compound (dilution series 0.1–500 pM). The rest of the procedure was the same as in case of P19/A15 cells. Cytotoxicity of tested dilutions of the samples was excluded using neutral red uptake assay [29].

Western blot analysis

The level of differentiation was confirmed by Western blot analysis of endoderm-specific cytokeratin Endo-A [30] and Oct-4, a marker of pluripotent cells [31]. We also assessed levels of AhR, RXRα, and RARα with the housekeeping protein lamin B as a control of loading. For Western blot analysis, cultured P19/A15 cells were briefly washed with phosphate-buffered saline and lysed in sodium dodecyl sulfate lysis buffer (50 mM Tris-HCl, pH 7.5, 1% sodium dodecyl sulfate, 10% glycerol). Protein concentrations were determined using the DC Protein assay kit (Bio-Rad, Hercules, CA, USA). Lysates were supplemented with bromphenol blue (0.01%) and β-mercaptoethanol (1%), and equal amounts of total protein (10 μg) were subjected to sodium dodecyl sulfate polyacrilamide gel electrophoresis in 10% gel. After being electrotransferred onto a nitrocelulose membrane (Sigma-Aldrich), proteins were immunodetected using appropriate primary and secondary antibodies and visualized by enhanced chemiluminescence using ECL-Plus kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions. The following primary antibodies were employed: rat monoclonal antibody against mouse endoderm–specific cytokeratin Endo-A (TROMA-I; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA) and Oct-4 (SC-9081; Santa Cruz Biotechnology, Heidelberg, Germany), lamin B SC-6217 (Santa Cruz Biotechnology), RXRα (SC-553; Santa Cruz Biotechnology), RARα 804-102-C050 (Alexis Biochemicals USA, San Diego, CA, USA), and AhR 804-421-R100 (Alexis Biochemicals USA). Horseradish peroxidase–second-

Table 1. Changes in protein levels after all-*trans* retinoic acid (ATRA)-induced differentiation of P19/A15 cells (Endo-A = mouse endoderm-specific cytokeratin; Oct-4 = marker of pluripotent cells; RARα = retinoic acid receptor α; RXRα = retinoid X receptor α; AhR = aryl hydrocarbon receptor; lamin B = housekeeping protein used for loading control)[a]

Proteins assessed by Western blotting	Nondifferentiated P19/ A15 cells	Differentiated P19/A15 cells	Nondifferentiated P19/A15 cells	Differentiated P19/A15 cells
Oct-4	***	—		
Endo-A	*	***		
RARα	*	**		
AhR	**	*		
RXRα	**	***		
Lamin B	**	**		

[a] Asterisks indicate relative amount of the analyzed protein.

ary antibody conjugates were from Sigma-Aldrich, anti-mouse (A9044), anti-rabbit (A4914), anti-goat (A4174).

Data analysis

To determine the response to treatments relative to the response to vehicle controls, statistical analyses were performed using a one-way analysis of variance (Statistica for Windows, StatSoft, Tulsa, OK, USA) from at least three independent experiments ($p < 0.05$). Results from H4IIE-*luc* cells were expressed as relative potencies with respect to TCDD. Relative potencies were calculated from median effective concentration (EC50) values. Toxic equivalents (TEQs) expressed as nanograms of TCDD per gram of sediment were calculated from EC50 values by use of the equieffective approach described by Villeneuve et al. [28]. Toxic equivalents of TCDD (TEQs) were calculated using the toxicity equivalence factors determined for the CALUX bioassay [32].

RESULTS

Characterization of the model cell line

The functionality of the P19/A15 model cell line was verified using a dilution series of the reference compound, ATRA, the natural ligand of the RAR. A dose–response dependence was observed to occur between 2 and 10,000 nM. Greater concentrations of ATRA were cytotoxic. The limit of detection was identical to the first point of linear part of the curve, 2 nM ATRA. The EC50 values were in the range of 512 ± 31 nM ATRA and 61 ± 19 nM ATRA in nondifferentiated and differentiated cells, respectively. Since no changes in transcriptional responsiveness were observed as a function of passage number, it was concluded that the transgene was stably integrated into the genome of the cells (J. Pacherník, unpublished data). Changes in expression of several receptors and protein markers were assessed after differentiation. Expression of Oct-4 and Endo-A was closely related to differentiation treatment with ATRA (Table 1). While nondifferentiated cells contained a great amount of Oct-4 and expressed little Endo-A, after ATRA-treatment this trend was reversed. Levels of RARα were slightly greater in differentiated cells, whereas the protein level of AhR was slightly less after differentiation. Lamin B did not change after differentiation (Table 1).

Effects of sediments

A gradient of contamination was observed with much lower concentrations of pollutants with dioxin-like activity in the sediment from the reference site. A similar gradient was also observed for the 16 U.S. EPA priority PAHs, seven indicator PCB congeners, and selected pesticides (Table 2). The total concentration of TEQ in the raw extracts and sulfuric acid–treated extracts determined by use of the H4IIE-*luc* cell line was very great except for samples 7 and 8, which were less contaminated, and the reference sediment extract, which contained almost no TEQ. The results from the assays with persistent fraction analysis corresponded to the data from chemical analysis except for sample 6, which had exhibited a lesser concentration of TEQ determined by the H4IIE-luc assay than the concentration of TEQ calculated from the results of chemical analysis (Table 2). The TEQ of sediments 2, 3, and 8 was

Table 2. Contaminant concentrations in sediment extracts; comparison of dioxin-like toxicity of raw extracts and sulfuric acid-treated extracts assessed in H4IIE-*luc* cells; 1 = reference sediment extract; 2–8 = extracts of Kymi River sediment (Finland)

	Chemical analysis results[a]							Bioassay results	
Extract no.	Σ PCBs (ng/g)	Σ HCH (ng/g)	Σ DDT + DDE (ng/g)	HCB (ng/g)	PeCB (ng/g)	Σ PAH (ng/g)	PCDD/ PCDF (Σ TEQ ng/g)	Raw extracts (TEQ ng/g)	H_2SO_4 treated (TEQ ng/g)
1	3.7	0.2	0.3	0.1	0.2	233	NA[b]	2	ND[c]
2	40.8	2	3.1	67	3.1	9,570	169	160	157
3	242.4	7.6	13	158.1	9.4	5,086	199	208	213
4	22.1	9.3	7.1	49.6	27.5	4,255	133	171	112
5	101.3	3.3	2.2	44.2	0	2,754	247	377	198
6	28.1	2.5	1.1	15.1	11.5	4,949	504	203	80
7	86	2.2	8.9	66.3	8.3	3,183	17	57	32
8	36.7	3	2.7	35.8	5.9	1,840	30	22	17

[a] PCBs = polychlorinated biphenyls; HCH = hexachlorocyclohexane; DDT = dichlorodiphenyltrichloroethane; DDE = dichlorodiphenyldichloroethylene; HCB = hexachlorobenzene; PeCB = pentachlorobenzene; PAH = polycyclic aromatic compounds; PCDD/PCDF = polychlorinated dibenzo-*p*-dioxins and dibenzofurans; TEQ = toxic equivalents of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.
[b] Not assessed.
[c] Not detected.

caused mostly by the persistent chemicals, while a significant part of the TEQ of samples 4 to 7 was caused by nonpersistent chemicals (Table 2). The same set of samples was used for evaluation of retinoid receptor–mediated effects in the P19/A15 cells. The experiments were conducted either with the extracts alone, which did not display any effect (data not shown), or in combination of the extracts with 32 nM ATRA. In this case we observed a significant increase of the luciferase activity with all samples of the contaminated sediments and no effect with the reference sediment sample (Fig. 1). The proretinoid activity of the sediment extract was mediated mainly by the nonpersistent fraction of the samples. The greatest effect was elicited by the raw extract of sample 5, which also exhibited the greatest TEQ as determined by H4IIE-*luc* cells. The sample caused a threefold increase in the effect of 32 nM ATRA alone, and it was comparable to the effect of 10,000 nM ATRA. Nevertheless, there was also significant induction of the ATRA response with sulfuric acid–treated samples that contained greater concentrations of AhR ligand–mediated luciferase activity (samples 2, 3, 4, and 6). However, this activation did not exceed 75% of 32 nM ATRA. No significant effect was found for sample 1 and persistent fractions of samples 7 and 8, which generally had lesser levels of contamination and especially lesser AhR ligand–mediated luciferase activity as well as calculated TEQ (Fig. 1 and Table 2).

Effects of PAHs

To better understand the effects of compounds in the sediment extracts, the action of model representatives of the predominant compounds present in the sediments was assessed. The greatest portion of the activity in most of the samples was mediated by the nonpersistent fraction (Fig. 1) containing significant amounts of PAHs. Thus, the activity of selected representative PAHs was assessed. The results demonstrate that some of the PAHs were able to increase the expression of luciferase when exposed together with 32 nM ATRA (Fig. 2). However, these same PAHs had no effect when exposed alone (data not shown). A concentration range from 185 nM (750 nM in case of fluoranthene) to the greatest noncytotoxic concentration was evaluated. The greatest effect was observed after exposure to 3.1 μM dibenz[*a,h*]anthracene (DB*a,h*A) and 12.5 μM benz[*a*]anthracene (B*a*A) with 3- and 2.5-fold increases of ATRA activity, respectively. Both compounds produced effects comparable to the maximal effect of ATRA (Fig. 2). Benzo[*a*]pyrene (B*a*P) caused nearly a twofold increase of ATRA activity at 25 μM concentration, while fluoranthene did not have any effect up to the same concentration (Fig. 2).

Effects of phthalates

The tested phthalate esters did not display any effects in nondifferentiated P19/A15 cells either with or without 32 nM

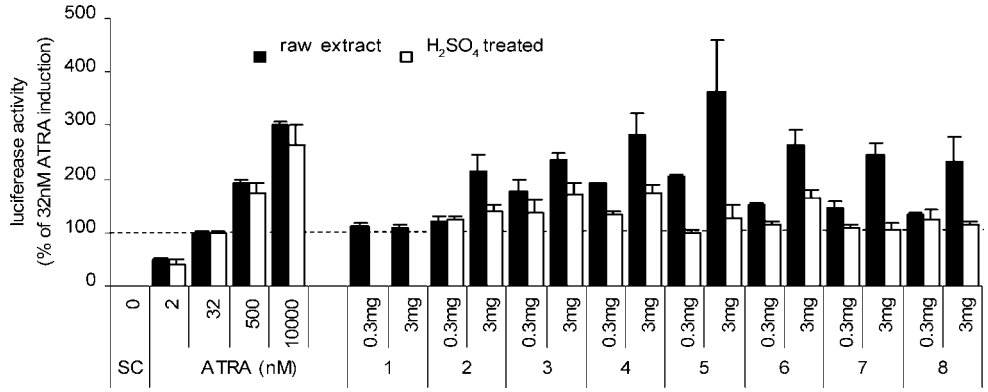


Fig. 1. Modulation of 32 nM all-*trans* retinoic acid (ATRA)–induced luciferase activity by simultaneous treatment with sediment extracts in nondifferentiated P19/A15 cells (expressed in percents of 32 nM ATRA + standard error of means). SC = solvent control; ATRA = calibration of ATRA (nM); 1 = reference sediment extract with 32 nM ATRA; 2–8 = contaminated sediment extracts with 32 nM ATRA (mg of sediment/well).

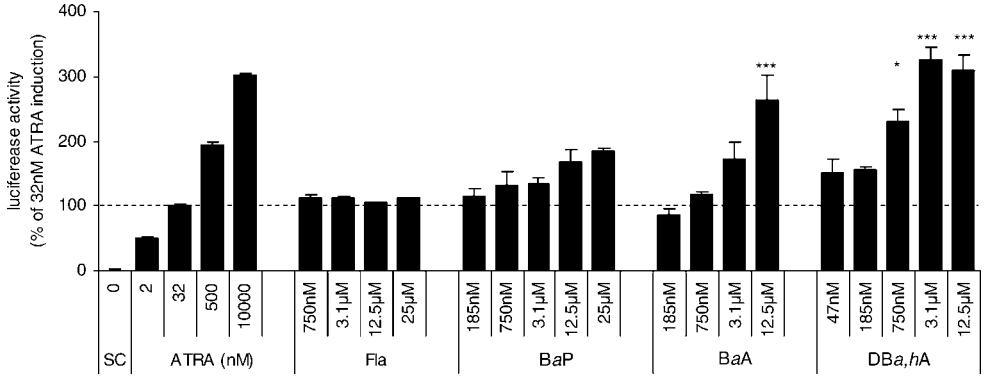


Fig. 2. Modulation of 32 nM all-*trans* retinoic acid (ATRA)–induced luciferase activity by simultaneous treatment with polycyclic aromatic hydrocarbons (PAHs) in nondifferentiated P19/A15 cells (expressed in percents of 32 nM ATRA + standard error of means). SC = solvent control; ATRA = calibration of ATRA; Fla = fluoranthene; B*a*P = benzo[*a*]pyrene; B*a*A = benzo[*a*]anthracene; DB*a,h*A = dibenzo[*a,h*]anthracene.

ATRA (data not shown), but all of them, except bis-decyl phthalate, inhibited ATRA-induced luciferase expression in differentiated cells at the concentration of 5 μM (Table 3). All the experiments were repeated five times, and similar inhibitory effects were consistently observed. The strongest effects were elicited by diethylhexyl phthalate, di-isononyl phthalate, and di-isoheptyl phthalate inhibiting ATRA activity by 30 to 40%, though these effects were not statistically significant and did not occur until concentrations close to cytotoxic levels.

Effects of TCDD

To elucidate the effect of the persistent fraction of the samples we tested the activity of TCDD as the most potent activator of AhR. The activity was evaluated with both nondifferentiated and differentiated P19/A15 cells. The TCDD alone was not able to induce any retinoid activity in either nondifferentiated or differentiated P19/A15 cells (data not shown). The only effect was weak inhibition at 5 nM (about 22%) of the effect of ATRA (32 nM) concentration in the differentiated cells. The dose-dependent inhibitory trend was uniform in six independent experiments, but the effect was not statistically significant.

DISCUSSION

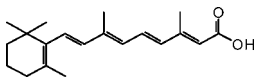
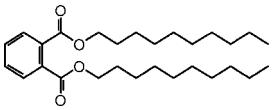
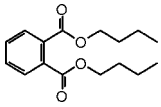
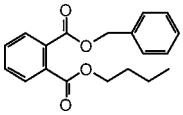
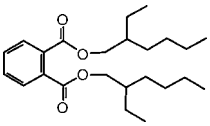
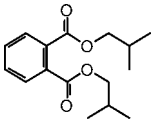
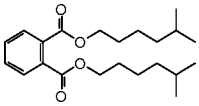
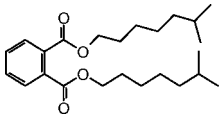
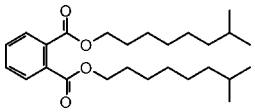
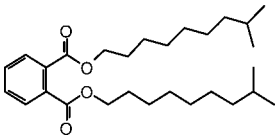
The relationship between the exposure to persistent organic pollutants and changes in retinoid homeostasis has been known for a relatively long time [5]. Modulations of retinoid signaling pathway activity and/or levels of retinoids have been described in animals exposed to contaminated waters or sediments. However, the mode of toxic action of organic compounds on retinoid signaling has not been elucidated yet [7,33]. Here we present a new tool for evaluation of the effects of individual chemicals or mixtures on the retinoid signaling pathway.

The model for assessment of retinoid activity is based on the P19 embryonic carcinoma cell line [22]. The clone P19/A15 prepared by transfection of the P19 cell line with p-RAREβ2-TK-luc plasmid retains the ability of the maternal cell line to differentiate. The differentiation procedure used in our study (exposure to ATRA in medium containing 10% fetal calf serum) was described to induce differentiation of the cells into primitive endoderm [34]. The differentiation was confirmed by a decrease in expression of Oct-4, which is a transcription factor connected with pluripotency of stem cells [31], and by increased expression of primitive endoderm-specific cytokeratin Endo-A (Table 1). The differentiation with ATRA slightly increased the expression of RARα and RXRα, but it led to a small decrease of AhR expression (Table 1). A similar decrease of AhR level caused by ATRA has been described for the adenocarcinoma cell line Caco-2 [35]. The differentiated P19/A15 cells seemed to be more sensitive than the nondifferentiated ones since they exhibited lower EC_{50} for ATRA, and they also responded to the model toxic compounds TCDD and phthalates, which did not have any effect in the undifferentiated cells. However, differentiated cells were also more prone to cytotoxicity, and the results were more variable. Thus, the experiments were performed preferentially with undifferentiated cells. The greater responsiveness of the differentiated cells may be attributed to altered expression of RXRα, RARα, and other components that affect the activity of the retinoid signaling pathway. Moreover, the model compounds could possibly induce CYPs and other drug-metabolizing enzymes in differentiated cells that decreased the level of ATRA. This possibility is supported by the indications from some studies that the pluripotent cells do not express drug-metabolizing enzymes even if they express AhR [36].

The results with retinoid action of the sediment extracts show that while the extracts from polluted sediments did not have any intrinsic retinoid activity, they seem to be able to potentiate the effect of retinoids. On the other hand, the clean reference sediment did not cause any activity by itself or in coexposure with ATRA (Fig. 1). The greatest effect was elicited by raw extract 5, which also had the greatest concentration of TEQs as determined by the H4IIE-*luc* bioassay. This finding suggests that the observed activity could be attributed to the pollutants present in the Kymi River sediments (Table 2), and the effect seems to be related to the amount of cocontaminants in the sample. Our results document that the activation of the retinoid signaling pathway is mediated mainly by nonpersistent compounds; nevertheless, the persistent fraction significantly contributed to the total effect in samples 3, 4, and 6. While extract 5 is the most potent in both AhR and RAR assays, the activity of its sulfuric acid–treated fraction (i.e., sample containing mostly persistent PCDD/Fs and PCBs) is small for the RAR assay but still significant for the AhR assay. Furthermore, raw extracts 2, 3, and 6 elicit similar activity in RAR assay as extracts 7 and 8 (Fig. 1), which contain relatively lesser concentrations of AhR ligands (Table 2). These results suggest that the alteration of ATRA signaling does not seem to be directly mediated by crosstalk with AhR.

Polycyclic aromatic hydrocarbons and their derivatives rep-

Table 3. Structures of all-*trans* retinoic acid (ATRA) and phthalates and inhibition of 32 nM ATRA-induced luciferase activity by 5 μM phthalates in differentiated P19/A15 cells. Inhibition is expressed as percent decrease of the luciferase activity induced by 32 nM ATRA

Compound	Formula	Inhibition (%)
ATRA		
Bis-decyl phthalate		NS[a]
Dibutyl phthalate		25
Benzyl butyl phthalate		20
Diethylhexyl phthalate		40
Di-isobutyl phthalate		20
Di-isoheptyl phthalate		30
Di-isooctyl phthalate		15
Di-isononyl phthalate		30
Di-isodecyl phthalate		15

[a] Not significant.

resent a significant part of the nonpersistent fraction in the extracts. Since individual PAHs were able to enhance the effect of natural ligands of retinoid signaling pathway, it is likely that the PAHs and their derivatives could significantly contribute to the effects caused by the sediment extracts. The potency of the PAHs does not seem to be related to the number of rings in the structure of PAHs because high effects were observed in DB*a,h*A and B*a*A (five and four rings, respectively), moderate effect was elicited by B*a*P (five rings), and no effect was produced by fluoranthene (four rings), which is one of the most abundant PAHs in sediments. This could be an important finding because PAHs and their derivatives are virtually ubiquitous pollutants of the environment. They are traditionally linked with carcinogenesis; moreover, they could mediate other effects, such as antiestrogenicity or effects on steroidogenesis [37], but their effects on retinoid signaling are not known yet.

Phthalates that possess a retinoid-like structure and could be possible ligands able to modulate retinoid signaling are also important nonpersistent environmental contaminants that can be found in waters, sediments, and fish [20]. These compounds were reported to cause several types of toxicity [38]. Our results show that at least some of the tested phthalates are able to inhibit the RAR-mediated response in differentiated P19/A15 cells (Table 3). Although the trends of response were uniform in all experiments, the effects were not statistically significant. Similar findings have been reported in previous studies where phthalates were able to inhibit nuclear localization of RARα and thus decrease its transcriptional activity in mouse Sertoli cell line MSC-1 [14]. It also might be possible that the differentiation leads to the increase of peroxisome proliferator–activated receptors that could be activated by the phthalates and that subsequent increase of CYPs activity would metabolize ATRA and decrease its levels. However, our results could be also attributed to sublethal changes in the cells because the effects were observed at concentrations near to cytotoxic levels. Nevertheless, phthalates do not seem to take part in the effects of the contaminated sediments because they showed the opposite effect.

Since the tested sediments were rich in AhR ligands (Table 2) and AhR presence in P19/A15 cells was confirmed by Western blotting (Table 1), the effect of TCDD on the activation of RAR-mediated response was tested. However, there was no observable effect after either TCDD alone or TCDD/ATRA exposure in undifferentiated cells. This finding agrees with results obtained in malignant human keratinocytes [39]. However, after differentiation of the cells to primitive endoderm, we observed a slight dose-dependent inhibition of luciferase activity by TCDD/ATRA coexposure, but these effects were detectable only at concentrations close to the cytotoxic levels. These results concur with previously reported results where an inhibition of retinoid signaling was observed to be caused by a decrease of ATRA binding to RARα after TCDD treatment in human keratinocytes [19]. Yet it is questionable whether the observed effect was elicited by the specific mechanism described by Lorick et al. [19] or just by nonspecific changes of the cell metabolism induced by sublethal doses of the TCDD since the differentiated cells were more prone to cytotoxicity than the undifferentiated ones. It is also possible that TCDD caused the breakdown of ATRA by induced CYPs, leading to a decrease of observed luciferase activity. The absence of the effect in nondifferentiated cells might be explained by the fact that pluripotent cells do not express drug-metabolizing enzymes even if they possess the AhR receptor [36].

The results reported here do not fully agree with the work of Widerak et al. [12], who described a transactivation of RARE-dependent genes through sequestration of silencing mediator of retinoid and thyroid receptors (SMRT) by activated AhR in MCF-7 breast cancer cells. We do not have any information about rate of expression of SMRT in the P19 cell line, and if it is naturally present in a large excess over AhR, SMRT might preclude the TCDD-mediated pseudoactivation of RARα. The negative result with TCDD exposure suggests

that it is not likely that the effects observed with sediments extracts would be produced just by simple crosstalk with AhR. This finding is confirmed by the significant decrease of the activity of sediment extracts after the sulfuric acid treatment (Fig. 1).

CONCLUSION

A new reporter gene model designed for fast evaluation of disrupting effects of chemicals on retinoid signaling was established, and its functionality was confirmed on complex samples of river sediment extracts and pure chemicals (TCDD, PAHs, and phthalates). The extracts from contaminated sediments did not have any intrinsic retinoid activity, but they strongly potentiated the RAR-mediated response when exposed together with ATRA. A similar effect was observed after the exposure to several PAH representatives. On the other hand, phthalates (substances with retinoid-like structure) and TCDD (AhR ligand) either did not have any effect or slightly down-regulated the effect of ATRA. Thus, it seems that at least part of the complex sample effects could be mediated by PAHs, with a possible contribution from other nonpersistent contaminants coming from the pulp bleaching industry. The results show that the novel in vitro bioassay is suitable for rapid screening and detection of compounds and mixtures disrupting retinoid endocrine regulation.

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REFERENCES

1. Zile MH. 2001. Function of vitamin A in vertebrate embryonic development. *J Nutr* 131:705–708.
2. Gardiner DM, Hoppe DM. 1999. Environmentally induced limb malformations in mink frogs (*Rana septentrionalis*). *J Exp Zool* 284:207–216.
3. Taylor B, Skelly D, Demarchis LK, Slade MD, Galusha D, Rabinowitz PM. 2005. Proximity to pollution sources and risk of amphibian limb malformation. *Environ Health Perspect* 113: 1497–1501.
4. Lemaire G, Balaguer P, Michel S, Rahmani R. 2005. Activation of retinoic acid receptor-dependent transcription by organochlorine pesticides. *Toxicol Appl Pharmacol* 202:38–49.
5. Spear PA, Bilodeau A, Branchaud A. 1992. Retinoids: From metabolism to environmental monitoring. *Chemosphere* 25:1733–1738.
6. Branchaud A, Gendron A, Fortin R, Anderson PD, Spear PA. 1995. Vitamin-a stores, teratogenesis, and EROD activity in white sucker, catostomus-commersoni, from Riviere-Des-Prairies near Montreal and a reference site. *Can J Fish Aquat Sci* 52:1703–1713.
7. Alsop D, Hewitt M, Kohli M, Brown S, Van der Kraak G. 2003. Constituents within pulp mill effluent deplete retinoid stores in white sucker and bind to rainbow trout retinoic acid receptors and retinoid X receptors. *Environ Toxicol Chem* 22:2969–2976.
8. Gardiner D, Ndayibagira A, Grun F, Blumberg B. 2003. Deformed frogs and environmental retinoids. *Pure Appl Chem* 75:2263–2273.
9. Berube VE, Boily MH, DeBlois C, Dassylva N, Spear PA. 2005. Plasma retinoid profile in bullfrogs, *Rana catesbeiana*, in relation to agricultural intensity of sub-watersheds in the Yamaska River drainage basin, Quebec, Canada. *Aquat Toxicol* 71:109–120.
10. Bastien J, Rochette-Egly C. 2004. Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene* 328:1–16.
11. Chambon P. 1996. A decade of molecular biology of retinoic acid receptors. *FASEB J* 10:940–954.
12. Widerak M, Ghoneim C, Dumontier MF, Quesne M, Corvol MT, Savouret JF. 2006. The aryl hydrocarbon receptor activates the retinoic acid receptor[alpha] through SMRT antagonism. *Biochimie* 88:387–397.
13. Palha JA, Goodman AB. 2006. Thyroid hormones and retinoids: A possible link between genes and environment in schizophrenia. *Brain Res Rev* 51:61–71.
14. Dufour JM, Vo MN, Bhattacharya N, Okita J, Okita R, Kim KH. 2003. Peroxisorne proliferators disrupt retinoic acid receptor alpha signaling in the testis. *Biol Reprod* 68:1215–1224.
15. Nilsson CB, Hakansson H. 2002. The retinoid signaling system —A target in dioxin toxicity. *Crit Rev Toxicol* 32:211–232.
16. Janosek J, Hilscherova K, Blaha L, Holoubek I. 2006. Environmental xenobiotics and nuclear receptors—Interactions, effects and in vitro assessment. *Toxicol In Vitro* 20:18–37.
17. Puga A, Tomlinson CR, Xia Y. 2005. Ah receptor signals crosstalk with multiple developmental pathways. *Biochem Pharmacol* 69:199–207.
18. Lind PM, Larsson S, Oxlund H, Hakansson H, Nyberg K, Eklund T, Orberg J. 2000. Change of bone tissue composition and impaired bone strength in rats exposed to 3,3′,4,4′,5-pentachlorobiphenyl (PCB126). *Toxicology* 150:41–51.
19. Lorick KL, Toscano DL, Toscano WA. 1998. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin alters retinoic acid receptor function in human keratinocytes. *Biochem Biophys Res Commun* 243:749–752.
20. Peijnenburg W, Struijs J. 2006. Occurrence of phthalate esters in the environment of the Netherlands. *Ecotoxicol Environ Saf* 63: 204–215.
21. Bhattacharya N, Dufour JM, Vo MN, Okita J, Okita R, Kim KH. 2005. Differential effects of phthalates on the testis and the liver. *Biol Reprod* 72:745–754.
22. van der Heyden MAG, Defize LHK. 2003. Twenty one years of P19 cells: What an embryonal carcinoma cell line taught us about cardiomyocyte differentiation. *Cardiovasc Res* 58:292–302.
23. Rossant J, Mcburney MW. 1982. The development potential of a euploid male teratocarcinoma cell-line after blastocyst injection. *J Embryol Exp Morphol* 70:99–112.
24. Pachernik J, Bryja V, Esner M, Kubala L, Dvorak P, Hampl A. 2005. Neural differentiation of pluripotent mouse embryonal carcinoma cells by retinoic acid: Inhibitory effect of serum. *Physiol Res* 54:115–122.
25. Wang HY, Kanungo J, Malbon CC. 2002. Expression of G alpha 13 (Q226L) induces p19 stem cells to primitive endoderm via MEKK1, 2, or 4. *J Biol Chem* 277:3530–3536.
26. Koistinen J, Paasivirta J, Suonpera M. 1995. Contamination of pike and sediment from the Kymijoki River by PCDES, PCDDS, and PCDFS—Contents and patterns compared to pike and sediment from the Bothnian Bay and seals from Lake Saimaa. *Environ Sci Technol* 29:2541–2547.
27. Isosaari P, Kankaanpaa H, Mattila J, Kiviranta H, Verta M, Salo S, Vartiainen T. 2002. Spatial distribution and temporal accumulation of polychlorinated dibenzo-*p*-dioxins, dihenzofurans, and biphenyls in the Gulf of Finland. *Environ Sci Technol* 36: 2560–2565.
28. Villeneuve DL, Blankenship AL, Giesy JP. 2000. Derivation and application of relative potency estimates based on in vitro bioassay results. *Environ Toxicol Chem* 19:2835–2843.
29. Freyberger A, Schmuck G. 2005. Screening for estrogenicity and anti-estrogenicity: A critical evaluation of an MVLN cell-based transactivation assay. *Toxicol Lett* 155:1–13.
30. Kanungo J, Potapova I, Malbon CC, Wang HY. 2000. MEKK4 mediates differentiation in response to retinoic acid via activation of c-Jun N-terminal kinase in rat embryonal carcinoma P19 cells. *J Biol Chem* 275:24032–24039.
31. Monti M, Garagna S, Redi C, Zuccotti M. 2006. Gonadotropins affect Oct-4 gene expression during mouse oocyte growth. *Mol Reprod Dev* 73:685–691.
32. Van Overmeire I, Clark GC, Brown DJ, Chu MD, Cooke MW, Denison MS, Baeyens W, Srebrnik S, Goeyens L. 2001. Trace contamination with dioxin-like chemicals: Evaluation of bioassay-based TEQ determination for hazard assessment and regulatory responses. *Environmental Science & Policy* 4:345.
33. Boily MH, Berube VE, Spear PA, DeBlois C, Dassylva N. 2005.

Hepatic retinoids of bullfrogs in relation to agricultural pesticides. *Environ Toxicol Chem* 24:1099–1106.
34. Rochette-Egly C, Chambon P. 2001. F9 embryocarcinoma cells: A cell autonomous model to study the functional selectivity of RARs and RXRs in retinoid signaling. *Histol Histopathol* 16: 909–922.
35. Fallone F, Villard PH, Seree E, Rimet O, Nguyen QB, Bourgarel-Rey W, Fouchier F, Barra Y, Durand A, Lacarelle B. 2004. Retinoids repress Ah receptor CYP1A1 induction pathway through the SMRT corepressor. *Biochem Biophys Res Commun* 322:551–556.
36. Trosko JE, Chang CC, Upham BL, Tai MH. 2004. Ignored hallmarks of carcinogenesis: Stem cells and cell-cell communication. *Signal Transduction and Communication in Cancer Cells* 1028: 192–201.
37. Evanson M, Van der Kraak GJ. 2001. Stimulatory effects of selected PAHs on testosterone production in goldfish and rainbow trout and possible mechanisms of action. *Comp Biochem Physiol* 130:249–258.
38. Corton JC, Lapinskas PJ. 2005. Peroxisome proliferator-activated receptors: Mediators of phthalate ester-induced effects in the male reproductive tract? *Toxicol Sci* 83:4–17.
39. Krig SR, Rice RH. 2000. TCDD suppression of tissue transglutaminase stimulation by retinoids in malignant human keratinocytes. *Toxicol Sci* 56:357–364.

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Endocrine effects of contaminated sediments on the freshwater snail *Potamopyrgus antipodarum in vivo* and in the cell bioassays *in vitro*

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ABSTRACT

Lake Pilnok located in the black coal-mining region Ostrava-Karvina, Czech Republic, contains sediments highly contaminated with powdered waste coal. Moreover, population of the endangered species of narrow-clawed crayfish *Pontastacus leptodactylus* with high proportion of intersex individuals (18%) was observed at this site. These findings motivated our work that aimed to evaluate contamination, endocrine disruptive potency using *in vitro* assays and *in vivo* effects of contaminated sediments on reproduction of sediment-dwelling invertebrates. Chemical analyses revealed low concentrations of persistent chlorinated compounds and heavy metals but concentrations of polycyclic aromatic hydrocarbons (PAH) were high (sum of 16 PAHs 10 μg/g dw). Organic extracts from sediments caused significant *in vitro* AhR-mediated activity in the bioassay with H4IIE-luc cells, estrogenicity in MVLN cells and anti-androgenicity in recombinant yeast assay, and these effects could be attributed to non-persistent compounds derived from the waste coal. We have also observed significant *in vivo* effects of the sediments in laboratory experiments with the Prosobranchian euryhaline mud snail *Potamopyrgus antipodarum*. Sediments from Lake Pilnok as well as organic extracts of the sediments (externally added to the control sediment) significantly affected fecundity during 8 weeks of exposure. The effects were stimulations of fecundity at lower concentrations at the beginning of the experiment followed by inhibitions of fecundity and general toxicity. Our study indicates presence of chemicals that affected endocrine balance in invertebrates, and emphasizes the need for integrated approaches combining *in vitro* and *in vivo* bioassays with identification of chemicals to elucidate ecotoxicogical impacts of contaminated sediment samples.

1. Introduction

Despite ongoing efforts of the European Union (EU) to control and ensure adequate surface water quality including its functioning as a habitat for wildlife, numerous freshwater ecosystems (especially in Eastern Europe), remain highly polluted. In particular, sediments are sinks/sources of contaminants such as heavy metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs), or organochlorine pesticides (OCPs) (Wirth et al., 1998; Hilscherová et al., 2001, 2002). Governments worldwide (including the European Commission via the European Water Framework Directive and others; FDEP, 2003) intend to set concentration limits or sediment quality criteria (SQC) for priority contaminants. These criteria, however, cover mostly "old" (traditional) persistent chemicals, whereas "modern" substances like hormones, pharmaceuticals, or personal care products (or various derivatives) are rarely included. Since these substances are known to occur in very low concentrations in the environment, they cannot easily be detected by routine analytical methods. Nevertheless, a number of studies have described the potential effects of these substances on aquatic organisms (Hallare et al., 2005; Verslycke et al., 2007).

The present study investigates reproduction-related endocrine disruptive effects of sediments from the contaminated Lake Pilnok, which is situated in the black coal-mining region of Ostrava-Karvina in the Czech Republic. Lake Pilnok is an artificial pond, which

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originated as flooded ground depression that has been used as a dumping site for powdered waste coal since the middle of the 20th century. In spite of intensive black coal-mining activities, basic parameters of water quality (oxygen content and transparency) supplied by underground springs remained stable, and the narrow-clawed crayfish *Pontastacus* (syn. *Astacus*) *leptodactylus* (Decapoda, Crustacea) Eschscholtz, 1823 lives in many reservoirs spread over the Ostrava-Karvina region. However, an abnormal population of this endangered species has been observed only in the Lake Pilnok with about 18% female-like individuals that possessed both female and male sexual characteristics (Ďuriš et al., unpublished results). Similar observations (abnormalities in external sexual characteristics or histologically determined ootestis) were previously described in crustaceans such as gammarids (Dunn et al., 1994; Ladewig et al., 2002) or decapods (Rudolph, 1999; Kozák et al., 2007) with proportion of intersex individuals about 10%. This abnormality was termed intersex in crustaceans and it was related to partial hermafroditism and plasticity of phenotypical sex determination or other factors such as parasitic infestation or environmental contamination (Medley and Rouse, 1993; Rudolph, 1999; Ford, 2008). The coincidence of the intersex and the waste coal powder suggested the presence of unknown compounds that might be causing endocrine disruption in this crayfish species.

The assessment of toxicity of sediments generally requires application of at least a single suitable biological test (biotest) along with chemical analyses. For instance, the combined TRIAD approach (chemical analyses of known compounds, whole-sediment toxicity testing and evaluation of benthic biodiversity) has been discussed and used (Chapman and Hollert, 2006; Sørensen et al., 2007). Thus, testing of *in vivo* effects plays a key role in sediment toxicity evaluation and several model organisms have been used to assess various groups of contaminants (Jobling et al., 2003; De Lange et al., 2005).

Some *in vivo* toxicity models have been shown to be particularly suitable for studying reproductive and developmental effects (Duft et al., 2007; Kusk and Wollenberger, 2007; Verslycke et al., 2007). Prosobranchian snails are sensitive organisms for the detection of (xeno-)hormones (Jobling et al., 2003), and bioassays with the euryhaline mud snail (*P. antipodarum* Gray, 1843) have been successfully used to study sediment toxicity (Duft et al., 2003; Oetken et al., 2005). The major advantages of this species are the continuous fertility of parthenogenic females, few maintenance requirements and relatively great sensitivity to compounds that may affect reproduction (Oetken et al., 2005).

The objectives of the study were: (1) to determine whether sediments of Lake Pilnok contain chemicals with endocrine disruptive potential and (2) to evaluate if the sediments can affect model invertebrate *P. antipodarum in vivo*. The approach used in this study combined chemical analyses (heavy metals and major organic contaminants), *in vitro* bioassays with cell lines (to study arylhydrocarbon (AhR), estrogen (ER) and androgen (AR) receptor-mediated effects) as well as *in vivo* experiments with *P. antipodarum* to assess mortality and reproduction in this sediment-dwelling animal. The comparison of effects observed in exposures to natural sediment versus control sediment spiked with its organic extract enabled evaluation of the importance of the extracted organic pollutants and their availability for the studied endpoints.

2. Methods

2.1. Experimental design

Samples of sediments were collected from a "contaminated" (Lake Pilnok) and "reference/control" site (Steinlach creek near Talheim, situated in a protected nature reserve, state of Baden-Württemberg, Germany). Extracts of sediments were analysed for (1) concentrations of chemical pollutants (metals, PAHs, PCBs, OCPs) and (2) the presence of compounds interfering with AhR, ER and AR using *in vitro* bioassays. Furthermore, *in vivo* effects of sediments on *P. antipodarum* snails were studied in two experimental settings: (1) whole-sediment toxicity assays with control sediment, contaminated Lake Pilnok sediment and two mixtures of both sediments, comprising 50% and 75% Lake Pilnok sediment, respectively and (2) toxicity assays using control sediment which was spiked with different volumes of organic extract from Lake Pilnok sediment (three doses equivalent to 50%, 75% and 100% of original Lake Pilnok sediment). All *in vivo* experiments were performed with 120 individuals for every exposure group (divided into two replicates of 60 animals each).

2.2. Sediment sampling and preparation of sediment organic extracts

Sediments of Lake Pilnok and Steinlach creek were collected from three places at each location, mixed, transported into the lab and prepared for use in studies. Sediments were stored frozen at −20 °C until further processing for analyses and experiments. A mass of 1.5 kg (fresh weight) of sediment from Lake Pilnok was extracted for 12 h with dichloromethane in a Soxhlet apparatus. Thawed sediment was ground with anhydrous sodium sulphate until it reached a paste-like consistency; the lump was placed in Soxhlet cartridges and extracted. The extract containing extractable organic fraction was concentrated by rotary evaporation and divided into two portions. The solvent of the first portion was changed to acetone. Acetone extract was used for *in vivo* experiments (fast evaporation after dosing). The second portion of the extract was transferred to dimethylsulfoxide, the carrier used during *in vitro* experiments with cells.

2.3. Analyses of organic contaminants

A portion of the organic extract was used for chemical analyses of 16 PAHs, 7 indicator PCBs and OCPs (hexachlorocyclobenzene, 4 HCH stereoisomers, 2 congeners of each DDE, DDD and DDT). Activated copper was used to remove sulphur from the extract prior to analyses. Fractionation was achieved on silica gel columns; a sulphuric acid modified silica gel column was used for PCB/OCP samples. Samples were analysed using GC–ECD (HP 5890) equipped with a Quadrex fused silica column 5% Phe for PCBs and OCPs. The 16 US EPA polycyclic aromatic hydrocarbons were determined in all samples using a GC–MS instrument (HP 6890–HP 5973) equipped with a J&W Scientific fused silica column DB-5MS. Samples were quantified using Pesticide Mix 13 (Dr. Ehrenstorfer, Augsburg, Germany) and PAH Mix 27 (LCG Promochem, Teddington, UK) standard mixtures. To assure quality of analyses, laboratory blanks and certified reference material BCR-536 were analysed in parallel, and surrogate recovery standards were used (D10-phenanthrene and D12-perylene for PAH analyses; *para*-terphenyl and PCB 121 for PCB/OCP analyses). Recoveries were 55% and 68% for PAHs analyses in control and Lake Pilnok sediment: 68% and 94% for PCBs in control and Lake Pilnok sediment. Blanks run in parallel always contained less than 1% of the concentrations determined in the studied samples.

2.4. Analyses of heavy metals

Concentrations of heavy metals (vanadium: V, chromium: Cr, cobalt: Co, nickel: Ni, copper: Cu, zinc: Zn, arsenic: As, cadmium: Cd, lead: Pb and mercury: Hg) in sediment samples were analysed according to ISO 11466, method adapted to analytical instrumen-

tation. Dry sediment (1 g dw) was leached with 2.3 ml HNO_3 and 7 ml HCl overnight followed by heating under reflux for 2 h, and after cooling the mixture was diluted for analyses using inductively coupled plasma-mass spectrometry (ICP-MS Agilent 7500ce, Agilent Technologies, Japan). Elements (isotopes) suffering from polyatomic interferences were measured in He collision mode using Octopole Reaction System. Ions of Ge, In and Bi were used as internal standards, methodology was verified by analyses of soil certified reference materials (ANA 7001–7004). Total content of mercury was determined by thermooxidation method using AMA-254 analyzer (Altec, Czech Republic).

2.5. In vitro assays

The potential of the sediment extracts to induce AhR-mediated (dioxin-like) effects were determined with the H4IIE.luc bioassay. The ER-mediated activity of the sediment extracts was evaluated using a bioassay with the MVLN cell line. Methodological details for both luciferase reporter gene-based assays have been described previously (Hilscherová et al., 2001, 2002). In brief, cells were seeded in 96-well culture ViewPlates™ (Packard, Meriden, CT, USA) and exposed to dilutions of sediment extracts for 24 h in three replicates. The activity of AhR- or ER-induced luciferase was quantified using Promega Steady Glo Kit (Promega, Mannheim, Germany). After the initial range-finding experiments, full concentration–response curves for induction of AhR- and ER-mediated responses were generated. Besides the effects of crude extract to induce AhR-mediated effects in the H4IIE.luc assay, responses were also determined for extracts that had been treated with sulphuric acid to remove labile compounds such as PAHs. The effects of sediment extracts were related to the luciferase induction by the reference compounds: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for AhR-mediated effects, 17β-estradiol (E2) for ER-mediated effects using methods described previously (Villeneuve et al., 2000).

The potency of sediment extracts to modulate AR-mediated responses was examined by a yeast reporter assay comprising a recombinant yeast cell line (Leskinen et al., 2005). Yeast seeded in 96-well culture ViewPlates™ (Packard, Meriden, CT, USA) were exposed to dilutions of sediment extracts in three replicates for 3 h. The effects of sediment extracts were assessed in comparison with the reference compound testosterone as AR-mediated luciferase activity. The anti-androgenicity (competitive activity) of sediment extract was measured as the decrease of AR-mediated luciferase activity in exposures using extracts supplemented with 10^{-8} M testosterone.

2.6. In vivo assays

The sediment biotest using parthenogenic females of the prosobranchian snail *P. antipodarum* was originally developed by Duft et al. (2003) and our studies followed these guidelines. The exposures were performed in 1.5 L glass aquaria using the thawed wet sediment to cover the ground (equivalents of 60 g dry weight, (dw), per aquarium). The aqueous medium (800 mL per aquarium) was a mixture of stream water from Steinlach creek and tap water at a ratio of 1:1. To establish equilibrium between sediment and water phase, sediment/water systems were set up 7 days prior to commencing experiments. A volume of 500 mL water was renewed weekly. The exposure (8 week) was performed under constant conditions at a temperature of $15.6 \pm 0.14\,^{\circ}C$ and a light:dark cycle of 14:10 h.

The exposure was performed with (1) natural sediments in quantitatively different mixtures and (2) control sediment spiked with different volumes of the organic extract from the Lake Pilnok sediment. The experiments with the natural sediments contained exposure of snails to Lake Pilnok sediment ("100% Lake Pilnok" = 60 g dw Lake Pilnok sediment), and to its mixtures with control sediment ("75% Lake Pilnok" = 45 g dw Lake Pilnok sediment + 15 g dw control Steinlach sediment; "50% Lake Pilnok" = 30 g dw Lake Pilnok sediment + 30 g dw control sediment). 60 g (dw) of Steinlach sediment served as control. The experiments with the organic extract used 60 g (dw) of Steinlach sediment to which different volumes of the extract prepared from the Lake Pilnok sediment was added. The sediment extract was dosed as 3 mL acetone solution in concentrations that corresponded to "50% Lake Pilnok" (total extract of 30 g dw Lake Pilnok sediment), "75% Lake Pilnok" (total extract of 45 g dw Lake Pilnok sediment), or "100% Lake Pilnok" (total extract of 60 g dw Lake Pilnok sediment). The control sediment spiked with 3 mL of acetone was used as the solvent control. After sediment spiking, the solvent was let to evaporate for 3 days in the dark before the aqueous medium was added to exposure systems.

All variants (control + 3 variants with whole sediment, solvent control + 3 variants with sediment extract) were performed in duplicate aquaria containing 60 *P. antipodarum* (parthenogenic females) per aquarium. Twenty animals were sampled from each aquarium at the end of the second week (after 14 days of exposure) and the 5th week (40 days exposure); all other surviving animals were examined at the end of the experiment (8 week exposure). Females were dissected and the embryos held in the brood pouch of each individual were counted under a stereomicroscope. Embryos were classified as either "early embryos" (without developed shell) or "further developed embryos" (after formation of a shell).

2.7. Data analysis

EC_{50} values (derived from H4IIE.luc, MVLN bioassays) were estimated using least-squares regression of the log-linear part of the full concentration–response curves. The assumptions of parallelism and equal efficacy of the unknown and standard curves were assessed by use of the method of comparing estimates of the EC20 and EC80 according to Villeneuve et al. (2000). TCDD equivalents (TEQ_{bio}) were calculated using the effect-equivalency approach by comparing the EC_{50} value of the TCDD standard calibration with the concentration of tested sample inducing the same bioassay response as the EC_{50} of TCDD (Hilscherová et al., 2000). Similarly, the estrogenicity was quantified as 17β-estradiol equivalents (E2-equivalents EEQs) by comparing the EC_{50} value of the E2 standard calibration with the concentration of tested sample inducing the same bioassay response as the EC_{50} of E2. Dioxin-equivalents (derived from the chemical analyses; TEQ_{chem}) were calculated from individual PAHs concentration and their relative potencies (REPs) calculated from H4IIE.luc bioassay according to Machala et al. (2001). Anti-androgenic effects of sediment extracts on the testosterone-induced luciferase were evaluated by analysis of variance (ANOVA) followed by Dunnet's test. *In vivo* experiments with *P. antipodarum* (all treatments) were performed in two duplicate aquaria. Differences between control aquaria and exposure variants were evaluated with the non-parametric Mann–Whitney *U* test. The threshold for significance of all statistical assays was set to $p < 0.05$.

3. Results

3.1. Chemical concentrations

Concentrations of both organic and inorganic chemicals were low in sediment from Steinlach creek while they were much higher

Table 1
Contamination of sediments by polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and metals

	Control (ng/g dw)	Lake Pilnok (ng/g dw)	FDEP-A/PEC[a] (ng/g dw)	REPs[b]
PAHs—sum of 16 PAHs	422	10,122	23,000	
Naphthalene	11	1071	560	n.a.
Acenaphthylene	5	40	130	n.a.
Acenaphthene	2	195	89	n.a.
Fluorene	6	1281	540	n.a.
Phenanthrene	57	3782	1200	n.a.
Anthracene	17	68	850	n.a.
Fluoranthene	101	356	2200	2.27×10^{-8}
Pyrene	72	594	1500	1.78×10^{-6}
Benzo[*a*]anthracene	37	434	1100	7.04×10^{-6}
Chrysene	37	900	1300	1.01×10^{-4}
Benzo[*b*]fluoranthene	23	541	n.a.	3.35×10^{-5}
Benzo[*k*]fluoranthene	12	58	n.a.	1.64×10^{-3}
Benzo[*a*]pyrene	20	278	1500	9.01×10^{-5}
Indeno[1,2,3-*cd*]pyrene	11	106	n.a.	2.96×10^{-4}
Dibenzo[*a*,*h*]anthracene	2	60	140	1.17×10^{-3}
Benzo[*g*,*h*,*i*]perylene	10	358	n.a.	6.19×10^{-6}
Sum of 7 PCBs[c]	0.86	4.30		
Sum of 8 OCPs[d]	0.52	2.94		
	(ng TCDD/g dw)	(ng TCDD/g dw)		
TEQ_{chem}[e]	0.032	0.338		
TEQ_{bio}[f]	2.4	70		
Metals	Control (μg/g dw)	Lake Pilnok (μg/g dw)	FDEP-TEC (μg/g dw)	
V	9.47	25.03	n.a.	
Cr	5.78	22.28	110	
Co	1.26	7.61	n.a.	
Ni	6.14	20.14	49	
Cu	2.94	27.76	150	
Zn	21.53	38.90	460	
As	1.38	3.37	33	
Cd	0.25	0.14	5	
Pb	3.46	45.85	130	
Hg	<0.01	0.06	1.1	

n.a.: data not available.

[a] Guideline values recommended by the Florida Department of Environmental Protection (FDEP, 2003) for the protection of sediment-dwelling organisms (anticipated/probable effect concentrations, A/PEC, for PAHs; threshold effects concentrations, TEC, for metals).

[b] Relative potencies (REPs) to induce AhR-mediated effects *in vitro* by PAHs (Machala et al., 2001).

[c] The sum of PCBs—seven indicator compounds: PCB 28, PCB 52, PCB 101, PCB 118, PCB 153, PCB 138, PCB 180.

[d] The sum of OCPs—hexachlorocyclobenzene, four hexachlorocyclohexane stereoisomers (α-HCH, β-HCH, γ-HCH, δ-HCH), *p*,*p*′- and *o*,*p*′-congeners of DDE, DDD and DDT.

[e] Calculated toxic equivalents (TEQ_{chem}).

[f] Toxic equivalents derived from the H4IIE.luc bioassay (TEQ_{bio}).

in sediment from Lake Pilnok (Table 1). The sum of analysed PAHs in Steinlach sediment was 422 ng ∑PAH/g dw, which is equivalent to 0.032 ng TEQ_{chem}/g dw. The concentration of ∑PAH in the Lake Pilnok was 1.0×10^4 ng/g dw, TEQ_{chem} = 0.34 ng TEQ_{chem}/g dw (Table 1). Concentrations of PCBs and OCPs in Lake Pilnok were about fivefold higher than in the control sediment. Also the concentrations of heavy metals were significantly higher in the Lake Pilnok sediment (e.g. 10-fold higher copper, lead, cobalt and molybdenum in comparison to the control).

3.2. *In vitro assays*

Concentrations of TEQ_{bio} were relatively low in Steinlach sediment (2.4 ng TEQ_{bio}/g dw) while concentrations of TEQ_{bio} were higher in sediments from Lake Pilnok (approximately 70 ng TEQ_{bio}/g dw sediment; Fig. 1A, Table 1). TEQ_{bio} were not detected in extracts treated with sulphuric acid to remove labile compounds, such as PAHs. This indicates that there was little contribution of persistent compounds such as PCBs and/or polychlorinated dibenzo-*p*-dioxins or dibenzofurans (PCDD/DF) to the observed dioxin-like activities.

While no significant (anti-)estrogenicity or (anti-)androgenicity were observed in sediments from Steinlach (Fig. 1B and C), there was significant activity in sediments from Lake Pilnok. Lake Pilnok sediment has elicited estrogenic potency approximately 4.5 ng EEQ/g dw (Fig. 1B). These extracts also displayed significant anti-androgenic effects (significant and dose-dependent inhibition of testosterone-induced AR-activation; Fig. 1C).

3.3. *In vivo test with P. antipodarum*

Mortality of *P. antipodarum* during the 8-week exposure was low. Six out of 60 individuals died in one of the control aquaria (control sediments and solvent controls), while no mortality was observed in the second replicate. Lake Pilnok sediment caused mortalities in the range of 1–13% of exposed animals (i.e. maximum 7 dead individuals out of 60). Steinlach sediment spiked with the extract from Lake Pilnok caused 0–18% mortality (i.e. maximum 11 dead individuals out of 60). The differences in mortalities between contaminated and control sediments were not statistically significant.

Fecundity of snails varied among treatments. The number of "early embryos" (with undeveloped, shell) was significantly increased after 2-week exposure to 50% Lake Pilnok sediment (Mann–Whitney *U* test, $p < 0.05$; Fig. 2A) followed by an apparent inhibition of reproduction at all concentrations at the end

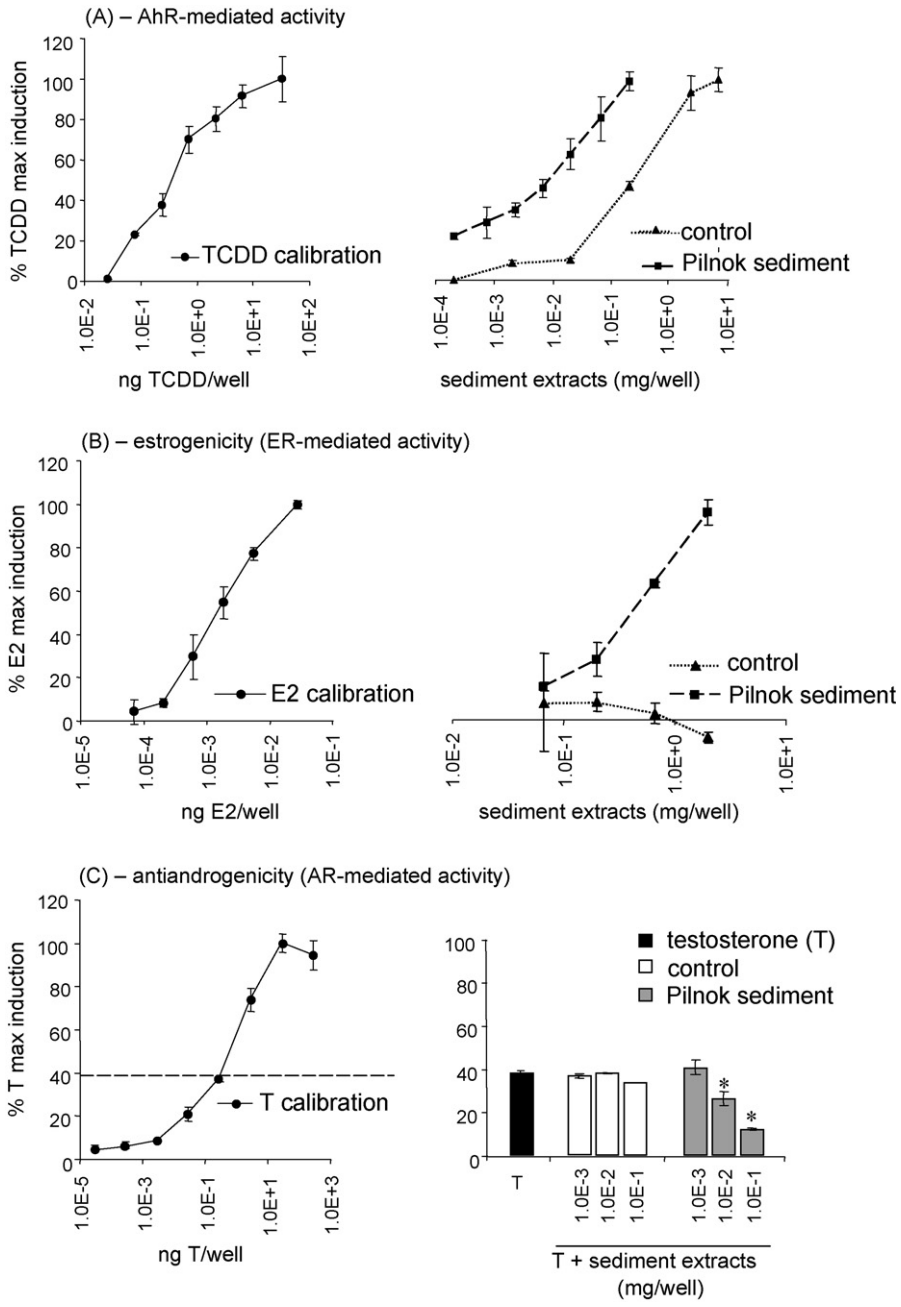

Fig. 1. Concentration–response curves of reference chemicals and sediment extracts. (A) Arylhydrocarbon receptor (AhR-) dependent luciferase activity in the H4IIE.luc cell bioassay; (B) estrogen receptor (ER-) dependent luciferase activity in the MVLN cell bioassay and (C) androgen receptor (AR-) dependent luciferase in recombinant yeast bioassay. Reference compounds were 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); 17β-estradiol (E2); testosterone (T). Graphs display means and standard deviation from three replicates. *Statistically significant anti-androgenicity of the Lake Pilnok sediment extract (suppression of the effect induced by 10^{-8} M testosterone; ANOVA followed by Dunnet's post-test; $p < 0.05$).

of exposure (8 week) ($p < 0.05$; Fig. 2A). More "further developed" (later stage with shell) embryos were observed at 5 week than 2 week of exposures to 50% and 75% Lake Pilnok, but prolonged (8 week) exposures suppressed numbers of embryos as well (Fig. 2B). Steinlach sediment spiked with organic extract from the Lake Pilnok sediment caused apparent reductions in the production of early embryos, and the effects were present both in the beginning of exposure (2 week) and at the end (8 week; 50%, 75% and 100% Lake Pilnok; $p < 0.05$; Fig. 3); numbers of "further developed" embryos were not affected (Fig. 3B).

4. Discussion

In our study, chemical and ecotoxicological investigations were combined to investigate contaminated sediments from the Lake Pilnok. Chemical analyses revealed high concentrations of PAHs. Concentrations of indicator PCBs and selected OCPs were about five times higher than in a control but contamination by these persistent organochlorine compounds seems to be generally lower than other polluted sites in the Czech Republic (Eljarrat et al., 2001; Hilscherová et al., 2001). Also concentrations of heavy metals (with the exception of cadmium) were higher in Lake Pilnok than

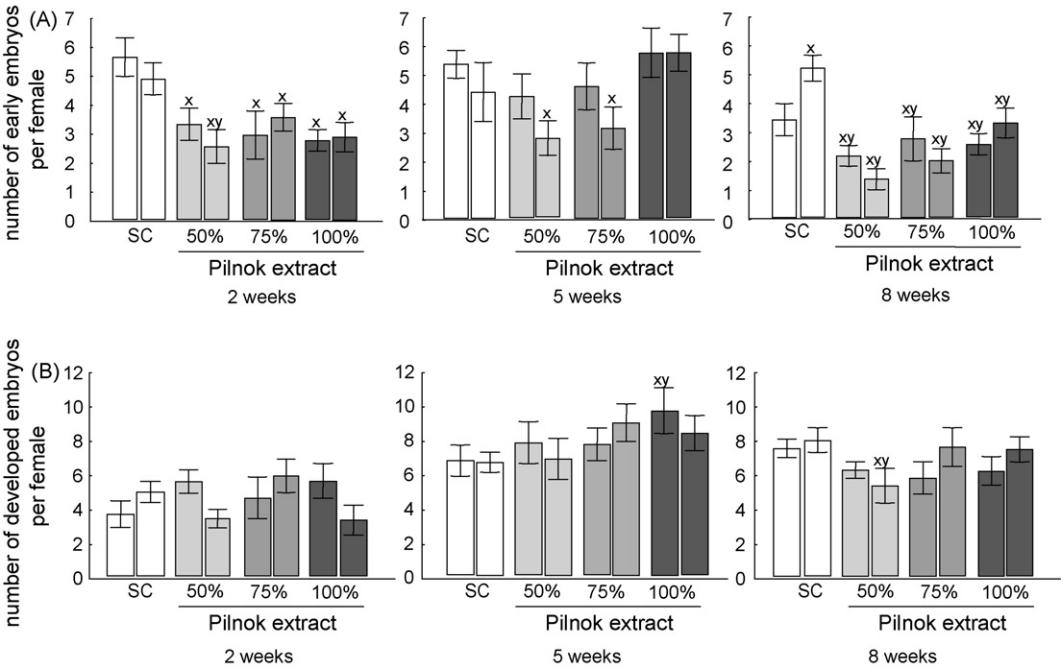

Fig. 2. Effects of Steinlach sediment spiked with the organic extract prepared from Lake Pilnok sediment. Number of (A) early embryos (i.e. embryos without developed shell) and (B) further developed embryos (with shell) in the brood pouch of female *Potamopyrgus antipodarum*; data given as numbers per individual. SC: solvent control (control Steinlach sediment with added and evaporated acetone); '50% Lake Pilnok', '75% Lake Pilnok' and '100% Lake Pilnok': three doses of the organic extract prepared from Lake Pilnok sediment, see Section 2). All variants were performed in duplicates—each column represents results from individual aquarium (average number of embryos per female; 20 females investigated; error bars: standard error of mean). Statistically significant differences from controls are marked with letters (*x*, *y*: significant difference from the first (*x*) and the second (*y*) control aquarium; Mann–Whitney *U* test; $p < 0.05$).

in Steinlach Creek but in comparison to other polluted sites they indicate minimally to moderately impaired sediments (Wiesner et al., 2001; Negri et al., 2006). The concentrations of metals were less than the threshold effect concentrations (TEC) proposed by FDEP (2003) indicating negligible effects on sediment organisms.

Concentrations of $\sum$PAH in Lake Pilnok were approximately 20-fold higher than in reference sediment from Steinlach Creek, and

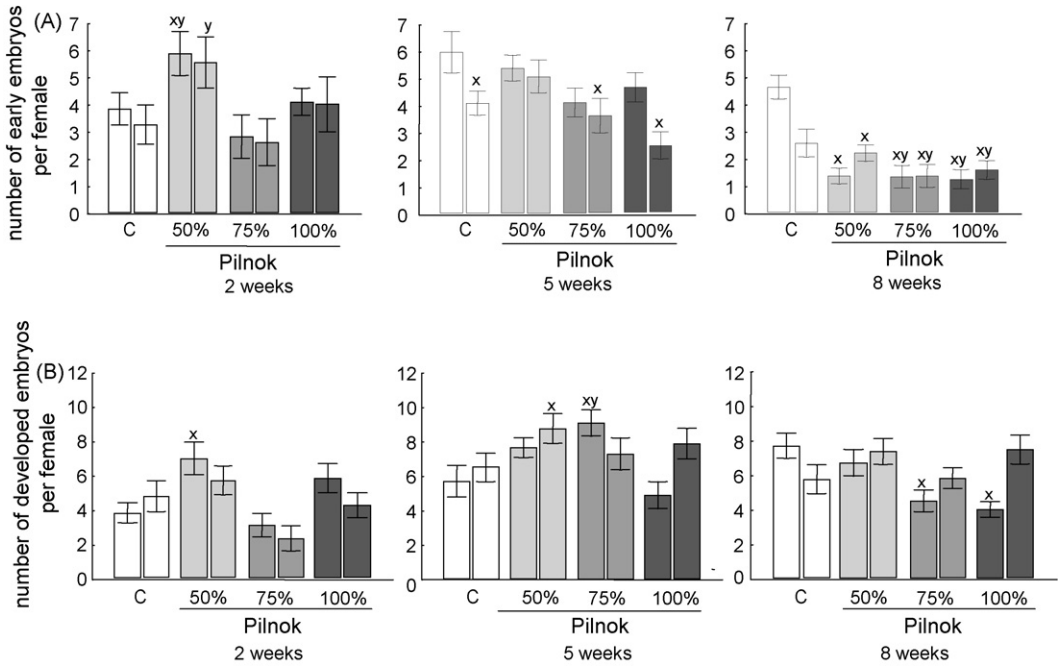

Fig. 3. Effects of the sediment exposures on the number of (A) early embryos (i.e. embryos without developed shell) and (B) further developed embryos (with shell) in the brood pouch of female *P. antipodarum*; data given as numbers per individual. (C) control sediment, '100% Lake Pilnok': untreated contaminated sediment; '50% Lake Pilnok' and '75% Lake Pilnok': appropriate mixtures of Lake Pilnok and control sediments. All variants were performed in duplicates—each column represents results from individual aquarium (average number of embryos per female; 20 females investigated; error bars: standard error of mean). Statistically significant differences from controls are marked with letters (*x*, *y*: significant difference from the first (*x*) and the second (*y*) control aquarium; Mann–Whitney *U* test; $p < 0.05$).

they were comparable to other contaminated sediments from the Czech Republic (Hilscherová et al., 2001) or worldwide (Giacalone et al., 2004). The PAHs in the two studied sediments, were, based upon the ratios of phenanthrene/anthracene and fluoranthene/pyrene, derived from different sources (Sanders et al., 2002). In sediments from Steinlach Creek, the ratios Phe/Ant = 3.4 and Flu/Pyr = 1.4 suggest a pyrogenic origin which may be preferentially derived from the traffic and farming activities in the vicinity, while the ratios Phe/Ant = 55.6 and Flu/Pyr = 0.6 confirmed the petrogenic source of PAHs in Lake Pilnok sediment. These conclusions are consistent with the historical use of Lake Pilnok for the storage of black coal waste.

Concentrations of PAHs measured in Lake Pilnok sediment (with the exception of anthracene and fluoranthene) were higher than the TEC used by FDEP (2003), and the concentrations of specific compounds (naphthalene, acenaphthene, fluorene and phenanthrene) were higher than probable effect limits (PEC) of the FDEP (2003). These findings represent one line of evidence that contamination of Lake Pilnok sediments with PAH may potentially cause adverse effects *in situ*.

The concentration of 70 ng TEQ_{bio}/g dw in extracts of Lake Pilnok sediment was relatively high, compared with sediments with similar concentrations of PAHs (Hilscherová et al., 2001) where concentrations ranged from 1.9 to 23 TEQ_{bio}/g dw. Furthermore, the concentration of TEQ_{bio} measured in the H4IIE.luc bioassay was approximately 200-fold higher than the concentration of TEQ_{chem} calculated from the concentrations of individual PAHs and their respective REP values. This result suggests that Lake Pilnok sediment contains more AhR-active compounds than can be accounted for by PAHs determined by instrumental analyses. However, it is unlikely that PCDD/Fs were responsible for this activity because treatment of the extract with sulphuric acid (which would remove only labile compounds and not PCDD/F), completely removed AhR-mediated activity. One explanation is the possible presence of AhR-active compounds originating from the deposited powdered waste coal that were not quantified in the PAHs studied here. A substantial part of coal is comprised of solid matter called maceral (ASTM, 1979), which is rich in organic compounds such as aliphatic hydrocarbons, cycloalkanes and also polyaromatics including miscellaneous substituted compounds (Schacht et al., 1999). Other studies (Orem et al., 1999; Frouz et al., 2005) have demonstrated that solid fossil organic substrates contain a bio-accessible fraction of organic compounds such as polyphenols. All these organic compounds (which are not analysed in routine chemical screenings) could contribute to unusually high AhR-mediated activity observed in our study, and they should be further explored as they may potentially harm living organisms *in vivo*.

Extracts from Lake Pilnok sediment were also estrogenic and anti-androgenic *in vitro*. PAHs could also play some role in these effects since previous studies have shown potential of some compounds (such as benzo[*a*]anthracene and dibenzo[*a*,*h*]anthracene) to activate ER and also act as anti-androgens (Vinggaard et al., 2000; Villeneuve et al., 2002). Furthermore, there is also evidence that anti-androgenic effects could be, at least in part, caused indirectly by high activation of AhR as shown for PAHs (Kizu et al., 2003). Similar to AhR-mediated effects, estrogenicity (EEQ) in Lake Pilnok extract was higher than previously reported for sediments with comparable concentrations of PAHs (Hilscherová et al., 2002). These results, taken together indicate the presence of organic compounds with estrogenic and anti-androgenic potential (derived most probably from the coal) that may display remarkable effects on aquatic organisms, possibly by means of affecting hormonally regulated processes.

The results of the *in vivo* studies of *P. antipodarum* were variable, even among individuals within the same exposure aquarium, including controls. However, some general trends were observed. For example, the number of "early embryos" was a more sensitive indicator of developmental toxicity than was the number of "further developed" (shelled embryos). This is similar to the results of previous studies (Duft et al., 2003). The early development of embryos in the brood pouch seems to reflect actual effects of those toxicants that immediately affect the general health condition and the reproductive status of the females.

The temporal profiles of effects exerted by the whole-sediment exposures (initial stimulations in the number of embryos followed by the decrease at higher doses or longer exposures) seem to correspond to the general character of the dose-response curve in the model species. Such effects have been observed in aquatic organisms exposed to 17β-estradiol, and complex estrogenic mixtures such as wastewater effluents (Jobling et al., 2003). The initial increase in fecundity was later suppressed by possible re-allocation of energy for processes involved in detoxifying xenobiotics or synthesizing functional or structural proteins damaged by toxicity. General inhibitory effects on fecundity were much stronger pronounced in animals exposed to sediments with external organic extracts. This may be most probably explained by a considerably higher fraction of readily bioavailable toxicants (rapidly released from the extract after spiking) in comparison with exposures to the natural 'undisturbed' contaminated sediment. Our *in vivo* experiments with *P. antipodarum* thus seem to suggest the presence of organic compounds that may stimulate fecundity, but such effects may be later masked by the toxicity of the complex contaminant mixture in the sediment.

5. Conclusions

Overall, our study provides evidence that organic sediment contaminants from the Lake Pilnok may affect reproductive ability of invertebrates. The results demonstrate that non-persistent organic compounds which originated from powdered waste coal but are not analysed by routine chemical analyses exert AhR-mediated activity, estrogenicity and anti-androgenicity *in vitro*, and that they also affect *in vivo* reproduction of a model invertebrate *P. antipodarum.* Our study emphasizes the need of integrated approaches including *in vitro* and *in vivo* toxicological studies along with detailed chemical analyses for the evaluation of complex contaminated environmental samples.

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References

ASTM, 1979. Annual Book of Standards. Part 26. Gaseous fuels; Coal and Coke; Atmospheric Analysis. American Society for Testing and Materials, Philadelphia, pp. 938.

Chapman, P.M., Hollert, H., 2006. Should the sediment quality triad become a tetrad, a pentad, or possibly even a hexad? J. Soils Sediment. 6, 4–8.

De Lange, H.J., De Haas, E.M., Maas, H., Peeters, E.T.H.M., 2005. Contaminated sediments and bioassay responses of three macroinvertebrates, the midge larva

Chironomus riparius, the water louse *Asellus aquaticus* and the mayfly nymph *Ephoron virgo*. Chemosphere 61, 1700–1709.
Duft, M., Schulte-Oehlmann, U., Weltje, L., Tillmann, M., Oehlmann, J., 2003. Stimulated embryo production as a parameter of estrogenic exposure via sediments in the freshwater mudsnail *Potamopyrgus antipodarum*. Aquat. Toxicol. 64, 437–449.
Duft, M., Schmitt, C., Bachmann, J., Brandelik, C., Schulte-Oehlmann, U., Oehlmann, J., 2007. Prosobranch snails as test organisms for the assessment of endocrine active chemicals—an overview and a guideline proposal for a reproduction test with the freshwater mudsnail *Potamopyrgus antipodarum*. Ecotoxicology 16, 169–182.
Dunn, A.M., Adams, J., Smith, J.E., 1994. Intersexuality in the crustacean *Gammarus duebeni*. Invert. Reprod. Dev. 25, 139–142.
Eljarrat, E., Caixach, J., Rivera, J., De Torres, M., Ginebreda, A., 2001. Toxic potency assessment of non- and mono-ortho PCBs, PCDDs, PCDFs, and PAHs in northwest Mediterranean sediments (Catalonia, Spain). Environ. Sci. Technol. 35, 3589–3594.
FDEP, 2003. Development and evaluation of numerical sediment quality assessment guidelines for Florida inland waters. Florida Department of Environmental Protection, Tallahassee, Florida, 150 pp.
Ford, A.T., 2008. Can you feminise a crustacean? Aquat. Toxicol. 88 (4), 316–321.
Frouz, J., Kristufek, V., Bastl, J., Kalcik, J., Vankova, H., 2005. Determination of toxicity of spoil substrates after brown coal mining using a laboratory reproduction test with *Enchytraeus crypticus* (Oligochaeta). Water Air Soil Pollut. 162, 37–47.
Giacalone, A., Gianguzza, A., Mannino, M., Orecchio, S., Piazzese, D., 2004. Polycyclic aromatic hydrocarbons in sediment of marine coastal lagoons in Messina, Italy: extraction and GC/MS analysis, distribution and sources. Polycyclic Aromat. Compd. 24, 135–149.
Hallare, A.V., Pagulayan, R., Lacdan, N., Kohler, H.R., Triebskorn, R., 2005. Assessing water quality in a tropical lake using biomarkers in zebrafish embryos: developmental toxicity and stress protein responses. Environ. Monit. Assess. 104, 171–187.
Hilscherová, K., Machala, M., Kannan, K., Blankenship, A.L., Giesy, J.P., 2000. Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. Environ. Sci. Pollut. Res. 7, 159–171.
Hilscherová, K., Kannan, K., Kang, Y.S., Holoubek, I., Machala, M., Masunaga, S., 2001. Characterization of dioxin-like activity of sediments from a Czech river basin. Environ. Toxicol. Chem. 20, 2768–2777.
Hilscherová, K., Kannan, K., Holoubek, I., Giesy, J.P., 2002. Characterization of estrogenic activity of riverine sediments from the Czech Republic. Arch. Environ. Contam. Toxicol. 43, 175–185.
Jobling, S., Casey, D., Rodgers-Gray, T., Oehlmann, J., Schulte-Oehlmann, U., Pawlowski, S., Baunbeck, T., Turner, A.P., Tyler, C.R., 2003. Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. Aquat. Toxicol. 65, 205–220.
Kizu, R., Okamura, K., Toriba, A., Kakishima, H., Mizokami, A., Burnstein, K.L., Hayakawa, K., 2003. A role of aryl hydrocarbon receptor in the antiandrogenic effects of polycyclic aromatic hydrocarbons in LNCaP human prostate carcinoma cells. Arch. Toxicol. 77, 335–343.
Kozák, P., Hulák, M., Policar, T., Tichý, F., 2007. Studies of annual gonadal development and gonadal ultrastructure in spiny-cheek crayfish (*Orconectes limosus*). Bull. Fr. Peche Piscic. 384, 15–26.
Kusk, K.O., Wollenberger, L., 2007. Towards an internationally harmonized test method for reproductive and developmental effects of endocrine disrupters in marine copepods. Ecotoxicology 16, 183–195.
Ladewig, V., Jungmann, D., Koehler, A., Schirling, M., Triebskorn, R., Nagel, R., 2002. Intersexuality in *Gammarus fossarum* Koch, 1835 (*Amphipoda*). Crustaceana 75, 1289–1299.
Leskinen, P., Michelini, E., Picard, D., Karp, M., Virta, M., 2005. Bioluminescent yeast assays for detecting estrogenic and androgenic activity in different matrices. Chemosphere 61, 259–266.
Machala, M., Vondráček, J., Bláha, L., Cigánek, M., Neca, J., 2001. Aryl hydrocarbon receptor-mediated activity of mutagenic PAHs determined using *in vitro* reporter gene assay. Mutat Res. Genet. Toxicol. Environ. Mutat. 497, 49–62.
Medley, P.B., Rouse, D.B., 1993. Intersex Australian red claw crayfish (*Cherax quadricarinatus*). J. Shellfish Res. 12, 93–94.
Negri, A., Burns, K., Boyle, S., Brinkman, D., Webster, N., 2006. Contamination in sediments, bivalves and sponges of McMurdo Sound. Antarctica. Environ. Pollut. 143, 456–467.
Oetken, M., Nentwig, G., Löffler, D., Ternes, T., Oehlmann, J., 2005. Effects of pharmaceuticals on aquatic invertebrates. Part I. The antiepileptic drug carbamazepine. Arch. Environ. Contam. Toxicol. 49, 353–361.
Orem, W.H., Feder, G.L., Finkelman, R.B., 1999. A possible link between Balkan endemic nephropathy and the leaching of toxic organic compounds from Pliocene lignite by groundwater: preliminary investigation. Int. J. Coal Geol. 40, 237–252.
Rudolph, E.H., 1999. Intersexuality in the freshwater crayfish Samastacus spinifrons (Philippi, 1882) (Decapoda, Parasticidae). Crustaceana 72 (13), 325–337.
Sanders, M., Sivertsen, S., Scott, G., 2002. Origin and distribution of polycyclic aromatic hydrocarbons in surfacial sediments from the Savannah River. Arch. Environ. Contam. Toxicol. 43, 438–448.
Schacht, S., Sinder, C., Pfeifer, F., Klein, J., 1999. Bioassays for risk assessment of coal conversion products. Appl. Microbiol. Biotechnol. 52, 127–130.
Sørensen, M., Conder, J., Fuchsman, P., Martello, L., Wenning, R., 2007. Using a sediment quality triad approach to evaluate benthic toxicity in the lower Hackensack river, New Jersey. Arch. Environ. Contam. Toxicol. 53, 36–49.
Verslycke, T., Ghekiere, A., Raimondo, S., Janssen, C., 2007. Mysid crustaceans as test models for the screening and testing of endocrine-disrupting chemicals. Ecotoxicology 16, 205–219.
Villeneuve, D.L., Blankenship, A.L., Giesy, J.P., 2000. Derivation and application of relative potency estimates based on *in vitro* bioassay results. Environ. Toxicol. Chem. 19, 2835–2843.
Villeneuve, D.L., Khim, J.S., Kannan, K., Giesy, J.P., 2002. Relative potencies of individual polycyclic aromatic hydrocarbons to induce dioxin-like and estrogenic responses in three cell lines. Environ. Toxicol. 17, 128–137.
Vinggaard, A.M., Hnida, C., Larsen, J.C., 2000. Environmental polycyclic aromatic hydrocarbons affect androgen receptor activation *in vitro*. Toxicology 145, 173–183.
Wiesner, L., Gunther, B., Fenske, C., 2001. Temporal and spatial variability in the heavy-metal content of *Dreissena polymorpha* (Pallas) (Mollusca: Bivalvia) from the Kleines Haff (Northeastern Germany). Hydrobiologia 443, 137–145.
Wirth, E.F., Fulton, M.H., Chandler, G.T., Key, P.B., Scott, G.I., 1998. Toxicity of sediment associated PAHs to the estuarine crustaceans: *Palaemonetes pugio* and *Amphiascus tenuiremis*. Bull. Environ. Contam. Toxicol. 61, 637–644.

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SEDIMENTS, SEC 3 • SEDIMENT MANAGEMENT AT THE RIVER BASIN SCALE • RESEARCH ARTICLE

Chronic toxicity of contaminated sediments on reproduction and histopathology of the crustacean *Gammarus fossarum* and relationship with the chemical contamination and in vitro effects

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Abstract
Purpose The aim of the present study was to investigate possible relationships between the sediment contaminants and the occurrence of intersex in situ. Two of the studied sediments were from polluted sites with increased occurrence of intersex crustaceans (Lake Pilnok, black coal mining area in the Czech Republic, inhabited by the crayfish *Pontastacus leptodactylus* population with 18% of intersex; creek Lockwitzbach in Germany with *Gammarus fossarum* population with about 7% of intersex).

Materials and methods Sediments were studied by a combined approach that included (1) determination of concentrations of metals and traditionally analyzed organic pollutants such as polychlorinated biphenyls, pesticides, and polycyclic aromatic hydrocarbons (PAHs); (2) examination of the in vitro potencies to activate aryl hydrocarbon (AhR), estrogen (ER), and androgen receptor-mediated responses; and (3) in vivo whole sediment exposures during a 12-week reproduction toxicity study with benthic amphipod *G. fossarum*.

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Results and discussion Investigations showed that Lake Pilnok was highly contaminated by powdered waste coal, contained high concentrations of PAHs (up to 12 μg/g dry weight), and exhibited various effects in biotests (high concentrations of AhR and ER agonists were determined by in vitro assays with H4IIE.luc cells and yeast luciferase reporter gene assays). Less pronounced effects were observed in Lockwitzbach and Steinlach creek sediments. Long-term in vivo laboratory exposures with *G. fossarum* resulted in significant mortalities and sex-specific toxicities (reflected in hepatopancreas histopathology). Significant effects on the reproduction-related parameters were observed at Lake Pilnok sediments, which elevated numbers of newly hatched individuals and stimulated reproduction cycle in females (larger portions of mature oocytes in comparison to other variants).
Conclusions Results of the present study indicate that sediments from Lake Pilnok contain a large portion of dioxin-like, estrogenic, and anti-androgenic compounds, which stimulated fecundity in *G. fossarum*. Although some effects might be attributed to PAHs, most of the bioactive compounds could not be detected by traditional instrumental analyses. Possibly, bioavailable fractions of the maceral (solid coal mass rich in organic compounds) could have contributed to the observed activities, but only few studies investigated its biological effects, and it will require further research. The present study emphasizes the need for integrated assessment of contaminated sediments to elucidate their ecotoxicological impacts.

Keywords Androgenicity · Estrogenicity · Fecundity · In vitro · Reproduction toxicity · Sediments

1 Introduction

Contamination and general degradation of freshwaters (including sedimentary material) is an important environmental and economical problem worldwide (Millennium Ecosystem Assessment 2005). Despite ongoing efforts of governments and local authorities to improve water quality, many ecosystems remain polluted, and sediments, in particular, serve as sinks and sources of various types of contaminants (Wirth et al. 1998; Sørensen et al. 2007). Sediment quality criteria (SQC) have been suggested for priority contaminants (such as heavy metals, polycyclic aromatic hydrocarbons (PAHs), or chlorinated persistent compounds), but other compounds are still neglected in SQC. For example, no SQC exist for derivatives or metabolites of persistent compounds, hormones, or pharmaceuticals, which can pose significant long-term effects on aquatic organisms including reproductive or developmental toxicity (Pane et al. 2008).

Assessment of contaminated sediments often combines various methods including in vivo biotests. For example, the TRIAD approach (chemical analyses of known compounds, whole sediment toxicity testing, and evaluation of benthic biodiversity) has been discussed and widely used for sediment evaluation (Chapman and Hollert 2006; Sørensen et al. 2007). Ecotoxicological studies with in vivo models play a key role in the assessment of whole sediment toxicity, and several model organisms have been used to assess the effects of different types of sediment contaminants (De Lange et al. 2005; Scarlett et al. 2007). Number of studies documented adverse chronic effects of contaminated sediments in mollusks, larvae of insects, amphipods, and many others, but causal links between the identity of the toxic compounds and their effects remain often uncovered (Sanchez et al. 2005; Mazurová et al. 2008a; Scarlett et al. 2007).

The motivation of the present study was to investigate relationships between the sediment contaminants and the occurrence of intersex using the model amphipod crustacean *Gammarus fossarum* Koch, 1835. *G. fossarum* has been previously used in ecotoxicological studies (Lieb and Carline 2000; Schill et al. 2003) and, due to the unique reproduction cycle and the maturation of hatched embryos spanning 1 month (Pöckl and Humpesch 1990), it is a valuable model organism for studies of reproductive endpoints (Schirling et al. 2006). Previous studies demonstrated the sensitivity of *Gammarus* sp. to the synthetic estrogen 17α-ethinylestradiol (Watts et al. 2002), the non-steroidal estrogen bisphenol A (Schirling et al. 2006), and other xenobiotics like the sunscreen blockers (Scheil et al. 2008). Moreover, the abundance of *G. fossarum* intersex individuals was observed in situ, and anthropogenic pollution was discussed as an important causal factor (Jungmann et al. 2004a; Ford et al. 2007).

The present study investigated sediments from two localities where crustacean populations with high proportions of intersex were reported. The first locality—Lake Pilnok—is an artificial pond in the black coal mining area in the Czech Republic. In spite of the waste coal pollution, endangered narrow-clawed crayfish *Pontastacus* (syn. *Astacus*) *leptodactylus* Eschscholtz, 1823 (Decapoda, Crustacea) lives in this area. However, an abnormal population with high occurrence of intersex (18% of fertile females with male gonopods) lived in Lake Pilnok (Zdeněk Ďuriš et al., University of Ostrava, Czech Republic, personal communication). The spatial coincidence of the abundance of intersex specimens and the coal contamination suggested the presence of unknown compounds that might be causing endocrine disruption in this crayfish species. Further, the present study included sediments from Lockwitzbach creek (the vicinity of the Dresden city, Germany) with high incidence of intersex in a natural population of *G. fossarum* (about 7% of intersex

individuals; Ladewig et al. 2002). Contamination-induced intersexuality as well as other types of reproduction toxicity in both vertebrates and invertebrates have broad ecological consequences with regard to the direct impact on the population growth (Watts et al. 2002; LeBlanc 2007; Mazurová et al. 2008a).

In this study, in vivo exposures with *G. fossarum* were combined with chemical analyses of major organic contaminants and heavy metals and in vitro bioassays addressing aryl hydrocarbon (AhR), estrogen, and androgen receptor-mediated potential of the contaminants present in sediments in order to elucidate possible exposure-effect relationships.

2 Materials and methods

2.1 Characterization of the studied localities

Lake Pilnok is an artificial pond formed by flooding of a terrain depression resulting from the black coal mining activities in the industrial region of Ostrava-Karvina in the Czech Republic. Lake Pilnok has also been used as a dumping site for waste coal powder since the middle of the twentieth century. In spite of industrial activities in the studied area, water quality (oxygen content and transparency) has remained stable in Lake Pilnok and similar ponds, and the endangered narrow-clawed crayfish *Pontastacus leptodactylus* lives in this area, but an abnormal population with inordinately high occurrence of intersex individuals was observed in Lake Pilnok. The second study site was the Lockwitzbach creek (Saxony, Germany; the vicinity of the Dresden city influenced by sewage treatment plant) with high incidence of intersex in a natural population of *G. fossarum*. Levels of toxicants coming from the sewage treatment plant were not investigated, but composition of the water in Lockwitzbach seemed to be responsible for intersex (Ladewig et al. 2002; Jungmann et al. 2004a). Sediments collected from the Steinlach Creek (situated in a landscape conservation area, Baden-Württemberg, Germany) were used as the reference (normal *G. fossarum population, low contaminant levels—see Section 3).*

2.2 Sediment sampling and experimental design

Representative sediment samples (surface 5–10 cm layers) were prepared by mixing and pooling several sub-samples manually collected at each studied location. Rocks and other pieces of sedimentary material larger than 0.5 cm were manually removed, and fine sediments (i.e., sediment particulate matter smaller than 5 mm) were used for experiments. Samples were transported into the laboratory and stored frozen at −20°C until further processing. Portions of the sediments were extracted with dichloromethane for analyses of chemical pollutants and assessment of androgen, estrogen, and AhR activities using in vitro bioassays. In parallel, whole sediment in vivo toxicity assays were performed using the amphipod *G. fossarum*, and various parameters were recorded (including survival, growth, histology, sex, number and size of neonates, and the presence of intersex).

2.3 Analyses of organic contaminants, metals, and organic carbon

Methods for the identification and quantification of chemical residues in sediments have been described previously (Mazurová et al. 2008a). Briefly, 20 g dry weight of sediments were extracted by an automated extraction unit (B-811, Büchi, Switzerland) using dichloromethane, treated with activated copper to remove sulfur and analyzed (simultaneously with laboratory blank and reference material; accuracy of the analyses lower than 15%) for concentrations of 16 PAHs, seven indicators polychlorinated biphenyls (PCBs), and organochlorine pesticides (OCPs; hexachlorocyclobenzene, four hexachlorocyclohexane stereo isomers, two congeners of each DDE, DDD, and DDT). GC-ECD (HP 5890) equipped with a Quadrex silica column was used for analyses of PCBs and OCPs. Concentrations of PAHs were determined by GC-MS (HP 6890–HP 5973) with a J&W Scientific fused silica column DB-5MS. Based on the analyses of organic contaminants, dioxin-equivalents derived from the chemical analyses (TEQ_{chem}) were calculated using WHO-recommended toxic equivalency factors for PCBs and relative potencies for PAHs (Machala et al. 2001). Concentrations of metals (vanadium V, chromium Cr, cobalt Co, nickel Ni, copper Cu, zinc Zn, arsenic As, cadmium Cd, lead Pb, and mercury Hg) were evaluated based on Aqua Regia leaching conducted according to ISO 11466 protocol. Inductively coupled plasma-mass spectrometry (Agilent 7500ce, Agilent Technologies, Japan) in He collision mode using Octopole Reaction System was used for quantification of metals for which polyatomic interferences were observed. Total mercury concentrations were determined by use of the thermo-oxidation method using AMA-254 analyzer (Altec, Czech Republic). Total organic carbon content in sediments (TOC, percentage of dry sediment weight) was determined by the use of a LiquiTOC analyzer (Elementar-Analysensysteme GmbH, Hanau, Germany). The volume of 50–100 mg dry weight of fine ground sediments was treated with 15% hydrochloric acid for 30 min to eliminate inorganic carbon present in the sample, and the treated sediments were then dried at 120°C for 30 min and analyzed with LiquiTOC.

2.4 In vitro assays

A portion of the dichloromethane extract prepared for chemical analyses was evaporated under the stream of nitrogen to the last drop and diluted in dimethylsulphoxide (DMSO; a nontoxic solvent traditionally used for in vitro studies) and tested for the presence of bioactive compounds. H4IIE.luc rat cells stably transfected with the luciferase gene under control of the AhR were used for analysis of dioxin-like activity of the samples, and the results were reported as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) equivalents (TEQ_{bio}; Villeneuve et al. 2002). The experimental protocol has been previously described (Hilscherová et al. 2001). Briefly, cells seeded in 96-well microplates were exposed to several dilutions of sediments extracts (and TCDD calibration) for 24 h. The intensity of luminescence corresponding to the AhR activation was measured using the Promega Steady Glo Kit (Promega, USA). Extracts treated with sulfuric acid (removal of labile non-persistent compounds) were assessed to evaluate contribution of both labile and stabile (persistent) compounds. The assays for (anti-)estrogenic and (anti-)androgenic activity of the sediment extracts used strains of yeast *Saccharomyces cerevisiae* stably transfected with human estrogen/androgen receptor genes along with the firefly luciferase under transcriptional control of estrogen/androgen-responsive element. Another yeast strain constitutively expressing luciferase served for the assessment of cytotoxicity as described by Leskinen et al. (2005). Blank control and solvent (DMSO) controls were tested at each individual microplate. The final concentration of the solvent in the exposure media did not exceed 1% *v*/*v*. Yeast cells were exposed to the calibrations of 17β-estradiol (estrogenicity) and testosterone (androgenicity), and to the sediment extracts (either alone or in the combination with competing physiological ligand to test for anti-estrogenicity/anti-androgenicity—2 nM 17β-estradiol or 10 nM testosterone, respectively). Exposure duration in the yeast assays was 2.5 h, and the luminescence signal was detected after addition of D-luciferin substrate. Experiments were performed in three replicates and repeated independently for at least two times, the results are expressed as mean (±standard error).

2.5 In vivo studies

Whole sediment bioassays were performed with the amphipod *G. fossarum* Koch, 1863 (Amphipoda, Crustacea). Individuals larger than 3 mm in length were collected at the reference locality (Steinlach Creek) and used in the experiments. Pairs of adult males and females in pre-copula position were collected to ensure that all sampled females were of the same reproductive status. The animals were acclimatized for 3 weeks in the laboratory prior to the experiments (15.6±0.14°C and a light:dark cycle of 14:10 h; feeding ad libitum with soaked leaves collected at the Steinlach Creek; leaves were properly washed in hot water and stored in aerated water medium under the same laboratory conditions). As a field reference, the proportion of intersex individuals (both male and female external characteristics) were investigated in the natural populations of *G. fossarum* at both Steinlach Creek and Lockwitzbach Creek (Supplementary Table 1).

Four exposure variants were carried out: (1) reference sediment from Steinlach Creek, (2) contaminated sediments from Lake Pilnok, and (3) sediment from the Lockwitzbach Creek. The fourth tested variant (4) consisted of sediment mixture of Lake Pilnok and Steinlach (1:1 based on dry weight) to investigate possible dose–response relationship (i.e., dilution of the contaminated Lake Pilnok sediments—lower impact on *G. fossarum* was expected in the mixture variant).

The experiments were performed in 10 L glass aquaria using the thawed wet sediment that formed a 2-cm layer (equivalents of 500 g dry weight per aquarium was used). The aqueous medium (8 L per aquarium) was a 1:1 mixture of the stream water from the reference Steinlach Creek and tap water (moderately hard water, 300 ppm calcium carbonate; preliminary experiments showed no effects on *G. fossarum* in the laboratory culture). To establish equilibrium, sediment/water systems were set up 7 days prior to the experiments. A volume of 5 L of the medium was renewed weekly in each aquarium. Every fourth week, animals were transferred into new aquaria with a new sediment/water system. The exposure was performed for 12 weeks under constant conditions at a temperature 15.6± 0.14°C and a light:dark regime 14:10 h. Animals were fed ad libitum with soaked leaves as described above.

For the Steinlach and Lake Pilnok exposures, three replicated aquaria were used, and the Lockwitzbach and Pilnok/Steinlach variants were performed in four replicates. Different numbers of replicates were used due to technical limitations. Each of the aquaria initially contained 90 animals, i.e., 45 males and 45 females.

The animals that survived until the end of the experiment were narcotized using carbon dioxide in mineral water, counted, and examined under a stereomicroscope (Leica MZ8, Heerbrugg, Switzerland). The length of the cephalothorax was measured by use of an eyepiece micrometer with an accuracy of 0.1 mm (to discriminate between larger parental individuals and smaller juveniles that may have hatched during exposure). The sex of specimens and/or the presence of individuals with mixed sexual characteristics were determined based on their external morphological characters (penis papillae in males, brood pouch formed from oostegites in females; Ladewig et al. 2002). All

individuals that appeared to have both male and female organs and also at least one male and one female from each aquarium were fixed in 2 M glutardialdehyde dissolved in 0.005 M cacodylate buffer (pH7.4) for histological examination.

2.6 Histology of the ovary and hepatopancreas

The tissues of specimens were decalcified in 5% trichloroacetic acid, dehydrated in graded series of ethanol (solutions of 70%, 80%, 90%, and 96% ethanol; Carl Roth GmbH & Co, Karlsruhe, Germany), and embedded in Technovit resin (Heraeus Kulzer, Germany). The tissues were cut into eight series with a Reichert Jung 2050 microtome (Heidelberg, Germany). Each series consisted of 20 longitudinal sections of 4-μm thickness. The sections were stained with methylene blue-azur II as described by Richardson et al. (1960). The ovarian maturity status and the histopathology of the hepatopancreas of both males and females were examined under a light microscope Axioscop 2 (Zeiss, Oberkochen, Germany).

The stage of the female reproduction cycle was estimated from the distribution of individual developmental stages of oocytes in the ovary. Oocyte stages were classified on the basis of previous study (Schirling et al. 2004) distinguishing classes of previtellogenic oocytes (PVO), vitellogenic oocytes, late vitellogenic oocytes (LVO), and mature oocytes (MO). Each oocyte stage can occur with an either "healthy" (intact) or "disturbed" (atretic) appearance. The atretic cells (characterized by an irregular cell shape with partially lyzed membrane compartments) do not progress in development, they undergo controlled autolysis, and their constituents are degraded and restored (Janz et al. 2001). For each maturation stage class, the frequencies of oocytes were estimated for individual ovaria (separately for intact and atretic oocytes). Each histological section was scored by use of a semi-quantitative scale: 0, no oocyte in the respective class observed; 1, only few oocytes in the respective class; 2, occurrence of the respective oocyte class is common; and 3, oocyte class is the most dominant in the examined section. The results from each tissue section series were averaged (eight values were counted for each individual female), and these values were used to characterize stages of ovarian reproduction cycle for each exposure variant (N=6 females per treatment).

The health status of the hepatopancreas was evaluated separately in males and females. The evaluation addressed (1) general tissue integrity, (2) distinct cellular integrity of resorption R-cells, and (3) nuclear pleomorphism (Harrison 1992). The impairment evaluation used an approach modified from the work of Scheil et al. (2008). Details on the evaluation criteria are in Supplementary Table 2.

2.7 Data analyses

Statistical significance of (anti-)estrogenic and (anti-)androgenic effects (i.e., up- or downregulation of estradiol/testosterone-induced luciferase in the yeast reporter assay) was tested by analysis of variance (ANOVA) followed by Dunnet's test (comparisons with controls/reference sediment exposure). Differences among exposure variants from in vivo experiments with *G. fossarum* were evaluated either by Pearson's Chi-square (for frequency data such as numbers of surviving animals) or by ANOVA followed by Dunnet's test (for parametric data). Results of the parametric ANOVA were further controlled by the non-parametric Kruskal–Wallis ANOVA (followed by the multiple comparisons of mean ranks for all groups). All calculations were performed with Statistica 8.0 (StatSoft, Inc., Tulsa, OK, USA), and p values less than 0.05 were considered statistically significant.

3 Results and discussion

Reproduction toxicity and/or related morphological changes of sexual organs such as intersex are known adverse effects in aquatic biota caused by sediment contaminants (Jungmann et al. 2004a; Scarlett et al. 2007). The present study aimed to investigate relationships between the levels of pollutants, in vitro biological activities, and chronic effects in model crustacean in vivo at sediment samples from specific localities, where intersex was reported at natural crustacean populations.

Chemical analyses showed that major organic contaminants in sediments from the Lake Pilnok (population of endangered crayfish *P. leptodactylus*, 18% intersex) were PAHs (1.0×10^4ng ∑PAH/g dry weight, Table 1). These values were more than 20 times higher than in sediments from Lockwitzbach creek (*G. fossarum* population with increased intersex) or Steinlach (reference site; see Table 1). Nevertheless, levels found in Lake Pilnok generally correspond to concentrations from other localities in the Czech Republic (Hilscherová et al. 2001, Babek et al. 2008), and the sum of PAHs in Lake Pilnok did not exceed the SQC of the Florida Department of Environmental Protection (for ∑PAH=2.3×10^4ng/g dry weight; FDEP 2003). Concentrations of persistent organic pollutants, such as PCBs and OCPs were lowest in sediments from the reference site—Steinlach Creek. Higher concentrations were found at other two sites (see Table 1), but the concentrations were still lower than expected for highly contaminated areas (Eljarrat et al. 2001; De Lange et al. 2005). Also, metal concentrations in studied sediments were low. For example, maximum Lake Pilnok concentrations (given in micrograms per gram dry weight) were

Table 1 Characterization of the studied sediments—levels of organic pollutants, content of total organic carbon (TOC), and TCDD equivalents (TEQs; ng TCDD/g dry weight)

	Steinlach Creek (reference)	Lockwitzbach Creek (Germany, close to Dresden city)	Lake Pilnok (Czech Republic, black coal mining)
Chemical contaminants (ng/g dry weight)			
Sum of 16 PAHs[a]	422	409	11,780
Sum of PCBs[b]	0.86	20.25	12.02
Sum of OCPs[c]	0.58	5.67	5.85
TOC (%)	6.3	0.6	33.7
TEQs (ng TCDD/g dry weight)			
TEQ_{chem}	0.032	0.035	0.309
TEQ_{bio} (crude extract)	2.4	3.0	93.4
TEQ_{bio} (H_2SO_4 treated)	0.010	0.231	1.160

TEQs were determined either as TEQ_{chem} (i.e., individual PAHs concentration multiplied by their relative potencies according to Machala et al. 2001) or TEQ_{bio} (AhR-mediated effects after 24 h exposure in H4IIE.luc cell bioassay)

PAHs polycyclic aromatic hydrocarbons, *PCBs* polychlorinated biphenyls, *OCPs* organochlorine pesticides

[a] The sum of 16 priority PAHs (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenzo[a,h]anthracene, and benzo[g,h,i]perylene)

[b] The sum of seven indicator PCBs (PCB 28, 52, 101, 118, 153, 138, and 180)

[c] The sum of OCPs—hexachlorobenzene, four hexachlorocyclohexane stereoisomers (α-HCH, β-HCH, γ-HCH, and δ-HCH), and p,p′- and o,p′-congeners of each DDE, DDD and DDT

0.14 for cadmium, 22 for chromium, 38 for zinc, 28 for copper, or three for arsenic (for details, see Supplementary Table 3), but these concentrations were below the levels expected to induce sublethal effects (Köhler et al. 1996; FDEP 2003).

Corresponding to the chemical analyses, TEQs for dioxin-like compounds calculated from PCB and PAH concentrations were highest in the Lake Pilnok (TEQ_{chem}= 0.3 ng TCCD/g dry weight, see Table 1). Interestingly, dioxin-like effects (TEQ_{bio}) determined with H4IIE.luc bioassay were unusually high with values higher than 90 ng TCDD/g dry weight for the Lake Pilnok sample (see Table 1). TEQ_{chem} and TEQ_{bio} values in the Steinlach and the Lockwitzbach sediments were lower and comparable with each other. These findings discriminated Lake Pilnok sediments and indicated that unknown compounds, which were not analyzed by routine methods, were responsible for the majority of the AhR-mediate effects. The derived TEQ_{bio} values were especially high in comparison with previous study of river sediments that found comparable or even higher concentrations of PAHs and chlorinated compounds but lower TEQ_{bio} values with maxima around 20 ng TCDD/g dry weight (Hilscherová et al. 2001).

Treatment of the samples with sulfuric acid prior to testing resulted in a substantial decrease of the AhR-mediated activity (TEQ_{bio}=1.16 ng TCDD/g dry weight for Lake Pilnok sediments, see Table 1), which demonstrated that labile (non-persistent) compounds were responsible for major part of the effects. Lake Pilnok sediments also displayed significant estrogenicity (estrogen-agonist activity, Fig. 1a) as well as anti-androgenicity (androgen-antagonist activity, suppression of the testosterone effects in yeast reporter bioassay, see Fig. 1b). In part, these effects could be related to contamination by PAHs. For example, benzo[a]anthracene and dibenzo[a,h]anthracene were shown to be estrogen-agonists and anti-androgens (Vinggaard et al. 2000; Villeneuve et al. 2002). Some studies also demonstrated a link between the activation of the AhR and anti-androgenicity (Kizu et al. 2003).

Besides chemical and in vitro analyses, long-term in vivo experiments further explored biological effects of the studied sediments. High mortalities were observed in all exposure variants (Fig. 2), which confirmed that *G. fossarum* is a sensitive organism in experimental biotests (Jungmann et al. 2004a, Schirling et al. 2006) when compared to other ecotoxicological models such as isopod *Asellus aquaticus* or *Chironomus* sp. larvae (De Lange et al. 2005). To fully demonstrate variability, Fig. 2 shows the raw data (numbers of surviving organisms in individual aquaria). The mean survival rates (males+females) ranged from 31% (Steinlach and Lockwitzbach variants) to 34.4% (Lake Pilnok) and did not differ among exposures. During the experiment, larger animals seemed to survive more likely, and this was more apparent for males. Also, the overall size distribution of animals was shifted towards larger individuals in comparison with the natural population

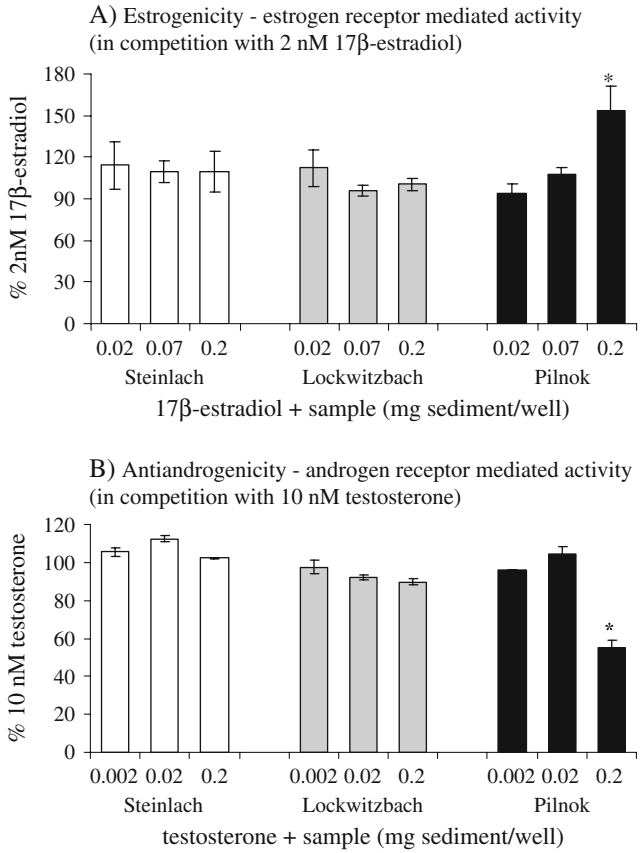

Fig. 1 Responses to sediment extracts in recombinant yeast cells transfected with a luciferase reporter gene under the control of (**a**) estrogen receptor and (**b**) androgen receptor. The cells were exposed for 2.5 h to dilutions of the sediment extracts (three concentrations 0.02–0.2 mg sediment/well) together with an appropriate competing ligand (i.e., 2 nM 17β-estradiol for estrogenicity and 10 nM testosterone for androgenicity). *Graphs* display means and standard error from three replicated experiments normalized to the response of the ligand alone (17b-estradiol or testosterone, i.e., 100% effect). *$P<$ 0.05; analysis of variance followed by Dunnet's post hoc test

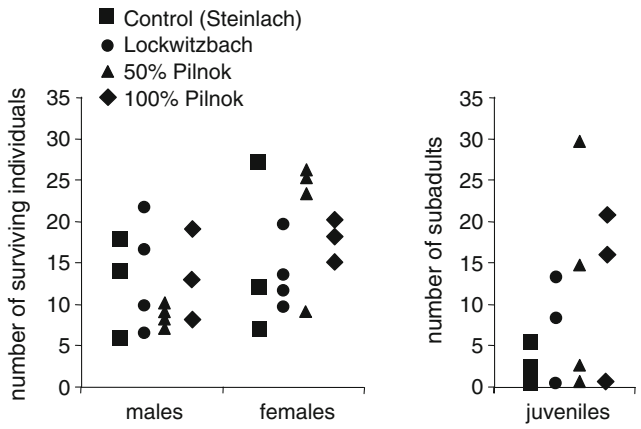

Fig. 2 Numbers of survived (males and females) and newly born individuals (juveniles) of *Gammarus fossarum* at the end of 12 weeks laboratory exposures to studied sediments. Each data point represents the result from individual aquarium (initial number of adult animals in each aquarium, males+females, was 90)

(see Supplementary Fig. 1). Females of aquatic crustaceans are expected to be more susceptible to environmental stress (with regard to higher energy demands to maintain an active reproduction status), while males can more easily allocate energy to cope with the stress (Lieb and Carline 2000). In the present study, however, females seemed to have higher (though not significantly different) survival rates than males, which is in agreement with findings of Schill et al. (2003); compare for example triangle and diamond symbols between male and female survival data in Fig. 2.

Sex-dependent differences were also found in the hepatopancreas histopathology (Figs. 3 and 4), which is an important parameter reflecting general stress and health condition (Pöckl and Humpesch 1990). While cellular and tissue integrity damage were dominant pathologies observed in females (see Figs. 3a and 4a–c), a different pattern was found in males (see Figs. 3b and 4d, e—nuclear pleomorphism—lyses of nuclear membrane or chromatin precipitation). Similar sex-specific sensitivity was also

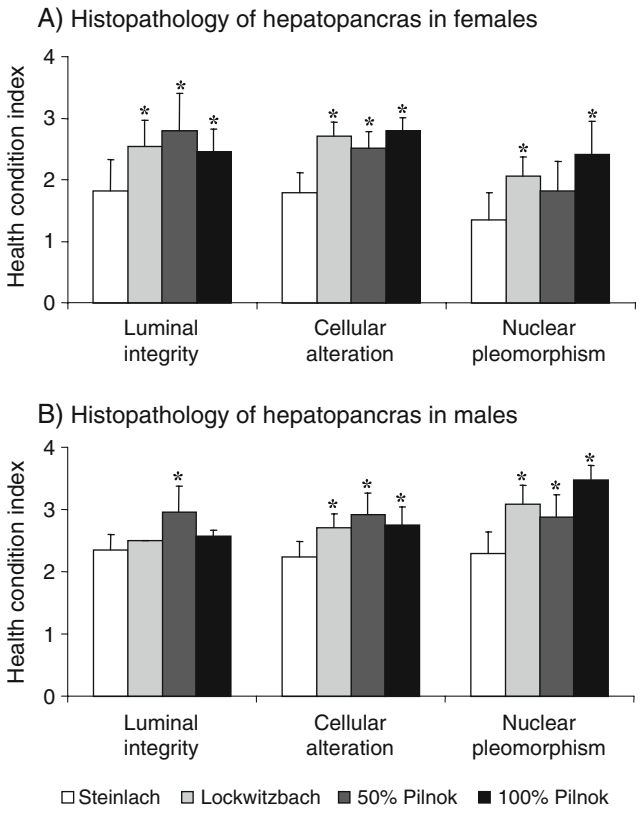

Fig. 3 Evaluation of the histopathology of hepatopancreas in females (**a**) and males (**b**) of *Gammarus fossarum* after 12 weeks exposures to contaminated sediments. Three parameters (see Supplementary Table 2) were evaluated for R-cells of hepatopancreas according to Scheil et al. (2008). The *vertical axis* shows the health condition of the examined parameters: *1*, good condition; *2*, weak alterations; *3*, obvious pathological changes; *4*, tissue destruction. Mean±standard deviation is displayed. *$P<0.05$; Kruskal–Wallis test

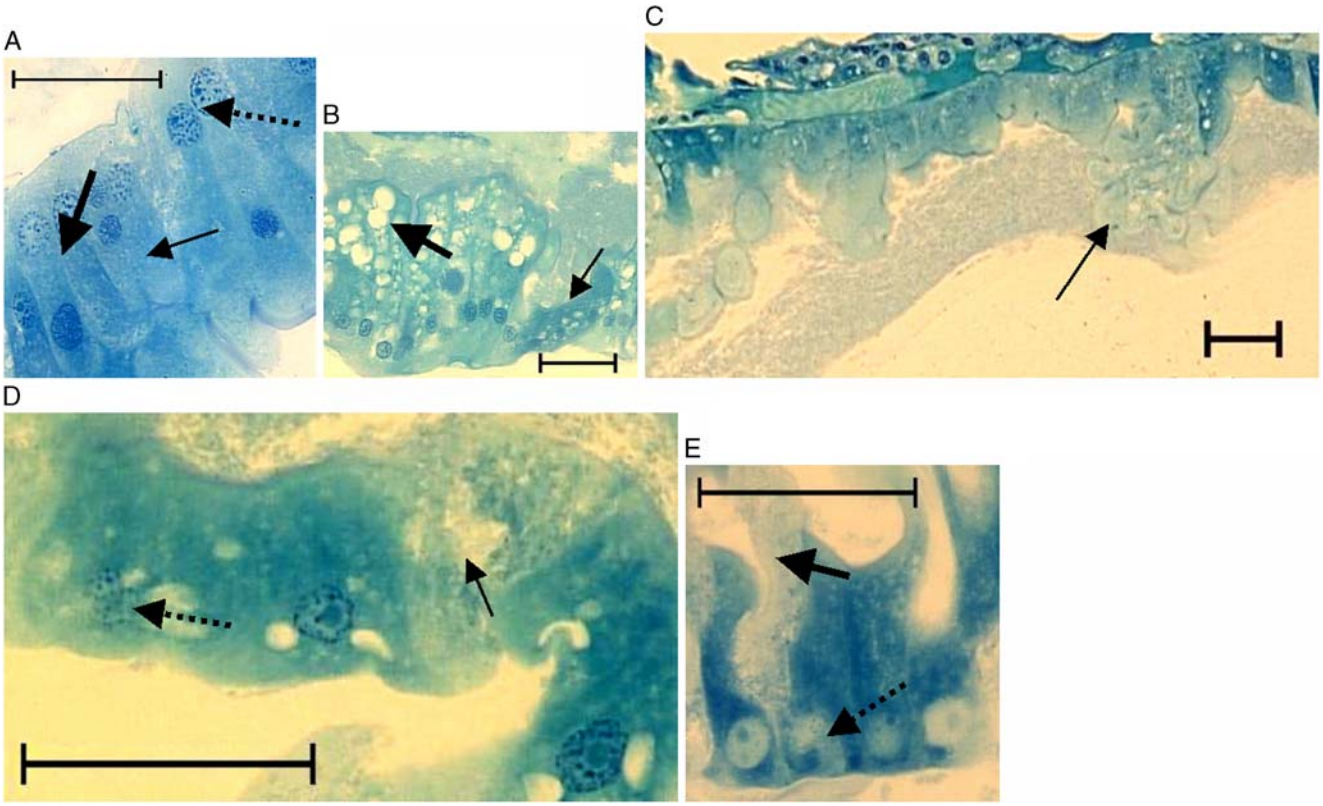

Fig. 4 Histopathology of hepatopancreas in females (**a**–**c**) and males (**d**, **e**) of *Gammarus fossarum* exposed for 12 weeks to sediments from reference Steinlach Creek (**a**), Lake Pilnok (**c**, **e**), and the mixtures of Pilnok/Steinlach sediments (**b**, **d**). Scale bar=50 μm. *Thin arrows* show parameters of general tissue integrity—epithelium with well-developed columnar cells epithelium (**a**), uneven epithelium thickness and irregularly shaped cells (**b**), and foci of disintegrated epithelium (**c**, **d**). *Thick arrows* show cellular parameters—fine vacuolization and well-stained cytoplasm (**a**), rough vacuolization (**b**), and lysed cell apex (**e**). *Dashed arrows* show parameters of nuclear pleomorphism—oval nucleus (**a**), pleomorphic nucleus (**d**), and completely lysed nucleus (**e**)

reported in *G. fossarum* exposed to a chemical stressor, a UV blocker compound (Scheil et al. 2008). It should be pointed out that observed effects (i.e., higher stress in males and the size shift towards larger individuals) might also be related to other factors such as aggressiveness (often observed especially among males) and cannibalism (larger individuals preying on the smaller ones). These behaviors were previously reported (Sexton 1928; Pöckl and Humpesch 1990), and they were observed also in the present study.

Evaluation of the reproduction-related parameters in *G. fossarum* showed other interesting results that discriminated Lake Pilnok from other exposure variants. Numbers of juveniles newly born during the 12-week exposures (mean±SEM) were 2±2.7, 5±3.2, 11±6.7, and 12±4.9 for Steinlach, Lockwitzbach, Pilnok 50%, and Pilnok 100%, respectively (see Fig. 2—right panel). Simple statistical comparison showed that both Lake Pilnok variants (50% and 100%) had significantly higher numbers of juveniles than the reference Steinlach exposure (Student's *t* test; $P<0.05$). However, high variability among aquaria did not allow confirmation by more robust statistics (ANOVA and Kruskal–Wallis test, $P>0.05$). Nevertheless, trend observed at Lake Pilnok was further supported by the evaluation of the juvenile size (Fig. 5), which was higher in both Pilnok/Steinlach 1:1 and Pilnok 100% variants (see Fig. 5c, d; distributions significantly different from both Steinlach and Lockwitzbach exposures; Chi-square, $P<0.05$). Relatively low numbers of juveniles at the end of exposure could be possibly related to the competition and canibalism discussed above. However, it does not explain the difference among exposures as the survival of adults (i.e., predation pressure) was comparable in all variants. Lake Pilnok sediments seem to contain unknown chemicals, which promoted the population growth of *G. fossarum*. Interestingly, comparable effects (increase in the population size, greater numbers of newly hatched animals, larger mature individuals) were previously observed during a 100-day-experiment with *Gammarus pulex* exposed to a model endocrine disrupter 17α-ethinylestradiol (Watts et al. 2002). Taken together, the presence of estrogenic compounds in Lake Pilnok, which was revealed by the in vitro assay, could contribute to the observed in vivo effects. Another possible explanation of the differences, which

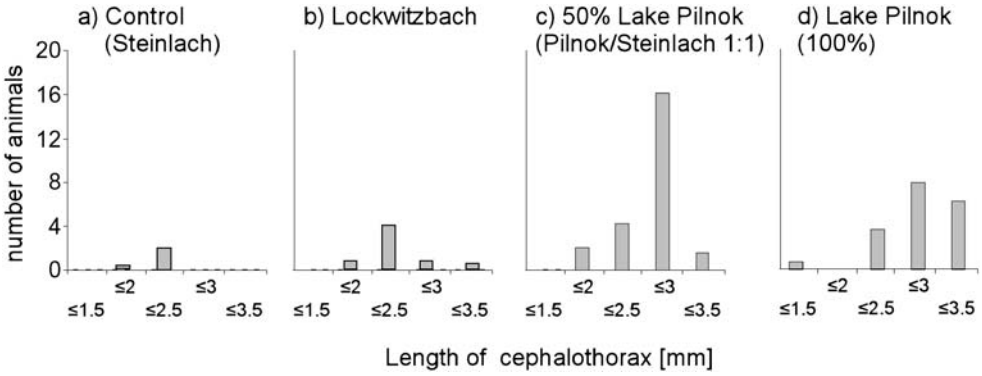

Fig. 5 Size distribution of newly born individuals (juveniles) of *Gammarus fossarum* (size of the cephalothorax <3.5 mm) at the end of 12 weeks exposures to sediments from reference Steinlach creek (**a**), Lockwitzbach Creek (**b**), and Lake Pilnok (**c**, **d**)

should be critically mentioned, is the difference in composition among sediments—particularly content of organic material (see Table 1). High TOC values in Pilnok sediment might have affected organisms (e.g., by providing an additional source of food), which could eventually promote higher reproduction.

Evaluation of female gonad histology (distribution of oocyte developmental stages) also demonstrated clear effects of sediment exposures on the reproduction status (Figs. 6 and 7). In the reference exposure, the early previtellogenic (PVO) stage was the most abundant (see Fig. 6, white bars). On the other hand, more developed ovaries were recorded at other variants (Lockwitzbach and Lake Pilnok sediments—higher proportions of LVO and fully MO). Also, the size of LVO in females exposed to Lake Pilnok sediments was often larger than in reference exposure (example shown in Fig. 7). This shift towards

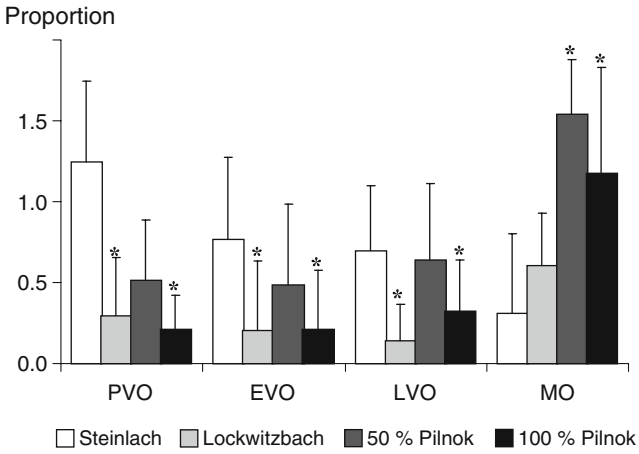

Fig. 6 Proportion of individual stages of oocytes in ovaries of females of *Gammarus fossarum* exposed for 12 weeks to contaminated sediments. *PVO* previtellogenic oocytes, *EVO* early vitellogenic oocytes, *LVO* late vitellogenic oocytes, *MO* mature oocytes. The proportion of oocytes in the maturity stage classes (*vertical axis*) was counted as follows: (*0*) no oocyte of appropriate class, (*1*) only a few oocytes of appropriate class, (*2*) considerable amount of oocytes of appropriate class, (*3*) the most dominant oocyte class. The atretic oocytes were not included. Data show mean±standard deviation (N= 6–7 individual females per group). *P<0.05; Kruskal–Wallis analysis of variance

oocyte maturity corresponds well with the results of previous studies with *G. fossarum* exposed to bisphenol A or wastewater effluents (Schirling et al. 2005, 2006). In the Lake Pilnok variant, there were also increased numbers of atretic oocytes, i.e., PVO that underwent controlled lyses (detailed results not shown). The proportion of atretic oocytes is an important histopathological biomarker with ecological relevance (Au 2004), and increased numbers were previously reported in studies with earthworms (Siekierska and Urbanska-Jasik 2002) or fish (Janz et al. 2001).

The present study focused on sediments from localities, where crustacean populations with high proportions of intersex were found in situ (Lockwitzbach and Lake Pilnok). Interestingly, only few intersex individuals were recorded at the end of laboratory exposure (in total, four specimens in all exposures), and according to histology investigations, they were fully developed females (see Supplementary Table 1). Comparing these observation to previous studies (Jungmann et al. 2004b, Ford et al. 2007), which also demonstrated induction of intersex in *G. fossarum* during relatively short exposure period, it may be hypothesized that the composition of the water (rather than sediment contaminants) might contribute to intersex development. However, other factors such as genetic variability or infection with parasites should also be considered (Dunn et al. 1993).

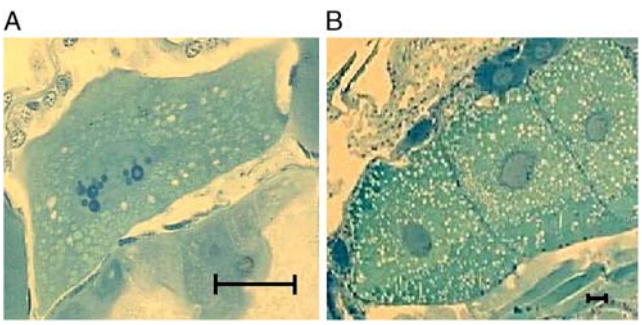

Fig. 7 Sizes of the late vitellogenic oocytes in females of *Gammarus fossarum*. Much smaller size was observed in controls/reference site (**a**) in comparison with females exposed to contaminated sediment from Lake Pilnok (**b**). Scale bars at both panels=50 μm

4 Conclusions

In summary, the combined results of chemical analyses and in vitro bioassays indicate that sediments from Lake Pilnok contain a large portion of dioxin-like, estrogenic, and anti-androgenic compounds, which seem to stimulate fecundity during in vivo exposures of *G. fossarum* (elevated numbers of newly hatched individuals, increased proportion of late vitellogenic and atretic oocytes in females). In vivo effects in *G. fossarum* might have an endocrine disruptive background, and they could be possibly caused by the same compounds that induced effects in vitro with respect to certain similarities between the vertebrate and crustacean endocrine systems (see reviews by LeBlanc 2007, Mazurová et al. 2008b). Although high PAHs concentrations were determined, they could not fully account for observed effects, and most of the bioactive compounds cannot be detected by traditional instrumental analyses. Possibly, bioavailable fractions of the maceral, i.e., solid coal mass rich in diagenic organic compounds (like polyaromatics, polyphenols, aliphatic hydrocarbons, and cycloalkanes; ASTM 1979) could have contributed to the observed activities (Orem et al. 1999; Schacht et al. 1999; Frouz et al. 2005). Interestingly, only few studies investigated biological effects of waste coal, and this issue will require further research. The present study documents that combination of chemical analyses, in vitro and in vivo experiments with in situ observations, is a necessary approach to obtain comprehensive information on the ecotoxicological effects and risks of contaminated sediment.

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References

ASTM (1979) Annual book of ASTM standards. Part 26—gaseous fuels, coal and coke, atmospheric analysis. American Society for Testing and Materials, Philadelphia

Au DWT (2004) The application of histo-cytopathological biomarkers in marine pollution monitoring: a review. Mar Pollut Bull 48:817–834

Babek O, Hilscherova K, Nehyba S, Zeman J, Famera M, Francu J, Holoubek I, Machat J, Klanova J (2008) Contamination history of suspended river sediments accumulated in oxbow lakes over the last 25 years. J Soils Sediments 8(3):165– 176

Chapman PM, Hollert H (2006) Should the sediment quality triad become a tetrad, a pentad, or possibly even a hexad? J Soils Sediments 6:4–8

De Lange HJ, De Haas EM, Maas H, Peeters ETHM (2005) Contaminated sediments and bioassay responses of three macro-invertebrates, the midge larva *Chironomus riparius*, the water louse *Asellus aquaticus* and the mayfly nymph *Ephoron virgo*. Chemosphere 61:1700–1709

Dunn AM, Adams J, Smith JE (1993) Is intersexuality a cost of environmental sex determination in *Gammarus duebeni*? J Zool 231:383–389

Eljarrat E, Caixach J, Rivera J, De Torres M, Ginebreda A (2001) Toxic potency assessment of non- and mono-ortho PCBs, PCDDs, PCDFs, and PAHs in northwest mediterranean sediments (Catalonia, Spain). Environ Sci Technol 35:3589–3594

FDEP (2003) Development and evaluation of numerical sediment quality assessment guidelines for Florida inland waters. Florida Department of Environmental Protection, Tallahassee

Ford AT, Read PA, Jones TL, Michino F, Pang Y, Fernandes TF (2007) An investigation into intersex amphipods and a possible association with aquaculture. Mar Environ Res 64:443–455

Frouz J, Kristufek V, Bastl J, Kalcik J, Vankova H (2005) Determination of toxicity of spoil substrates after brown coal mining using a laboratory reproduction test with *Enchytraeus crypticus* (Oligochaeta). Water Air Soil Pollut 162:37–47

Harrison FW (1992) Crustacea. In: Harrison FW, Humes AG (eds) Microscopic anatomy of invertebrates. Wiley-Liss, New York, pp 529–617

Hilscherová K, Kannan K, Kang YS, Holoubek I, Machala M, Masunaga S (2001) Characterization of dioxin-like activity of sediments from a Czech river basin. Environ Toxicol Chem 20:2768–2777

Janz DM, McMaster ME, Weber LP, Munkittrick KR, Van Der Kraak G (2001) Recovery of ovary size, follicle cell apoptosis, and HSP70 expression in fish exposed to bleached pulp mill effluent. Can J Fish Aquat Sci 58:620–625

Jungmann D, Köhler A, Köhler H-R, Ladewig V, Licht O, Ludwichowski K-U, Schirling M, Triebskorn R, Nagel R. (2004a) Umweltchemikalien mit Wirkung auf das Hormonsystem—TV 5: Wirkung von Xenohormonen in aquatischen Ökosystemen. Report nr F+E-Vorhaben 299 65 221/05, German Federal Environmental Agency (UBA), Berlin

Jungmann D, Ladewig V, Ludwichowski KU, Petzsch P, Nagel R (2004b) Intersexuality in *Gammarus fossarum* KOCH—a common inducible phenomenon? Arch Hydrobiol 159:511–529

Kizu R, Okamura K, Toriba A, Kakishima H, Mizokami A, Burnstein KL, Hayakawa K (2003) A role of aryl hydrocarbon receptor in the antiandrogenic effects of polycyclic aromatic hydrocarbons in LNCaP human prostate carcinoma cells. Arch Toxicol 77:335–343

Köhler H-R, Hüttenrauch K, Berkus M, Gräff S, Alberti G (1996) Cellular hepatopancreatic reactions in *Porcellio scaber* (Isopoda) as biomarkers for the evaluation of heavy metal toxicity in soils. Appl Soil Ecol 3:1–15

Ladewig V, Jungmann D, Koehler A, Schirling M, Triebskorn R, Nagel R (2002) Intersexuality in *Gammarus fossarum* Koch, 1835 (Amphipoda). Crustaceana 75:1289–1299

LeBlanc GA (2007) Crustacean endocrine toxicology: a review. Ecotoxicology 16:61–81

Leskinen P, Michelini E, Picard D, Karp M, Virta M (2005) Bioluminescent yeast assays for detecting estrogenic and androgenic activity in different matrices. Chemosphere 61:259–266

Lieb DA, Carline RF (2000) Effects of urban runoff from a detention pond on water quality, temperature and caged *Gammarus minus* (Say) (Amphipoda) in a headwater stream. Hydrobiology 441:107–116

Machala M, Vondráček J, Bláha L, Cigánek M, Neca J (2001) Aryl hydrocarbon receptor-mediated activity of mutagenic PAHs determined using in vitro reporter gene assay. Mutat Res Genet Toxicol Environ Mutagen 497:49–62

Mazurová E, Hilscherová K, Triebskorn R, Köhler H-R, Maršálek B, Bláha L (2008a) Endocrine regulation of the reproduction in crustaceans: identification of potential targets for toxicants and environmental contaminants. Biologia 63:139–150

Mazurová E, Hilscherová K, Jálová V, Köhler H-R, Triebskorn R, Giesy JP, Bláha L (2008b) Endocrine effects of contaminated sediments on the freshwater snail *Potamopyrgus antipodarum* in vivo and in the cell bioassays in vitro. Aquat Toxicol 89:172–179

Millennium Ecosystem Assessment (2005) Ecosystems and human well-being: Wetlands and water. Synthesis. World Resources Institute, Washington

Orem WH, Feder GL, Finkelman RB (1999) A possible link between Balkan endemic nephropathy and the leaching of toxic organic compounds from Pliocene lignite by groundwater: preliminary investigation. Int J Coal Geol 40:237–252

Pane L, Giacco E, Corra C, Giuliano G, Mariottini GL, Varisco F, Faimali M (2008) Ecotoxicological evaluation of harbour sediments using marine organisms from different trophic levels. J Soils Sediments 8:74–79

Pöckl M, Humpesch UH (1990) Intra- and interspecific variants in egg survival and brood development time for austrian populations of *Gammarus fossarum* and *Gammarus roeseli* (Crustacea: Amphipoda). Freshw Biol 23:441–455

Richardson KC, Jarret L, Finke EH (1960) Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Technology 35:313–325

Sanchez P, Alonso C, Fernandez C, Vega MM, Garcia MP, Tarazona JV (2005) Evaluation of a multi-species test system for assessing acute and chronic toxicity of sediments and water to aquatic invertebrates—effects of pentachlorophenol on *Daphnia magna* and *Chironomus prasinus*. J Soils Sediments 5(1):53–58

Scarlett A, Galloway TS, Rowland SJ (2007) Chronic toxicity of unresolved complex mixtures (UCM) of hydrocarbons in marine sediments. J Soils Sediments 7:200–206

Schacht S, Sinder C, Pfeifer F, Klein J (1999) Bioassays for risk assessment of coal conversion products. Appl Microbiol Biotechnol 52:127–130

Scheil V, Triebskorn R, Köhler H-R (2008) Cellular and stress protein responses to the UV filter 3-benzylidene camphor in the amphipod crustacean *Gammarus fossarum* (Koch 1835). Arch Environ Contam Toxicol 54:684–689

Schill RO, Görlitz H, Köhler H-R (2003) Laboratory stimulation of a mining accident: acute toxicity, hsc/hsp70 response, and recovery from stress in *Gammarus fossarum* (Crustacea, Amphipoda) exposed to a pulse of cadmium. Biol Metal 16:391–401

Schirling M, Triebskorn R, Köhler HR (2004) Variation in stress protein levels (hsp70 and hsp90) in relation to oocyte development in *Gammarus fossarum* (Koch 1835). Invertebr Reprod Dev 45:161–167

Schirling M, Jungmann D, Ladewig V, Nagel R, Triebskorn R, Köhler HR (2005) Endocrine effects in *Gammarus fossarum* (Amphipoda): Influence of wastewater effluents, temporal variability, and spatial aspects on natural populations. Arch Environ Contam Toxicol 49:53–61

Schirling M, Jungmann D, Ladewig V, Ludwichowski KU, Nagel R, Köhler HR, Triebskorn R (2006) Bisphenol A in artificial indoor streams: II. Stress response and gonad histology in *Gammarus fossarum* (Amphipoda). Ecotoxicol 15:143–156

Sexton EW (1928) On the rearing and breeding of Gammarus in laboratory conditions. J Mar Biol Assoc UK 15:33–55

Siekierska E, Urbanska-Jasik D (2002) Cadmium effect on the ovarian structure in earthworm *Dendrobaena veneta* (Rosa). Environ Pollut 120:289–297

Sørensen M, Conder J, Fuchsman P, Martello L, Wenning R (2007) Using a sediment quality triad approach to evaluate benthic toxicity in the lower Hackensack river, New Jersey. Arch Environ Contam Toxicol 53:36–49

Villeneuve DL, Khim JS, Kannan K, Giesy JP (2002) Relative potencies of individual polycyclic aromatic hydrocarbons to induce dioxinlike and estrogenic responses in three cell lines. Environ Toxicol 17:128–137

Vinggaard AM, Hnida C, Larsen JC (2000) Environmental polycyclic aromatic hydrocarbons affect androgen receptor activation in vitro. Toxicology 145:173–183

Watts MM, Pascoe D, Carroll K (2002) Population responses of the freshwater amphipod *Gammarus pulex* (L.) to an environmental estrogen, 17 alpha-ethinylestradiol. Environ Toxicol Chem 21:445–450

Wirth EF, Fulton MH, Chandler GT, Key PB, Scott GI (1998) Toxicity of sediment associated PAHs to the estuarine crustaceans, *Palaemonetes pugio* and *Amphiascus tenuiremis*. Bull Environ Contam Toxicol 61:637–644

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Retinoid-like activity and teratogenic effects of cyanobacterial exudates

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ABSTRACT

Retinoic acids and their derivatives have been recently identified by chemical analyses in cyanobacteria and algae. Given the essential role of retinoids for vertebrate development this has raised concerns about a potential risk for vertebrates exposed to retinoids during cyanobacterial blooms. Our study focuses on extracellular compounds produced by phytoplankton cells (exudates). In order to address the capacity for the production of retinoids or compounds with retinoid-like activity we compared the exudates of ten cyanobacteria and algae using *in vitro* reporter gene assay. Exudates of three cyanobacterial species showed retinoid-like activity in the range of 269–2265 ng retinoid equivalents (REQ)/L, while there was no detectable activity in exudates of the investigated algal species. The exudates of one green alga (*Desmodesmus quadricaudus*) and the two cyanobacterial species with greatest REQ levels, *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii*, were selected for testing of the potential relation of retinoid-like activity to developmental toxicity in zebrafish embryos. The exudates of both cyanobacteria were indeed provoking diverse teratogenic effects (*e.g.* tail, spine and mouth deformation) and interference with growth in zebrafish embryos, while such effects were not observed for the alga. Fish embryos were also exposed to all-*trans* retinoic acid (ATRA) in a range equivalent to the REQ concentrations detected in exudates by *in vitro* bioassays. Both the phenotypes and effective concentrations of exudates corresponded to ATRA equivalents, supporting the hypothesis that the teratogenic effects of cyanobacterial exudates are likely to be associated with retinoid-like activity. The study documents that some cyanobacteria are able to produce and release retinoid-like compounds into the environment at concentrations equivalent to those causing teratogenicity in zebrafish. Hence, the characterization of retinoid-like and teratogenic potency should be included in the assessment of the potential adverse effects caused by the release of toxic and bioactive compounds during cyanobacterial blooms.

1. Introduction

In eutrophic conditions cyanobacteria can form dense blooms, which represent an unwanted ecological state due to various negative impacts on ecosystem function and environmental and human health. For instance, increasing pH and low oxygen levels associated with cyanobacterial blooms in surface waters and a reduced light penetration in water columns impact algae and macrophytes, and also fish populations (Scheffer et al., 1997; Wiegand and Pflugmacher, 2005). Moreover, cyanobacteria produce a wide spectrum of toxic metabolites. Cyanobacteria have been implicated in causing adverse effects in humans and other vertebrates (Hitzfeld et al., 2000; Ibelings and Havens, 2008; Kuiper-Goodman et al., 1999; Lévesque et al., 2013). Toxins produced by cyanobacteria include neurotoxins, hepatotoxins, cytotoxins, dermatotoxins and irritants (Aráoz et al., 2010; Kinnear, 2010; Stewart et al., 2006; Wiegand and Pflugmacher, 2005). Furthermore, compounds causing gastrointestinal tract and respiratory distress, immunotoxicity, carcinogenicity, genotoxicity and mutagenicity (Rastogi and Sinha, 2009) have been identified. The most studied cyanobacterial toxins are the hepatotoxic and tumor promoting microcystins (Bláha et al., 2009). Some recent studies have indicated the potential of cyanobacterial metabolites to interfere with the endocrine system (Rogers et al., 2011; Stěpánková et al., 2011). Because of the simultaneous presence of various bioactive compounds in cyanobacteria it is important to investigate both particular cyanotoxins and

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the toxicity of mixtures of compounds released from cyanobacteria. Numerous studies (Berry et al., 2009; Oberemm et al., 1997; Rogers et al., 2011) have shown that the toxicity of biomass extracts often cannot be explained by the level of the known cyanotoxins and have therefore suggested that other bioactive compounds contribute to the toxicity. Published studies investigating cyanobacterial metabolite mixtures have mostly focused on extracts of biomass (Berry et al., 2009; Rogers et al., 2011). However, limited information is available for cyanobacterial exudates, *i.e.* mixtures of extracellular compounds excreted during common physiological processes (Nováková et al., 2013). For instance, a recent study indicated high mortality in zebrafish embryos exposed to exudates of the cyanobacterium *Fischerella ambigua*, and this toxicity could not be explained by the level of known active compounds which were tested simultaneously (ambigol A, ambigol C, 2,4-dichlorobenzoic acid, and tjipanazole D) (Wright et al., 2006).

Recently, several retinoid compounds have been chemically identified in both biomass and exudates of some phytoplankton species (Wu et al., 2013, 2012). The same compounds were detected in water samples obtained from a eutrophic lake with cyanobacteria blooms (Taihu Lake, China) suggesting that these retinoids were probably produced by cyanobacteria. Retinoid-like activity was also detected in biomasses of seven cyanobacterial species using an *in vitro* yeast bioassays (Kaya et al., 2011). Retinoic acid (RA) plays an important role in vertebrate development and the pathways and proteins involved in retinoic acid signalling are highly conserved in vertebrates. RA is important for hindbrain, forebrain, fin and limb development and it is required to establish body axis symmetry (Rhinn and Dollé, 2012). Furthermore, germ layer formation, cardiogenesis, pancreas, eye and lung development are regulated by RA (Kam et al., 2012). Excessive amounts of retinoids, as well as their deficiency, cause teratogenicity (Collins and Mao, 1999).

High levels of retinoids might explain previous observations of diverse malformations, including several types of oedema, tail bents, undeveloped eyes or neural tube malformations in zebrafish embryos exposed to extracts of cyanobacteria *Microcystis aeruginosa*, *Anabaena flos-aquae*, *Cylindrospermopsis raciborskii*, *Aphanizomenon ovalisporum*, *Planktothrix agardhii*, and *Aphanizomenon flos-aquae* (Acs et al., 2013; Berry et al., 2009; Ghazali et al., 2009; Oberemm et al., 1999). Retinoic acids are known to cause various types of malformations in zebrafish embryos, such as yolk sac and heart edemas, brain and tail malformations, duplication of otic placodes and otoliths (Herrmann, 1995), elongated heart chambers, small intestine, absence of liver tissue (Haldi et al., 2011), and neurotoxicity (Parng et al., 2007).

The goal of this study was to determine *in vitro* retinoid-like activity of phytoplankton exudates and their effects on zebrafish embryo development and reveal the potential relation of the *in vitro* activity to *in vivo* effects. Exudates (metabolites produced and released into water by living cells) of ten phytoplankton species, including both algae and cyanobacteria were studied using *in vitro* assay for retinoid-like activity. The two most potent and one negative exudate were then tested in detail in zebrafish embryos. Fish embryos were also exposed to all-trans retinoic acid (ATRA) in a range corresponding to the retinoic acid equivalents (REQ) detected in exudates by *in vitro* bioassays. ATRA was used as a positive control due to its frequent detection in cyanobacterial extracts and exudates (Wu et al., 2012), reported highest teratogenicity among retinoids in zebrafish (Herrmann, 1995) and its use as standard ligand in *in vitro* assays for total retinoid-like activity, which is generally expressed as concentration equivalents of ATRA (Kaya et al., 2011; Novák et al., 2007). The phenotypes provoked by the exudates in zebrafish embryos and the effective concentrations of *in vitro* determined REQ were compared to those from exposure to ATRA.

2. Materials and methods

2.1. Cyanobacterial strains and culture conditions

The identification and source of investigated cyanobacterial and algal strains and the microcystin content of their exudates are listed in Table 1. All strains were cultivated in a mixture of Zehnder (Schlosser, 1994) and Bristol (modified Bold) medium (Stein, 1973) with distilled water in the ratio of 1:1:2 (v/v/v). Organisms were grown for 21 days at 22 °C ± 2 °C under continuous light (cool white fluorescent tubes, 3000 lx) and aeration with air filtered through a 0.22 μm membrane (Labicom, Czech Republic). The cultivations were started with a 20% (v/v) inoculum of a previous culture.

2.2. Exudate preparation

Spent growth media were separated from the cyanobacterial and algal cells (biomass) by centrifugation (2880 × *g*, 10 min, 25 °C) after 21 days of culture and filtered through a 0.6 μm glass fiber filter (Fisher Scientific, Czech Republic). Organic compounds present in the media (exudates) were concentrated by solid phase extraction (SPE) using an Oasis HLB column (Waters, USA) and Carbograff column (Alltech, USA) in sequence. The SPE procedure was performed according to the manufacturer's instructions for HLB and Carbograff columns. Each sample was first passed through the HLB, then through the Carbograff column. Both columns were then eluted with 100% MeOH. The eluates were concentrated using a rotary evaporator at room temperature (22 ± 1 °C). For exposure, eluates from both columns were pooled to obtain maximal recovery. A final concentration of exudates that corresponded to 2000-fold concentrated original media was reached using evaporation under a stream of inert gas (nitrogen) at room temperature and the addition of 100% methanol (Nováková et al., 2011).

2.3. Microcystin analyses

Microcystins were analysed in exudates after SPE extraction by HPLC Agilent 1100 Series coupled with a PDA detector (Agilent Technologies, Germany) using C18 Supelcosil ABZ + Plus column, 150 × 4.6 mm, 5 μm (Supelco, USA), and gradient elution with acetonitrile (Babica et al., 2006). Microcystins were identified by comparing the UV spectra and retention times with standards of microcystin-LR, -YR, -RR (MW 995, 1045, 1038 g/mol, respectively, Enzo Life Sciences, Switzerland) and quantified using calibration standards (limit of detection 0.025 μg/L).

2.4. Reporter gene assay

For the study of *in vitro* retinoid-like activity, we used the murine embryonic carcinoma cell line P19 (European Collection of Cell Culture, UK) transfected with a luciferase reporter pRAREβ2-TK-luc plasmid (P19/A15 clone) (Novák et al., 2007). The plasmid contains a reporter luciferase gene under the control of a retinoic acid-responsive element. Cells were cultured in plastic tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum Mycoplex (PAA, Austria) at 37 °C in a humidified atmosphere of 5% CO_2.

For the RAR/RXR transactivation assay, 10,000 cells per well were seeded into 96-well microplates in DMEM with gentamicin (1%) and incubated overnight under above described conditions. After 24 h, the cells were exposed to tested samples and calibration standard diluted in dimethylsulphoxide (DMSO), which was also used as a solvent control. The exudates of six cyanobacteria and four

Table 1
List of investigated phytoplankton species, their origin, microcystin content and total retinoid equivalent (REQ) of their exudates determined by *in vitro* assays.

Species	Source[a]	Place of origin		MCs concentration (μg/L)[b]			REQ[c]
		Country	Water Body	MC-RR	MC-YR	MC-LR	ng ATRA/L
Cyanobacteria							
Nostocales							
Cylindrospermopsis raciborskii	SAG 1.97	Hungary	Lake Balaton	n.d.	n.d.	n.d.	2265
Aphanizomenon gracile	RCX 06[d]	Ireland	Lough Neagh	n.d.	n.d.	n.d.	269
Anabaena flos-aquae	UTEX 1444	USA	Mississippi River	n.d.	n.d.	n.d.	n.d.
Aphanizomenon klebahnii	CCALA 009	UK	Queen Elizabeth Reservoir	n.d.	n.d.	n.d.	n.d.
Chroococcales							
Microcystis aeruginosa	PCC 7806	Netherlands	Braakman Reservoir	n.d.	n.d.	232.4	414
Oscillatoriales							
Planktothrix aghardii	CCALA 159	Czech Republic	Unknown	n.d.	0.085	0.025	n.d.
Chlorophyta							
Sphaeropleales							
Desmodesmus quadricaudatus	CCALA 463	Germany	Greifswald	n.d.	n.d.	n.d.	n.d.
Ankistrodesmus falcatus	CCALA 211	Unknown	Unknown	n.d.	n.d.	n.d.	n.d.
Chlorellales							
Chlorella kessleri	CCALA 253	Russia	Unknown	n.d.	n.d.	n.d.	n.d.
Chlamydomonadales							
Chlamydomonas reinhardtii	UTEX 2246	USA	Amherst	n.d.	n.d.	n.d.	n.d.

[a] Culture collection ID for laboratory cultured strains: CCALA—Culture Collection of Autotrophic Organisms, Institute of Botany, Academy of Sciences of the Czech Republic; RCX—RECETOX Culture Collection of Cyanobacteria and Algae; PCC—Pasteur Culture Collection of Cyanobacteria; SAG–Culture Collection of Algae at University of Göttingen; UTEX—Culture Collection of Algae at University of Texas in Austin.
[b] Limit of detection: 0.025 μg/L; MCs—microcystins: microcystin-LR, -YR, -RR (MW 995, 1045, 1038 g/mol, respectively); n.d.—not detected (below limit of detection).
[c] Retinoid equivalent of ATRA (MW 300.4 g/mol) in exudate (method limit of detection was 30 ng/L).
[d] This species originates from CCALA (strain 008), but has been long-term cultivated at RECETOX.

algae (Table 1) were exposed in a dilution series corresponding to a range of 1×–10× concentrated samples. Each plate also contained an exposure with a calibration standard of all-*trans* retinoic acid (ATRA, MW 300.4 g/mol) at concentration range of 0.5–10,000 nM (0.15–3004 μg/L). The final concentration of the solvent did not exceed 0.5% v/v (corresponding to the addition of 1 μL concentrated exudate/ATRA standard per 200 μL media/well). The activity of the reporter luciferase induced in the presence of RAR/RXR ligands was measured after 24 h exposure using Promega Steady Glo Kit (Promega, USA) with a microplate luminometer (Luminoskan Ascent, Thermo Electron Corp., USA). At least three independent experiments were performed for each cyanobacterial or algal exudate sample, with three technical replicates per each concentration.

2.5. Zebrafish husbandry and embryo collection

Adult zebrafish of UFZ-OBI strain were maintained in a recirculated flow-through system with local tap water, the temperature adjusted to 26 ± 1 °C, and the photoperiod set to 14 h light and 10 h dark. Fish were fed by live brine shrimp (*Artemia salina*) twice a day. Fish embryos were collected immediately after spawning in the morning. Fertilised embryos were rinsed with tank water and transferred to standard test medium (ISO, 2008, 1996). Details of zebrafish husbandry and embryo production are described elsewhere (*e.g.* Nagel, 2002).

2.6. Exposure of zebrafish embryos

Exudates of *Desmodesmus quadricaudatus*, *M. aeruginosa* and *C. raciborskii* were used for zebrafish embryos exposure. The appropriate amount of exudate sample in methanol was added into empty exposure dishes (80 mL crystallization dishes). The methanol was left to evaporate at room temperature in a fume hood. Twenty milliliter of standard test medium was added to each dish immediately after methanol evaporation to dissolve the dried exudates to meet 1, 3.3, 10, 17 (*D. quadricaudatus*, *M. aeruginosa*) or 1, 3.3, 10, 33 (*C. raciborskii*) fold concentrations of the original exudates. Exposure media with exudates were mixed by agitation, briefly ultrasonicated and mixed again. Subsequently, 20 zebrafish embryos at the stage of 24 h post fertilization (hpf) were added into each prepared exposure dish. Exposure media were renewed after two days of exposure. The exposure was terminated at 5 days post fertilization (dpf). The temperature was kept at 26 ± 1 °C and the photoperiod was set to 12 h light and 12 h dark. Each exudate was tested in three independent experiments on different days. Each independent experiment included three negative controls (standard medium). Mortality and teratogenicity were analysed daily. The length of embryos was only measured at the end of the exposure. pH and dissolved oxygen (measured by Fibox 3 trace; PreSens, Germany) were measured at 72 and 120 hpf.

For comparison, an exposure of zebrafish embryos with ATRA was performed with the same experimental setup as used for exudate testing. Concentrations of 0.4, 1.3, 4, 12, 36 and 108 μg/L (1.3–360 nM) ATRA were analysed in parallel with appropriate negative (standard medium) and solvent (DMSO 0.01%) controls. ATRA was added to the test medium using DMSO stock solutions with final DMSO concentrations of 0.01%. ATRA effects were analysed in two independent experiments.

2.7. Toxicity, teratogenicity and length

Mortality and teratogenicity (*i.e.* any deviation from normal development) were analysed daily using a stereomicroscope and observation of morphological endpoints as described by Nagel (2002). Furthermore, craniofacial disorders (particularly mouth deformities) were recorded. Spontaneous movement and growth retardation were only analysed at 48 hpf and 120 hpf, respectively. Standard length of embryos as defined by OECD guideline 210 (length of the fish without the caudal fin, OECD, 2013) was measured with the software QuickPhoto Micro 2.3 (PROMICRA, Czech Republic) using digital images of embryos.

2.8. Data analysis

Statistical analysis was conducted with the software Statistica version 10 (StatSoft, USA) if not specified otherwise. Total retinoid-like activity was determined using the equi-effective approach and the results were expressed as retinoic acid equivalents (REQ) with

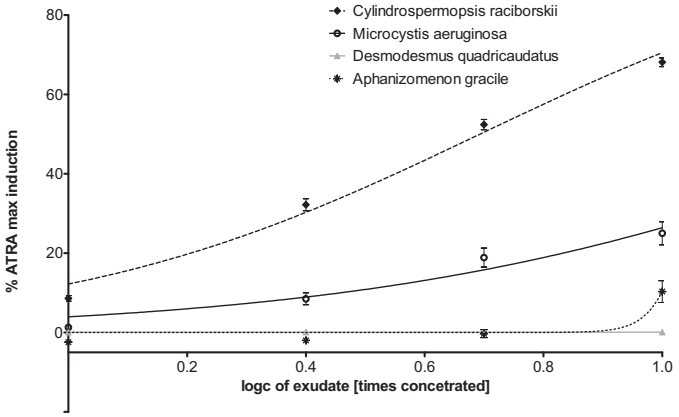

Fig. 1. Concentration-response curves of the retinoid-like activity (expressed as % of maximal RAR-mediated induction caused by all-*trans* retinoic acid = ATRAmax, 500 nM) in the P19/A15 cell line after 24 h exposure to exudates of cyanobacteria and algae.

respect to the ATRA standard (Villeneuve et al., 2000). Relative luminescence units obtained from *in vitro* cellular reporter assay were converted to percent of maximum response of the standard curves with ATRA. EC_X values were calculated from nonlinear logarithmic regression of dose–response curves of calibration standards and samples (GraphPad Prism, GraphPad Software, USA). REQs for exudate samples where luminescence measured at the highest tested concentration exceeded 20% of maximal induction reached by ATRA were calculated by relating the EC_{20} value of standard calibrations with the concentration of the tested sample inducing the same response (Villeneuve et al., 2000). In case of *Aphanizomenon gracile*, where only the highest tested concentration caused a significant induction, the REQ was derived as a point estimate from the effect of this concentration according to EC_X ATRA/EC_X sample, where *X* represents percentage induction caused by this effective concentration. The significance of differences in responses among exposures and controls was tested by ANOVA with Dunnett's post hoc test.

Fisher exact chi-square test was used for the calculation of significance of teratogenic effects and mortality (Wiegand et al., 2001). Statistically significant differences in length were identified by ANOVA and Dunnett's *post-hoc* test. The EC_{50} of ATRA for malformations in zebrafish embryos was calculated using the software GraphPad Prism with Hill slope model.

3. Results

Exudates from only two of the tested species contained microcystins levels above the detection limit (>0.025 μg/L). Relatively low levels of the microcystin variants MC-LR and MC-YR (0.11 μg/L in total) were detected in exudates from *P. agardhii*. Two thousand fold greater levels of microcystin-LR (232 μg/L) were found in exudates of *M. aeruginosa* (Table 1).

3.1. In vitro retinoid-like activity

Exudates of six cyanobacterial and four algal species were tested in order to determine their retinoid-like activity. None of the algal exudates showed retinoid-like activity up to the highest concentrations tested (10×) (limit of detection 30 ng REQ/L). In contrast, three out of the six tested cyanobacterial exudates elicited retinoid-like activity (Fig. 1). The *in vitro* assay revealed the highest concentrations of retinoid-like compounds in the exudates of *C. raciborskii* (2265 ng REQ/L equivalent); retinoid-like activity was detected at as low as the 1-fold concentration of original exudates. These REQ levels were about one order of magnitude higher than those determined for the other species where retinoid-like activity was detected. Lower concentrations of REQ were detected in exudates of *M. aeruginosa* and *A. gracile* (414 and 269 ng/L, respectively, Table 1).

3.2. Toxicity and teratogenicity in zebrafish embryos

Based on the *in vitro* analysis of retinoid-like activity, the exudates of two cyanobacterial species (*C. raciborskii* and *M. aeruginosa*) with high levels of REQs and one negative algal species (*D. quadricaudatus*) were selected for assessment of teratogenicity in zebrafish embryos. Mortality and teratogenic effects in zebrafish embryos exposed to exudates were only detected for the two selected REQ-positive cyanobacteria species (Table 2, Fig. 2). *C. raciborskii* exudate caused tail tip deformation (15% of embryos) at as low as 1× concentrations of exudates. At 3.3× concentration spine and mouth deformations were observed in all embryos from 96 hpf. At the end of exposure, all surviving embryos exposed to 10× exudate concentration exhibited heart edema and gross malformation (*e.g.* Fig. 2B) characterised by the simultaneous occurrence of several types of malformations, such as smaller deformed head, elongated heart chambers, trunk edema and pectoral fin deformities. This concentration also caused yolk deformation and mortality. The highest tested concentration of this exudate (33×) caused 100% mortality at 96 hpf. *M. aeruginosa* exudate caused tail tip deformation, gross malformation and heart edema (10× concentrated sample), and tail tip and yolk deformations and more than 50% mortality as early as 72 hpf (17×). Additionally, 30% of embryos exposed to 3.3× concentrated exudates had tail tip deformation, but this effect was not statistically significant. In general the malformations appeared to be concentration-dependent, *i.e.* an increase in the frequency of phenotypes was observed with higher concentrations. Comparisons of the malformation rates indicate weaker effects with respect to the fold concentration for exudate of *M. aeruginosa*. However, for this species approximately 5 fold lower REQ levels compared to *C. raciborskii* were determined by *in vitro* assays.

Exposure to ATRA caused similar phenotypes as observed for exudates (Table 3, Fig. 2). Tail tip, spine and mouth deformation represented the malformations that were observed at the lowest concentration (1.3 μg/L, 4.3 nM). At higher concentrations heart edema and gross malformation (12 μg/L, 40 nM), yolk deformation (36 μg/L, 120 nM) and mortality (108 μg/L, 360 nM) were also observed. An EC_{50} of 0.76 μg/L (2.53 nM) and a corresponding teratogenic index (LC_{50}/EC_{50}) of 142 were calculated for ATRA based on a cumulative frequency analysis of all malformations, which often occurred simultaneously in the same embryos (Table 3, Figure S1 in Supplementary material).

Hatching rates were significantly affected by all exudates in the majority of tested concentrations at 72 hpf. Most exposure variants caused an earlier hatching (Table 2). However, exposure to low concentrations of *D. quadricaudatus* (1 and 3.3 times concentrated exudate) led to a delayed hatching at 72 hpf. No effect on hatching was observed for ATRA.

As a further indicator of interference with development, the length of embryos was analysed at 5 dpf (Table 4). The length was significantly increased by about 3–5% in exposures to 1× and 3× exudates of *M. aeruginosa* and to 1× exudates of *C. raciborskii*. This increase was observed at similar REQ levels (0.4–1.3 and 2.3 μg/L REQ). A decrease in length by 9.4% and 16.6% was observed for higher sublethal concentrations (10×) of *M. aeruginosa* and *C. raciborskii*, respectively. *D. quadricaudatus* exudates did not significantly affect the length of embryos. In the case of ATRA exposure the length was statistically significantly increased by about 3% in

Table 2
Toxicity and teratogenic effects observed in zebrafish embryos exposed to cyanobacterial exudates in relation to the exudate concentration and REQ levels. Frequency of effects (in %) represents means ± standard deviation of three replicates.

Time		Control	*Cylindrospermopsis raciborskii*				*Microcystis aeruginosa*				Desmodesmus quadricauda			
	Fold concentration		1×	3.3×	10×	33×	1×	3.3×	10×	17×	1×	3.3×	10×	17×
	REQ[a] (μg/L)	0	2.3	7.5	22.7	74.7	0.4	1.4	4.1	7.0	0	0	0	0
	Endpoint													
48 hpf	Yolk deformation	1 ± 2	0	0	93 ± 8*	75 ± 15*	0	2 ± 3	17 ± 29	77 ± 21*	0	0	0	0
	Tail tip deformation	0	0	72 ± 26*	98 ± 3*	0	0	10 ± 10	57 ± 10*	98 ± 3*	0	0	0	0
	Heart edema	0	2 ± 3	0	22 ± 38	0	0	0	0	0	0	0	0	0
	Mortality	0	0	0	2 ± 3	25 ± 15*	0	0	0	0	0	0	0	0
	Hatched	0	13 ± 23	7 ± 12	20 ± 23	0	0	0	2 ± 3	0	0	0	2 ± 3	0
72 hpf	Tail tip deformation	0	0	100*	100*	–	0	12 ± 10	100*	45 ± 36*	0	0	0	0
	Heart edema	0	0	0	90 ± 17*	–	0	0	0	33 ± 49	0	0	0	0
	Spine deformation	0	0	30 ± 52	0	–	0	0	0	0	0	0	0	0
	Mortality	0	0	0	2 ± 3	97 ± 6*	0	0	0	55 ± 36*	0	0	0	0
	Hatched	34 ± 31	70 ± 18*	80 ± 5*	67 ± 28*	–	45 ± 48	87 ± 8*	58 ± 21*	–	13 ± 15*	13 ± 8*	58 ± 45*	63 ± 33*
96 hpf	Tail tip deformation	0	0	100*	90 ± 5*	–	0	0	98 ± 3*	17 ± 29	0	0	0	0
	Heart edema	0	0	0	90 ± 5*	–	0	0	72 ± 28*	17 ± 29	0	0	0	0
	Spine deformation	1 ± 2	0	100*	0[b]	–	0	0	0	0	0	0	0	0
	Mouth deformation	0	0	100*	0[b]	–	0	0	0	0	0	0	0	0
	Mortality	0	0	0	10 ± 5*	100*	3 ± 6	0	3 ± 3	80 ± 26*	0	0	0	0
	Hatched	78 ± 31	100	98 ± 2	90 ± 5	–	95	98 ± 3	98 ± 3	–	100	100	100	100
120 hpf	Tail tip deformation	0	15 ± 9*	100*	80 ± 15*	–	0	30 ± 15	98 ± 3*	0	0	0	0	0
	Heart edema	0	0	0	80 ± 15*	–	0	0	72 ± 28*	15 ± 26	0	0	0	0
	Spine deformation	0	0	100*	0[b]	–	0	0	0	0	0	0	0	0
	Mouth deformation	0	0	100*	0[b]	–	0	0	0	0	0	0	0	0
	Gross malformation	1 ± 2	2 ± 3	0	80 ± 15*	–	0	0	65 ± 18*	17 ± 25	0	0	0	0
	Mortality	0	0	0	20 ± 15*	100*	0	0	3 ± 3	83 ± 25*	0	0	0	0
	Hatched	94 ± 6	100	100	80 ± 15	–	100	100	100	–	100	100	100	100

* Significantly different from control ($p \leq 0.05$).
– Not assessed due to mortality.
[a] Retinoid equivalent of ATRA (MW 300.4 g/mol) in exudate (limit of detection 30 ng/L).
[b] Specific mouth and spine malformations could not be evaluated since they were masked by more severe malformations (Fig. 2).

Table 3
Lowest observed effect concentrations (LOEC) and median effective concentration (EC50) in exposure of zebrafish embryos to ATRA and LOEC based on ATRA equivalents (REQ) for cyanobacterial exudates.

	Mortality	Deformations				Gross malformation	Heart edema	Length	
		Spine	Tail tip	Mouth	Yolk			Decrease	Increase
LOEC (μg/L ATRA[a])	108.0	1.3	1.3	1.3	36.0	12.0	12.0	36.0	0.4
EC50 (μg/L ATRA)	108.0	2.1	1.8	1.1	20.8	13.6	12.6		
Cylindrospermopsis raciborskii LOEC (μg/L REQ)	22.7	7.5	2.3	7.5	22.7	22.7	22.7	22.7	2.3
Microcystis aeruginosa LOEC (μg/L REQ)	7.0	4.1	4.1	–	7.0	4.1	4.1	4.1	0.4

[a] ATRA, MW 300.4 g/mol.

Table 4
Comparison of length of embryos exposed to all-*trans* retinoic acid (ATRA) and cyanobacterial exudates relative to the mean length in controls at the end of the experiment (5 days post fertilisation). The first column shows concentration of all-*trans* retinoic acid or retinoid equivalents (REQ) in exudates. The numbers in parenthesis show fold concentration of original exudate. Results shown as means ± standard deviation.

ATRA or REQ[a] (μg/L)	ATRA	Exudates	
		Cylindrospermopsis raciborskii	*Microcystis aeruginosa*
Control	100 ± 2.4	100 ± 1.7	100 ± 1.7
0.4	103.3 ± 2.1*		103.7 ± 1.2* (1×)
1.3	103.5 ± 7.9*		104.8 ± 0.0* (3.3×)
2.3		103.9 ± 1.9* (1×)	
4	99.0 ± 3.6		90.6 ± 2.5* (10×)
7.5		101.0 ± 1.5 (3.3×)	
12	94.7 ± 8.1		
22.7		83.4 ± 4.8* (10×)	
36	79.9 ± 7.0*		
108	72.4 ± 18.2*		

[a] Retinoid equivalent of ATRA (MW 300.4 g/mol) in exudate.
* $p \leq 0.05$.

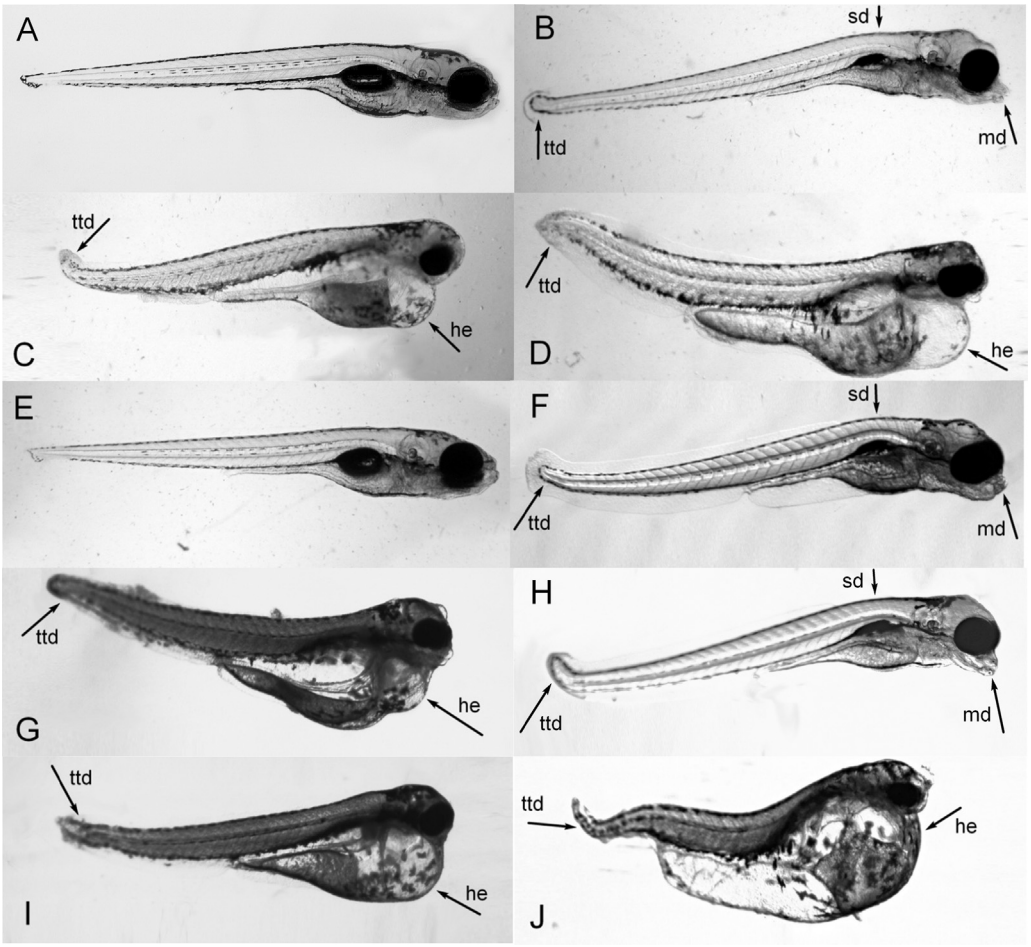

Fig. 2. Comparison of phenotypes of control and zebrafish embryos exposed to all-*trans*-retinoic acid (ATRA) and cyanobacterial and algal exudates. Images were taken at 120 h post fertilisation. Control (A), exudates of *C. raciborskii* 3.3× (B) and 10× (C), *M. aeruginosa* 10× (D) and *D. quadricaudatus* 17× (E). Exposures to ATRA at 4 μg/L (13.3 nM) (F), two variants of phenotype at 12 μg/L (40 nM) ((G) and (H)), 36 μg/L (120 nM) (I) and 108 μg/L (360 nM) (J). Zebrafish embryos depicted in figures (C), (D), (G), (I) and (J) exhibit gross malformation. Specific effects are marked by an arrow and appropriate abbreviations: ttd—tail tip deformation, he—heart edema, sd—spine deformation, md—mouth deformation.

exposure to 0.4 μg/L and 1.3 μg/L (1.3 and 4.3 nM) and decreased at higher test concentrations (≥36 μg/L, Table 4).

4. Discussion

Similarities in malformations observed in wild frogs and frogs exposed to retinoids in laboratory had raised concerns about the presence of retinoid-like compounds in the environment (Gardiner and Hoppe, 1999). However, the potential relevance of retinoids was controversially discussed, and the need to provide evidence for the occurrence and sources of retinoids in water bodies was emphasized (Stocum, 2000). For instance, REQ levels up to 10.9 ng/L and 1.7 ng/L were detected in influents and effluents of waste water treatment plants (WWTP), respectively, and up to 8.3 ng/L was detected in receiving rivers (Zhen et al., 2009). Concentrations of six retinoids not exceeding 1.23 ng/L in other rivers were attributed to untreated sewage effluents (Wu et al., 2010). However, this concentration was considered not high enough to cause developmental disorders in frogs.

As documented in our and a few previous studies, cyanobacteria and algae could also represent a possible source of retinoid compounds. Retinoids have been discovered in various species indicating a potential risk to animals and human health—particularly in eutrophic environments and during phytoplankton blooms (Kaya et al., 2011; Wu et al., 2013, 2012). The retinoid compounds may be released *via* exudates or from intracellular sources after cell death. Chemical analysis documented the presence of several retinoids in both biomass and exudates of some cyanobacteria, and retinoid-like activity has been detected by *in vitro* assay in biomass (Kaya et al., 2011; Wu et al., 2012, 2013). Our study, however, is the first to report total retinoid-like activity using an *in vitro* bioassay also for exudates of several phytoplankton species. Cyanobacterial exudates exhibited detectable retinoid-like activity, but none of the tested algal exudates did. More compounds than those previously determined by chemical analysis (Wu et al., 2012, 2013) can contribute to total retinoid-like activity detected by *in vitro* bioassay. Despite this, the previously analytically-determined contents of retinoids correspond to our results on retinoid-like bioactivity for the exudates of the species included in both our and previous studies. There is a good agreement especially considering that the comparable model species of the previous studies originated from China, while strains of European and North American origin from international collections were used in our study (Table 1). No retinoid-like compounds were detected in exudates of *Desmodesmus*, *Chlorella* and *Chlamydomonas* species in any of these studies, while *M. aeruginosa* represented the species with the highest retinoid content (Wu et al., 2013, 2012). Our study also included additional species not investigated before. Of these species exudates of *Planktothix agardhii* and *Aphanizomenon klebanii* did not reveal REQ above the detection limit, while for *A. gracile* and *C. raciborskii* REQ levels of 269 ng/L and 2265 ng/L, respectively, were observed. The REQ of *C. raciborskii* exudate is more than 100-fold

higher than the highest REQ measured in WWTP effluents or their receiving rivers (Zhen et al., 2009) and more than 1000-fold higher than concentrations in river water from another study (Wu et al., 2010), indicating that indeed cyanobacteria could represent a relevant sources of retinoids in the environment.

The REQs detected in phytoplankton exudates by *in vitro* assay were paralleled by diverse developmental effects observed in exposed zebrafish embryos. These were only observed in REQ-positive species. The only endpoint affected by both cyanobacteria and algae exudates was hatching. However, the hatching pattern differed between cyanobacteria and algae. This provided additional evidence for a different composition of exudates from the investigated algae and cyanobacteria. The strongest teratogenic effects on zebrafish embryos were observed for *C. raciborskii*, which contained the highest REQ levels. The tail tip deformation was observed even in 1× concentrated exudates of *C. raciborskii*, which can be released into water by normal physiological processes.

Comparison of zebrafish developmental effects caused by exposure to cyanobacterial exudates with effects caused by ATRA showed a strong concordance of phenotypes. For both exudates and ATRA, malformations of tail tip, spine, yolk and mouth, heart edemas, and at higher concentrations, gross malformations and mortality were observed. Similar effects had also been observed for ATRA in other studies (Haldi et al., 2011; Herrmann, 1995; Selderslaghs et al., 2009). Furthermore, there was a strong coincidence in exudates and ATRA effects on embryonic growth (indicated by length), with a similar concentration dependency indicating growth stimulation at low and retardation at high REQ concentrations (Table 4). Since RA is known to regulate and increase growth hormone expression *in vitro* in human and fish (carp, salmon, and zebrafish) (Bedo et al., 1989; Guibourdenche et al., 1997; Sternberg and Moav, 1999) the increased length at low concentrations could be related to an elevation of embryonic growth hormone levels. Further, the growth stimulation could be linked to the fact that retinoids are related to and may act similarly to vitamin A, which is important for growth (Collins and Mao, 1999; Bedo et al., 1989). The decreased length of embryos observed at greater concentrations of ATRA as well as in ten times concentrated exudates of *C. raciborskii* and *M. aeruginosa* is probably related to overall malformations and toxic effects. The observation of reduced length corresponds to a previous study with zebrafish embryos exposed to ATRA (Herrmann, 1995). The ATRA effective concentrations are very similar to the levels of REQs corresponding to the LOECs of exudates (Tables 3 and 4), which provides further support that retinoid-like compounds are responsible for the teratogenic effects. The accuracy of LOEC determination is relatively sensitive to aspects of test design including the number of replicates and the number and spacing of concentration tested. In this particular application, when investigating the effect of unknown mixtures with very limited sample volumes, the use of such metric was necessary. Care was taken to use appropriate test design to minimize the uncertainties and reach the goals of our study.

Taken together, in the case of *C. raciborskii* the effects were in line with equivalent ATRA concentrations indicating that the retinoids may represent the most important compounds causing toxicity of this exudate. This also applies to sublethal effects of *M. aeruginosa* exudate. However, *M. aeruginosa* exudate caused mortality at lower REQ concentrations than ATRA, indicating the potential influence of other toxic compound/s, including microcystins or compounds modifying the toxicity of ATRA. Even though microcystins might have contributed to some effects of *M. aeruginosa* exudate, it did not correspond to the developmental effects. The greatest teratogenicity was observed for exudates of *C. raciborskii*, for which chemical analysis did not indicate any microcystins. Furthermore, zebrafish embryos are known to be only weakly affected by microcystins in water-borne exposure, possibly because of a restricted uptake of this high molecular weight compound through biological membranes or the chorion (Berry et al., 2007; Wang et al., 2005). Microcystins, often related to harmful effects caused by cyanobacteria in mammals and fish (Ibelings and Havens, 2008; Malbrouck and Kestemont, 2006), may not represent the most important compounds with respect to toxicity of cyanobacterial metabolites for some species or developmental stages of fish and amphibians (Ibelings and Havens, 2008; Jaja-Chimedza et al., 2012; Wang et al., 2010).

In conclusion, our findings stress the importance of testing the effects of cyanobacterial exudates, which have so far only rarely been addressed. We demonstrated that the observed teratogenicity of cyanobacterial exudates is likely related to retinoids. Further investigations are needed for the identification of the compounds responsible for the observed effects. Given the high levels of REQs, exudates from cyanobacterial blooms may represent a possible risk for the development of fish and other vertebrate species in surface waters. Hence, the characterization of their retinoid-like and teratogenic potency should be included in the assessment of the potential adverse effects caused by release of toxic and bioactive compounds during cyanobacterial blooms. It was shown that the zebrafish embryo provides a suitable model for developmental toxicity studies with phytoplankton exudates. Since the zebrafish embryo exhibits a similar sensitivity to various RA as mammals (Herrmann, 1995) it may be used as a routine whole organism model to study the hazard of retinoid-like metabolites. Our findings, together with high conservation of retinoic acid signalling among vertebrates, contribute to concern about potential risks of retinoid-like cyanobacterial metabolites also to mammals and humans (Wu et al., 2013, 2012).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2014.06.022.

References

Acs, A., Kovács, A.W., Csepregi, J.Z., Törő, N., Kiss, G., Győri, J., Vehovszky, A., Kováts, N., Farkas, A., 2013. The ecotoxicological evaluation of *Cylindrospermopsis raciborskii* from Lake Balaton (Hungary) employing a battery of bioassays and chemical screening. Toxicon 70C, 98–106, doi: 10.1016/j.toxicon.2013.04.019.

Aráoz, R., Molgó, J., Tandeau de Marsac, N., 2010. Neurotoxic cyanobacterial toxins. Toxicon 56, 813–828, doi: 10.1016/j.toxicon.2009.07.036.

Babica, P., Kohoutek, J., Blaha, L., Adamovsky, O., Marsalek, B., 2006. Evaluation of extraction approaches linked to ELISA and HPLC for analyses of microcystin-LR, -RR and -YR in freshwater sediments with different organic material contents. Anal. Bioanal. Chem. 385, 1545–1551.

Bedo, G., Santisteban, P., Aranda, A., 1989. Retinoic acid regulates growth hormone gene expression. Nature 339, 231–234, doi: 10.1038/339231a0.

Berry, J.P., Gantar, M., Gibbs, P.D.L., Schmale, M.C., 2007. The zebrafish (*Danio rerio*) embryo as a model system for identification and characterization of developmental toxins from marine and freshwater microalgae. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 145, 61–72, doi: 10.1016/j.cbpc.2006.07.011.

Berry, J.P., Gibbs, P.D.L., Schmale, M.C., Saker, M.L., 2009. Toxicity of cylindrospermopsin, and other apparent metabolites from *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum*, to the zebrafish (*Danio rerio*) embryo. Toxicon 53, 289–299.

Bláha, L., Babica, P., Maršálek, B., 2009. Toxins produced in cyanobacterial water blooms—toxicity and risks. Interdiscip. Toxicol. 2, 36–41, doi: 10.2478/v10102-009-0006-2.

Collins, M.D., Mao, G.E., 1999. Teratology of retinoids. Annu. Rev. Pharmacol. Toxicol. 39, 399–430, doi: 10.1146/annurev.pharmtox.39.1.399.

Gardiner, D.M., Hoppe, D.M., 1999. Environmentally induced limb malformations in mink frogs (*Rana septentrionalis*). J. Exp. Zool. 284, 207–216.

Ghazali, I., El, Saqrane, S., Carvalho, A.P., Ouahid, Y., Oudra, B., Del Campo, F.F., Vasconcelos, V., 2009. Compensatory growth induced in zebrafish larvae after pre-exposure to a *Microcystis aeruginosa* natural bloom extract containing microcystins. Int. J. Mol. Sci. 10, 133–146, doi: 10.3390/ijms10010133.

Guibourdenche, J., Djakouré, C., Porquet, D., Pagésy, P., Rochette-Egly, C., Peillon, F., Yuan Li, J., Evain-Brion, D., Li, J.Y., 1997. Retinoic acid stimulates growth hormone synthesis in human somatotrophic adenoma cells: characterization of its nuclear receptors. J. Cell. Biochem. 65, 25–31.

Haldi, M., Harden, M., D'Amico, L., DeLise, A., Seng, W.L., 2011. Developmental toxicity assessment in zebrafish. In: Zebrafish. John Wiley, Sons, Inc., pp. 15–25, doi: 10.1002/9781118102138.ch2.

Herrmann, K., 1995. Teratogenic effects of retinoic acid and related substances on the early development of the zebrafish (*Brachydanio rerio*) as assessed by a novel scoring system. Toxicol. In Vitro 9, 267–283.

Hitzfeld, B.C., Höger, S.J., Dietrich, D.R., 2000. Cyanobacterial toxins: removal during drinking water treatment, and human risk assessment. Environ. Health Perspect. 108 (Suppl), 113–122.

Ibelings, B.W., Havens, K.E., 2008. Cyanobacterial toxins: a qualitative meta-analysis of concentrations, dosage and effects in freshwater, estuarine and marine biota. In: Hudnell, H.K. (Ed.), Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs. Springer Science+Business Media, LLC, New York, Chapter 32. doi: 10.1007/978-0-387-75865-7_32.

ISO, 1996. ISO 7346—Water Quality—Determination of the Acute Lethal Toxicity of Substances to a Freshwater Fish [Brachydanio rerio Hamilton-Buchanan (*Teleostei, Cyprinidae*)]—Part 1: Static method; Part 2—Semi-Static Method. ISO.

ISO, 2008. International Standard Organization, Water Quality—Determination of the Acute Toxicity of Waste Water to Zebrafish Eggs (*Danio rerio*). Eur. Stand. EN ISO 15088. ISO.

Jaja-Chimedza, A., Gantar, M., Gibbs, P.D.L., Schmale, M.C., Berry, J.P., 2012. Polymethoxy-1-alkenes from *Aphanizomenon ovalisporum* inhibit vertebrate development in the zebrafish (*Danio rerio*) embryo model. Mar. Drugs 10, 2322–2336, doi: 10.3390/md10102322.

Kam, R.K.T., Deng, Y., Chen, Y., Zhao, H., 2012. Retinoic acid synthesis and functions in early embryonic development. Cell Biosci. 2, 11, doi: 10.1186/2045-3701-2-11.

Kaya, K., Shiraishi, F., Uchida, H., Sano, T., 2011. A novel retinoic acid analogue, 7-hydroxy retinoic acid, isolated from cyanobacteria. Biochim. Biophys. Acta, Gen. Subj. 1810, 414–419, doi: 10.1016/j.bbagen.2010.11.009.

Kinnear, S., 2010. Cylindrospermops: a decade of progress on bioaccumulation research. Mar. Drugs 8, 542–564.

Kuiper-Goodman, T., Falconer, I., Fitzgerald, J., 1999. Human health aspects. In: Chorus, I., Bartram, J. (Eds.), Toxic Cyanobacteria in Water: A Guide to their Public Health Consequences. Monitoring and Management. WHO, London and New York, Chapter 4.

Lévesque, B., Gervais, M.-C., Chevalier, P., Gauvin, D., Anassour-Laouan-Sidi, E., Gingras, S., Fortin, N., Brisson, G., Greer, C., Bird, D., 2013. Prospective study of acute health effects in relation to exposure to cyanobacteria. Sci. Total Environ. 466–467C, 397–403.

Malbrouck, C., Kestemont, P., 2006. Effects of microcystins on fish. Environ. Toxicol. Chem. 25, 72–86.

Nagel, R., 2002. DarT: The embryo test with the zebrafish *Danio rerio*—a general model in ecotoxicology and toxicology. Altex-Alternativen Zu Tierexperimenten 19, 38–48.

Novák, J., Beníšek, M., Pacherník, J., Janošek, J., Šidlová, T., Kiviranta, H., Verta, M., Giesy, J.P., Bláha, L., Hilscherová, K., 2007. Interference of contaminated sediment extracts and environmental pollutants with retinoid signaling. Environ. Toxicol. Chem. 26, 1591–1599, doi: 10.1897/06-513R.1.

Nováková, K., Babica, P., Adamovský, O., Bláha, L., 2011. Modulation of gap-junctional intercellular communication by a series of cyanobacterial samples from nature and laboratory cultures. Toxicon 58, 76–84, doi: 10.1016/j.toxicon.2011.05.006.

Nováková, K., Kohoutek, J., Adamovský, O., Brack, W., Krauss, M., Bláha, L., 2013. Novel metabolites in cyanobacterium *Cylindrospermopsis raciborskii* with potencies to inhibit gap junctional intercellular communication. J. Hazard. Mater. 262, 571–579, doi: 10.1016/j.jhazmat.2013.09.007.

Oberemm, A., Becker, J. Codd, Steinberg, G.A., Nu, C., 1999. Effects of cyanobacterial toxins and aqueous crude extracts of cyanobacteria on the development of fish and amphibians. Environ. Toxicol. 14, 77–88.

Oberemm, A., Fastner, J., Steinberg, C.E.W.W., 1997. Effects of microcystin-LR and cyanobacterial crude extracts on embryo-larval development of zebrafish (*Danio rerio*). Water Res. 31, 2918–2921, doi: 10.1016/S0043-1354(97)00120-6.

OECD, 2013. Test No. 210: Fish, Early-life Stage Toxicity Test, OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing, doi: 10.1787/9789264203785-en.

Parng, C., Roy, N.M., Ton, C., Lin, Y., McGrath, P., 2007. Neurotoxicity assessment using zebrafish. J. Pharmacol. Toxicol. Methods 55, 103–112, doi: 10.1016/j.vascn.2006.04.004.

Rastogi, R.P., Sinha, R.P., 2009. Biotechnological and industrial significance of cyanobacterial secondary metabolites. Biotechnol. Adv. 27, 521–539, doi: 10.1016/j.biotechadv.2009.04.009.

Rhinn, M., Dollé, P., 2012. Retinoic acid signalling during development. Development 139, 843–858, doi: 10.1242/dev.065938.

Rogers, E.D., Henry, T.B., Twiner, M.J., Gouffon, J.S., McPherson, J.T., Boyer, G.L., Sayler, G.S., Wilhelm, S.W., 2011. Global gene expression profiling in larval zebrafish exposed to microcystin-LR and microcystis reveals endocrine disrupting effects of cyanobacteria. Environ. Sci. Technol. 45, 1962–1969.

Selderslaghs, I.W.T., Van Rompay, A.R., De Coen, W., Witters, H.E., 2009. Development of a screening assay to identify teratogenic and embryotoxic chemicals using the zebrafish embryo. Reprod. Toxicol. 28, 308–320, doi: 10.1016/j.reprotox.2009.05.004.

Scheffer, M., Rinaldi, S., Gragnani, A., Mur, L., van Nes, E., 1997. On the dominance of filamentous cyanobacteria in shallow, turbid lakes. Ecology 78, 272–282.

Schlosser, U.G., 1994. Sag—Sammlung-Von-Algenkulturen at the University-of-Gottingen—catalog of strains 1994. Bot. Acta 107, 113–186.

Stein, J., 1973. Hanbook of Phycological Methods. Culture Methods and Growth Measurements. Cambridge University Press, Cambridge.

Stěpánková, T., Ambrožová, L., Bláha, L., Giesy, J.P., Hilscherová, K., 2011. In vitro modulation of intracellular receptor signaling and cytotoxicity induced by extracts of cyanobacteria, complex water blooms and their fractions. Aquat. Toxicol. 105, 497–507, doi: 10.1016/j.aquatox.2011.08.002.

Sternberg, H., Moav, B., 1999. Regulation of the growth hormone gene by fish thyroid retinoid receptors. Fish Physiol. Biochem. 20, 331–339.

Stewart, I., Webb, P., Schluter, P., Shaw, G., 2006. Recreational and occupational field exposure to freshwater cyanobacteria—a review of anecdotal and case reports, epidemiological studies and the challenges for epidemiologic assessment. Environ. Health A Glob. Access Sci. Source 5, 6.

Stocum, D.L., 2000. Frog limb deformities: an "eco-devo" riddle wrapped in multiple hypotheses surrounded by insufficient data. Teratology 62, 147–150, doi: 10.1002/1096-9926(200009)62:3<147::AID-TERA2>3.0.CO;2-2.

Villeneuve, D.L., Blankenship, A.L., Giesy, J.P., 2000. Derivation and application of relative potency estimates based on in vitro bioassay results. Environ. Toxicol. Chem. 19, 2835–2843.

Wang, M., Chan, L.L., Si, M., Hong, H., Wang, D., 2010. Proteomic analysis of hepatic tissue of zebrafish (*Danio rerio*) experimentally exposed to chronic microcystin-LR. Toxicol. Sci. 113, 60–69, doi: 10.1093/toxsci/kfp248.

Wang, P.-J., Chien, M.-S., Wu, F.-J., Chou, H.-N., Lee, S.-J., 2005. Inhibition of embryonic development by microcystin-LR in zebrafish, *Danio rerio*. Toxicon 45, 303–308, doi: 10.1016/j.toxicon.2004.10.016.

Wiegand, C., Krause, E., Steinberg, C., Pflugmacher, S., 2001. Toxicokinetics of atrazine in embryos of the zebrafish (*Danio rerio*). Ecotoxicol. Environ. Saf. 49, 199–205, doi: 10.1006/eesa.2001.2073.

Wiegand, C., Pflugmacher, S., 2005. Ecotoxicological effects of selected cyanobacterial secondary metabolites: a short review. Toxicol. Appl. Pharmacol. 203, 201–218, doi: 10.1016/j.taap.2004.11.002.

Wright, A.D., Papendorf, O., König, G.M., Oberemm, A., 2006. Effects of cyanobacterium *Fischerella ambigua* isolates and cell free culture media on zebrafish (*Danio rerio*) embryo development. Chemosphere 65, 604–608, doi: 10.1016/j.chemosphere.2006.02.004.

Wu, X., Hu, J., Jia, A., Peng, H., Wu, S., Dong, Z., 2010. Determination and occurrence of retinoic acids and their 4-oxo metabolites in Liaodong Bay, China, and its adjacent rivers. Environ. Toxicol. Chem. 29, 2491–2497, doi: 10.1002/etc.322.

Wu, X., Jiang, J., Hu, J., 2013. Determination and occurrence of retinoids in a eutrophic lake (Taihu Lake, China): cyanobacteria blooms produce teratogenic retinal. Environ. Sci. Technol. 47, 807–814, doi: 10.1021/es303582u.

Wu, X., Jiang, J., Wan, Y., Giesy, J.P., Hu, J., 2012. Cyanobacteria blooms produce teratogenic retinoic acids. Proc. Nat. Acad. Sci. U.S.A. 109, 9477–9482, doi: 10.1073/pnas.1200062109.

Zhen, H., Wu, X., Hu, J., Xiao, Y., Yang, M., Hirotsuji, J., Nishikawa, J., Nakanishi, T., Ike, M., 2009. Identification of retinoic acid receptor agonists in sewage treatment plants. Environ. Sci. Technol. 43, 6611–6616, doi: 10.1021/es9000328.

Článek XXV:

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Endocrine, teratogenic and neurotoxic effects of cyanobacteria detected by cellular *in vitro* and zebrafish embryos assays

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HIGHLIGHTS

- Retinoid-like activity newly identified in two cyanobacterial species.
- Estrogenic and retinoid-like activity can occur simultaneously in cyanobacteria.
- Teratogenicity of cyanobacteria in zebrafish likely associated with retinoids.
- Mixture toxicity probably masked estrogenicity in transgenic fish.
- Cyanobacteria affected the locomotion of zebrafish embryos.

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ABSTRACT

Cyanobacteria contain various types of bioactive compounds, which could cause adverse effects on organisms. They are released into surface waters during cyanobacterial blooms, but there is little information on their potential relevance for effects *in vivo*. In this study presence of bioactive compounds was characterized in cyanobacteria *Microcystis aeruginosa* (Chroococcales), *Planktothrix agardhii* (Oscillatoriales) and *Aphanizomenon gracile* (Nostocales) with selected *in vitro* assays. The *in vivo* relevance of detected bioactivities was analysed using transgenic zebrafish embryos tg(*cyp19a1b*-GFP). Teratogenic potency was assessed by analysis of developmental disorders and effects on functions of the neuromuscular system by video tracking of locomotion. Estrogenicity *in vitro* corresponded to 0.95–54.6 ng estradiol equivalent (g dry weight (dw))$^{-1}$. In zebrafish embryos, estrogenic effects could not be detected potentially because they were masked by high toxicity. There was no detectable (anti)androgenic/glucocorticoid activity in any sample. Retinoid-like activity was determined at 1–1.3 μg all-trans-retinoic acid equivalent (g dw)$^{-1}$. Corresponding to the retinoid-like activity *A. gracile* extract also caused teratogenic effects in zebrafish embryos. Furthermore, exposure to biomass extracts at 0.3 g dw L^{-1} caused increase of body length in embryos. There were minor effects on locomotion caused by 0.3 g dw L^{-1} *M. aeruginosa* and *P. agardhii* extracts. The traditionally measured cyanotoxins microcystins did not seem to play significant role in observed effects. This indicates importance of other cyanobacterial compounds at least towards some species or their developmental phases. More attention should be paid to activity of retinoids, estrogens and other bioactive substances in phytoplankton using *in vitro* and *in vivo* bioassays.

1. Introduction

Blooms of cyanobacteria have become a serious problem in surface waters throughout the world. Their occurrence is associated with poor water quality, accumulation of biomass and low content of oxygen in water (Wiegand and Pflugmacher, 2005). Furthermore, cyanobacteria produce a wide spectrum of substances, some of which can cause various adverse effects on organisms (Kuiper-Goodman et al., 1999). Cyanobacterial toxins are categorised into five functional groups: hepatotoxins, neurotoxins, cytotoxins, dermatotoxins and irritant toxins (Wiegand and Pflugmacher, 2005). The hepatotoxic microcystins have been investigated in the greatest detail (Bláha et al., 2009). Great attention has also been paid to the diverse group of neurotoxins produced by cyanobacteria (Aráoz et al., 2010). Effects of complex blooms often cannot be attributed

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solely to the activity of individual cyanotoxins (Berry et al., 2009, 2007; Oberemm et al., 1997; Bláha et al., 2009). This could be due to the effect of unknown substances and/or the mutual interactions of the mixture components and environmental factors.

Recent results have indicated the ability of compounds produced by cyanobacteria to interfere with signalling of several intracellular receptors, which play important roles in physiological processes and are of relevance for potential adverse effects in vertebrates including humans (Klejdus et al., 2010; Kaya et al., 2011; Rogers et al., 2011; Wu et al., 2013, 2012). Signalling pathways, in which these receptors are engaged, play roles in hormonal regulation, reproduction and development of vertebrates (Janosek et al., 2006). Results of several studies have indicated the presence of estrogenic compounds in cyanobacteria (Klejdus et al., 2010; Stěpánková et al., 2011; Rogers et al., 2011). Furthermore, a potential interference of compounds from cyanobacterial blooms with androgen receptor signalling has been observed (Stěpánková et al., 2011). However, there is little information on potential of cyanobacterial compounds to affect signalling of other important endocrine receptors, such as glucocorticoid receptors that regulate genes controlling development, metabolism, stress and immune response (Odermatt and Gumy, 2008).

Recently, retinoic acid derivatives were identified by chemical analysis in cyanobacterial blooms from Tai Lake, China, and in several laboratory cultures of cyanobacteria (Wu et al., 2013, 2012). Extracts of a few cyanobacteria were shown to exhibit retinoid-like activity in a yeast reporter gene assay (Kaya et al., 2011). Retinoic acid (RA) signalling is crucial for normal vertebrate development and highly conserved among different species (Rhinn and Dollé, 2012). However, RAs are potent teratogens (Selderslaghs et al., 2009) when normal physiological concentrations are exceeded. Hence, the gross malformations reported for zebrafish embryos exposed to crude extracts of cyanobacteria *Microcystis aeruginosa*, *Anabaena flos-aquae, Cylindrospermopsis raciborskii* and *Aphanizomenon flos-aque* (Oberemm et al., 1997; Berry et al., 2009; Ghazali et al., 2009; Acs et al., 2013) might be related to the presence of retinoids. These malformations could not be explained by the known toxins considered in these studies, such as microcystins or cylindrospermopsin (Oberemm et al., 1997; Berry et al., 2009; Acs et al., 2013).

The objective of this study was to investigate extracts of biomass from several cyanobacterial species for the presence of bioactive compounds *in vitro* and *in vivo*, using reporter cell assays and zebrafish embryos. This approach aimed to determine the relevance of the detected *in vitro* bioactivity for *in vivo* situation. Several *in vitro* cellular reporter assays were used to examine estrogenic, retinoid-like, anti/androgenic and glucocorticoid activity. Correspondingly, estrogenic activity was also assessed by a transgenic zebrafish strain tg(*cyp19a1b*-GFP). In order to identify teratogenic effects possibly related to retinoid-like compounds the frequency of malformations was analysed. Potential interference with neuromuscular development and function was assessed in zebrafish embryos using a locomotion analysis. The selection of cyanobacterial species for testing was based on our previous results which indicated endocrine disrupting potency of biomass extracts (Stěpánková et al., 2011) and designed to represent different cyanobacterial orders. The test species included cyanobacteria *M. aeruginosa* (Chroococcales), *Planktothrix agardhii* (Oscillatoriales) and *Aphanizomenon gracile* (Nostocales).

2. Materials and methods

2.1. Preparation of cyanobacterial samples

The source and characteristics of cyanobacterial strains used in this study are given in Table 1. Cyanobacteria were cultured as described previously (Nováková et al., 2013). Details of cultivation and preparation of samples for testing are given in Supplementary Materials (Section S1).

Ultrasound was used to extract 200 mg of lyophilized biomass with 6 mL 75% MeOH. The final extract was centrifuged and the debris re-extracted with 2 × 2 mL 75% MeOH. Organic compounds in samples were pre-cleaned and concentrated by solid phase extraction (SPE) using Oasis HLB and Carbograff cartridges. Eluates from both columns were pooled to obtain maximal recovery. Concentrations of microcystins were determined as previously described (Bláhová et al., 2008).

2.2. In vitro estrogenic, (anti-)androgenic, glucocorticoid and retinoid-like activity

Complete description of the used bioassays and testing procedures is given in Supplementary Materials (Section S2). Reporter gene assays stably transfected with luciferase gene under control of estrogen-, androgen-, glucocorticoid- and retinoid-receptor activation, respectively, were used to assess the interference of the samples with signalling of the endogenous ligands. All *in vitro* assays were performed in 96 well microplates. Cells were exposed for 24 h to cyanobacterial biomass extracts in the concentration range of 0.03125–2 g dw L^{-1}, calibration standards, blanks and solvent controls. Cytotoxicity of samples was assessed using two fluorescent indicator dyes (Schirmer et al., 1997). The activity of induced reporter luciferase was measured using luciferase substrate.

2.3. In vivo experiments with zebrafish embryos

Experiments with zebrafish embryos were performed at the UFZ Leipzig using the transgenic zebrafish strain tg(*cyp19a1b*-GFP) (Tong et al., 2009; Brion et al., 2012). The strain was kindly provided by O. Kah, University of Rennes and was crossed to the in-house wild-type strain UFZ-OBI prior to use. Details on zebrafish culture and embryo production as well as on exposure experiments are included in Supplementary Materials (Section S3.1–S3.2). Zebrafish embryos at the stage of 24hpf were exposed to extracts of biomass prepared in methanol. Extracts were added to the test vessels and methanol was allowed to evaporate. Standard test medium (ISO, 2008) was added to the exposure dishes immediately after methanol evaporation. Exposure media were mixed by gentle agitation, briefly ultrasonicated and mixed again. The exposure was conducted for 96 h at 26 ± 1 °C and a photoperiod 12 h light: 12 h dark. Exposure media were replaced after 48 h. Dissolved oxygen (Fibox 3 trace, PreSens, Germany) and pH were recorded at the beginning and end of each exposure interval.

Due to limited availability of biomass, an initial screening experiment for reduction of oxygen levels, mortality and malformations was conducted. The screening concentrations were selected based on previous experience and literature data, which have indicated lower oxygen content at greater biomass concentrations (Burýšková et al., 2006). Based on this screening appropriate concentrations for further detailed assessment were defined. For screening, fish embryos were exposed in 6 mL glass vials containing 2 mL exposure medium. Biomass concentrations of 0.3, 1, 3 and 10 g dw L^{-1} were tested for each species. As a positive control embryos were exposed to 1 nM ethinylestradiol (EE2). The vials were incubated on a shaker to promote oxygen exchange. The percentage of dead and malformed embryos was assessed daily. The induction of GFP reporter fluorescence was measured at the end of exposure at 120hpf.

Based on the results of the screening test detailed test was carried out in three independent replicated experiments conducted on different days. Twenty embryos were exposed per replicate and

Table 1
List of cyanobacterial strains used in this study, their origin, cyanotoxin content and total retinoid (REQ in ng ATRA (g dw)$^{-1}$) and estrogenic equivalents (EEQ in ng E2 (g dw)$^{-1}$).

Order *Species*	Source[a]	Place of origin		MCs concentration (μg g^{-1})[b]			Relative equivalent *in vitro*	
		Country	Water body	MC-RR	MC-YR	MC-LR	ng ATRA (g dw)$^{-1}$	ng E2 (g dw)$^{-1}$
Oscillatoriales								
Planktothrix agardhii	CCALA 159	Germany	Lake Plussee	n.d.	22.9	7.8	1163	54.6
Nostocales								
Aphanizomenon gracile	RCX06[c]	Ireland	Lake Lough Neagh	n.d.	n.d.	n.d.	1322	1.2
Chroococcales								
Microcystis aeruginosa	PCC 7806	Netherlands	Reservoir Braakman	n.d.	n.d.	325	1092	0.95

[a] Culture collection ID for laboratory cultured strains: CCALA – Culture Collection of Autotrophic Organisms, Institute of Botany, Academy of Sciences of the Czech Republic; RCX – RECETOX Culture Collection of Cyanobacteria and Algae; PCC – Pasteur Culture Collection of Cyanobacteria.
[b] Method limit of detection was 5 μg (g dw)$^{-1}$; MCs – microcystins; n.d. – not detected (below limit of detection).
[c] This species originates from CCALA (strain 008), but has been long-term cultivated at RECETOX.

test concentration in exposure dishes in 20 mL exposure medium. Each independent experiment included three negative controls (test medium) and three positive controls (1 nM EE2). In the detailed test, hatching, teratogenicity and mortality were assessed daily. Locomotion, length and estrogenic effects were evaluated at the end of the exposure.

Details on the methods for evaluating these parameters as well as on conducted data analyses are included in Supplementary Materials (Section S3.3–S4).

3. Results

A. gracile did not contain detectable levels of microcystins. Microcystin-LR was detected in *M. aeruginosa,* microcystin-LR and -YR in *P. agardhii* (Table 1).

The *in vitro* assay revealed concentration-dependent retinoid receptor mediated activity in the biomass extracts of all tested cyanobacteria (Fig. 1A). Similar potencies were detected for all three species (Table 1). The LOEC for retinoid-like activity was in the range of 0.25–0.5 g dw L^{-1} for the three cyanobacterial species.

Estrogenic potency was detected in all samples (Fig. 1B and Table 1). The greatest estrogenicity was observed for *P. agardhii*. Approximately 50-fold lower concentrations of estrogen equivalents were detected for *M. aeruginosa* and *A. gracile*. The LOECs for the detection of an estrogenic response ranged from 0.03 g dw L^{-1} (*P. agardhii*) to 1 g dw L^{-1} in case of *M. aeruginosa* and *A. gracile*. No androgenic, glucocorticoid or antiandrogenic activity was detected in any of the tested biomass extracts up to the highest tested concentration (2 g dw L^{-1}).

The initial screening test revealed 100% mortality in embryos exposed to extracts of *P. agardhii* or *M. aeruginosa* at 3 or 10 g dw L^{-1} or to 10 g dw L^{-1} of the extract of *A. gracile*. Since mortality was observed during the first 24 h after initiation of exposure, when oxygen levels in these treatments dropped to 5–31%, it might have been associated with hypoxia. However, the mortality rate of 20% at concentrations of 1 g dw L^{-1} of *M. aeruginosa* occurred at oxygen levels greater than 80% saturation, which indicates that components in the biomass caused reduced survival. No teratogenic effects were observed in embryos exposed to the least concentration (0.3 g dw L^{-1}) of all samples and in 1 g dw L^{-1} *P. agardhii*. Deformities of the tip of the tail were observed in zebrafish embryos exposed to all other extracts at 1 and 3 g dw L^{-1}. Extracts of *A. gracile* at 3 g dw L^{-1} also caused edema in hearts of all embryos (Table S1).

A potential estrogenic activity was indicated in the screening experiment by greater GFP fluorescence in comparison to controls (>1) for the extracts of *A. gracile*, *P. agardhii* and *M. aeruginosa* at 0.3 g dw L^{-1} and *A. gracile* at 1 g dw L^{-1}. At the highest sublethal test concentrations, however, i.e. *A. gracile* at 3 g dw L^{-1} and *M. aeruginosa* at 1 g dw L^{-1}, significantly lower GFP fluorescence was observed indicating a potential toxic interference with GFP protein synthesis (Table S1).

Given the mortality and decrease in oxygen content and the fact that the screening test indicated the greatest estrogenicity at concentration 0.3 g dw L^{-1}, the maximum test concentration in the subsequent detailed testing was limited to a biomass concentration of 0.3 g dw L^{-1} for *P. agardhii* and *M. aeruginosa* and to 0.3–3 g dw L^{-1} for *A. gracile*.

In the detailed testing dissolved oxygen levels were greater than 90% saturation and pH was between 7 and 8 in all test

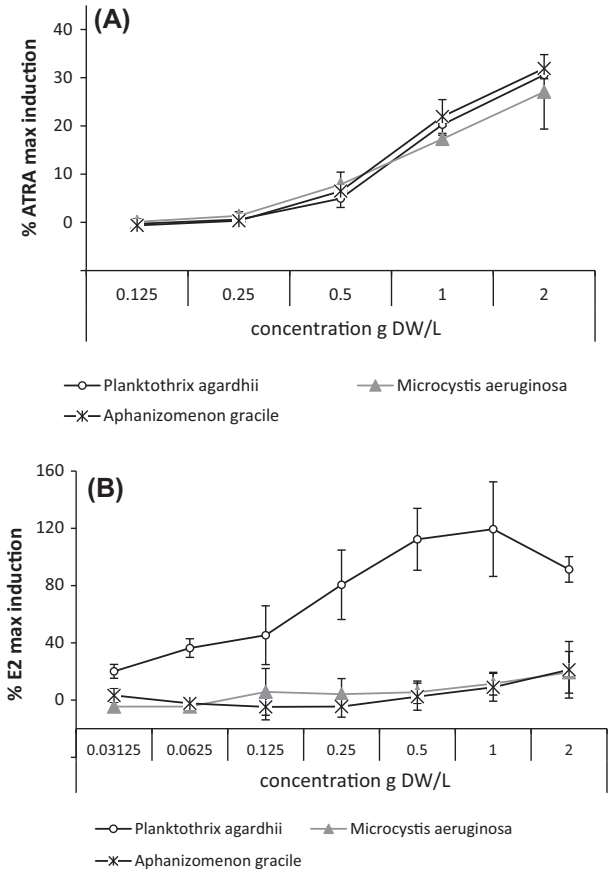

Fig. 1. Concentration–response curves of (A) the retinoid-like activity (expressed as % of maximal RAR-mediated induction caused by standard all-trans retinoic acid – ATRAmax, 500 nM) in P19/A15 cell line (B) the estrogenic activity (expressed as % of maximal ER-mediated induction caused by standard estradiol – E2max, 500 pM) in MVLN cell line after 24 h exposure to extracts from cyanobacterial and algal biomass.

Table 2
Toxicity, teratogenic and estrogenic effects and effects on locomotion and length observed during the detailed experiments on zebrafish embryos after exposure to biomass extracts. Shading emphasizes significant effects.

		control	*Aphanizomenon gracile*			PA[a]	MA[a]
	Concentration (g dwL^{-1})		0.3	1	3	0.3	0.3
	REQ[b] ngL^{-1}		397	1322	3966	349	328
Time	Endpoint						
48hpf	mortality[c]	0	0	0	21.7 ±38	0	0
	tail tip deformation	0	0	8.33 ±6*	68.3 ±33*	0	0
	yolk deformation	0	0	1.67 ±3	28.3 ±26	0	0
	heart edema	0	0	0	11.7 ±20	0	0
	hatched	1 ±2	7 ±12	17 ±29	45 ±17*	0	3 ±6
72hpf	mortality	0	0	0	30 ±48	0	0
	tail tip deformation	0	0	83.3 ±29*	70 ±48*	0	0
	heart edema	0	0	5 ±5	5 ±9	0	0
	spine deformation	0	0	33.3 ±57	0	0	0
	hatched	33 ±43	63 ±40*	75 ±35*	62 ±43*	62 ±40*	82 ±24*
96hpf	mortality	0	0	0	70 ±40*	0	0
	tail tip deformation	0	0	100 *	38.3 ±54	0	0
	heart edema	0	0	1.67 ±3	38.3 ±54	0	0
	spine deformation	0	0	33.3 ±58	0	0	0
	mouth deformation	0	0	33.3 ±58	0	0	0
	hatched	76 ±21	100	97 ±3	-	100	100
120hpf	mortality	0	0	0	88.3 ±13*	0	0
	tail tip deformation	0	0	100 *	11.7 ±13	0	0
	heart edema	0	0	3.33 ±3	11.7 ±13	0	0
	spine deformation	0	0	100 *	0	0	0
	mouth deformation	0	0	36.7 ±55	0	0	0
	hatched	90 ±10	100	100	-	100	100
	Estrogenicity[d]	1.01 ±0.3	1.06 ±0.1	0.61 ±0.3	-	0.96 ±0.2	1.22 ±0.5
	Locomotion[e]						
	mean distance	0.8 ±0.2	3.3 ±1.6	2.7 ±1.4	-	5.5 ±1.8*	4.1 ±1.3*
	OA	1	0.60 ±0.13	0.62 ±0.22	-	0.44 ±0.04*	0.52 ±0.07*
	Length (μm)[f]	3755 ±21	3952 ±93*	3842 ±94	3165 -	3943 ±77*	3958 ±53*

[a] PA is *Planktothrix agardhii* and MA *Microcystis aeruginosa*.
[b] REQ = equivalent concentration of ATRA in exposure media.
[c] Frequency of effects (in %) for mortality and malformations are shown as an average of three replicate experiments (60 embryos each) with standard deviation.
[d] Induction of fluorescence in transgenic cyp19a1b-GFP zebrafish embryos as an indicator of *in vivo* estrogenicity shown as means and standard deviation.
[e] Locomotion expressed as mean moved distance and overlapping area (OA, see material and methods for details) with corresponding standard deviation.
[f] Length of embryos (μm) shown as an average length and standard deviation of three independent experiments.
* Statistically significant difference from control ($p < 0.05$) – not assessed due to mortality.

concentrations and controls. Hatching rates were particularly affected by all extracts and test concentrations at 72hpf due to an earlier hatching of exposed embryos. The effect on hatching at 48hpf appeared to be dependent on the concentration of biomass as indicated by exposure to different concentrations of *A. gracile* extracts (Table 2).

Teratogenic effects were only observed in zebrafish embryos exposed to extract of *A. gracile* at $\geqslant 1$ g dw L^{-1}. Specifically, deformities of the tip of the tail and spine were observed (Table 2). Edema of the heart and trunk, small head and yolk retention, were only noted at greater concentrations (3 g dw L^{-1}) that already caused mortality. Details of types of malformations along with the time of their occurrence are shown in Table 2.

Embryos measured at 5dpf were significantly ($p \leqslant 0.05$; by about 5%) longer when exposed to extracts of *P. agardhii*, *M. aeruginosa* and *A. gracile* at 0.3 g dw L^{-1} (Table 2, Fig. S1). Lengths of embryos exposed to 1 g dw L^{-1} of *A. gracile* extract were not significantly different from controls. Surviving embryos exposed to 3 g dw L^{-1} of *A. gracile* extract (8 out of 60) were on average 16% shorter than in controls, but this difference was not statistically significant due to greater mortality at this concentration.

The weak estrogenic effects initially observed in the screening test could not be confirmed in the detailed test (Table 2). Mean GFP levels relative to control from the three independent experiments ranged from 0.6 to 1.2, but the differences compared to control were not statistically significant.

Statistically significant differences in locomotion compared to control were observed for 0.3 g dw L^{-1} extracts of *M. aeruginosa* and *P. agardhii* (Table 2). Effects were observed only in the second light phase of the behavioural assays, when an increase in the moved distance was noted for exposed embryos.

4. Discussion

Receptor transactivation studies have demonstrated the occurrence of bioactive compounds in cyanobacteria. However, there is a

lack of information on relevance of the *in vitro* potencies for *in vivo* situations. Therefore, in the present study *in vitro* bioactivities of cyanobacteria were compared to effects in a whole-organism model.

The species selected for this study are common in the environment and can dominate aquatic habitat in case of cyanobacterial blooms (Stěpánková et al., 2011; Wu et al., 2012). The lower concentrations used in this study (0.3 g dw L^{-1}) can be considered environmentally relevant during cyanobacterial blooms with densities over 1 000 000 cells mL^{-1}, which occur frequently in the water bodies. Our field studies document concentrations of biomass up to 3 g dw L^{-1} (approximately 70 000 000 cells $(mL)^{-1}$) in lakes with massive cyanobacterial water bloom dominated by *M. aeruginosa* (unpublished data).

The mortality results showed the importance of measuring concentrations of dissolved oxygen in testing of cyanobacterial biomasses extracts because of the biological oxygen demand (BOD) they exerted. In the initial screening test oxygen deficiency in some samples might have contributed to mortality, which is indicated by association of oxygen content of less than 35% saturation with 100% mortality (Table S1). A minimum oxygen concentration of 80% is suggested by fish embryo testing guidelines (OECD, 2013) and supported by experimental observations (Küster and Altenburger, 2008). Difficulties in maintaining sufficient concentration of dissolved oxygen and appropriate pH was indicated previously as a possible limitation in biotests with cyanobacteria samples (Burýšková et al., 2006). However, the mortality caused by insufficient oxygen due to the BOD of cyanobacterial blooms represents an environmentally relevant effect (Kuiper-Goodman et al., 1999). Therefore, oxygen depletion should be measured and considered in order to assess the environmental relevance of toxicants versus oxygen insufficiency.

Despite that the tested species belong to distant orders (Oscillatoriales, Nostocales, Chroococcales) of both unicellular (*M. aeruginosa*) and filamentous (*A. gracile, P. agardhii)* cyanobacteria they contained similar concentrations of retinoid-like compounds. Among these species previous information on total retinoid-like activity had been available only for a different strain of *M. aeruginosa* analyzed by *in vitro* yeast RA activity assay (Kaya et al., 2011). There is a similarity in observed total retinoid-like potency with the previous study (2500 and 1092 ng ATRA $(g\ dw)^{-1}$) despite the different origin and different bioassays used for the assessment. Also chemical analyses documented the presence of a few analogues of retinoic acids in *M. aeruginosa* strains isolated from two Chinese lakes (Wu et al., 2012). Our study is the first to report the presence of compounds with retinoid-like activity also for the cyanobacterial species *P. agardhii* and *A. gracile*.

Potentially linked to the retinoid compounds and activities reported in phytoplankton species, teratogenic effects in zebrafish embryos were found at greater concentrations of *A. gracile* extracts. Effects included tail tip, spine, mouth and yolk deformation and heart edema (Fig. 2C). These effects were also observed in exposure tests with ATRA (Jonas et al., 2014; Herrmann, 1995; Haldi et al., 2011). Concentrations of REQ detected in cyanobacteria by *in vitro* tests corresponded to concentrations of ATRA that were shown to cause teratogenicity in zebrafish embryos. Content of retinoid equivalents in the least extract concentration causing malformations (1.3 μg ATRA L^{-1} in 1 g dw L^{-1} *A. gracile*) corresponded with the LOEC of ATRA (Jonas et al., 2014; Herrmann, 1995) for some of these malformations.

All exposures to 0.3 g dw L^{-1} cyanobacterial biomass equivalents (containing 0.3–0.4 μg ATRA L^{-1}) caused a small but statistically significant increase in the length of embryos. A similar effect was observed after exposure to 0.4 and 1.3 μg L^{-1} ATRA (Jonas et al., 2014). RAs are known to increase growth hormone levels and mRNA *in vitro* in human- and fish-cells (Guibourdenche et al., 1997; Sternberg and Moav, 1999) and the observed greater length could be related to the stimulation of growth hormone synthesis. For greater concentrations of *A. gracile* extracts as well as for greater ATRA concentrations no increased body length was reported, which might be due to interfering toxic effects as indicated by the high rates of malformations and mortality. The lesser length indicated at 3 g dw L^{-1} of *A. gracile* corresponds to observations in zebrafish embryos exposed to greater concentrations of ATRA (Jonas et al., 2014; Herrmann, 1995).

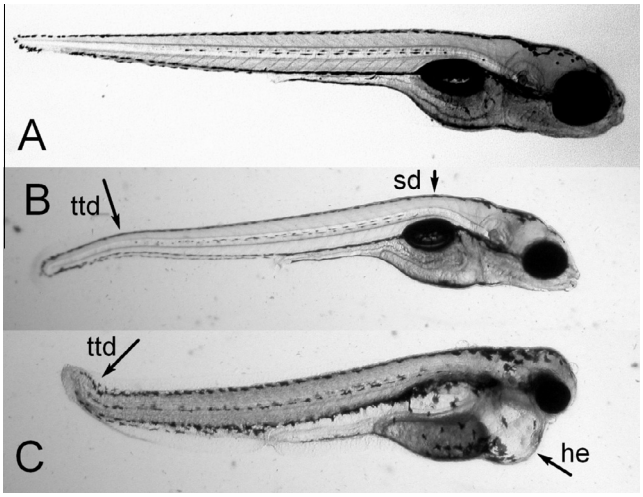

Fig. 2. Comparison of control and exposed zebrafish embryos (120hpf): control (A) compared to embryos exposed to biomass extracts of *Aphanizomenon gracile* 1 g dry weight L^{-1} (B) and 3 g dry weight L^{-1} (C). Arrows and abbreviations indicate heart edema (he), tail tip (ttd) and spine deformation (sd).

Exposures to cyanobacterial extracts caused earlier hatching compared to controls, particularly at 72hpf, while no such affect was observed for ATRA (Jonas et al., 2014). Hence, effects on hatching are probably not associated with RA activity, but possibly with some other compounds produced by cyanobacteria.

Given the complexity of extracts of cyanobacteria other compounds than retinoids or their analogues may have contributed to the observed teratogenicity. Microcystins were found in two cyanobacteria species in this study, i.e. *P. agardhii* and *M. aeruginosa*. Microcystins probably did not play a significant role in most of the effects observed in this experiment. *In vitro* potencies of the species with greatest microcystins content (*M. aeruginosa*) were lesser (estrogenicity) or comparable (retinoid-like activity) than in species with no or lesser concentrations of microcystins. Mortality and malformations in embryos were induced by greater concentrations of the extract of *A. gracile*, which does not contain these toxins. This is consistent with previously published reports where it was argued that the weak effect of microcystins on zebrafish embryos was due to a limited uptake (Wang et al., 2005; Berry et al., 2007).

A small *in vitro* estrogenic potential was detected for extracts of *M. aeruginosa* and *A. gracile* (near limit of detection). More than 50-fold higher estrogenic potency was observed for extract of *P. agardhii*. An estrogenic activity of different types of extracts from cyanobacterial species used in this study has been reported previously (Rogers et al., 2011; Stěpánková et al., 2011). Particularly, cyanobacterial species forming massive water blooms could contribute estrogenic compounds into water bodies, which might interfere with the hormonal control of aquatic organisms.

Although estrogenicity was detected *in vitro,* no significant estrogenic effects were detected *in vivo*. Potencies of estrogenic compounds in the exposures might have been too small to cause effect in zebrafish embryo assay. The EC_{50} of 17β-estradiol for

GFP induction in transgenic fish is 130 ng L^{-1} causing approximately 10-fold induction. Since the exposure to 0.3 g dw L^{-1} biomass corresponds to concentrations of 0.3–16.4 ng EEQ L^{-1}, these concentrations might only cause low levels of GFP induction that could in addition be mitigated by other factors (see below). *In vivo* estrogenicity could be expected from greater concentrations of extracts, particularly from *P. agardhii* samples (546 ng EEQ L^{-1} in 10 g dw L^{-1}). However, at these concentrations mortality prevented detection of estrogenic activity. Several other reasons could also account for the lack of estrogenic effects in fish embryos. For instance, fish embryos might have greater capacity for metabolisation and deactivation of estrogenic compounds in phytoplankton biomasses. Furthermore, other compounds present in the extracts such as RA could reduce the estrogenic effects. RA is known to regulate the development of the brain (Rhinn and Dollé, 2012) and impact on differentiation of stem cells into the neural radial glial cells progenitors (Plachta et al., 2004). This could potentially affect production of aromatase B and consequently GFP, because aromatase B is mainly produced in the radial glial cell progenitors in the brain of zebrafish embryos (Tong et al., 2009).

Among the various toxins known to be produced by cyanobacteria also an array of different neurotoxins can be found (Aráoz et al., 2010). The neuroactivity or –toxicity is difficult to detect *in vitro* since a functional nervous system would be required. However, behavioural assays (i.e. analysis of movements) represent a simple and efficient tool to detect functional interference in zebrafish embryos (Selderslaghs et al., 2010). We detected a weak increase of the moved distance in zebrafish embryos exposed to biomass extracts of *M. aeruginosa* and *P. agardhii*. The observed effects might not necessarily represent a specific neurotoxic response but could also indicate sublethal effects, subtle morphological changes or avoidance reactions rather than specific interaction with e.g. neuronal receptors or ion channels. Increased locomotion of 120hpf old embryos was observed after exposure to 600 ng L^{-1} ATRA (Wang et al., 2014), which indicates possible influence of retinoids on increased locomotion in our samples. It has been also reported that exposure to 0.5 and 5 μg L^{-1} microcystin-LR resulted in an increased day-time locomotion of adult zebrafish (Baganz et al., 1998). The strains, of which the extract increased movement of zebrafish embryos, contain microcystins. Hence, non-neurotoxic compounds such as microcystins may have contributed to the response in behavioural assays. Acetylcholin esterase (AChE) inhibitor anatoxin-a was reported in several species from genera Aphanizomenon, Microcystis and Planktothrix (Aráoz et al., 2010). The potential presence of anatoxin in samples used in our study is unknown. Nevertheless, AChE inhibitor would rather decrease the locomotion of zebrafish embryos as was shown with AChE inhibitor diazinon (Yen et al., 2012). It will require further investigations to identify the compounds responsible for the observed effects and their mechanism of action.

In conclusion, this study demonstrates the diversity of bioactive compounds in biomass extracts. Our findings indicate that endocrine activities detected by *in vitro* assays might not be directly reflected by whole organism assays. This could, however, be provoked by various relevant mitigating factors (metabolism, down-regulation via other pathways, toxic effects, see above) not present in *in vitro* assays. We confirmed that phytoplankton species produce estrogenic, retinoid-like and/or teratogenic compounds, which could be released into the aquatic environment and affect the development of organisms living in surface waters. Fish embryos often live in littoral zones where greater accumulation of cyanobacterial water blooms can be expected (Malbrouck and Kestemont, 2006). Hence, retinoic acid-like effects of phytoplankton indicate the need to consider early life stages, which could be more vulnerable than adults due to sensitive developmental processes, for the estimation of the environmental impact of cyanobacterial blooms. More attention should be paid to the activity of retinoids, estrogens and other bioactive compounds in cyanobacteria using *in vitro* and *in vivo* bioassays.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2014.07.074.

References

Acs, A., Kovács, A.W., Csepregi, J.Z., Törő, N., Kiss, G., Győri, J., Vehovszky, A., Kováts, N., Farkas, A., 2013. The ecotoxicological evaluation of Cylindrospermopsis raciborskii from Lake Balaton (Hungary) employing a battery of bioassays and chemical screening. Toxicon 70C, 98–106.

Aráoz, R., Molgó, J., Tandeau de Marsac, N., 2010. Neurotoxic cyanobacterial toxins. Toxicon 56, 813–828.

Baganz, D., Staaks, G., Steinberg, C., 1998. Impact of the cyanobacteria toxin, microcystin-LR on behaviour of zebrafish, Danio rerio. Water Res. 32, 948–952.

Berry, J.P., Gantar, M., Gibbs, P.D.L., Schmale, M.C., 2007. The zebrafish (Danio rerio) embryo as a model system for identification and characterization of developmental toxins from marine and freshwater microalgae. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 145, 61–72.

Berry, J.P., Gibbs, P.D.L., Schmale, M.C., Saker, M.L., 2009. Toxicity of cylindrospermopsin, and other apparent metabolites from Cylindrospermopsis raciborskii and Aphanizomenon ovalisporum, to the zebrafish (Danio rerio) embryo. Toxicon 53, 289–299.

Bláha, L., Babica, P., Maršálek, B., 2009. Toxins produced in cyanobacterial water blooms – toxicity and risks. Interdiscipl. Toxicol. 2, 36–41.

Bláhová, L., Babica, P., Adamovský, O., Kohoutek, J., Maršálek, B., Bláha, L., 2008. Analyses of cyanobacterial toxins (microcystins, cylindrospermopsin) in the reservoirs of the Czech Republic and evaluation of health risks. Environ. Chem. Lett. 6, 223–227.

Brion, F., Le Page, Y., Piccini, B., Cardoso, O., Tong, S.-K., Chung, B., Kah, O., 2012. Screening estrogenic activities of chemicals or mixtures in vivo using transgenic (cyp19a1b-GFP) zebrafish embryos. PLoS One 7, e36069.

Burýšková, B., Hilscherová, K., Babica, P., Vršková, D., Maršálek, B., Bláha, L., 2006. Toxicity of complex cyanobacterial samples and their fractions in Xenopus laevis embryos and the role of microcystins. Aquat. Toxicol. 80, 346–354.

Ghazali, I.El., Saqrane, S., Carvalho, A.P., Ouahid, Y., Oudra, B., Del Campo, F.F., Vasconcelos, V., 2009. Compensatory growth induced in zebrafish larvae after pre-exposure to a *Microcystis aeruginosa* natural bloom extract containing microcystins. Int. J. Mol. Sci. 10, 133–146.

Guibourdenche, J., Djakouré, C., Porquet, D., Pagésy, P., Rochette-Egly, C., Peillon, F., Yuan Li, J., Evain-Brion, D., Li, J.Y., 1997. Retinoic acid stimulates growth hormone synthesis in human somatotrophic adenoma cells: characterization of its nuclear receptors. J. Cell. Biochem. 65, 25–31.

Haldi, M., Harden, M., D'Amico, L., DeLise, A., Seng, W.L., 2011. Developmental Toxicity Assessment in Zebrafish. in: Zebrafish, John Wiley&Sons, Inc., pp. 15–25.

Herrmann, K., 1995. Teratogenic effects of retinoic acid and related substances on the early development of the zebrafish (Brachydanio rerio) as assessed by a novel scoring system. Toxicol. in Vitro 9, 267–283.

ISO, 2008. International Standard Organization, Water quality – Determination of the acute toxicity of waste water to zebrafish eggs (Danio rerio). Eur. Stand. EN ISO. 15088.

Janosek, J., Hilscherová, K., Bláha, L., Holoubek, I., 2006. Environmental xenobiotics and nuclear receptors-interactions, effects and in vitro assessment. Toxicol. in Vitro 20, 18–37.

Jonas, A., Buranova, V., Scholz, S., Fetter, E., Novakova, K., Kohoutek, J., Hilscherova, K., 2014. Retinoid-like activity and teratogenic effects of cyanobacterial exudates. Aquat. Toxicol. 155, 283–290.

Kaya, K., Shiraishi, F., Uchida, H., Sano, T., 2011. A novel retinoic acid analogue, 7-hydroxy retinoic acid, isolated from cyanobacteria. Biochim. Biophys. Acta – Gen. Subjects 1810, 414–419.

Klejdus, B., Lojková, L., Plaza, M., Snóblová, M., Stěrbová, D., 2010. Hyphenated technique for the extraction and determination of isoflavones in algae: ultrasound-assisted supercritical fluid extraction followed by fast chromatography with tandem mass spectrometry. J. Chromatogr. A 1217, 7956–7965.

Kuiper-Goodman, T., Falconer, I., Fitzgerald, J., 1999. Human Health Aspects. in: Chorus, I., Bartram, J. (Eds.), Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management. WHO, London and New York.

Küster, E., Altenburger, R., 2008. Oxygen decline in biotesting of environmental samples — Is there a need for consideration in the acute zebrafish embryo assay? Environ. Toxicol. 23, 745–750.

Malbrouck, C., Kestemont, P., 2006. Effects of microcystins on fish. Environ. Toxicol. Chem. 25, 72–86.

Nováková, K., Kohoutek, J., Adamovský, O., Brack, W., Krauss, M., Bláha, L., 2013. Novel metabolites in cyanobacterium *Cylindrospermopsis raciborskii* with potencies to inhibit gap junctional intercellular communication. J. Hazard. Mater. 262, 571–579.

Oberemm, A., Fastner, J., Steinberg, C.E.W., 1997. Effects of microcystin-LR and cyanobacterial crude extracts on embryo-larval development of zebrafish (Danio rerio). Water Res. 31, 2918–2921.

Odermatt, A., Gumy, C., 2008. Disruption of glucocorticoid and mineralocorticoid Receptor-mediated responses by environmental chemicals. Chim. (Aarau). 62, 335–339.

OECD, 2013. Test No. 236: Fish Embryo Acute Toxicity (FET) Test, OECD Guidelines for the Testing of Chemicals, Section 2 1-22.

Plachta, N., Bibel, M., Tucker, K.L., Barde, Y.-A., 2004. Developmental potential of defined neural progenitors derived from mouse embryonic stem cells. Development 131, 5449–5456.

Rhinn, M., Dollé, P., 2012. Retinoic acid signalling during development. Development 139, 843–858.

Rogers, E.D., Henry, T.B., Twiner, M.J., Gouffon, J.S., McPherson, J.T., Boyer, G.L., Sayler, G.S., Wilhelm, S.W., 2011. Global gene expression profiling in larval zebrafish exposed to microcystin-LR and microcystis reveals endocrine disrupting effects of Cyanobacteria. Environ. Sci. Technol. 45, 1962–1969.

Schirmer, K., Chan, a.G., Greenberg, B.M., Dixon, D.G., Bols, N.C., 1997. Methodology for demonstrating and measuring the photocytotoxicity of fluoranthene to fish cells in culture. Toxicol. in Vitro 11, 107–119.

Selderslaghs, I.W.T., Van Rompay, A.R., De Coen, W., Witters, H.E., 2009. Development of a screening assay to identify teratogenic and embryotoxic chemicals using the zebrafish embryo. Reprod. Toxicol. 28, 308–320.

Selderslaghs, I.W.T., Hooyberghs, J., De Coen, W., Witters, H.E., 2010. Locomotor activity in zebrafish embryos: a new method to assess developmental neurotoxicity. Neurotoxicol. Teratol. 32, 460–471.

Stěpánková, T., Ambrožová, L., Bláha, L., Giesy, J.P., Hilscherová, K., 2011. In vitro modulation of intracellular receptor signaling and cytotoxicity induced by extracts of cyanobacteria, complex water blooms and their fractions. Aquat. Toxicol. 105, 497–507.

Sternberg, H., Moav, B., 1999. Regulation of the growth hormone gene by fish thyroid/retinoid receptors. Fish Physiol. Biochem. 20, 331–339.

Tong, S.-K., Mouriec, K., Kuo, M.-W., Pellegrini, E., Gueguen, M.-M., Brion, F., Kah, O., Chung, B., 2009. A cyp19a1b-gfp (aromatase B) transgenic zebrafish line that expresses GFP in radial glial cells. Genesis 47, 67–73.

Wang, P.-J., Chien, M.-S., Wu, F.-J., Chou, H.-N., Lee, S.-J., 2005. Inhibition of embryonic development by microcystin-LR in zebrafish, Danio rerio. Toxicon 45, 303–308.

Wang, Y., Chen, J., Du, C., Li, C., Huang, C., Dong, Q., 2014. Characterization of retinoic acid-induced neurobehavioral effects in developing zebrafish. Environ. Toxicol. Chem. 33, 431–437.

Wiegand, C., Pflugmacher, S., 2005. Ecotoxicological effects of selected cyanobacterial secondary metabolites: a short review. Toxicol. Appl. Pharmacol. 203, 201–218.

Wu, X., Jiang, J., Wan, Y., Giesy, J.P., Hu, J., 2012. Cyanobacteria blooms produce teratogenic retinoic acids. Proc. Natl. Acad. Sci. USA 109, 9477–9482.

Wu, X., Jiang, J., Hu, J., 2013. Determination and occurrence of retinoids in a eutrophic lake (Taihu Lake, China): cyanobacteria blooms produce teratogenic retinal. Environ. Sci. Technol. 47, 807–814.

Yen, J., Donerly, S., Levin, E.D., Linney, E.A., 2012. Differential acetylcholinesterase inhibition of chlorpyrifos, diazinon and parathion in larval zebrafish. Neurotoxicol. Teratol. 33, 735–741.

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Retinoid compounds associated with water blooms dominated by *Microcystis* species

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ABSTRACT

Retinoic acids play a critical role in vital physiological processes and vertebrate development, and their derivatives can be produced by some cyanobacterial species into surface waters. This study presents important environmentally-relevant information on total retinoid-like activity of field cyanobacterial biomasses and their surrounding waters. Intracellular and extracellular levels of total retinoid-like activity and retinoic acids have been investigated at a set of independent sites with the occurrence of water bloom dominated by widespread species *Microcystis aeruginosa*. Twelve samples of biomass and surrounding water from seven localities affected by blooms were studied in comparison with samples from *M. aeruginosa* laboratory cultures. The method for biomass extraction was optimized and final extracts and samples of surrounding water concentrated by solid phase extraction were assessed using *in vitro* reporter gene bioassay and chemical analyses for all-*trans*-retinoic acid (ATRA), 9-*cis* retinoic acid (9-*cis* RA) and microcystins RR, LR and YR. Methanol was the most efficient solvent for the extraction of compounds with retinoid-like activity. An *in vitro* bioassay with the P19/A15 transgenic cell line revealed retinoid-like activity in all cyanobacterial biomasses in the range of 356–2838 ng of retinoid acid equivalents (REQ)/g dry mass (dm), while only three of surrounding water samples exhibited detectable retinoid-like activity, in the range of 12.8–28.7 ng REQ/L. Microcystins were detected in all samples, but they elicited no detectable retinoid-like activity up to 10 mg/L. Chemical analyses detected concentrations up to 340 ng/g dm of all-*trans*-retinoic acid (ATRA) and 84 ng/g dm 9-*cis* retinoic acid (9-*cis* RA) in bloom extracts, and up to 19 ng/L ATRA and 2.2 ng/L 9-*cis* RA in surrounding water. In most samples, ATRA and 9-*cis* RA contributed relatively little to the total REQs, which indicates the presence of significant amounts of other compounds with retinoic acid receptor-mediated modes of action. The impact of retinoid-like cyanobacterial metabolites could be of importance namely in smaller water bodies with dense water blooms and low dilution.

1. Introduction

Worldwide occurring expansion of harmful water blooms has been linked with increased eutrophication, particularly due to the intensification of agricultural and industrial activities associated with the growth of the human population. Water blooms lead to undesirable ecological conditions due to various negative impacts on ecosystem functions. Ecological impairment can be caused by reduced light penetration through the mass of cyanobacteria in water columns or *via* the increasing pH and low oxygen levels associated with cyanobacterial bloom decomposition (Scheffer et al., 1997). Moreover, cyanobacteria produce a wide spectrum of biologically active intra- and extra-cellular substances. Some of them have been recognized as human and animal health hazards, since they have been shown to cause adverse effects on invertebrates, fish, amphibians, birds and mammals (De Figueiredo et al., 2004; Kuiper-Goodman et al., 1999; Malbrouck and Kestemont, 2006; Skocovska et al., 2007; Wiegand and Pflugmacher, 2005). Various biologically active compounds are synthesized during the growth phase of cyanobacteria. The largest amounts of bioactive compounds are released after cell lysis or from actively expanding cyanobacterial populations into the water.

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Microcystis aeruginosa is one of the most harmful species of cyanobacteria due to the occurrence of frequent and abundant water blooms and the production of toxic microcystins (Chorus and Bartram, 1999; Kolmakov, 2006; Paerl et al., 2011). *M. aeruginosa* is probably the most widely-distributed cyanobacterium, and is responsible for major toxic bloom problems in Europe (Via-Ordorika et al., 2004), Asia (Zhang et al., 2012), North America (Wilson et al., 2005) and other regions. The ecological advantage of *Microcystis* species is their lower dependence on high light intensities compared to some other cyanobacteria, because semi-active vertical movement enables them to find optimal light conditions. The genus thus occurs in mesotrophic, eutrophic and hypertrophic waters, but the amount of biomass produced depends on the level of eutrophication (Chorus and Bartram, 1999). *Microcystis* is a known producer of a wide variety of bioactive metabolites such as hepatoxin microcystin, alkaloids anatoxin-a, β-methylamino-L-alanine, odorous compounds (geosmins and terpenoids) and some other peptides (aeruginosins, cyanopeptolins and microviridins, *etc.*) (Isaacs et al., 2014; Suurnäkki et al., 2015; Welker et al., 2012; Zhang et al., 2013).

Next to the production of known cyanotoxins, some compounds able to interfere with the endocrine system have also been shown to be associated with complex cyanobacterial samples (Rogers et al., 2011; Stěpánková et al., 2011). Recently, studies of laboratory cultivated cyanobacteria have reported retinoid-like activity in extracts (Jonas et al., 2015; Kaya et al., 2011) as well as in exudates of several species (Jonas et al., 2014) including Microcystis sp. Chemical analyses have documented the presence of several analogues of retinoic acids in *Microcystis aeruginosa* strains isolated from two Chinese lakes (Wu et al., 2012). *M. aeruginosa* and *M. flos-aquae* were suggested as the two species mainly responsible for the presence of retinoic acid derivatives in blooms of Taihu Lake, China (Wu et al., 2012), which indicates the environmental importance of *Microcystis* sp. in the production of retinoid compounds.

The significance of the potential presence of retinoid-like compounds in surface waters is related to their important role in controlling vital physiological processes such as reproduction and development in vertebrates (Grenier et al., 2007). The physiological activity of retinoid-like compounds is mediated *via* the retinoid acid receptor (RAR). All-*trans*-retinoic acid (ATRA), a low molecular weight lipophilic metabolite of retinol (vitamin A), is the most potent natural ligand of RAR. Retinoic acids (RAs) are significant teratogens when normal physiological concentrations are exceeded (Bryant and Gardiner, 1992; Selderslaghs et al., 2009). RAs were found to cause malformations and mortality to tadpoles of African clawed frogs (*Xenopus laevis*) (Degitz et al., 2000), as well as deformities in zebrafish (*Danio rerio*) embryos, such as yolk sac and heart edemas, brain and tail malformations, duplication of otic placodes and otoliths (Herrmann, 1995), elongated heart chambers, small intestine deformities, absence of liver tissue (Haldi et al., 2011) and neurotoxicity (Parng et al., 2007).

Recently, teratogenic effects were reported in embryos of *Danio rerio* after exposure to cyanobacterial extracts and exudates from laboratory cultivations, with malformations remarkably similar to deformities caused by retinoic acids (Jonas et al., 2015, 2014).

It is necessary to take into account that complex metabolite production is affected by abiotic as well as biotic factors in the natural environment of the organisms. Also, when cultivated in laboratory conditions, the spectrum and levels of the secondary metabolites produced could be different than in the environment (Halstvedt et al., 2008; Repka et al., 2004; Rohrlack and Utkilen, 2007). This leads to difficulties with estimation of cyanobacterial toxicity risks from laboratory cultivations. Thus, field studies in water reservoirs are needed for better understanding of the production retinoid-like compounds during cyanobacterial water blooms.

The presence of retinoid compounds associated with cyanobacterial water blooms in the environment has so far only been reported in a study of samples collected from Taihu Lake in China (Wu et al., 2012). That study detected retinoic acids and a few of their derivatives by chemical analyses, but there was no information on total retinoid-like activity of cyanobacterial biomass and surrounding water. Moreover, there are no studies from sites other than Taihu Lake. Also, there is no report of seasonal changes or time development of retinoid-like compounds occurrence.

The aim of this study was to investigate the presence of compounds with retinoid-like activity in water blooms dominated by *Microcystis* species across independent ecosystems, and to identify the contribution of known individual chemicals, *i.e.* microcystins (MCs) and RAs, to the assessed total retinoid-like activity. The paper focuses on the retinoid-like potency of both cyanobacterial biomass and its surrounding water. Samples were taken from seven separate reservoirs across the South Moravian region in the Czech Republic, and selected sites were sampled periodically to reveal possible seasonal variability. The extraction process was optimized for maximal yields of retinoid-like activity. The data obtained with field samples were compared to the samples from laboratory culture.

2. Materials and methods

2.1. Sampling sites and samples collection

Samples of biomass from cyanobacterial water blooms and surrounding water were collected from seven localities across the Moravia region of the Czech Republic (Fig. 1), where water blooms frequently occur. Each locality represented independent separated reservoir. Sampling campaigns took place between 16 July and 11 September 2012. In order to examine the variability during bloom season and possible time trends, the Nové Mlýny site (N) was sampled five times, and Bítov was sampled twice, at 14 day intervals. Samples of cyanobacterial biomass were collected from the water with a plankton net (20 μm mesh), transported on ice to the laboratory, and stored frozen (−20° C) prior to further processing. Samples of water surrounding the water bloom (*i.e.* surrounding water) were collected in 2.5 L amber-glass bottles, transported on ice to the laboratory, and stored in the dark at 4 °C for up to 24 h before processing.

2.2. Processing of water samples

Surrounding water samples were concentrated using solid phase extraction (SPE). The samples were vacuum filtered through 0.6 μm paper filters (Macherey-Nagel, Dueren, Germany). Filtrates were passed through two SPE columns connected in tandem: 1 g Oasis HLB column (Waters, Milford, USA) and 1 g Carbograff column (Alltech, Deerfield, USA). The samples were dosed to the cartridges through PTFE tubes with a flow rate of approximately 4–6 mL/min. Subsequently, the cartridges were dried for 10 min by negative pressure, and then eluted with 20 mL of methanol. The extracts were evaporated to near dryness under gentle stream of nitrogen with a sample concentrator (LabEva Visible, Labicom, Czech Republic). Finally, samples were reconstituted in methanol to obtain extracts with concentration factor of 4000×.

2.3. Optimization of biomass samples extraction

In order to select the most efficient method for extraction of retinoid-like substances, the extraction method was optimized through several steps: selection of most efficient solvent, optimal duration of ultrasonic homogenization and maximizing effectiveness by re-extraction and prolonged extraction with shaking.

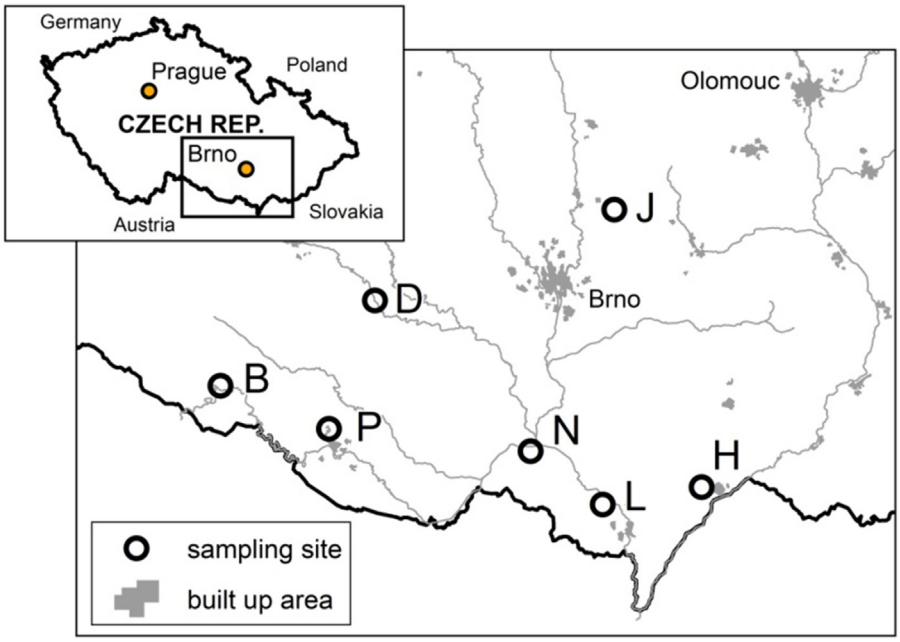

Fig. 1. Map of sampling sites in South Moravian region, Czech Republic.

The extraction efficiency of various solvents was tested on biomass samples from localities D, J and N1 (Supplementary Fig. S1). The solvents used were acetone, ethyl acetate, methanol, 75% (v/v) and 50% (v/v) methanol/water, and hexane/chloroform (1:1, v/v). For each sample, 200 mg of freeze-dried biomass were extracted in a glass test tube with 6 mL of solvent. Cells were disintegrated by sonication with an ultrasonic disintegrator (100% power, cycle 0.9, Bandelin Sonopuls HD 2070, Germany) for 3×3 min in a cooling bath. Test tubes containing biomasses were held in a cooling bath during the sonication to prevent thermal degradation of sensitive compounds. The disintegration of the cells was always checked under a microscope. To remove cellular debris, extracts were centrifuged at $3050 \times g$ for 5 min. The liquid phase was transferred to glass vials and evaporated to near dryness by a nitrogen stream. Samples extracted by 100% methanol, acetone, ethyl acetate and hexane/chloroform were reconstituted in 500 μL of 100% methanol to obtain biomass concentration of 400 g dry mass (dm)/L, and tested for *in vitro* retinoid-like activity at final concentrations of 0.25–2 g dm/L. Extracts prepared with 50% and 75% methanol/water became gelatinous when evaporated and difficult to reconstitute in 100% methanol, therefore the final volume was 1 mL with concentration of 200 g dm/L. These extracts were tested *in vitro* at final concentrations of 0.25–1 g dm/L.

To investigate extraction dynamics, samples B1 and N3 were extracted with different durations of sonication and subsequently by shaking on an orbital shaker. 200 mg of freeze-dried biomass were extracted in glass test tubes with 6 mL of 100% methanol and sonicated for 2×1, 2×2 and 3×3 min periods (as described above). After the sonication, some extraction variants were placed on an orbital shaker (100 RPM, GFL 3020, GFL, Burgwedel, Germany) for further extraction. At time points of 5 and 60 min, the appropriate test tubes were taken from the shaker. The samples were centrifuged ($3050 \times g$ for 3 min), extracts were transferred to glass vials, and final volumes were adjusted to obtain concentrations of 400 g dm/L.

To evaluate extraction efficiency, re-extraction with fresh solvent and prolonged extraction with shaking was tested. 200 mg of freeze-dried biomass from localities B1 and N3 were extracted in glass test tubes with 5 mL of methanol and sonicated for 2×2 min. After the sonication, test tubes were centrifuged at $3050 \times g$ for 3 min. Extracts were transferred to a glass vial, 1 mL of methanol was added to the test tubes with biomass, samples were re-extracted by sonication (30 s), centrifuged again ($3050 \times g$ for 3 min), and re-extracts were added to the appropriate primary extracts from the first extraction. After that, another 5 mL of methanol was added to the test tubes, sonicated briefly to resuspend cellular mass, and placed on the orbital shaker (100 RPM). After 2 h, the extract was transferred to a glass vial and biomass re-extracted with 1 mL of methanol. This process was repeated twice more for shaking durations of 5 and 24 h. Final volumes were adjusted to obtain concentrations of 400 g dm/L.

2.4. Optimized extraction procedure

Based on the optimization experiments, all samples were finally extracted with the following procedure. In a glass test tube 200 mg of freeze-dried biomass was extracted with 5 mL of methanol by sonication for 2×2 min (100% power, cycle 0.9) in a cooling bath. Then, test tubes were centrifuged ($3050 \times g$ for 3 min), extracts were transferred to glass vials and biomass re-extracted with 1 mL of methanol and 30 s sonication. After the centrifugation ($3050 \times g$ for 3 min), re-extracts were added to the first extracts. Then, another 5 mL were added to the test tubes, sonicated for 30 s and test tubes were placed on orbital shaker (100 RPM). After 2 h shaking, samples were centrifuged and supernatants added to previous corresponding extracts. Biomasses were re-extracted with 1 mL methanol and 30 s sonication, centrifuged and supernatants pooled with extracts from the previous extraction steps. The final volumes were adjusted to obtain exact concentrations of 400 g dm/L. Obtained extracts were tested for *in vitro* retinoid-like activity at concentrations of 0.25–2 g dm/L. All extracts were stored at −20 °C.

2.5. Preparation of samples from laboratory cultured Microcystis and microcystins

Cyanobacterium *Microcystis aeruginosa* PCC 7806 was purchased from Pasteur Collection of Cyanobacteria (PCC, Paris, France), and cultivated over the long-term in RECETOX labs in a 50% mixture of Zehnder medium (Schlösser, 1994) and Bristol Bold

medium (Stein, 1975). Organisms were grown at 22 ± 2 °C under continuous light and aeration with air filtered through a 0.22 μm filter (Labicom, Czech Republic). The cultivations were started with 20% (v/v) of the inoculum with optical density 0.3 at 680 nm. After 21 days, cells were harvested by centrifugation, and supernatant (medium with exudates) extracted by SPE in the same manner as surrounding water from field samples. The harvested cells were freeze-dried and extracted according to the optimized extraction procedure used for the environmental biomasses.

To assess retinoid-like potential of pure cyanotoxins, microcystins MC-LR, MC-RR and MC-YR (Enzo Life Sciences, Inc., USA) were dissolved in methanol and tested *in vitro* at concentrations of 3 μg/L–10 mg/L.

2.6. In vitro *bioassay*

The retinoid receptor-mediated response was tested on cell line P19/A15 derived from murine embryonic carcinoma cells P19 (European Collection of Cell Culture, Wiltshire, UK) with endogenous expression of retinoid receptors by stable transfection with reporter luciferase gene under the control of retinoic acid-responsive element (pRAREβ2-TK-luc plasmid) (Novák et al., 2007).

Cells were cultured in plastic tissue culture flasks (TPP, Austria) in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Prague, Czech Republic) with phenol red containing 10% fetal calf serum Superior (Biochrom, Berlin, Germany). Cells were grown in a humidified atmosphere with 5% CO_2 at 37 °C.

After the cells subculturing, 10,000 cells per well were seeded into 96-well microplates with a final volume of 100 μL of cultivation media. After 24 h, extracts, solvent controls and standard calibration were dosed into the wells containing 100 μL of cultivation medium. A series of dilutions of all-*trans*-retinoic acid (ATRA), a known potent ligand of RAR, in the 1–1000 nM range, was used for calibration of retinoid-like response. Extracts of cyanobacterial biomasses were tested at final concentrations of 0.25, 0.5, 1 and 2 g dm/L. Surrounding water extracts were tested at concentration factors of 2.5×, 5×, 10× and 20× relative to the environmental water.

After 24 h exposure, the medium was removed, cells were gently washed with phosphate buffered saline (PBS) and luminescence was measured on a luminometer (Luminoskan Ascent, Thermo Scientific, Waltham, MA, USA) after the addition of luciferase reagent (Steady-Glo® Luciferase Assay System, Promega, Mannheim Germany) following the manufacturers recommendations.

All samples were tested in triplicate, and each experiment was independently repeated at least twice. The relative luminescence units were converted to a percentage of the maximal luminescence response induced by 500 nM ATRA for easier comparison among experiments.

2.7. Evaluation of cytotoxicity

The cytotoxicity of samples was determined by using neutral red uptake assay (Freyberger and Schmuck, 2005). Briefly, cells were treated as described in Section 2.6 and exposed to solvent control and samples. After a 24 h incubation period, neutral red solution (0.5 mg/mL of media) was added and cells were incubated for 1 h at 37 °C. The medium was removed, cells were washed with PBS and lysed with 1% acetic acid in 50% ethanol. Absorbance was measured in a microplate spectrophotometer (POLARstar OPTIMA, BMG Labtech, Ortenberg, Germany) at 540 and 690 nm. Extract dilutions were determined as cytotoxic in case the absorbance at tested concentration was less than three times the standard deviation of solvent control.

2.8. Liquid chromatography electrospray ionization mass spectrometry analyses for microcystins

Analyses of microcystins were performed with an Agilent 1290 series HPLC (Agilent Technologies, Waldbronn, Germany) consisting of a vacuum degasser, a binary pump, an autosampler, and a thermostatted column compartment kept at 30 °C. The column was a Phenomenex LUNA C-18 endcapped (3 μm) 100 × 2 mm i.d., equipped with a Phenomenex SecureGuard C18 guard column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of 5 mM ammonium acetate in water, pH 4 (A) and methanol-acetonitrile mixture (1:1) with 5 mM ammonium acetate (B). The binary pump gradient was non-linear (increase from 25% B at 0 min to 80% B at 2 min, then increase to 95% B at 10 min, then 95% B for 4 min and 4 min column equilibration to initial conditions (25% B)); the flow rate was 0.25 mL/min. 5 μL of individual sample was injected for the analyses.

The mass spectrometer AB Sciex Qtrap 5500 (AB Sciex, Concord, ON, Canada) with electrospray ionization (ESI) was used for detection. Ions were detected in the positive mode. The ionization parameters were as follows: capillary voltage, 5.5 kV; desolvation temperature, 350 °C; curtain gas 15 psi, gas 1 40 psi, gas 2 30 psi. In scheduled MRM mode the following *m/z* transitions were monitored (with corresponding values of declustering potential—DP (V), entrance potential—EP (V) and collision energy–CE (V)): MC-RR 519.8 > 135.1 (DP 156, EP 10, CE 37) and 102.9 (DP 156, EP 10, CE 91), MC-YR 1045.5 > 102.9 (DP 241, EP 10, CE 129) and 212.9 (DP 241, EP 10, CE 69), MC-LR 995.5 > 102.9 (DP 171, EP 10, CE 129) and 105.1 (DP 171, EP 10, CE 127). The quantification of analytes was based on external standards of MC-RR, MC-YR and MC-LR.

2.9. Liquid chromatography electrospray ionization mass spectrometry analyses for RAs

Analyses of retinoic acids were performed with a Waters Acquity HPLC (Waters, Manchester, U.K.) consisting of a vacuum degasser, a binary pump, an autosampler, and a thermostatted column compartment kept at 40 °C. The column was a Waters Acquity UPLC BEH C18, 1.7 μm, 100 × 2.1 mm i.d., equipped with a Waters Acquity UPLC BEH C18 VanGuard Pre-column (Waters, Manchester, U.K.). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The binary pump gradient was non-linear (increase from 20% B at 0 min to 70% B at 1 min, then increase to 100% B at 5 min, then 100% B for 1 min and 2 min column equilibration to initial conditions (20% B)). The flow rate was 0.3 mL/min. 5 μL of individual sample was injected for analysis.

The mass spectrometer used for detection was Waters XEVO TQ-S (Waters, Manchester, U.K.) with electrospray ionization (ESI). Ions were detected in the positive mode. The ionization parameters were as follows: capillary voltage, 1.0 kV; desolvation temperature, 450 °C; cone gas 150 L/h, drying gas 600 L/h, nebulizer 7.0 bar. In scheduled MRM mode the following *m/z* transitions were monitored (with corresponding values of cone voltage—CV (V), source offset—SO (V) and collision energy—CE (V)): ATRA 301.2 > 205.2 (CV 30, SO 60, CE 12) and 159.1 (CV 30, SO 60, CE 23), 9 *cis*-RA 301.2 > 205.2 (CV 30, SO 60, CE 13) and 159.1 (CV 30, SO 60, CE 23). The quantification of analytes was based on external standards of ATRA and 9cis-RA.

2.10. Data analyses

To derive EC_{10} values, concentration-response models were fitted on *in vitro* data using R software (version 3.1.0 for Windows, www.R-project.org, R Core Team, 2014) with the following

packages: Dose-response curve (drc; Ritz and Streibig, 2005), Epidemiological calculator (epicalc; Chongsuvivatwong, 2012), Multiple Comparisons (multicomp; Hothorn et al., 2008), Harrell Miscellaneous (Hmisc; Harrell, 2014) and Calibration functions for analytical chemistry (chemCal; Ranke, 2014).

The Hill (*i.e.* four-parametric log-logistic) model was used to fit ATRA as a positive control. In the case of concentration-response models for samples, the best-fitting regression model was always selected from six fitted models to properly describe measured concentration-response trend of each sample. As a selection criterion, the Akaike's information criterion (AIC) was used to choose from linear, linear-log, exponential, Hill, Weibull I and Weibull II models (more details in Supplementary Table S1) (Ritz and Streibig, 2005).

Retinoid Equivalents (REQs) for extracts of cyanobacterial biomass were calculated as the ratio of $EC_{10(ATRA)}/EC_{10(sample)}$ presented as ng of equivalent ATRA per g of dry mass (g dm) of freeze-dried cyanobacterial biomass/cells. For the few samples which did not reach an EC_{10} effect, REQs were interpreted based on point interpolation of their statistically significant maximal response using the ATRA calibration curve. REQs are expressed as equivalents of ng ATRA per liter of sampled water (ng/L), or equivalents of ng ATRA per gram of cyanobacterial dry mass (ng/g dm).

3. Results

The taxonomic composition of collected environmental biomass samples was dominated by *Microcystis aeruginosa*, with the occasional presence of *Planktothrix agardhii*, *Microcystis viridis*, *Phormidium* spp. and *Microcystis ichtyoblabe* (Table 1). *M. aeruginosa* reached 100% dominance in eight out of twelve samples. The density of biomass in sampled reservoirs ranged from 135 500 (N1) to 76 375 000 (D) cells/mL. The density of laboratory cultures of *M. aeruginosa* (6 660 000–22 920 000 cells/mL) were in the same range as the field biomasses of higher densities.

3.1. Retinoid-like activity in surrounding water samples

All extracts of surrounding water samples were tested *in vitro* for retinoid-like activity at concentrations 2.5, 5, 10 and 20 times higher than the original environmental waters. Two samples from Nové Mlýny (N3 and N4) and one from Bítov (B1) showed significant responses (Fig. 2). No detectable RAR-mediated activity was observed for other samples (<10 ng REQ/L). The strongest retinoid-like activity corresponding to 28.7 ng REQ/L was found in the Nové Mlýny sample from August 28, 2012 (N4). No cytotoxicity was observed for any water extract up to the highest tested concentration.

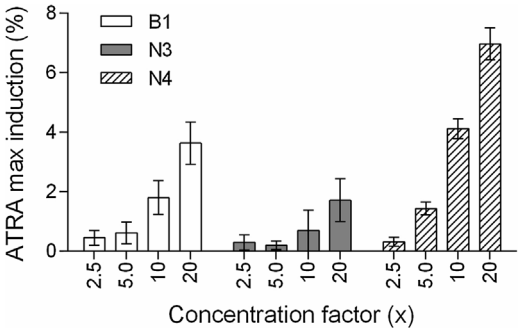

Fig. 2. Retinoid-like activity of water surrounding the cyanobacterial blooms. Three out of twelve samples exhibited detectable retinoid-like activity. Effects are shown as percentage of maximal induction caused by ATRA. Results are presented as mean ± SD of two independent measurements.

Exudates of the laboratory cultivated strain *Microcystis aeruginosa* PCC 7806 elicited retinoid-like activity as high as 474–1081 ng REQ/L across nine cultivations (details in Sychrova et al., submitted).

3.2. Optimization of extraction method

Retinoid-like activity for concentrations of 0.25, 0.5 and 1 g dm/L of extracts from five biomasses from localities Bítov (B1), Dalešice (D), Jedovnice (J), and Nové Mlýny (N1 and N3) using various solvents is shown in Fig. 3. All five samples showed the highest retinoid-like responses after extraction with 100% methanol, with maximal response ranging from 3 to 15% of maximal ATRA induction. Lower responses were observed for extracts prepared with other solvents. Extracts in 75% methanol showed dose-dependent responses in two samples (D and J); weak effects were observed also in samples extracted by acetone (samples B1, D, J), ethyl acetate (sample N3) and hexane-chloroform (sample B1).

Table 1
Characterization of the environmental samples, species composition, biomass density and sites descriptions.

Sample	Date of sampling	Locality	Total biomass (cells/mL)	Percentage of cyanobacteria in total biomass	Species composition (% of cyanobacterial content)
B1	28 August 2012	Bítov–Vranov reservoir	1.23×10^7	100	*Microcystis aeruginosa* (100)
B2	11 September 2012	Bítov–Vranov reservoir	1.03×10^6	100	*Microcystis aeruginosa* (100)
D	23 August 2012	Dalešice	7.64×10^7	100	*Microcystis aeruginosa* (49.75) *Microcystis viridis* (48.94) *Microcystis ichtyoblabe* (1.31)
H	16 July 2012	Hodonín–pond Písečenský	2.43×10^5	97.2	*Microcystis aeruginosa* (97) *Phormidium* spp. (3)
J	23 August 2012	Jedovnice	1.84×10^5	78.4	*Microcystis aeruginosa* (100)
L	28 August 2012	Lednice	–	100	*Microcystis aeruginosa* (100)
N1	16 July 2012	Nové Mlýny	1.35×10^5	98.7	*Microcystis aeruginosa* (60) *Planktothrix agardhii* (40)
N2	30 July 2012	Nové Mlýny	1.36×10^5	97.1	*Microcystis aeruginosa* (100)
N3	14 August 2012	Nové Mlýny	1.02×10^7	99.8	*Microcystis aeruginosa* (100)
N4	28 August 2012	Nové Mlýny	1.49×10^5	100	*Microcystis aeruginosa* (100)
N5	11 September 2012	Nové Mlýny	4.0×10^5	100	*Microcystis aeruginosa* (100)
P	28 August 2012	Přížřenice	1.54×10^5	89.7	*Microcystis aeruginosa* (100)
M. aeruginosa PCC 7806[a]		Laboratory culture	$6.66–22.92 \times 10^6$	100	*Microcystis aeruginosa* (100)

–: Invalid sample, cell counting was not possible.
[a] Laboratory culture, values are shown as range obtained by nine separated cultivations.

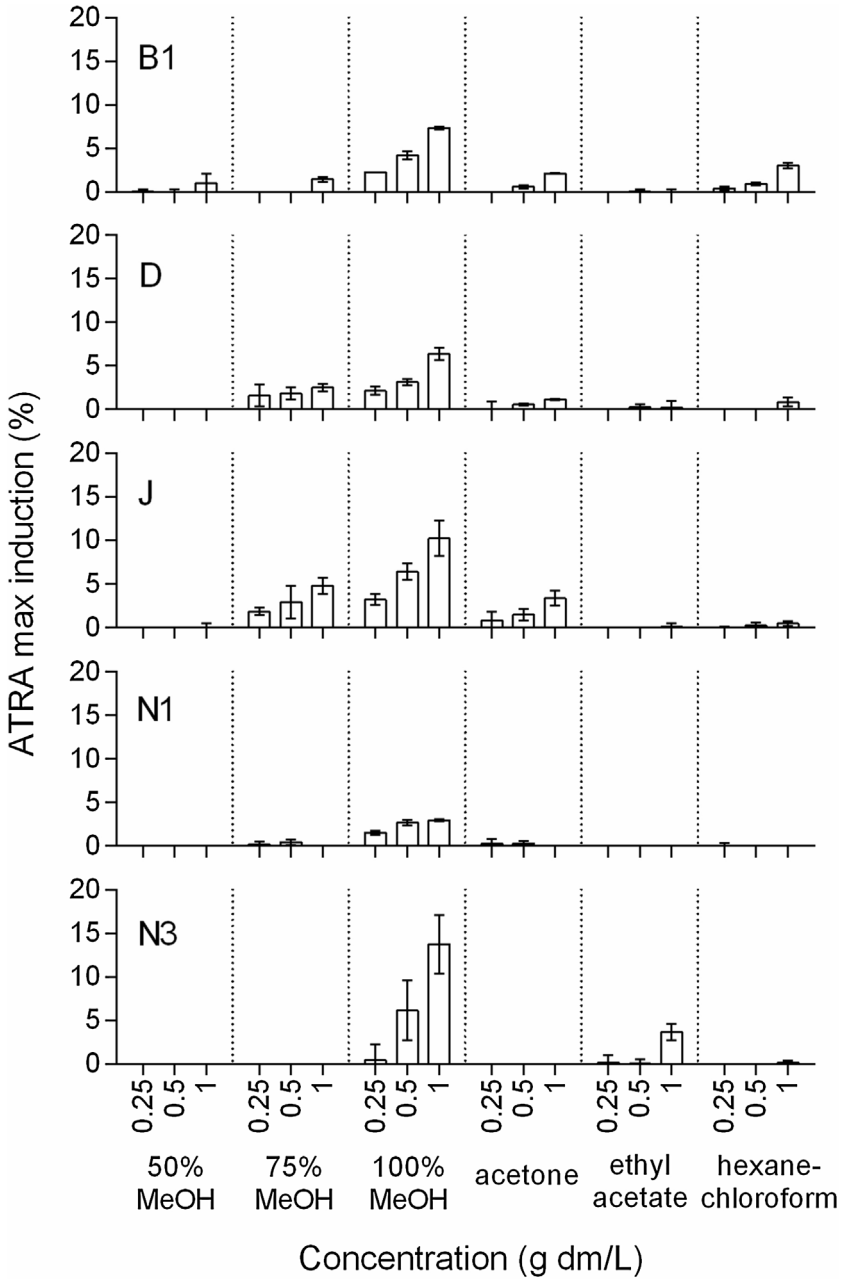

Fig. 3. Comparison of *in vitro* retinoid-like activity of biomasses from localities Bítov (B1), Dalešice (D), Jedovnice (J) and Nové Mlýny (N1 and N3) extracted by various solvents. Retinoid-like activity is shown as a percentage of maximal ATRA induction. Results are presented as mean ± SD of two independent measurements.

Therefore, methanol was chosen as the most efficient extraction solvent for further experiments.

In the assessment of the influence of sonication and extraction duration, two biomasses (B1 and N3) were extracted with methanol. Despite the use of various durations of sonication and extraction, all methods showed similar efficiency (Supplementary Fig. S1). The variant with a 2 × 2 min sonication period was chosen for further extractions. Longer extraction time (*i.e.* duration of sonication or shaking) had no positive influence on the resulting retinoid-like activity.

To maximize extraction efficiency, two biomasses from localities B1 and N3 were re-extracted with methanol at four time points. The highest responses were caused by primary extracts collected immediately after the first sonication and centrifugation (23% of maximal ATRA response for B1 and 32% for N3, tested concentrations 2 g dm/L), followed by re-extracts shaken for 2 h (5.5 and 9.8% of maximal ATRA, respectively). Further re-extractions (5 and 24 h) showed no significant *in vitro* responses (Fig. 4). Therefore, extraction with 2 h re-extraction to fresh solvent was chosen for extracting all samples.

3.3. Retinoid-like activities of biomass extracts

Extracts of all twelve environmental biomasses dominated by *Microcystis aeruginosa* from seven separate lakes showed significant dose-dependent retinoid-like activity *in vitro* (Fig. 5). Two samples (H and N5) did not reach 10% of maximal ATRA induction due to their greater cytotoxicity. Most of the samples elicited effect in the range of 10–30% of maximal ATRA induction at the highest non-cytotoxic concentrations (usually 2 g dm/L; H, N1 and N5 at 1 g dm/L). The greatest induced responses reached up to 42% (B1) and 34% (P). The total retinoid-like activity of extracts from

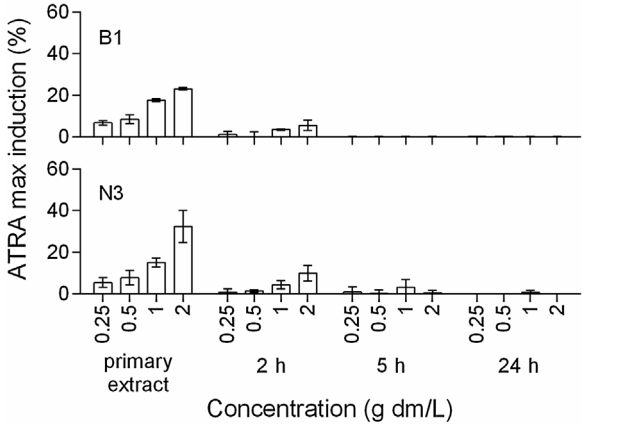

Fig. 4. Comparison of retinoid-like activity of extracts and re-extracts from localities Bítov (B1) and Nové Mlýny (N3). Primary extracts and re-extracts after 2, 5 and 24 h of shaking were tested. Results are presented as mean ± SD of two independent measurements.

environmental biomasses ranged from 356 to 2838 ng REQ/g dm (Table 2). The retinoid-like activity from locality N sampled at five time points during bloom season varied from 492 to 2838 ng REQ/g dm in biomass and from below the limit of detection (LOD) of 10 to 28.7 ng REQ/L in surrounding water.

Extracts of biomass from nine laboratory cultivations of *Microcystis aeruginosa* PCC 7806 tested *in vitro* for retinoid-like activity showed dose-dependent retinoid-like responses ranging from 1066 to 1837 ng REQ/g dm (Table 2).

3.4. Chemical analysis

Chemical analyses of water and cyanobacterial extracts by HPLC/MS/MS identified both microcystins and retinoic acids in most samples. Concentrations of individual microcystins (MC-RR, MC-YR, MC-LR) ranged from below LOD to 1114 μg/g dm in biomasses and to 33.5 μg/L in surrounding waters (highest concentration of MC-RR was in sample J; Table 2). The sum of the three studied microcystins ranged from 51 to 1506 μg/g dm in environmental biomasses and from 0.0012 to 44.2 μg/L in surrounding waters. Microcystin RR was the predominant structural variant in environmental samples while it was MC-LR in laboratory cultivations. Nevertheless, no significant *in vitro* retinoid-like effect was observed for any of the microcystins MC-LR, MC-RR and MC-YR at concentrations ranging from 3 μg/L–10 mg/L (data not shown).

Retinoid acids were detected in eight biomasses extracts at concentrations up to 340 ng/g dm ATRA (sample N4) and 84 ng/g dm 9-*cis* RA (sample H). They were also detected in eight water extracts reaching up to 19 ng/L ATRA and 2.2 ng/L 9-*cis* RA (sample N4). The sum of both ATRA and 9-*cis* RA was from below LOD to 394 ng/g dm in environmental biomasses and to 20 ng/L in surrounding water.

Extracts of laboratory cultured *Microcystis aeruginosa* biomass contained retinoic acids at concentrations ranging from below LOD up to 123 ng/g dm ATRA and up to 32 ng/g dm 9-*cis* RA, respectively. Levels in surrounding media reached up to 12 ng/L of ATRA and up to 0.7 ng/L 9-*cis* RA, respectively.

4. Discussion

In a few recent papers, cyanobacterial water blooms have been associated with the occurrence of retinoic acids and their derivatives. These compounds can be produced into surface waters as exudates from living cells or released from intracellular sources after cell death. They can be also directly uptaken by organisms feeding on phytoplankton. Given the essential role of retinoids for development of vertebrates (Bryant and Gardiner, 1992; Selderslaghs et al., 2009), and consequently their teratogenic effects at greater concentrations (Bryant and Gardiner, 1992; Selderslaghs et al., 2009; Degitz et al., 2000), this has raised concerns about their potential risks during cyanobacterial blooms. The information regarding retinoids in environmental water blooms is very limited (originating from one lake) and there was no knowledge on the total retinoid-like activity of environmental cyanobacterial biomasses and their surrounding waters, needed for understanding of environmental toxicity risks caused by these compounds.

There are only few studies describing the presence of retinoids in cyanobacteria and surrounding water. Wu et al. (2012, 2013) provided information on several individual retinoids in environmental biomasses, surrounding water extracts, and laboratory cultures of phytoplankton species. In 32 out of 39 laboratory cultures (22 Cyanobacteria, 6 Chlorophyta, 3 Bacillariophyta, and 1 Euglenophyta), they detected ATRA at levels up to 250 ng/g dm, and 9-*cis* RA at levels up to 520 ng/g dm. Specifically in *Microcystis aeruginosa* culture, the content of RAs was 90 ng ATRA/g dm, and 140 ng 9-*cis* RA/g dm. The content of RAs in environmental cyanobacterial biomasses dominated by Microcystis sp. was, in case of ATRA, at levels up to 220 ng/g dm, but 9-*cis* RA was not detected at all. Extracellular concentrations in surrounding lake water reached up to 5.9 ng ATRA/L and 3.2 ng 9-*cis* RA/L (Wu et al., 2012). In the field study discussed in this paper, which used

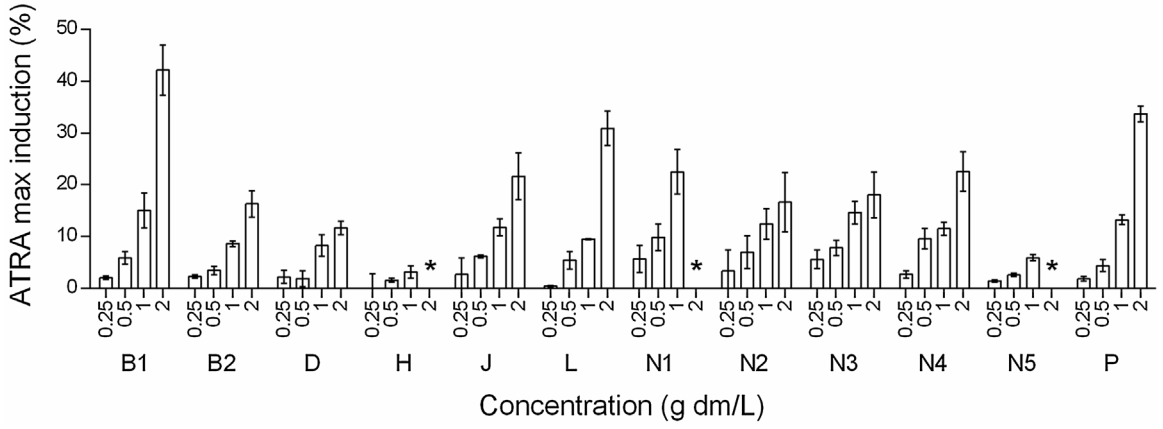

Fig. 5. Retinoid-like activity of extracts from all samples of biomass collected during summer 2012. Extract of biomass from each locality showed dose-dependent retinoid-like activity. Results are presented as mean ± SD of two independent measurements. *—Cytotoxic effect, data excluded.

Table 2
Retinoid equivalents (REQs) and concentrations of microcystins and retinoic acids (RAs) detected in water and biomass samples.

Sample	Biomass REQ [ng/g dm]	Water REQ [ng/L]	MC in biomass [μg/g dm]			MC in water [μg/L]			RAs in biomass [ng/g dm]		RAs in water [ng/L]	
			MC-RR	MC-YR	MC-LR	MC-RR	MC-YR	MC-LR	ATRA	9-*cis* RA	ATRA	9-*cis* RA
B1	1565	19.29	807	40	371	0.001	<0.00061	0.0006	59	16	<0.15	<0.15
B2	1095	<10	900	39	334	0.017	0.0013	0.0084	<1.5	6.9	<0.15	0.83
D	1039	<10	213	86	191	0.004	<0.0021	0.0032	3.2	<1.5	1.6	0.88
H	356[a]	<10	66	24	31	0.003	<0.00094	0.0012	310	84	<0.15	0.25
J	1487	<10	1114	60	222	33.5	3.09	7.58	<1.5	<1.5	<0.15	0.64
L	1371	<10	508	70	213	0.0006	<0.00097	0.001	76	33	0.95	<0.15
N1	2838	<10	553	129	214	0.004	<0.0013	0.0014	15	19	<0.15	2.2
N2	1715	<10	709	270	527	0.0012	<0.00042	<0.00085	<1.5	<1.5	<0.15	<0.15
N3	2208	12.79	329	83	373	2.22	0.68	1.29	<1.5	<1.5	<0.15	0.255
N4	1459	28.66	36	<5	15	0.0022	<0.0012	<0.0012	340	<1.5	19	1
N5	492[a]	<10	966	95	327	0.18	0.014	0.056	16	<1.5	<0.15	<0.15
P	1415	<10	543	41	152	0.0014	<0.00074	0.0007	<1.5	<1.5	<0.15	<0.15
M. aeruginosa PCC 7806[b]	1066–1837	474–1081	<0.5–3.9	<5	127–708	0.2–0.4	<0.25	16–66	<1.5–123	<1.5–32	<0.3–12	<0.3–0.7

[a] Weak dose-dependent retinoid-like activity, REQs were calculated by point estimation.
[b] Results are shown as range of values obtained from nine separate laboratory cultivations.

optimized extraction by methanol and two-phased sonication/shaking, RAs were detected in 8 out of 12 environmental cyanobacterial biomasses at levels comparable to the previous study (ATRA up to 340 ng/g dm, and 9-*cis* RA up to 84 ng/g dm). Additionally, the concentrations in surrounding water samples corresponded to previously reported values (in 8 out of 12 water samples, ATRA content was up to 19 ng/L, and 9-*cis* RA content up to 2.2 ng/L).

A study by Kaya et al. (2011) focused on biomass of laboratory-cultured cyanobacteria described new RA analogue (7-hydroxy retinoic acid), and also provided information about the total *in vitro* retinoid-like activity of extract from several cyanobacteria analyzed by yeast RA activity assay. The detected total retinoid-like potency of extract from *Microcystis aeruginosa* biomass (2500 ng REQ/g dm) was comparable to another more recent study reporting 1092 ng REQ/g dm (Jonas et al., 2014), despite the different origin of cyanobacteria cultures, different extraction methods and different bioassays used for the assessment.

The methods of sample extraction in previous studies varied, and to the best of our knowledge, no detailed optimization of extraction for retinoids from cyanobacterial samples was performed. *In vitro* assessment of environmental cyanobacterial samples prepared by optimized extraction in P19/A15 cells revealed significant retinoid-like activity in all twelve biomasses in the present study. All samples from different lakes showed relatively comparable retinoid-like activity (Table 2); in case of the biomasses consisting of 100% *Microcystis aeruginosa* ranging from 492 to 2208 ng REQ/g dm. Together with results from laboratory cultivations (1066–1837 ng REQ/g dm), it may imply that compounds with retinoid-like activity are present in *M. aeruginosa* cells at relatively stable levels. The greatest REQ (2838 ng REQ/g dm) was found in case of N1 sample consisting of 60% *M. aeruginosa* and 40% *Plantothrix agardhii*, indicating retinoid-like potential also for the latter species. This is in agreement with recent study that reported 1163 ng REQ/g dm in extract (75% MeOH) of *P. agardhii* from laboratory cultivations (Jonas et al., 2015). Retinoid-like activities in surrounding waters (<10 to 28.7 ng REQ/L) did not correlate strongly with cell density and they were lower than in laboratory culture media. Considering that *M. aeruginosa* was cultivated in the lab culture in small volumes (1 L) with cell densities of 6.66–22.9 × 10^6 cells/mL, it is not surprising that retinoid-like activity in their culture media is greater than in environmental waters, since the exudates can be diluted in much larger amounts of water in ponds and lakes compared to laboratory cultivations. Moreover, the sampled reservoirs were relatively large water bodies, and local characteristics such as reservoir depth, circulation and mixing of lake water, as well as the actual condition of the biomass cells could significantly influence the resulting content of retinoid-like compounds in water.

Periodical sampling from locality Nové Mlýny (N1-5, July 16–August 14) documented seasonal development of retinoid-like activity associated with water blooms. The greatest biomass REQs were observed at the beginning and in the middle of the sampling season (2838 ng/g dm), with a decrease at the end of the bloom season (492 ng/g dm in September). During the main bloom season (July–August) REQs were comparable (N1-4), indicating that production of retinoid-like compounds was relatively constant, and decreased when population reached its final phase (Fig. 5). The peak of biomass abundance at this locality was in the middle of season (N3), with cell density two orders of magnitude higher than in other sampling periods (10^7 cells/mL). During this sampling, retinoid-like activity was also detected in surrounding water (12.8 ng REQ/L). Even greater REQ (28.7 ng/L) was detected in water sample from the following sampling period (N4), when the biomass density was 0.15 × 10^6 cells/mL. This greatest REQ corresponded to the greatest ATRA concentrations both in water and biomass from this sampling period (Table 2).

The comparison of REQ values to concentration of RAs determined by chemical analyses document different rates of contribution of these compounds to retinoid-like activity. In majority of samples, most of the *in vitro* activity was probably caused by chemicals others than the examined RAs. REQs of extracts from environmental biomasses were mostly one or two orders of magnitude greater than the sum of detected RAs (except sample H), which is in agreement with results of the REQs/RAs ratio in cyanobacteria lab cultures. Exceptions were namely the N4 samples of biomass and surrounding water and also the H biomass sample, where the contribution of ATRA and 9-*cis* RA to retinoid-like activity reached 23, 70 and 100%, respectively. The contribution of other RA analogues and other metabolites should be examined. Microcystins, abundant cyanotoxins found in extracts and exudates of *Microcystis* sp., do not contribute to retinoid-like effects. Results showed no retinoid-like potency of any of the tested microcystin structural variants (LR, RR, and YR) that were assessed *in vitro* at both environmentally relevant and also much higher levels compared to their natural occurrence.

Although retinoids are potent teratogens with adverse effects on aquatic organisms (Haga et al., 2002; Herrmann, 1995; Jonas et al., 2014; Mohanty and Boettger-Tong, 2005; Zhang et al., 1996), known *in vivo* effects in fish appear at concentrations generally

higher than 28 ng/L of equivalent ATRA, which was the highest REQ detected in water in this study. Most information on *in vivo* effects is available for ATRA, less for cis RA, while there is very limited information for other retinoids. For example, both ATRA and 9-*cis* RA induced deformities in larvae of Japanese flounder (*Paralichthys olivaceus*), when exposed to concentrations 7.5 μg/L (the only concentration tested) (Haga et al., 2002). Embryos of Japanese medaka (*Oryzias latipes*) showed impaired development when exposed to concentrations higher than 3 μg/L of ATRA or 9-*cis* RA, and most of the embryos treated to 30 μg/L died prior to hatching (Mohanty and Boettger-Tong, 2005). In zebrafish embryos, exposure to ATRA leads to several adverse effects on development such as tail, spine, mouth and neural system deformities, heart edema and gross malformations (Herrmann, 1995; Jonas et al., 2014; Zhang et al., 1996), but no effects occurred at concentrations below 900 ng/L (Herrmann, 1995). This concentration was also a LOEC for developmental effects of wider spectra of retinoids on zebrafish embryos characterized by Herrmann (1995). These results suggest that REQs of surface waters detected in this study might be relatively low to pose significant risk for fish. On the other hand, the risk posed to sensitive organisms during susceptible periods (*e.g.* amphibian tadpoles) cannot be neglected. For example, the amphibians undergo complex development including multiple life stages, which can be much more sensitive than others. Stage and species specific increase in dysmorphogenesis and mortality after 24 h exposure starting from 6.25 and 12.5 μg/L ATRA was demonstrated in *Xenopus laevis* and four ranids (*Rana clamitans*, *Rana pipiens*, *Rana septentrionalis* and *Rana sylvatica*) by Degitz et al. (2000). The following study of Degitz et al. (2003) tested wider concentration range and longer exposures. Mortality in *X. laevis* embryos and larvae after chronic exposure to ATRA was 60% at 144 ng/L and 100% at 240 ng/L, while mortality in 52 ng/L exposure variant was comparable to controls. The concentration causing 60% mortality is only several fold higher than highest water REQ reported in this study. Taking into the account the ability of cyanobacteria to rapidly appear at very dense blooms, followed by massive recession with release of cellular metabolites and also potential complex character of pollution at many sites with other compounds than just retinoids contributing to the exposures, this raises concerns. The actual concentration of retinoid-like compounds in water will always depend on a number of factors, including water bloom species composition and density, its stage of development, weather conditions, characteristics of the pond/reservoir *etc.* Peak concentrations especially in shallow water bodies with lesser water volumes heavily affected by cyanobacterial water blooms might possibly reach levels sufficient for adverse effects on organisms.

5. Conclusions

This is the first study providing important environmentally relevant information on total retinoid-like activity of both field cyanobacterial biomasses and their surrounding waters. Levels of total activity in biomass were generally greater than concentrations of the two analyzed retinoic acids indicating that biomasses can serve as a significant source of other retinoid-like compounds. Two recent studies have provided information on retinoid-like activity *in vitro* and teratogenicity *in vivo* of cultured cyanobacterial biomasses (Jonas et al., 2015) and their exudates (Jonas et al., 2014). Compared to available information on *in vivo* effects of retinoids, levels of REQs detected in environmental surrounding waters in the present study were generally lower than ATRA concentrations reported to cause *in vivo* teratogenicity in studied fish and amphibian models, but the information on possible relevance to other sensitive species and also regarding other retinoids is limited. As discussed above, environmental factors such as *e.g.* dilution and degradation can play important roles for total REQs in surrounding water. Therefore, the impact of retinoid-like activity of cyanobacterial metabolites could be of importance especially in smaller water bodies with dense water blooms but limited possibilities of dilution. Moreover, the organisms in the environment affected by cyanobacteria are co-exposed also to other types of bioactive compounds, *e.g.* microcystins in case of *Microcystis aeruginosa*, and other stressors such as limitation of oxygen levels, which might together lead to more pronounced adverse effects.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.hal.2015.06.006.

References

Bryant, S.V., Gardiner, D.M., 1992. Retinoic acid, local cell-cell interactions, and pattern formation in vertebrate limbs. Dev. Biol. 152, 1–25, http://dx.doi.org/10.1016/0012-1606(92)90152-7.

Chongsuvivatwong, V., 2012. epicalc: Epidemiological calculatorIn: R package version 2.15.1.0. , ⟨http://CRAN.R-project.org/package=epicalc⟩

De Figueiredo, D.R., Azeiteiro, U.M., Esteves, S.M., Gonçalves, F.J.M., Pereira, M.J., 2004. Microcystin-producing blooms—a serious global public health issue. Ecotoxicol. Environ. Saf. 59, 151–163, http://dx.doi.org/10.1016/j.ecoenv. 2004.04.006.

Degitz, S.J., Holcombe, G.W., Kosian, P.a., Tietge, J.E., Durham, E.J., Ankley, G.T., 2003. Comparing the effects of stage and duration of retinoic acid exposure on amphibian limb development: chronic exposure results in mortality, not limb malformations. Toxicol. Sci. 74, 139–146, http://dx.doi.org/10.1093/toxsci/kfg098.

Degitz, S.J., Kosian, P.A., Makynen, E.A., Jensen, K.M., Ankley, G.T., 2000. Stage- and species-specific developmental toxicity of all-trans retinoic acid in four native North American ranids and *Xenopus laevis*. Toxicol. Sci. 57, 264–274, http://dx.doi.org/10.1093/toxsci/57.2.264.

Freyberger, A., Schmuck, G., 2005. Screening for estrogenicity and anti-estrogenicity: a critical evaluation of an MVLN cell-based transactivation assay. Toxicol. Lett. 155, 1–13, http://dx.doi.org/10.1016/j.toxlet.2004.06.014.

Grenier, E., Maupas, F.S., Beaulieu, J.-F., Seidman, E., Delvin, E., Sane, A., Tremblay, E., Garofalo, C., Levy, E., 2007. Effect of retinoic acid on cell proliferation and differentiation as well as on lipid synthesis, lipoprotein secretion, and apolipoprotein biogenesis. Am. J. Physiol. Gastrointest. Liver Physiol. 293, G1178–G1189, http://dx.doi.org/10.1152/ajpgi.00295.2007.

Haga, Y., Suzuki, T., Takeuchi, T., 2002. Retinoic acid isomers produce malformations in postembryonic development of the Japanese flounder, *Paralichthys olivaceus*. Zool. Sci. 19, 1105–1112, http://dx.doi.org/10.2108/zsj.19.1105.

Haldi, M., Harden, M., D'Amico, L., DeLise, A., Seng, W.L., 2011. Zebrafish, Zebrafish. John Wiley & Sons, Inc., Hoboken, NJ, http://dx.doi.org/10.1002/9781118102138.ch2.

Halstvedt, C.B., Rohrlack, T., Ptacnik, R., Edvardsen, B., 2008. On the effect of abiotic environmental factors on production of bioactive oligopeptides in field populations of *Planktothrix* spp. (Cyanobacteria). J. Plankton Res. 30, 607–617, http://dx.doi.org/10.1093/plankt/fbn025.

Harrell, F.E., 2014. Hmisc: Harrell Miscellaneous. R package version 3.14-4 ⟨http://CRAN.R-project.org/package=Hmisc⟩

Herrmann, K., 1995. Teratogenic effects of retinoic acid and related substances on the early development of the zebrafish (*Brachydanio rerio*) as assessed by a novel scoring system. Toxicol. Vitro 9, 267–283, http://dx.doi.org/10.1016/0887-2333(95)00012-W.

Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biometr. J. 50 (3), 346–363.

Chorus, I., Bartram, J., 1999. Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management. WHO, E&FN Spon, London, New York.

Isaacs, J.D., Strangman, W.K., Barbera, A.E., Mallin, M.a., McIver, M.R., Wright, J.L.C., 2014. Microcystins and two new micropeptin cyanopeptides produced by unprecedented Microcystis aeruginosa blooms in North Carolina's Cape Fear River. Harmful Algae 31, 82–86, http://dx.doi.org/10.1016/j.hal.2013.09.010.

Jonas, A., Buranova, V., Scholz, S., Fetter, E., Novakova, K., Kohoutek, J., Hilscherova, K., 2014. Retinoid-like activity and teratogenic effects of cyanobacterial exudates. Aquat. Toxicol. 155, 283–290, http://dx.doi.org/10.1016/j.aquatox.2014.06.022.

Jonas, A., Scholz, S., Fetter, E., Sychrova, E., Novakova, K., Ortmann, J., Benisek, M., Adamovsky, O., Giesy, J.P., Hilscherova, K., 2015. Endocrine, teratogenic and neurotoxic effects of cyanobacteria detected by cellular in vitro and zebrafish embryos assays. Chemosphere 120C, 321–327, http://dx.doi.org/10.1016/j.chemosphere.2014.07.074.

Kaya, K., Shiraishi, F., Uchida, H., Sano, T., 2011. A novel retinoic acid analogue, 7-hydroxy retinoic acid, isolated from cyanobacteria. Biochim. Biophys. Acta 1810, 414–419, http://dx.doi.org/10.1016/j.bbagen.2010.11.009.

Kolmakov, V.I., 2006. Methods for prevention of mass development of the cyanobacterium *Microcystis aeruginosa* Kutz emend. Elenk. in aquatic systems. Microbiology 75, 115–118, http://dx.doi.org/10.1134/S0026261706020019.

Kuiper-Goodman, T., Falconer, I., Fitzgerald, J., 1999. Human health aspects. In: Chorus, I., Bartram, J. (Eds.), Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management. WHO, E&FN Spon, London, New York.

Malbrouck, C., Kestemont, P., 2006. Effects of microcystins on fish. Environ. Toxicol. Chem. 25, 72–86.

Mohanty, N., Boettger-Tong, H., 2005. Comparative effects of all-trans and 9-*cis* retinoic acid on Medaka (*Oryzias latipes*) development. Bios, http://dx.doi.org/10.1893/0005-3155(2005)076[0001:RACEOA]2.0.CO;2.

Novák, J., Beníšek, M., Pacherník, J., Janošek, J., Šídlová, T., Kiviranta, H., Verta, M., Giesy, J.P., Bláha, L., Hilscherová, K., 2007. Interference of contaminated sediment extracts and environmental pollutants with retinoid signaling. Environ. Toxicol. Chem. 26, 1591, http://dx.doi.org/10.1897/06-513R.1.

Paerl, H.W., Xu, H., McCarthy, M.J., Zhu, G., Qin, B., Li, Y., Gardner, W.S., 2011. Controlling harmful cyanobacterial blooms in a hyper-eutrophic lake (Lake Taihu, China): the need for a dual nutrient (N & P) management strategy. Water Res. 45, 1973–1983, http://dx.doi.org/10.1016/j.watres.2010.09.018.

Parng, C., Roy, N.M., Ton, C., Lin, Y., McGrath, P., 2007. Neurotoxicity assessment using zebrafish. J. Pharmacol. Toxicol. Methods 55, 103–112, http://dx.doi.org/10.1016/j.vascn.2006.04.004.

R Core Team, 2014. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria ⟨http://www.R-project.org/⟩ (last accessed November 25th 2014).

Ranke, J., 2014. chemCal: calibration functions for analytical chemistryIn: R package version 0.1–34. , ⟨http://CRAN.R-project.org/package=chemCal⟩ (last accessed November 25th 2014).

Repka, S., Koivula, M., Harjunpä, V., Rouhiainen, L., Sivonen, K., 2004. Effects of phosphate and light on growth of and bioactive peptide production by the *Cyanobacterium* anabaena strain 90 and its anabaenopeptilide mutant. Appl. Environ. Microbiol. 70, 4551–4560, http://dx.doi.org/10.1128/AEM.70.8.4551-4560.2004.

Ritz, C., Streibig, J., 2005. Bioassay analysis using R. J. Stat. Softw. 12.

Rogers, E.D., Henry, T.B., Twiner, M.J., Gouffon, J.S., McPherson, J.T., Boyer, G.L., Sayler, G.S., Wilhelm, S.W., 2011. Global gene expression profiling in larval zebrafish exposed to microcystin-LR and microcystis reveals endocrine disrupting effects of Cyanobacteria. Environ. Sci. Technol. 45, 1962–1969, http://dx.doi.org/10.1021/es103538b.

Rohrlack, T., Utkilen, H., 2007. Effects of nutrient and light availability on production of bioactive anabaenopeptins and microviridin by the cyanobacterium *Planktothrix agardhii*. Hydrobiologia 583, 231–240, http://dx.doi.org/10.1007/s10750-006-0536-y.

Selderslaghs, I.W.T., Van Rompay, A.R., De Coen, W., Witters, H.E., 2009. Development of a screening assay to identify teratogenic and embryotoxic chemicals using the zebrafish embryo. Reprod. Toxicol. 28, 308–320, http://dx.doi.org/10.1016/j.reprotox.2009.05.004.

Scheffer, M., Rinaldi, S., Gragnani, A., Mur, L.R., van Nes, E.H., 1997. On the dominance of filamentous cyanobacteria in shallow, turbid lakes. Ecology 78, 272–282, http://dx.doi.org/10.1890/0012-9658(1997)078[0272:OTDOFC]2.0.CO;2.

Schlösser, U.G., 1994. SAG—Sammlung von Algenkulturen at the University of Göttingen Catalogue of Strains 1994. Bot. Acta 107, 113–186, http://dx.doi.org/10.1111/j.1438-8677.1994.tb00784.x.

Skocovska, B., Hilscherova, K., Babica, P., Adamovsky, O., Bandouchova, H., Horakova, J., Knotkova, Z., Marsalek, B., Paskova, V., Pikula, J., 2007. Effects of cyanobacterial biomass on the Japanese quail. Toxicon 49, 793–803, http://dx.doi.org/10.1016/j.toxicon.2006.11.032.

Stein, J., 1975. Handbook of phycological methods: culture methods and growth measurements. In: Handbook of Phycological MethodsCambridge University Press, Cambridge.

Stěpánková, T., Ambrožová, L., Bláha, L., Giesy, J.P., Hilscherová, K., 2011. In vitro modulation of intracellular receptor signaling and cytotoxicity induced by extracts of cyanobacteria, complex water blooms and their fractions. Aquat. Toxicol. 105, 497–507, http://dx.doi.org/10.1016/j.aquatox.2011.08.002.

Suurnäkki, S., Gomez-Saez, G.V., Rantala-Ylinen, A., Jokela, J., Fewer, D.P., Sivonen, K., 2015. Identification of geosmin and 2-methylisoborneol in cyanobacteria and molecular detection methods for the producers of these compounds. Water Res. 68, 56–66, http://dx.doi.org/10.1016/j.watres.2014.09.037.

Via-Ordorika, L., Fastner, J., Kurmayer, R., Hisbergues, M., Dittmann, E., Komarek, J., Erhard, M., Chorus, I., 2004. Distribution of microcystin-producing and non-microcystin-producing Microcystis sp. in European freshwater bodies: detection of microcystins and microcystin genes in individual colonies. Syst. Appl. Microbiol. 27, 592–602, http://dx.doi.org/10.1078/0723202041748163.

Welker, M., Dittmann, E., von Döhren, H., 2012. Cyanobacteria as a source of natural products. Methods Enzymol. 517, 23–46, http://dx.doi.org/10.1016/B978-0-12-404634-4.00002-4.

Wiegand, C., Pflugmacher, S., 2005. Ecotoxicological effects of selected cyanobacterial secondary metabolites: a short review. Toxicol. Appl. Pharmacol. 203, 201–218, http://dx.doi.org/10.1016/j.taap.2004.11.002.

Wilson, A.E., Sarnelle, O., Neilan, B.A., Salmon, T.P., Gehringer, M.M., Hay, M.E., 2005. Genetic variation of the bloom-forming Cyanobacterium *Microcystis aeruginosa* within and among lakes: implications for harmful algal blooms. Appl. Environ. Microbiol. 71, 6126–6133, http://dx.doi.org/10.1128/AEM.71.10.6126-6133.2005.

Wu, X., Jiang, J., Hu, J., 2013. Determination and occurrence of retinoids in a eutrophic lake (Taihu Lake, China): Cyanobacteria blooms produce teratogenic retinal. Environ. Sci. Technol. 47, 807–814, http://dx.doi.org/10.1021/es303582u.

Wu, X., Jiang, J., Wan, Y., Giesy, J.P., Hu, J., 2012. Cyanobacteria blooms produce teratogenic retinoic acids. Proc. Natl. Acad. Sci. U.S.A. 109, 9477–9482, http://dx.doi.org/10.1073/pnas.1200062109.

Zhang, K., Lin, T.F., Zhang, T., Li, C., Gao, N., 2013. Characterization of typical taste and odor compounds formed by *Microcystis aeruginosa*. J. Environ. Sci. 25, 1539–1548, http://dx.doi.org/10.1016/S1001-0742(12)60232-0.

Zhang, P., Zhai, C., Chen, R., Liu, C., Xue, Y., Jiang, J., 2012. The dynamics of the water bloom-forming *Microcystis aeruginosa* and its relationship with biotic and abiotic factors in Lake Taihu, China. Ecol. Eng. 47, 274–277, http://dx.doi.org/10.1016/j.ecoleng.2012.07.004.

Zhang, Z., Balmer, J.E., Løvlie, A., Fromm, S.H., Blomhoff, R., 1996. Specific teratogenic effects of different retinoic acid isomers and analogs in the developing anterior central nervous system of zebrafish. Dev. Dyn. 206, 73–86, http://dx.doi.org/10.1002/(SICI)1097-0177(199605)206:1<73::AID-AJA7>3.0.CO;2-Y.