Research centre for toxic compounds in the environment

Ecotoxicological bioassays

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CHEMICAL ENTERS THE ENVIRONMENT

LEVELS, FATE, PROCESSES

Bioavailable fraction

"EXPOSURE"

acute

chronic

CHEMICAL ENTERS THE ORGANISM

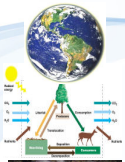
biomonitoring

Toxikokinetics

biotransformation
bioactivation
excretion / sequestration

Target site

"EFFECT"



Biosphere

Ecosystem

Community

Population

Individual

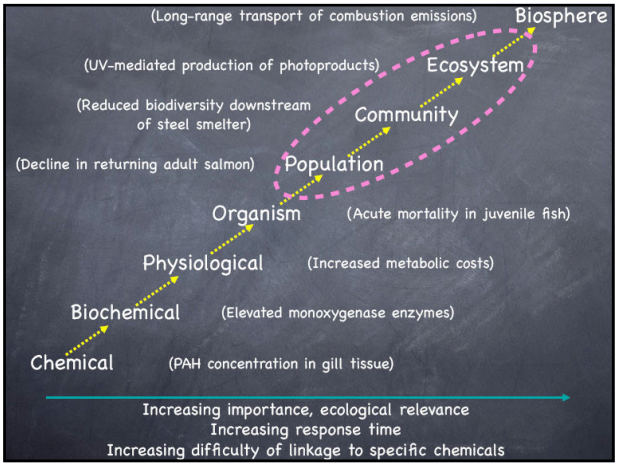
System of organs
Organ
Tissue
Cell
Organell
Biomolecule

LOW → HIGH

Ecological relevance
Response duration
Longer-term effects

HIGH → LOW

Flexibility
Ability to determine cause
Specifity
Sensitivity



Effect of Chemicals

Binding of pollutant to receptor	Bio-chemical response	Physiol-ogical alterations	Whole organism	Population and community
		Time scale		
Seconds to minutes	Minutes to days	Hours to weeks	Days to months	Months to years

Least >>>**Difficulty in relating observed effects to a specific chemical**>>>→ Greatest

Least >>>**Importance**>>> Greatest

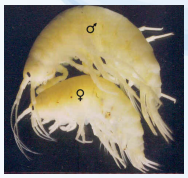
Note: On the far right of the diagram, changes in structure and function of ecosystems occur, and the chasm that separates this impact from the stages on the left is too great to demonstrate graphically.

Figure 13.1. Levels of organization to evaluate the effects of chemicals

Biotest

- Bioassay is a process where a test system (tissue,organism, population) is exposed under defined conditions to different known concentrations of tested compound or sample.

In vivo effects?



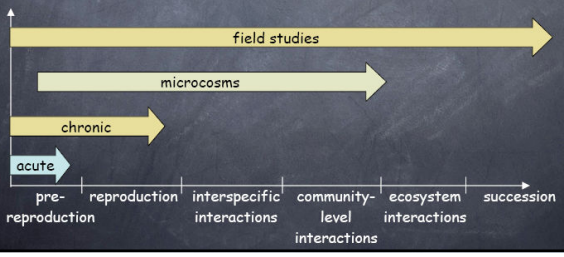
Toxicity Tests

- Toxicity Tests/Bioassays
- Microcosm & Mesocosm Studies
- Provide a direct measure of biological uptake of the toxicants
- Establish link between site contamination and adverse ecological effects
- May provide info on synergistic or antagonistic interactions among chemicals
- Direct extrapolation of lab to field should be carefully evaluated
- May do an *in situ* toxicity test under field conditions

Toxicity Test Duration

Acute = short-term toxicity
-usually defined as <96h
-endpoints: mortality, photosynthesis, germination

Chronic = long-term
>96h, up to multigenerational
-endpoints: mortality, reproduction, growth rate, teratogenesis



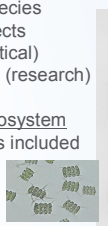
Toxicity Tests

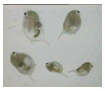
- Toxicity tests can be used for both aquatic and terrestrial systems
- Aquatic tests are more developed
- Endpoints are mortality, growth and/or reproduction
- Vertebrates
  - Rodents
  - Fish
  - Birds
- Invertebrates
  - Insects
  - Amphipods (crustacea related to shrimp and krill)
  - Plankton
- Microbes
  - Luminescent bacteria (Microtox)
- Plants
  - Aquatic or terrestrial
  - Vascular or non-vascular

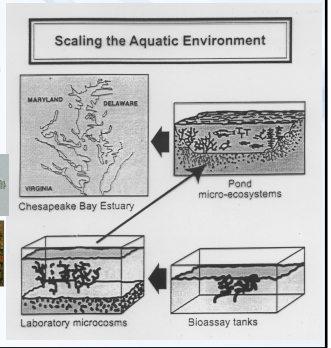
Ecotoxicology: Laboratory studies

Bioassays
- single / multiple species
- acute / chronic effects
- standardized (practical)
  vs. experimental (research)

Simulation of the ecosystem
- major trophic levels included
  - producers
  - consumers
  - destruents







Standardized methods

- **ISO**: International Organization for Standardization (www.iso.ch)
- **OECD**: Organization for Economic Cooperation and Development (www.oecd.org)

- EPA (US EPA) – Environmental Protection Agency USA
- US Army Corps of Engineers
- ASTM American Society of Testing and Materials
- CEN - European Commitee for Standardization (Comité Européen de Normalisation) (www.cenorm.be)
- EEC: European Economic Community
- WHO – World Health Organisation

OECD Guidelines for the Testing of Chemicals
Section 2: Effects on Biotic Systems

Test No. 223: Avian Acute Oral Toxicity Test - describes procedures designed to estimate the acute oral toxicity of substances to birds, and it provides three testing options: (1) limit dose test, (2) LD50-slope test, and (3) LD50-only test. The LD50-slope and LD50-only...

Test No. 233: Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment - designed to assess the effects of prolonged exposure of chemicals to the life-cycle of the sediment-dwelling freshwater dipteran Chironomus sp. First instar chironomid larvae are exposed to five concentrations of the test chemical...

Test No. 229: Fish Short Term Reproduction Assay - describes an in vivo screening assay for fish reproduction where sexually mature male and spawning female fish are held together and exposed to a chemical during a limited part of their life-cycle.

Test No. 230: 21-day Fish Assay - describes an in vivo screening assay for certain endocrine active substances where sexually mature male and spawning female fish are held together and exposed to a chemical during a limited part of their life-cycle.

Test No. 231: Amphibian Metamorphosis Assay - describes an amphibian metamorphosis assay intended to screen substances which may interfere with the normal functioning of the hypothalamo-pituitary-thyroid axis. The assay was validated with the species Xenopus laevis, which is...

Test No. 232: Collembolan Reproduction Test in Soil

Test No. 226: Predatory mite (Hypoaspis (Geolaelaps) aculeifer) reproduction test in soil - describes a method to assess the effects of chemical substances in soil on the reproductive output of the soil mite species Hypoaspis (Geolaelaps) aculeifer Canestrini (Acari: Laelapidae).

Test No. 211: Daphnia magna Reproduction Test - described in this Test Guideline assesses the effect of chemicals on the reproductive output of Daphnia magna Straus.

Test No. 225: Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment - designed to assess the effects of prolonged exposure to sediment-associated chemicals on the reproduction and the biomass of the endobenthic oligochaete Lumbriculus variegatus (Müller).

Test No. 208: Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test - designed to assess effects on seedling emergence and early growth of higher plants following exposure to the test substance applied to the soil surface or into the soil.

Test No. 221: Lemna sp. Growth Inhibition Test - designed to assess the toxicity of substances to freshwater aquatic plants of the genus Lemna (duckweed).

Test No. 201: Alga, Growth Inhibition Test - purpose of this test is to determine the effects of a substance on the growth of freshwater microalgae and/or cyanobacteria.

Test No. 202: Daphnia sp. Acute Immobilisation Test - describes an acute toxicity test to assess effects of chemicals towards daphnids (usually Daphnia magna Staus).

Notes on practical testing

- Testing chemicals
  - Traditional approach - bioassays developed to assess **chemicals**
  - **Standardized and validated approaches**
    - OECD – Guideline methods - series „2" Effects on biota
    - ISO methods
      - E.g. Fish tests - OECD 203 / ISO 7346
      - E.g. D. magna - OECD 202 / ISO 6341
    - Limited ecological relevance
      - often acute tests only, „too standardized…"
      - does not assess bioavailability, no consideration of mixtures
      - no consideration of specific modes of action
- Testing toxicity of natural matrices
  - Rather new in ecotoxicology – many open challenges
  - More complex and more complicated
    - „cause-effects" often not clear (natural variability …)

Testing strategy

- **Battery of assays**
  - Fast screening tests (inhibition of *Vibrio fisheri* bioluminiscence, MICROTOX – 30 min toxicity)
  - Standardized acute toxicity tests
  - Further studies with chronic assays
- **Various purposes -> guidelines and recommendations**
  - REACH
  - Plant protection products + biocides
  - Veterinary and human pharmaceuticals
  - Waste materials …

Basic principles of bioassays

- Reproducibility
- Standard design
- Possibility of data extrapolation on field conditions
- Cost and time feasibility

Tested matrix	Sample type
Water Soil Air Sediment Waste Chemical compound	Single compounds (hydrophobic, hydrophilic, volatile) Mixture of compounds (known and/or unknown) Environmental samples (usually unknown, mixtures of different compounds with different properties – complicated interpretation)

Used to develop Water Quality Criteria (WQC) for different uses

Endpoints

- Lethal effects (mortality)
- sub lethal effects (immobilisation)
- Physiological activity (photosynthetic activity, enzymatic activity, biomass increase, resistance to diseases, pests and/or parasites)
- Reproductive activity, malformations, reproductive activity
- Mutagenicity/genotoxicity (microbial, vascular plants, wildlife animals)
- Teratogenity (amphibian- *Xenopus laevis*)
- Embryotoxicity
- Reproduction bioassays

Factors influencing results of bioassays

For reproducibility of results these main factors have to be standardized:

Exposure duration
Temperature
Light:dark period
Volume
Oxygen content
Composition of cultivation media
Age of organism

Biotests … to be considered

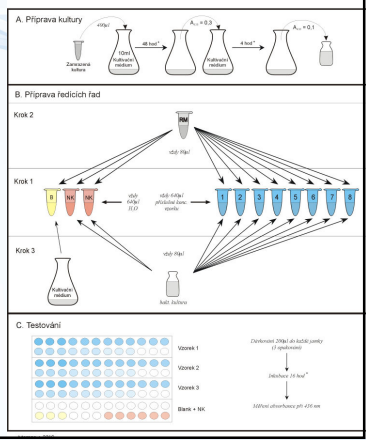
- ***Parameters of the biological system***
  - *Complexity / in vitro, in vivo, population, microcosm …*
  - *Population characteristics – sex, age …*
  - *Aquatic vs. Terrestrial (soil)*
- ***Exposure duration & effects***
  - *Acute (often mortality), sub-acute, chronic (other endpoints) (4 days - algae / 4 generations, fish / acute toxicity)*
- ***Exposure setup***
  - *Static / with exchange of media / flow-through*
  - *Depends on the compound stability (should be measured!)*
- ***Bioassay endpoints***
  - *Lethality, immobilization (Daphnia), growth, reproduction …*
- ***Abiotic factors in the experiment***
  - *Validity criteria (pH, oxygen, temperature, humidity, water hardness …)*

Steps to conduct the biotest

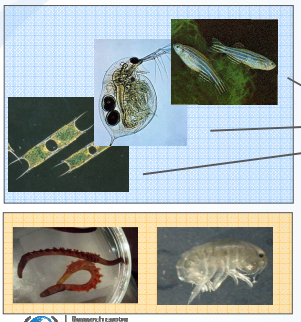
- **1) Prepare the organism**
  - Culture media, standardized numbers, age, etc.
- **2) Prepare the sample**
  - Dilution series
    - water/culture media – direct organism exposure
      - Include BLANK (medium only)
    - solvent for organic compounds – minimum to be added (1% vol)
      - Include SOLVENT CONTROL
- **3) Expose organisms**
  - … for appropriate time, number of repetitions, under specified conditions
- **4) Evaluate and report results**
  - measure the endpoint / count organisms
  - statistical evaluation (means, ANOVA, dose-response …)

- **1) Prepare organism**
- **2) Prepare sample**
- **3) Expose**
- **4) Evaluate**

Schema inhibičního testu na *Pseudomonas putida*

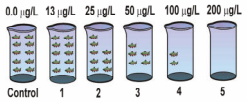


Ecotoxicology – laboratory studies – experimental design



Cu addition

Concentration:



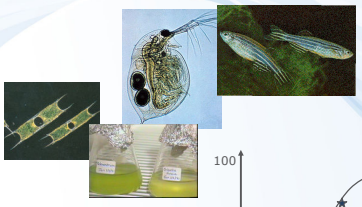
96-hour LC50 = 50 µg/L

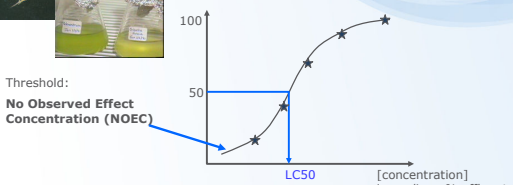
Effect concentrations expressed in total/dissolved Cu

Extrapolation =

PNECs or EQCs expressed in total / dissolved Cu

Laboratory ecotoxicology – data and results





Threshold:

No Observed Effect Concentration (NOEC)

Acute Aquatic Toxicity Tests

- Most frequently used (short = less expensive)
- Relates dose (C_w x time of exposure) to time of death for a particular test organism
- Produce concentration/response curve
- Ranges from 1 to 4 days for aquatic tests and up to 10 days for assessment of sediment toxicity
- Done in laboratory under controlled conditions

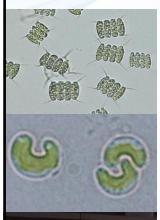
Table 3.2. Some freshwater acute toxicity tests

Species	*Ceriodaphnia dubia, Daphnia pulex and Daphnia magna*, fathead minnow, rainbow trout
Endpoint	Mortality
Duration	24, 48, or 96 hours
Temperature (°C)	20 or 25 for *Daphnia* and minnow; 12 for trout
Conditions	Static non-renewal and renewal, flow-through
Level of effort	Low
Citation	USEPA, 1991b

Table 3.3. Some estuarine and marine acute toxicity tests (USEPA, 1991b)

Species	Mysid shrimp (*Mysidopsis bahia*), sheepshead minnow (*Cyprinodon variegatus*) and silverside (*Menidia* sp.)
Endpoint	Mortality
Duration	24, 48, or 96 hours
Temperature (°C)	20 or 25
Conditions	Static non-renewal, static renewal, and flow-through
Level of effort	Low

AQUATIC BIOTESTS with PRODUCERS

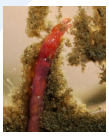


- **Growth inhibition assays with algae and macrophyta (72 h)** (*Scenedesmus quadricauda, Raphidocelis subcapitata, Selenastrum capricornutum, Lemna minor*)
  - ISO 8692/2004 Water quality -- Freshwater algal growth inhibition test with unicellular green algae
  - OECD 201 Alga, Growth Inhibition Test
  - microplate miniaturization

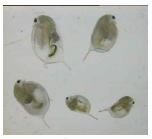
- **Germination tests and root elongation with higher plants** – testing toxicity in the aquatic media (*Lepidium sativum, Sinapis alba, Lactuca sativa*)
  - OECD 208 Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test

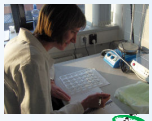
BIOTESTS with CONSUMERS - invertebrates

AQUATIC ASSAYS



- *Daphnia magna* immobilisation (24 – 48h)
  - ISO 6341/1996 Water quality - Determination of the inhibition of the mobility of Daphnia magna Straus (Cladocera, Crustacea) - Acute toxicity test
  - OECD 202 Daphnia sp. Acute Immobilisation Test
- crustacea *Ceriodaphnia dubia*
- rotifer *Brachionus calyciflorus*
- *ToxKit assays*
- *Thamnotoxkit (Thamnocephalus platyurus)*
- *Artoxkit Artemia salina*



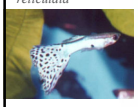


BIOTESTS with CONSUMERS – fish (acute 96h)

OECD 203 **Fish, Acute Toxicity Test**

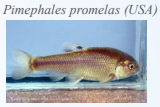
ISO 7346 **Water quality - Determination of the acute lethal toxicity of substances to a freshwater fish**
Static/semi-static/flow through method

Guppy, *Poecilia reticulata*

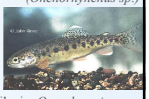


Zebrafih, *Danio rerio* (syn. *Brachydanio rerio*)

Fathead minnow, *Pimephales promelas (USA)*



(Rainbow) trout *(Onchorhynchus sp.)*



Carassius (Goldfish)

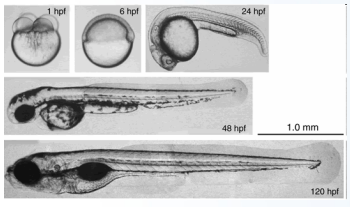
Medaka, *Oryzias latipes*

Nile tilapia, *Oreochromis niloticus*

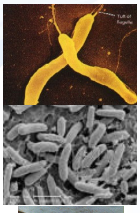
BIOTESTS with CONSUMERS - amphibians

FETAX – Frog Embryo Teratogenicity Assay Xenopus (ASTM E1439-98)
African clawed frog (*Xenopus laevis*)

96 h / egg and embryo exposure



BIOTESTS with DESTRUENTS - microorganisms

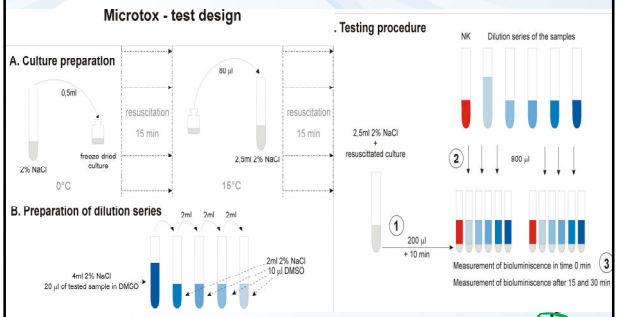


- **Toxicity to luminescent bacteria** Vibrio fisheri (MICROTOX®)
  - ISO 11348 Water quality - Determination of the inhibitory effect of water samples on the light emission of Vibrio fischeri
- **Growth inhibitions** (Pseudomonas putida, Toxi-Chromotest, Toxi-ChromoPad)
- **Toxicity assays** with SOIL BACTERIA

Microtox

EN ISO 11348-(1)

- based on inhibition of bioluminiscence of marine bacteria *Vibrio fischeri*



Chronic Aquatic Toxicity Tests

- Longer tests: 7 - 30 days
- Objective is to expose for at least 1/10th of lifetime
- Effect of different C_w on growth, reproduction, behavioral, physiological or other biological function
- Sub-chronic: only exposed during part of life-cycle (usually early stages)
- Life-cycle tests have been done for only a few contaminants

Chronic Aquatic Toxicity Tests

Table 3.4. Some freshwater chronic toxicity tests (USEPA, 1989)

Species/test	1. Fathead minnow larval survival and growth test 2. Fathead minnow embryo larval survival and tetratogenicity test 3. *Ceriodaphnia dubia* survival and reproduction test 4. Algal (*Selenastrum capricornutum*) growth test
Duration	7 days for tests 1, 2, and 3; 96 hours for test 4
Temperature (°C)	25
Conditions	Static renewal for tests 1, 2, and 3; static non-renewal for test 4
Level of Effort	Low

Chronic Aquatic Toxicity Tests

Table 3.5. Some estuarine and marine chronic toxicity tests

Species/test:	1. Sheepshead minnow or Island Silverside larval survival and growth test 2. Sheepside minnow embryo/larval survival and tetratogenicity test 3. *Mysidopsis bahia* survival, growth, and fecundity test 4. Sea urchin fertilization test 5. Algal sexual reproduction test
Duration:	7 days for tests 1, 2, and 3; 1.3 hours for test 4; 7–9 days for test 4
Temperature (°C):	25 for tests 1 and 2; 26–27 for test 3; 20 for test 4; 22–24 for test 5
Conditions:	Static renewal for tests 1, 2, and 3; static non-renewal for tests 4 and 5
Level of Effort:	Medium for tests 1, 3, 4, and 5; high for test 4
Citation:	USEPA, 1988

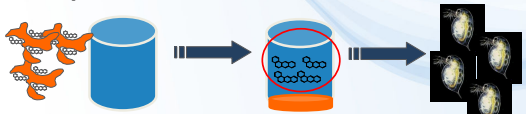
Toxicity Tests

Table 3.2 Aquatic Toxicity Tests Required by U.S. EPA for the Development of Water Quality Criteria

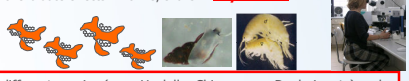
Type of Testing	Recommended Aquatic Tests
Acute Toxicity Tests	Eight different families must be tested for both freshwater and marine species (16 acute tests): Freshwater 1. A species in the family Salmonidae 2. A species in another family of the class Osteichthyes 3. A species in another family of the phylum Chordata 4. A plankton species in the class Crustacea 5. A benthic species in the class Crustacea 6. A species in the class Insecta 7. A species in a phylum other than Chordata or Arthropoda 8. A species in another order of Insecta or in another phylum Marine 1. Two families in the phylum Chordata 2. A family in a phylum other than Arthropoda or 3. Chordata 4. Either the Mysidae or Penaeidae family 5. Three other families not in the phylum Chordata (may include Mysidae or Penaeidae, whichever was not used above) 6. Any other family
Chronic Toxicity Tests	Three chronic or partial life cycle studies are required: One invertebrate and one fish One freshwater and one marine species
Plant Testing	At least one algal or vascular plant test must be performed with a fresh-water and a marine species
Bioconcentration Testing	At least one bioconcentration study with an appropriate freshwater and saltwater species is required

Sediment ecotoxicity tests

- **Toxicity of pore water/eluates** (several ISO / OECD standards)
  : 100 g d.w./L water, 24h slow shake, filter, test
  V. fisheri (30 min), Algae, Invertebrates - D. magna **(2 days)**
  ? Aquatic eluate vs. Sediment



- **Direct (contact) toxicity** (only few standards ...)
  : sediment+organisms & evaluate effects - worms, snails ... **days-weeks**



- Recommended at least 2 different species (e.g., Hyalella, Chironomus, Daphnia, etc) and two different endpoints (e.g., growth, survival, reproduction, etc.)

Toxicity Test Species Freshwater Sediments

Amphipods

Hyalella azteca

Midges

Chironomus tentans
Chironomus riparius

Oligochaetes

Tubifex tubifex

Mayfly

Hexagenia limbata

Acute toxicity tests

Pore water

Sediment eluates

48-96 h exposure

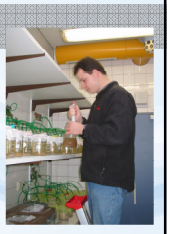
- Preparation of eluates: 24h shaking, 100 g sediment/1L water
- Species: *Daphnia magna*, *Daphnia pulex*, *Ceriodaphnia dubia*, fathead minnow (*Pimephales Promelas*), rainbow trout (*Oncorhynchus mykiss*)
- Endpoints: survival, immobilization

Contact tests - whole sediment

96h – 10 day exposure

- Species: amphipod (*Hyalella azteca*), mayfly (*Hexagenia limbata*), chironomids (*C.tentants/riparius*)
- 10-day test with *Hyalella azteca* and *Chironomus tentans*
- Endpoint: survival

Chronic toxicity tests



Pore water

Eluate from sediment

7-35 day exposure

- Species: *Ceriodaphnia dubia*, fathead minnow (*Pimephales Promelas*), rainbow trout (*Oncorhynchus mykiss*)
- Endpoints: survival, immobilization, growth, reproduction, time to the first reproduction, time of death, offspring survival

Contact tests - whole sediment

about 28 days exposure

- Species: *Hyalella azteca*, Chironomids (*C.tentants/riparius*)
- Endpoints: survival, immobilization, growth, reproduction, time to the first reproduction, time of death, offspring survival
- 28- and 42-day tests with *H. azteca*
- Sub-chronic and lifecycle tests with *Chironomus tentans*
- 10-day short term chronic test with amphibian larvae

Tests for sediment toxicity - EPA

Test Medium	Species	Common Name
Freshwater benthic	*Chironomus dilutus*	Chironomid, midge larvae
	Chironomus riparius	Chironomid, midge larvae
	Hyalella azteca	Amphipod, scud
	Lumbriculus variegatus	Oligochaete, "worm"
	Gammarus pulex	Amphipod
	Hexagenia limbata	Ephemeroptera, mayfly
	Tubifex tubifex	Oligochaete
	Diporeia sp	Amphipod, Great Lakes
Marine Benthic	*Americamysis bahia*	Mysid shrimp
	Ampelisca abdita	Amphipod (Atlantic)
	Eohaustorius estuarius	Amphipod (Pacific)
	Leptocheirus plumulosus	Amphipod (Atlantic)
	Rhepoxynius abronius	Amphipod (Pacific)
	Grandidierella japonica	Amphipod

www.epa.gov

Tests for sediment toxicity - EPA

	Psammechinus miliaris	Shore urchin
	Mercenaria mercenaria	Hard shell clam
	Mulinia lateralis	Dwarf surf clam
	Microtox (*Vibrio fischerii*)	Bacteria
Freshwater Pelagic	*Ceriodaphnia dubia*	Cladoceran, water flea
	Daphnia magna	Cladoceran, water flea
	Daphnia pulex	Cladoceran, water flea
	Pimephales promelas	Fish, fathead minnow
	Salvelinus fontinalis	Fish, brook trout
	Oncorhynchus mykiss	Fish, rainbow trout
Marine Pelagic	*Atherinops affinis*	Fish, topsmelt
	Cyprinodon variegatus	Fish, sheepshead minnow
	Menidia beryllina	Fish, silverside

www.epa.gov

Tests for sediment toxicity - ASTM

Table 3.6. Some freshwater sediment toxicity tests (ASTM E1383, 1993)

Species:	1. Amphipod (*Hyalella azteca*) 2. Midges: *Chironomus tentans*, *Chironomus riparius* 3. *Daphnia magna* and *Ceriodaphnia dubia* 4. Mayflies (*Hexagenia* spp.)
Endpoints:	1. Number of young; survival, growth & development; reproductive capacity 2. Larval survival and growth, adult emergence 3. Survival and reproduction 4. Mortality, growth, burrowing behaviour, moulting frequency
Duration:	10–30 days for tests 1 and 2; 2–7 days for test 3; 7–21 days for test 4
Temperature (°C):	20–25 for test 1; 20-23 for test 2; 25 for test 3; 17-22 for test 4
Conditions:	Static for all tests; flow-through for tests 1 and 2; recirculating for test 4
Level of effort:	Medium for all tests

Sediment Toxicity - ASTM

Table 3.7. Some marine and esturine sediment toxicity tests (ASTM E1383, 1993)

Species:	1. Amphipods 2. Fish, crustaceans, zooplanctons, or bivalves 3. Infaunal amphipods, burrowing polycheates, mollusks, crustaceans, or fish
Material:	1. Whole sediment 2. Dredged material (elutriate) 3. Dredged material (whole sediment)
Endpoints:	1. Mortality, emergence, renurial 2. Mortality 3. Survival
Duration:	10 days for tests 1 and 3; 2 days for zooplancton and fish larvae in test 2 and 4 days for bivalves and crustaceans in test 2
Temperature (°C):	20–25 for test 1; 20–23 for test 2; 25 for test 3; 17–22 for test 4
Conditions:	Static for all tests; flow-through for tests 1 and 2; recirculating for test 4

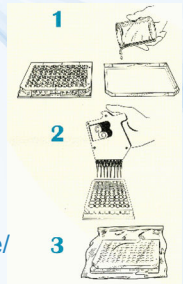
Sediment Toxicity Test – confounding factors

- **Potential Non-Contaminant Factors**
  - Sediment grain size
  - Content and type of clay
  - Organic carbon content and character
  - Humic substances/organic matter structure and properties
  - pH
  - Oxygen content
  - Ammonia / Sulfide toxicity
  - Nutrition
- **Changing sediment toxicity due to sampling and experimental procedures**
  - Mixing of more contaminated sediments with the thin layer at the sediment-water interface
  - Oxidation and precipitation of redox metals from the reaeration required for the sediment toxicity testing

Microbioassay

- Saving of
  - Time
  - Space
  - Work
  - Chemicals

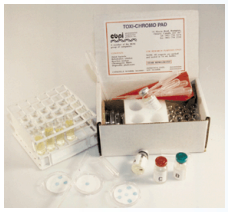
1. Batching
2. Inoculation
3. Exposure



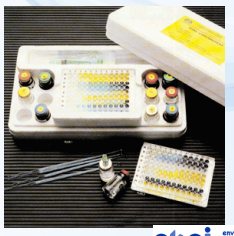
http://www.microbiotests.be/

MICROBIOTESTS

Toxichromo-Pad ®
Solid samples



Toxichromo-test ®
Water samples



http://www.ebpi-kits.com/

Test for specific *in vivo* effects

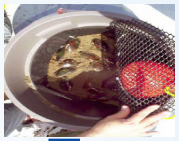
- embryotoxicity
- teratogenesis
- developmental disorders
- endocrine disruption
- reproductive disorders

- Aquatic tests or contact tests with sediment-dwelling invertebrates, amphipods, molluscs, fish, amphibian and mollusc eggs

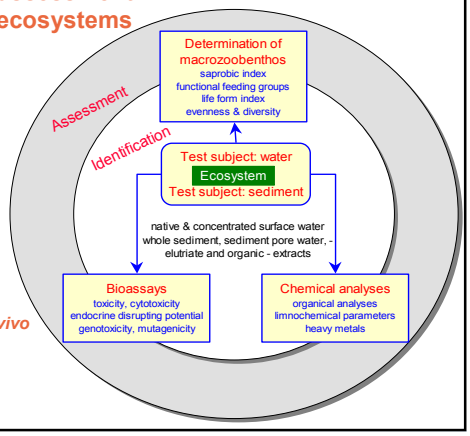
- Specific sublethal endpoints, histology

In situ tests

- Caging – bivalves, fish, molluscs
- Health status and specific biomarkers assessment in species collected on site
- Sublethal biomarkers, histology



Integrated assessment of aquatic ecosystems



... in vitro and *in vivo* bioassays

Terrestrial Toxicity Tests

- Direct exposure of test biota to media samples from a site
- Indirect exposure to filtered water exposed to soil or sediments samples
- Exposure to leachates from a site
- Controlled exposure to a specific contaminant using soil from the site
- Test biota
  - soil microbes and fungi - critical role in C, N, S, P cycling, plus production of SOM and other organics
  - invertebrates (earthworms and insects): provide essential ecosystem functions
- These tests are fast, simple and relatively inexpensive, with relevant results to evaluate effects on ecosystem biogeochemical functions

Terrestrial Toxicity Tests

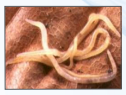
- Vertebrates:
  - amphibian: survival, growth and reproductive success
  - avian and small mammal: reproductive success and body burden
- Feeding studies (small mammal and avian toxicity tests) are useful to determine potential uptake and transfer within the food web - potential human exposure route
- Standard protocols have been derived from veterinary studies and FDA methods, but many are still under development
- Longer than invertebrate tests

Table 3.10. Vertebrate, invertebrate, and microbial test methods to assess the toxicity to terrestrial ecosystems

Test/species	Chemical sensitivity	References
Earthworm survival *Eisenia foetida, Lumbricus terrestris*	Water-soluble chemicals, metals, pesticides, organics, mixtures	Callahan *et al.*, 1985; Edwards, 1983; Goats and Edwards, 1982
Insect tests Ants, crickets, fruit flies, mites, beetles	Pesticides, chemical mixtures (not for metals or herbicides)	Gano *et al.*, 1985; OECD, 1984; James and Lighthart, 1990
Amphibian tests *Xenopus laevis*	Metals, pesticides, organics	ASTM E1439
Small mammal tests Rodents, voles, ferrets	Any substance capable of contaminating feed stocks	ASTM protocols: 552, 555, 593, 757, 758, 1103, 1163, 1372, 1373
Avian tests Bobwhite, quail, mallard, pheasant	Any substance capable of contaminating feed stocks	ASTM E857 and E1062
Vertebrate immunotoxicity Birds and mammals	Selenium, pentachlorophenol	Rose and Friedman, 1976; Oppenheim and Schechter, 1976; Gewurz and Suyehira, 1976
Invertebrate immunotoxicity Earthworms	PCBs	Stein and Cooper, 1988; Eyambe *et al.*, 1990; Rodriguez-Grau *et al.*, 1989
Chromosomal aberration tests Small mammals residing on site	Any known genotoxicant	Brusick, 1980; McBee *et al.*, 1987
Bacterial luminescence test *Photobacterium phosphoreum*	Metals, pesticides, herbicides, volatile and semi-volatile organics, hydrocarbons	Bulich, 1982, 1986; Ribo and Kaiser, 1987; Ahn and Morrison,1991
Soil biota metabolic activity Soil bacteria and fungi	Metals	Burns, 1986; Ladd, 1985; Nannipieri *et al.*, 1986a, 1986b
Soil biota respiration rates Soil bacteria and fungi	Metals and pesticides	Doelman and Haanstra, 1984; Dumontet and Mathur, 1989
Soil biota nitrogen cycling Soil bacteria and fungi	Insecticides, herbicides	Parr, 1974

Terrestrial bioassays - exposure in soil

•Folsomia candida



•Enchytraeus crypticus

Eisenia fetida

- •Earthworms: ***Eisenia fetida/andrei*** *(OECD 222, 2000)*
- •Enchytraeids: ***Enchytraeus albidus, E.crypticus*** *(OECD 220, 2000)*
- •Collembolans: ***Folsomia candida, F.fimetaria*** *(ISO 11267, 1999)*

➢**Test substrates: OECD artifical soil, real soils**
➢**10 adults (synchronized) in test vessel**
➢**Test duration: 28 days – 56 days**
➢**Endpoints: survival, reproduction – number of juveniles, weight changes**
➢**Preliminary test => Final test**

Terrestrial Toxicity Tests

- Vegetation
  - mostly crops
  - primary endpoints are:
    - survival: seed germination test
    - growth: seedling growth rate and root elongation test
    - reproduction success: vascular plant toxicity
    - photosynthesis rates: chlorophyll fluorescence assay
  - can be applied in lab or in the field
  - nutrient,water and light limitations can complicate analysis of results
  - longer term studies

Table 3.11. Vegetation toxicity test methods to assess chemical impacts to terrestrial ecosystems

Test/species	Chemical sensitivity	References
Seed germination test: Lettuce *Lactuca sativa*	Metals, insecticides, herbicides, volatile and semi-volatile organics, hydrocarbons	US Code of Federal Regulations, 1985; USFDA, 1987b; Gorsuch *et al.*, 1990; Linder *et al.*, 1990; USEPA, 1989, 1992
Root elongation test: Lettuce, *Lactuca sativa*	Metals, insecticides, herbicides, volatile and semi-volatile organics, hydrocarbons	US Code of Federal Regulations, 1985; USFDA, 1987b
Seedling growth tests: Purchased lettuce seeds or site-specific collected seeds	Metals, insecticides, herbicides, volatile and semi-volatile organics, hydrocarbons	US Code of Federal Regulations, 1985; USFDA, 1987c; OECD, 1984
Whole plant toxicity tests: Purchased lettuce seeds or site-specific collected seeds	Highly mobile, water-soluble compounds	Pfleeger *et al.*, 1991
Vascular plant toxicity tests: Plants from purchased seeds (cress, mustard) or site-specific collected seeds	Water-soluble compounds only	Ratsch, *et al.*, 1986; Shimabuku *et al.*, 1991
Photosynthetic inhibition tests/ chlorophyll fluorescence assay: Terrestrial plants	Water-soluble compounds only (if using soil eluate); all types of substances evaluated in field	Judy *et al.*, 1990, 1991; Miles, 1990

Battery of bioassays

- Different
  - Trophic level
  - Sensitivity
  - Target effect/organ
  - Specific toxic effect (mutagenity, neurotoxicity, etc…)
- The negative response in test with one species does not mean that substance is not toxic.
- Toxicity can be observed after longer exposure and/or in different species.
- Simple battery: algae, zooplankton, fish, bacteria.

Battery of bioassays:

- Standard acute toxicity tests representing different ecology groups – different levels of food chain – microorganism, algae, invertebrates, fish
- Different guidelines: *Vibrio fisheri, Thamnocephalus, Daphnia, Scenedesmus,*
- USEPA – crustacean *Ceriodaphnia dubia*, algae *Selenastrum capricornutum*, fish *Pimephales promelas*
- Chronic toxicity – crustaceans *Daphnia magna*, *Ceriodaphnia dubia*
- Specific endpoints: survival, reproduction, growth, activity, heartbeat, respiration, biochemical markers

Micro and Mesocosm

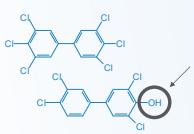
- Controlled experiments in lab or field to study changes at any level:
  - population
  - community
  - ecosystem
- Microcosm are small studies, usually in lab
- Mesocosm are large, containing many species, usually outdoors
- Advantages of microcosm studies:
  - Better than single-species studies
  - More space efficient
  - Easier to maintain controlled conditions
  - Replication and standardization easier
  - Low chance of contaminating the environment
- Issues with Microcosm:
  - Can't simulate certain processes (e.g. acid deposition from environment)
  - small population sizes => extinctions?
  - Extrapolation of results
  - May leave out a critical and/or sensitive component of ecosystem

Chronic effects

- endocrine disruption (compounds interfere with hormonal regulation in organism), (anti)estrogenicity ...
- reproductive failure, teratogenicity
- neurotoxicity
- immunosuppressions
- carcinogenicity (mutagenicity / tumor promotion)

How to study chronic toxicity ?

- **Chronic toxicity is difficult to study and predict**
  - time and cost consuming experiments
  - limited number of species (laboratory vs. natural species)
  - effect = combination of chemical exposure and life style, habits ...
  - metabolites or derivatives (*not parent compounds*) are often the active substances



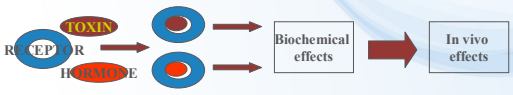
Ecotoxicological bioassays for chronic endpoints

How to study (chronic) toxicity ?

- **In vitro studies (biochemical mechanisms)**
  - + easy to perform, short-term
  - + highly controlled conditions
  - + lower amounts of chemicals needed (new compounds screening)
  - - ecotoxicological relevancy
  - - mostly with vertebrate cells
- **In vivo biotest testing**
  - + unique whole organisms
  - + controlled conditions
  - + better ecological interpretation
  - - only few (ecologically nonrelevant) organisms used
  - - mostly ACUTE assays
  - - chronic: long exposures
- **Field and *in situ* observations, epidemiological studies**

MECHANISMS of toxicity

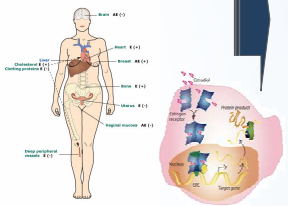
- **Various chronic effects have uniform biochemical basis**
  - principal studies with mechanistically based *in vitro* techniques



  - estimation of *in vitro* effects of individual compounds
    - understanding the mechanisms, prediction of hazard
  - application for risk assessment or monitoring
    - derivation of relative potencies ("toxic equivalents")

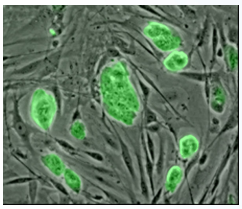
SINGLE mechanism -> SEVERAL effects
=> understanding to mechanisms may predict effects

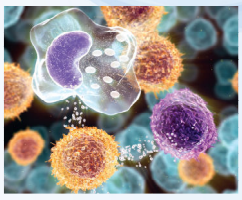
Estrogen receptor activation



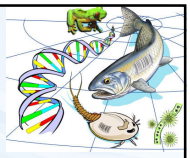
1) **female reproduction disorders**
2) **male feminisation**
3) **tumor promotion**
4) **immunomodulations**
5) **developmental toxicity**

Effects *in vitro* ?



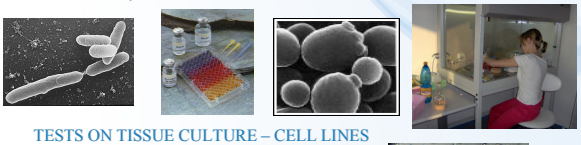


In vitro models



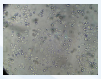
Original or genetically modified prokaryotic or eukaryotic cells

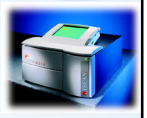
BACTERIAL, YEAST TESTS



TESTS ON TISSUE CULTURE – CELL LINES

In vitro bioassays




Principle

- Mechanism of action based
- Mechanism related to toxic effects

➢ **Using biological system as if it was an instrumental detector and/or integrator**

Screening tests

Toxicity/genotoxicity

Toxicity:
- Bacterial models
  Vibrio fisheri (Microtox) – 0.5 h
  Escherichia coli (Toxichromotest) – 2 h
- Fish/mammalian cell lines

Genotoxicity :
- SOS chromotest, umuC test
- Comet assay
- GFP test etc.

Contact test
- Flash test with *Vibrio Fisheri* – kinetic test

Specific mode of action

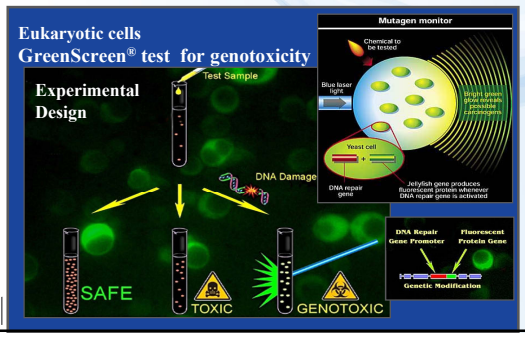
Yeast models
- Fish/mammalian cell lines

Tests for presence of compounds with hormone-like effects :
- Anti/estrogenicity
- Anti/androgenicity
- Retinoid-like activity
- Dioxin-like potency

In vitro assays for genotoxic effects

GENOTOXICITY = toxic modification or alteration of the structure or function of genetic material

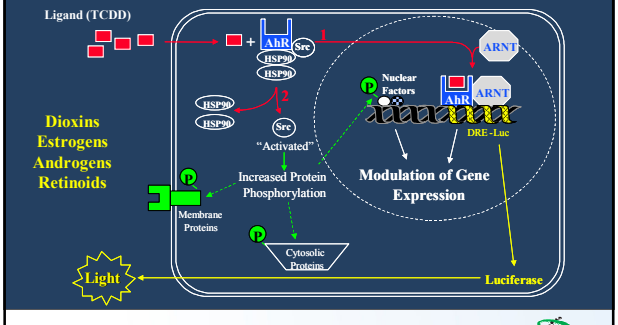
Bacterial or yeast assays with reporter genes



NUCLEAR RECEPTOR MEDIATED EFFECTS – important mechanisms involved in chronic toxicity

- **Dioxin-like activity: Aryl hydrocarbon receptor (AhR)-mediated effects**
  PCDDs/Fs, PAHs, PCBs
- **Xenoestrogenity / Antiestrogenity: Estrogen receptor (ER)-mediated effects**
  PCDDs/Fs, PAHs, PCBs, OH-PCBs, alkylphenols, natural and synthetic hormones ...
- **Xenoandrogenity / Antiandrogenity: androgen receptor (AR) - mediated effects** pesticides
- **Interactions with retinoic acid receptor (RAR)**

Receptor-mediated effects luciferase reporter assays



NUCLEAR RECEPTORS IN TOXICITY

- Nuclear receptors (**AhR, ER, AR, RAR/RXR**) play an important role in toxic effects of many pollutants
  - DIOXIN-like toxicity
  - Anti / estro-, Anti / andro- … -genicity
  - **Common mechanism - transcription factors:**
    - development of mechanistically based bioassays
  - ***In vitro* luciferase reporter bioassays – studies of …**
    - individual chemicals (toxicity identification, IEF calculation)
    - complex environmental samples (estimation of toxic potential)

BIOMARKERs

- Sublethal effects, studied in organisms from biotests or sampled in the environment
- Early warning signals of potential damage in organism and/or the whole population, early marker of toxicity (prior to any morphological alterations)
- Changes in cellular or biochemical components, structures or functions caused by xenobiotics
- Sensitive, fast responses, can show the mechanism of effect, precede any visible toxicity symptoms
- Most studied in vertebrates
- Possible to study also in plants and invertebrates from standard biotests (algae, macrophytes, invertebrates)

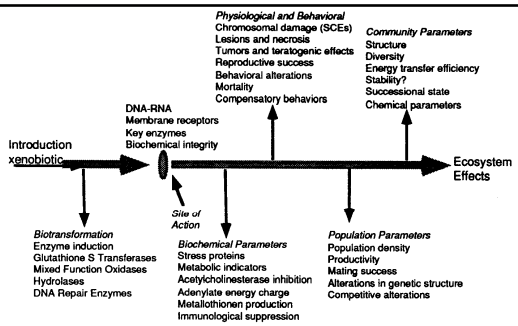
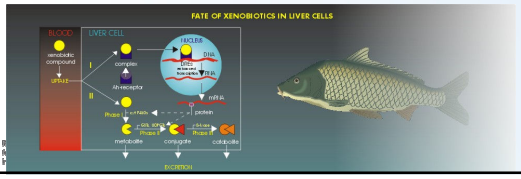


Figure 2.2 Parameters and indications of the interaction of a xenobiotic with the ecosystem. The examples listed are only a selection of the parameters that need to be understood for the explanation of the effects of a xenobiotic upon an ecosystem. However, biological systems appear to be organized within a hierarchy and that is how environmental toxicology must frame its outlook upon environmental problems.

Biotransformation enzymes (phase I&II)

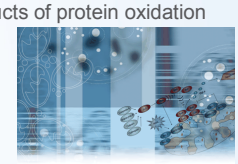
Induction of detoxification enzymes in plants and animals
A. Enzymes of the 1st phase of biotransformation – MFO enzymes (mixed function monooxygenases) – induction of P450 cytochrome enzymes (EROD, MROD, PROD)

B. Enzymes of the 2nd phase of biotransformation – glutathione transferases (GST), uridinedifosfoglukuronosyl transferases, sulphotransferases



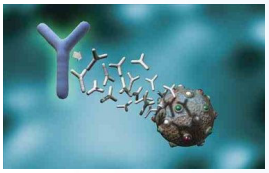
Oxidative stress parameters

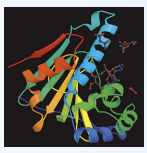
- Production of reactive oxygen species
- Activity of antioxidant enzymes – glutathion peroxidase, glutathion reductase, superoxidase, catalase
- Concentration of nonenzymatic antioxidants
- Oxidative damage to macromolecules – lipid peroxidation, oxidative DNA aducts, products of protein oxidation



Protective proteins

- Stress proteins: heat shock proteins (HSP), glucose-regulated proteins (GRP)
- Metallothioneins (MT): metal binding
- Multi Xenobiotic Resistance (MXR): excretion of xenobiotics; induction or inhibition by chemisensitizers

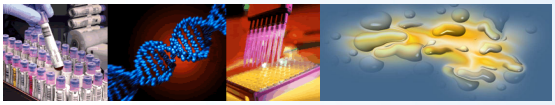




Hematological parameters

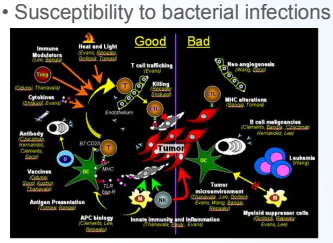
- Serum transaminases: Alanine transaminase (ALT), aspartate transaminase (AST); membrane disruption or organ damage

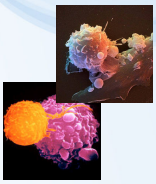
- Blood values: haematocrit, haemoglobin, blood sugars (glucose), plasma lipids and proteins (albumin)



Immunological parameters

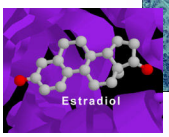
- White blood cell count
- Lymphocyte status
- Morphology of spleen, thymus and kidney
- Macrophage function
- Susceptibility to bacterial infections

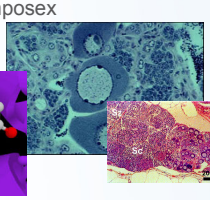


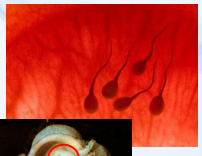


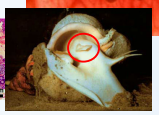
Reproductive & endocrine parameters

- Biochemical: Fish vitellogenin (VTG), Zona radiata Protein (ZRP), Cytochrome aromatase, spiggin (stickleback)
- Morphology of gonads; sperm condition
- Reproductive success (eggs, larvae)
- Intersex, Imposex



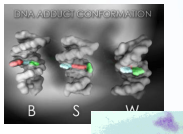


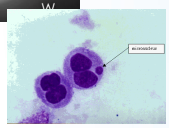


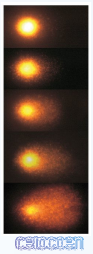


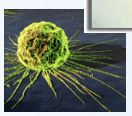
Genotoxic parameters

- DNA adducts
- Comet assay
- Micronucleus assay, sister-chromatid exchange
- Flow cytometric screening (DNA, RNA, protein)



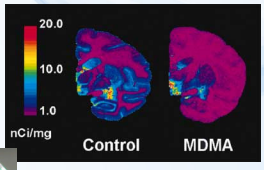






Neurotoxic parameters

- Acetyl cholinesterase inhibition assay (ACHE)
- Neurotransmitter impairment (e.g. SERT)
- Behavioral studies



SUMMARY – BIOASSAYS

BIOASSAYS are needed to test effects of …

1) Individual chemicals
   - Understanding toxicity + prospective studies for R.A.

2) Environmental samples
   - Routine analytical data (PAHs, PCBs, OCPs) provide only partial information
   - Biological experiments complement chemical analyses and may suggest elevated levels of unknown toxic chemicals (e.g. EDs)
   - In vitro assays are screening tools that help to understand mechanisms (e.g. „feminization“ / anti-androgenicity)
   - In vivo assays – ecologically relevant results

„Real ecotoxicology“ needed

1) Use non-standardized organisms
- Laboratory - aquatic snails, chironomids, soil organisms …
- Natural – sample natural organisms and test ecotoxicity immediately

2) Assess parameters important for populations
- Reproduction
- Life cycle effects (including early life stages)

3) Consider natural situations
- Addapt test conditions (temperature?, water hardness? …)
- Simulate real exposures (e.g. peaks during pesticide spraying)

Summary

- Methods for assessing effect vary from
  - single chemical/single species
  - multiple stressors/multiple species
  - short-term/long-term
- Ability to relate cause and effect varies accordingly (easier for simpler system)
- Need studies at all scales (temporal and spatial) to have better understanding
- Be critical of a standard developed with just one methodology!

Research centre for toxic compounds in the environment