Research centre for toxic compounds in the environment

Ecotoxicological bioassays

Klara Hilscherova, Ludek Blaha, Jakub Hofman & co.

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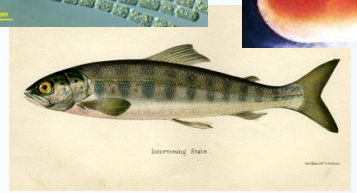
CHEMICAL ENTERS THE ORGANISM

biomonitoring

LEVELS, FATE, PROCESSES

Bioavailable fraction



Toxikokinetics

biotransformation
bioactivation
excretion / sequestration

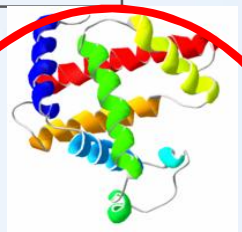
Target site

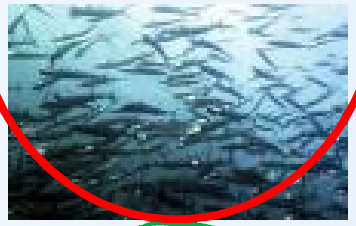
"EFFECT"

"EXPOSURE"

acute

chronic





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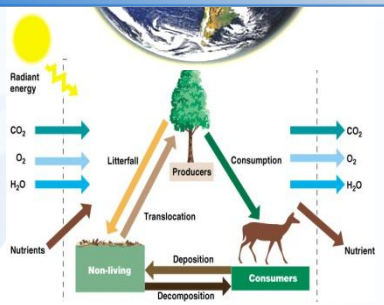

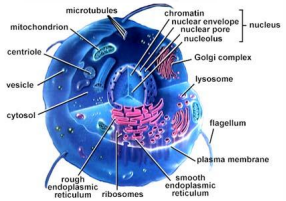

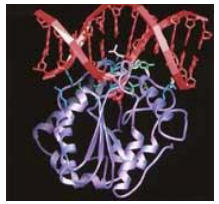

Biosphere

Ecosystem

Community

Population

Individual

System of organs
Organ
Tissue
Cell
Organell
Biomolecule

LOW

HIGH

Flexibility
Ability to determine cause
Specifity
Sensitivity

HIGH

Ecological relevance
Response duration
Longer-term effects

LOW

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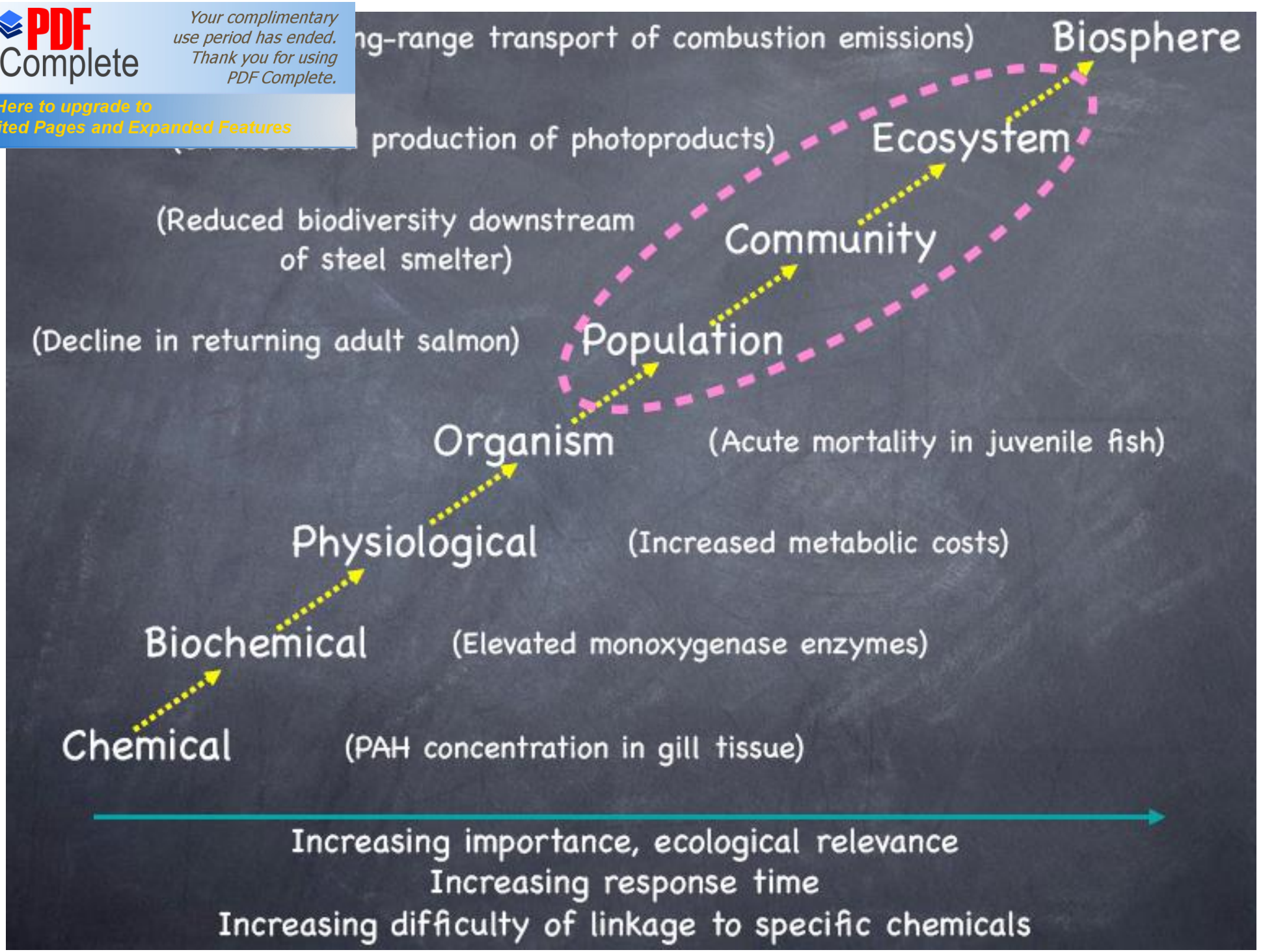


Effect of Chemicals

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

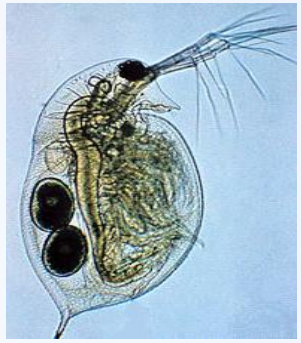
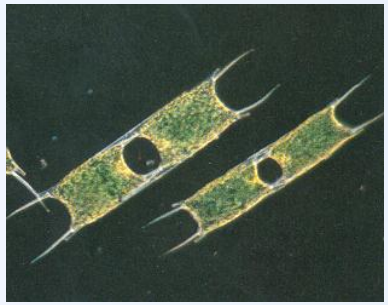
Note: On the far right of the diagram, changes in structure and function of ecosystems occur, and the chasm that separates this impact from the stages on the left is too great to demonstrate graphically.

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.



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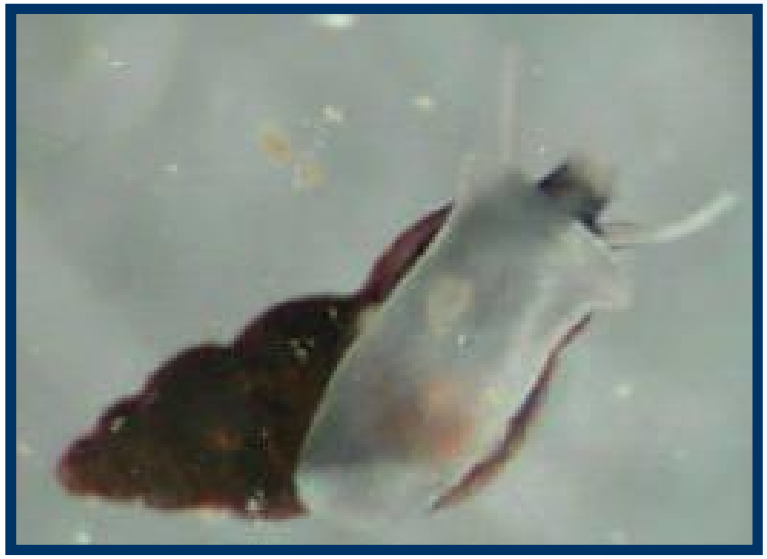
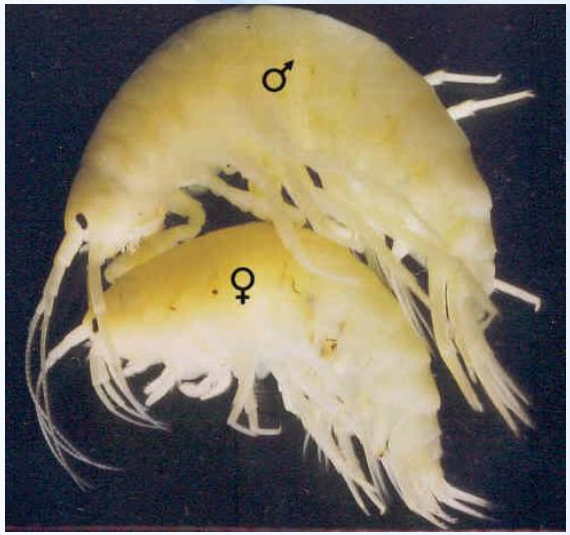
In vivo effects?





Ecotoxicity Tests

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

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

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

Ecotoxicity 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

Toxicity Test Duration

rm toxicity

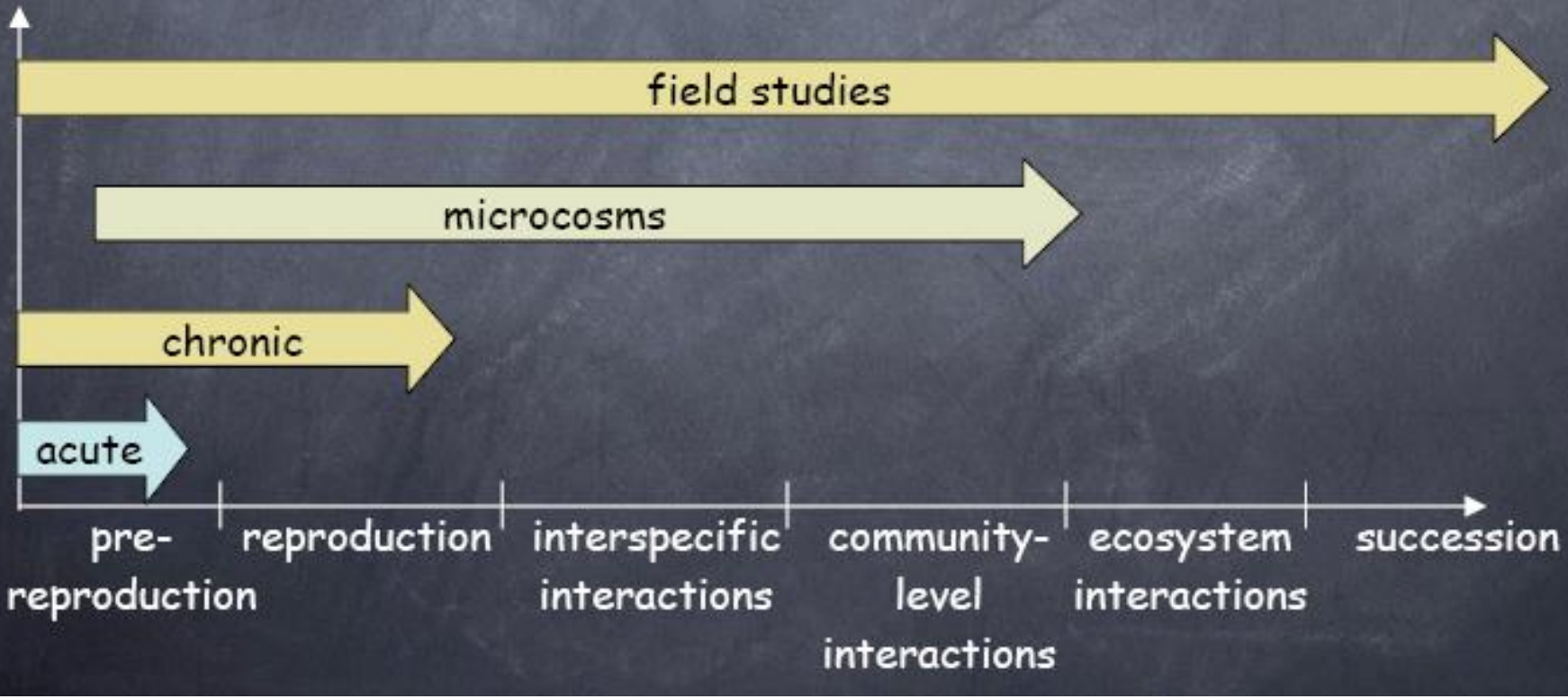
-usually defined as <96h

-endpoints: mortality, photosynthesis, germination

Chronic = long-term

>96h, up to multigenerational

-endpoints: mortality, reproduction, growth rate, teratogenesis



Standardized methods associated with biotests and standardized guidelines

- **OECD = Organization for Economic Cooperation Development**
- **ISO = International Standardization Organization**
- **US EPA = US Environmental Protection Agency**
- SETAC = Society for Environmental Toxicology and Chemistry
- IOBC = International Organisation for Biological and Integrated Control of Noxious Animals and Plants
- EPPO = European and Mediterranean Plant Protection Organization
- ASTM = American Society of Testing and Materials
- ANSI = American National Standards Institute
- CEN = European Commitee for Standardization
- AFNOR = Association Francaise de Normalisation
- EEC = European Economic Community
- WHO = World Health Organisation
- BBA = Biologische Bundesanstalt für Land- und Forstwirtschaft
- OPPTS = The Office of Prevention, Pesticides and Toxic Substances (EPA)
- DIN = German Deutsches Institut für Normung

s for the Testing of Chemicals
ts on Biotic Systems

Aquatic organisms

Test No. 201: Alga, Growth Inhibition Test	11 July 2006
Test No. 221: Lemna sp. Growth Inhabition Test	11 July 2006
Test No. 202: Daphnia sp. Acute Immobilisation Test	23 Nov 2004
Test No. 211: Daphnia magna Reproduction Test	16 Oct 2008
Test No. 203: Fish, Acute Toxicity Test	17 July 1992
Test No. 204: Fish, Prolonged Toxicity Test: 14-Day Study	04 Apr 1984
Test No. 210: Fish, Early-Life Stage Toxicity Test	17 July 1992
Test No. 212: Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages	21 Sep 1998
Test No. 215: Fish, Juvenile Growth Test	21 Jan 2000
Test No. 229: Fish Short Term Reproduction Assay	08 Sep 2009
Test No. 230: 21-day Fish Assay	08 Sep 2009
Test No. 231: Amphibian Metamorphosis Assay	08 Sep 2009

Tests with sediment

Test No. 218: Sediment-Water Chironomid Toxicity Using Spiked Sediment	23 Nov 2004
Test No. 219: Sediment-Water Chironomid Toxicity Using Spiked Water	23 Nov 2004
Test No. 233: Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment	23 July 2010
Test No. 225: Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment	15 Oct 2007

OECD guidelines

Soil organisms

Test No. 208: Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test	17 Aug 2006
Test No. 227: Terrestrial Plant Test: Vegetative Vigour Test	17 Aug 2006
Test No. 207: Earthworm, Acute Toxicity Tests	04 Apr 1984
Test No. 220: Enchytraeid Reproduction Test	23 Nov 2004
Test No. 222: Earthworm Reproduction Test (Eisenia fetida/Eisenia andrei)	23 Nov 2004
Test No. 228: Determination of Developmental Toxicity of a Test Chemical to Dipteran Dung Flies(Scathophaga stercoraria L. (Scathophagidae), Musca autumnalis De Geer (Muscidae))	16 Oct 2008
Test No. 232: Collembolan Reproduction Test in Soil	08 Sep 2009
Test No. 226: Predatory mite (Hypoaspis (Geolaelaps) aculeifer) reproduction test in soil	16 Oct 2008
Test No. 216: Soil Microorganisms: Nitrogen Transformation Test	21 Jan 2000
Test No. 217: Soil Microorganisms: Carbon Transformation Test	21 Jan 2000

Other tests

Test No. 213: Honeybees, Acute Oral Toxicity Test	21 Sep 1998
Test No. 214: Honeybees, Acute Contact Toxicity Test	21 Sep 1998
Test No. 205: Avian Dietary Toxicity Test	04 Apr 1984
Test No. 206: Avian Reproduction Test	04 Apr 1984
Test No. 223: Avian Acute Oral Toxicity Test	23 July 2010

ISO guidelines

Aquatic microorganisms

ISO 10712:1995	**Water quality -- Pseudomonas putida growth inhibition test (Pseudomonas cell multiplication inhibition test)**
ISO 11348-1:2007	**Water quality -- Determination of the inhibitory effect of water samples on the light emission of Vibrio fischeri (Luminescent bacteria test) -- Part 1: Method using freshly prepared bacteria**
ISO 11348-2:2007	**Water quality -- Determination of the inhibitory effect of water samples on the light emission of Vibrio fischeri (Luminescent bacteria test) -- Part 2: Method using liquid-dried bacteria**
ISO 11348-3:2007	**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 13641-1:2003	**Water quality -- Determination of inhibition of gas production of anaerobic bacteria -- Part 1: General test**
ISO 13641-2:2003	**Water quality -- Determination of inhibition of gas production of anaerobic bacteria -- Part 2: Test for low biomass concentrations**
ISO 13829:2000	**Water quality -- Determination of the genotoxicity of water and waste water using the umu-test**
ISO 16240:2005	**Water quality -- Determination of the genotoxicity of water and waste water -- Salmonella/microsome test (Ames test)**
ISO/DIS 11350	**Water quality -- Determination of the genotoxicity of water and waste water -- Salmonella/microsome fluctuation test (Ames fluctuation test)**
ISO 15522:1999	**Water quality -- Determination of the inhibitory effect of water constituents on the growth of activated sludge microorganisms**
ISO 21338:2010	**Water quality -- Kinetic determination of the inhibitory effects of sediment, other solids and coloured samples on the light emission of Vibrio fischeri (kinetic luminescent bacteria test)**
ISO 8192:2007	**Water quality -- Test for inhibition of oxygen consumption by activated sludge for carbonaceous and ammonium oxidation**
ISO 9509:2006	**Water quality -- Toxicity test for assessing the inhibition of nitrification of activated sludge microorganisms**

ISO guidelines

Aquatic plants

ISO 20079:2005	**Water quality -- Determination of the toxic effect of water constituents and waste water on duckweed (Lemna minor) -- Duckweed growth inhibition test**
ISO 8692:2004	**Water quality -- Freshwater algal growth inhibition test with unicellular green algae**
ISO/CD 16191	**Water quality - Determination of the toxic effect of sediment and soil on the growth behaviour of Myriophyllum aquaticum - Myriophyllum test**
ISO 10253:2006	**Water quality -- Marine algal growth inhibition test with Skeletonema costatum and Phaeodactylum tricornutum**
ISO 10710:2010	**Water quality -- Growth inhibition test with the marine and brackish water macroalga Ceramium tenuicorne**
ISO 14442:2006	**Water quality -- Guidelines for algal growth inhibition tests with poorly soluble materials, volatile compounds, metals and waste water**
ISO/DIS 13308	**Water quality -- Toxicity test based on reproduction inhibition of the green macroalga Ulva pertusa**
ISO/TR 11044:2008	**Water quality -- Scientific and technical aspects of batch algae growth inhibition tests**

ISO guidelines

Aquatic invertebrates

ISO 6341:1996	**Water quality -- Determination of the inhibition of the mobility of Daphnia magna Straus (Cladocera, Crustacea) -- Acute toxicity test**
ISO 10706:2000	**Water quality -- Determination of long term toxicity of substances to Daphnia magna Straus (Cladocera, Crustacea)**
ISO/DIS 14380	**Water quality -- Determination of the acute toxicity to Thamnocephalus platyurus (Crustacea, Anostraca)**
ISO/CD 16303	**Water quality -- Determination of toxicity of fresh water sediments using Hyalella azteca**
ISO 10872:2010	**Water quality -- Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of Caenorhabditis elegans (Nematoda)**
ISO 16712:2005	**Water quality -- Determination of acute toxicity of marine or estuarine sediment to amphipods**
ISO 20665:2008	**Water quality -- Determination of chronic toxicity to Ceriodaphnia dubia**
ISO 20666:2008	**Water quality -- Determination of the chronic toxicity to Brachionus calyciflorus in 48 h**
ISO 14669:1999	**Water quality -- Determination of acute lethal toxicity to marine copepods (Copepoda, Crustacea)**
ISO/DIS 14371	**Water quality -- Determination of freshwater-sediment subchronic toxicity to Heterocypris incongruens (Crustacea, Ostracoda)**
ISO 7828:1985	**Water quality -- Methods of biological sampling -- Guidance on handnet sampling of aquatic benthic macro-invertebrates**
ISO 8265:1988	**Water quality -- Design and use of quantitative samplers for benthic macro-invertebrates on stony substrata in shallow freshwaters**
ISO 8689-1:2000	**Water quality -- Biological classification of rivers -- Part 1: Guidance on the interpretation of biological quality data from surveys of benthic macroinvertebrates**
ISO 8689-2:2000	**Water quality -- Biological classification of rivers -- Part 2: Guidance on the presentation of biological quality data from surveys of benthic macroinvertebrates**
ISO/DIS 10870	**Water quality -- Guidelines for the selection of sampling methods and devices for benthic macroinvertebrates in fresh waters**
ISO/WD 16778	**Water quality -- Calanoid copepod development test with Acartia tonsa**

ISO guidelines

Aquatic vertebrates

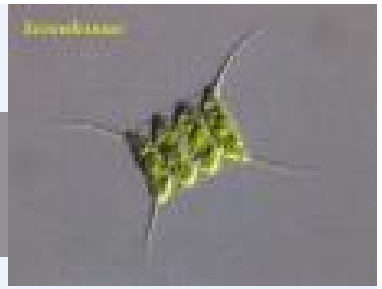
ISO 15088:2007	**Water quality -- Determination of the acute toxicity of waste water to zebrafish eggs (Danio rerio)**
ISO 7346-1:1996	**Water quality -- Determination of the acute lethal toxicity of substances to a freshwater fish [Brachydanio rerio Hamilton-Buchanan (Teleostei, Cyprinidae)] -- Part 1: Static method**
ISO 7346-2:1996	**Water quality -- Determination of the acute lethal toxicity of substances to a freshwater fish [Brachydanio rerio Hamilton-Buchanan (Teleostei, Cyprinidae)] -- Part 2: Semi-static method**
ISO 7346-3:1996	**Water quality -- Determination of the acute lethal toxicity of substances to a freshwater fish [Brachydanio rerio Hamilton-Buchanan (Teleostei, Cyprinidae)] -- Part 3: Flow-through method**
ISO 10229:1994	**Water quality -- Determination of the prolonged toxicity of substances to freshwater fish -- Method for evaluating the effects of substances on the growth rate of rainbow trout (Oncorhynchus mykiss Walbaum (Teleostei, Salmonidae))**
ISO 12890:1999	**Water quality -- Determination of toxicity to embryos and larvae of freshwater fish -- Semi-static method**
ISO 21427-1:2006	**Water quality -- Evaluation of genotoxicity by measurement of the induction of micronuclei -- Part 1: Evaluation of genotoxicity using amphibian larvae**
ISO 21427-2:2006	**Water quality -- Evaluation of genotoxicity by measurement of the induction of micronuclei -- Part 2: Mixed population method using the cell line V79**
ISO 23893-1:2007	**Water quality -- Biochemical and physiological measurements on fish -- Part 1: Sampling of fish, handling and preservation of samples**
ISO/TS 23893-2:2007	**Water quality -- Biochemical and physiological measurements on fish -- Part 2: Determination of ethoxyresorufin-O-deethylase (EROD)**
ISO/CD 23893-3	**Water quality -- Biochemical and physiological measurements on fish -- Part 3: Determination of vitellogenin**

n of bioassays in ecotoxicology

- Testing chemicals
  - Traditional approach - bioassays developed to assess **chemicals**
  - **Standardized and validated approaches**
    - OECD Guideline methods - series Effects on biota
    - ISO methods
      - E.g. Fish tests - OECD 203 / ISO 7346
      - E.g. D. magna - OECD 202 / ISO 6341
    - Other standard guidelines
    - 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 environmental matrices
  - Relatively new in ecotoxicology many open challenges
  - More complex and more complicated
    - cause-effectsoften not clear (many confounding factors)

Testing strategy



- **Battery of assays**
  - Fast screening tests (inhibition of Vibrio fisheri bioluminiscence, MICROTOX ï 30 min toxicity)
  - Standardized acute toxicity tests
  - Further studies with chronic assays

- **Various purposes -> guidelines and recommendations**
  - REACH (EU - Registration, Evaluation and Authorisation of Chemicals)
  - Plant protection products + biocides
  - Veterinary and human pharmaceuticals
  - Waste materials é

- **The most common set up for aquatic environment**
  - *algae / D. magna / fish*

sic principles of bioassays

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

Tested matrix

Water
Soil
Air
Sediment
Waste
Chemical compound

Sample type

Single compounds (hydrophobic, hydrophilic, volatile)
Mixture of compounds (known and/or unknown)
Environmental samples (usually unknown, mixtures of different compounds with different properties ï 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
- Mutagenicity/genotoxicity (microbial, vascular plants, wildlife animals)
- Teratogenity (amphibian- *Xenopus laevis*)
- Embryotoxicity
- Reproduction bioassays
- Growth

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

ests é to be considered

- ***Parameters of the biological system***
  - *Complexity / in vitro, in vivo, population, microcosm* ***é***
  - *Population characteristics* ***ï*** *sex, age* ***é***
  - *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* ***é***
- ***Abiotic factors in the experiment***
  - *Validity criteria (pH, oxygen, temperature, humidity, water hardness* ***é*** *)*

ps to conduct the biotest

- **1) Prepare the organism**
  - Culture media, standardized numbers, age, etc.
- **2) Prepare the sample**
  - Dilution series
    - water/culture media ï direct organism exposure
      - Include BLANK (medium only)
    - solvent for organic compounds ï minimum to be added (1% vol)
      - Include SOLVENT CONTROL
- **3) Expose organisms**
  - é 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 é)

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

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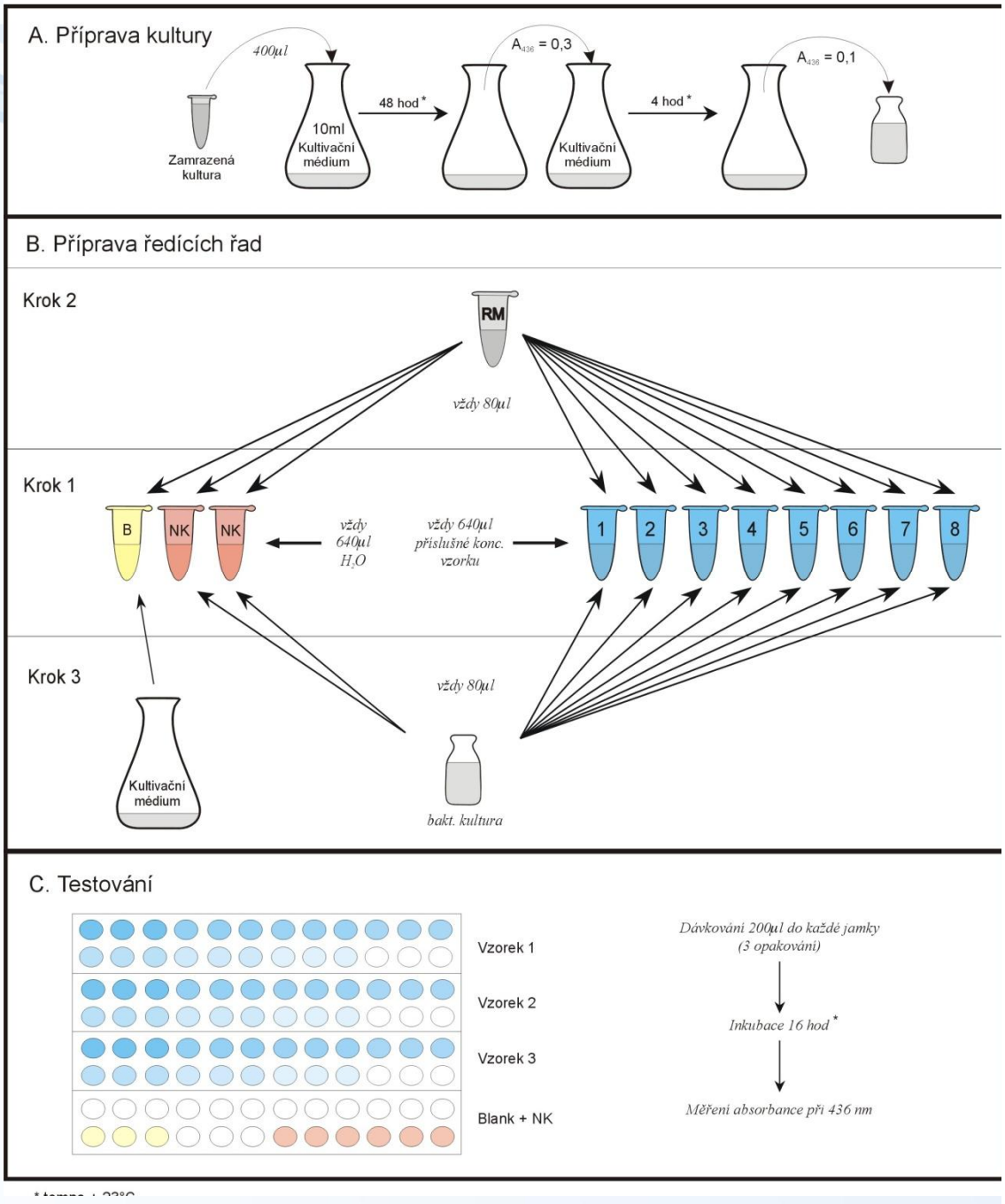
Schema inhibičního testu na *Pseudomonas putida*



ory ecotoxicology ï data and results

Parameters:

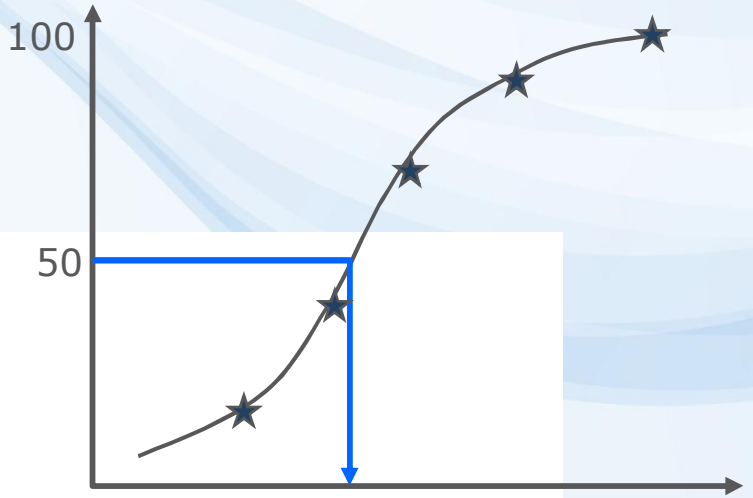
No Observed Effect Concentration (NOEC)

Lowest Observed Effect Concentration (LOEC)

ECx (x % effects concentration)

LCx (x % lethal concentration)

Dose-response curve



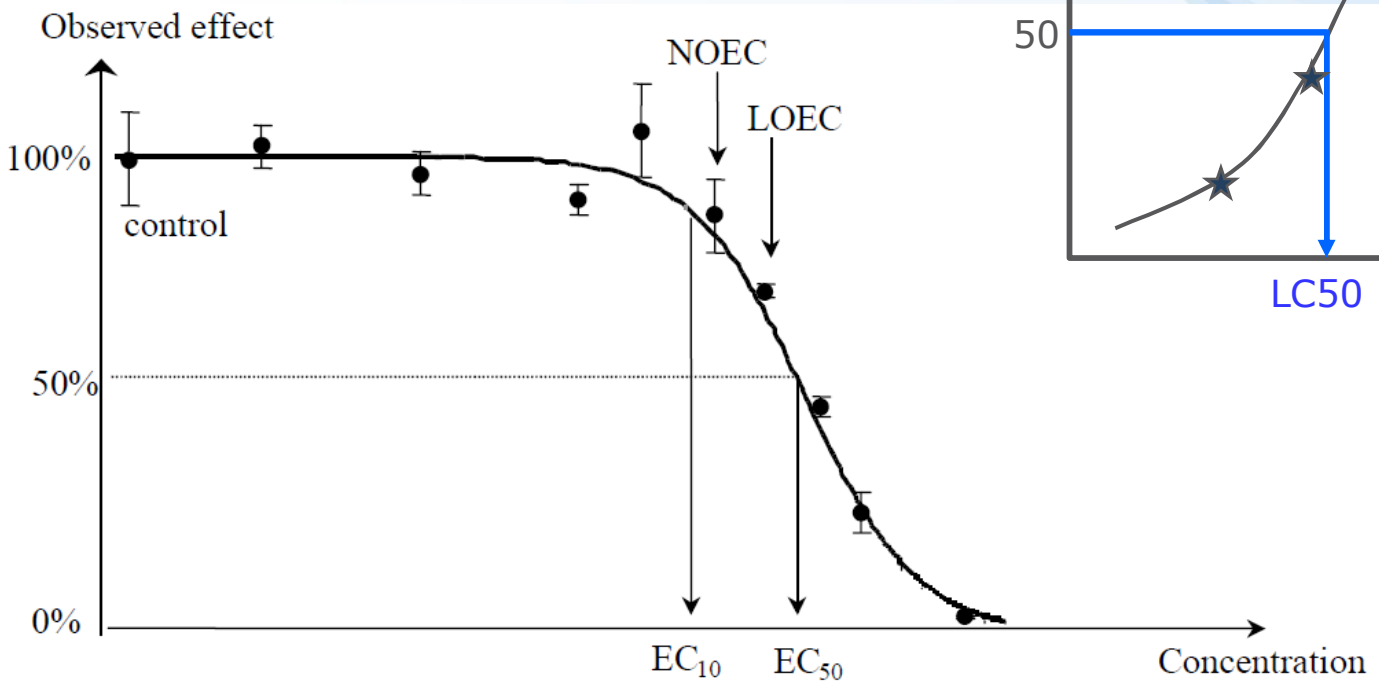
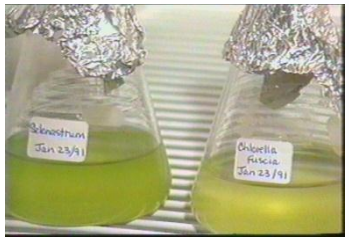


Figure 3.2 Illustration of a concentration-response relationship and of the estimates of the ECx and NOEC/LOEC. The order of the parameters given in this figure has been taken at random.

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



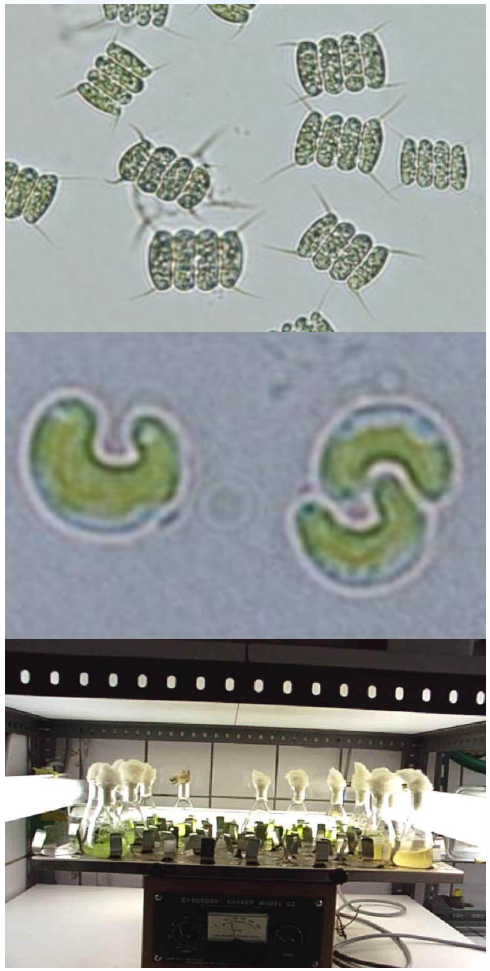
reshwater acute toxicity tests

	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

ATIC 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** ï testing toxicity in the aquatic media (*Lepidium sativum, Sinapis alba, Lactuca sativa*)
  - OECD 208 Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test

TS with CONSUMERS - invertebrates

AQUATIC ASSAYS

- *Daphnia magna* immobilisation (24 ï 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*

S with CONSUMERS ï 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

prolonged tests embryolarval tests
chronic tests ï reproduction, growth
Specific endpoints ï genotoxicity, endocrine disruption

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*

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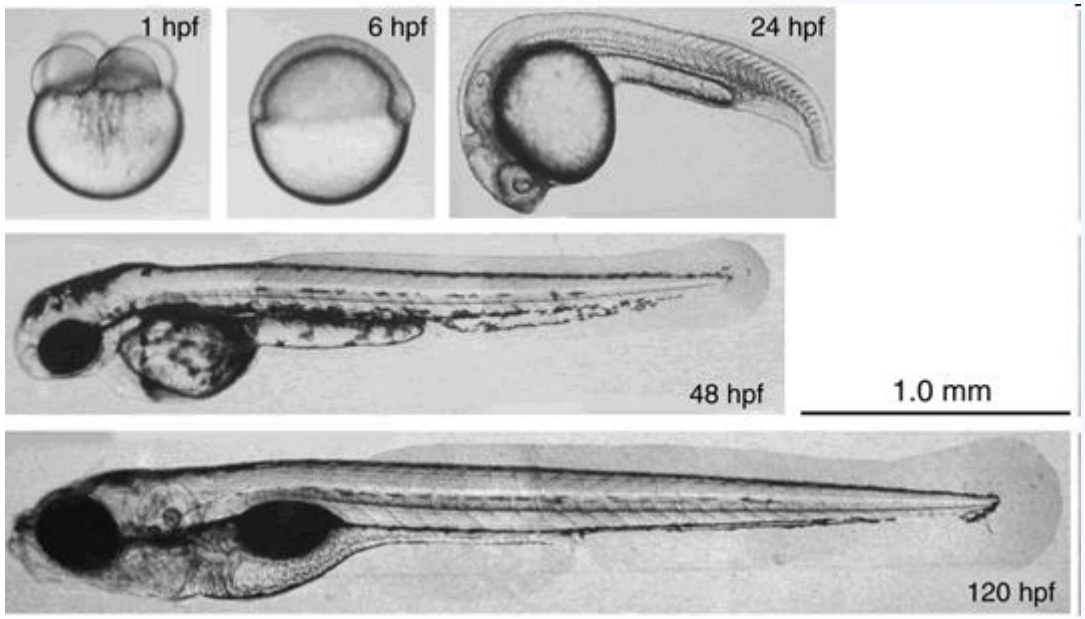
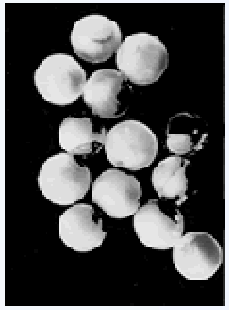
STS with CONSUMERS - amphibians

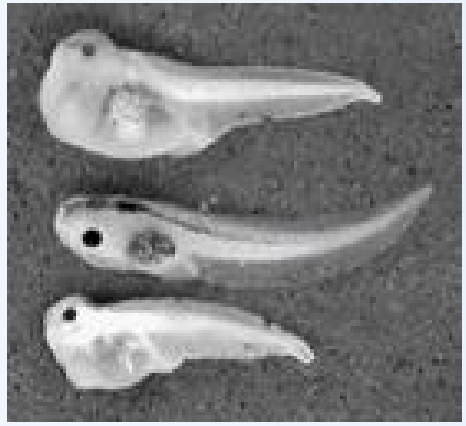
FETAX ï Frog Embryo Teratogenicity Assay Xenopus (ASTM E1439-98)

African clawed frog (*Xenopus laevis*)

96 h / egg and embryo exposure







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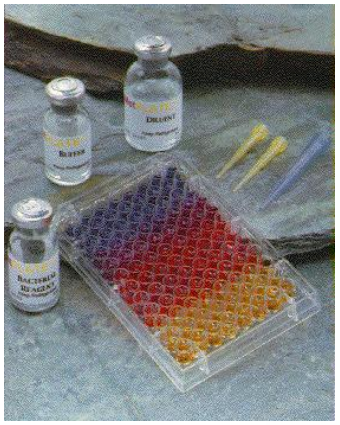
S with DESTRUENTS - microorganisms



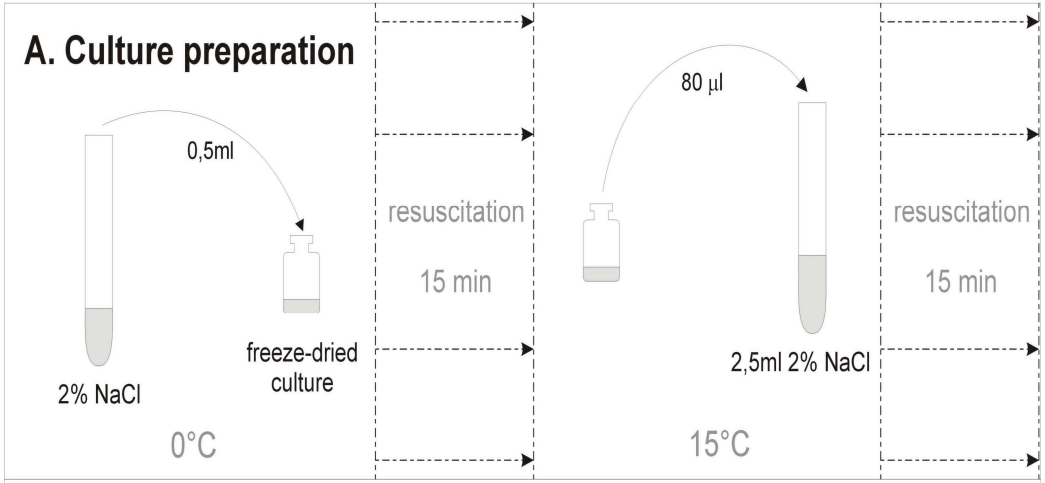
- **Toxicity to luminescent bacteria** Vibrio fisheri (MICROTOX®)
  - ISO 11348 Water quality - Determination of the inhibitory effect of water samples on the light emission of Vibrio fischeri
- **Growth inhibitions** (Pseudomonas putida, Toxi-Chromotest, Toxi-ChromoPad)
- **Toxicity assays** with SOIL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

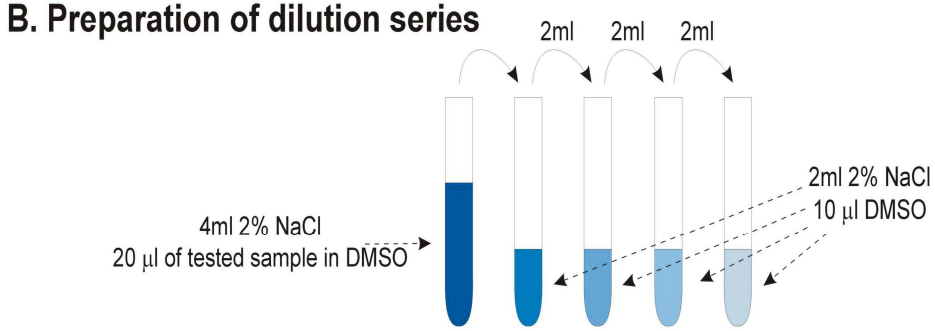
Microtox

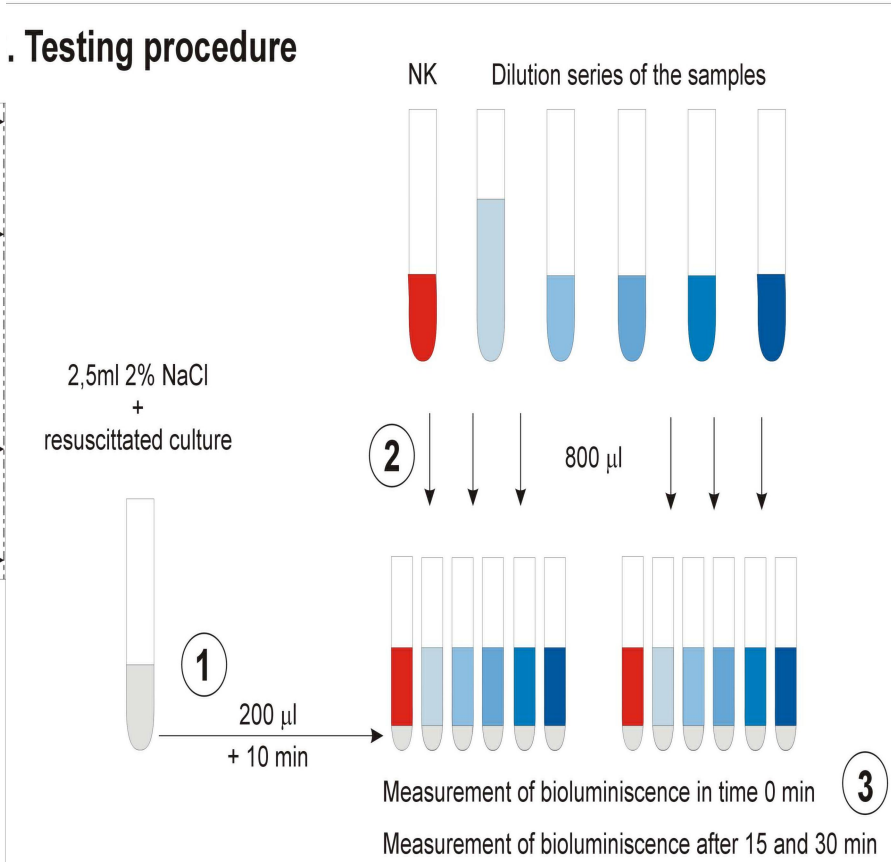
EN ISO 11348-(1)

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

Microtox - test design







hronic 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

hronic 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

Aquatic 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 freshwater and a marine species
Bioconcentration Testing	At least one bioconcentration study with an appropriate freshwater and saltwater species is required

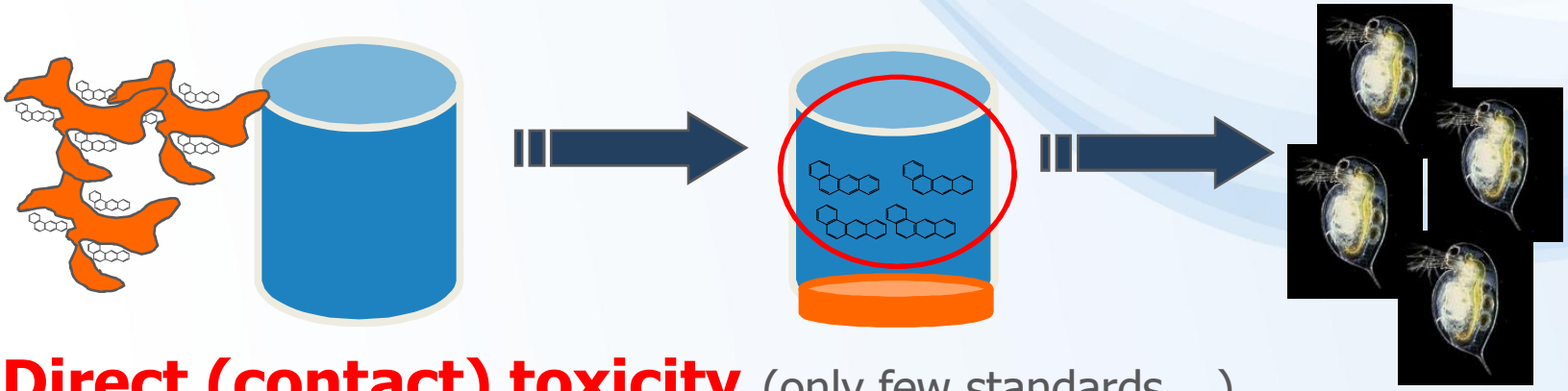
Sediment ecotoxicity tests

f pore water/eluates (several ISO / OECD standards)

: 100 g d.w./L water, 24h slow shake, filter, test

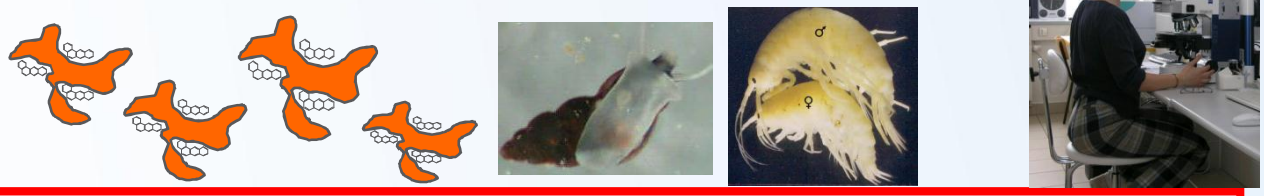
V. fisheri (30 min), Algae, Invertebrates - D. magna **(2 days)**

? 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

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ment toxicity ï Acute 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 ï 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

ment toxicity ï Chronic 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

sts 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

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ests 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

s 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

t Toxicity Test ï confounding factors

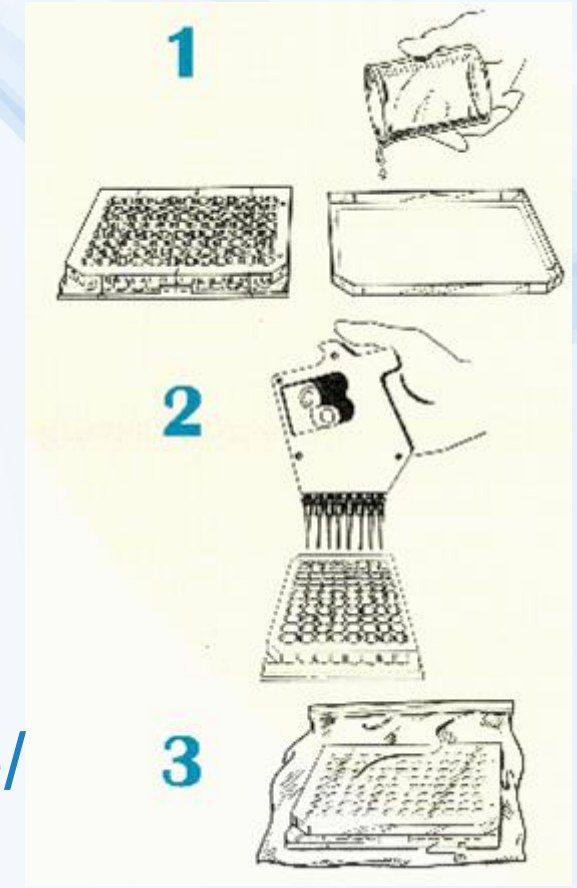
- **Potential Non-Contaminant Factors**
  - ÅSediment grain size
  - ÅContent and type of clay
  - ÅOrganic carbon content and character
  - ÅHumic substances/organic matter structure and properties
  - ÅpH
  - ÅOxygen content
  - ÅAmmonia / Sulfide toxicity
  - ÅNutrition

- **Changing sediment toxicity due to sampling and experimental procedures**
  - ï Mixing of more contaminated sediments with the thin layer at the sediment-water interface
  - ï 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

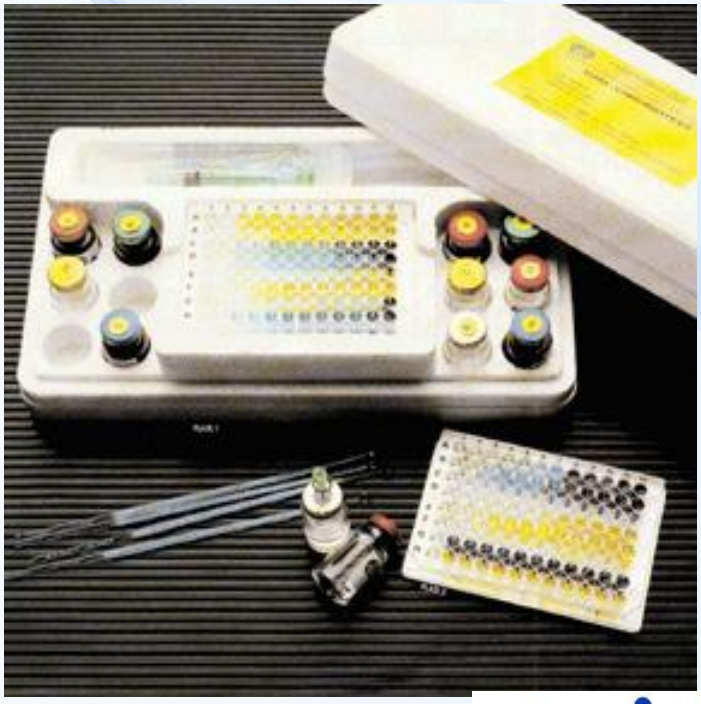


http://www.microbiotests.be/

MICROBIOTESTS

Toxichromo-Pad ®
Solid samples

Toxichromo-test ®
Water samples



ebpi environmental bio-detection products inc.

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

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s 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

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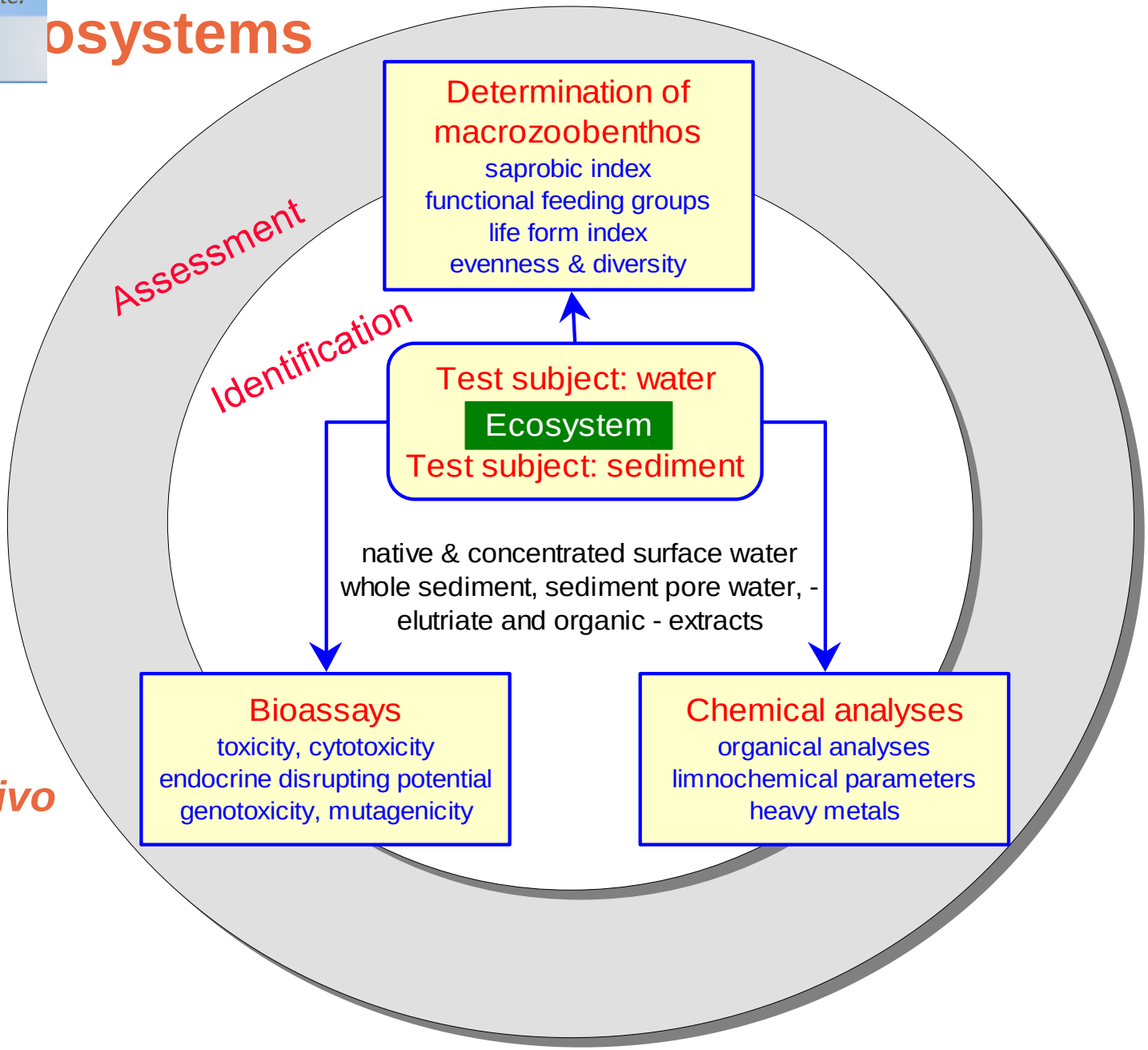
In situ tests

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

sessment
osystems



... *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

strial 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 ï 56 days**
- **Endpoints: survival, reproduction ï 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

ation toxicity test methods to assess chemical impacts to
ns

	Chemical sensitivity	References
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 lettude 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

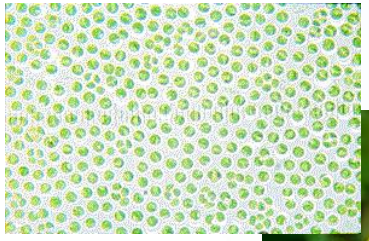
Battery of bioassays

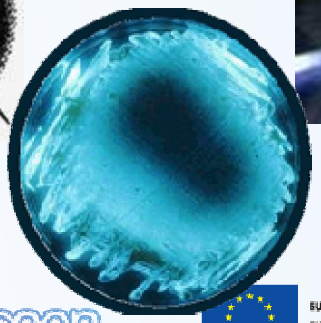
- Different
  - Trophic level
  - Sensitivity
  - Target effect/organ
  - Specific toxic effect (mutagenity, neurotoxicity, etcé)

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

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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

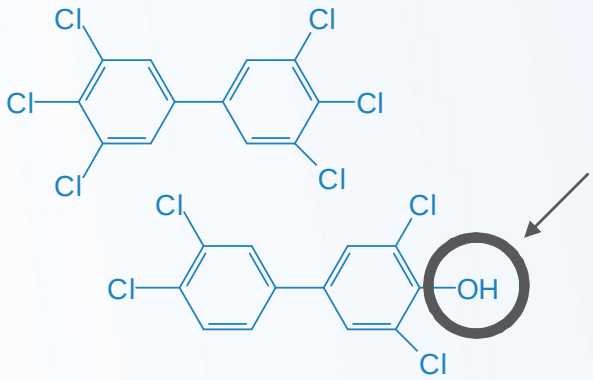
Chronic effects

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

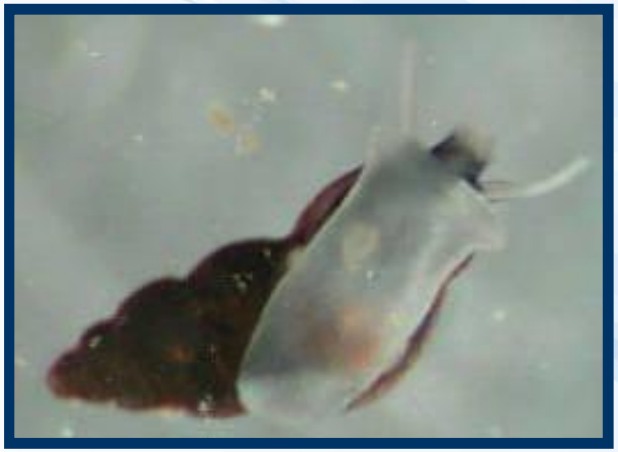
o study chronic toxicity ?

Å Chronic toxicity is difficult to study and predict

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



cotoxicological bioassays for chronic endpoints



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to study (chronic) toxicity ?

Å 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

Å In vivo biotest testing

+ unique whole organisms
+ controlled conditions
+ better ecological interpretation

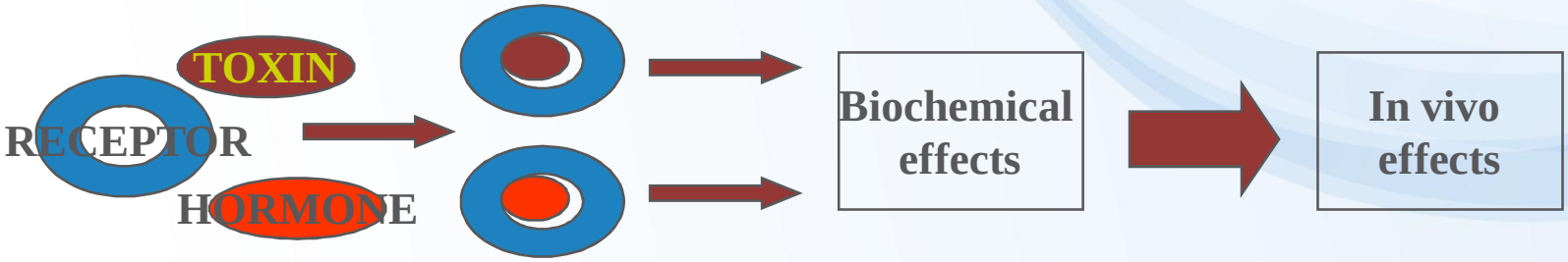
- only few (ecologically nonrelevant) organisms used
- mostly ACUTE assays
- chronic: long exposures

Å 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

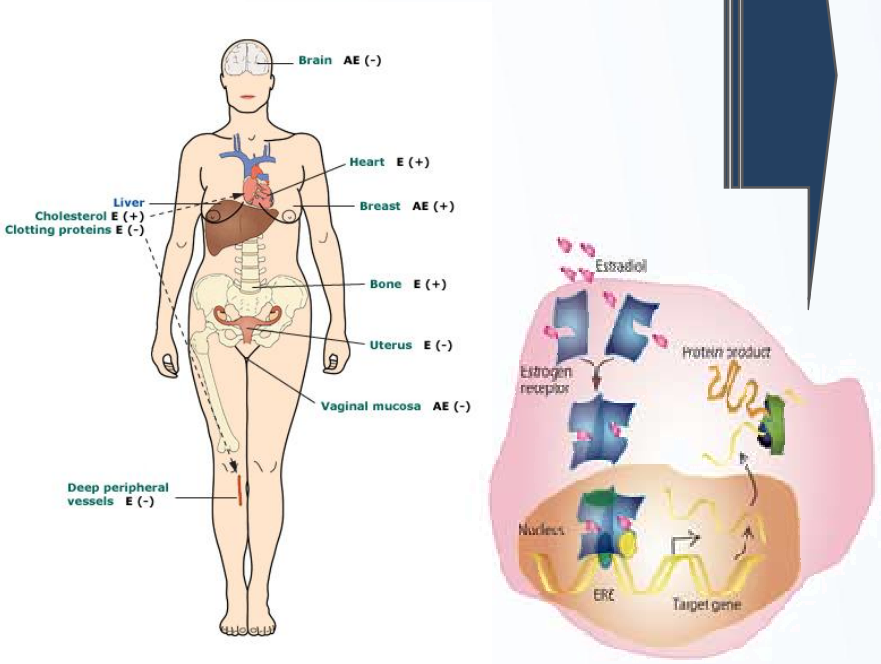


- estimation of *in vitro* effects of individual compounds
  - understanding the mechanisms, prediction of hazard
- application for risk assessment or monitoring
  - derivation of relative potencies ("toxic equivalents")

SINGLE mechanism -> SEVERAL effects

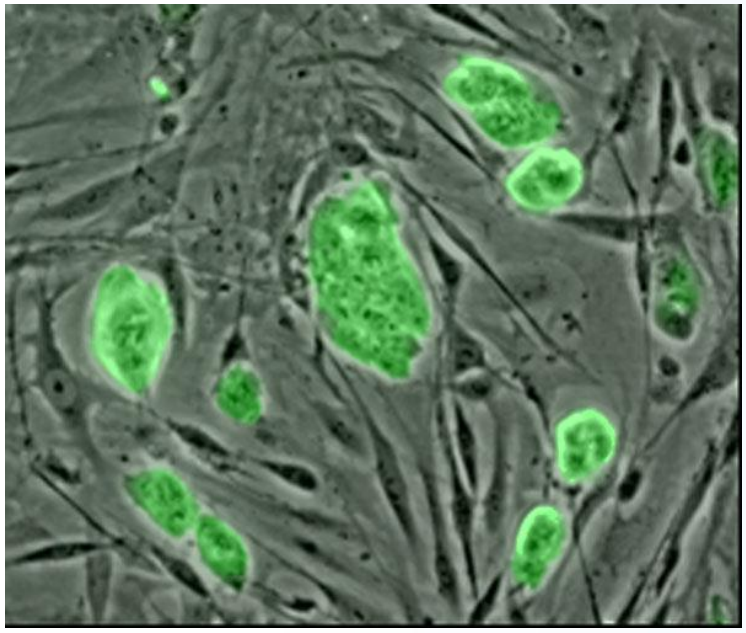
=> understanding to mechanisms may predict effects

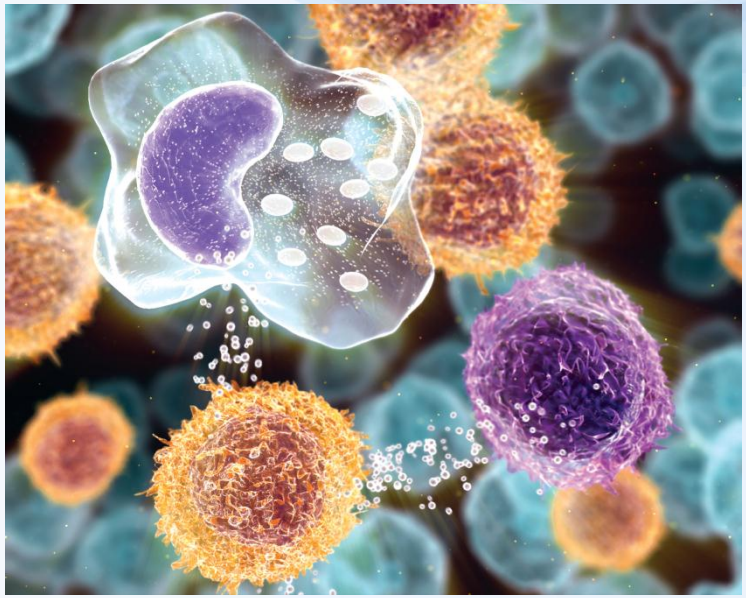
Estrogen receptor activation



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

Effects *in vitro* ?





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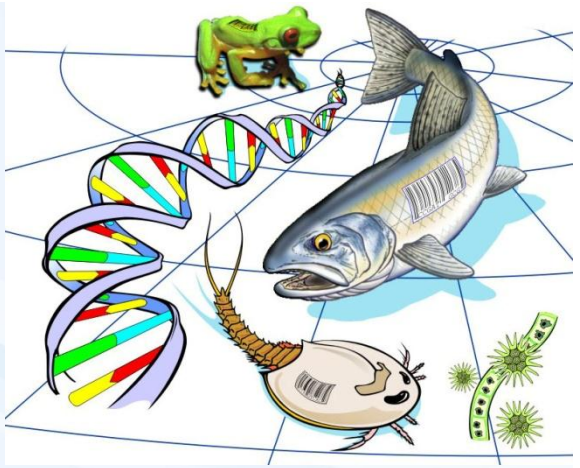
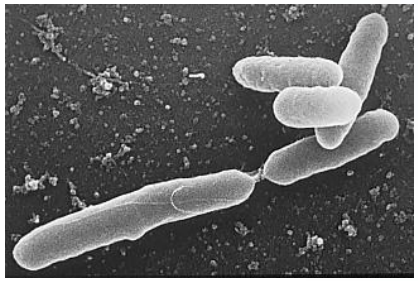
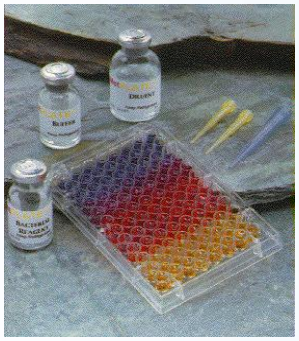
In vitro models

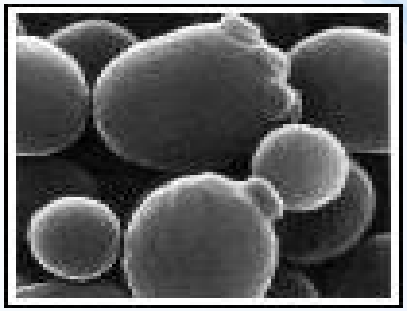


Original or genetically modified prokaryotic or eukaryotic cells

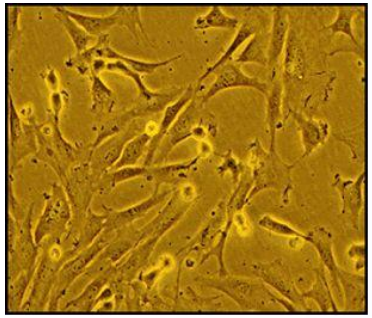
BACTERIAL, YEAST TESTS

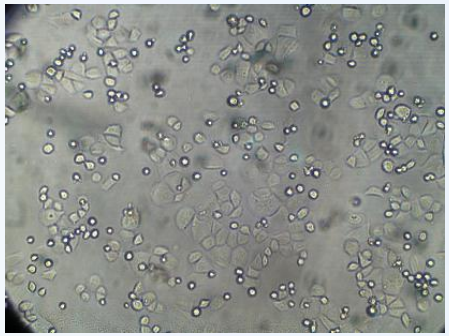






TESTS ON TISSUE CULTURE ï CELL LINES





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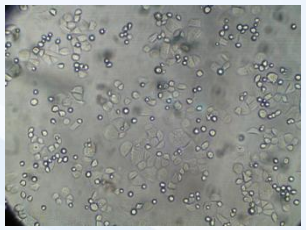
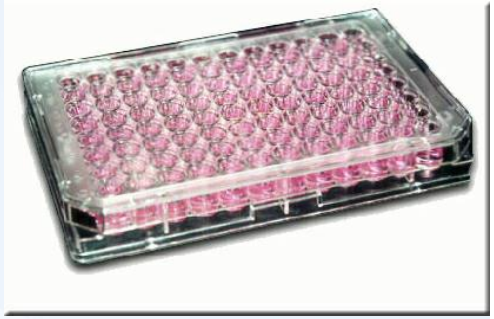
bassays

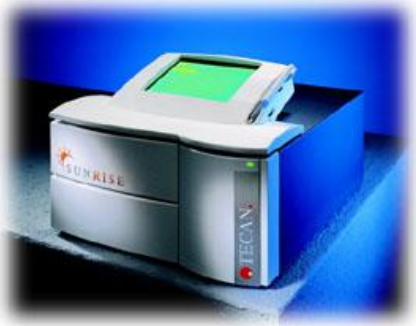




Principle

ÅMechanism of action based

ÅMechanism related to toxic effects



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

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Screening tests

Toxicity/genotoxicity

Toxicity:

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

Genotoxicity :

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

Contact test

- Flash test with *Vibrio Fisheri* ï kinetic test

Specific mode of action

Yeast models

- Fish/mammalian cell lines

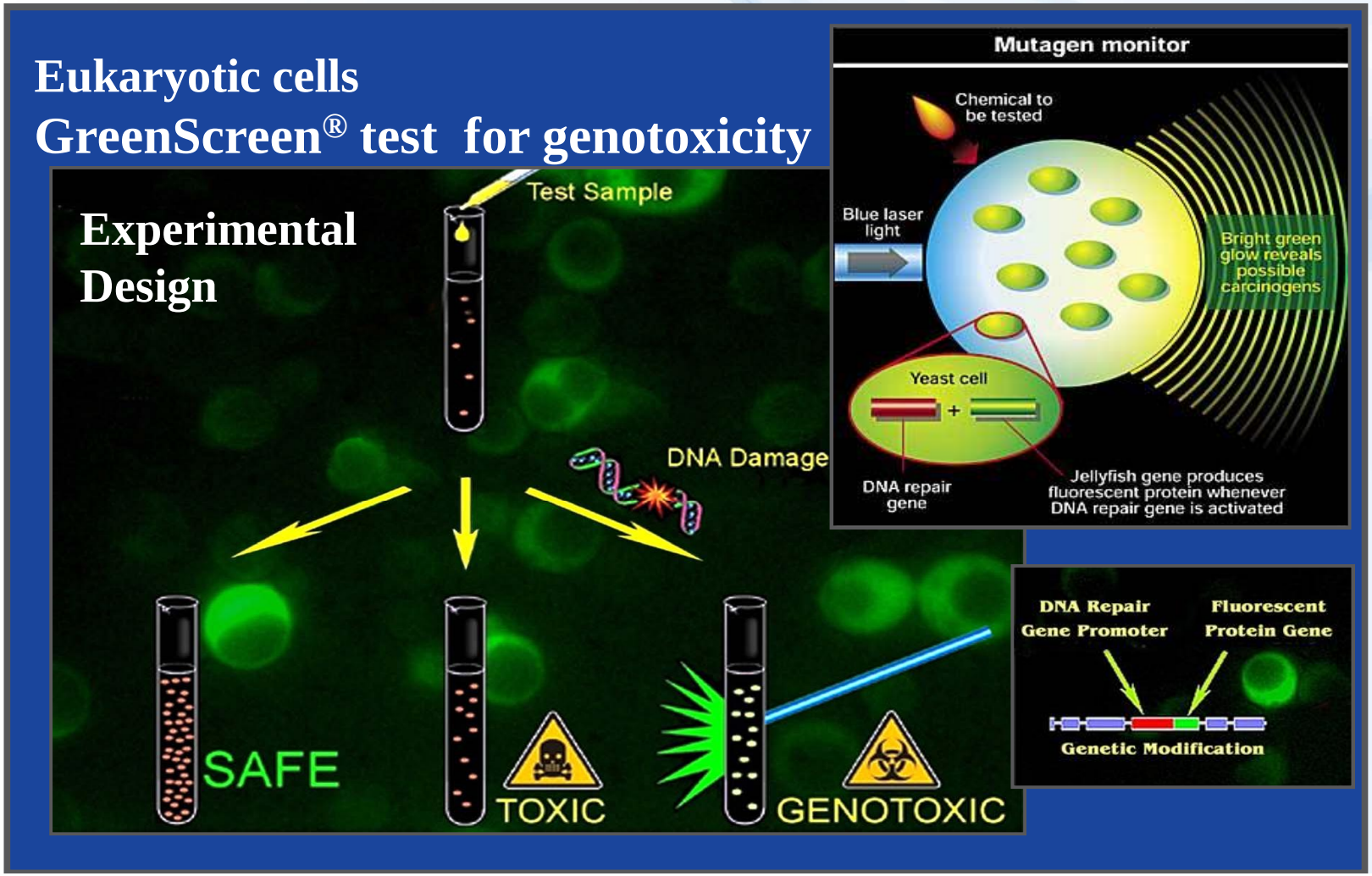
Tests for presence of compounds with hormone-like effects :

- Anti/estrogenicity
- Anti/androgenicity
- Retinoid-like activity
- Dioxin-like potency

for genotoxic effects

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

Bacterial or yeast assays with reporter genes



RECEPTOR MEDIATED EFFECTS

Ì important mechanisms involved in chronic toxicity

- **Dioxin-like activity: Aryl hydrocarbon receptor (AhR)-mediated effects**

  PCDDs/Fs, PAHs, PCBs

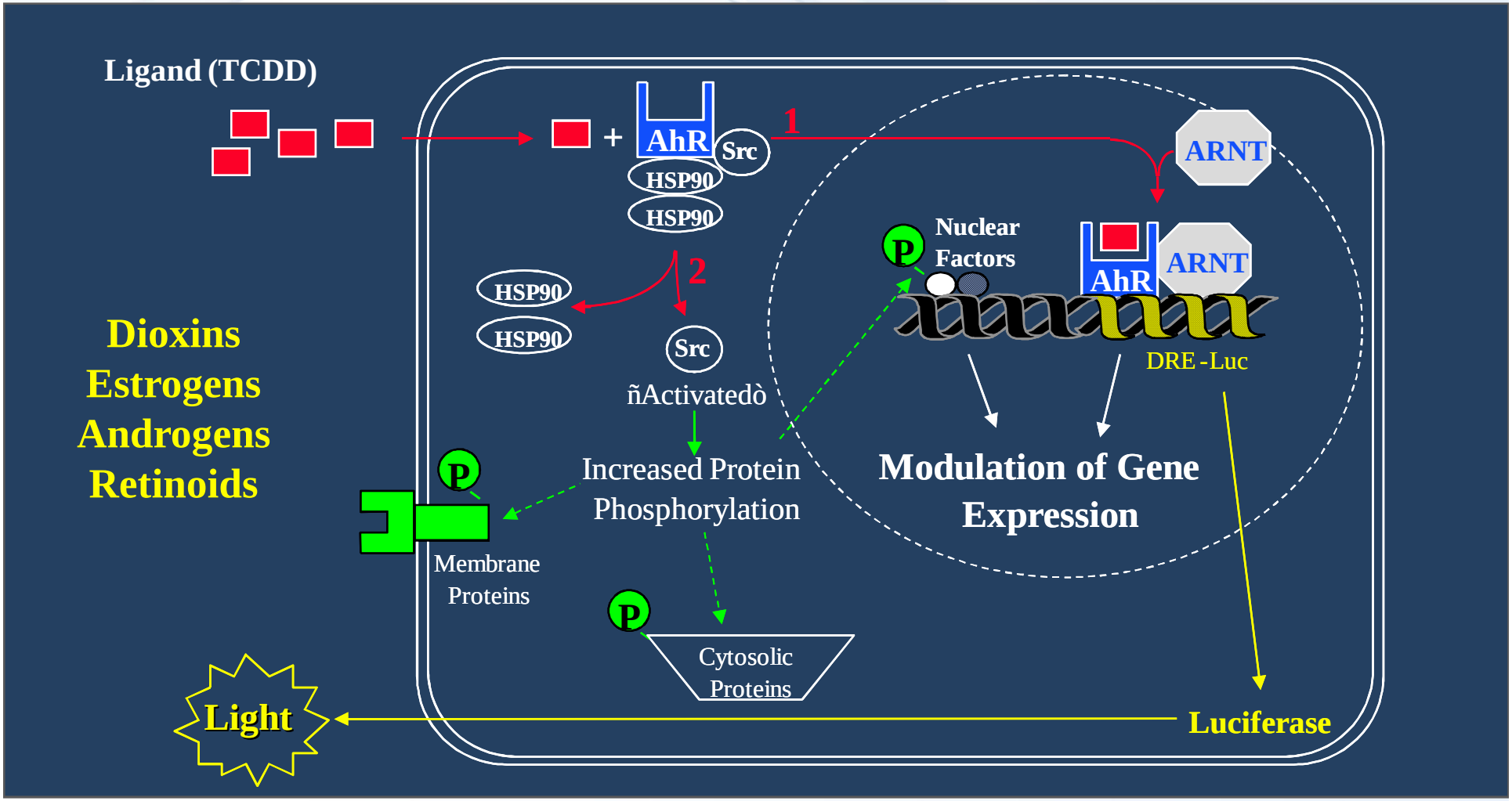
- **Xenoestrogenity / Antiestrogenity: Estrogen receptor (ER)-mediated effects**

  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)**

eceptor-mediated effects
uciferase reporter assays



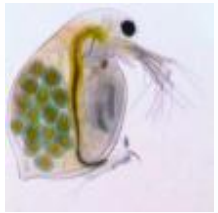
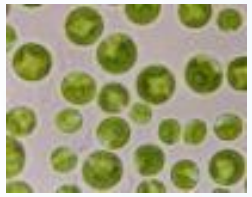
CLEAR RECEPTORS IN TOXICITY

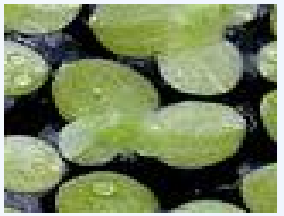
- Nuclear receptors (**AhR, ER, AR, RAR/RXR**) play an important role in toxic effects of many pollutants
  - DIOXIN-like toxicity
  - Anti / estro-, Anti / andro- é -genicity

- **Common mechanism - transcription factors:**
  - development of mechanistically based bioassays
- ***In vitro* luciferase reporter bioassays ï studies of é**
  - 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)

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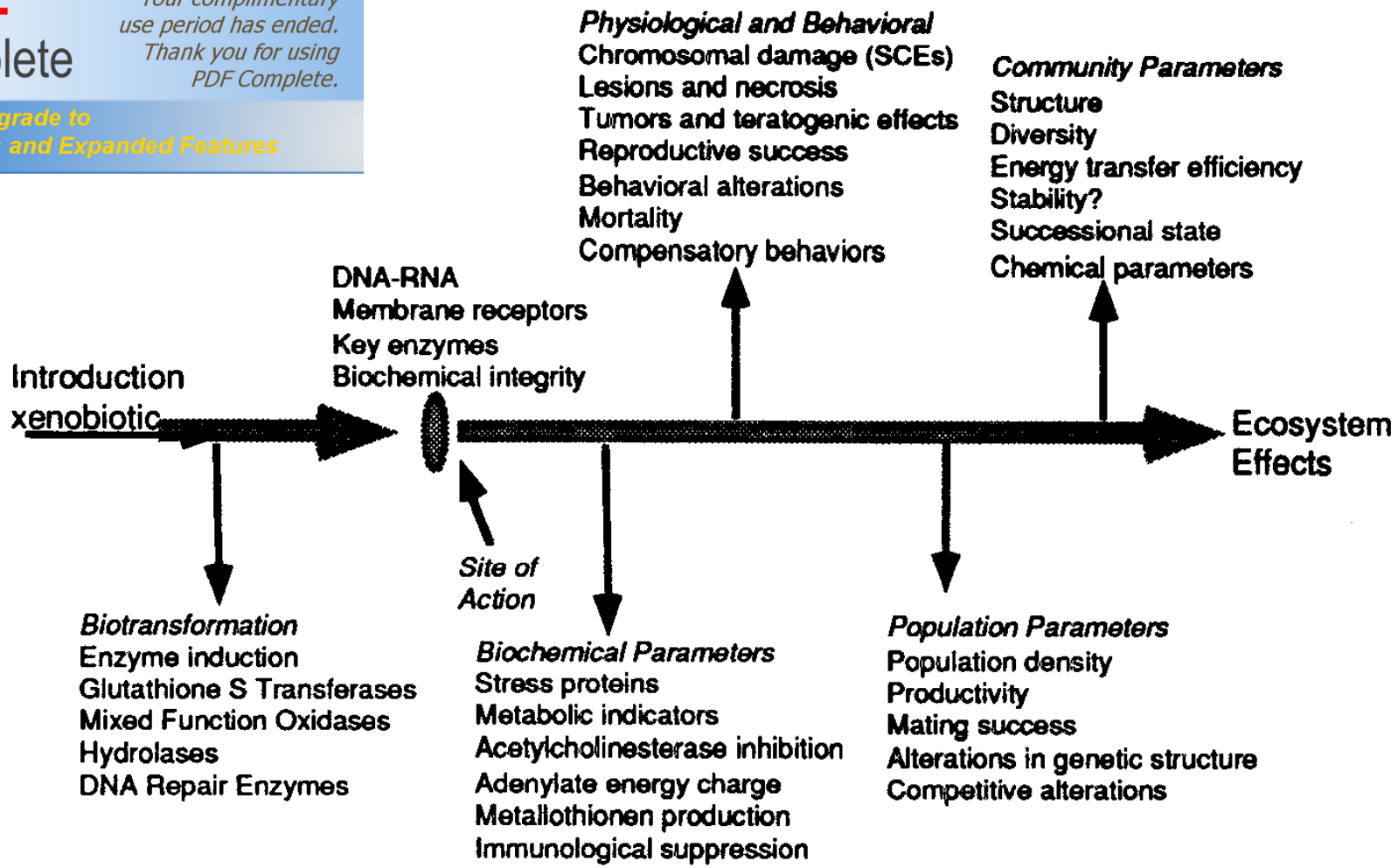


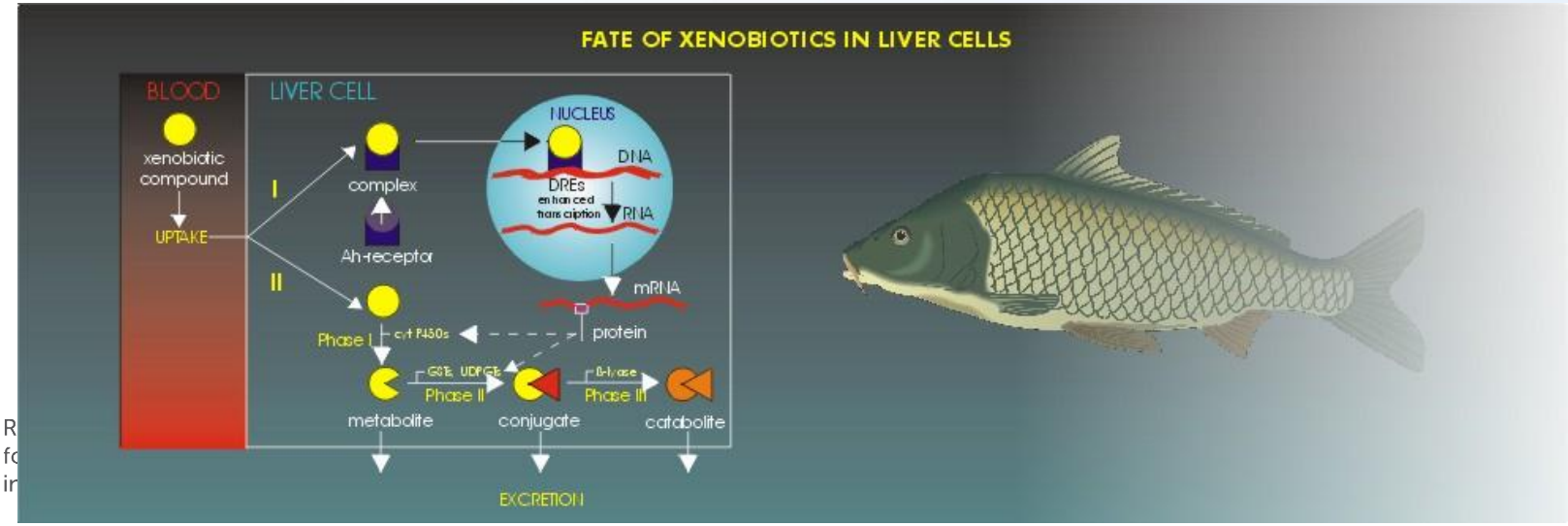
Figure 2.2 Parameters and indications of the interaction of a xenobiotic with the ecosystem. The examples listed are only a selection of the parameters that need to be understood for the explanation of the effects of a xenobiotic upon an ecosystem. However, biological systems appear to be organized within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

ormation enzymes (phase I&II)

Induction of detoxification enzymes in plants and animals

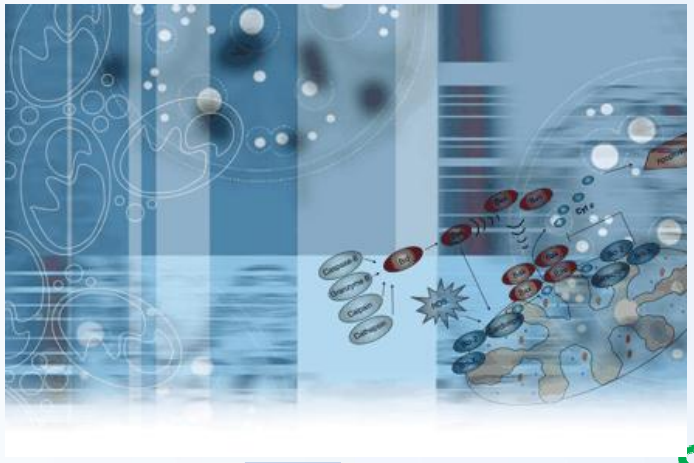
A. Enzymes of the 1st phase of biotransformation ï MFO enzymes (mixed function monooxygenases) ï induction of P450 cytochrome enzymes (EROD, MROD, PROD)

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



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

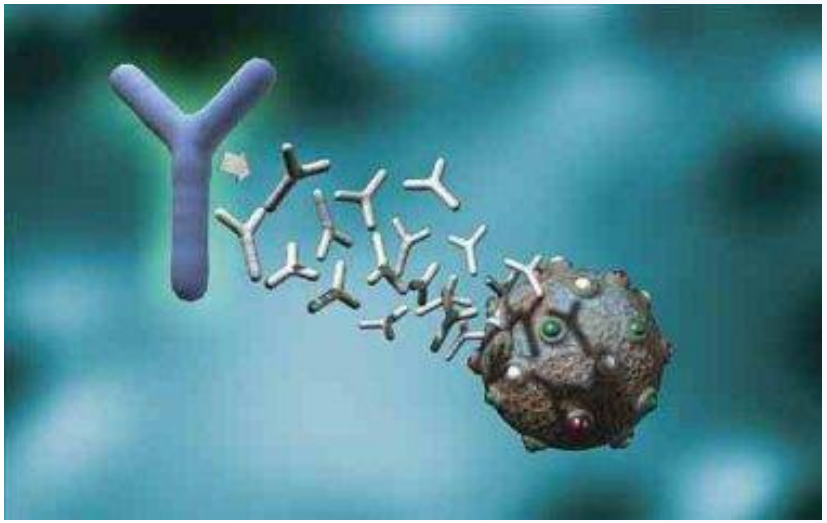


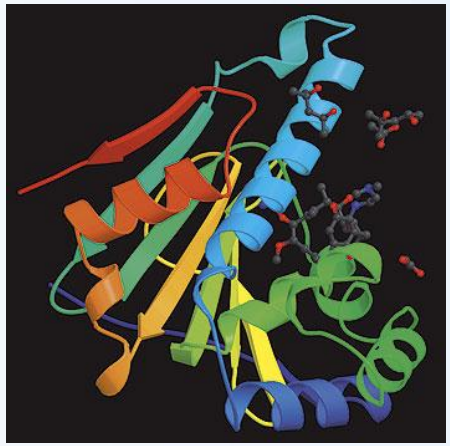
e proteins

ÅStress proteins: heat shock proteins (HSP), glucose-regulated proteins (GRP)

ÅMetallothioneins (MT): metal binding

ÅMulti Xenobiotic Resistance (MXR): excretion of xenobiotics; induction or inhibition by chemisensitizers

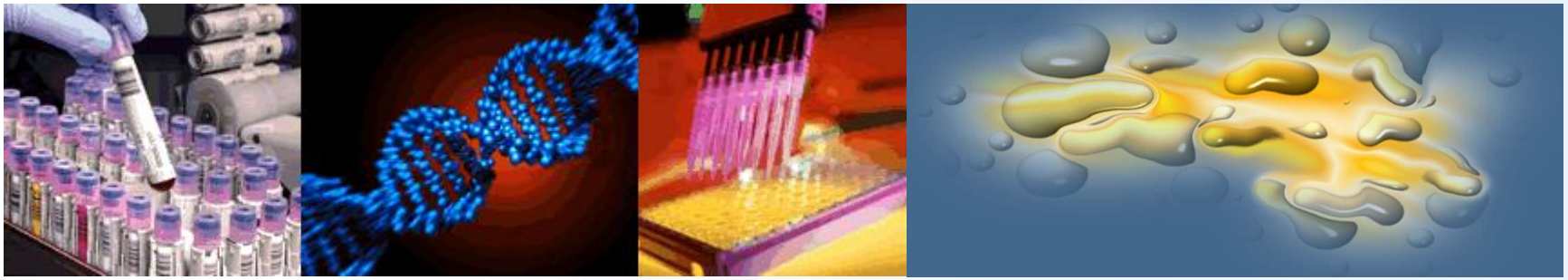




gical parameters

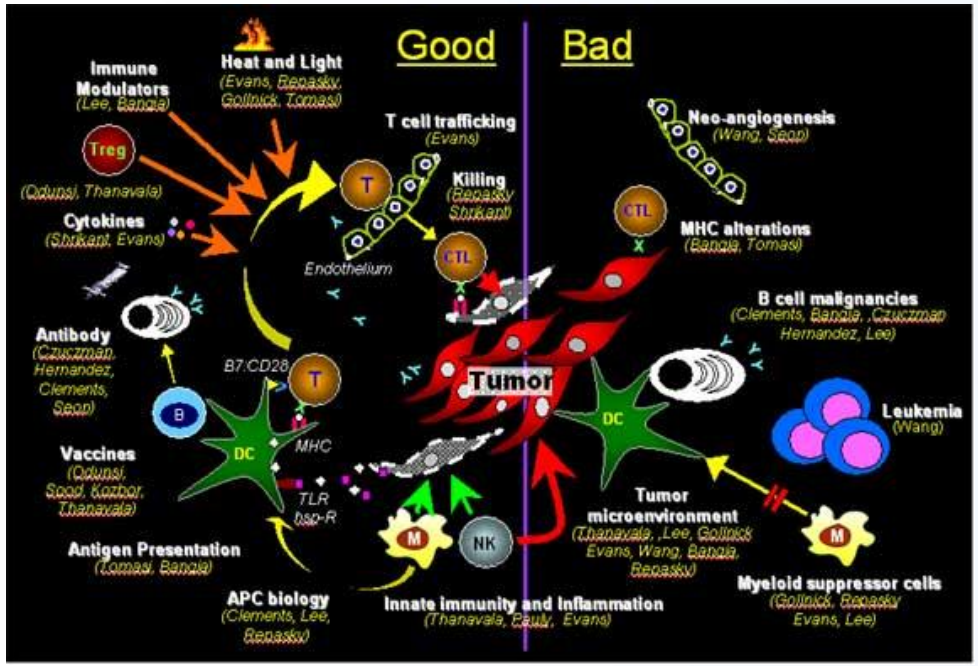
ÅSerum transaminases: Alanine transaminase (ALT), aspartate transaminase (AST); membrane disruption or organ damage

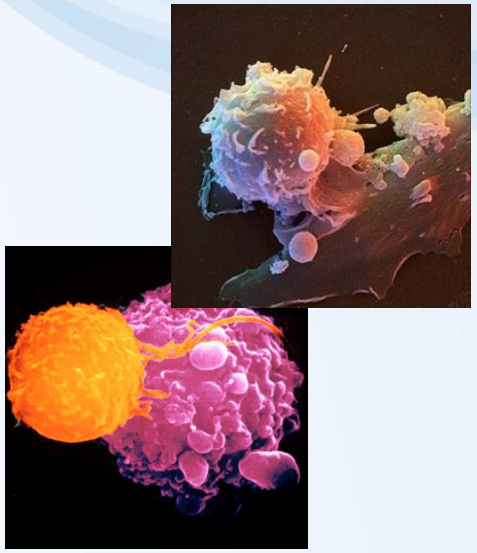
ÅBlood values: haematocrit, haemoglobin, blood sugars (glucose), plasma lipids and proteins (albumin)



ogical parameters

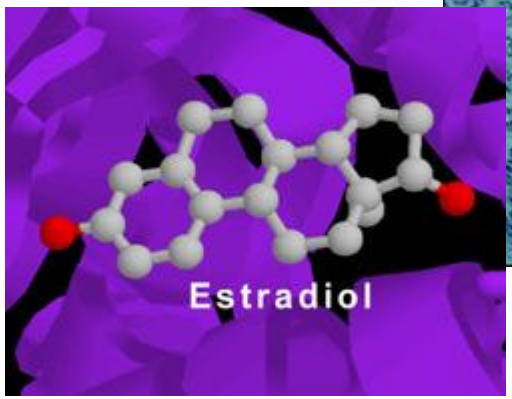
- White blood cell count
- Lymphocyte status
- Morphology of spleen, thymus and kidney
- Macrophage function
- Susceptibility to bacterial infections

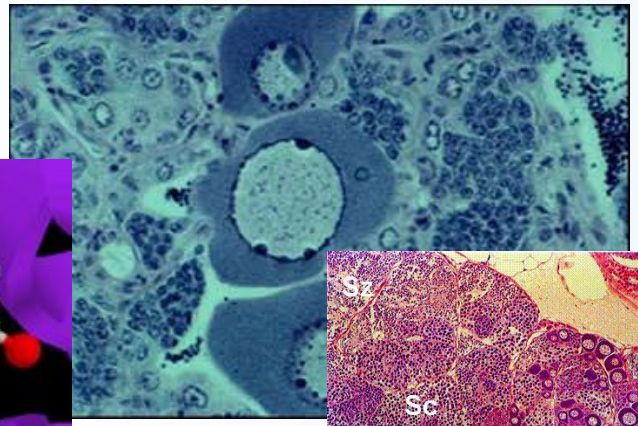




tive & endocrine parameters

ÅBiochemical: Fish vitellogenin (VTG), Zona radiata Protein (ZRP), Cytochrome aromatase, spiggin (stickleback)
ÅMorphology of gonads; sperm condition
ÅReproductive success (eggs, larvae)
ÅIntersex, Imposex





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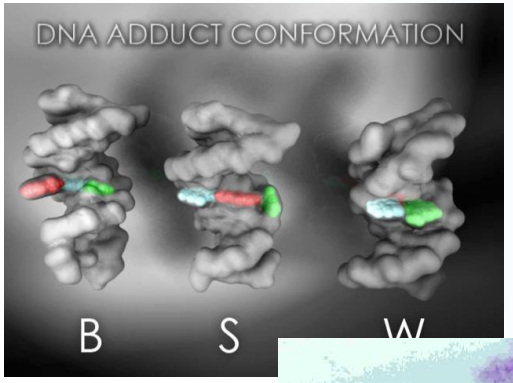
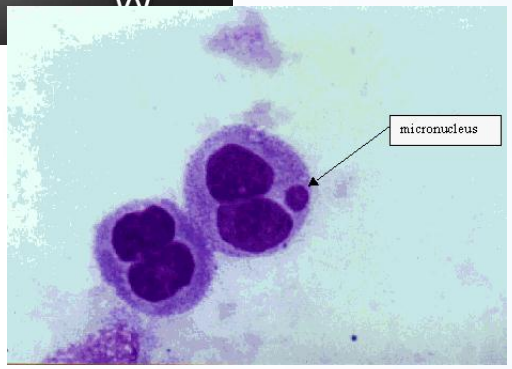
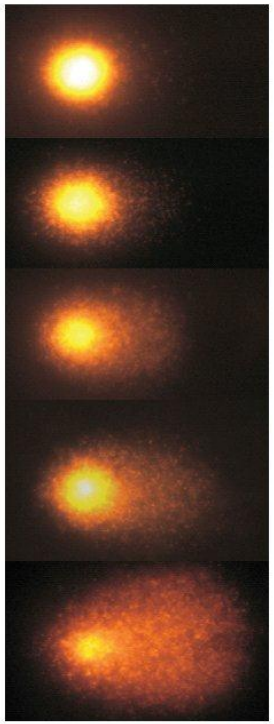
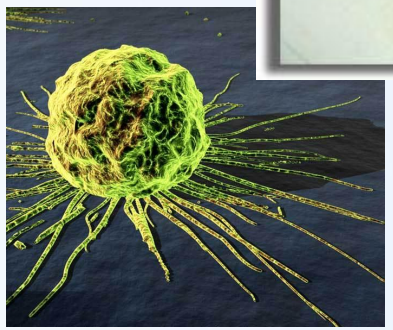
c parameters

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









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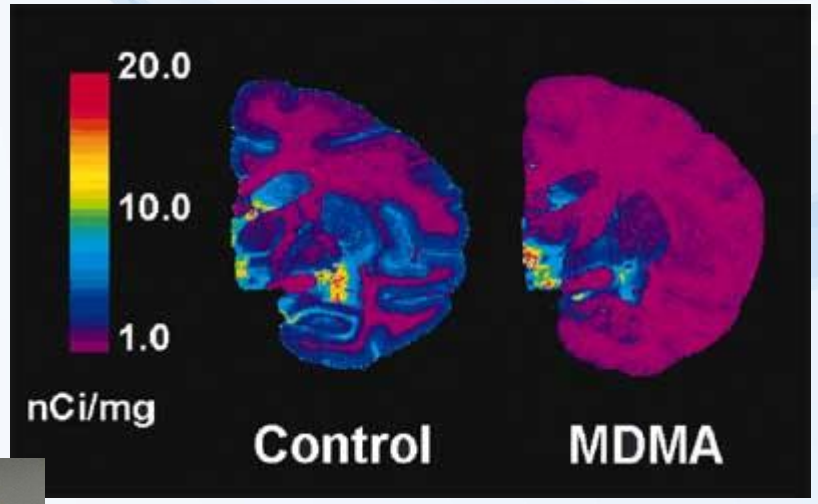
ic parameters

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



SUMMARY ï BIOASSAYS

BIOASSAYS are needed to test effects of é

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

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

Real ecotoxicologyñneeded

1) Use non-standardized organisms

- Laboratory - aquatic snails, chironomids, soil organisms é
- Natural ï 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? é)
- 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!