Analysis and Assessment of Contaminated Sediments

- Sampling techniques for sediments, pore water and suspended matter *(Andreas Müller)*
- Chemical analysis *(Werner Brack)*
- Ecotoxicological effect assessment *(Nadja Metzner)*
- Assessment of water and sediment quality using benthic invertebrates *(Jan von Baumbach, Klaus Swarowsky, Florian Burgis)*
- Sediment triad *(Julian Monka, Christian Löb)*
- Effect-directed analysis *(Werner Brack)*
Chemical Analysis
Many sediment assessment procedures are based on chemical analysis of sediment contaminants.

**Qualitative**: Are distinct chemicals present? or What chemicals can be found?

**Quantitative**: How much of the chemicals can be found?

**Target analysis**: Analysis of pre-selected individual chemicals. Typically quantitative.

**Screening analysis**: Analysis of all chemicals that are accessible with the selected method. Typically qualitative.
Compartments that are typically analysed:

**Sediment** (solid phase, total concentration)
**Pore water** (freely dissolved)
**Benthic organisms** (bioaccumulated)

**What chemicals should be monitored in sediments?**
Typical criteria: Persistence, bioaccumulation/adsorption, relevance for the water body, ecotoxicity
Monitor organic compounds with

\[ \log K_{\text{OW}} < 3 \] in water

\[ \log K_{\text{OW}} > 5 \] in sediments

For \( 3 < \log K_{\text{OW}} < 5 \) both compartments are relevant
European Water Framework Directive (WFD) defines 41 Priority Pollutants that need to be monitored in all European River Basins.

For 33 of these compounds sediments are the preferred or optional matrix. These include:

**Polycyclic aromatic hydrocarbons (PAHs)**
- benzo[a]pyrene (log $K_{OW}$ 6.04)
- benzo[ghi]perylen (log $K_{OW}$ 7.23)
- benzo[b]fluoranthene (log $K_{OW}$ 6.57)
- indeno[1,2,3-cd]pyrene (log $K_{OW}$ 7.66)
- benzo[k]fluoranthene (log $K_{OW}$ 6.84)
Chemical Analysis

Further PAHs
- Naphthalene (log K\text{OW} 3.3)
- Anthracene (log K\text{OW} 4.4)

Pesticides
- Alachlor (herbicide) (log K\text{OW} 3.5)
- Chlorfenvinphos (insecticide) (log K\text{OW} 5.13)
- Hexachlorocyclohexane (γ isomere lindane, insecticide) (log K\text{OW} 3.7)
- DDT (insecticide) (log K\text{OW} 6.19)
- Chlorpyrifos (insecticide) (log K\text{OW} 5.11)
- Endosulfan (insecticide) (log K\text{OW} 3.8)
**Chemical Analysis**

**Pesticides**
- Aldrin/ stereoisomer isodrin (insecticide) (log $K_{OW}$ 6.75)
- Dieldrin/ stereoisomer endrin (insecticide) (log $K_{OW}$ 6.2)
- Trifluralin (herbicide) (log $K_{OW}$ 5.3)
- Pentachlorophenol (pesticide, wood preservative) (log $K_{OW}$ 4)

**Other chemicals**
- Pentachlorobenzene (by-product) (log $K_{OW}$ 5)
- Hexachlorobutadiene (organic chemical industry) (log $K_{OW}$ 5.5)
- Chlorinated paraffines (mixtures, metal working fluids, paints, flame retardents…)
- Hexachlorobenzene (organic chemical industry) (log $K_{OW}$ 5)
Chemical Analysis

Other chemicals

- Polychlorinated dibenzodioxins and dibenzofurans
- Polybrominated diphenylethers (PBDEs, flame retardants)
- 2,4,6-Trichlorophenol
- Nonylphenol, octylphenol (mixtures of many isomers, surfactants and others)

Metals

- Cd, Pb, Hg, Ni
Typical procedure (organic analysis of solid phase)

1) Pre-treatment of sediments
   - (freeze) drying
   - sieving (typically < 63 µm to remove sand and coarse material)
   - homogenization
2) Extraction

Separation of organic chemicals from sediment matrix dissolving them in organic solvents. Efficiency enhanced by

- Repeated percolation of solvents through the sample (Soxhlet)
- Enhanced pressure and temperature (ASE)
- Ultrasonication
- Microwaves

.....
Accelerated solvent extraction (ASE)
3) Clean up

Organic extracts typically need to be cleaned before analysis.
⇒ Removal of matrix components
⇒ Removal of compounds not in the target of the analysis

- to preserve sensitive analytical instruments
- to allow a chromatographic isolation and detection
- to improve detection limits

For target analysis typically solid-liquid adsorption chromatography
3) Clean up

**Target analysis**: Typically solid-liquid adsorption chromatography is used

Different retention of target and non-target analytes/ matrix components

**Screening analysis**: To avoid losses of compounds typically only a clean up exploiting different molecular volumes of analytes and matrix components (humic compounds, lipids..)

⇒ Size exclusion chromatography, ultrafiltration...
4) Instrumental analysis (of organic compounds)

Two steps:
1) Separation (Chromatography)
2) Detection, quantification, identification

1) Separation tools in instrumental analysis

Gas-liquid chromatography (= gas chromatography)

Partitioning of analytes between a liquid stationary phase (thin film in a capillary) and a gas phase (carrier gas) under defined (and programmable) temperature and flow conditions.
Chemical Analysis

Liquid chromatography (in most cases “high performance liquid chromatography”, HPLC)

Partitioning between a solid stationary phase and a liquid mobile phase.
Liquid chromatography (in most cases “high performance liquid chromatography”, HPLC)

Discrimination normal-phase (NP) HPLC and reversed-phase (RP) HPLC

**NP-HPLC:**

Polar stationary phase and non-polar mobile phase (typically: alkanes, dichloromethane, toluene)

Typical stationary phases:

Today rarely used for analytical purposes
Chemical Analysis

Liquid chromatography (in most cases “high performance liquid chromatography”, HPLC)

**RP-HPLC:**
Non-polar stationary phase (typically silica capped with C18, C8 or phenyl and polar mobile phase (typically: water and methanol or acetonitril)
Often gradients with increasing share of organic solvents
Separation according to hydrophobicity ($\log K_{OW}$)
The more hydrophobic compounds are, the more they partition into the stationary phase and the later they elute.
2) Detection

**GC detectors**

1) flame ionization detector (FID)

Sensitive and selective for easily flammable analytes such as hydrocarbons

Analytes are pyrolyzed in a small hydrogen (C)-air (D) flame

Production of positively charged ions and electrons

Analytes travel up to the nozzle head (E) with positive voltage repelling the ions to a collector plate (G)
Chemical Analysis

GC detectors

2) electron capture detector (ECD)

Sensitive and selective for electron-absorbing components
Radioactive β-particle (electron) emitter e.g. $^{63}$Ni
Flow to a positively charged electrode (anode) ⇒ steady current
Analyte molecules capture electrons and reduce this current
Analyte concentration proportional to degree of current reduction

Sensitive to halogens, organometallic and nitro compounds
Chemical Analysis

GC detectors

3) thermoionic detector, Nitrogen/Phosphorus Detector (NPD)

Sensitive and selective for N and P containing compounds
Thermic energy to produce ions
Thermoionic bead coated with an alkali metal Li, Na, K promotes ionization
Hydrogen and air flows create hydrogen plasma
Catalytic surface chemistry reaction producing thermoionic electrons
Attracted to a positively charged collector electrode
GC/HPLC detectors

4) Mass spectrometer

today most often applied detection techniques in GC and HPLC provides additional information on molecular weight and structure (mass spectra)
⇒helps to identify compounds
⇒selective detection and quantification of compounds with defined molecular weights.

Many types of MS but same basic composition:
1) Ion source: Analytes are ionized
2) Analyzer: Separation of ions according to their mass/charge ratio
3) Detector: Detection of ions
Ion sources/Ionization techniques

1) Electron impact (EI)

Ionization with of electrons with high energy (in most cases of 70 eV = 5000 km/s).
Primary ions are energy rich and of low stability
⇒ Fragmentation in a reproducible and partly predictable way.

**Advantage**: mass spectra

**Disadvantage**: sometimes no molecular ions (molecular weight)

Only applicable to compounds that can be evaporated (GC/MS)
Mass spectrum after EI ionization: Fingerprint of the compound

2,3,7,8-Tetrachloridibenzofuran
Ion sources/Ionization techniques

2) Chemical Ionization (CI)

Introduction of a reagent gas that is ionized by EI (⇒ positively charged ions) and reacts with analytes by ⇒ protonation or ⇒ charge transfer forming ions.

Typical protonating reagent gases: H₂, CH₄, H₂O, CH₃OH, NH₃
Reagent gases for charge transfer: C₆H₆, Xe, CO₂, CO, N₂, Ar, He
Requires evaporated analyte molecules (GC/MS)
Ion sources/Ionization techniques

2) Chemical Ionization (CI)

Advantages:
Detection of molecular ions (no fragmentation)
Targeted ionization of substance classes by selection of reagent gas
(e.g. with NH3 only basic compounds are ionized)
Chemical Analysis

Ion sources/Ionization techniques

3) Electrospray Ionization (ESI)

Typically for LC/MS and ionic and polar compounds
Mobile phase with analytes is forced through a very small, charged capillary.
Aerosols are formed, the mobile phase is evaporated and ions (repelling each other) break up the droplet.
Ion sources/Ionization techniques

4) Atmospheric Pressure Chemical Ionization (APCI)

Typically for LC/MS
Mobile phase is sprayed at high temperature and ionized by a corona discharge electrode in a plasma.

Then gas phase ionization of analytes by reaction with the ionized solvent molecules.
Less soft ionization than ESI ⇒ more fragmentation.
Ion sources/Ionization techniques

5) Atmospheric Pressure Photoionization (APPI)

typically for LC/MS
Evaporation of analytes and subsequent ionization with photons
Good method for aromatic compounds
**Analyzers**

1) **Quadrupole MS**

Ions are accelerated by a static electric field and selected in a quadrupole field of four electrodes.
Only ions with a defined m/z ratio reach the detector.
Defined by the voltage in the electrodes

2) **Time-of-Flight MS**

Since all ions have the same energy when entering the analyzer heavy ions are slower and reach the detector later than lighter ones ⇒ separation.
High resolution.
Chemical analysis is able to detect and quantify even traces of pre-selected compounds. However, it does not provide information on overall hazards of a sediment because:

- sediments contain thousands of chemicals and only few of them can be analyzed
- are toxicologically characterized
- it normally does not consider bioavailability of the compounds
- it cannot consider mixture effects

Thus: **Methods are required to directly measure effects of complex environmental samples such as sediments on biota**

⇒ Biotests
Biotesting

Characterization of biotests according to

the **phases** that are analyzed:
- pore water, aqueous elutrients
- whole sediments
- organic extracts

the **level of biological complexity** that is used:
- *in vitro*
- *in vivo*
- *community tests*

the **trophic level** that is considered
Biotesting

Test phases
pore water, aqueous elutrients

basic idea: pore water as major route of exposure

advantages:
- classical biotest procedures, test organisms and endpoints developed for whole effluent testing can be applied.
- only little sample manipulation
- bioavailability is considered (correctly?)

disadvantages:
- ignores other routes of exposure such as ingestion of sediments or direct contact
- losses due to uptake in organisms, adsorption to test vessels, degradation… are not balanced by delivery from sediments
- low O₂, NH₃, H₂S may mask toxicity of chemicals
Test phases

whole sediments

basic idea: attempt to consider all routes of exposure in contaminated sediments in an organism specific way

advantages:

- realistic exposure scenario
- no sample manipulation
- relevance for *in situ* situation

disadvantages:

- no rapid, high throughput test systems
- difficult to quantify exposure and to establish concentration-response relationships
- problems with confounding factors such as O₂ depletion
- limited possibilities to identify causes of effects
- many benthic test organisms used to low oxygen contents are also tolerant to toxicants
Biotesting

Test phases
whole sediments

Typical test systems (invertebrates):

**Hyalella azteca**
endpoints: mortality, growth, development, reproduction

**Lumbriculus variegatus**

**Chironomus**
Example: nematode assay

Rose bengale (free acid)

Caenorhabditis elegans (eingefärbt)

Biotesting

Vorgehensweise Nematodentest

- 10 Juvenile
- kolloidales SO₂ in Wasser (Dichte 1,14 g/cm³)
- Inhalation 96 h bei 20°C
- Extraktion über Dichte
- Auswertung

0,5 g Sediment
0,5 ml E. coli Suspension

Juvenile Würmer: Anzahl
Adulter Würmer: Länge, Eibildung, Eizahl

Bild: Gerhard Kotschik
Detection of endocrine disruption in a sediment contact test with the mudsnail *Potamopyrgus antipodarium*

- exposure of snails to sediments for two weeks
- open the shell and count the embryos
- enhanced numbers of embryos indicate the presence of estrogenic compounds

Example: sediments from three different rivers
Biotesting

Test phases
whole sediments

Typical test systems (plants):

- *Hydrilla verticillata*
- *Lemna minor*

Toxicological endpoints:
- mortality
- growth measured via biomass, chlorophyll content, number of roots...
Biotesting

Test phases

whole sediments

Typical test systems (vertebrates):

Sediment contact test with fish eggs (development of embryos of *Danio rerio*)

- Exposure for 48 hours
- Aeration to prevent oxygen depletion

Microscopic analysis for:
- developmental retardation
- oedemas
- lack of pigmentation
- missing heart beat
- detachment of the tail
- formation of somites (Segmentierung)
- deformities
- early hatching
- coagulation
Development of *Danio rerio* embryos

**Biotesting**

- Day 1
- Day 2
- Day 3
- Day 4
Biotesting

Test phases

sediment extracts

basic idea: dosing of the full amount of organic toxicants present in a sediment

advantages:

- no interference of confounding factors
- good compatibility with fractionation and chemical analysis
- classical aqueous phase biotests can be used
- compatible with in vitro assays
- worst case scenario in agreement with precautionary principle

disadvantages:

- no reflection of real exposure conditions
- no consideration of bioavailability
- overestimation of hazards due to particle bound lipophilic compounds
Biotesting

Level of biological complexity

*in vitro* assays "im Glas" (today mostly in microtiter plates)

- biological processes run outside a living organism
- assays on a cellular or even molecular level
- help to understand mechanisms and to detect and discriminate specific effects rather than observing black box effects like mortality, growth…
- however, does not reflect a real organism with repair systems and physiological interactions \(\Rightarrow\) relevance for hazards needs to be proved
Level of biological complexity

in vitro assays
toxicological endpoints useful for sediment assessment:
cytotoxicity damage and death of cells
neutral red uptake

Vital cells take up the dye neutral red into their lysosomes (organelles containing digestive enzymes (pH4.5))
In damaged or death cells the ability to take up and bind neutral red is reduced e.g. by damage of lysosome membranes
Amount of bound neutral red discriminates vital, damaged and dead cells.

\[
\text{3-amino-7-dimethylamino-2-methylphenazine}
\]
\[pK_s \approx 5.89\]

Uptake of neutral form by diffusion \(\Rightarrow\) trapping in protonized form in lysosomes
Biotesting

_in vitro_ assays

neurotoxicity

Inhibition of acetylcholinesterase

Acetylcholinesterase hydrolyses the neurotransmitter acetylcholin in the central nervous system
Inhibition e.g. by organophosphate insecticides like parathion
Molecular enzyme inhibition test

Ah-receptor mediated activity
PolychloAh receptor binding mediates the induction of cytochrome P450 (CY
in vitro assays

genotoxicity
Induction of DNA repair mechanisms

SOS Chromotest

*Escherichia coli* (K12 PQ 37) with a β-galactosidase gen (*lac Z*) fused with a gene of the SOS repair system
⇒ Induction of SOS repair system after exposure to genotoxic compounds
⇒ Induction of β-galactosidase
⇒ determination of β-galactosidase activity using a chromogenic substrate
⇒ photometric measurement

umu test

similar principle as SOS-Chromotest
other bacteria: *Salmonella typhimurium* (TA1535/pSK 1002)
Biotesting

**Ames test**

*Salmonella typhimurium* (different strains) with mutations in genes involved in histidine synthesis ⇒ unable to synthesize histidine ⇒ do not grow in media without histidine

Mutagens may cause a reversion of this mutation ⇒ number of colonies growing on histidine-free media as a measure for mutagenicity

Different strains for diagnostic purposes (different metabolic activity)

Addition of rat liver (S9) homogenate to simulate metabolic activity in mammals

With sediment extracts often problems with cytotoxicity or other processes masking mutagenicity

⇒ clean up and fractionation

Procaryotic cells are no perfect models for humans ⇒ alternatives e.g. human breast cancer cell lines
in vitro assays

Ah-receptor mediated activity

Polychlorinated dibenzo-\(p\)-dioxins and furans (PCDD/F) and other halogenated aromatic hydrocarbons (PCBs, PCNs) bind to the Arylhydrocarbon (Ah) receptor.

Binding mediates the induction of cytochrome P450 (CYP1A) → ethoxyresorufin-O-deethylase (EROD) but is also closely related to dioxin-like effects in mammals

⇒ **EROD induction** in rat liver or rainbow trout liver cells as a tool for estimating the exposure to dioxin-like compounds and other AhR binding compounds (e.g. PAHs)

\[
\text{ethoxyresorufin} + 2\text{NADPH} + \text{O}_2 \rightarrow \text{resorufin} + \text{H}_3\text{C}-\text{CHO} + 2\text{NADP}^+ + \text{H}_2\text{O}
\]
in vitro assays

Ah-receptor mediated activity

**DR CALUX**: Alternative tool to detect Ah-receptor-binding toxicants
*(Chemical Activated Luciferase gene expression)*

Rat hepatoma cell line stably transfected with luciferase reporter gene
⇒ light emission related to Ah-receptor binding

Estrogenic endocrine disruption

**ER CALUX**
⇒ light emission related to estrogen receptor binding (similar to DR CALUX)

**YES (Yeast Estrogen Screen)**

Human estrogen receptor integrated in yeast genome
β-Galactosidase (Lac Z) gene is expressed as a result of receptor binding
Measurement of enzyme activity
Biotesting

*in vitro* assays

Androgenic endocrine disruption

**AR CALUX**

**YAS (Yeast Androgen Screen)**

similar to ER CALUX and YES but with androgen receptor
Effect-Directed Analysis (EDA) of Sediments
Key Stressors

Driving forces and pressures that prevent the achievement of good status.

Providing evidence on cause-effect relationships is a prerequisite for efficient river basin management.

Driving forces

- sources of contamination
- insufficient ecological status
- monitoring programmes
- river basin management
- chemical quality
- hydromorphological quality

Effect-Directed Analysis - Motivation

Key Stressors

Driving forces and pressures that prevent the achievement of good status.

Providing evidence on cause-effect relationships is a prerequisite for efficient river basin management.
Effect-Directed Analysis - Motivation

Evaluation of the ecological status in European surface waters – Example Germany (WFD Scoreboard 2004/2005)

For 60 % of all German surface waters an achievement of good ecological status is unlikely!

Why? Chemicals? Which chemicals?
A priori selected target compounds:

- Tiny portion of possible compounds (>16 million)
- Often no explanation of observable effects

Chemical analysis of all compounds impossible and not helpful (no data on effects)

⇒ Chemical analysis needs to be directed on those compounds with adverse effects

⇒ Concept of „Effect-directed analysis“ combines biotesting, fractionation and chemical analysis

**16 mio known chemicals**

**thousands of compounds in real world samples**

**33 priority pollutants (WFD)**
Effect-Directed Analysis - Approach

Complex environmental sample

Effect-directed analysis

Toxicant

effect

Cause-effect-relationships

Cause

Effect
Effect-Directed Analysis - Approach

- **Step:** complex env. sample
- **Aim:** identification of tox. groups

**Toxicity Characterisation**
- Volatiles
- NH₃
- Lipophilic comp.
- Metals

**Toxicity Identification**
- Fractionation
- Biological analysis

**Toxicity Confirmation**
- Analytical confirmation
- Effect confirmation
- Hazard confirmation

- Confirmation of cause-effect relationships
Toxicity Characterisation

Characterisation of toxicants responsible for effects (TIE, US-EPA)

Starting point: Pore water or bulk sediment

Basic techniques: Biotesting + Indirect fractionation
Selective removal of distinct compound groups and subsequent biotesting
Toxicity Characterisation

Characterisation of toxicants responsible for effects (TIE, US-EPA)

Indirect fractionation
Selective removal of distinct compound groups and subsequent biotesting

-Pore water samples

-Stripping of volatiles at different pH
- EDTA and thiosulfate complexation of metals
- pH-manipulation → impact on NH$_3$
- Solid phase extraction (at different pH) to remove organic chemicals
Toxicity Characterisation

Characterisation of toxicants responsible for effects

Pore water samples

Examples for interpretation:

- Solid phase extraction (SPE) removes toxicity. Recovery of toxicity in eluate 
  $\Rightarrow$ lipophilic organic compounds
- Stripping, filtration, SPE, EDTA have no impact on toxicity 
  $\Rightarrow$ salt
- Filtration, stripping, SPE reduce toxicity but not EDTA 
  $\Rightarrow$ surfactants
- EDTA and thiosulfate reduce toxicity 
  $\Rightarrow$ metals
Characterisation of toxicants responsible for effects

Sediment samples

- Mixing with selective adsorbents in order to remove distinct groups of toxicants

Examples

- zeolite to remove ammonia
- resins or activated carbon to remove organic toxicants
- ion exchange resins to remove metals

- 100% selectivity cannot be achieved, e.g. zeolite also removes portions of organic compounds, ion exchange resins also affect ammonia concentration
Toxicity Characterisation

Sediment sample

Example: sediment spiked with several organic toxicants and ammonia

Biotesting with fish embryos (*Danio rerio*)

<table>
<thead>
<tr>
<th>organic toxicants</th>
<th>ammonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>73.3</td>
</tr>
<tr>
<td>spiked sediment</td>
<td>63.6</td>
</tr>
<tr>
<td>zeolite</td>
<td>71.8</td>
</tr>
<tr>
<td>adsorption resin</td>
<td>67.9</td>
</tr>
<tr>
<td>ion exchange resin</td>
<td>26.3</td>
</tr>
</tbody>
</table>
In-situ toxicity characterisation

Pore water is pumped in situ through different sorbents and a chamber with organisms (e.g. Daphnia magna)

Burton et al. (2004) ET&C 23
Effect-directed analysis of organic toxicants

Starting point: extract

Toxicity Identification

complex mixture

fractionation

biological analysis

chemical analysis

confirmation

toxicant
Toxicity Identification

Extraction:
- Separation of toxicants from the matrix (water, soil, sediment ...)
- Facilitates or enables concentration, biotesting, separation, analysis
- Disadvantages: Alteration of the exposure situation
  ⇒ alteration of bioavailability,
  ⇒ possible transformation, losses or input of toxicants by
  - altered redox conditions
  - loss by evaporation or adsorption
  - contamination from laboratory glass ware
Extraction:

Approaches for soils and sediments:

1) **No extraction.** In stead: biotesting and fractionation of original samples (bulk sediment testing, specific adsorbents → toxicity characterisation)

2) **Use of pore water or aqueous elutriates.** (pore water as main route of exposure. However, often underestimation of toxicity since
- other exposure routes are ignored (e.g. ingestion),
- losses due to adsorption, evaporation, uptake into the organism without further delivery by the sediment

3) **Mild extraction procedures focusing on bioaccessible fractions**
   Example: TENAX extraction
Toxicity Identification

Extraction of rapidly desorbing fraction with TENAX

4) Exhaustive extraction with organic solvents

- Attempt to extract the total amount of organic compounds.
- Not related to actual bioavailability.
- Worst case approach

Highest extraction efficiency:
Accelerated solvent extraction (ASE)
At enhanced temperature and pressure
5) Fractionated extraction with solvents of different polarity
e.g. water at different temperature
Increasing temperature ⇒
Decreasing polarity

⇒ Temperature modification allows fractionated extraction with only one solvent (H₂O)

Kubatova et al. (2004) ET&C 23
Effect-directed analysis of organic toxicants

Starting point: extract

Toxicity Identification

complex mixture

biological analysis

chemical analysis

fractionation

biological analysis

confirmation

toxicant
Toxicity Identification

What is toxic?

“All Ding’ sind Gift und nichts ohn’ Gift; allein die Dosis macht, dass ein Ding kein Gift ist.“

⇒ No use to look for toxic compounds in general
- All compounds exhibit (baseline) toxicity according to their lipophilicity
- If many (all) components of a mixture contribute to toxicity to a similar degree, EDA does not work

⇒ EDA looks for a limited number of dominant toxicants because of –specific/excess toxicity or –high concentrations
Effect:

-all compounds at least baseline toxic according to their membrane-water partition coefficient (often estimated from log $K_{ow}$)

-Most components in environmental samples are baseline toxic

- Some compounds much more toxic (excess toxicity) ⇒ specific toxicity

Example: Tox. to Daphnia
Toxicity Identification

*in vivo*:

analysis of effects on cells or organisms representing natural biological systems

**primary producers:**

*Scenedesmus obliquus*

**destruents:**

*Lemna minor*

*Vibrio fischeri*
in vivo:

customers:

Caenorhabditis elegans

Daphnia magna

Danio rerio
EDA may be not only based on biotests with whole organisms but often specific *in vitro* assays are used. Examples:

- genotoxicity/mutagenicity
- dioxin-like effects
- tumor promotion
- estrogenic/androgenic effects
- neurotoxicity
- photosynthesis inhibition
Toxicity Identification

-Selection of test organism and toxicological endpoint is crucial for EDA
-There is no toxicity as such (always interaction between a specific organism and a specific chemical at a distinct dose)
-Comprehensive picture only with test battery

Example: Bitterfeld sediments
Toxicity Identification

Sediment extract
Bitterfeld:
Primary fractions (Alumina)

Secondary fractions
(NP-HPLC)

Chemical identification
identified fractions and key toxicants

F3.4
N-phenyl-2-naphthylamine
reactive toxicant

F4
prometryne
herbicide, inhibitor of photosynthesis

F3.6
methyl parathion
insecticide
Effect-directed analysis of organic toxicants

Starting point: extract

Toxicity Identification

complex mixture

fractionation

biological analysis

chemical analysis

confirmation

toxicant
**Aim:** Reduce complexity of a mixture by separation of different components

⇒ Several fractions with lower complexity (less compounds)

**Wanted:**
- Low overlap between fractions (high resolution)
- High recovery of compounds in fractions
- High throughput (many samples/time) and high capacity (much sample)
- Conclusion on physico-chemical properties from fractionation results → helps to identify compounds
Aproaches: distillation, filtration, centrifugation, membrane diffusion, chromatographic approaches

Basic principle: Partitioning of analytes between a mobile phase (liquid or gas) and a stationary phase (liquid or solid) depending on the physico-chemical properties of the analyte, and both phases.
Preparative capillary gas chromatography (pcGC)

Thin-layer chromatography

HPLC: High performance liquid chromatography

Liquid chromatography
Liquid Chromatography

Types:
- Reversed phase chromatography (RP, polar mobile phase, non-polar stationary phase)
- Normal phase chromatography (NP, non-polar mobile phase, polar stationary phase)
- Size exclusion chromatography (SEC)
RP-LC:

Partitioning between lipophilic stationary phase (e.g. C18) and aqueous mobile phase. Often **gradient** with increasing ratios of miscible organic solvents (methanol, acetonitril) for elution of lipophilic compounds,

First approximation: Separation according to **lipophilicity** represented by log $K_{OW}$
Ocurrence of toxicity in a specific RP-LC fraction gives information on the lipophilicity of causing compounds. Models available to predict log $K_{OW}$ from structure. Allows plausibility check for tentatively identified compounds!

However: Silica-C18 is no octanol
Deviations may occur (e.g. interactions of analyte functional groups – free silica groups)

Alternative: consider specific interactions
Example: Abraham equation

$$\log k' = c + nR_2 + s\pi^H_2 + a\sum a^H_2 + b\sum b_2 + \nu V_x$$

dipolarity/polarizability
hydrogen-bond basicity
refraction (polarizability)
hydrogen-bond acidity
volume (cavity formation)

Problem: Descriptors available for few compounds. General models to predict from structure are work in progress.
NP-LC:
Partitioning between polar stationary and non-polar mobile phase

Primary: Separation according to interactions between polar functional groups (amino, nitro, hydroxy, keto…..) and functional groups of the stationary phase.

Frequently used phases:
Electron-donor-acceptor interactions for separation of aromatic compounds

Example: Electron-acceptor: nitrophenyl phase

- Electron-deficiency in aromatic ring because of negative inductive (-I) and mesomeric (-M) effect of the nitro group.
- Interaction with electron-rich planar aromatic molecules
- Increasing retention of PAHs with increasing numbers of aromatic carbons
Example: Electron-acceptor: nitrophenyl phase
Example: Electron-donor: pyrenyl phase (PYE)

- Electron-rich $\pi$-electron system
- Ideal for separation of planar aromatic compounds with electron deficiency (e.g. halogens (-I effect))
- **Examples**: polychlorinated naphthalenes (PCNs), biphenyls (PCBs), dibenzo-p-dioxins and furans (PCDD/Fs)
- Increasing retention with increasing halogenation and planarity
Example: Electron-donor: pyrenyl phase

Liquid Chromatography (NP)

Helps to separate dioxin-like highly toxic planar PCBs with four and more Cl-atoms, PCDD/Fs and PCNs from much less toxic bulk compound.

retention time
- Separation according to molecular size
- The smaller a molecule, the larger the available volume and the longer the retention time

Example: separation of PCDDs from PCNs
Typical problem: complexly contaminated sediments exhibiting dioxin-like effects

Typical compounds: Alkanes, PAHs, PCBs, PCDD/Fs, PCNs, nitro- and oxy-PAHs, alkylphenols, pesticides…

Typical question: How to do group-specific separation of all relevant compound groups in an efficient way?

Possible answer: Combine several separation steps according to polarity, aromaticity, planarity, halogenation.
Example: off-line combination

**EXTRACT**

- Open column NP-LC (AlO)_{23}
- F1: non-polar aliphatic com.
- F2: non-polar aromatic com.
- F3, F4: polar fractions

**NP-LC (NO-phenyl)**

- F2.1 PCDD/Fs
  - PCBs, PCNs
- F2.2 PAHs
  - MW 152-166
- F2.3 PAHs
  - MW 178
- F2.4 PAHs
  - MW 202
- F2.5 PAHs
  - MW 226/228
- F2.6 PAHs
  - MW 252
- PAHs
  - MW 276

**Electron-donor-acceptor-LC (PYE)**

- F2.1.1 non-halogenated
- F2.1.2
- F2.1.3
- F2.1.4
- F2.1.5
- F2.1.6
- F2.1.7
- F2.1.8
- F2.1.9
- F2.1.10
- F2.1.11

**PCBs: Cl +, ortho Cl -**

**Tetra- to octa-CN, CDD/F**

**Size-exclusion chromatography**
Example: dioxin-like effects, Bitterfeld sediments

Example: off-line combination

EXTRAKT

open column NP-LC (AIO)

F1: unpolare Aliphaten
F2: unpolare Aromaten
F3, F4: polare Fraktionen

NP-LC (NO₂-Phenyl)

F2.1 PCDD/Fs
F2.2 PAHs MW152-166
F2.3 PAHs MW178
F2.4 PAHs MW202
F2.5 PAHs MW226/228
F2.6 PAHs MW252
PAHs MW > 276

RP-LC (C18, endcapped, Polymer)

F2.5.1 e.g. TRP
F2.5.2 e.g. BaA
F2.5.3 e.g. CHR
F2.5.4 e.g. 2MBA
F2.5.5 e.g. 10MBA
F2.5.6 e.g. 4MCHR
F2.5.7 e.g. 5MCHR
F2.5.8 e.g. 9MBA
F2.5.9 e.g. 2MCHR
F2.5.10 e.g. 1212D

RP-LC (PYE)

F2.5.8.1 9MBA
F2.5.8.2 22NBT
F2.5.8.3 2123DNF
F2.5.8.4 1MCHR
Example: dioxin-like effects, Bitterfeld sediments

Dioxin-like effects of fractions

NP-fractions

RP-fractions
Example: dioxin-like effects, Bitterfeld sediments

fraction F2.5.8

GC/MS analysis
Example: dioxin-like effects, Bitterfeld sediments

HPLC/DAD analysis

Fraction F2.5.8

Retention time [min]
Example: dioxin-like effects, Bitterfeld sediments

identified and confirmed as cause of effects

by-products of naphthol production

2-(2-Naphthalenyl)-benzothiophen

Dinaphtho[2,1-b; 2',3'-d]furan

Dinaphtho[1,2-b; 1',2'-d]furan
Multistep fractionation: powerful tool for toxicant isolation

However:
- Laborious and time-consuming
- Risk of artefacts (losses and contamination) during concentration and solvent exchange
Liquid Chromatography

⇒ novel on-line combination of three different NP-columns

Lübcke-von Varel et al. 2008 J Chrom A
Liquid Chromatography
With LC-methods you can exploit
- lipophilicity
- polar interactions
- interactions of π-electron systems
- planarity...
for separation.

Volutility is another useful chemical property for separation ⇒ gas chromatography
Preparative Capillary Gas Chromatography

- Separation based on partitioning between mobile gas phase and stationary liquid phase
- Fractions are collected with cooled traps
- High resolution
- Good reproducibility

⇒ Separation of difficult mixtures of isomers e.g. technical nonylphenol
- Not for non-volatiles and thermally labile compounds

Preparative Capillary Gas Chromatography
Effect-directed analysis of organic toxicants

Starting point: extract

complex mixture

fractionation

biological analysis

chemical analysis

confirmation

toxicant
Toxicant identification

Most frequently used: gas chromatography with mass selective detection (GC-MS)

Comparison with library data e.g. NIST (190000 mass spectra of 163000 compounds)
Is the problem solved now?

Fraction xy is toxic.

In fraction xy compounds A, B and C are identified (library search).

⇒ Compounds A, B and C are responsible for toxicity

There are good reasons to be sceptical!
There are good reasons to be sceptical!

- Good match with library entries no proof for correct identification (isomers with similar or identical spectra)

- The compounds we detected may not be responsible at all. Maybe the "real" toxicant could not be seen (not detectable with applied method, below detection limit, co-eluting with other compounds).
Problems of library use for compound identification:

Only a small portion of possible compounds may be found in the library.

Example:
Compounds with MW = 150

⇒ Even with good matches you cannot be sure that you have identified correctly.

- 376 mass spectra in NIST
- 5,300 in the Beilstein database
- 615,977,591 possible compounds
Alternative:

- generation of all possible structures fitting to the MW e.g. with MOLGEN
- stepwise reduction of number of candidates
- final list of candidates
- mass spectrum
- classifiers from fragmentation
- retention indices (chromatography)
- other spectra (UV, IR)
Toxicant identification

40 compounds generated
no C=C in ring
25 compounds left
C=C-C=C
4 compounds left

Example: unknown compound from a groundwater fraction

available in NIST
Toxicity Confirmation

Tiered approach

- Tentatively identified toxicant
  - Analytical confirmation
    - Effect confirmation \(\textit{in vitro/in vivo}\)
      - Hazard confirmation
  - Evidence on chemical structures
  - Evidence of cause-effect relationships
    - Quantification of individual toxicants' contribution to toxicity
    - Estimate of unexplained effects
  - Evidence of cause-effect relationships at realistic exposure conditions on populations and communities
Confirm the chemical structure of tentatively identified compounds.

- Extensive fractionation and high chromatographic resolution before mass spectra recording.
- Confirm with neat standards where available (agreement of spectrum and retention).
- Without neat standards: lines-of-evidence approach (retention on different GC and LC columns, UV, fluorescence spectra)
- Be aware that there are much more chemicals on earth than in library! Structure generation may help.
Toxicity Confirmation

Confirm that identified compounds are actually responsible for measured effects. Estimate how much of effect is explained.

- compounds (names, quantities)
- predicted mixture toxicity
- mixture of neat compounds
- measured mixture toxicity
- toxic data + mixture toxicity models
- tox. data + mixture toxicity models
- candidate toxicants
- literature tox. data
- compounds (names, quantities)

confirmation quality
Toxicity Confirmation

Do identified toxicants have an impact *in situ*?

**Example: Pollution induced community tolerance (PICT)**

**Basic idea:** Communities that have been affected by a specific toxicant are more tolerant to the same compound if exposed again.

**Reason:** Sensitive species disappeared

⇒ **PICT confirms effects on community level**
Example: experimental confirmation on community level: e.g. PICT

- prometryne identified as key toxicant for green algae in the creek Spittelwasser

⇒ confirmation by higher tolerance of Spittelwasser biofilms to prometryne compared to Mulde

Schmitt-Jansen et al. 2008