Consideration of Bioavailability in the Effect-Directed Analysis of Contaminated Sediment Samples

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Content

Abstract **1**
Zusammenfassung **3**
Chapter 1 ..... **5**
1.1 Toxicants in Freshwater Sediments 6
1.2 Excursus Background Partitioning and concentration measurements 15
1.3 Objectives of the study 20
Chapter 2 ..... **28**
2.1 Introduction 29
2.2 Materials and Methods 31
2.2.1 Chemicals and Materials 31
2.2.2 Chemical Analysis 32
2.2.3 Non equilibrium Solid Phase Microextraction 33
2.2.4 Algae Cultivation and Harvest 33
2.2.5 Determination of Negligible Depletion 34
2.2.6 Determination Limit of Quantification (LOQ) 34
2.2.7 Influence of Fouling 35
2.2.8 Mass Balances 36
2.2.9 Algae-GB-Medium Partition Coefficient Determination 37
2.3 Results and Discussion 38
2.3.1 Non-Depletive Solid Phase Micro Extraction 38
2.3.2 LOQ 39
2.3.3 Impacts of Algae Cells on SPME 40
2.3.4 Mass Balances 42
2.3.5 Partition Coefficients 43
2.4 Conclusions 45
Chapter 3 ..... **48**
3.1 Introduction 49
3.2 Material and Methods 50
3.2.1 Chemicals 50
3.2.2 GC-MS 51
3.2.3 Silicone Rods 52
3.2.4 Loading Efficiency 52
3.2.5 Depletion Kinetics 53
3.2.6 Toxicity Test 54
3.2.7 Concentration Stability during 24 h 54
3.2.8 Sampling and Extraction 54
3.2.9 Fractionation 55
3.3 Results and Discussion 56
3.3.1 Loading 56
3.3.2 Correlation between Partitioning Coefficients 57
3.3.3 Equilibrium Partitioning 57
3.3.4 Constant Concentrations 60
3.3.5 Concentration Effect Relationships in Algae Test 61
3.3.6 Toxicity Patterns 62
Chapter 4 ..... **67**

4.1 Introduction 68
4.2 Material and Methods 69
4.2.1 Chemicals 69
4.2.2 Sampling and Extraction 70
4.2.3 Normal Phase Fractionation 71
4.2.4 Reversed Phase Fractionation 71
4.2.5 Toxicity Test and Dosing 72
4.2.6 GC-MS 73
4.2.7 Confirmation of Identified Chemicals 74
4.3 Results and Discussion 75
4.3.1 Toxicity Pattern for Normal Phase Fractionation 75
4.3.2 Toxicity Pattern Derived for RP-HPLC Sub-Fraction 77
4.3.3 Identified Compounds 80
4.3.4 Confirmation 81
4.3.5 Influence of Dosing Technique on Fraction Prioritization 85
Chapter 5 88
List of publications 101
Acknowledgements 103
CV 105

List of Figures

Figure 1-1	Schematic overview of Effect Directed Analysis from [18].	**9**
Figure 1-2	Schematic overview over all processes, which contribute to the bioavailability of sediment contaminants (adapted from ISO/DIS 2006) [32].	**12**
Figure 1-3	Boundary Layer model showing the concentration gradient in a sample containing a Silicon Rod (SR) as dosing device and aqueous test medium. The boundary layer (BL) is a static layer around the SR, where no convection occurs.Schematic overview over all processes, which contribute to the bioavailability of sediment contaminants (adapted from ISO/DIS 2006) [32].	**17**
Figure 1-4	Uptake kinetic of analytes in the coating of SPME fibers.	**19**
Figure 2-1	Double chamber scheme. Compounds loaded on the Silicone-plate partition to both sides. ⊡ algae suspension, □ Grimme-Boardmann growth medium.	**35**
Figure 2-2	Picture of chamber for loading siloxane-plates with organic compounds.	**36**
Figure 2-3	Measured medium concentrations (n = 3) of consecutive SPME measurements for three different sample concentrations (µg/L). The samples were extracted three times. ■ first extraction ■ second extraction □third extraction. ATR (atrazine), PRO (prometryn), MPA (methyl parathion), ANT (anthraquinone) and DDD (p'p-DDD) were measured with a DVB/CAR/PDMS fibre. LIN (lindane), FLU (fluoranthene), PYR (pyrene), PCB (PCB 101), PNA (N-phenyl-2-naphthylamine), BEN (benz[a]anthracene) and MET (methoxychlor) were measured with a PA fibre.	**39**
Figure 2-4	Peak areas of the MS-signal (abundance) with and without algae of a.) prometryn, b.) PCB 101, c.) N-phenyl-2-naphthylamine, d.) methoxychlor, and e.) atrazine. The measurements were performed in double chambers divided by a poly(dimethylsiloxane)-plate, which dose identical concentrations of the test compound to the two halves. One half contains algae suspension , while the other half was filled with growth medium without algae. ** Significant difference between the two treatments; $p < 0.01$, Wilcoxon-Mann-Whitney test.	**41**
Figure 2-5	Correlation between K_{OW} and the partition coefficient between lipid of the algae and the aqueous phase (K_{AGB}) ($y = 0.776 (\pm 0.163) \times x - 0.388 (\pm 0.764)$) $R^2 = 0.885$.	**44**
Figure 3-1	Loading efficiency in percent (n = 3) of methyl-parathion (MP), prometryn (PROM), N-phenyl-2-naphthylamine (PNA) and p,p'-DDD on SRs.	**56**
Figure 3-2	Correlation ($r^2 = 0.90$), between log K_{OW} and log K_{SW} (partitioning coefficient between silicone rod and water) (n = 3).	**57**
Figure 3-3	Depletion kinetics from SRs into the aqueous phase a.) p,p'-DDD, b.) fluoranthene, c.) prometryn, d.) pyrene, e.) benzo[a]anthracene, f.) anthraquinone, g.) methoxychlor, h.) PCB 101, i.) methyl-parathion j.) lindane k.) N-phenyl-2-naphthylamine. Concentration in mg/L.	**59**
Figure 3-4	Concentration of model compounds over 24 h, dosed to a bioassay using the green algae Scenedesmus vacuolatus with DMSO solution of the compounds (▭) or loaded SRs (▬). Concentrations are measured in percent relative to the measured concentration at t = 0.	**61**

Figure 3-5	Concentration dependent response in the 24 h growth inhibition assay with the green algae Scenedesmus vacuolatus of a.) prometryn and b) of a sediment extract from the Elbe River (Přelouč, Czech Republic). The sediment extract and prometryn were loaded on SRs. Prometryn concentrations have been measured with SPME in combination with GC-MS. For the fit a sigmoid model with three parameters was used for the whole sediment extract (y = a/(1+b*e(-kx))) and a Hill model (y = (a*xn)/(kn + xn) was used for the prometryn sample.	**62**
Figure 3-6	Overview of growth inhibition of green algae Scenedesmus vacuolatus (▭0-20 %; ▨ 20-50 % ; ■ over 50 %) exposed to fractions of sediment extracts of sediments from Elbe River Přelouč (Pre), Bilina River Most (Bil), and Spittelwasser Bitterfeld (Spi) for dosing with loaded SRs (load 1.03 g OCEQ/SR Most, 0.42 g OCEQ/SR Přelouč and 0.75 g OCEQ/SR Spittelwasser) and conventional dosing with DMSO (c = 2.06 g OCEQ/L Most, 0.84 g OCEQ/L Přelouč and 1.5 g OCEQ/L Spittelwasser).	**63**
Figure 4-1	Growth inhibition of the green algae Scenedesmus vacuolatus exposed 24 h to fractions gained with NP fractionation of a sediment extract and dosed to the test system either by solvent dosing with DMSO□ (c = 20 g SEQ/L) or partitioning based dosing with loaded silicone rods■ (c = 10 g SEQ/SR).	**76**
Figure 4-2	Growth inhibition (n=3) of the green algae Scenedesmus vacuolatus exposed for 24 h to sub-fractions of a sediment extract dosed with DMSO to the test system (c = 40 g SEQ/L). Recombination of all sub-fractions (recomb) and original fraction (c = 20 g SEQ/L). The sediment extract was first fractionated with NP HPLC and then with RP HPLC as second fractionation step.	**77**
Figure 4-3	Growth inhibition of the green algae Scenedesmus vacuolatus exposed 24 h to sub-fractions of a sediment extract dosed to the test using silicone rods (SRs). The sediment extract was first fractionated with NP HPLC and then with RP RPLC as second fractionation step. The sub-fractions from the RP HPLC of fraction 14, the residue (RS) and the recombination of all sub-fractions (recomb) are shown.□ 6.25 g SEQ/SR, ▩12.5 g SEQ/SR,■ 25 g SEQ/SR,⊟ 10 g SEQ/SR.	**79**
Figure 4-4	Dose-response curves of fractions (●) and corresponding artificial mixtures (▲) in a 24 h growth inhibition test with the green algae Scenedesmus vacuolatus. The samples were either dosed with DMSO a.) F 10-6, b.) 10-5 c.) F11 d.) 8-7 e.) 15-16 or with silicone rods (SRs) f.) 14-7 g.) 15-RS h.)15-15. The concentrations are given as g sediment equivalents (g SEQ).	**82**
Figure 4-5	Index of Confirmation Quality of sub-fractions for dosing with DMSO (▬F10-6, ▬ ▬ F10-5, ▬ . ▬ F11, ... F8-7 and ▬ ▬ F15-16) and for dosing with SRs (▬ F14-7, ... F15-15 and ▬ . ▬ F15 RS). The range of ICQ between 0.5 and 2 is shaded in grey.	**84**

Figure 5-1 Overview of developed method. The silicone rods (SR) are loaded with sediment extract containing compounds with different hydrophobicity: ▬ hydrophobic compound, ▶ medium hydrophobic compound and ● hydrophilic compound. The partition of the compounds from the SR into the test medium of the bio assay is analog to the partition in the natural sediment water system. The exposure concentration is determined with non depletive solid phase micro extraction (nd-SPME) in combination with gas chromatography with mass selective detector (GC-MS). **90**

List of Tables

Table 2-1	Model substances and their physico-chemical properties.	**32**
Table 2-2	EC50 for green algae and the limit of quantification (LOQ) of the SPME-method for selected model compounds. The LOQ refers to the freely dissolved concentration of the compound in the GB-medium.	**40**
Table 2-3	Mass balance with recoveries [%] for N-phenyl-2-napththylamine (PNA), methoxychlor (MET), PCB 101 (PCB), prometryne (PRO) and atrazine (ATR).	**42**
Table 2-4	Model substances, the concentration in water and the consequential calculated concentration in air using the air/water partition coefficients from table 2-1. The absolute amount in water (MH20) and in air (Mair) was calculated using the volumes of the two phases (VH20= 2 mL; Vair= 6 mL). The percental losses in the gaseous phase are based on the absolute amounts in the two phases.	**43**
Table 2-5	Log K_{OW} of compounds and the logarithm of the partitioning coefficient between algae and water log K_{AG}.	**44**
Table 3-1	Model substances and their physico-chemical properties.	**51**
Table 3-2	Used chemicals, calculated equilibrium times and parameters of the curves fitted to the data. The overall kinetics are described using the following formula: $c_t = c_{eq}*(1-e^{-kt})$ [10].	**58**
Table 4-1	Identified compounds, supplier, used columns for separation during the GC-MS analysis and used SPME-fibres for concentration measurements in aqueous samples.	**70**
Table 4-2	Description of the gradients of the RP HPLC-method. The ration ACN/H2O (v/v) is given at specified times.	**71**
Table 4-3	Compounds and their log K_{OW} value used to determine the log K_{OW} windows of the RP HPLC.	**72**
Table 4-4	Compounds identified and quantified in individual fractions; applied dosing techniques are given in brackets, the sediment concentration c (µg/g sediment dry weight) is listed.	**80**
Table 4-5	Tentatively identified compounds in fractions 15-6, log K_{OW} values and expected log K_{OW} range from RP HPLC for sub-fraction 15-6.	**81**
Table 4-6	Parameters for dose-response curves fitted to fractions (F) and corresponding artificial mixtures (Mix). A three parameter sigmoid model was used for fitting ($y = a/ (1+\exp((x-x_0)/b))$).	**83**
Table 4-7	Identified compounds and EC_{50} values observed using the solvent dosing technique (DMSO) in a 24 h growth inhibition test with the green algae Scenedesmus vacuolatus; partitioning coefficients between silicone rod (SR) and water (K_{SW}), and subsequently calculated exposure concentrations (c_W) using SR for dosing with actually applied loads.	**85**
Table 5-1	Overview of the identified key toxicants in this study from sediments from the Bilina River, Elbe River and Spittelwasser. The used dosing technique solvent carrier dosing with dimethylsulfoxid (DMSO) or partition based dosing with silicone rods (SR) is specified.	**95**

Abbreviations used

a_i	chemical activity
ACN	acetonitrile
ACT	acetone
ASE	accelerated solvent extraction
BL	bulk layer
c_0	initial concentration
c_A	concentration in algae
c_{eq}	concentration at equilibrium
c_f	concentration in fibre
c_{GB}	concentration in GB-medium
c_h	concentration in headspace
c_i	concentration of compound i
c_{PDMS}	concentration in PDMS
c_s	concentration in sample
c_t	concentration at time t
c_W	concentration in water
CAS	chemical abstract service
δ_W	thickness of boundary layer
δ_S	thickness of polymer
D	diffusion coefficient
D_w	diffusion coefficient in water
D_s	diffusion coefficient in silicone
DCM	dichlormethane
DMSO	dimethylsulfoxide
DVB/CAR/PDMS	divenylbenzene-carboxene-poly(dimethylsiloxane)
EC_{50}	effect concentration 50 %
EC_x	effect concentration x
EDA	effect-directed analysis
EqP	equilibrium partitioning theory
EPA	environmental protection agency
EU	European Union
G	Gibbs free energy
GB	Grimme-Boardmann
GC	gas chromatography
GC-MS	gas chromatography with mass selective detector
HPLC	high performance liquid chromatography

HX	hexane
ICQ	index of confirmation quality
ISO	International organization for standardization
k_u	relative uncertainty of analysis
K_{AGB}	partition coefficient between algae and GB-medium
K_{AW}	partition coefficient between air and water
K_{fh}	partition coefficient between fibre and headspace
K_{hs}	partition coefficient between headspace and sample
K_{OW}	partition coefficient between octanol and water
K_{SW}	partition coefficient between silicone and water
l	length
LC-MS	liquid chromatography with mass selective detector
LC_{50}	lethal concentration 50 %
LOQ	limit of quantification
μ_i	chemical potential
μ^*	standard chemical potential
MeOH	methanol
MS	mass selective detector
n_j	chemical composition
nd-SPME	non depletive solid phase microextraction
NP	normal phase
OCEQ	organic carbon equivalents
OECD	organization for economic co-operation and development
p	pressure
PA	polyacrylate
PAH	poly aromatic hydrocarbons
PAK	polyaromatischer Kohlenwasserstoff
PCB	poly chlorinated biphenyl
PCDD	poly chlorinated dibenzo-*p*-dioxin
PCDF	poly chlorinated dibenzofuran
PCN	poly chlorinated naphthalene
PDMS	poly(dimethylsiloxane)
PGC	porous graphitized carbon
PNA	N-phenyl-2-naphthylamin
p,p'-DDD	1,1-dichloro-2,2-bis-(p-chlorophenyl)ethane
QSAR	quantitative structure active relationship
R	gas constant

R_0	total transport resistance
RP	reversed phase
RPM	rounds per minute
RS	residue
s_{x0}	process standard deviation
S_{XX}	sum of squared deviations
SEQ	sediment equivalent
SIM	single ion mode
SPMD	semi permeable membrane device
SPME	solid phase microextraction
SR	silicone rod
T	temperature
$t_{1-\alpha,v}$	students factor
TIE	toxicity identification and evaluation
TOC	total organic carbon
TOL	toluene
TU	toxic unit
UTM	universal transverse mercator
V_f	volume fiber coating
V_h	volume headspace
V_s	volume sample
WDF	water frame work directive
x_i	concentration
$\overline{x}_i$	mean value
x_{LOQ}	Concentration at limit of quantification
y_i	activity coefficient

Abstract

The scope of the presented PhD thesis is on the incorporation of bioavailability into effect directed analysis (EDA) of contaminated sediment samples. EDA has been successfully applied in the last years to identify key toxicants in environmental samples. One of the major challenges in EDA is how to consider bioavailability of investigated chemicals. By using crude sediment extracts and solvent dosing the ecotoxicological effects may be overestimated and prioritisation of fractions may be biased.

The equilibrium approach is often used for bioavailability estimation. A new dosing technique has been developed to simulate the partitioning between sediment particles and the surrounding aqueous phase. Costumer made stirrer bars (SR) consisting of poly(dimethylsiloxane) were suggested as a novel tool and evaluated for the application in a cell multiplication inhibition test with green algae *Scenedesmus vacuolatus* using a set of model compounds covering a broad range of physico-chemical properties. The SRs have been shown to have a high loading capacity and in general a fast achievement of equilibrium. This subsequent delivery compensates for possible losses due to e.g. adsorption or evaporation and leads to constant concentrations. Good dose-response curves were achieved using this method.

To measure and monitor the aqueous concentration during the bioassay a pre-equilibrium solid phase extraction (SPME) method was developed and validated. This method is non depletive and thus does not change the concentration in the sample. Evaluation of the limit of detection (LOQ) confirms that sufficiently low concentrations compared to EC_{50} concentrations observed in the algae assay can be measured. The presence of the algae has no observable overall effect on the concentration measurements. Therefore, bio fouling of the SPME fibers and enhanced uptake kinetics due to the presence of another strong adsorbing phase (algae) do not play an important role in this system. This SPME method was also used for determination of algae growth medium partition coefficients.

The two developed methods were applied in EDA studies of contaminated sediment samples. The sediment extracts from hot spots from the Elbe River basin were fractionated using a multistep normal phase HPLC method. The

fractions were dosed to the algae assay either by conventional solvent dosing via dimethylsulfoxid (DMSO) or by using the partition based dosing method using SR. The results reveal that the consideration of the bioavailability has a strong influence on the prioritization of fractions and that the differences between the three sediment samples is less pronounced than between the two dosing techniques. Dosing with DMSO identifies mainly fractions, in which polyaromatic hydrocarbons (PAHs) and more polar compounds are expected to elute, as most toxic fractions, while for dosing with SRs the fractions co-eluting with polar compounds are the most toxic ones. From the three sediments one from a tributary from the Elbe River was chosen. As second fraction step a reversed phase HPLC was used to fractionate the most toxic fraction from the first step. Sub-fractions showing highest toxicity in the bioassay with green algae were analyzed by GC-MS. The identity of the compounds was confirmed with pure standards. Effect confirmation was based on the effects of artificial mixtures with the same composition and concentrations as the sub-fractions. For DMSO dosing PAHs and hexadecanol were confirmed as toxicants, while for the new dosing technique more polar compounds as e.g. triclosane and benz[*c*]acridine were confirmed. These results support the need for increasing environmental realism in EDA to identify those toxicants that pose major hazards.

Zusammenfassung

Die vorliegende Arbeit befasst sich mit der Berücksichtigung der Bioverfügbarkeit in der Wirkungsorientierten Analytik (EDA) von belasteten Sedimentproben. Die EDA ist in den letzen Jahren erfolgreich angewendet worden, um Schlüsselschadstoffe in Umweltproben zu identifizieren. Eine der größten Herausforderungen der EDA ist die Veränderung der Bioverfügbarkeit der Substanzen durch die Verwendung von Gesamtextrakten von Sedimenten und die damit einhergehende Veränderung der relativen Toxizität der Sedimentinhaltsstoffe. Dies kann zu einer fehlerhaften Priorisierung von Fraktionen und Substanzen führen.

Die Gleichgewichtsverteilungstheorie wird häufig verwendet, um Bioverfügbarkeit abzuschätzen. Um die Verteilung zwischen dem Sediment und der umliegenden wässrigen Phase zu simulieren, wurde eine verteilungsbasierte Dosierungstechnik entwickelt. Selbst gefertigte Magnetrührstäbe aus Silikon wurden hinsichtlich ihrer Anwendung im Algenwachstumshemmtest mit der Grünalge *Scenedesmus vacuolatus* untersucht. Dazu wurden ausgewählte Modellsubstanzen verwendet, die eine Vielzahl von unterschiedlichen physikalisch-chemischen Eigenschaften abdecken. Die Ergebnisse zeigen, dass die Magnetrührstäbe eine hohe Beladekapazität und eine schnelle Gleichgewichtseinstellung haben. Die kontinuierliche Nachlieferung von Testsubstanzen, deren freie gelöste Konzentration durch z.B. Adsorption an Gefäßwänden sinkt, führt zu konstanten Konzentrationen und guten Dosiswirkungsbeziehungen im Biotest.

Um die Konzentrationen in der wässrigen Phase während des Biotest messen und verfolgen zu können, wurde eine Festphasenmikro-extraktionsmethode (SPME) entwickelt und validiert. Diese Extraktion ist nicht erschöpfend und verändert daher die Konzentrationen in der Probe nicht. Die Bestimmungsgrenzen liegen weit genug unter den EC_{50} Konzentrationen für diesen Biotest und erlauben damit die Messung der realen Expositionskonzentrationen über den ganzen Konzentrationsbereich der Dosiswirkungskurven. Die Anwesenheit der Algen hat keinen messbaren Effekt auf die Konzentrationsmessung. Biologische Ablagerungen oder die Beeinflussung der Aufnahmekinetik in die SPME durch die Anwesenheit

einer zweiten absorbierenden Phase (Algen) spielen bei diesem Testdesign offensichtlich keine Rolle. Die SPME Methode wurde auch dazu verwendet, um Verteilungskoeffizienten zwischen Algen und dem Nährmedium zu bestimmen.

Beide hier entwickelten Methoden wurden in der EDA von Sedimentproben angewendet. Sedimentproben von drei stark belasteten Standorten im Elbeeinzugsgebiet wurden extrahiert und mit einer Hilfe einer mehrstufigen Normalphasen HPLC Methode fraktioniert. Die so gewonnen Fraktionen wurden im Algentest entweder konventionell mit Dimethylsulfoxid (DMSO) als Lösungsvermittler oder mit der neu entwickelten verteilungsbasierten Methode mit Magnetrührstäben dosiert. Die Ergebnisse zeigen, dass die Art der Dosierung einen großen Einfluss auf die Priorisierung der Fraktionen hat und dass die Unterschiede zwischen den Dosierungstechniken größer sind als zwischen den Proben. Die Verwendung von DMSO führt zur Priorisierung der Fraktionen, die mit polyzyklischen aromatischen Kohlenwasserstoffen (PAKs) sowie derjenigen, die mit polaren Substanzen ko-eluieren. Dagegen sind bei Verwendung der Magnetrührstäbe nur noch die Fraktionen mit polareren Substanzen entscheidend.

Aus den drei Sedimentproben wurde eine aus einem Zufluss der Elbe für weitere Fraktionierungsschritte ausgewählt und mit Umkehrphasen HPLC als zweitem Fraktionierungsschritt weiter aufgetrennt. Subfraktionen, welche die höchste Wachstumshemmung im Algentest hervorriefen, wurden mit Hilfe von GC-MS sowohl quantitativ als auch qualitativ analysiert. Die chemische Identität wurde mit Standards verifiziert. Die biologische Wirkung wurde mit Hilfe von künstlichen Mischungen bestätigt, die in ihrer Zusammensetzung und Konzentration den Subfraktionen entsprachen. Bei Dosierung mit DMSO wurden PAKs und Hexadecanol als Schlüsselschadstoffe identifiziert, während bei verteilungsbasierter Dosierung mit Magnetrührstäben polarere Substanzen wie z.B. Triclosan und Benz[*c*]acridin die Toxizität dominieren. Diese Ergebnisse unterstreichen die Notwendigkeit EDA Studien unter realistischeren Bedingungen durchzuführen, um diejenigen Substanzen zu identifizieren, die in der Umwelt die größten Gefährdungspotentiale zeigen.

Chapter 1

Introduction

1.1 Toxicants in Freshwater Sediments

Impact of Sediments on Water Quality

In the year 2000 the water frame work directive (WDF) of the European Union became effective aiming for the establishment of a good ecological and chemical status of the European water bodies until 2015 [1]. Despite the increasing awareness that the water resources are at risk and the progress achieved in the water quality during the last two decades, the monitoring for the WDF reveals that more than 62 % of the surface water bodies in Germany and 40 % in total Europe are not in the required good ecological and chemical status. They probably will fail to match the criteria until 2015. For additional 30 % of the surface water bodies in the EU there is a lack of data and it is unclear if the goal will be reached [2].

The insufficient ecological status is caused by human activities and can have different reasons: increasing nutrient content due to intensive agricultural land use, the discharge of chemicals and agrochemicals during production, use or waste disposal and the change of the morphological profile by river regulations including canalization and the construction of dams. The identification of the relevant stressors is the first step to take efficient measures to achieve the good ecological and chemical status. The monitoring program revealed that 50 % of the water bodies in Germany are affected by chemicals [3]. In contrast to the problems caused by morphological changes or high nutrient content the chemical stressors are not that obvious and not easy to identify due to the high diversity of chemicals produced and used.

Besides the water phase itself the sediments are in the focus of interest. Sediments serve as sink as well as source for contaminants and may have a strong influence on the water quality. Many anthropogenic chemicals accumulate in sediments [4]. However, sediments do not only act as a sink but also as a source for water contamination as a result of partitioning processes between the solid phase and the water phase. Changes in environmental conditions such as temperature, pH-value, salinity or flood events and resulting re-suspension of contaminated particles may enhance the release from the sediment [5]. Contaminated sediments may act as a secondary source even if the primary source such as an industrial process

has been closed down decades ago. As over the last decades the emission from such point sources was effectively regulated, the importance of the sediment-bound pollutants for the water quality will increase [6]. Thus, the consideration of sediments in the risk assessment of the WDF is recommended [7].

The high number of chemicals and the high number of contamination sources hampers the establishment of cause-effect-relationships in the case of observed ecotoxicity.The evaluation of the sediment or water quality can in general be based on chemical data from monitoring program or on observed effects in bioassays. Bioassays alone can only provide information about the toxicological impact but not about the source or responsible toxicant. The selection of the chemicals for the monitoring programs is rather difficult in view of over 50 millions different organic and inorganic chemicals registered at the chemical abstract service (CAS) today. The number of chemicals possibly present in the environment may be even higher if we assume that numerous compounds including degradation products and metabolites are still not identified and registered. Thus a complete surveillance of all the chemicals is neither possible nor reasonable. The WFD [1] solved the problem by using only a limited set of chemicals (priority compounds) to determine the chemical status. These compounds of high concern show high ecotoxicological effects, are persistent or mutagenic and are produced and used in high amounts. Heavy metals such as lead and mercury, polyaromatic hydrocarbons (PAHs) including benzo[*a*]pyrene and benzo[*k*]fluoranthene, pesticides such as endosulfane and nonylphenols are part of the list. In mass balance approaches the chemical concentrations of the priority compounds and the effects in the bioassays show often a discrepancy between the results [8-10]. Thus, it can be concluded that due to the restriction to the rather limited set of priority pollutants parts of observed toxicity remains unexplained.

Identification of Toxicants in complex mixtures

Identifying only those compounds that cause the observed effect, is one solution to overcome this discrepancy. The identification of unknown toxicants is still a challenging task, which needs the combination of chemical and biological methods. First steps have been made by the US

Environmental Protection Agency (EPA), who invented the Toxicity Identification Evaluation (TIE), which was mainly focussing on water samples and was designed for effluents [11-14]. This approach is divided in three phases. Phase I tries to characterize the toxicity by separating the toxicants into representative classes such as volatile toxicants, particle-associated toxicants, oxidants, non polar organics, metals and pH-dependent toxicants. The classification is done by comparing the toxicity of the sample before and after manipulations including aeration, filtration, oxidant reduction, solid phase extraction, cation exchange, chelation or graduated pH change. Phase II tries to identify the toxicants by means of analytical chemistry in case of metals and by a combination of fractionation and analytical methods for non-polar organics. Phase III deals with the confirmation of the identified toxicants. Today TIE studies are not restricted only to effluents, but are also applied for sediments, pore waters and seawater [15, 16]. Recently a new guidance document for TIE studies based on sediment samples was published and reflects the increasing awareness for the importance of sediment-bound contaminants [17]. Effect-directed analysis (EDA) is based on the same idea as TIE however, with a stronger focus on the isolation and identification of organic chemicals. The majority of EDA studies focused on solid samples such as air particulate matter, soils or sediments often based on organic extracts [18]. Like TIE, EDA studies combine biological and chemical methods (Figure 1-1). Extracts are fractionated to separate the compounds according to their physico-chemical properties. These may include polarity, hydrophobicity, planarity, molecular size and the presence of functional groups [19]. The fractionation does not only separate the compounds, but also gives first information about their physico-chemical properties and may be used for the identification and confirmation of the compounds. The biological response of every fraction is tested to prioritize the most toxic fractions for the further EDA procedure. Other fractionation steps based on other properties can be applied, if the toxic fractions still contains complex mixtures of chemicals.

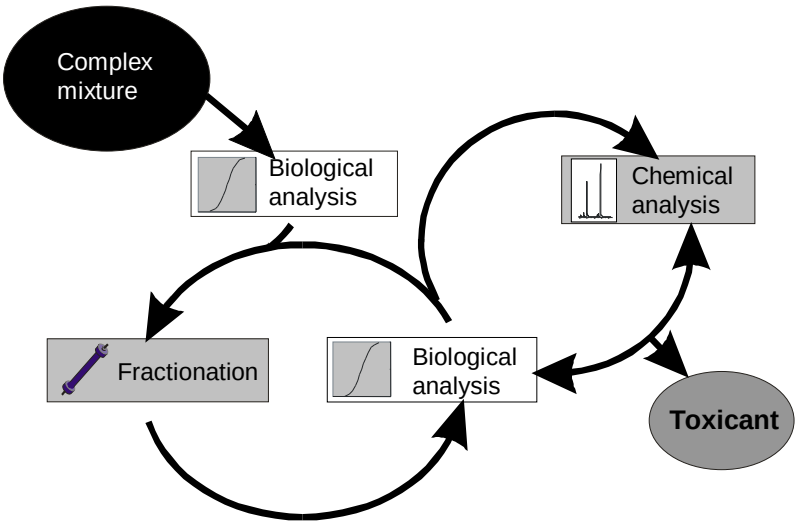

Figure 1-1: Schematic overview of Effect Directed Analysis from [18].

The compounds in the active fractions are identified mainly by gas-chromatography-mass spectrometry (GC-MS), but for more polar compounds the use of liquid-chromatography-mass-spectrometry (LC-MS) can be more useful. For the GC-MS analysis spectral libraries as the NIST Library [20] can be used for identification. Although this library contains spectra of 163.000 compounds, this amount is small compared to the 50 mil of known chemicals. That means that for the majority of compounds no library entry exists. For such cases the use of computer tools for the structure generation from the spectra and selection of reliable candidates can be useful [21].

Confirmation of Toxicants

After toxicant identification the confirmation of these compounds as the cause of the effect is a crucial part of every EDA study. The confirmation can also be divided in several steps which include the analytical confirmation and the effect confirmation in the bioassay [22]. For the analytical confirmation the retention time and the mass spectra of an unknown is compared with the parameters of pure standards. If this confirmation step is passed, the effects of the toxicants are confirmed in the bioassay. If a fraction still contains several toxicants toxicity of these mixtures may be predicted on the basis of mixture effect models. The most frequently used model is based on concentration addition and often expressed as toxic units (TU). This concept is based on the assumption that the compounds act with similar mode of

action. Although, this does not generally hold, predictions are mostly quite realistic.

$$\sum TU = \sum_{i=1}^{n} \frac{c_i}{EC_{50,i}} \qquad \textbf{(1-1)}$$

C_i is the concentration of the compound i and $EC_{50;i}$ is the effect concentration where compound i causes a 50 % effect. If standards are available artificial mixtures mimicking the fractions can be prepared and toxicity can be compared. This is often done on the basis of one effect level (often the EC_{50}). If the dose-response curves of the sample and the artificial mixtures are not parallel, then the degree of accordance depends on the effect levels [23] and the interpretation of the results is hampered. The index of confirmation quality (ICQ) [24], using the same theoretical background as the concept of TUs, is a possibility to overcome this problem by comparing effects over the full range of effect levels. The ICQ is calculated according the following formula:

$$ICQ = \frac{EC_{x,mixture}}{EC_{x,sample}} \qquad \textbf{(1-2)}$$

An ICQ value of 1 indicates 100 % overlap of the dose-response curves of the fraction and the corresponding mixture, while a smaller or greater value is a measure for deviation.

Several freshwater and marine sediments have been successfully investigated using EDA. The following toxicants have been identified: non polar PAHs as major mutagens in estuary sediments (United Kingdom) [25], polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs) in freshwater sediments (Western Scheldt basin, Netherlands) [26], nonylphenol (Neckar river, Germany) [27] and PAHs, N-phenyl-2-naphthylamine, promethryn and tributyltin (Spittelwasser creek, Elbe Basin, Germany) [28].

However, it should be considered that these studies were based on effects only without any attempt to consider bioavailability. Thus, the present study attempts to include the concept of bioavailability in EDA for further enhancement of environmental realism.

Bioavailability of Sediment Pollutants

According to the precautionary principle dosing of total extract may be generally acceptable and seen as a worst case scenario of complete bioavailability. It also reflects the use of total concentrations for regulatory purposes [29]. However, bioavailability is compound-specific and thus, ignoring bioavailability may bias the prioritisation of fractions and compounds with respect to hazards and risks. Hazards due to very hydrophobic compounds in a sediment-water-system may be overestimated while less hydrophobic toxicants with a great potential to cause adverse effects because of significant toxicity and great bioavailability may be ignored. Thus, incorporation of bioavailability into EDA methodologies is required for a realistic toxicity identification and assessment in sediments.

According to the ISO 11074 (2005) the bioavailability is defined as “degree to which chemicals present in the soil/sediment may be absorbed by human or ecological receptors or are available with biological systems”. In addition to this definition there exists a lot more definitions, because bioavailability is not a well defined term and is used for different processes addressing different aspects: for toxicologists the term represents the fraction of active components crossing the cell membranes and reaching the site of action, while for environmental scientists the term often represents the accessibility of compounds for uptake in the organism and possible toxicity [30]. In total the term bioavailability of sediment contaminants includes all processes from desorption of a compound from the sediment until it reaches the target site in the organism (Figure 1-2). This process can be separated in three phases: (i) desorption of the compound from the sediment, (ii) the uptake into the organism and (iii) the transportation or metabolism in the organism itself. Bioavailability of sediment-associated chemicals therefore depends on sediment characteristics, the physico-chemical properties of the compounds and the test organism itself [29]. This multi parameter dependency of the bioavailability leads to a bunch of different approaches to consider bioavailability in the toxicity evaluation of sediments [31]. In the next paragraph the different possibilities for incorporation of these methods in EDA studies will be discussed.

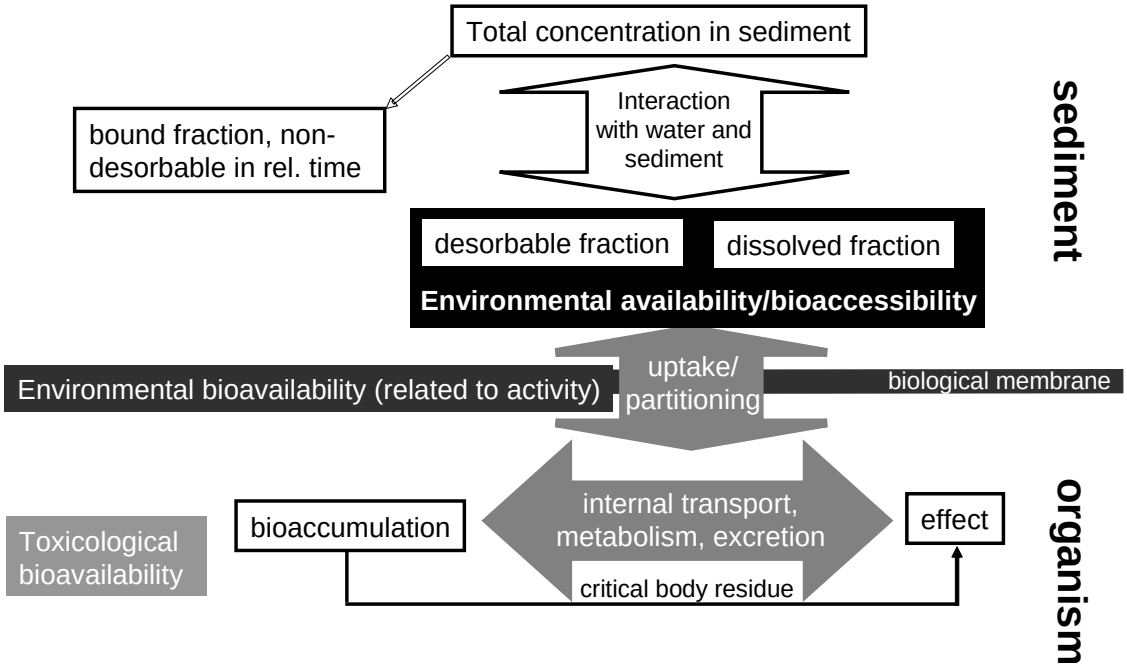

Figure 1-2: Schematic overview over all processes, which contribute to the bioavailability of sediment contaminants (adapted from ISO/DIS 2006) [32].

Sediment Contact Tests

All three processes are considered, if organisms are exposed to whole sediment samples. *In situ* exposure of test organisms such as midges (*Chironimus riparius* and *Chironimus tentans*) and amphipods (*Hyalella azteca*) grown under standardized laboratory conditions may be a good way to avoid artefacts caused by sediment pre-treatment [33, 34]. Alternatively, sediment contact tests with sediment samples under controlled laboratory conditions can be performed. The assays are either using fresh sediments or freeze-dried sediments without further treatment to minimize disturbance and change of the sample during preparation. They have been developed for several test organisms as e.g. mudsnails (*Potamopyrgus antipodarum*), fish embryos (*Danio rerio*), plants (*Myriophyllum aquaticum*) and nematodes (*Caenorhabditis elegans*) [35-38]. However, these methods focus on toxicity assessment and not on chemical analysis and identification of responsible toxicants. Separation of toxicants, a crucial part of every EDA study, is difficult to realize when whole sediments are tested. EDA in tissues of organisms, which have been in contact with the sediment of concern and act as a biological passive sampler would overcome the problem. The extracts of the exposed organisms might be used for the EDA studies, because they

include all compounds, which have been bio available, and hence taken up by the organism. Until now there are only few examples for this kind of extractions in EDA studies including estrogenic compounds in fish bile and aromatic hydrocarbons from crude oil in mussel tissues and compounds binding to aryl hydrocarbon and sex steroid receptors extracted from hepatic tissues from fish [39-42].

Chemical Passive Samplers

The limited amount of published studies reflects the limitations of tissue EDA: The compounds are distributed in the organism and can be metabolised leading to toxification or detoxification and in the last step to excretion or accumulation of the parent compounds or metabolites. These toxicokinetics are highly species depended and cannot be transferred to other organisms. Metabolisation produces new compounds and decreases the concentration of the parent ones and thus, leads to difficulties in the identification and quantification of the toxicants present in the sediment. To avoid these problems the organisms may be substituted by chemical passive samplers.

Chemical passive samplers such as thin silicone films and SPME were shown to simulate the uptake of lipophilic organic compounds into worms, midge larvae and amphipods avoiding the influence of species specific toxicokinetics of the real organism [43-46]. This includes neglecting of species specific pathways of uptake as ingestions followed by an acid digestion .Thus the exposition of the organism might be underestimated [47]. The samplers work on the basis of equilibrium partitioning. In order to simulate partitioning processes in the sediment-water-organism system they are designed to be of low volume and non-depletive without significant impact on sediment concentrations. Thus, the extracted amount of sediment contaminants is low but sufficient for analytical purposes while it fails to be sufficient for EDA. Thus, the application of a high volume depletive extraction to gain sufficient amounts of contaminants with subsequent simulation of partitioning processes in the biotest may be more promising.

Selective Extraction Techniques

Extraction techniques may focus either on total amounts or on rapidly desorbing and thus bioaccessible fractions only. Desorption from the sediment in a time frame that is relevant for benthic organisms to the surrounding aqueous phase is a prerequisite for the uptake into organisms. The bound compounds can be divided in three fractions: the freely dissolved fraction, the fraction, that desorbs in ecotoxicologically relevant time frames, and a fraction which is tightly bound and is desorbing in month to millennia [48, 49]. Therefore the kinetics have been characterized by a rapidly, a slowly and a very slowly desorbing fraction. The rapidly desorbing fraction is mainly adsorbed to easily accessible sites at the surface of the sediment particles, while the slow desorbing fractions is bound to more remote sites or absorbed into the organic matter [50]. Previous studies suggested that only the fast desorbing fraction is bioavailable and relevant for the uptake in organism [51]. The slow and very slow desorbing fraction were not available to the organisms [52].

Extraction procedures which extract only the rapidly desorbing fraction are a tool to focus sediment assessments on bioaccessible contamination only. Several methods have been developed to extract only the bio accessible amount: the use of mild solvents [53-56], subcritical water and CO_2 extraction with biotic fluids [57] and adsorption with an adsorbent as for example TENAX® after desorption into the aqueous phase [52]. Recently, the TENAX® method has been scaled up to extract sufficient amounts of sediments for application in the EDA [58]. This approach has been used to obtain sediment extracts containing only the bio available compounds. The limited bioaccessibility of PAHs bound to sediments is shown by the lower toxicity of TENAX® extracts compared to a sample using total extracts gained by extraction with accelerated solvent extraction (ASE) [59]. The PAHs in the sediment are less bio available because they are bound to condensed and aromatic material, which is commonly termed as black carbon. Black carbon consists of unburned coal, kerogen, coke, soot and charcoal [60] and has a stronger binding capacity for hydrophobic chemicals than organic material from biological origin as amorphous organic matter [61].

Partition-based Dosing

The partitioning of rapidly desorbable compounds between sediment, water and biota is driven by chemical activity and characterized by partition coefficients [62]. This process can be simulated by transferring extracted compounds on equilibrium passive samplers as a surrogate of organic matter and using them for dosing the biotests. The partition process between the organic carbon of the sediment and the aqueous phase is often correlated to the partitioning between octanol and water (K_{OW}) [63-65]. The K_{OW} is easily available for a lot of compounds and thus, a good parameter to predict the partition behaviour.

In the last years several dosing techniques as poly(dimethylsiloxane) (PDMS) films [66, 67], empore disks [68], semipermeable membrane devices (SPMDs) [69] and teflon-coated stirrer bars [70] have been established to control and maintain concentrations of mainly poorly water soluble compounds or sediment extracts in different bioassays. The single compounds or extracts are loaded on the dosing device via different techniques as addition of compounds during film preparation [66, 67], soaking the material with solvent [67, 68, 70, 71] and partitioning from methanol-water mixtures [72, 73]. Partition-based dosing was shown to be successful in maintaining constant concentrations during the whole bioassay and to dose poorly water soluble compounds to bioassays without the need of a solvent carrier.

1.2 Excursus Background Partitioning and concentration measurements

An ideal dosing device is characterized by a fast achievement of the equilibrium and a reliable prediction of the system at equilibrium. In the following paragraph the theoretical background of these two parameters will be given. In this work PDMS was used as material of silicone rods (SR) as dosing device. Thus this excursus is based on this material.

Chemical Potential

The partition process between the PDMS of the SR and the water phase is driven by the chemical potential μ of the compounds in the two phases. The direction of processes like partition and diffusion is always from high to low

chemical potential until equilibrium with equal chemical potential in both phases is reached [74]. The chemical potential is related to the energetically status of the system and depends on the pressure (p), the temperature (T) and the chemical composition (n_j). It reflects the Gibbs free energy (G) added to the system at constant T, P, and composition with each added increment of compound of the individual compound i:

$$\mu_i = \left[\frac{\partial G}{\partial n_i}\right]_{T,p,nj\neq i} \quad \textbf{[75].} \qquad \textbf{(1-3)}$$

The chemical potential cannot directly be measured. Therefore the closely related concepts of chemical activity and fugacity were introduced. Both concepts define a standard chemical potential μ^* as a reference state at a standard pressure of commonly 1 bar. The fugacity as the fleeing tendency from a system can be measured as a pressure for gaseous compounds. In contrast to fugacity the chemical potential is a relative value keyed to a reference state (relative to the compound's escaping tendency from its own pure liquid) and is often used for compounds in aqueous solutions.

$$\mu_i = \mu^* + RT \ln a_i \qquad \textbf{(1-4)}$$

with the gas constant R and the chemical activity (a_i) which is the product of the real concentration (x_i) and the activity coefficient (γ_i):

$$a_i = x_i \gamma_i \qquad \textbf{(1-5)}$$

The activity of a compound is always a relative measure, which is connected to a certain reference state. It was suggested that as reference state the pure liquid of the compound at 298 K and 1 atm is chosen [75]. The concept of chemical activity is not limited to special systems, but can also be applied in systems with high complexity and with a diversity of phases with different properties.

Diffusion Processes

The equilibration time is controlled by the diffusion of the compound in the different media. The diffusion in the PDMS is mainly controlled by molecular diffusion, while diffusion in the water column is mainly turbulent. Thus, the diffusivity between the fully turbulent fluid and the non fluid medium changes

abrupt at the boundary between the two layers. This kind of boundary is defined as Wall Boundary [75]. Between the two phases a third layer the Prantl or water boundary layer exists. This layer is part of the aqueous phase, but in this part no convection occurs and therefore the properties are different to the bulk water phase e.g. the concentration is not evenly distributed, but a gradient occurs (see Figure 1-3). The thickness of this layer depends on the agitation of the system and the viscosity of the liquid. In the ideal case with a perfect agitation the layer is zero.

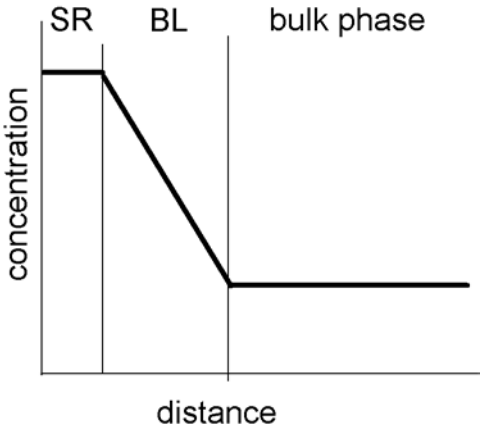

Figure 1-3: Boundary Layer model showing the concentration gradient in a sample containing a Silicon Rod (SR) as dosing device and aqueous test medium. The boundary layer (BL) is a static layer around the SR, where no convection occurs.

The mass transport in a dynamic system with concentration gradients in time and place can be described by Fick's second law of diffusion:

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} \quad \textbf{(1-6)}$$

Where D is the diffusion coefficient and C is the concentration of the analyte. The slowest part controls the overall mass transfer and thus the equilibration time can, in general, be limited by either the diffusion in the polymer, the diffusion through the water boundary layer or the diffusion in the bulk water phase. The diffusion in the bulk water phase can be excluded as the rate limiting step due to our experimental conditions such as turbulence by agitation in a small sample volume. The total transport resistance (R_0) then may be calculated using the following formula [76] and is the sum of the transport resistance in the polymer and in the water boundary layer:

$$R_o = \frac{\delta_W}{D_W} + \frac{\delta_S}{D_S * K_{SW}} \qquad \textbf{(1-7)}$$

where D_w and D_S are the diffusion coefficients in the boundary layer and in the silicone respectively, δ_W and δ_S are the thickness of the boundary layer and the polymer and K_{SW} is the partitioning coefficient between the silicone and water. The log K_{SW} is well correlated with the log K_{OW} of the compounds as shown by several previous works [77-79]. With increasing K_{OW} the influence of the resistance in the polymer decreases and the kinetic of partitioning for compounds above a specific K_{OW} may get dominated by the water boundary layer, while it is dominated by the silicone for compounds with a low K_{OW} [80]. The K_{OW} value for which this shift can be observed depends on the conditions in the test system and compound and polymer specific parameters [81].

Under unstirred conditions it was shown that the diffusion through the boundary water layer is normally the rate limiting step for the partitioning between water and silicone [82], but also for agitated system the water boundary layer was reported to be the limiting step [73, 76].

In equilibrium the activity of the compounds is equal in all of the equilibrated compartments. This situation can then be described by the partitioning coefficient between the aqueous phase and the PDMS:

$$K_{PDMS/W} = \frac{C_{PDMS}}{C_W} \qquad \textbf{(1-8)}$$

SPME Kinetic and Equilibrium Sampling

The knowledge of this coefficient allows estimating water concentrations from the loading concentrations. Evaluation of the equilibrium partitioning includes the need to control and measure the concentration in the test medium e.g. for characterization of the equilibrium situation. Solid phase micro extraction (SPME), invented by Pawliszyn and co-workers, has been shown to be a simple and reliable method to measure concentrations in aqueous samples. This methods uses chemically modified fused silica fibres for adsorption of the analyte together with thermal desorption of the analytes in the inlet of a GC-MS [83]. As an equilibrium sampler SPME is a non exhaustive extraction method.

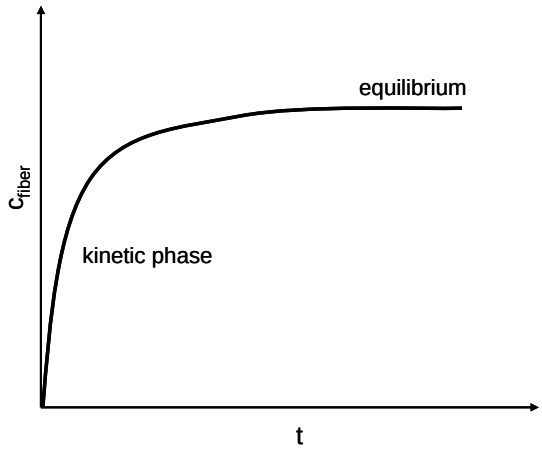

Figure 1-4: Uptake kinetic of analytes in the coating of SPME fibers.

The uptake kinetics (Figure 1-4) can be divided in two phases: the kinetic phase and the equilibrium. The time needed to reach equilibrium depends on the analyte and is in general increasing with increasing molecular size and K_{OW} of the compounds. Thus, for very hydrophobic compounds the fibres are often operated in the kinetic phase to shorten the time of analysis. In this case care has to be taken to hold all parameters (agitation, temperature, vessel geometry), that may influence the up-take kinetics constant. Special care should be taken that the presence of other adsorbing phases do not impact the uptake kinetics [84]. Equilibrium sampling does not have these disadvantages. The equilibrium can be described by the following mass balance:

$$C_0 V_S = C_f^{\infty} V_f + C_h^{\infty} V_h + C_s^{\infty} V_s \qquad \textbf{(1-9)}$$

Where C_0 is the initial concentration of the analyte in the sample, C_f, C_h and C_s are the equilibrium concentrations of the analyte in the coating of the fiber, in the headspace and in the sample. V_f, V_h and V_s are the volume of the fiber coating, the headspace and the sample. The amount n adsorbed by the fiber at equilibrium can be described by:

$$n = C_f^{\infty} V_f \qquad \textbf{(1-10)}$$

Using the partitioning coefficient between the fiber coating and the headspace ($K_{fh} = C_f^{\infty} / C_h^{\infty}$) and between the sample and the headspace ($K_{hs} = C_h^{\infty} / C_s^{\infty}$), equation 1-10 can be converted to

$$n = \frac{K_{fh} K_{fs} V_f V_s C_0}{K_{fh} K_{fs} V_f + K_{hs} V_h V_s} \quad \textbf{(1-11)}$$

Where n is the extracted amount of the analyte, which is adsorbed in the coating. If the headspace is sufficiently small the partition to the headspace can be neglected and the equations can be written as:

$$n = \frac{K_{fs} V_f V_s C_0}{K_{fs} V_f + V_s} \quad \textbf{(1-12)}$$

When the partition coefficients of the compounds are known it is possible to calculate the aqueous concentration in the sample from the detected amount in the fibre.

1.3 Objectives of the study

While partition-based dosing to date focused on individual chemicals only in one study more complex samples such as sediment extracts were dosed [69]. The present thesis is the first attempt to apply partition-based dosing in EDA. Thus, the aim of this work is to develop a method to include the bioavailability in the EDA of sediment by simulation of the partition process between sediment and the surrounding aqueous phase with a dosing device. As test organism algae were chosen, because algae play an important role as primary producers in the aquatic food web and benthic algae communities are strongly affected by contaminations. Green algae are besides cyanobacteria, diatoms and red algae one of the dominant taxon of benthic algae in freshwater habitats [25]. The relevance of algae is also reflected in the regulation of chemicals as the new European chemical law REACH, which chose algae together with fish and daphnia as test organisms to investigate the impact of chemicals on the aquatic environment.

The development and usage of a partition controlled delivery method includes the need for a simple way of measuring the aqueous concentrations in the sample. The knowledge of exposure is elementary for the assessment of the observed effects. Algae tests are often performed in low volume test vessels or even in microtiter plates. Therefore the demands on analytical methods to measure in such test systems are high: They have to deal with small sample volumes, low concentrations and complex mixtures of

compounds. To allow measurements during the bioassay the procedure should neither disturb nor harm the algae cells. Accordingly the first chapter will deal with the following question:

- **Can the concentrations in the bioassay be measured without affecting the sample?**

As mentioned above several approaches have been developed for partition-based dosing in bioassays. These approaches were designed to control and establish constant concentrations in different bioassays and to delivery hardly water soluble compounds. All of them were only used for a limited set of compounds often with similar physico-chemical properties. Thus, despite this existing work it was necessary to develop and validate a new dosing technique to fulfil the needs for the application in the EDA of sediment samples. This chapter wants to answer

- **What are requirements on a dosing technique used in EDA studies and how can these requirements be realized?**

Sediments from highly industrialized areas contain lipophilic compounds as PAHs and halogenated hydrocarbons as classical sediment contaminants. The extraction of the sediments is changing the bioavailability of the compounds. As explained above the degree of this change is not the same for compounds with different physico-chemical properties. In this chapter the influence of the bioavailability on the results of the EDA study is investigated. The sediments extracts are either dosed to bioassay using conventional solvent dosing as DMSO or using partition based dosing to answer the following questions:

- **Does toxicant identification and prioritisation depend on the dosing technique?**
- **Can the predominance of lipophilic compounds with respects to hazards be confirmed or do other more polar compounds can be identified as key toxicants?**

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Chapter 2

Application of nd-SPME to determine freely dissolved concentrations in the presence of green algae and algae-water partition coefficients

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2.1 Introduction

Effect assessment of chemicals to aquatic organism is commonly based on standardised laboratory toxicity tests. In these biotests the response of a test organism to a chemical at a certain concentration is tested. Most test results are based on nominal rather than actually measured freely dissolved concentrations in the water phase. Actual concentrations may be lower than the nominal concentration. Underlying processes may be adsorption to test vessels and suspended matter, uptake and metabolism by the test organisms, evaporation and degradation of the test compounds [1]. Thus, the use of nominal concentrations may lead to an overestimation of EC_{50} values and thus to an underestimation of the risk chemicals may pose to the environment. It was shown recently by comparing literature values that for example for hexachlorobenzene the LC_{50} decreases by the factor of 20 when the test is evaluated on the basis of freely dissolved concentrations instead of nominal concentrations [2]. Heringa et al. [3] reported that the nominal EC_{50} depends on the serum content in an estrogenicity reporter gene assay, while the EC_{50} based on measured free concentrations are independent from the serum content. These examples underline the need for methods to measure freely dissolved concentrations in biotest systems for a realistic estimate of effect concentrations but also for corresponding estimation of internal dose in the test organisms. We developed such a method for a small volume cell multiplication inhibition test with the green algae *Scenedesmus vacuolatus* often utilised as a standard test organism for phytotoxicity.

Despite the evidence of possibly significant misinterpretations caused by nominal concentration-based biotesting there are only few examples where freely dissolved concentrations have been measured in biotests with green algae. Examples are studies applying analytical concentration control of nitrobenzene, 2-methyl-4-nitroaniline, dinitroamine [4], 3-nitroaniline and diuron [5], a suite of quinolines [6] and several heterocyclic compounds [7] using high performance liquid chromatography (HPLC). This technique requires for a separation of algae and aqueous phase prior to analysis and thus is relatively time consuming. With miniaturization of biotests the effective amounts of toxicants approximate analytical limits of quantification (LOQ) and

challenge analysis. Analysis by HPLC requires the removal of significant volumes from the algal suspensions in order to meet analytical LOQs. Thus, it can only be applied after termination of the biotest rather than for monitoring of exposure during the performance of the test. Solid phase microextraction (SPME) in combination with GC-MS [8] is thought to overcome these problems. The SPME-fibre can be directly inserted into the aqueous samples without further sample preparation. Previous studies proved that SPME techniques actually sample freely dissolved concentrations [9-11]. More recently non depletive solid phase extraction (nd-SPME) has been developed to measure concentrations in samples without disturbing the partition equilibrium in the sample (Heringa and Hermens, 2003). This technique was shown to be well suited to measure concentrations in *in vitro* bioassays [3].

SPME analysis can be performed either at equilibrium conditions or by applying a pre-equilibrium approach [12, 13]. Under equilibrium conditions the concentration in the aqueous sample can be calculated using partition coefficients between water and the fibre coating. To achieve non depletive conditions in small volume test systems, the SPME-fibre is preferably used under pre-equilibrium conditions. Under these conditions external calibration with several standard solutions is required for quantification [12]. The approach assumes that adsorption to glass walls from aqueous calibration standards is negligible. The pre-equilibrium approach has been applied frequently and with satisfying reproducibility [14-16]. Although equilibrium is not reached a linear relationship between the amount sampled by the fibre and thus the GC-MS signal and the concentration in the sample was observed [13].

Analyses using SPME in aqueous suspensions of biological materials are always challenging because of the complexity, the interference with other substances present in the sample and binding of the analyte to biopolymers [13]. The presence of strong binding matrices can result in enhanced uptake kinetics and thus increased analytical signal [17]. Biofouling was identified as reason for a change in the analytical signal either by hindering the uptake of the analytes, which would lead to a decrease of the analytical signal or by an overestimation of the freely dissolved concentration due to analytes adsorbed to the matrix which is bound to the fibre [18]. For volatile compounds the

exposure concentrations can also be measured with headspace SPME avoiding damages of the fibre as it was done for kerosene in an algae test [1].

The aim of this study was to develop and to validate a pre-equilibrium nd-SPME method for monitoring freely dissolved concentrations of pollutants in the presence of green algae *Scenedesmus vacuolatus* and for determination of algal lipid-growth medium partition coefficients of a suite of compounds with log K_{OW} between 2 and 6. The method was tested for possible effects of green algae on the concentration measurement by biofouling of the fibre. For this purpose specially made glass chambers, which provide two half vessels with identical concentrations were used.

2.2 Materials and Methods

2.2.1 Chemicals and Materials

All solvents used are from Merck (Darmstadt, Germany) and of LiChrosolv grade (purity > 95 %). Methyl parathion, prometryn, lindane, methoxychlor, fluoranthene and *p,p'*-DDD were obtained from Riedel de Haen, anthraquinone and benz[*a*]anthracene were bought from Fluka (Basel, Switzerland), N-phenyl-2-naphthylamine was obtained from Aldrich (Seelze, Germany), pyrene from Merck (Darmstadt, Germany) and the PCB 101 from Promochem (Wesel, Germany). The polyacrylate (PA) fibre (coating 85 µm) and the divinylbenzene-carboxene-polydimethylsiloxane (DVB/CAR/PDMS) fibre (coating 50 µm DVB/CAR on PDMS 30 µm), both with a length of 1 cm, were purchased from Supelco (Bellefonte, USA).

Table 2-1: Model substances and their physico-chemical properties.

Chemical	CAS number	Log K_{OW}*	Solubility* in water (mg/L)	Log K_{AW}*
Atrazine	1912-24-9	2.61[1]	34.7	-7.02
Methyl-parathion	298-00-0	2.80[1]	37.7	-5.39
Prometryn	7287-19-6	3.51[1]	33.0	-6.27
Anthraquinone	84-65-1	3.39[1]	1.35	-6.02
Lindane	58-89-9	3.72[1]	7.3	-4.76
N-Phenyl-2-naphthylamine	135-88-6	4.40[1]	6.31	-5.38
Pyrene	129-00-0	4.88[1]	0.135	-3.31
Methoxychlor	72-43-5	5.08[1]	0.1	-5.08
Fluoranthene	206-44-0	5.16[1]	0.26	-3.44
Benz[*a*]anthracene	56-55-3	5.79[2]	0.0094	-3.30
p,*p*'-DDD	72-54-8	6.02[3]	0.09	-3.57
PCB 101	37680-73-2	6.80[1]	0.0154	-2.43

* Epi Suite v.3.20, 2007, U.S. Environmental Protection Agency.[1][19], [2][20], [3][21]

2.2.2 Chemical Analysis

A gas chromatograph (GC, Agilent 6890, Agilent Technologies, Santa Clara, USA) coupled to a mass selective detector (Agilent 5973) equipped with a HP5MS fused silica capillary column (30 m × 0.25 mm i.d., 0.25 µm) was used for chemical analysis. An autosampler (Agilent 7683 Series) was used for injection of liquid samples. The carrier gas was helium at a constant flow of 1.3 mL/min. An aliquot of 1 µL of the sample was injected at pulsed splitless mode at 250 °C. The following oven program was used: 60 °C to 150 °C with a rate of 30 °C/min, 150 °C to 186 °C with a rate of 6 °C/min and from 186 °C to 280 °C with a heating rate of 4 °C/min. The final temperature of 280 °C was held for 7 min. The mass selective detector was operated either in SCAN mode or in selective ion monitoring mode (SIM).

The following oven programs were used for SPME samples: The carrier gas was helium at a constant flow of 1.3 mL/min. When single compounds were measured the following oven program was used: The oven was held for five minutes at 60 °C and heated with a rate of 120 °C/min to the final temperature of 280 °C, which was held for seven minutes. For the recovery experiments the oven was held for five minutes at 60 °C and heated with a rate of 40 °C/min to the final temperature of 280 °C, which was held for eleven minutes. For the nd-SPME experiments the oven was held for five

minutes at 60 °C and then heated with 6 °C min until 250 °C (DVB/CAR/PDMS fibre) or 260 °C (PA-fibre).

2.2.3 Non equilibrium Solid Phase Microextraction

Concentrations in aqueous samples were measured with pre-equilibrium SPME in combination with GC-MS. The fibres were calibrated using external standard calibration. For calibration of the SPME-fibres standard stock solutions of the analytes in methanol were prepared. The standard stock solution was diluted with GB-Medium for green algae (Grimme and Boardman, 1972) to reach different aqueous concentrations. The methanol content of the diluted samples did not exceed 1 %. All calibration measurements were performed without the addition of algae cells in glass vessels. Five concentrations with three replicates were extracted with the SPME-fibre. Attention was paid to keep the conditions during the extraction (temperature, agitation, size of stirrer bars, fibre position and size of used vessels) and the measurement with the GC-MS exactly the same for all standards and the corresponding samples. The resulting peak area of the GC-MS signal was used for calculation of calibration curves.

The fibres were chosen according to results of preliminary experiments as the best compromise between reproducibility and sensitivity. The PA fibre used for N-phenyl-2-naphthylamine, fluoranthene, pyrene, PCB 101, lindane, methoxychlor and benzo[*a*]anthracene and the DVB/CAR/PDMS fibre used for methyl parathion, atrazine, prometryn, *p'p'*-DDD and anthraquinone were loaded for five minutes at 28 °C under agitation with glass covered stirrer bars at 200 r.p.m. in the aqueous sample by inserting the fibre with a special made holder through the lid, which ensures that the fibre is located at the same position in the vessel. The agitation was controlled by a Variomag Telemodul 40 (H+P Labortechnik GmbH, Munich, Germany) The loaded fibre were desorbed in the inlet of the GC-MS for five minutes in the splitless mode at 270 °C (DVB/CAR/PDMS fibre) and 300 °C (PA fibre), respectively.

2.2.4 Algae Cultivation and Harvest

The synchronous cultivation of the green algae *Scenedesmus vacuolatus* (strain 211-15, SAG, Göttingen, Germany) is described elsewhere [22]. At the time of harvest the algae suspension had a density of approximately 1-2

$\times 10^7$ cells/mL. The suspension was diluted with GB-Medium [23], a salt solution without any organic components, to achieve lower cell densities, and the actual cell density was measured using a CASY II particle counter (Schärfe Systems, Reutlingen, Germany). If higher concentrations were needed, the algae suspension was concentrated by centrifugation at 1948 g and 4 °C for three minutes. The alga pellet was separated from the overlying water and re-suspended in GB-Medium. The cell density was also measured using the CASY II particle counter.

2.2.5 Determination of Negligible Depletion

Two standard stock solutions in methanol were prepared containing the following model compounds: solution 1 atrazine (164 mg/L), anthraquinone (13.0 mg/L), methyl parathion (127 mg/L), prometryn (14 mg/L) and *p,p'*-DDD (4.25 mg/L); solution 2 fluoranthene (16.0 mg/L), lindane (134 mg/L), pyrene (6.0 mg/L), methoxychlor (4.5 mg/L), benzo[*a*]anthracene (0.55 mg/L), PCB 101 (1.5 mg/L) and N-phenyl-2-naphthylamine (100 mg/L). The concentrations were chosen according to the water solubility of the compounds. These solutions were diluted with GB-medium with three different dilution factors (100, 200 and 800). The concentration in the solution (V=2 mL) was measured with SPME in combination with GC-MS as it is described above. To investigate whether the extraction with the SPME-fibre was non depletive the same sample was extracted three times and the concentrations were compared. All experiments were done with three replicates per concentration. This way of measuring the depletion was chosen, because it reflects the situation in the bioassay, where concentrations are measured several times in the sample.

2.2.6 Determination Limit of Quantification (LOQ)

The limits of quantification were calculated from calibration data according to Danzer [24] with a level of significance ($p<0.05$) and a relative accuracy of analysis of 33.3 %. The LOQ refers to the nominal concentrations in the GB-medium in the standard solutions used for the calibration. The limit of quantification was calculated using the following formula:

$$x_{LOQ} = k_u \times s_{x0} \times t_{1-\alpha,\nu} \times \sqrt{\frac{1}{N} + \frac{1}{n} + \frac{(x_{\min} - \bar{x}_i)^2}{S_{xx}}} \qquad \textbf{(2-1)}$$

Whit x_{LOQ}: limit of quantification, k_u: relative uncertainty of analysis, set as 3 (33.3 %), s_{x0}: process standard deviation, $t_{1-\alpha,v}$: student factor for the chosen level of significance (P=0.95), N: number of replicates, n: number of calibration levels, x_{min}: minimal value of signal, near the expected detection limit, $x_{i:}$ mean value., S_{XX}: Sum of Squared Deviations.

2.2.7 Influence of Fouling

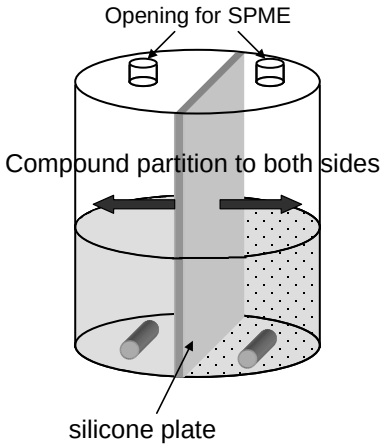

Figure 2-1: Double chamber scheme. Compounds loaded on the Silicone-plate partition to both sides. algae suspension Grimme-Boardmann growth medium.

The aim of this experiment was to investigate if the presence of the algae influences the concentration measurement. A special glass chamber with two identical halves (Figure 2-1) was used to have two sample aliquots with identical concentrations. The two halves of the chamber are divided by a plate of silicone. The plates were obtained from Good Fellow (Friedberg, Germany) and are made of poly(dimethylsiloxane) with a thickness of 3 mm. These plates were loaded with compounds to deliver them into the two halves and provide identical and constant concentrations. For loading, the plates were soaked with 7 mL of a solution of prometryn (49 mg/L), methoxychlor (515 mg/L), atrazine (100 mg/L), PCB 101 (1 g/L) or N-phenyl-2-naphthylamine (860 mg/L) in hexane over night in custom-made glass tubes (Figure 2-2). These loads were selected in order to obtain similar concentrations in the aqueous solutions compensating for the differences in the silicone water partition coefficients. The silicone swelled during the loading process and the compound was distributed in the material. The plates were dried in an oven with circulating air at 30 °C for 4 h to evaporate the hexane. The silicone plate was fixed in the middle of the two halves and

each half was filled with 7.5 mL GB-Medium together with a glass covered stirrer bar. To achieve equilibrium partitioning between the silicone plates and the aqueous phase the solution was stirred for 24 h. After that 1 mL GB-Medium was added to one side, while to the other side 1 mL algae suspension was added. Every experiment was done with four different concentrations. Both halves were analyzed with nd-SPME and GC-MS six times as described above. The peak areas of compound signal were compared for the two halves.

Figure 2-2: Picture of chamber for loading siloxane-plates with organic compounds.

2.2.8 Mass Balances

The aim of the following experiment is to verify whether a simple extraction method is suited to determine the amount accumulated in algae. Mass balances were established for five replicates including the freely dissolved concentration, the amount accumulated in algae, as well as fractions that adsorbed to the glass test vessel. Mass balance experiments were performed with N-phenyl-2-naphthylamine, methoxychlor, PCB 101, prometryn and atrazine in three replicates using 20 µL (c = 100 mg/L N-phenyl-2-naphthylamine, 87 mg/L methoxyhlor, 5 mg/L PCB 101, 100 mg/L prometryn and 100 mg/L atrazine) standard stock solutions in dimethylsulfoxid (DMSO) added to 20 mL of algae suspension (atrazine and promethryn: 0.95×10^9 cells/mL, N-phenyl-2-napththylamine, methoxychlor and PCB 101: 2×10^7 cells/mL) and stirred for 2 h in amber vials. Free aqueous concentrations in the GB-medium were measured with GC-MS after extraction with SPME externally calibrated with 20 mL standard solutions. The algae suspensions were double filtered using a Büchner flask with a glass fibre filter (GF/F, Whatman, Maidstone, England). The filters containing the algae cells were washed with bi-distilled water, freeze dried and extracted

under ultrasonication three times with acetone at 50 °C for 15 min and three times with methylene chloride for 15 min at room temperature. The extracts for each sample were combined and evaporated until dryness under a nitrogen stream. The residue was re-dissolved in 50 µL of toluene and measured with GC-MS as described above. After use, glass vessels were dried at 40 °C and rinsed with methylene chloride to determine the amount, which adsorbs to the glass surface. The methylene chloride was evaporated until dryness under a gentle nitrogen stream and the residue was re-dissolved in 50 µL toluene. The concentrations in the extracts were measured with GC-MS as described above. The possible losses due to evaporation to air during the incubation were estimated using the air-water partition coefficients of the compounds (Table 2-1).

2.2.9 Algae-GB-Medium Partition Coefficient Determination

For the determination of partition coefficients, algal suspensions were prepared with cell densities adapted to the selected chemical and expected partition coefficient in order to ensure a freely dissolved concentration above the nd-SPME GC-MS detection limit for the individual compound. Cell densities were 5×10^8 cells/mL for the experiment with atrazine, 3×10^8 cells/mL for prometryn, 2×10^7 cells/mL for N-phenyl-2-naphthylamine, 7×10^6 cells/mL for methoxychlor and 6×10^5 cells/mL for PCB 101. Aliquots of 100 µL of the suspension were used for the control of cell density and the mean cell volume with the CASY II, while the rest was subjected to partition experiments by spiking with standard stock solutions of the chemicals above (DMSO/water 1/1000). All experiments were performed in five replicates with 20 mL of sample volume. Amber vials were used to prevent photodegradation of the compounds and algal growth. The mixture was stirred for 2 h at 200 r.p.m. and 28 °C. Earlier experiments have shown that this time is sufficient to achieve equilibrium partitioning between the aqueous phase and the algae. Freely dissolved concentration and concentrations in algae were determined as described above in the mass balance section. The measured concentrations in the extracts were converted to concentrations in the algae by using the cell density and the lipid content. The partitioning coefficient was calculated using the following formula (c_A= concentration in the algae, c_{GB} = measured free concentration in aqueous GB-Medium):

$$K_{AGB} = \frac{c_A}{c_{GB}} \qquad \textbf{(2-2)}$$

2.3 Results and Discussion

2.3.1 Non-Depletive Solid Phase Micro Extraction

In order to allow for concentration monitoring in the algae test without disturbing the system a pre-equilibrium non-depletive extraction method was established. There is no generally agreed maximum extracted percentage justifying the use of the term non depletive. The percentage may vary between 1 and 10 % and should be set according to the requirements of the experiment [25]. We set the limit of 7.5 % as a reasonable compromise between not disturbing the system and extracting a sufficient amount for proper detection of the compounds. This setting allowed for two extractions during the exposure time with a maximum depletion of 15 % and is in the range set by the OECD, which requires a concentration stability of ± 20 % [26].

Standard solutions with three different concentrations were extracted three times each with a SPME-fibre and the resulting concentrations were compared to assess if non-depletive conditions according to the criteria defined above were met (Figure 2-3). In most of the cases the extractions fulfil the requirements for a non depletive extraction. A trend to fail non-depletion criteria was observed for N-phenyl-2-napththylamine, methoxychlor, pyrene, benz[*a*]antracene and PCB 101 after two or three extractions although most of these deviations are still within the analytical error of the method. The percentage of an analyte taken up by the fibre should be independent of the aqueous concentration. Thus, failure of non-depletion observed only for one of three concentrations such as for *p,p'*-DDD, pyrene and N-phenyl-2-naphthylamine was probably due to analytical errors rather than to systematic problems.

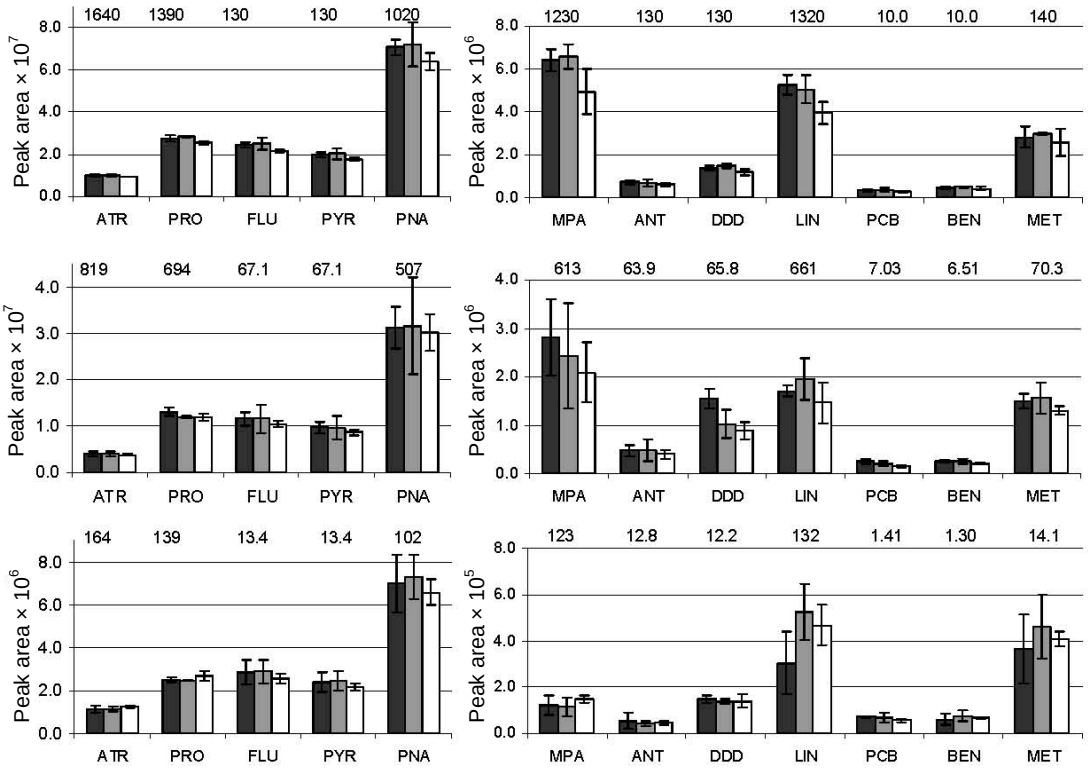

Figure 2-3: Measured medium concentrations (n = 3) of consecutive SPME measurements for three different sample concentrations (µg/L). The samples were extracted three times. ■ first extraction ■ second extraction □ third extraction. ATR (atrazine), PRO (prometryn), MPA (methyl parathion), ANT (anthraquinone) and DDD (p'p-DDD) were measured with a DVB/CAR/PDMS fibre. LIN (lindane), FLU (fluoranthene), PYR (pyrene), PCB (PCB 101), PNA (N-phenyl-2-naphthylamine), BEN (benz[a]anthracene) and MET (methoxychlor) were measured with a PA fibre.

2.3.2 LOQ

A comparison of the limit of quantification (LOQ) of our SPME-method and the EC_{50} for green algae for the model compounds indicates that even for compounds with a specific mode of action and a low EC_{50} (30 µg/L) value such as prometryn and N-phenyl-2-napththylamine the LOQ was smaller by about a factor of 10 (Table 2-2). This shows that nd-SPME is suitable for concentration control over whole dose-response curves in cell multiplication assays with green algae. The amount extracted by the fibre varies for every combination of compounds and fibre types and cannot be generalized. Thus, the non-depletion criterion has to be tested for every individual compound. However, the general suitability of the method to measure concentrations in small volume test vessels has been demonstrated using a broad set of compounds as examples.

Table 2-2: EC_{50} for green algae and the limit of quantification (LOQ) of the SPME-method for selected model compounds. The LOQ refers to the freely dissolved concentration of the compound in the GB-medium.

compound	EC_{50} µg/L	LOQ µg/L
Atrazine	25.9 [a]	2.76
Prometryn	30.2 [b]	5.16
N-phenyl-2-naphthylamine	30.6 [c]	4.60
Fluoranthene	36.4 [d]	5.95
Lindane	1396 [d]	3.61
Methyl parathion	8160 [e]	95.0

[a] [27], [b] [28], [c] [29], [d] [30], [e] [22]

2.3.3 Impacts of Algae Cells on SPME

The impact of algae on SPME was determined in double chambers separated by a silicone-plate loaded either with prometryn, PCB 101, N-phenyl-2-naphthylamine, methoxychlor or atrazine. One half contained algae suspension, while the other contained only the growth-medium. No significant differences between the two treatments were observed for most experiments (Figure 2-4). Only for PCB 101 (1.4×10^7 cells/mL and 7.5×10^4 cells/mL) the peak areas of the treatment with algae were smaller than without algae. For N-phenyl-2-naphtylamine at a cell density of 1.0×10^6 cells/mL algae significantly enhanced concentrations relative to the pure medium. The observed differences for the PCB 101 might be explained by non-equilibrium conditions at high cell densities due to rapid partitioning from the medium into the algae cells that could not be compensated rapidly enough by delivery from the silicone plate. PCB 101 has the highest log K_{OW} (Table 2-1) of the used compounds and therefore the expected bioaccumulation is greater than for other compounds.

In general, reduced analytical signals in SPME-based approaches may be due to damages of the fibre by biofouling by attached algae cells. This phenomenon was observed when human blood plasma was investigated using SPME fibres. The formation of a protein coating could be visibly observed and led to a reduction of extraction reproducibility [31]. To detect effects like this in our experiment, the concentrations in the two halves (one with, one without algae) were measured alternately. In case of fibre damages the concentration measurement in both halves should be affected and the reduction in reproducibility should be visible as increasing standard deviations with increasing algae density.

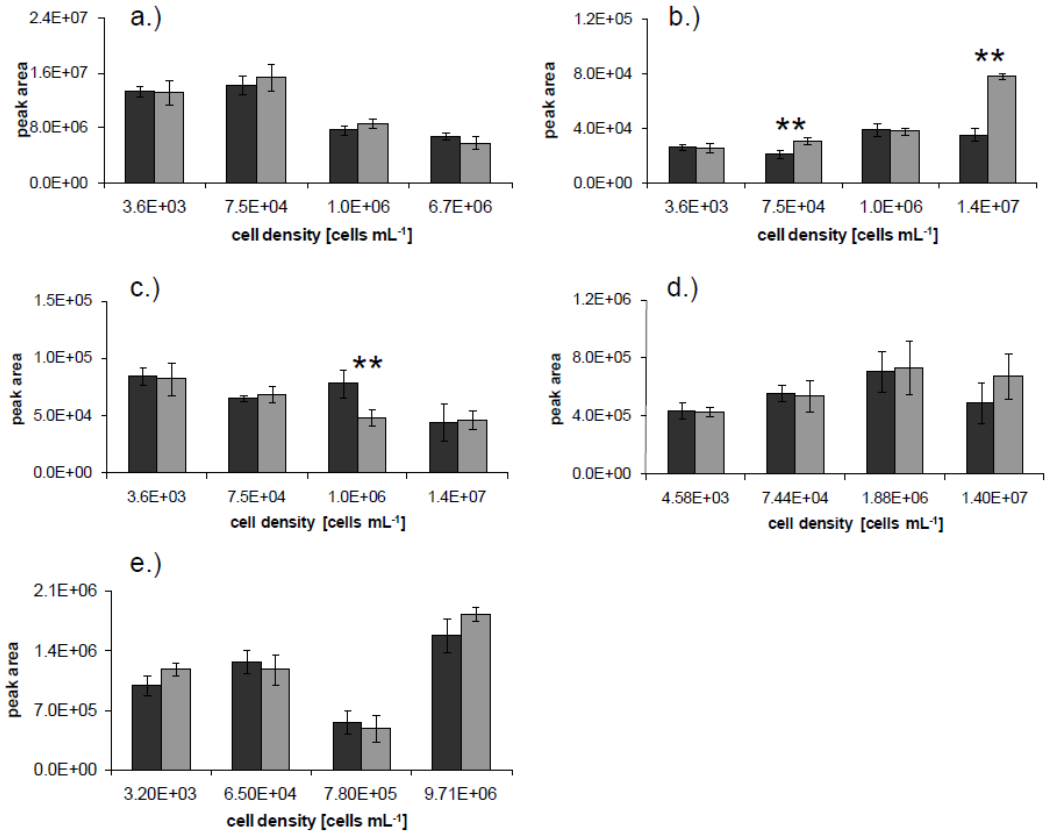

Figure 2-4: Peak areas of the MS-signal (abundance) with and without algae of a.) prometryn, b.) PCB 101, c.) N-phenyl-2-naphthylamine, d.) methoxychlor, and e.) atrazine. The measurements were performed in double chambers divided by a poly(dimethylsiloxane)-plate, which dose identical concentrations of the test compound to the two halves. One half contains algae suspension ■, while the other half was filled with growth medium without algae □. ** Significant difference between the two treatments; $p < 0.01$, Wilcoxon-Mann-Whitney test.

However, the results do not show such a trend (Figure 2-4) and therefore we suggest that biofouling is not the cause for the differences in concentrations between the two treatments in the PCB 101 experiment. Another possible impact of the algae cells might be enhanced kinetics for the uptake in the SPME fibres due to the presence of an additional binding matrix. A previous study showed that the release of pyrene from SPME fibres is facilitated when bovine serum albumin (BSA) as binding matrix is present [17]. If the enhanced kinetics caused the increase in the N-phenyl-2-napthylamine signal, this effect should become even more pronounced for the highest algae densities. In our experiment we do not find these algae density depending deviations in concentration measurements and therefore we suggest that this mechanism does not play a significant role in our test system. Kramer and co-workers also state that the data found in the literature are rather unclear with some studies showing an influence on the kinetics

while others do not. With our experiment it is not possible to distinguish between the different mechanism as biofouling or enhanced kinetics, but the overall effect can be examined. As the aim of this study is to proof the usefulness of the nd-SPME method for concentration measurement in an algae assay and not the exploration of the mechanisms, the overall effect is exactly that what we need. In conclusion the experiments suggest the SPME-method as a reliable method to measure the freely dissolved concentration of the compounds in medium of the alga assay.

2.3.4 Mass Balances

The mass balances of partitioning experiments indicate total recoveries of 72.5 (± 10.6) % for prometryn and 84.9 (± 9.27) % for atrazine, respectively (Table 2-3). For methoxychlor and PCB 101 recoveries were slightly greater than 100 % (113 ± 7.8 % and 128 ± 14.1 %). Only for N-phenyl-2-napththylamine the recoveries were low with 40.9 (± 5.93) %. The mass balances indicate that adsorption to the glass vessels and the Büchner flask played a minor role in our experiment and did not disturb the calculation of the partition coefficients between the algae and the aqueous phase. Only for methoxychlor the amount, which adsorbed to the glass surface of the vessels (12 ± 5.17 %) was not negligible.

Table 2-3: Mass balance with recoveries [%] for N-phenyl-2-napththylamine (PNA), methoxychlor (MET), PCB 101 (PCB), prometryne (PRO) and atrazine (ATR).

	PNA	MET	PCB	PRO	ATR
water	32.5 (± 5.2)	79.6 (± 6.1)	93.6 (± 9.4)	72.5 (± 10.6)	84.9 (± 9.3)
glass vessel	n.d.	12.0 (± 5.2)	n.d.	n.d.	n.d.
algae	8.33 (± 2.4)	21.6 (± 4.7)	34.2 (± 5.0)	6.34 (± 0.7)	4.53 (± 0.7)
Sum recovered	**40.9 (± 5.9)**	**113 (± 7.8)**	**128 (± 14.1)**	**79.6 (± 9.8)**	**90.8 (± 9.9)**

n.d. not detected

Evaporation to the headspace can also effect the free concentrations. We worked with closed vials and, thus the losses were estimated using air-water partition coefficients (Table 2-1). It should be noted that partition behaviour between air/GB-medium is not exactly the same as between air/water, but this calculation still allows a reasonable estimation. Losses were always smaller than 1.2 % (Table 2-4) and were therefore regarded as insignificant.

Table 2-4: Model substances, the concentration in water and the consequential calculated concentration in air using the air/water partition coefficients from table 2-1. The absolute amount in water (M_{H20}) and in air (M_{air}) was calculated using the volumes of the two phases (V_{H20}= 2 mL; V_{air}= 6 mL). The percental losses in the gaseous phase are based on the absolute amounts in the two phases.

Chemical	C_{H20} mg/L	C_{Air} mg/L	M_{H20} µg	M_{Air} µg	Air %
Atrazine	2.18	1.92E-09	4.36	1.26E-06	2.89E-05
Methyl-parathion	1.69	6.91E-06	3.38	4.15E-05	1.23E-03
Prometryn	0.19	1.00E-07	0.37	6.03E-07	1.62E-04
Anthraquinone	0.17	1.66E-07	0.35	9.98E-07	2.88E-04
Lindane	1.78	3.13E-05	3.56	1.88E-04	5.26E-03
N-phenyl-2-naphthylamine	1.33	5.60E-06	2.66	3.36E-05	1.26E-03
Pyrene	0.08	3.88E-05	0.16	2.33E-04	1.46E-01
Methoxychlor	0.21	4.97E-07	0.12	2.98E-06	2.49E-03
Fluoranthene	0.21	7.71E-05	0.43	4.63E-04	1.09E-01
Benz[*a*]anthracene	0.01	3.63E-06	0.02	2.18E-05	1.49E-01
p,*p*'-DDD	0.06	1.52E-05	0.11	9.15E-05	8.09E-02
PCB 101	0.02	7.34E-05	0.04	4.41E-04	1.10

2.3.5 Partition Coefficients

The bioaccumulation is often related to the lipid content of the organisms and therefore the partition coefficient refers to the lipid phase of the organism. The total lipid content of *Scenedesmus vacuolatus* has been determined as 11.5 % of the total volume [32]. Thus, we determined the average cell volume and corrected it for the lipid content. The logarithmic partitioning coefficients for the model compounds between the algal lipids and the GB-medium K_{AGB} range between 1.3 and 4.5 and are well correlated with the log K_{OW} of the compounds (R^2 = 0.885, Figure 2-5, for exact values of K_{AGB} Table 2-5).

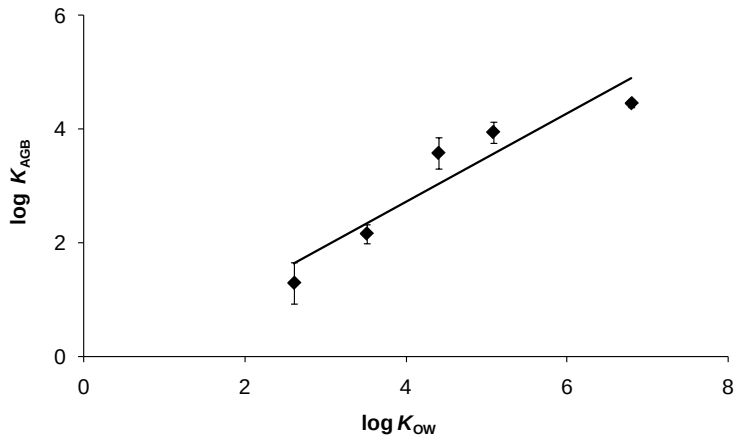

Figure 2-5: Correlation between K_{OW} and the partition coefficient between lipid of the algae and the aqueous phase (K_{AGB}) (y = 0.776 (± 0.163) ×x – 0.388 (± 0.764)) R^2 = 0.885.

A partition coefficient between algae and water greater than the K_{OW} was suggested to indicate that non-lipid compartments (e.g. cellulose of the cell membranes) contribute to absorption (Swackhamer and Skolglund, 1993). In our study K_{AGB} values are always smaller than K_{OW}. Thus, we found no evidence for a significant contribution of non-lipids to the accumulation of the investigated compounds in algae. Comparable studies are quite scarce. Under comparable conditions the bioaccumulation in a former study with the green algae *Scenedesmus sp.* based on lipid content is well in agreement with our results [33]. Others used partition coefficients based on dry weight and found species-specific bioaccumulation [34]. Algal growth can influence the partitioning of the compounds by dilution of the compounds [35]. We used amber vials for the uptake experiments to prevent this. Non-depletive SPME together with analysis of lipid based concentrations in algae proved to be suitable to determine algae/water partition coefficients for an improved evaluation of dose-effect relationship in green algae.

Table 2-5: Log K_{OW} of compounds and the logarithm of the partitioning coefficient between algae and water log K_{AGB}.

Chemical	Log K_{OW}*	Log K_{AGB}
Atrazine	2.61	1.29
Prometryn	3.51	2.16
N-phenyl-2-naphthylamine	4.40	3.58
Methoxychlor	5.08	3.95
p,*p*'-DDD	6.02	4.45

* Epi Suite v.3.20, 2007, U.S. Environmental Protection Agency.

2.4 Conclusions

A pre-equilibrium nd-SPME method for exposure monitoring in a miniaturized cell multiplication inhibition test is presented and uptake into SPME fibres proved to be actually non-depletive. The LOQs were by a factor of about 10 smaller than the EC_{50} of compounds with a specific mode of action and therefore whole dose-response curves can be investigated using pre-equilibrium nd-SPME. The presence of algae cells does not influence the concentration measurement by damaging the fiber or influencing the partitioning between fiber and aqueous phase. The results indicate that nd-SPME is a suitable method to determine partition coefficients and to monitor real exposure concentrations in cell multiplication inhibition tests with *Scenedesmus vacuolatus*. The labor-intensive and time consuming manual SPME method might gain further acceptance by automation, which was recently reviewed by Risticevic et al. [36] including other advancements such as multi fiber SPME autosampler in a multi-well plate format. Due to its high flexibility the method can be also used in other biotest systems (e.g. well plates) as it was done by Heringa and coworkers [3].

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Chapter 3

Partitioning based dosing – an approach to include bioavailability in the effect directed analysis of contaminated sediment samples

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3.1 Introduction

Sediments are a major sink for a multitude of environmental pollutants, including lipophilic organic compounds. Particulate organic matter and organic coatings on mineral particles play a dominant role for the accumulation of these compounds in sediments. However, contaminated sediments may also act as a source of contamination for water and biota and may cause adverse effects to benthic organisms. According to the equilibrium partitioning approach [1], the exposure of benthic organisms to sediment-bound contaminants and thus bioavailability of these compounds may be seen as the result of equilibrium partitioning between organic matter, water and biota, driven by activity gradients [2, 3].

Sediments in densely populated and industrialized areas are often contaminated with complex mixtures of compounds rather than with single well-known toxicants. Deciding which of these compounds may pose a major risk to benthic organisms is not a straight-forward procedure. Toxicity Identification Evaluation (TIE) and Effect Directed Analysis (EDA) have been developed to identify major toxicants in complex mixtures [4-9]. Contaminated sediments and extracts thereof are subjected to a procedure combining biotesting, fractionation and chemical analysis to sequentially reduce the complexity of the mixture and finally to isolate and identify the toxicants that cause the measured effects. This approach helps to focus on ecotoxicologically-relevant compounds rather than pre-defined priority pollutants of concern.

A major shortcoming of EDA procedures of sediment extracts is the absence of a realistic exposure scenario. Exhaustive solvent extraction and dosing of extracts and fractions in organic solvents to biological tests attempt to expose the test organisms to the entire amount of sediment-bound toxicants independent of their physico-chemical properties and partitioning behaviour. This may result in a shift in freely dissolved mixture composition between sediment pore water and test medium and in decreasing exposure during the run of a bioassay due to adsorption, evaporation or transformation processes. Pore water concentrations reflect activity-driven partitioning with lower proportions of lipophilic compounds in the water phase compared with

less lipophilic compounds. Conventional dosing of mixtures in biological tests fails to maintain these proportions and tends to overestimate the hazards due to lipophilic toxicants.

Thus, the development of bioassay and EDA procedures for sediment-associated toxicants based on more realistic exposure conditions are a major challenge towards higher relevance of EDA for hazard and risk assessment of sediment-associated contaminants. In the last decade, first attempts to involve partitioning behaviour in the development of new dosing techniques have been made using lipophilic solid phases such as polydimethylsiloxane (PDMS) films [10, 11], empore disks [12], semipermeable membrane devices (SPMDs) [13] and teflon-coated stirrer bars [14]. In this paper, a cost-effective and promising alternative is presented using silicone rods (SRs) equipped with a magnetic wire, which can be used as a stirrer bar. The SRs combine a fast achievement of equilibrium for a broad range of compounds with a high carrying capacity in the solid phase for quick compensation for losses due to adsorption, volatilization, transformation and uptake by test organisms and to provide constant concentrations during the biotest [10]. PDMS/water and silicone/water partitioning coefficients are well correlated with log K_{OW} [15-17], suggesting a reasonable simulation of partitioning processes in sediments. The technique was applied and validated for dosing in a growth inhibition test with the green algae *Scenedesmus vacuolatus.* The applicability in EDA studies was shown for three sediment extracts separated with a novel automated multistep fractionation procedure developed recently [18]. A clear shift in toxicity ranking of fractions compared to conventional dosing underlines the need for partition-based dosing in EDA.

3.2 Material and Methods

3.2.1 Chemicals

All solvents were purchased from Merck (Darmstadt, Germany) and were of LiChrosolv grade (purity ≥ 99.8 %). The following abbreviations are used: dimethylsulfoxide (DMSO), acetonitrile (ACN) and methanol (MeOH). All other chemicals used (see Table 3-1) were of analytical grade. The chemicals were chosen to cover a broad range of K_{OW} and different chemical structures. All compounds have been identified as relevant toxicants in

previous studies on sediments from Bitterfeld [5], which were selected as one of the sampling sites in the present study.

Table 3-1: Model substances and their physico-chemical properties.

Chemical	CAS number	Log K_{OW}*	Solubility* in H_2O (mg/L)	Supplier
Methyl-parathion	298-00-0	2.80	37.7	Riedel de Haen
Promethryn	7287-19-6	3.51	33.0	Riedel de Haen
Anthraquinone	84-65-1	3.39	1.35	Fluka
Lindane	58-89-9	3.72	7.3	Riedel de Haen
N-phenyl-2-naphthylamin	135-88-6	4.40	6.31	Aldrich
Pyrene	129-00-0	4.88	0.135	Merck
Methoxychlor	72-43-5	5.08	0.1	Riedel de Haen
Fluoranthene	206-44-0	5.16	0.26	Riedel de Haen
Benz[*a*]anthracene	56-55-3	5.76	0.0094	Fluka
p,*p*'-DDD	72-54-8	6.02	0.09	Riedel de Haen
PCB 101	35065-28-2	6.22	0.0154	Promochem

* Epi Suite v.3.20, 2007, U.S. Environmental Protection Agency.

3.2.2 GC-MS

An Agilent 6890 GC coupled to an Agilent 5973 mass selective detector equipped with a HP5MS fused silica capillary column (30 m x 0.25 mm i.d., 0.25 µm), all from Agilent Technologies (Böblingen, Germany), was used. Concentrations in aqueous samples were measured using solid phase microextraction (SPME) in combination with the GC-MS. The polyacrylate fibre (PA) used for N-phenyl-2-naphtylamine, fluoranthene, pyrene, PCB 101, lindane, methoxychlor and benzo[*a*]anthracene and the divinylbenzene-carboxene-polydimethylsiloxane (DVB/CAR/PDMS) fibre used for methyl-parathion, prometryn, *p,p'*-DDD and anthraquinone were purchased from Supelco (Bellefonte, USA). The fibres were loaded for five minutes at 28 °C and 200 r.p.m. in the aqueous sample and then transferred to the inlet of the GC-MS. The loaded SPME-fibres were desorbed in the inlet of the GC-MS for five minutes in the splitless mode at 270 °C (DVB/CAR/PDMS fibre) and 300 °C (PA), respectively. The carrier gas was helium at a constant flow of 1.3 mL/min. The oven was heated from 60 °C to 280 °C with a rate of 120 °C/min, to the final temperature of 280 °C, which was held for seven minutes. Solid phase microextraction based analysis was calibrated with external standards dissolved in methanol and diluted with GB-medium to 0.1 % solvent content. In order to avoid any disturbing of equilibration

processes non-depletive extraction in the kinetic phase for 5 min at a stirring velocity of 200 r.p.m. and a temperature of 28 °C was applied. For all standard compounds (see table 1) extracted fractions remained below 7.5 %, which was taken as a criterion for non-depletive extraction.

For liquid samples an Agilent 7683 Series Autosampler (Böblingen, Germany) was used. The carrier gas was helium at a constant flow of 1.3 mL/min. 1 µL of the sample was injected using pulsed splitless mode at 250 °C. The following oven program was used: 60 °C to 150 °C with a rate of 30 °C/min, 150 °C to 186 °C with a rate of 6 °C/min and from 186 °C to 280 °C with a heating rate of 4 °C/min. The final temperature of 280 °C was held for 7 min. The mass selective detector was operated either in SCAN mode or in selective ion monitoring mode (SIM).

3.2.3 Silicone Rods

The silicone rods (d = 5 mm) were purchased from Good Fellow (Friedberg, Germany). The silicone rod was cut into cylindrical pieces of 0.84 ± 0.01 cm length and magnetic Cr-Ni-steel wires (d = 0.2 mm) (Svenska Stållinor, Sweden) were inserted in the middle of these rods so that they could be used as stirrer bars with a dosing opportunity. The rods prepared as described above are called SRs and have a volume of 165 ± 3.5 µL. The SRs were cleaned three times with ACN:MeOH (80:20) for 15 min in an ultrasonic bath and were heated in the oven at 240 °C in a nitrogen stream overnight. During loading they were soaked overnight in 150 µL solution of the sample in hexane. During this process the silicone of the rods swells and the compounds are evenly distributed in the polymer. The loaded SRs were dried for 75 min under a nitrogen stream to evaporate the hexane. SRs used in the bioassays were dried for 15 min under a nitrogen stream and 2 h at 30 °C in an oven with circulating air to ensure that the hexane was completely evaporated. This modification was necessary, as drying with nitrogen alone resulted in traces of hexane remaining in the rods. This did not interfere with partitioning but resulted in the occasional occurrence of toxic blanks.

3.2.4 Loading Efficiency

Five SRs were loaded with 150 µL of a mixture containing methyl-parathion, N-phenyl-2-naphthylamine, *p,p'*-DDD and prometryn (c = 24 mg/L, 30 mg/L,

34 mg/L and 23 mg/L, respectively) in hexane as described above. The loaded and dried SRs were extracted by stirring them three times in 4 mL ACN/MeOH (80:20). The extracts of one SR were combined and evaporated under a nitrogen stream until dryness and the residue was redissolved in toluene. Aliquots of the loading solutions, which were treated analogous to the extracts, were used as reference for the concentration measurement. All samples were analyzed using GC-MS, as described above.

3.2.5 Depletion Kinetics

SRs loaded with single compounds were stirred in 2 mL Grimme-Boardmann medium (GB-medium) for green algae [19] at 200 r.p.m. and 28 °C for 0.5, 1, 2, 4, 7.5, 15, 30, 60, 180, 360 and 1440 min. All experiments were performed with three replicates. The stirring conditions were chosen to be equal to the conditions in the bioassay with the green algae *Scenedesmus vacuolatus.* The SRs were replaced by a stirrer bar covered with glass after reaching the desired depletion time and the concentration in the water phase was measured with SPME as described above. Exponential functions ($c_t = c_{eq}*(1-e^{-kt})$) [10] were fitted to the resulting kinetics using the software Origin 7.5 G (Origin lab corporation, Northampton, USA). C_t is the concentration in the water and t is the time. Since the kinetics show an asymptotic progression towards equilibrium, the equilibrium time was set at the time, when 99 % of equilibrium concentration (c_{eq}) is reached. For determination of the partitioning coefficient between silicone and water, the SRs were extracted three times with ACN/MeOH (80:20). The extracts of one SR were combined, evaporated under nitrogen until dryness and the residue was re-dissolved in toluene. The concentrations of the compounds were analyzed using GC-MS. The partitioning coefficient (K_{SW}) was then calculated using the following formula:

$$K_{SW} = \frac{c_S}{c_w} \qquad \textbf{(3-1)}$$

c_S is the concentration in the SR and c_W is the concentration in the water phase.

3.2.6 Toxicity Test

The unicellular green algae *Scenedesmus vacuolatus* (strain 211-15, SAG, Göttingen, Germany) was cultivated as described elsewhere [19]. In this study the test compounds were dosed into the test system either dissolved in DMSO or by loading them onto SRs. In the test system, loaded SRs were stirred for 24 h with 200 r.p.m. in 1.95 mL GB-medium at 28 °C in the dark to reach equilibrium between the silicone and the aqueous phase. By adding 50 µL algae suspension (cell density approx. $3x10^6$ cells/mL) to a total volume of 2 mL, an initial cell density of approx. $7.5x10^4$ cells/mL for both the SR and the DMSO-dosed test was established. As negative control samples, SRs loaded with pure hexane were used. For the DMSO dosing DMSO controls were used. All other test conditions were equal for both dosing techniques and have been described by Altenburger et al. [19]. The algal growth was determined by measuring the fluorescence at a wavelength of 685 nm (Backscat Fluorometer, Haardt, Kiel) at the beginning (t = 0) and the end of the test (t = 24 h). For fractions with high natural fluorescence, which can lead to false results in the algae assay, fluorescence controls analogous to the normal samples by replacing the algae suspension with culture medium were prepared.

3.2.7 Concentration Stability during 24 h

The concentrations in the algae test were measured during 24 h for both dosing techniques. The samples were prepared and incubated as described above. The concentration in the aqueous phase was measured with SPME in combination with GC-MS (see above) at t = 0, 2, 4, 6, 12 and 24 h. Previous experiments with the standard compounds as listed in table 3-2 have shown that the selected extraction time of 5 min does not change sample concentrations by more than 7.5 % and thus considered as non-depletive. For all standard compounds after 5 min extraction is still in the kinetic phase and far from equilibrium.

3.2.8 Sampling and Extraction

The sediment samples were taken at the Elbe River and its tributaries with an Ekman-Birge-grab sampler. Sampling locations were Přelouč (Elbe, Czech Republic, coordinates UTM: N 5543600, E 541124, May 2006), Most (Bilina,

Czech Republic, coordinates UTM: N 5595617, E 406603, July 2006) and Bitterfeld (Spittelwasser, Germany, coordinates UTM: N 575033, E 1358673, May 2007). The samples were homogenized on a roller mill, freeze-dried and sieved to 63 µm. One aliquot was used for measurement of the total organic carbon (TOC). The TOC was determined in an elemental analyzer (RC-412, Leco, Mönchengladbach, Germany) by heating a freeze-dried sub-sample to 580 °C after removal of inorganic carbon with hydrochloric acid. The measurements were carried out in triplicate. The rest of the sediment was extracted using accelerated solvent extraction (ASE 300, Dionex, Idstein, Germany) with three extraction cycles of dichloromethane/acetone (3:1) at 50 °C and 103.4 bar with 10 min static time. For clean-up the sediment extracts were dialysed in polyethylene-foil bags (80 µm, Polymer Synthese Werk, Rheinberg, Germany) with 16 cycles of dichloromethane:hexane (1:1) and 16 cycles of dichloromethane:acetone (7:3) at 40 °C and 35.5 bar using the accelerated solvent extractor ASE 300 [20]. During this step humid acids and other macromolecules from the sediment matrix were removed.

3.2.9 Fractionation

The cleaned extracts were fractionated with an online multi-step normal phase HPLC-method using the following stationary phases: nitrophenylpropyl silica, cyanopropyl silica and a porous graphitised carbon column. The three columns were sequentially eluted and 18 fractions were collected [18]. The fractions were evaporated under a nitrogen stream until dryness, redissolved in dichloromethane and divided into two aliquots. One aliquot of each fraction was re-dissolved in hexane and 1.03 g, 0.42 g and 0.72 g organic carbon extract equivalent (g OCEQ) from the samples Most, Přelouč and Bitterfeld, respectively, were loaded on the single SR. A value of 1 g OCEQ corresponds to the amount of compounds extracted from 1 g organic carbon of the dry sediment. One SR has a weight of 200 mg and therefore the following loads were achieved: 5.15 g OCEQ/g SR (Most), 2.1 g OCEQ/g SR (Přelouč) and 3.75 g OCEQ/g SR (Spittelwasser). The loads were selected to be in the order of magnitude of the load of native organic matter exceeding them by a maximum of factor five. The remaining aliquot was solvent exchanged to DMSO (c = 2.06 g OCEQ/mL Most, 0.84 g OCEQ/mL Přelouč

and 1.5 g OCEQ/mL Spittelwasser). The fractions were tested in the bioassay as described above.

3.3 Results and Discussion

3.3.1 Loading

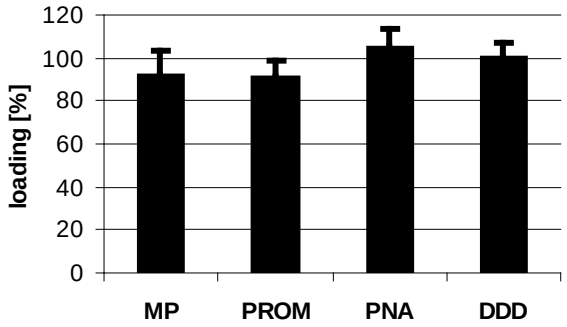

Figure 3-1: Loading efficiency in percent (n = 3) of methyl-parathion (MP), prometryn (PROM), N-phenyl-2-naphthylamine (PNA) and p,p'-DDD on SRs.

The results of the loading experiments are shown in Figure 3-1. Between 91 % (prometryn) and 105 % (N-phenyl-2-naphthylamine) of the compounds, with standard deviations below 12 %, were loaded on the SRs. These results confirm the high efficiency and good reproducibility of the loading method. The small losses of compounds are within the analytical error of the method. Furthermore, as long as volatile compounds are excluded the applied loading procedure is obviously not dependent on the physico-chemical properties of the compounds and does not discriminate single compounds. Volatile organic compounds may be lost during the drying process. However, there are neither indications that these compounds play a significant role for sediment toxicity, nor are volatiles expected in sediment extracts any more after freeze drying of sediments and solvent exchanges.

3.3.2 Correlation between Partitioning Coefficients

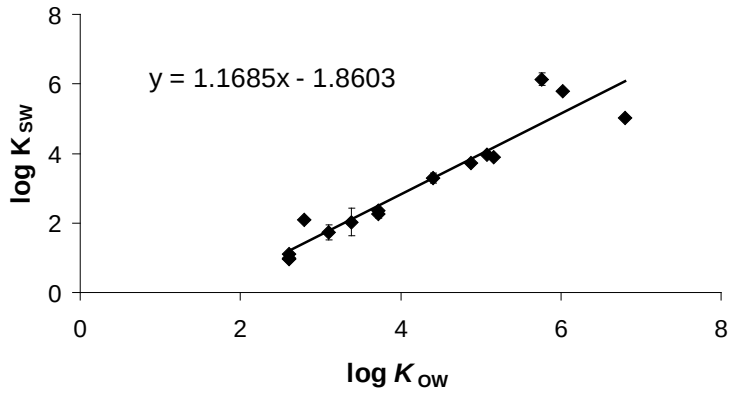

Figure 3-2: Correlation (r^2 = 0.90), between log K_{OW} and log K_{SW} (partitioning coefficient between silicone rod and water) (n = 3).

The correlation between the log K_{OW} and the log K_{SW} of the model compounds is shown in Figure 3-2. The two partitioning coefficients correlate well over 4 orders of magnitude (r^2 = 0.90), but error bars for individual compounds may be up to 20 % for the log transformed data. This is in good agreement with previous results [15-17, 21]. As a first estimate partition coefficients of sediment organic carbon and water (log K_{OC}) can be estimated on the basis of the log K_{OW} of the compounds of interest [1, 22]. Thus, a good correlation of the logarithm of the solid phase water partition coefficient with log K_{OW} is a crucial criterion for its application in partition-based dosing. However, we should be aware that substitutes like silicone only mimic partitioning in generic sediment and do not consider site specific sediment characteristics that impact real world partitioning. Sediment characteristics with known impacts on partitioning that cannot be simulated with silicone include sequestration [23], black carbon contents [24] and H-donor and acceptor properties [25]. Despite these limitations we suggest that partitioning processes in sediments are better simulated with silicone partition-based dosing than with organic solvent dosing.

3.3.3 Equilibrium Partitioning

The equilibrium between the SRs and the aqueous phase is reached after 5-50 min (see Figure 3-3 and Table 3-2), except for lindane. For this compound experimental data did not fit sufficiently well to the depletion kinetic model. Thus, it was difficult to determine the exact equilibrium time for lindane. The

apparent equilibration time of 5 h for this compound is probably due to the difficulties fitting the regression curve and to quantification problems.

Table 3-2: Used chemicals, calculated equilibrium times and parameters of the curves fitted to the data. The overall kinetics are described using the following formula: $c_t = c_{eq} * (1-e^{-kt})$ [10].

Chemical	c_{eq} [µg/L]	k	t [min]
Methyl-parathion	0.162	4.47	1.03
Prometryn	0.20363	0.05753	80.0
Anthraquinone	0.3166	0.337	13.7
Lindane	0.15761	1.81649	470
N-phenyl-2-naphthylamine	0.15161	0.155	29.7
Pyrene	0.04734	0.09489	48.5
Methoxychlor	0.04737	0.087	52.9
Fluoranthene	0.083	1.81649	1.89
Benz[*a*]anthracene	0.00044	0.17937	25.7
p,*p*'-DDD	0.04824	0.40808	11.3
PCB 101	0.00234	1.48667	3.10

The desorption of *p,p'*-DDD, fluoranthene, prometryn and anthraquinone leads to an initial overshoot before the equilibrium conditions are reached. This phenomena was also observed by others [10, 11], who applied equilibrium partitioning from a solid phase. This observation, together with the quantification problems leads to difficulties in defining the exact equilibration time, but the kinetics clearly show that the equilibration partitioning is fast enough for the desired use of the SRs. The fast equilibrium times are in accordance with other experiments with agitated systems.

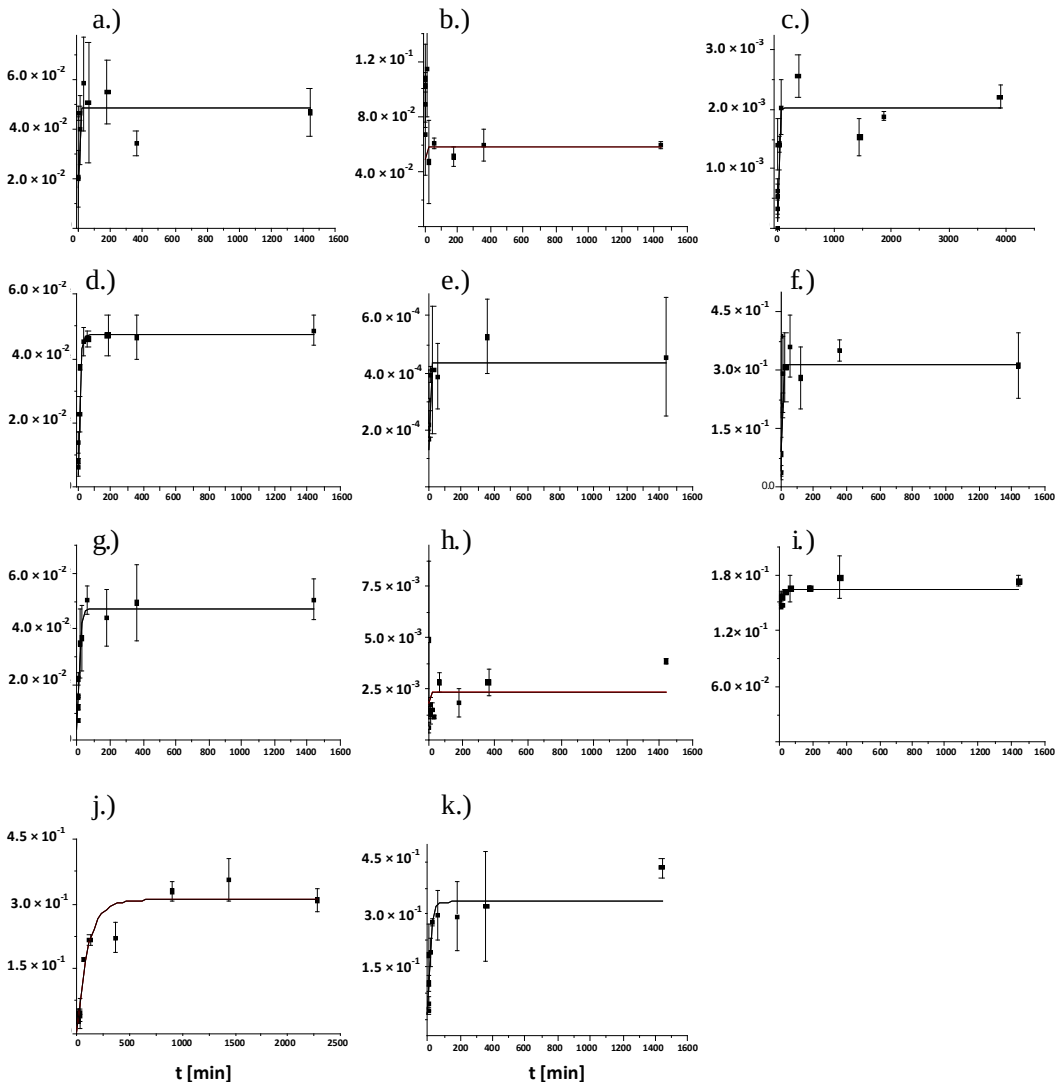

Figure 3-3: Depletion kinetics from SRs into the aqueous phase a.) p,p'-DDD, b.) fluoranthene, c.) prometryn, d.) pyrene, e.) benzo[*a*]anthracene, f.) anthraquinone, g.) methoxychlor, h.) PCB 101, i.) methyl-parathion j.) lindane k.) N-phenyl-2-naphthylamine. Concentration im mg/L.

Baltussen et al. [26] reported equilibrium times between 30 and 60 min for coated stirrer bars and Kiparissis et al. [11, 21] reported 45 min equilibrium times for the release of compounds from PDMS-films. The focus of this study was on the development of a partitioning-based dosing system with sufficiently rapid equilibration compared to the duration (24 h) of the bioassay. The developed dosing device was shown to be well suited to fulfil this requirement.

3.3.4 Constant Concentrations

The concentration of four different compounds was monitored during the algae test for the full test duration of 24 h for both dosing techniques, SR and conventional DMSO dosing (Figure 3-4). The concentration of prometryn varies for SR dosing between 87 (± 8.3) % and 101 (± 17.0) % of initial concentration and for dosing with DMSO between 84 (± 14.0) % and 103 (± 8.0) %. For methoxychlor and PCB 101 this stability of concentrations is only reached with SR dosing. This dosing technique maintains the concentration between 67 (± 13) % and 91 (± 4.5) % for methoxychlor and between 82 (±11) % and 95 (± 24) % for PCB 101. When DMSO dosing is used, the concentration of methoxychlor and PCB 101 decreases continually to 50 (± 9.5) % and less than 20 (± 8.13) % of initial concentrations, respectively. The concentration of N-phenyl-2-naphthylamine decreased for both dosing techniques to less than 50 % of initial concentrations. At the end of the experiment, the concentration of N-phenyl-2-naphthylamine was 40 (±10) % for SR dosing and 50 (±13) % of initial concentrations for DMSO dosing. The trend in the concentration stabilities clearly depends on the hydrophobicity of the compounds and on the applied dosing technique. Compounds with high log K_{OW} tend to adsorb to the glass walls of the test tubes or to the Teflon coated stirrer bars [27], while bioconcentration in the algae also increases with increasing log K_{OW}. These partitioning processes deplete the aqueous phase. In the samples containing the SRs, the losses are compensated by the reservoir of the compounds on the SRs and therefore constant concentrations are also achieved for the compounds with a higher log K_{OW}. For N-phenyl-2-naphthylamine this compensation is not effective despite its only medium-range log K_{OW}. This is likely caused by a fast chemical degradation of this compound in the water phase. One of its isomers, N-phenyl-1-naphthylamine is degraded quickly under ultraviolet light (300 nm), with a half-life of 5.7-8.4 min [28] and it is likely that the other isomer also undergoes photolysis. If the photolysis of the compound is faster than the delivery from the SRs to the water phase, constant concentrations in the water phase cannot be achieved with SR dosing. For such special cases the SRs cannot compensate the loss of compounds in the water phase, despite the relatively fast release kinetics of the SRs. However, they can compensate

the losses of hydrophobic compounds due to partitioning to other phases in the test system.

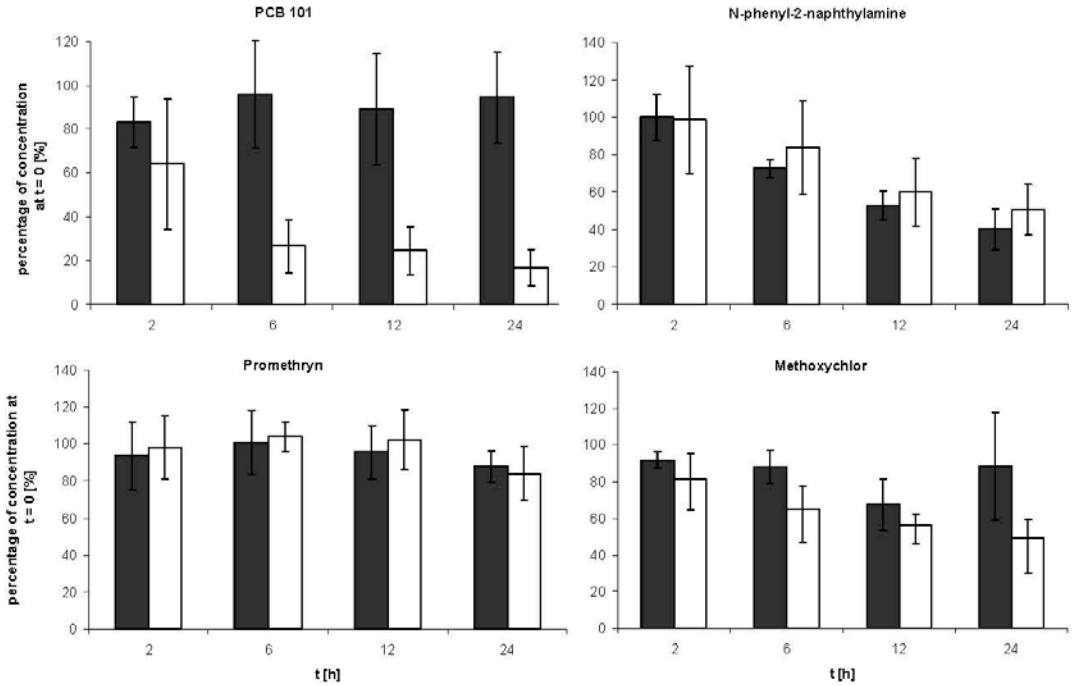

Figure 3-4: Concentration of model compounds over 24 h, dosed to a bioassay using the green algae *Scenedesmus vacuolatus* with DMSO solution of the compounds (▭) or loaded SRs (■). Concentrations are measured in percent relative to the measured concentration at t = 0.

3.3.5 Concentration Effect Relationships in Algae Test

The aim of the following experiment was to examine whether it is possible to achieve dose-effect relationships with SR dosing. SRs were loaded with different amounts of prometryn and with a whole sediment extract from Přelouč. The SRs were used to perform a growth inhibition assay with the green algae *Scenedesmus vacuolatus*. Prometryn as well as the sediment extracts show load dependent response in the bioassay (Figure 3-5). For sediment extract testing a maximum load of the silicone rods corresponding to the load in the native organic carbon was used (1.1 g OCEQ/g SR). This resulted in a maximum inhibition of 77 %. Greater concentrations are of course testable, however, taking the risk of reduced environmental realism and increasing risk of artefacts due to limited solubility in silicone that may result in nano-crystals inside PDMS pores. Concentrations in the test medium can be calculated for compounds with known log K_{SW}. For other compounds concentrations can only be measured. In the case of prometryn

the measurable range was limited to concentrations higher than 0.22 µg/L by the limit of quantification for the analytical method using non-depletive SPME in combination with GC-MS. Thus, no effects of less than 20 % inhibition could be detected.

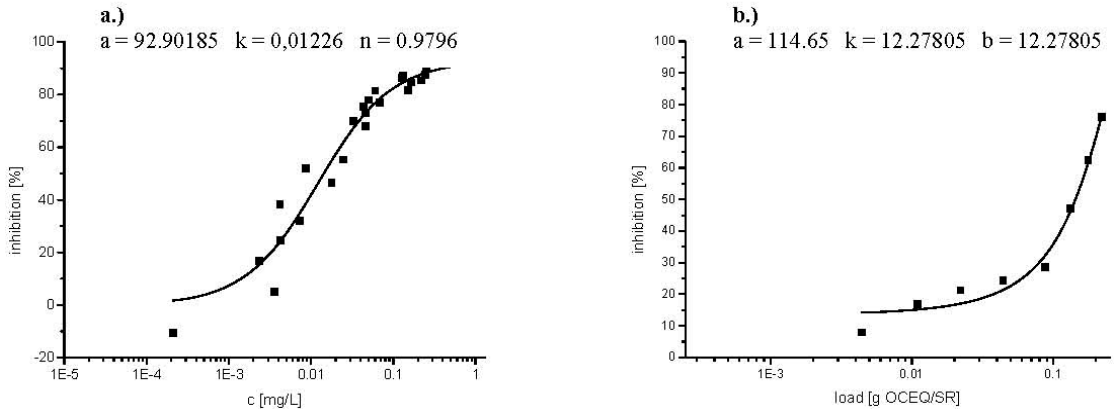

Figure 3-5: Concentration dependent response in the 24 h growth inhibition assay with the green algae *Scenedesmus vacuolatus* of a.) prometryn and b) of a sediment extract from the Elbe River (Přelouč, Czech Republic). The sediment extract and prometryn were loaded on SRs. Prometryn concentrations have been measured with SPME in combination with GC-MS. For the fit a sigmoid model with three parameters was used for the whole sediment extract (y = a/(1+b*e(-kx))) and a Hill model (y = (a*xn)/(kn + xn) was used for the prometryn sample.

3.3.6 Toxicity Patterns

The growth inhibition of the green algae *Scenedesmus vacuolatus,* which has been exposed for 24 h to the 18 fractions of three different sediment extracts, is summarized in Figure 3-6. Blanks of the fractionation procedure were also prepared and tested, but showed no effect in the biotest. The fractionation method is described elsewhere in detail [18] and was evaluated for a broad range of model compounds. Briefly, fractions 1 to 5 coelute with polychlorinated biphenyls (PCBs), naphthalenes (PCNs), dibenzo-*p*-dioxins (PCDDs) and furans (PCDFs) with increasing chlorine substitutions, fraction number 6 to 12 coelute with polyaromatic hydrocarbons (PAHs) with increasing number of aromatic rings and fractions 13 to 18 are characterized by compounds with increasing polarity. For DMSO dosing a concentration of 0.84 – 2.06 g OCEQ/L with a total volume of 2 mL was tested, while for the SRs the dose can only be expressed as a load on the SRs because the concentration in the aqueous phase depends on the partitioning coefficients of the sediment constituents. 0.42-1.03 g OCEQ were loaded on a single SR

to dose 2 mL GB-medium. Thus the concentrations for the two dosing techniques cannot be compared directly.

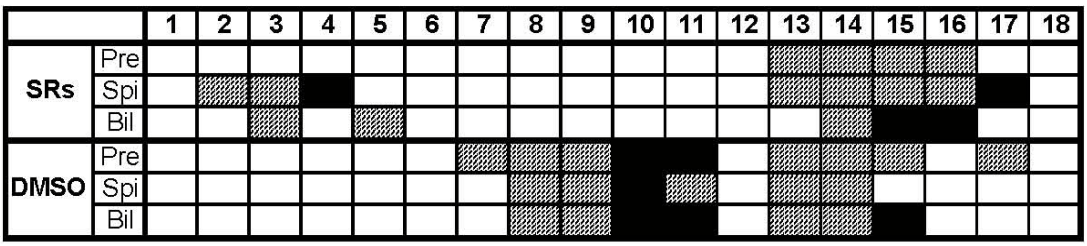

Figure 3-6: Overview of growth inhibition of green algae Scenedesmus vacuolatus (▭ 0-20 %; ▨ 20-50 % ; ■ over 50 %) exposed to fractions of sediment extracts of sediments from Elbe River Přelouč (Pre), Bilina River Most (Bil), and Spittelwasser Bitterfeld (Spi) for dosing with loaded SRs (load 1.03 g OCEQ/SR Most, 0.42 g OCEQ/SR Přelouč and 0.75 g OCEQ/SR Spittelwasser) and conventional dosing with DMSO (c = 2.06 g OCEQ/L Most, 0.84 g OCEQ/L Přelouč and 1.5 g OCEQ/L Spittelwasser).

However, concentrations in DMSO and SR were selected that exhibited similar effects in selected active fractions (F13 to F15) for both dosing techniques in order to demonstrate the prioritisation of fractions. Fractions 13-17 (generally polar contaminants) were toxic for both dosing methods, while fractions containing PAHs were only toxic for the DMSO dosing. This is in accordance with the idea that solvent extraction of sediment samples may overestimate the bioavailability and thus the hazard of hydrophobic toxicants [29]. Fractions 2 to 5 of two of the three sediment extracts only showed effects when dosed with SR. Although designed as PCB and PCDD/F fractions [18] in cases with high concentrations of long chain alkanes and other non-polar aliphatic these compounds may elute in significant amounts in fractions 2 to 5. This was the case in the present sediment samples. In contrast to polyaromatic compounds long chain alkanes are almost insoluble in DMSO. Thus, these compounds were probably not dosed with DMSO but with hexane and thus SR. This might explain the higher effect with SR dosing compared to DMSO dosing.

The results clearly indicate that the toxicity patterns are quite different for the two dosing techniques, while the differences between the three sediment samples are less pronounced. These differences in the biological response can be explained by the availability of the compounds, which depends on the dosing technique. The differences in toxicity patterns for the two dosing methods confirm the importance of considering partitioning based dosing techniques for an improved prioritisation of fractions and toxicants and a

better risk assessment of contaminated sediments. The dosing technique with SRs can be also used in all other test systems where stirrer bars are applied. The battery of bioassays might be extended further to assays using micro plates by applying PDMS-films which were developed in the last years [10, 11] and are available commercially now e.g. as so-called Immobilized Liquid Extraction™ well plates.

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Chapter 4

Effect-Directed Analysis of contaminated sediments with partition-based dosing using green algae cell multiplication inhibition

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4.1 Introduction

Sediments are well known sinks of hydrophobic organic chemicals in aquatic ecosystems but may also act as a long term source of contamination for interstitial and surface water, benthic organisms and finally for whole aquatic food webs. The concentrations in the different compartments, and thus bioavailability are a result of partitioning processes driven by activity gradients [1]. Typically, sediments are contaminated with a mixture of many compounds with different toxicity and bioavailability that may cause adverse effects to benthic organisms. Benthic algae play a key role in aquatic food webs [2] and may be highly sensitive to many contaminants [3]. Risk to benthic primary production is a result of exposure and thus bioavailability and algal toxicity of the contaminants.

Since an analysis of all compounds associated to a sediment is impossible and target analysis of pre-selected compounds often misses site specific toxicants, effect-directed analysis (EDA) has been developed combining chemical and biological methods in order to direct chemical analysis to those compounds that actually cause effects [4-6]. The complexity of samples is sequentially reduced by removing non-toxic fractions while major toxicants are isolated and identified.

EDA of sediment samples is commonly done with organic solvent extracts obtained by exhaustive sediment extraction finally transferring all extracted compounds to the test medium resulting in an enhanced bioavailability of lipophilic compounds compared to the availability resulting from partitioning in the original sediment-water-system. This may bias the prioritization of fractions and compounds and result in an overestimation of hydrophobic toxicants relative to more hydrophilic ones that are more bio available. Recently, we suggested an approach to simulate partitioning processes in sediments in EDA by applying partition-based dosing using silicone rods (SRs) [7].

The aim of the present study is to apply EDA based on partition-based dosing technique in comparison to conventional DMSO dosing to identify major algal toxicants in a contaminated sediment from the Bílina River downstream of Most, Czech Republic, which is influenced by petrochemical industry and waste water treatment plants. The sediment extract was first fractionated using an on-line combination of three preparative normal phase

HPLC columns [8]. According to the resulting toxicity pattern using growth inhibition test with the green algae *Scenedesmus vacuolatus* the most relevant fractions have been chosen and were further fractionated using a preparative RP-HPLC method. In the algae toxic sub-fractions of the second fractionation step the present compounds have been identified and quantified using gas chromatography-mass spectrometry (GC-MS). A crucial part of every EDA study is the confirmation of the identified toxicants [9]. The index of confirmation quality (ICQ) has been calculated from the effects of sub-fractions and corresponding artificial mixtures [10].

4.2 Material and Methods

4.2.1 Chemicals

All solvents were purchased from Merck (Darmstadt, Germany) and were of LiChrosolv grade (purity ≥ 99.8%). The following abbreviations are used: dimethylsulfoxide (DMSO), acetonitrile (ACN), dichloromethane (DCM), hexane (HX), acetone (ACT), toluene (TOL) and methanol (MeOH). Ammoniumacetat (≥ 98 %) was obtained from Riedel de Haen (Seelze, Germany) and glacier acid (99.99 %) from Sigma Aldrich (Steinheim, Germany). The purity and the supplier of the standards used for identification and toxicity confirmation of the compounds can be found in Table 4-1.

Table 4-1: Identified compounds, supplier, used columns for separation during the GC-MS analysis and used SPME-fibres for concentration measurements in aqueous samples.

compound	CAS	supplier	column	SPME –fibre[1]
Hexadecanol	36653-82-4	Aldrich	VF200MS	-
Triclosan	3380-34-5	Merck	DB-17 MS	PA
Benz[*c*]acridine	225-11-6	Chiron	VF200MS	PA
Diphenylsulfone	127-63-9	Alfa Aesar	DB-17 MS	PA
N-Butylbenzensulfonamid	3622-84-2	Ehrenstorferr	DB-17 MS	Mix
Palmitic acid	629-73-2	Aldrich	VF200MS	-
Pentadecanol	629-76-5	Merck	VF200MS	-
2-Methylanthraquinone	84-54-8	TCI	DB-17 MS	PA
7-H-Benzo[*de*]-anthracen-7-one	82-05-3	Fluka	DB-17 MS	PA
Cyclopenta[*def*]phenanthren-4-one	5737-13-3	Chiron	DB-17 MS	PA
fluoranthene	206-44-0	Fluka	DB-17 MS	-
Benzo[*b*]fluoranthene	205-99-2	Promochem	DB-17 MS	-
Benzo[*e*]pyrene	192-97-2	Aldrich	DB-17 MS	-
Benzo[*j*]fluoranthene	205-82-3	Promochem	DB-17 MS	-
Benzo[*k*]fluoranthene	207-08-9	Promochem	DB-17 MS	-
Benzo[*a*]fluoranthene	56-55-3	Promochem	DB-17 MS	-
Perylene	198-55-0	Fluka	DB-17 MS	-
Benzo[*a*]pyrene	50-32-8	Aldrich	DB-17 MS	-
Indeno[123,*cd*]fluoranthene	193-43-1	Promochem	DB-17 MS	-
Indeno[123,*cd*]pyrene	193-39-5	BCR	DB-17 MS	-
Benzo[*ghi*]perylene	191-24-2	Aldrich	DB-17 MS	-
Dibenzo[*ah*]anthracene	53-70-3	Fluka	DB-17 MS	-

[1] PA: polyacraylate fibre, Mix: divenylbenzene/carbowax/polydimethylsiloxane fibre

4.2.2 Sampling and Extraction

The sediment sample was taken at the Bílina River (Czech Republic) with an Ekman-Birge-grab sampler near Most (coordinates: 50° 30’ 18.29 N, 13° 40’59.03’’ E, June 2007). The sample was homogenized on a roller mill, freeze-dried and sieved to 63 µm. One aliquot was used for measurement of total organic carbon (TOC) with an elemental analyzer (RC-412, Leco, Mönchengladbach, Germany) by heating a freeze-dried sub-sample to 580 °C after removal of inorganic carbon with hydrochloric acid. The measurements were carried out in triplicate. The rest of the sediment (640 g dry weight) was extracted using accelerated solvent extraction (ASE 300, Dionex, Idstein, Germany) with three extraction cycles of DCM/ACT (3:1) at 50 °C and 103.4 bar with 10 min static time. The sediment extract was

dialysed for clean up in polyethylene-membrane bags (80 µm, Polymer Synthese Werk, Rheinberg, Germany) with 16 cycles of DCM/HX (1:1) and 16 cycles of DCM/ACT (7:3) at 40 °C and 35.5 bar using the accelerated solvent extractor ASE 100 [11]. During this step humid acids and other macromolecules from the sediment matrix were removed.

4.2.3 Normal Phase Fractionation

The cleaned extracts were fractionated with an online multi-step normal phase (NP) HPLC-method using the following stationary phases: nitrophenylpropyl silica (21 x 250 mm, 5 µm Nucleosil 100-5 NO_2, Macherey-Nagel, Düren, Germany), cyanopropyl silica (21 x 125 mm, 5 µm Nucleosil 100-5 CN, Macherey-Nagel) and porous graphitised carbon (Hypersil PGC, 10 x 50 mm, 7 µm, ThermoFisher Scientific, Waltham, MA, USA). The three columns were sequentially eluted with the following solvents HX/DCM (95:5 v/v), 100 % DCM, 100 % ACN (cyanopropyl silica), HX/DCM (95:5 v/v) and 100 % HX (nitrophenylpropyl silica) and HX/TOL (60:40 v/v) and 100 % TOL (porous graphitised carbon). 18 fractions were collected. The whole method is described in more detail elsewhere [8]. The fractions were evaporated under a nitrogen stream until dryness, re-dissolved in DCM and divided into the following aliquots: One aliquot of each fraction was solvent-exchanged to hexane for loading the silicone rods (see below) and the other was solvent-exchanged to DMSO for the conventional bioassay.

4.2.4 Reversed Phase Fractionation

Table 4-2: Description of the gradients of the RP HPLC-method. The ration ACN/H_2O (v/v) is given at specified times.

fraction	t (min)	ratio ACN/H_2O
F8, F10	0	40/60
	59	100/0
F11	0	50/50
	49	100/0
F14	0	30/70
	70	70/30
	90	100/0
F15, F16	0	20/80
	90	60/40
	110	100/0

Selected active NP-fractions were further fractionated using reversed phase (RP) HPLC together with a diode array detector and a fraction collector (all

Dionex Corp., CA, USA). The compounds were separated on a C18 stationary phase (Nucleosil 100-5 C18 HD, 250 x 21 mm, Macherey-Nagel) using ACN and a buffer solution (0.05 % CH_3COONH_4/acetic acid, pH 4.75) as mobile phase at a flow rate of 10 mL/min (more information on the gradients in Table 4-2). For all gradients lipophilicity-dependent elution windows were defined using retention of nine compounds covering a broad range of log K_{OW} values (Table 4-3).

Table 4-3: Compounds and their log K_{OW} value used to determine the log K_{OW} windows of the RP HPLC.

compound	log K_{OW}*
Thiocarbamate	-1.31
1,2-Benzenediol	1.03
Nitrobenzene	1.81
Chlorobenzene	2.90
Biphenyl	3.76
Fluoranthene	4.93
Hexachlorobenzene	5.86
PCB153	7.62
PCB 209	9.60

* Epi Suite v.3.20, 2007, U.S. Environmental Protection Agency.

While ACN fractions were directly subjected to solvent exchange aqueous fractions were solid phase extracted after dilution to less than 5 % ACN using glass cartridges packed with 200 mg of an end-capped C18 stationary phase (Discovery DSC-18, Supelco, Taufkirchen, Germany) and 60 mg of polystyrene-divinylbenzene (Chromabond Easy, Macherey-Nagel). The first RP-fraction was extracted once more at pH = 10 to minimize the risk of losing basic compounds (F 1B). The cartridges were rinsed with 5 mL bi-distilled water, freeze dried and eluted with 5 mL HX, 10 mL DCM and 10 mL ACN. Solid phase extracts of aqueous fractions and ACN fractions were solvent-exchanged to TOL for chemical analysis and to DMSO or HX for toxicity testing. The residue, which could not be dissolved in the solvent of the RP HPLC, was solvent exchanged to DMSO or HX for toxicity testing.

4.2.5 Toxicity Test and Dosing

Cultivation and test conditions for unicellular green algae *Scenedesmus vacuolatus* (strain 211-15, SAG, Göttingen, Germany) are described elsewhere [12]. Cell multiplication was determined via the fluorescence at a wavelength of 685 nm (Backscat Fluorometer, Haardt, Kleinbarkau,

Germany) at the beginning (t = 0) and the end of the test (t = 24 h). For fractions (F14) with high background fluorescence additional fluorescence controls were prepared by replacing the algae suspension with culture medium.

Test compounds were dosed either dissolved in DMSO or by loading them onto SRs (d = 5 mm, l = 0.84 ± 0.01 cm) using silicone from Good Fellow (Friedberg, Germany) as described recently [7]. Briefly, the cleaned SRs were soaked with 150 µL of the sample in HX over night and dried to evaporate the hexane. The loads of the SRs are referred as g SEQ/SR. A value of 1 g SEQ corresponds to the amount of compounds extracted from 1 g dry sediment. In the test system, loaded SRs were stirred for 24 h with 200 RPM in 1.95 mL modified Grimme-Boardman-medium [12] at 28 °C in the dark to reach equilibrium between the silicone and the aqueous phase. By adding 50 µL algae suspension (cell density approx. $3x10^6$ cells/mL) to a total volume of 2 mL, an initial cell density of approx. $7.5x10^4$ cells/mL for both the SR and the DMSO-dosed test was established. As negative control samples, SRs loaded with pure HX and DMSO controls were used.

4.2.6 GC-MS

Samples were analysed on an Agilent 6890 GC equipped with an HP 7682 Series Autosampler and coupled to an Agilent 5973 mass selective detector (all from Agilent Technologies, Böblingen, Germany) using 1.3 mL/min of He as carrier gas and the following columns for separation: DB 17 MS (Agilent, 30 m x 0.25 mm i.d., 0.25 µm) and VF 200MS (Varian, Darmstadt, Germany, 30 m x 0.25 mm i.d. 0.25 µm). Table 4-1 lists columns used for the quantification of the compounds in the sub-fractions.

Liquid samples were analysed after injection of 1 µL using pulsed splitless mode at 250 °C. The oven was programmed from 60 °C to 150 °C with a rate of 30 °C/min, 150 °C to 186 °C with a rate of 6 °C/min and from 186 °C to 280 °C with a heating rate of 4 °C/min. The final temperature of 280 °C was held for 16.5 min, in the case of non volatile compounds as in F11 for 30 min to elute the compounds from the column. For identified compounds, which caused toxicity dosed with SRs, the partitioning coefficient between water and the SRs has been determined [7]. The aqueous concentrations were measured using non-depletive solid phase microextraction (SPME) in

combination with the GC-MS. The polyacrylate fibre (PA) and the divinylbenzene-carboxene-polydimethylsiloxane fibre (DVB/CAR/PDMS) were purchased from Supelco (Bellefonte, USA). The fibres were immersed for 5 minutes or 10 minutes (triclosan) at 28 °C and 200 RPM into the aqueous sample. Then, the loaded SPME-fibres were introduced into the injector of the GC-MS operated in the splittless mode. Compounds were allowed to desorb for 5 minutes at 270 °C (DVB/CAR/PDMS fibre) and 300 °C (PA fibre), respectively. The oven was heated from 60 °C to 280 °C with a rate of 120 °C/min to the final temperature of 280 °C, which was held for 7 minutes.

4.2.7 Confirmation of Identified Chemicals

In order to confirm whether the compounds identified in the fractions are the likely cause of observed toxicity, dose response curves obtained from individual fractions and corresponding mixtures of pure standards (artificial mixtures) were compared. Dose-response curves were fitted to these experimental data using a three parameter sigmoid model:

$$inhibition = \frac{a}{1 + e^{-\frac{(x - x_0)}{b}}} \qquad \textbf{(4-1)}$$

a,b and x_0 are the parameters of the model and x is the concentration. Identified chemicals were confirmed as the cause of measured effects by comparing full fractions and corresponding artificial mixtures. To gain a measure for the confirmation quality over the whole effect range the Index of Confirmation Quality (ICQ) was calculated from the EC_x of the fractions and the associated artificial mixtures according to the following formula [10]:

$$ICQ = \frac{EC_x^{fraction}}{EC_x^{mixture}} \qquad \textbf{(4-2)}$$

For the SR-dosed tests aqueous concentrations were estimated based on partitioning coefficients between the silicone and water determined as described recently [7].

4.3 Results and Discussion

4.3.1 Toxicity Pattern for Normal Phase Fractionation

The goal of the first set of experiments was the identification of the most potent fractions to reduce the number of fractions, which have to be processed in further EDA steps. The results of the 24 h growth inhibition test with the green algae *Scenedesmus vacuolatus* exposed to the 18 NP fractions are shown in Figure 4-1. When the fractions were dosed with DMSO, algal growth was mainly inhibited by F8, F10 and F11, which co-elute with PAHs as model compounds, and F15, which is characterized by more polar compounds. The latter fraction as well as F14 and F16 caused growth inhibition when they were dosed using SRs. In contrast to DMSO dosing, the SR-dosed PAH fractions did not cause any algal toxicity, but an additional inhibition of approx. 30% could be observed in F3 and F5. The toxicity patterns are in accordance with earlier findings [7] and show the good reproducibility of the whole procedure including sample preparation, extraction, fractionation and dosing. The differences between the two dosing techniques can be explained by different availability of the compounds. Dosing with DMSO exposes the algae to the whole extracted amount soluble in DMSO, while for the SRs exposure depends on the partition coefficient between silicon and water, which correlates well with the K_{OW} of the compounds [7]. When DMSO dosing was used, a concentration of 20 g SEQ/L was tested. For the SRs the concentration can only be given as a load per SR (10 g SEQ/SR), because the aqueous concentration of the single compound depends on the partitioning coefficient between SR and water. 10 g SEQ equals 1.03 g organic carbon of the sediment. Regarding the weight of a single SR (200 mg) the load on the SRs is five time higher than the load on the organic carbon of the sediment, but still in same range as the load in the sediments. Thus, direct quantitative comparison of both dosing techniques is neither possible nor intended. Our focus is on relative prioritisation of compounds and fractions. To achieve this goal, concentrations were chosen to achieve similar overall effect levels for both methods. Following this approach DMSO dosing prioritised the fractions F8, F10, F11 and F15 while F14-F16 were prioritised by SR for further investigation.

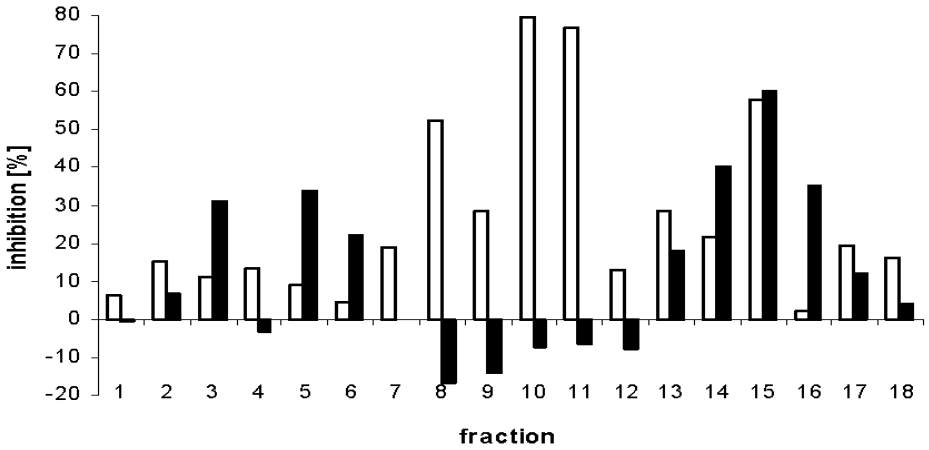

Figure 4-1: **Growth inhibition of the green algae *Scenedesmus vacuolatus* exposed 24 h to fractions gained with NP fractionation of a sediment extract and dosed to the test system either by solvent dosing with DMSO □ (c = 20 g SEQ/L) or partitioning based dosing with loaded silicone rods ■ (c = 10 g SEQ/SR).**

4.3.2 Toxicity Pattern Derived for RP-HPLC Sub-Fraction

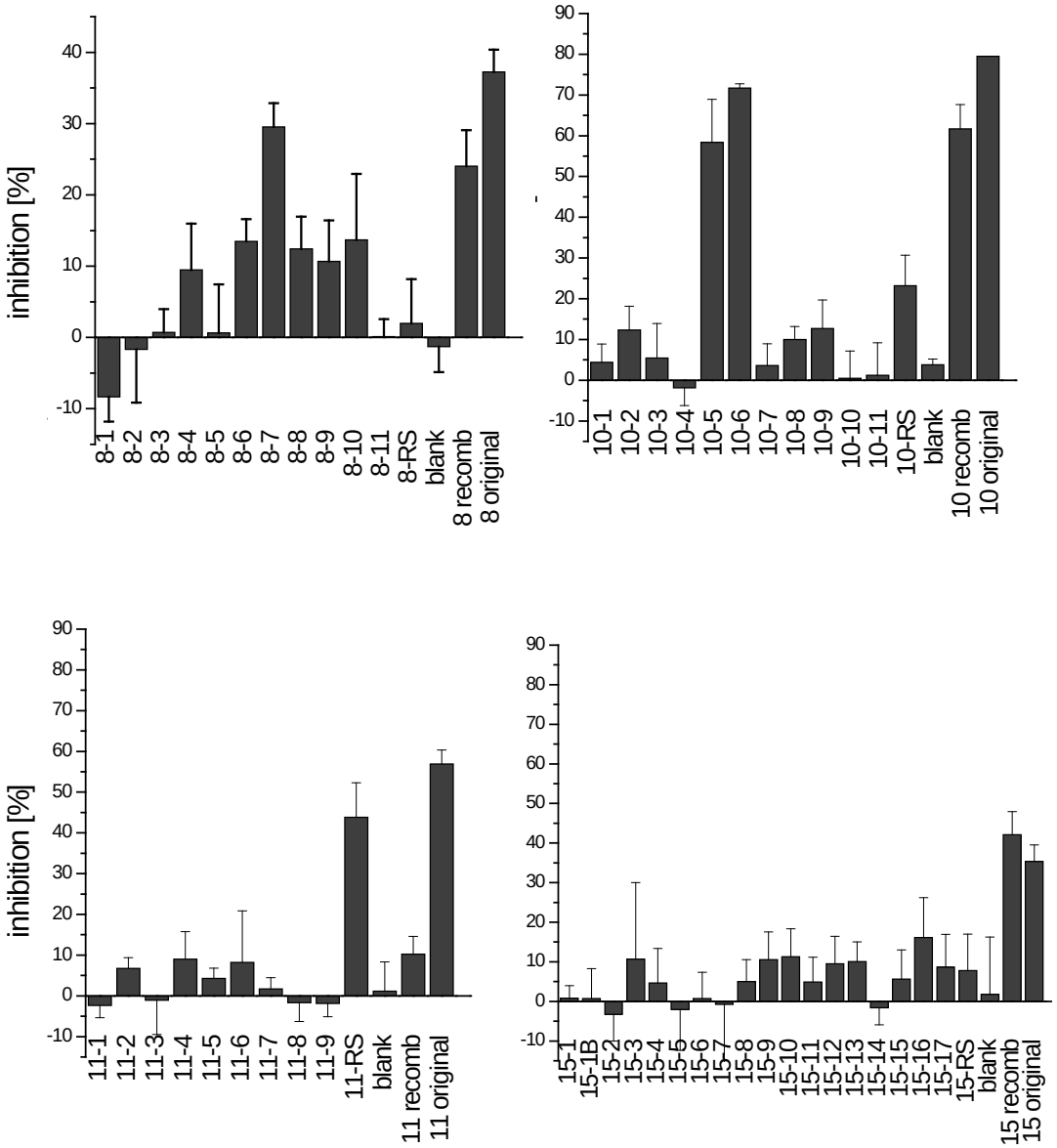

Figure 4-2: **Growth inhibition (n=3) of the green algae *Scenedesmus vacuolatus* exposed for 24 h to sub-fractions of a sediment extract dosed with DMSO to the test system (c = 40 g SEQ/L). Recombination of all sub-fractions (recomb) and original fraction (c = 20 g SEQ/L). The sediment extract was first fractionated with NP HPLC and then with RP HPLC as second fractionation step.**

The toxicity pattern of the RP-sub-fractions of F8, F10, F11 and F15 dosed with DMSO are shown in Figure 4-2. Applying DMSO-dosing RP-fractionation of F8 and, F10 resulted in one (F8-7) and two (F10-5, F10-6) toxic sub-fractions with more than 20 % inhibition, respectively. RP-fractionation of F15 resulted in a rather even distribution of low toxicity over many fractions with a maximum inhibition of less than 20 % by F15-16. Quality control comparing

effects of parent fractions and reconstituted mixtures of sub-fractions indicate good agreement and only negligible losses of toxicity during fractionation. After RP-fractionation of F11 no toxicity was observed in the sub-fractions, but in the residue that was not soluble in ACN/water. The expected compounds in F11 are five ring PAHs, which exhibit a poor solubility in this solvent mixture. Thus, toxicity identification was based on the parent NP fraction F11.

Partition-based SR-dosing prioritised F14 to F16. The RP-fractionation of F14 resulted in one outstanding sub-fraction (F14-7, figure 4-3) with dose-dependent toxicity and 80 % inhibition at 25 g SEQ/SR. RP-fractionation of F15 and F16 resulted in a more even distribution of inhibition potency of 10 to 30 % over many sub-fractions, allowing however a prioritisation of F15-6, F15-15 and F16-4 with about 30 % inhibition each (Figure 4-3). The comparison of F15 parent fraction toxicity with the reconstituted mixture indicates a loss of about 30 % of the toxic potency which may be at least partly explained by toxicity remaining in the residue (F15-RS). Relative to the other sub-fractions F15-RS was the most toxic and was prioritised for toxicant identification. Quality control of F16 indicated high recovery of toxicity after RP-fractionation without significant toxicity remaining in the residue.

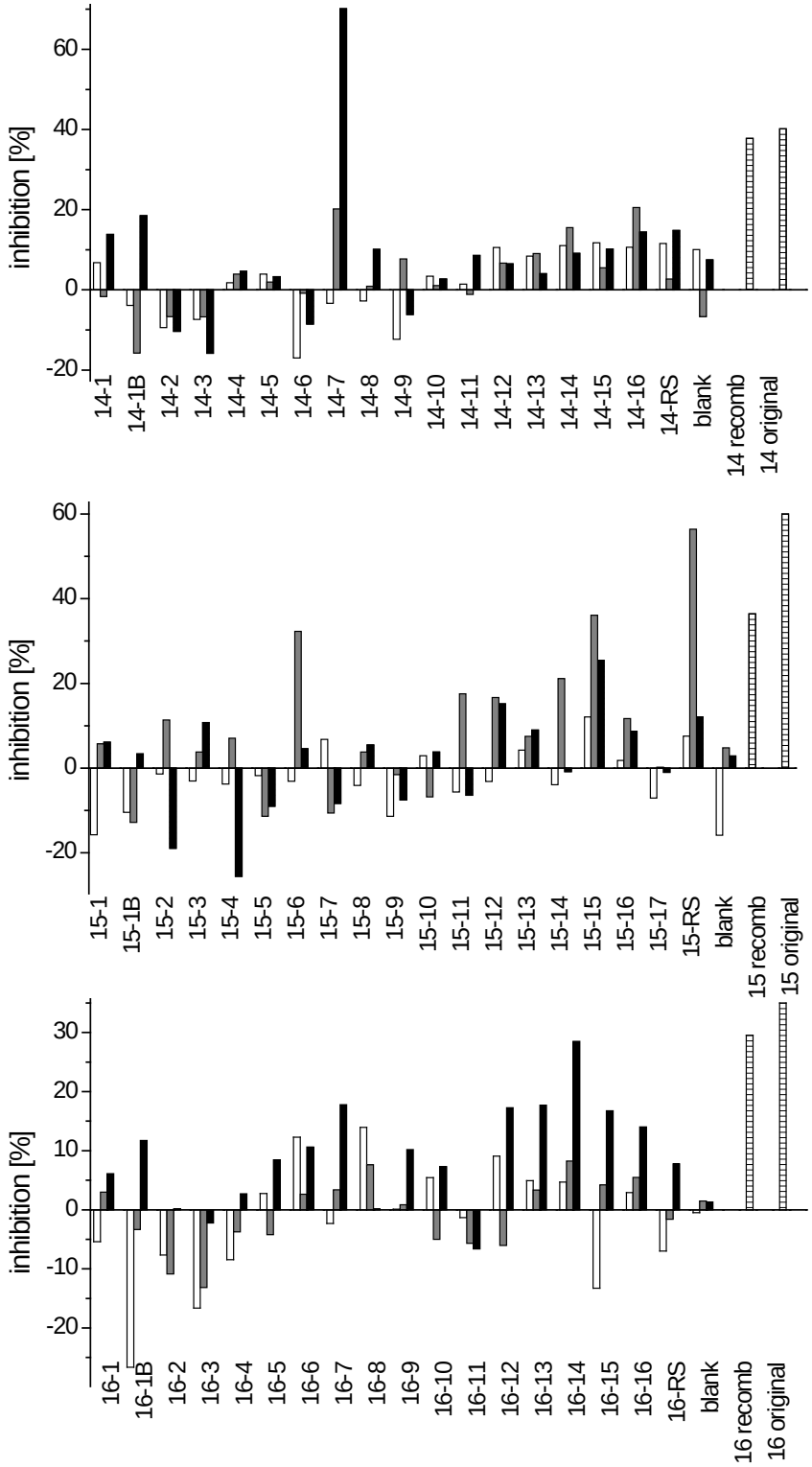

Figure 4-3: Growth inhibition of the green algae *Scenedesmus vacuolatus* exposed 24 h to sub-fractions of a sediment extract dosed to the test using silicone rods (SRs). The sediment extract was first fractionated with NP HPLC and then with RP RPLC as second fractionation step. The sub-fractions from the RP HPLC of fraction 14, the residue (RS) and the recombination of all sub-fractions (recomb) are shown. □ 6.25 g SEQ/SR, ■ 12.5 g SEQ/SR, ■ 25 g SEQ/SR, ▤ 10 g SEQ/SR.

The distribution of toxicity in F15 and F16 over many sub-fractions may indicate a significant role of many baseline toxic compounds that cause toxicity according to their log K_{OW}, even though in some of the fractions also specific toxicity of individual toxicants may contribute. RP-fractionation pattern of F14 indicates the presence of a clearly dominant probably specifically acting toxicant.

4.3.3 Identified Compounds

Table 4-4: Compounds identified and quantified in individual fractions; applied dosing techniques are given in brackets, the sediment concentration c (µg/g sediment dry weight) is listed.

fraction (dosing)	compound	c
8-7 (DMSO)	Fluoranthene	1.570
10-5 (DMSO)	Benzo[*b*]fluoranthene	0.477
	Benzo[*e*]pyrene	0.581
	Benzo[*j*]fluoranthene	0.189
	Benzo[*k*]fluoranthene	0.379
	Benzo[*a*]fluoranthene	0.056
	Perylene	0.132
10-6 (DMSO)	Benzo[*a*]pyrene	0.274
	Benzo[*b*]fluoranthene	0.017
	Benzo[*j*]fluoranthene	0.051
	Benzo[*a*]fluoranthene	0.001
	Benzo[*e*]pyrene	0.027
	Perylene	0.011
11 (DMSO)	Indeno[123,*cd*]fluoranthene	0.020
	Indeno[123,*cd*]pyrene	0.473
	Benzo[*ghi*]perylene	3.012
	Dibenzo[*ah*]anthracene	0.014
15-16 (DMSO)	Hexadecanol	11.42
14-7 (SRs)	Triclosan	0.181
	2-Methylanthraquinone	0.077
	Benzanthrone	0.094
	Cyclopenta(*def*)phenanthren-4-one	0.040
15-15 (SRs)	Pentadecanol	5.290
	Palmetic acid	16.86
15-RS (SRs)	Benz[*c*]aridine	0.100

Toxic fractions were analysed by GC-MS and major peaks were identified using the NIST Library [13]. Identified compounds were confirmed and quantified with standards (Table 4-4). In sub-fractions F8-7, F10-5, F10-6 and F11 PAHs with increasing number of aromatic rings have been detected. In

F14-7 triclosan has been identified as major compound. Further identified compounds were oxy-PAHs such as 1-methylanthraquinone, 7-H-benzo[*de*]-anthracene-7-one and cyclopenta[*def*]phenanthrenone. In F15-16 hexadecanol was identified as a major compound, in F15-15 also long chain aliphatic ketones and alcohols such as hexadecanone and pentadecanol have been found. In the residue of the RP-HPLC of fraction F15 benzo[*c*]acridine was found. In F15-6 several peaks were detected and some of them tentatively identified by NIST MS library search including benzophenone, anthrone, p-tert-butyl-phenol and sulfones as N-butyl-sulfonamide and diphenylsulfone (Table 4-5). Because only for half of components standards were available no attempts were made to confirm or to quantify.

Table 4-5: **Tentatively identified compounds in fractions 15-6, log K_{OW} values and expected log K_{OW} range from RP HPLC for sub-fraction 15-6.**

log K_{OW} expected	compound	log K_{OW}*
2.93-3.37	p-Tert-butyl-phenol	3.42
	1,1'-(1,2-Ethanediyl)bisbenzene	4.74
	2(3H)Furanone,dihydro-5 -pentyl	2.08
	Benzophenone	3.15
	1(2H)Acenaphthylenone	2.79
	2,3-Dihydro-1-oxo-1H-phenalene	3.28
	N-Butyl-benzenesulfonamide	2.31
	Anthrone	3.81
	Diphenylsulfone	2.61
	7H-Benz[*de*]anthracen-7-one	4.73

* Epi Suite v.3.20, 2007, U.S. Environmental Protection Agency.

4.3.4 Confirmation

Quantitative effect confirmation of identified compounds was based on whole dose-response curves of the toxic fractions and artificial mixtures of pure compounds simulating these fractions (Figure 4-4). The parameters of the curves are given in Table 4-6. Except for DMSO-dosed F11 and silicone-dosed F15-15 fractions and corresponding artificial mixtures were in good agreement suggesting that major toxicants have been identified.

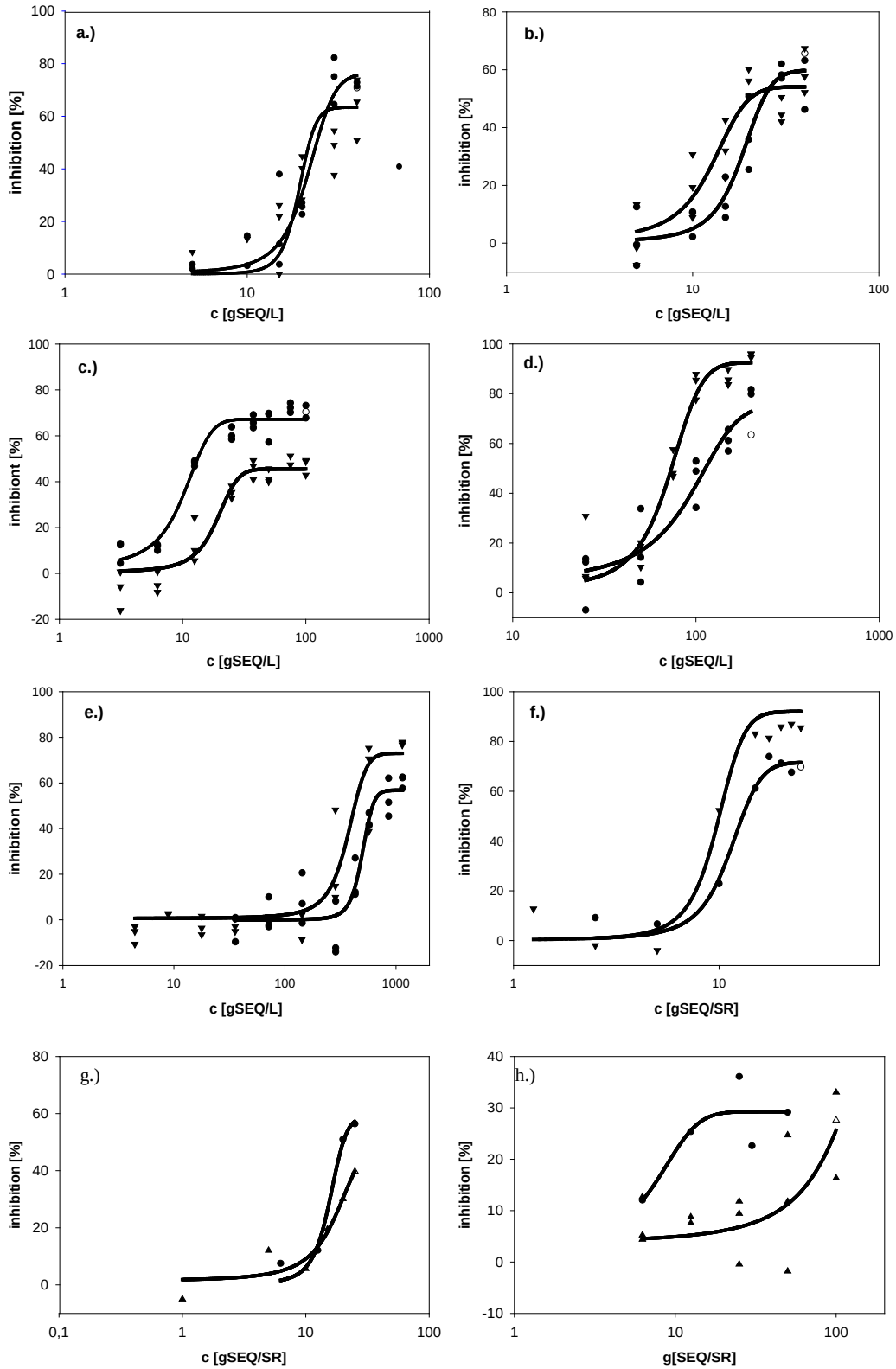

Figure 4-4: **Dose-response curves of fractions (●) and corresponding artificial mixtures (▲) in a 24 h growth inhibition test with the green algae *Scenedesmus vacuolatus*. The samples were either dosed with DMSO a.) F 10-6, b.) 10-5 c.) F11 d.) 8-7 e.) 15-16 or with silicone rods (SRs) f.) 14-7 g.) 15-RS h.)15-15. The concentrations are given as g sediment equivalents (g SEQ).**

Table 4-6: Parameters for dose-response curves fitted to fractions (F) and corresponding artificial mixtures (Mix). A three parameter sigmoid model was used for fitting ($y = a/(1+\exp((x-x_0)/b))$).

Sample	a	b	x_0
F10-6	75.93	3.77	21.51
Mix 10-6	60.75	4.73	18.53
F10-5	59.79	3.49	18.32
Mix 10-5	55.87	5.78	11.12
F11	67.22	2.98	10.03
Mix 11	45.54	4.15	18.75
F 8-7	75.34	32.56	90.42
Mix 8-7	92.50	16.08	70.85
F15-16	56.77	61.68	493.97
Mix 15-16	73*	76.00	365
F14-7	71.60	2.05	11.29
Mix14-7	92*	1.59	9.74
F15 RS	58.82	2.60	15.58
Mix 15 RS	48.91	5.10	17.51
F15-15	29.26	2.70	7.24
Mix 15-15	57.94	41.96	109.59

* These values have been fixed for the fitting, because otherwise no meaningful fits were achieved.

Quantitative confirmation was based on ICQ using ICQ according to equation 2 [9, 10]. An ICQ of 1 indicates 100% overlap of the dose-response curves and thus unambiguous confirmation. If the ICQ is > 1 the fraction is less toxic than the artificial mixture for example due to limited availability of the compounds. An ICQ < 1 indicates that the mixture could not fully explain the observed toxicity and further, not yet identified toxicants are possibly present. Recently Schwab et al. [14] proposed in agreement with a previous study [15] that dose-response-plots of samples and artificial mixtures should be regarded as significantly different if their 95 % confidence intervals do not overlap.

This corresponded for several cases to an ICQ < 0.5 or ICQ > 2 in the study of Schwab et al. In our study we found a similar variance. Thus, we consider identified toxicants as confirmed by standards or artificial mixtures thereof to cause the measured effect if 2 > ICQ > 0.5. ICQs were calculated for five prioritised DMSO dosed (F8-7, F10-5, F10-6, F11 and F15-16) and for three prioritised SR-dosed fractions (F14-7, F15-15 and F15 RS) (Figure 4-6). For all sub-fractions nearly the whole curve is in range between 0.5 and 2 indicating reasonable confirmation of identified compounds. SR-dosed F15-15 and DMSO-dosed F11 achieved only ICQs of about 0.1 and 0.3, respectively, indicating presence of additional non-identified contributors to

toxicity. For DMSO-dosing non-polar compounds such as PAHs and hexadecanol were confirmed as responsible toxicants.

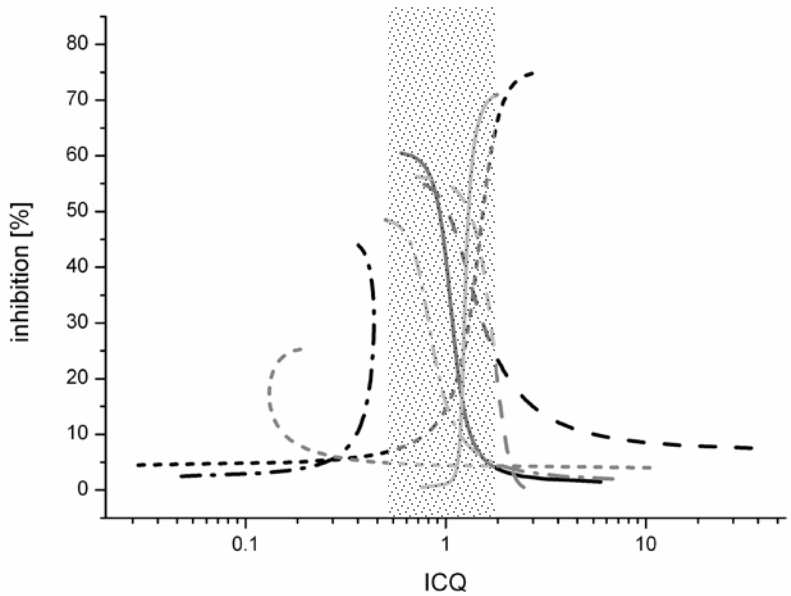

Figure 4-5: **Index of Confirmation Quality of sub-fractions for dosing with DMSO (—F10-6, – – F10-5, – . – F11, ... F8-7 and – – F15-16) and for dosing with SRs (— F14-7, ... F15-15 and – . – F15 RS). The range of ICQ between 0.5 and 2 is shaded in grey.**

PAHs were frequently identified as key toxicants in sediments [16, 17] contaminants and are well known for their algal toxicity [17]. Hexadecanol is used as emulgator in personal care products and probably have a high baseline toxicity due to its high K_{OW} value. Hexadecanone and pentadecanol dosed with SRs did not explain much of the observed toxicity. Their sediment concentration was in the same range as for hexadecanol and hexadecanone showed algae toxicity in another study with *Selenastrum capricornutum* (EC_{50} = 3.87 mg/L) [18]. The non observance of toxicity is possibly due to the high log K_{OW} of the compounds and the resulting small water concentrations when they are SR-dosed. Water concentrations (C_W) of F14-7 components triclosan, 2-methylanthraquinone, benz[*a*]anthracene and cyclopenta(*def*)phenantrone predicted from SR-water partitioning coefficient were related to their EC_{50} (Table 4-7). Triclosan predicted C_W exceeds its EC_{50} by a factor of 4 providing strong evidence that this compound is responsible for a major part of the effect, while for 2-methylanthraquinone predicted C_W (15 µg/L) is still in the order of magnitude of its EC_{50} suggesting also a contribution to the measured effects. Concentrations of the other compounds were more than one order of magnitude below EC_{50} values. The

major toxicant triclosan is used as a bactericide in pharmaceuticals and personal care products and was shown to be specifically toxic to green algae [19].

Table 4-7: **Identified compounds and EC_{50} values observed using the solvent dosing technique (DMSO) in a 24 h growth inhibition test with the green algae *Scenedesmus vacuolatus;* partitioning coefficients between silicone rod (SR) and water (K_{SW}), and subsequently calculated exposure concentrations (c_W) using SR for dosing with actually applied loads.**

compound	EC_{50} [µg/L]	K_{SW}	c_W [µg/L]
Triclosan	4.03	3.23	16.2
2-Methylanthraquinone	28.1	2.90	14.7
Benz[*a*]anthrone	38.1	2.00	1.34
Cyclopenta(def)phenanthren-4-one	- [1]	2.34	2.36
Benz[*c*]acridine	270	2.29	84.2
Hexadecanol[2]	296	-	-
Pentadecanol	380 (EC 30)	5.43	11.9

[1] No effects were observed, highest tested concentration 282 µg/L. [2] hexadecanole was not toxic with SRs dosing, and thus no partitioning coefficient was determined.

4.3.5 Influence of Dosing Technique on Fraction Prioritization

Our study suggests that the prioritisation of fractions and toxicants in EDA may strongly depend on the dosing technique. Conventional solvent dosing that ignores bioavailability prioritises highly lipophilic toxicants such as PAHs in agreement with priority pollutants according to the European Frame Work Directive [20]. In contrast EDA combined with partition-based dosing may direct the focus on more polar and bioavailable toxicants such as triclosan. This may increase environmental realism of EDA and help to identify those compounds posing major risks. Remaining limitations of partition-based dosing with surrogates such as silicone are the non consideration of the diversity of interactions with native organic matter such as the binding to black carbon [21] or H-bonding or ionic interactions of more polar compounds [22], that cannot be described with simple partition coefficients related to log K_{OW}. Limited accessibility of sequestered lipophilic sediment contaminants due to slow desorption kinetics are not considered in partition-based dosing, but may be considered combining this technique with bioaccessibility-directed extraction methods e.g. using TENAX® [14, 23].

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Chapter 5

Conclusions

The present study successfully developed methods for exposure control and bioavailability simulation in EDA studies of contaminated sediment samples. It was shown that nd-SPME is an appropriate method to monitor the freely dissolved concentrations during exposure of green algae *Scenedesmus vacuolatus* in a cell multiplication inhibition test. Nd-SPME has no impact on the sample concentration and the analysis is not influenced by the cells in the sample. Thus, the method enables the determination of EC_{50} values based on a more realistic basis (see Chapter 2).

Bioavailability can be included in EDA studies by using partition-based dosing as shown in Chapter 3. Partition-based dosing with SRs is well suited to deal with complex mixtures of unknowns. SRs exhibit a high loading capacity, fast equilibrium times and achievement of constant concentrations as shown for a set of compounds with a broad range of physico-chemical properties. Good dose-response relationships were achieved when loading single compounds or complex samples as sediment extracts. Figure 5-1 gives a schematic overview of the partition based dosing method developed in this thesis.

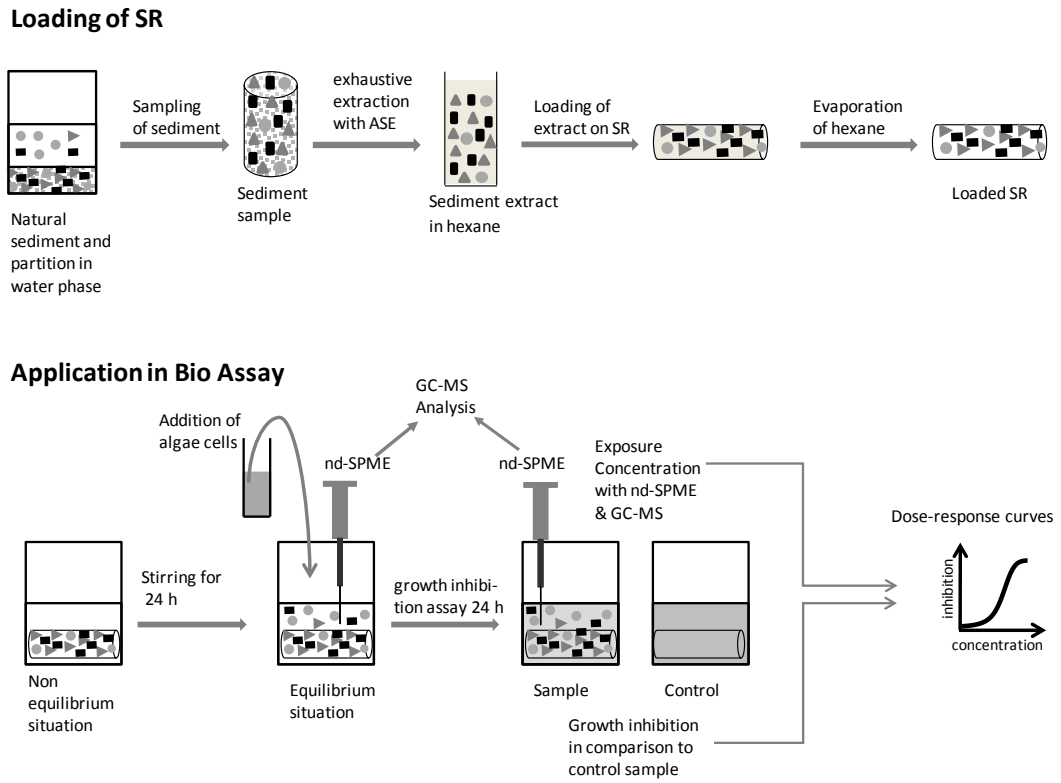

Figure 5-1: **Overview of developed method. The silicone rods (SR) are loaded with sediment extract containing compounds with different hydrophobicity: ▬ hydrophobic compound, ▶ medium hydrophobic compound and ● hydrophilic compound. The partition of the compounds from the SR into the test medium of the bio assay is analog to the partition in the natural sediment water system. The exposure concentration is determined with non depletive solid phase micro extraction (nd-SPME) in combination with gas chromatography with mass selective detector (GC-MS).**

The results of Chapter 3 and 4 confirm that the prioritized fractions and, therefore, the identified key toxicants depend strongly on the set up of the EDA procedure. The differences in fraction prioritization between the two dosing methods were more pronounced than the differences between the three sediment samples. This lead to the conclusion that differences in identified pre-dominant toxicants in sediments may not be caused only by site specific contamination profiles but rather by the way how bioavailability is assessed.

However, it is obvious that SR-dosing of exhaustive extracts cannot fully cover all relevant processes determining real world bioavailability. Several relevant aspects are not considered by this technique so far. In the following part the advantages and limitations of the developed method will be discussed.

Organism

The aim of studies on bioavailability is to predict the concentration of compounds in the organism at the target site, which is decisive for the observed toxic effect [1]. The often used equilibrium partitioning theory (EqP) assumes that the distribution of hydrophobic chemicals is in equilibrium between the sediment normalized to the organic carbon content, the aqueous phase and the organism normalized to the lipid content and can be predicted using partition coefficients as e.g. the K_{ow} of the compounds [2]. Under real environmental conditions there are several processes, which lead to non equilibrium situations: i.) fluctuating environmental and thus exposure concentrations [3, 4], ii.) kinetic limitations caused by slow uptake kinetics due to unfavorable surface to volume ratio of the organism or high hydrophobicity of the compounds [5] and iii.) decreasing concentrations in the organism by growth and metabolism [6]. Kinetic models assuming first order kinetics try to consider this by including uptake and release of compounds as well as metabolic pathways and can thus be used to predict concentrations in the organism in non equilibrium situations [6].

Besides the environmental conditions the bioavailability of compounds strongly depends on the organism under study itself. According to their habitat and feeding behavior different exposure routes are possible and should be taken into consideration. Organism living directly in the sediment may uptake the compound not only from the aqueous phase, but also from direct contact with sediments [7]. Ingestion of sediment particles often lead to enhanced uptake of the adsorbed chemicals by facilitated release of the compounds by the gut fluids and by a preferred uptake of small sediment particles with a higher load of contaminants [8-10]. Another shortcoming of the equilibrium theory is that organism are not passive, membrane transporters can actively reduce the concentration in the cells [11] and some organism can avoid uptake by behavioral response like escaping from the contaminated site [12] or changing feeding behavior [10]. Sediment contact tests may overcome a lot of the above mentioned problems and would directly include bioavailability in the testing of sediments. These tests have been already developed for a broad range of test species, such as zebra fish, snails, nematodes and macrophytes [13-17], and could, therefore, amplify

the range of possible used organism. As it was shown that differences in metabolic pathways strongly influence the final concentration at the receptor at the target site [18, 19], a bio assay battery analogous to toxic effect evaluation is recommended rather than using a single species test to gain a clear picture of the effects.

For the green algae *Scenedesmus vacuolatus* used in this work the uptake from the water is the most important exposure route and thus, the free concentration in the water phase is the key parameter. Therefore, it is possible to use partition based dosing for establishing this free concentration in the laboratory tests and exposing the organism only to the available fraction of the contaminants. This experimental set up is limited to simulate uptake from the water phase. Before transferring it to other organisms it is essential to evaluate critically if the other exposure routes can be neglected or if another experimental setup is necessary for proper simulation of bio availability.

Biomemitika

Extraction methods providing extracts of sediments for further investigations are normally optimized for depletive extractions. However, even if these extracts are dosed to the test system via partition-based dosing, the exposure concentration may be overestimated. This is due to strong binding sites such as black carbon and soot which cause limitation of the desorption kinetics in sediments [20, 21]. These kinetic limitations of desorption may be overcome by extraction techniques that yield only rapidly desorbable and, thus bioaccessible fractions of sediment-associated compounds. Several extraction methods have been suggested to achieve that goal including supercritical fluid extraction [22], extraction with cyclodextrin [23] or with solid adsorbents such as TENAX® [24]. As already discussed in the introduction the use of such biomemitic techniques are better suited than using real organism as a kind of living passive sampler due to the non-existence of metabolilsm. The partition based dosing technique developed in this study can be combined with such bioaccessibility-directed extraction techniques in order to exclude those fractions that fail to desorb in ecotoxicologically relevant time frames. Provided that the extraction method is fully validated for the whole range of sediment-binding compounds the combination of partition-

based dosing with bioaccessibility-directed extraction will be a reasonable next step to further enhance environmental realism of EDA of sediments.

Combination of extraction with sediment contact tests may be used to confirm that the compounds extracted with the adsorbens are the responsible toxicants in the field. Sediment TIE studies using whole sediments are based on this principle [25-27]. For TIE studies the sediments are manipulated by mixing with different specific resins to reduce the bio availability of compound classes. Up to now resins or special treatments are available to specifically bind ammonia, organic contaminants and metals [28]. First attempts have been made in separating the resin from the sediment by sieving for further exploration of the adsorbed chemicals [29]. After back extraction they are accessible for further separation procedures as fractionation steps and also for chemical analysis. The fractionated extracts are tested via aquatic toxicity tests. Therefore, only in the first step real sediment contact test are performed and the bioavailability is not included in the later toxicity tests. Extracting the sediments with bioaccessibility-directed extraction and re-dosing fractions to the sediments could open the possibility to include sediment contact tests in further steps of EDA procedures.

Draw back tests using whole sediments are co-founding factors as possible oxygen depletion, which can also cause toxic effects [30, 31], effects to different hardness/salinity of the sample and problems to consider the dilution by the resin [28]. Regarding organism with particle ingestion as an important exposure route, it has to be evaluated carefully if binding to the resin results in a decrease in bioavailability or not.

Partition based Dosing

The K_{ow} is often used to describe and predict the binding of compounds to the organic carbon of sediments [2, 32]. For silicon this correlation also was found and is, therefore, often used for partition experiments. Most of the work done in this field focuses on the delivery of hydrophobic compounds. Studies using different compound classes e.g. PAHs, chlorinated hydrocarbons and pharmaceuticals revealed that partition coefficients often show this correlation only for one compound class [33] and cannot be generalized. Despite the generally good relationship it is possible that especially for polar

compounds also other parameters and interactions play an important role [34, 35]. This interaction does not only change the behavior in respect of partition in the silicone, but also the interactions with the sediment [36]. In the past the behavior of polar compounds has been less investigated than that of non polar contaminants and should gain more attention.

The loading is very important for the effectiveness of this method and should allow establishing the same relative concentrations between the compounds as observed under field conditions. Different loading procedures are described in literature: loading from an water-methanol mixture [37], loading from methanolic solutions according to the activity of compounds [38] and loading by swelling of the material [39]. Loading from aqueous solutions is limited by the solubility of the compounds and by the partition coefficient resulting in a different proportional load of the compounds compared to the original situation in the sediment. The method using swelling of the material does not have these problems, but the material might be overloaded resulting in the formation of crystals [40]. Before the crystallized compound can be released they have to be dissolved requiring additional energy. In this case, the partition would depend on another parameter than the partition coefficient alone. These problems could be prevented if the compounds are loaded from a saturated methanolic solution. For real samples this method is not suited due to high amount of sample needed and the swelling method should be preferred. Nevertheless, further experiments with model compounds should be conducted to critically investigate the hypothetic crystal formation and effects on the partition behavior caused by overloading.

Identified Compounds

As toxicants will be identified in the prioritized fractions only, the design of the study preselects the possible identified toxicants. This underlines the need for a realistic exposure scenario in the bioassay, as it strongly impacts the outcome of the whole study. This aspect was elaborated in Chapter 4, which shows that the importance of polar substances as e.g. triclosane and benz[*c*]acridine is increasing when the bioavailability is considered. Lipophilic compounds like e.g. PAHs, which have been regarded as major toxicants in sediments so far, may be of less relative importance compared to more polar compounds if the bioavailability is considered. Thus, bioavailability simulation

in EDA may help to enhance environmental realism of the analysis and assessment of contaminated sediments.

A recent EDA study based on TENAX® and solvent extraction identified PAHs together with more polar compounds such as N-phenyl-2-naphthylamine or 7H-benzo[*de*]anthracene-7-one as key toxicants and also suggested a shift from non-polar to more polar fractions when bioaccessibility-directed TENAX® fractionation is applied [41]. Therefore, the dominance of the fractions containing more polar compounds will increase even more if desorption kinetics and equilibrium partitioning are both considered by extraction and dosing.

Table 5-1: Overview of the identified key toxicants in this study from sediments from the Bilina River, Elbe River and Spittelwasser. The used dosing technique solvent carrier dosing with dimethylsulfoxid (DMSO) or partition based dosing with silicone rods (SR) is specified.

Confirmed Toxicants	Dosing DMSO	Partition based dosing	Priority compound
PAHs	X	-	yes
Hexadecanol	X	-	no
Triclosan		X	no
Benz[*c*]acridin		X	no
2-Methylanthraquinone		X	no
Benzanthrone		X	no
Cyclopenta(*def*)phenanthren-4-		X	no
Benzanthrone		X	no
N-phenyl-2-naphtylamin		X	no

Until now non polar contaminants with a high accumulation potential are in focus in research on contaminated sediments [42] and are also part of regulations as the list of priority substances defined by the water framework directive [43]. Comparing the compounds (table 5-1) identified in this study with these traditional sediment contaminants reveals the shortcoming of this set of compounds. Especially when the lower availability of non polar compounds is considered by using partition based dosing real sample key toxicants may differ significantly from those that are included in the list. Therefore, it is not surprising that in many cases observed effects and thus,

an insufficient ecological status cannot be explained by the chemical status [44].

These outcomings are supported by the results of the integrated EU project MODELKEY (models for assessing and forecasting the impact of environmental key pollutants on marine and freshwater ecosystems and biodiversity). A variety of bioassays using several endpoints as mutagenicity, tumor promotion and endrocrine disruption (estrogenic, androgenic, arylhydrogencrabon-receptor mediated, thyroid hormone disturbing) also identified the polar fractions and, thus, polar toxicants as major cause. Identified compounds were e.g. polar PAHs deritivates as keto- or nitro-PAHs, musk compounds and the flame retardand tri(2-chloroisopropyl)phosphate [42]. Altogether the results of this work and of the MODELKEY project show that alternative approaches besides the today used priority list have to be applied, if effects cannot be explained by the presence of priority compounds. The methodology presented here can be seen as a part of a toolbox, which also includes exhaustive and bioaccessibility directed extractions [45], multistep fractionation procedures [46] and preparative gas chromatography [47] as well as computer tools for structure elucidation [48]. This toolbox may help water managers to identify causes in the case of bad chemical status of their water bodies. Only when these causes are identified properly, a reasonable risk and hazard assessment is possible.

Research needs

This work and the outcome of the whole MODELKEY project show that polar compounds should receive more attention during toxicity assessment of sediments. This includes that more methods and knowledge special for polar compounds has to be generated. In contrast to non polar toxicants e.g. PAHs and PCBs, which have been considered as important environmental toxicants for more than two decades and are subject of numerous studies, polar compounds are a relatively new group of environmental pollutants. Therefore, there still is a lack of methods for sampling, sample preparation and measurement of these compounds. Especially the parameters describing the partition between different phases are important and until now the

prediction of the partition behavior is less reliable than for the non polar compounds.

This thesis can be seen as a first step, which helps to increase *in situ* relevance of laboratory experiments for EDA studies in regard to the bioavailability. As discussed in the last paragraphs, this relevance can be increased stepwise:

1.) extraction methods providing only the bio-accesible fraction, which desorbs in relevant timeframes as e.g. TENAX® should be combined with the partition-based dosing method. This includes a validation of the extraction methods for a broad range of polar toxicants.

2.) Combination of this extraction with sediment contact tests by performing it with and without resin can confirm that the identified toxicants were in the mixture causing effects in the field.

3.) Transferring the method to organisms with other exposure routes than the water phase by re-dosing the fractionated extracts to sediments and applying it in that manner in sediment contact tests.

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Tuikka AI, Schmitt C, Höss S, Bandow N, von der Ohe PC, de Zwart D, de Deckere E, Streck G, Mothes S, van Hattum B, Kocan A, Brix R, Brack W, Barcelo D, Sormunen AJ, Kukkonen JVK. 2010. Toxicity assessment of sediments from three European river basins using a sediment contact test battery. *Ecotox Environ Saf* 74:123-131.

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Platform Presentations

Bandow N, Altenburger R, Schwab K, Streck G, Brack Werner, Consideration of bioavailability in the effect-directed analysis of sediments. MODELKEY meeting, December 2009, Leipzig.

Bandow N, Altenburger R, Lübcke-von Varel U, Pascke A, Streck G, Brack Werner, Partitioning based dosing in the EDA. MODELKEY meeting, November 2008, Venice

Bandow N, Lübcke-von Varel U, Altenburger R, Brack Werner, Partitioning based dosing - including the bioavailability in the effect directed analysis of contaminated sediment samples, SETAC Europe, May 2008, Warschau.

Bandow N, Lübcke-von Varel, Paschke A, Altenburger R, Brack W, PDMS coated stirring bars - a new method to include the bioavailability in the effect-directed analysis of contaminated sediments, SETAC Europe, May 2007, Porto.

Bandow N, Altenburger R, Paschke A, Brack W, Verteilungsbasierte Dosierung von Modellsubstanzen: Einsatz von Polydimethylsiloxan beschichteten Rührfischen im Algentest (*Scenedesmus vacuolatus*), GDCh Tagung, Fachgruppe Umweltchemie und Ökotoxikologie, October 2006, Halle.

Bandow N, Altenburger R, Pascke A , Brack W, Application of PDMS-coated stirring bars as a dosing technique in the bio assay with green algae *Scenedesmus vacuolatus*, MODELKEY (EU project) meeting, October 2006, Leipzig.

Poster Presentations

Bandow N, Egerer S, Schaumann GE, Development of an EDA method to identify hydrophobicity causing compounds in olive oil mill waste waters, SETAC Europe , Mai 2010, Seville.

Bandow N, Streck G, Lübcke-von Varel, Paschke A, Altenburger R, Brack W, Partitioning-based dosing – an approach to simulate bioavailability in the effect directed analysis of contaminated sediments, Workshop Water framework Directive,2008 Koblenz.

Bandow N, Streck G, Lübcke-von Varel, Paschke A, Altenburger R, Brack W, Partitioning-based dosing – an approach to simulate bioavailability in the effect directed analysis of contaminated sediments, SETAC World, August 2008, Sydney.

Bandow N, Streck G, Lübcke-von Varel, Altenburger R, Brack W, Effect-directed analysis of a sediment sample from the Bilina River (Most, Czech Republic) using green algae *Scenedesmus vacuolatus*, SETAC Europe, Mai 2008, Warzaw.

Bandow N, Küster E, Brack W, Toxicity characterization of Freshwater Sediments using a Sediment Contact Test with Embryos of the Zebrafish *Danio rerio*, SETAC Europe, Mai 2006, Den Haag.

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