Development of a passive sampling device for combined chemical and toxicological long-term monitoring of groundwater

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Abbreviations

1-MNap	1-methyl-naphthalene
2-MNap	2-methyl-naphthalene
Ace	acenaphthene
Ant	anthracene
Any	acenaphthylene
BaA	benzo[a]anthracene
BaP	benzo[a]pyrene
BbF	benzo[b]fluoranthene
BkF	benzo[k]fluoranthene
BghiP	benzo[g,h,i]perylene
BTEX	benzene, toluene, ethylbenzene and xylenes
CFDA-AM	5-carbonxyfluorescein diacetate acetoxymethyl ester
CHC	chlorinated hydrocarbon
Chr	chrysene
CYP1A	subfamily of cytochrome P450 (monooxygenase enzymes involved in the phase I metabolic pathway)
DBA	dibenzo[a,h]anthracene
DGT	diffusive gradient in thin films
DMLS	diffusive multi layer sampler
DMSO	dimethylsulfoxide
DVB	divinylbenzene
EC_{50}	effect concentration where 50 % of the measured effect is detected
equ	equilibrium
EROD	7-ethoxyresorufin-*O*-deethylase
FA	fluoranthene
FBS	fetal bovine serum
Flu	fluorene
FU	fluorescent unit
IndP	indeno[1,2,3-cd]pyrene
K_{bw}	Biosilon/water partition coefficient
K_{ow}	octanol/water partition coefficient
L-15	Leibovitz's L-15 medium
LOQ	limit of quantification
MESCO	membrane enclosed sorptive coating
MF	melamine resin
Milli-Q water	purified deionized water
NA	Natural Attenuation
Nap	naphthalene
NR	neutral red fluorescent dye
PAH	polycyclic aromatic hydrocarbon
PC	polycarbonate
PCB	polychlorinated biphenyl
PDBS	passive diffusion bag samplers
PDMS	polydimethylsiloxane
PDBE	polybrominated diphenylether
PDPO	poly-2,6-diphenyl-*p*-phenyleneoxide
PE	polyethylene
Phe	phenanthrene
POCIS	polar organic integrative sampler
PRC	performance reference compound
PS	polystyrene
Pyr	pyrene
R1	rainbow trout liver cell line (Ahne 1985)
RTL-W1	rainbow trout liver cell line (Lee 1993)
SPE	solid phase extraction
SPMD	semipermeable membrane device
SPME	solid phase microextraction
SVOC	semivolatile organic compound
TEC	toxic equivalency concentrations
TEF	toxic equivalency factor
TLC	thin layer chromatography
TWA	time weighted average
VOC	volatile organic compound

Summary

Sampling is an important aspect in groundwater monitoring which may influence all following steps involved in water quality assessment. Most common is the use of conventional snap-shot sampling, where a certain amount of water is pumped or bailed. Time-integrative passive sampling, in contrast, accumulates analytes out of the water on a special receiving phase along a diffusion gradient. Using such passive sampling devices enables the determination of time weighted average aqueous contaminant concentrations over extended sampling periods without any intermittent sampling steps. Thus, passive sampling devices are a valuable alternative for long-term monitoring of groundwater.

Another important aspect in conventional groundwater monitoring is that long-term monitoring is usually based on chemical analyses alone. The focus generally is on contaminants known or suspected to be present, for instance from site history. Thus, unexpectedly occurring compounds, which might be of toxicological relevance, might be overlooked. In contrast, biological analyses can assess effects elicited by complex mixtures in environmental samples as a whole.

On this background it was the scope of this thesis to develop a passive sampling device for combined chemical and toxicological long-term monitoring of groundwater. It thereby was a special aim to perform the toxicological analysis in a solvent-free, solid phase bioassay (bead assay) using adherence-dependent permanent vertebrate cell cultures as reporter system. Various solid phase sorbent materials were investigated concerning (1) their compatibility with fish cell cultures, which were used as model vertebrate cultures (2) their ability to sorb polycyclic aromatic hydrocarbons (PAHs) as hydrophobic model contaminants, and (3) the availability of these sorbed compounds to fish cells growing on the contaminated sorbents for detection of toxicologically relevant effects. From ten tested materials, Biosilon (a polystyrene polymer, usually used as cell culture carrier) was found to be most suitable because it enabled a reliable cell attachment and the detection of reproducible dose-response curves using PAH contaminated Biosilon as a cell culture surface.

Biosilon was then used as a receiving phase in the newly developed passive sampler, the Toximeter. This sampler is based on an earlier developed device, called the Ceramic Dosimeter. The Toximeter uses the same ceramic tube as diffusion barrier and container holding the solid receiving phase. Biosilon filled ceramic tubes were evaluated concerning their sampling behavior in three different laboratory exposure scenarios. Aqueous PAH concentrations derived from the exposed Toximeters were generally in good accordance with directly analyzed water samples with best results for compounds with a log K_{ow} 4.5-6.

In a third step, Toximeter sampling behavior was validated under field conditions. Toximeters were deployed in three groundwater wells at a PAH contaminated site. Toximeters were

exposed for one to six months, with monthly sampling. In parallel, snap-shot water samples were collected every two weeks. Aqueous PAH concentrations derived from the Toximeters were within a median factor of four of truly measured aqueous concentrations based on snap-shot sampling. In general, Toximeter-derived aqueous PAH concentrations underestimated those determined using the snap-shot water samples. Underestimation occurred mainly for lower K_{ow} PAHs (log K_{ow}<4.5) which might be attributed to the lower binding affinity of these compounds to Biosilon. Beyond the chemical analyses, Toximeter samples were investigated in the EROD bead assay developed during this project. Up to a two fold induction of EROD enzyme activity compared to the control was detected with Biosilon previously exposed in the field. The detected effects could only partly be explained by the analyzed PAH concentrations, so that other PAHs not detected in the standard analytical procedure, or completely different substance classes must have been responsible.

Taken together, the combined passive-sampling and chemical-biological analysis approach represents a novel alternative to current water sampling and analysis methods. The developed device and methodologies are amenable to a multitude of aqueous sampling and assessment scenarios. As developed in this thesis, they will be most useful in situations where a cost-efficient, integrative long-term monitoring strategy is required.

Zusammenfassung

Die Probennahme stellt einen wichtigen Aspekt bei der Überwachung von Grundwasser dar, der alle nachfolgenden Schritte der Überwachung der Wasserqualität beeinflussen kann. Üblicherweise werden konventionelle Stichprobennahmeverfahren angewandt, bei denen eine definierte Wassermenge gepumpt oder geschöpft wird. Im Gegensatz dazu werden bei der Probennahme mit zeit-integrierenden Passivsammlern Substanzen entsprechend ihres Massentransfergradienten ohne aktive Handlung aus dem Wasser aufgenommen und an einer Sammelphase akkumuliert. Der Einsatz solcher Passivsammler ermöglicht die Bestimmung von zeitlich gemittelten Schadstoffkonzentrationen im Wasser über lange Expositionszeiten ohne zusätzliche Schritte während des Beprobungszeitraums.

Ein anderer wichtiger Aspekt in der konventionellen Grundwasserüberwachung ist, dass meist nur chemische Analysen in der Langzeit-Überwachung zum Einsatz kommen. Dabei wird der Fokus überwiegend auf Kontaminanten gerichtet, deren Auftreten bereits bekannt ist oder z.B. aufgrund der Standortsgeschichte vermutet wird. Somit können unerwartet auftretende Substanzen, die von toxikologischer Relevanz sein können, übersehen werden. Im Gegensatz dazu können biologische Tests Wirkungen, die durch komplexe Mischungen in einer Umweltprobe ausgelöst werden, gesamtheitlich erfassen.

Vor diesem Hintergrund war es das Ziel der vorliegenden Dissertation, einen Passivsammler für die kombinierte chemische und toxikologische Langzeit-Überwachung von Grundwasser zu entwickeln. Ein spezieller Aspekt war dabei die Anwendung eines lösemittelfreien Festphasen-Biotests, unter Verwendung anheftungsabhängiger permanenter Wirbeltierzellkulturen als Reportersystem. Im ersten Schritt wurden verschiedene Sorbentien bezüglich (1) ihrer Kompatibilität mit Fischzellkulturen, die als Modell-Wirbeltierzellkulturen verwendet wurden, (2) ihrer Fähigkeit, Polyzyklische Aromatische Kohlenwasserstoffe (PAKs) als hydrophobe Modellschadstoffe zu sorbieren, und (3) der Verfügbarkeit dieser sorbierten Substanzen für Fischzellen, die auf der Oberfläche der kontaminierten Sorbentien wachsen, um toxikologisch relevante Effekte zu detektieren, untersucht. Von zehn untersuchten Materialien erwies sich Biosilon (ein Polystyrol-Polymer, das als Trägermaterial für die Zellkultur verwendet wird) als am besten geeignet, weil es zuverlässig die Zellanheftung ermöglichte und eine reproduzierbare Aufnahme von Dosis-Wirkungskurven mit PAK-kontaminiertem Biosilon als Zellkulturoberfläche gewährleistete.

In dem neu entwickelten Passivsammler, dem Toximeter, wurde dann im zweiten Schritt Biosilon als Sammelphase eingesetzt. Dieser Sammler basiert auf einem früher entwickelten Probennehmer, dem so genannten Keramik-Dosimeter. Das Toximeter verwendet das gleiche Keramik-Röhrchen als Diffusionsbarriere und als Behälter für die Sammelphase. Biosilon-gefüllte Keramik-Röhrchen wurden bezüglich ihres Sammelverhaltens in drei verschiedenen

Expositionsansätzen im Labor untersucht. Über das Toximeter bestimmte PAK-Konzentrationen im Wasser stimmten allgemein gut mit den direkt analysierten Konzentrationen überein, wobei die besten Ergebnisse für Substanzen mit einem log K_{ow} Wert von 4,5-6 erzielt wurden.

In einem dritten Schritt wurde das Sammelverhalten des Toximeters unter Feldbedingungen untersucht. Dazu wurden Toximeter an einem PAK-belasteten Standort in drei Grundwasserbrunnen ausgebracht. Die Toximeter wurden für Zeiträume von ein bis sechs Monaten exponiert, wobei monatlich Sammler entnommen wurden. Parallel dazu wurden die Brunnen im Abstand von je zwei Wochen konventionell beprobt, um Stichproben zum Vergleich heranziehen zu können. Über das Toximeter bestimmte PAK-Konzentrationen im Wasser wichen mit einem mittleren Faktor von 4 von den in den Stichproben bestimmten Konzentrationen ab. Generell unterschätzten die Toximeter die PAK-Konzentrationen der Stichproben. Die Unterschätzung trat vor allem für PAKs mit einem niedrigeren K_{ow} Wert (log K_{ow}<4.5) auf, was durch die geringere Sorptionsaffinität dieser Substanzen an Biosilon erklärt werden kann. Zusätzlich zur chemischen Analyse wurden Toximeter-Proben im EROD Festphasen Test, der in dieser Arbeit entwickelt wurde, untersucht. Dabei wurde eine bis zu zweifache Induktion der EROD Enzymaktivität gegenüber der Kontrolle mit feldexponiertem Biosilon gemessen. Die beobachteten Effekte konnten nur teilweise durch die gemessenen PAK Konzentrationen erklärt werden. Andere PAKs, die nicht in der Standardanalytik erfasst wurden, oder andere Substanzklassen müssen dafür verantwortlich gewesen sein.

Der kombinierte Ansatz aus chemisch-biologischer Analyse verknüpft mit der Passivsammlertechnologie stellt eine neue Alternative zu den gegenwärtigen Wasserprobennahme- und Analysenmethoden dar. Der hier entwickelte Probennehmer und die Methoden können für eine Vielfalt von Wasserprobennahme- und Überwachungs-Maßnahmen angewandt werden. Einsatzmöglichkeiten sind vor allem im Bereich der kostengünstigen, integrierenden Langzeit-Überwachung vorstellbar, wie anhand der Feldstudie der vorliegenden Arbeit gezeigt wurde.

Chapter 1

General Introduction

In recent years there has been a growing awareness of the need to improve the ability to assess groundwater quality. Groundwater is an important source for drinking water. In Germany nearly 80 % of drinking water is gained from groundwater resources (TEUTSCH 1998). Therefore, one focus is on the survey of groundwater quality for drinking water production. The second aspect is the monitoring of contaminated sites in order to remediate them or prevent the spreading of contaminant plumes. An overview on current restoration technologies can be found in KHAN *et al.* (2004). It is often impossible to reach environmentally sound levels, compatible with health-based standards, by active remediation strategies for groundwater at contaminated sites. However, restoration of contaminated aquifers to pristine levels is not always necessary and in most cases neither technically nor economically feasible (TRAVIS AND DOTY 1990). Thus, other strategies such as natural attenuation (NA) or intrinsic bioremediation are gaining increasing importance (TEUTSCH *et al.* 1997). For both the evaluation of the success of active remediation as well as plume control in long-term monitoring in the NA approach, adequate methodologies for site investigation are crucial. Two important aspects in site investigations and groundwater monitoring are firstly the application of suitable sampling techniques, and secondly the approaches pursued for sample evaluation.

1.1 Current sampling strategies

Sampling is an important aspect in groundwater monitoring because it may influence all subsequent steps (WILSON 1995). Most common is the use of conventional snap-shot sampling techniques, where a certain amount of water is pumped or bailed. This is a very cost- and labor-intensive method, as large sample numbers have to be dealt with for long-term monitoring. As well, particularly for groundwater monitoring, natural water flow regimes are easily disturbed (fig. 1.1 A). Time-integrative passive sampling, in contrast, accumulates analytes out of the water on a special receiving phase along a diffusion gradient without disturbances of water flow (fig. 1.1 B). Using such passive sampling devices enables the determination of time weighted average aqueous contaminant concentrations over extended sampling periods, whereas snap-shot sampling can only determine instantaneous aqueous concentrations (KOT *et al.* 2000). Thus, passive sampling devices are a valuable low-cost

alternative for long-term monitoring of groundwater. A detailed overview on passive sampling theory, devices, and applications is presented in Chapter 2 and forms one of the bases of the research presented here.

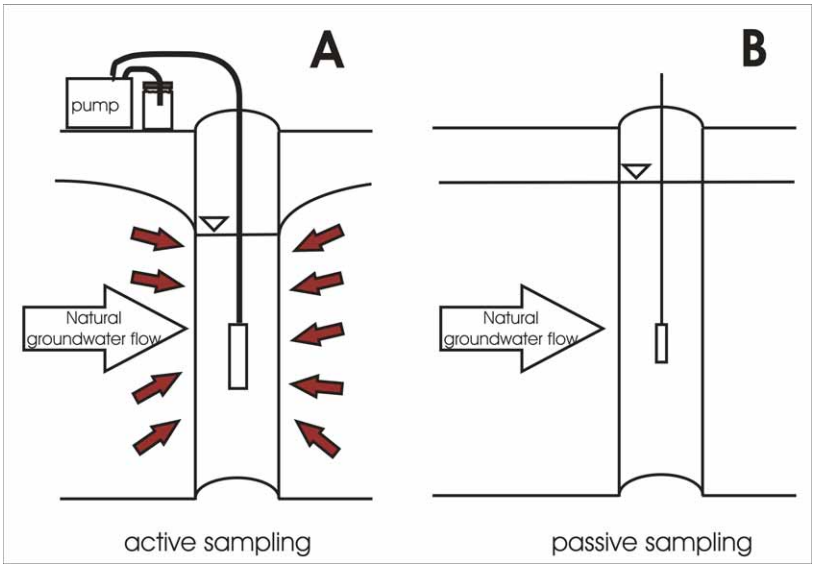

Figure 1.1 Comparison of active (A) and passive (B) groundwater sampling.

1.2 Approaches for combined chemical-biological monitoring

Most common in water survey programs is the use of chemical analysis to identify hazardous pollutants. Progressing methodologies in environmental chemical analytics enable a fast and sensitive detection of a broad variety of compounds. However, in chemical analysis, the focus is generally on pollutants known or suspected to be present, for instance from site history. Thus, unexpectedly occurring compounds, which could be of toxicological relevance, might be overlooked. Consequently, biological analyses performed alone or in addition can be an important alternative or complement in order to assess the sample as a whole (fig. 1.2). While biological or a combined chemical-biological analysis is nowadays often pursued in surface water, biological monitoring of groundwater is still rarely applied.

HELMA *et al.* (1998) performed three genotoxicity and three ecotoxicological assays, using bacteria, plants, daphnia, and rat hepatocytes, with groundwater samples. They could not detect a causal relationship between the measured biological effects and the physical / chemical parameters determined. A prediction of toxic potential of the samples from chemical analysis could not be deduced. Therefore they suggested to include toxicity assays in routine monitoring studies. BAUN *et al.* (2003) reported that nonvolatile organic xenobiotics determined by chemical analysis could not explain toxic effects on algae and luminescent bacteria caused by testing of SPE extracts of contaminated groundwater from a landfill leachate plume. They showed that in this case study, where monitored natural attenuation was applied, toxic effects could still be detected more than 135 m downstream of the landfill.

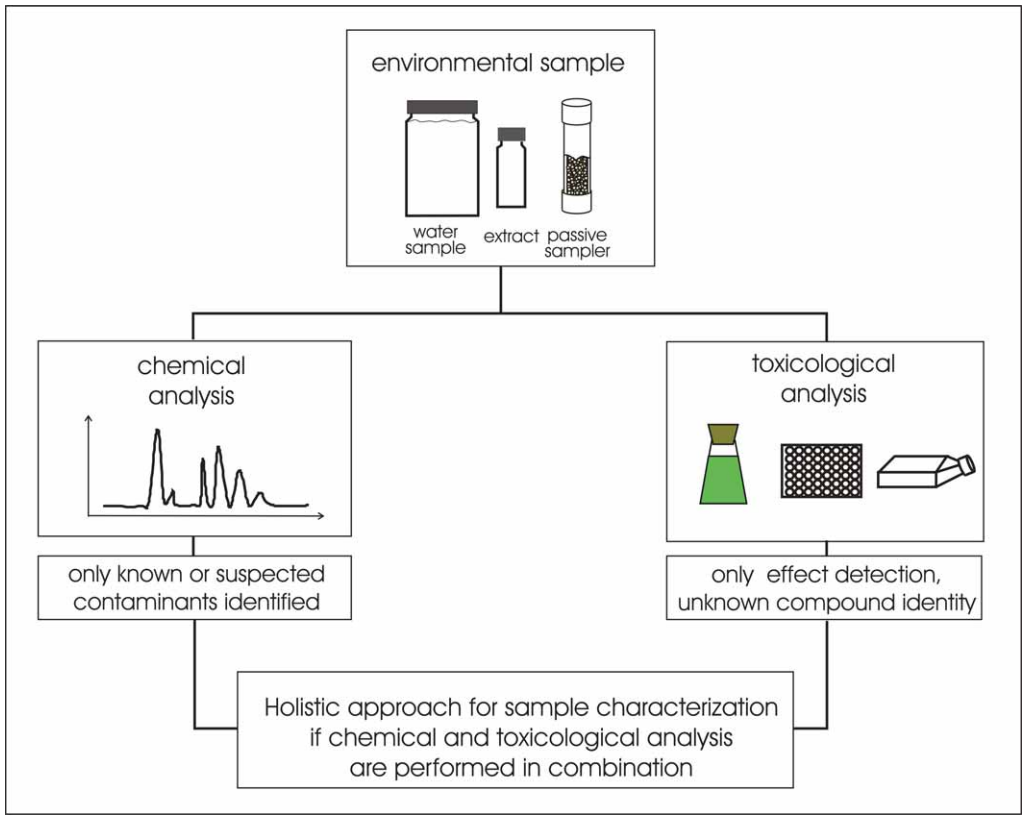

Figure 1.2 Holistic approach for sample characterization combining chemical and biological analysis.

In another study, investigating toxic effects elicited by groundwater downstream of another landfill, toxic effects decreased with increasing distance to the contamination source and background levels were reached within 80 m (BAUN *et al.* 2000). They showed that the application of batteries of biotests can be a useful tool for toxicity characterization and hazard ranking of sites polluted with complex mixtures of contaminants. GUSTAVSON *et al.* (2000) proved that *in vitro* biological assays can be an important complement to chemical analysis during monitoring remediation processes at contaminated sites. SCHIRMER *et al.* (2004) applied fish cell culture *in vitro* assays in a monitoring program of a benzene contaminated site and compared the results from biological and chemical analysis. Chemical analysis detected mainly high concentrations of benzene and toluene. Direct testing of groundwater samples in a specific EROD enzyme induction test, which detects the presence of dioxin like compounds, showed high induction levels for some groundwater wells, which could not be explained by the presence of compounds which were analyzed in chemical standard procedures. Compounds like benzofurane, indane and indene were identified as potential new EROD inducing contaminants. The combination of toxicological and chemical analysis in this case showed the importance of investigating samples beyond the main or known contaminants and in addition, helped to discover transport phenomena in the aquifer affected by different contaminant source zones (RUSSOLD 2003).

1.3 Aim and structure of this thesis

Based on the valuable mutually complementary nature of chemical and biological investigations, a passive sampling device was developed within the current study in order to provide a simple and cost-effective alternative for such a combined approach. Up to now, toxicological analyses have been limited to two passive sampling devices (PETTY *et al.* 2004). In these cases, solvent extracts of passively collected contaminants were used for exposure in bioassays which may bear problems due to toxic solvent effects or interactions of the solvent with sample constituents (CHAURET *et al.* 1998; YIN *et al.* 2001).

Thus the major goals of the present study were to develop a solvent-free, solid-phase bioassay as a pre-requisite to establish a passive sampling device, called the Toximeter, directly designed to combine chemical and biological analysis of passively collected samples. In the course of this work, five major steps were pursued.

Firstly, a detailed overview on passive sampling was developed and is summarized in **CHAPTER 2**. A short introduction into the function and analysis as well as various currently available sampling devices is presented.

Next, Chapters 3 to 5 summarize the design and results of the experimental part of this work. **CHAPTER 3** aimed at investigating the availability of sorbed contaminants to adhering fish cells. The development of a solvent-free, solid-phase bioassay with cultured fish cells growing on contaminated sorbent beads is delineated.

CHAPTER 4 deals with the laboratory evaluation of the Toximeter. Biosilon, which was identified to be a suitable receiving phase from a biological point of view in Chapter 3, was explored concerning its chemical suitability in the passive sampling device. The sampling behavior of Biosilon filled Toximeters for polycyclic aromatic hydrocarbons (PAHs) as model compounds was investigated in three different exposure scenarios.

A field validation of the new sampling system is presented in **CHAPTER 5**. Toximeters were deployed at a PAH contaminated gas works site for periods between one to six months. Chemical and biological analyses were performed after field exposure.

In the final **CHAPTER 6**, conclusions from the whole project are summarized and ideas for future research provided in order to aid further improvement and implementation of the new approach.

Chapter 2

Aqueous phase passive sampling – an overview

2.1 Introduction

One of the most important steps in analyzing water samples for contaminants is the sampling of water itself (WILSON 1995). Disturbances of water composition during sampling as well as alterations during transport and storage, all can irreversibly affect the outcome and subsequent interpretation of water analysis results. This sparks a common interest by scientists, engineers and regulators to standardize and improve water sampling techniques. The conventional, and most common, sampling technique is the *active* removal of a defined volume of water at a given time using bailers or pumps. The caveats of such an active sampling technique are well known. Contaminants can sorb to tubes and sampling containers. As well, they may be degraded and / or may decay during transport. For groundwater in particular, disturbed flow regimes due to pumping may lead to the pulling in of clean or contaminated water from zones not intended for sampling. Pumping can also cause losses of volatile compounds (POWELL AND PULS 1997). Knowledge about these and other problems associated with active sampling helps in the standardization of procedures in an attempt to reduce irreversible sample alterations. Yet, due to the many steps involved in active sampling and with the many sampling events needed to obtain information in space and time, minimization of sampling errors will always be a challenge.

In contrast to active sampling, the *passive* sampling approach relies on the passive uptake of contaminants into appropriate sampling devices without the use of bailers or pumps. Many passive sampling devices have been developed throughout the past years. However, while gaining significant acceptance by industry and regulators in the monitoring of air (BROWN 2000), the application of passive sampling devices in water is still limited to research use. Considering the potential of this technology, its current restricted use can likely be attributed to its comparably early stage in development and to a lack of widespread information, both scientifically and commercially. Several reviews have summarized the philosophy underlying passive sampling in general and have highlighted details of selected samplers. For example, GÓRECKI AND NAMIESNIK (2002) provided an excellent, detailed overview of the principles of passive sampling along with a brief summary of sampling in gas, liquid, soil and the use of living organisms as passive samplers. Further, Namieŝnik and colleagues were the first to

explicitly outline passive samplers used for the long-term monitoring of organic pollutants in water, focusing specifically on Semipermable Membrane Devices (SPMDs) as the most widely used samplers (KOT *et al.* 2000; ZABIEGALA *et al.* 2000). Since then, significant advances have been reported with regard to sampler optimization and data interpretation. As well, a number of new devices have been developed for passive water monitoring.

This review provides important general information with regard to passive sampling in water. Specifically, it presents an overview of the passive samplers developed and applied to water to date, along with facts regarding their availability and practical considerations. As well, current needs for further passive sampler development and more widespread application are presented and, at least in part, form the basis of this thesis.

2.2 Principles underlying passive samplers

2.2.1 Assembly

Most passive sampling devices consist of a container or diffusion barrier through which the contaminants of interest can traverse. The interior of the container is filled with a receiving phase, i.e. a sorbent material, capable of binding the contaminants of interest (fig. 2.1). The collection phase can be a liquid or a solid material. In the simplest case, the sampling container is filled with deionized water (VROBLESKY AND HYDE 1997; VROBLESKY AND CAMPBELL 2001; NAVFAC 2000). For most passive sampling devices, the sampling container itself serves as a barrier, limiting collection of contaminants to those that are truly dissolved in water and enter the sampling container by means of diffusion. At the end of the sampling period which, depending on the type of sampler and the aim of the investigation, can take weeks to months, the samplers are removed and contaminants collected are analyzed in the laboratory.

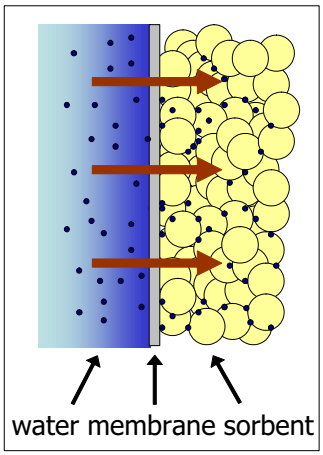

Figure 2.1 General passive sampling principle: substances (black dots) traverse through a diffusion barrier into the passive sampling device where they are entrapped by a receiving phase.

2.2.2 Equilibrium vs. non-equilibrium samplers

Based on the concentration gradient of contaminants in the water and on / in the collection phase, contaminants can diffuse into passive sampling devices until an equilibrium is reached. Upon achieving an equilibrium, further enrichment of contaminants within the sampler can no longer take place. Thus, the time span available until an equilibrium is reached depends on the capacity of the collection phase for the contaminants of interest. The capacity is defined by the amount and affinity of the collection material: the greater the amount of collection material and / or the greater its affinity for the contaminants, the greater the capacity. This implies that the distinction between equilibrium and non-equilibrium sampling is not always clear. Especially if a large number of contaminants with a broad spectrum of physico-chemical characteristics is to be sampled, it is conceivable that some contaminants may be present at equilibrium at the end of the sampling period while others are not. Despite this ambiguity, passive sampling devices can, for practical reasons, be divided into equilibrium- and non-equilibrium samplers (fig. 2.2).

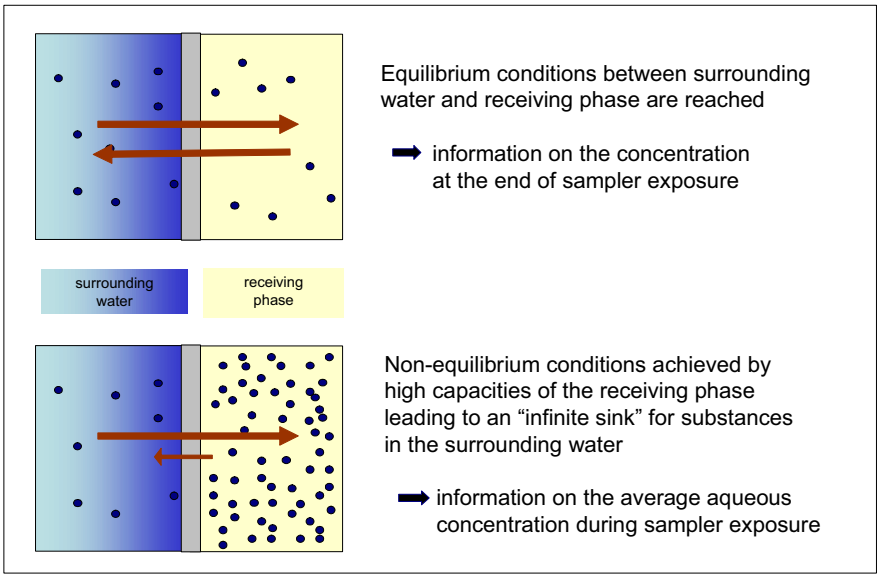

Figure 2.2 Comparison of the equilibrium and non-equilibrium passive sampling concepts.

Equilibrium samplers are characterized by a rapid achievement of equilibrium between the contaminants in the water to be sampled and the contaminants inside the passive sampler. One consequence of achieving an equilibrium rapidly is that contaminants are also capable of diffusing back into the surrounding water should aqueous concentrations of contaminants decline. Two frequently used equilibrium samplers are water-filled polyethylene (PE) bags (PDBS, passive diffusion bag samplers) and the Diffusive Multi-Layer Sampler (DMLS) (e.g. RONEN *ET AL.* 1987) (tab. 2.1). For both systems, equilibrium can be generally assumed to be reached within 7 days.

Non-equilibrium samplers are those that do not reach equilibrium with the surrounding water within the sampling period. These samplers are characterized by a high capacity to collect the contaminants of interest. The high capacity ensures that contaminants can be enriched continuously throughout the sampling period. As well, the high capacity means that contaminants are much less prone to diffuse back out of the sampler in case of decreasing aqueous concentrations. Thus, the high capacity of non-equilibrium samplers for contaminants of interest forms the pre-requisite for determining average contaminant concentrations present in the water over the entire sampling period. These concentrations are also referred to as time-weighted average concentrations (TWA) (GRATHWOHL AND SCHIEDEK 1996). Most passive samplers are being employed as non-equilibrium, i.e. time-integrating samplers, for periods of 2 weeks to about 3 months (tab. 2.1 & 2.2).

Whether a passive sampler behaves as an equilibrium or non-equilibrium sampler is also dependent on the partitioning properties of the chemicals. It might occur that samplers are in equilibrium for some environmental pollutants during field sampling while they are still in the non-equilibrium phase for other compounds (e.g. VRANA *et al.* 2001b).

2.3 Overview of passive samplers suitable for ground- and surface water

The monitoring of ground and surface water using passive sampling devices has been reported since the 1970s (BENES AND STEINNES 1974; MAYER 1976; HESSLEIN 1976). First sampler assemblies consisted of water filled dialysis tubes. Later on, organic solvent filled bags were used which allow the time-integrated sampling of hydrophobic substances (e.g. SÖDERGREN 1987). A similar type of sampling device is the SPMD (Semipermeable Membrane Device), which contains triolein, a neutral lipid, as the receiving phase (HUCKINS *et al.* 1990; PETTY *et al.* 2000b). SPMDs are often referred to as "virtual fish", as they mimic the uptake of contaminants over a membrane into a lipid phase. A number of passive samplers exist that rely on a solid sorbent as a receiving phase. These samplers are generally characterized by a high sorption capacity and ease of handling for chemical analysis, e.g. enabling direct thermodesorption of analytes from the receiving sorbent. More detailed information on individual passive samplers can be found in selected review articles such as in (KOT *et al.* 2000; BOPP AND SCHIRMER 2002). Although up to now, most passive samplers described in the international literature are still restricted to research use, several are commercially available and validated in the field. An overview is provided by separating passive sampling devices that are both commercially available and validated in the field under true environmental exposure conditions (tab 2.1) and samplers still significantly under development and evaluation (tab. 2.2).

Table 2.1 Overview on commercially available, field validated passive sampling devices

passive sampler type	collected compounds [1]	assembly	field deployment	recommended exposure periods [2,3]	preparation for chemical analysis	exemplary references web-site of provider
water filled passive diffusion bag sampler (PDBS)	more polar organic substances and metals (VOCs, metals, trace elements)	dialysis or PE-tubings (Ø 3-5 cm x 30-50 cm length) filled with distilled water (300 mL)	in surface water, sediments or groundwater, attached to a string; deployment with or without protective cages of PE or stainless steel	14 d (equ)	direct analysis of water filling, ion analysis	VROBLESKY et al. (1997, 2001) Innovative Messtechnik Weiß: www.im-weiss.de Columbia Analytical Services : www.caslab.com Eon products: www.eonpro.com
DMLS (diffusive multi layer sampler)	more hydrophilic substances (metals, sulfate, nitrate, chloride, BTEX)	several cylindrical water filled cells (30-160 mL), closed with a membrane; separated for depth-determined sampling (total length variable, up to 200 cm)	in groundwater boreholes, spacers between cells are adjusted to borehole diameter	2-4 weeks (equ, mostly within 7 d)	direct water analysis	PULS & PAUL (1997), RONEN et al. (1987) Margan Physical Diagnostics: www.margan.com
SPMD (Semipermeable Membrane Device)	hydrophobic substances (PCBs, dioxins, PAHs, organochlorine pesticides, PBDE)	PE tubing (91.4 x 2.54 cm) filled with triolein (1 mL)	in metal protective cage; sometimes winding of samplers in the cage; deployment in surface and groundwater, sediment and	ca. 28 d (TWA, for most substances)	outer clean-up, dialysis, gel permeation chromatography, GC or HPLC	PETTY et al (2000), GUSTAVSON & HARKIN (2000) EST Environmental Sampling Technologies : www.spmds.com Exposmeter: www.exposmeter.com
Ceramic Dosimeter	PAHs, BTEX, (CHCs)	ceramic tube (5 cm x 1 cm) filled with solid loose sorptive material, closed with teflon caps	in metal protective cage, deployment only in groundwater up to now	several months (TWA)	solvent extraction, GC or HPLC analysis	GRATHWOHL (1999), MARTIN et al. (2003) Innovative Messtechnik Weiß: www.im-weiss.de
Gore-Sorber®	BTEX, MTBE, PAHs, VOCs, SVOCs	loose sorptive material in modules of Gore-Tex®-membrane (ca. 2.5 x 0.3 cm, 40 mg sorbent), several moduls with different sorbents contained in one Gore-Tex® strap	strap with modules is exposed in boreholes in soil or groundwater	14 d for determination of concentration trends (equ)	thermodesorption, GC analysis	SORGE et al. (1994) Gore & Associates www.gore.com/surveys/index.html
Gaiasafe sampler	metals, anions (sulfate, phosphate), undissociated substances (hydrogen sulfide), organic compounds	fibers (paper, wood wool, textiles) impregnated with sorptive agents (2.5 g fiber per sampler)	impregnated fibers are exposed in net bags (9 x 22 cm)	2 d - 2 months (TWA, determination of contaminant freights)	extraction and analysis depend on sampled substances	HAAS & OESTE (2001) Gaiasafe www.gaiasafe.de
SPME (solid phase microextraction)	VOCs, BTEX, pesticides, PAHs, PCBs,	sorptive coated fibers, coupled to a syringe	field sampler mainly as *on site* sample preparation method; rarely used as real passive sampler in water	30 min (equ)	thermodesorption, GC analysis	NILSSON et al. (1998) MÜLLER et al. (1999) Supelco: www.sigmaaldrich.com/Brands/Supelco_Home.html
DGT (diffusive gradients in thin films)	metals	outer gel as diffusive layer and second gel with ion-chelating resin as receiving phase (gel-Ø 2.5 cm), protected by an additional membrane	deployment as single samplers or with several DGTs attached to a plate	up to 1 moth (TWA)	extraction	DAVISON & ZHANG (1994), MCNEE & ROBERTSON (2000), MURDOCK et al. (2001) Exposmeter: www.exposmeter.com

[1] VOCs = volatile organic compounds, SVOC = semivolatile organic compounds, CHCs = chlorinated hydrocarbons, PAHs = polycyclic aromatic hydrocarbons, PCBs = polychlorinated biphenyls, BTEX = benzene, toluene, ethylbenzene, xylene, PBDE = polybrominated diphenylether, MTBE = methyl-*tert*-butyl-ether

[2] Recommended exposure periods depend on target compounds. Times given here refer to the listed substances, collected up to now.

[3] Sampler behavior throughout the given deployment time: as equilibrium sampler (equ) or as time integrative sampler for derivation of time weighted average concentrations (TWA).

Table 2.2 Overview on further passive sampler developments

passive sampler type	collected compounds [1]	assembly	field deployment	recommended exposure periods [2,3]	preparation chemical analysis	for	exemplary references
MESCO (membrane enclosed sorptive coating)	hydrophobic substances (oragnochlorine pesticides, PAHs, PCBs)	sorptive coated rod (1.5 cm) enclosed in a water filled cellulose membrane (3 x 1.8 cm)	in protective metal cage (Ø 5 cm)	20 d (TWA for some substances only up to 7 d, for e.g. PCBs several months)	thermodesorption, GC analysis		VRANA et al. (2001b)
organic solvent filled tubes	hydrophobic substances; (PCBs, organochlorine pesticides)	solvent filled tubings of cellulose acetate or PE (containing between 40 and 200 ml solvent)	in protective metal cage	4 weeks (TWA)	direct analysis of solvent filling, sometimes concentration step for solvent		SÖDERGREN (1987), SABALIUNAS & SÖDERGREN (1996), LEONARD (2002)
POCIS (Polar Organic Chemical Integrative Sampler)	for more hydrophilic organic substances (pesticides, xenoestrogenes, pharmaceuticals)	mixture of sorbents covered by a membrane in a metal clamp (7 cm outer diameter)	several samplers together in one metal cage	30 d (TWA)	solvent extraction		ALVAREZ et al. (1999, 2000)
Greenwood Sampler	for more hydrophilic organic substances (pesticides, metals)	C_{18} or chelating disk covered by a membrane in a Teflon body	deployment upside down directly in water	7 - 14 d (TWA)	extraction depending on target substances and sorbent used		KINGSTON et al (2000), BJÖRKLUND BLOM et al. (2002)

[1] PAHs = polycyclic aromatic hydrocarbons, PCBs = polychlorinated biphenyles

[2] Recommended exposure periods depend on target compounds. Times given here refer to the listed substances, collected up to now.

[3] Sampler behavior throughout the given deployment time:
as equilibrium sampler (equ) or as time integrative sampler for derivation of time weighted average concentrations (TWA).

2.4 Chemical analysis and evaluation of passively collected contaminants

The receiving phases of passive samplers are commonly analyzed in the laboratory upon termination of sampler exposures. For samplers where analytes substantially accumulate in the diffusion barrier, such as in the membranes of SPMDs, the diffusion barrier and sampling container are both extracted and analyzed. The type of information gathered differs depending on the application of the passive sampler as an equilibrium or a time-integrated, non-equilibrium sampler.

Devices used as *equilibrium samplers*, which are in rapid exchange with the aqueous surrounding, are deployed in the field for periods safely ensuring the achievement of an equilibrium between the receiving phase and the surrounding water. Based on the amounts of analytes found in the sampler, aqueous concentrations can be derived that reflect true contaminant concentrations toward the end of the deployment period. Thus, equilibrium passive samplers yield information similar to conventional snap-shot sampling, which also determine instantaneous aqeuous concentrations (fig. 2.3). In contrast to snap-shot sampling, however, equilibrium samplers bring along advantages linked to passive sampling, such as the sampling under undisturbed groundwater flow conditions based on the elimination of sampling pumps (see below). In the simplest case, if water filled samplers are used, the concentration found in the water filling is identical to the freely dissolved contaminant concentrations in the surrounding water. For other equilibrium passive samplers, such as SPMEs, or for time-integrating samplers for which some analytes reach equilibrium conditions at the end of the exposure period, partition coefficients have to be applied for calculation of aqueous contaminant concentrations. These partition coefficients have to be determined in laboratory experiments.

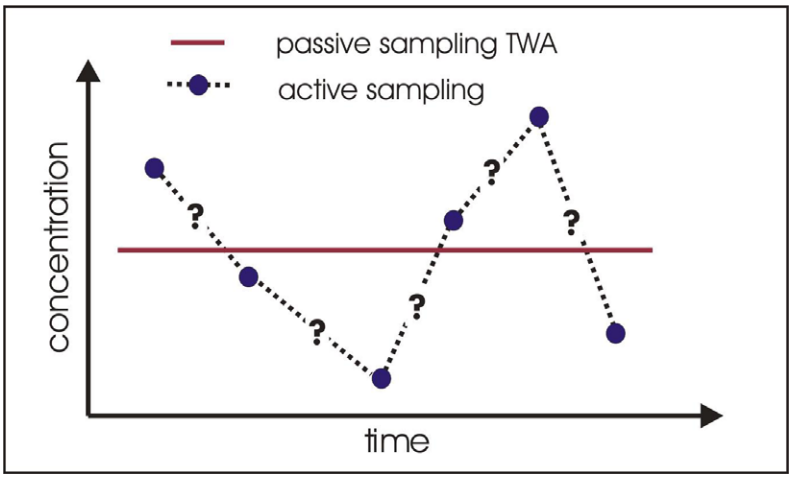

Figure 2.3 Comparison of information on aqueous contaminant concentrations derived from active snap-shot sampling and from time integrative passive sampling (modified from GRATHWOHL AND SCHIEDEK 1997).

Time integrating passive sampling devices accumulate substances from the surrounding water over extended periods. Thus concentration fluctuations during sampling are registered integratively and a time weighted average concentration can be derived. This is advantageous compared to snap-shot sampling where only momentary information can be derived and nothing is known about concentrations between sampling events. With snap-shot sampling, outliers can additionally significantly skew true average aqueous contaminant concentration calculations (fig. 2.3). The determination of average concentrations for time integrating, diffusion controlled passive samplers with an inert diffusion barrier is based on Fick's first law (equation 1, (FICK 1855)). A pre-requisite for applying Fick's law for deriving aqueous contaminant concentrations from passive samplers is that the "lag-phase" is negligible compared to the monitoring period. The "lag-phase" is the initial retardation which might occur due to interactions of analytes with the diffusion barrier and which corresponds to the time until a constant diffusion gradient is achieved (steady state). Such retardation effects are generally in the range of a few hours. From the samplers listed in the tables, the gaiasafe sampler cannot be calculated in the way presented below, as the uptake is not diffusion controlled.

$$F = D\frac{dC}{dz} \tag{1}$$

The mass flux F [g/(cm²·s)] is determined by the effective diffusion coefficient D [cm²/s] in the diffusion limiting barrier as well as by the concentration gradient dC/dz. For time integrative passive samplers with suitable receiving phases comprising a high capacity for the target analytes, the freely dissolved, unbound concentration within the sampler can be neglected and concentrations in the inner part assumed to be zero. Thus only the concentration of the surrounding water C_w is used in equation 2. The diffusion pathway dz is represented by the thickness of the diffusion barrier or membrane. The total accumulated mass M [g] in a passive sampling device is the product of the mass flux F across the surface available for diffusion A [cm²] and the time of exposure t [s] (see also equation 2, MARTIN *ET AL.* 2003). This enables the calculation of surrounding aqueous contaminant concentrations C_w [g/cm³] from accumulated masses determined in the laboratory following sampler exposure (equation 3, MARTIN *ET AL.* 2003).

$$M = F \cdot A \cdot t = D\left(\frac{dC}{dz}\right) \cdot A \cdot t \tag{2}$$

$$C_w = \frac{M \cdot z}{D \cdot A \cdot t} \tag{3}$$

The Ceramic Dosimeter (GRATHWOHL 1999) and the DGT samplers (diffusive gradients in thin films, DAVISON AND ZHANG 1994) (tab. 2.1) are examples for such purely diffusion determined samplers, where substances pass a porous barrier with negligible retardation of analytes (lag-phase).

For *time integrative permeation samplers*, no diffusion through a porous barrier but a permeation through non-porous material takes place. Those non-porous barriers are mostly membranes often consisting of organic polymers. Calculations for these permeation samplers are also based on Fick's first law. The pre-requisite for the application is the assumption of a "steady-state-flux" of the analytes through the various layers that have to be traversed on the way of the analytes into the sampler. In addition to membranes, such layers include e.g. a diffusive boundary layer. Depending on the mixing of surrounding water, diffusive boundary layers form on the outer surface of the membrane thus constituting an additional barrier. For permeation samplers the effective diffusion coefficient D is insufficient for calculations. A factor comprising the mass transfer coefficients for the several barriers of the particular sampler types has to be used instead. As it is not feasible to calculate or experimentally determine all single coefficients, a factor which includes all these parameters, the sampling rate R, is determined in laboratory experiments. Average aqueous contaminant concentrations C_w can then be derived from concentrations C_s determined in the sampler using sampling rates R, sampling time t and the sampler volume V_s (equation 4) (see e.g. HUCKINS *ET AL.* 1993).

$$C_s = C_w \cdot R \cdot t / V_s \qquad (4)$$

For several permeation samplers, e.g. for the SPMDs, sampling rates can nowadays be derived from the literature for a number of analytes.

2.5 Factors to be considered in the application of passive sampling devices

Passive sampling technology bears a number of advantages compared to the conventional snap-shot sampling approach. Passive samplers do not require energy and thus can easily be applied even in remote regions. No collection, transport and storage of large sampling volumes is required. Because tubing and sampling containers are not employed, losses during sampling due to sorption are avoided. As well, losses of more volatile analytes, as is often observed e.g. in active sampling of groundwater using vacuum- or peristaltic pumps, are minimized. Sorption of analytes into / on the receiving phase leads to a stabilization and thus a reduced risk for biodegradation during transport and storage.

Numerous substances occurring as contaminants in the aquatic environment are present at very low concentrations. Based on their accumulation in time-integrating (non-equilibrium) samplers, such analytes become detectable using standard analytical techniques without the need to extract large volumes of water in the laboratory. For example, GUSTAVSON AND HARKIN (2000) reported the detection of contaminants in groundwater that were below the limit of detection using conventional sampling techniques.

Another advantage of passive sampling is the reduction of interfering matrix effects by the selective accumulation of analytes. Matrix effects often result from non-target analytes in conventionally obtained water samples and have the potential to impact on chemical or biological water analysis. Along these lines, passively obtained samples do not need to be filtered in order to remove particles. Thus, the loss of analytes due to sorption onto filter material is avoided (HESSLEIN 1976; MAYER 1976; BENES & STEINNES 1974). Further, passive samplers, for which the receiving phase is separated from the sampling environment by a fine- or non-porous barrier, minimize the uptake of particle-bound analytes, thus providing an approximate measure of the rather truly dissolved analytes only. These are the ones considered to account for the bioavailable fraction (KOT *et al.* 2000). In many cases, such as the assessment of potential toxic effects, these concentrations are much more useful than total concentrations, which are not available to organisms.

A particular advantage in the application of passive samplers exists for groundwater sampling. With conventional groundwater sampling, a significant distortion of water composition due to pumping of larger water volumes with mixing of clean and contaminated water from various aquifer layers can never be completely ruled out. Passive samplers in contrast do not influence the natural groundwater flow regime. In addition they avoid the necessity to dispose large volumes of contaminated water pumped to the surface in conventional techniques.

Finally, the time-integrative passive sampling can greatly reduce time and labor because only a few sum analyses are needed. These sum analyses integrate over possible deviations in concentrations during the entire sampling period (fig 2.3). These specific advantages of passive sampling appear especially valuable in long-term monitoring programs.

Several aspects have to be taken into account in order to effectively apply passive samplers. It firstly is advantageous to know the target substances or substance classes and their approximate range of concentrations to properly choose sampler type and exposure time. For this purpose a rough site characterization using conventional, active sampling techniques can be helpful. This is particularly important for time-integrative samplers in order to approximate the period in which the uptake remains linearly correlated with time, a pre-requisite for reliably calculating time weighted average aqueous contaminant concentrations. The uptake remains approximately linear until half-saturation is reached in the sampler (HUCKINS *et al.* 1993; VRANA *et al.* 2001b). For some samplers with a particularly high uptake rate, such as the SPMDs, exceeding of half-saturation in higher contaminated water can be a problem, whereas such

effects can be practically excluded for devices such as the Ceramic Dosimeter (MARTIN *et al.* 2003) and solvent filled devices (SÖDERGREN 1990; JOHNSON 1991) (tab. 2.1 & 2.2). In general, uptake rates will always depend on the combination of chemical and sampler properties and thus saturation effects need to be considered for each individual application.

One problem in time-integrating passive sampling technology is external factors which might impact on sampling rates in the field. Factors include temperature, flow velocity and the formation of biofilms on the surface, which may lead to altered uptake rates and biodegradation of contaminants. The application of devices that are less prone to impacts elicited by these factors is one way of avoiding such difficulties. For example, passive samplers that are purely diffusion controlled are unaffected by flow velocities, whereas higher alterations can occur for permeation samplers. VRANA AND SCHÜÜRMANN (2002) showed that uptake into SPMDs is independent of flow velocity only for compounds with a log K_{ow} < 4. The formation of biofilms can be avoided using a biocidal impregnation of the outer sampler surface. When organic solvent filled samplers are deployed, a small portion of the solvent filling slowly diffuses out through the membrane and thus prevents biofouling, so that no additional biocides are needed (LITTEN *et al.* 1993). For application of passive samplers in groundwater, a lower potential for biofilm formation can be assumed. For example, when Ceramic Dosimeters were deployed in groundwater for 3 months no biofilm formation was observed (MARTIN *et al.* 2003). The so called "Performance Reference Compound" (PRC) approach enables the monitoring of altered sampling rates due to environmental factors (HUCKINS *et al.* 2002). PRCs are substances that are similar to the target compounds but not suspected to be present at the sampling site. They are spiked into the sampler prior to deployment and are then continuously released from the sampler. The release of PRCs is assumed to be affected by exposure conditions in the same way as the uptake, so that an exposure adjustment factor for the sampling rates can be determined *in situ*.

2.6 Current trends in passive water sampling

Recent developments show three main trends concerning passive sampling technology. The first is the miniaturization of sampling devices. This is especially important for groundwater monitoring because of continuously reduced borehole diameters for new monitoring wells, using e.g. "direct-push" technique for well construction. Another goal linked to miniaturization is to provide a balance between sampling rates and continuous delivery of contaminants dissolved in water. GUSTAVSON AND HARKIN (2000) postulated that the sampling rates of SPMDs can be limited in slow flowing groundwater because the sampling exceeds delivery of the contaminants toward the sampling device. The use of miniaturized devices, such as in this case shorter SPMDs, or samplers with lower uptake rate can help to overcome this problem.

The second important trend in further development of passive sampling devices is the broadening of the spectrum of target analytes. In addition to the more classical focus on very hydrophobic contaminants, recently more attention has been paid to more polar organic compounds (ALVAREZ *et al.* 2000), such as several pesticides, BTEX, pharmaceuticals, and personal care products.

A third tendency is the coupling of passive samplers to biological assays, aiming at identifying the presence of toxicologically relevant components. Unexpectedly occurring compounds might be overlooked if chemical analyses are used alone (e.g. HELMA *et al.* 1998). The first samplers coupled to biological analysis were the SPMDs, where solvent extracts were tested in various biological systems (e.g. PETTY *et al.* 2000a; PARROTT *et al.* 1999; WHYTE *et al.* 2000). Problems with high background toxicity from unexposed SPMDs were reported for the Microtox assay with *Vibrio fischeri* and attributed to co-extracted impurities from the triolein receiving phase (SABALIUNAS *et al.* 1999). These problems were recently solved by an additional clean-up step for triolein (LEBO *et al.* 2004), which however further complicates the clean-up procedure for SPMDs. The latest development is the coupling of solvent extracts derived from the POCIS (Polar organic chemical integrative sampler) to assays for e.g. the detection of estrogenic potential in the yeast screen assay (YES) (PETTY et al. 2004).

2.7 Conclusions with regard to this thesis

The above overview on various aspects of passive sampling technology shows the potential and manifold possibilities for passive sampler applications in monitoring water quality. One area largely ignored thus far, however, is the application of biological analysis in passive sampling approaches. Two samplers (SPMDs and POCIS, see above) have recently served as sources of samples to be tested in bioassays, but the applications were limited to surface water and depended on solvent extraction. Thus, this thesis focused on the development of a passive groundwater sampling device using a solid sorbent such that this sorbent can be applied directly to miniaturized toxicological tests without the need for solvent extraction.

Chapter 3

Development of a solvent-free, solid-phase bioassay

3.1 Introduction

Throughout the past decade, significant efforts have been devoted to decrease assay sizes in ecotoxicological studies. There are obvious advantages using miniaturized test systems, such as lower sample consumption and the avoidance of large volumes of toxic waste. Additionally, testing of smaller volumes using e.g. multi-well microplates allows the examination of many replicates and samples simultaneously and enables the coupling to automated systems. The "Microtox" test using *Vibrio fisheri*, the algal reproduction test using e.g. *Selenastrum capricornutum* as well as the use of vertebrate cells in culture (*in vitro*) (see also Chapter 1) to detect adverse effects, all are examples of test systems that can be used at a miniaturized scale. However, despite their many advantages, miniaturized test systems do bear caveats.

The first difficulty is to maintain constant exposure concentrations in the test vessels throughout the exposure period. A decrease in exposure concentrations can have a number of reasons including sorption to test vessels, volatilization or photodegradation (RUFLI *et al.* 1998). Although by no means unique to miniaturized systems (see for example, BASU *et al.* 2001; BILLIARD *et al.* 1999), sorption to vessel surfaces is augmented in miniaturized systems due to a relatively large surface to volume ratio. Along these lines, LONGMAN AND BUEHRING (1986) found considerable losses of steroids (28-43 %) due to sorption to tissue culture plasticware (24-well polystyrene microplates). GELLERT AND STOMMEL (1999) reported the detection of lower EC_{50} values for three organic substances for growth inhibition tests with bacteria in microplates made of quartz glass than in plates made of polystyrene. A lower EC_{50} value implies that higher exposure concentrations occurred in quartz plates, likely due to the lower extent of sorption of contaminants compared to polystyrene plates. Another aspect affecting the outcome of miniaturized assays, particularly when working with vertebrate cell lines, is the composition of culture media. DRENTH *et al.* (1998) and HESTERMANN *et al.* (2000) reported changes in the sensitivity of cultured cells to respond to dioxin-like compounds via the induction of ethoxyresorufin-*O*-deethylase (EROD) if the percentage of fetal bovine serum (FBS) was altered. FBS is a cell culture additive that provides growth factors and other constituents generally required for cell attachment and survival *in vitro*. However, the serum content present

in the medium alters the distribution of hydrophobic compounds in the test system such that higher amounts of contaminants remain in the culture medium rather than sorbing to the cell culture vessels. Likely, this can be attributed to the binding of hydrophobic compounds to serum proteins or similarly acting constituents (HESTERMANN *et al.* 2000; SCHIRMER *et al.* 1997; LONGMAN AND BUEHRING 1986).

The various factors affecting contaminant distribution in miniaturized systems lead to significant uncertainties in true exposure concentrations. They thus call for new methodologies to provide constant delivery of test substances so that constant exposure concentrations can be maintained. Flow-through settings, as are suggested e.g. in the exposure of fish, are usually unfeasible for small-scale test systems. In other approaches, lipophilic substances are specifically sorbed to a solid phase, which is then used as a delivery phase to sustain constant exposure levels in test systems. One example is partitioning driven administration, where a C_{18} phase is used as generator disk which can equilibrate with the solution either from the headspace through the vapor phase (URRESTARAZU RAMOS *et al.* 1997) or directly from the solid phase to the water, when the disk is inserted into solution (MAYER *et al.* 1999). MAYER *et al.* (1999) confirmed the applicability of this approach by showing that the generator disks could rapidly react to concentration changes and compensate even strong decreases, which were created by the addition to the system of XAD sorbent acting as a sink. The method was applied to algal growth inhibition tests with hexachlorobenzene. Another approach, using contaminated stir bars, was presented by GEROFKE *et al.* (2004). Teflon coated stir bars contaminated with PCBs were used to maintain constant exposure concentrations with the scope to provide a reliable system for determination of bioconcentration factors of hydrophobic organic compounds in algae. Another system reported in the literature is the use of polydimethylsiloxane (PDMS) films which are loaded with the contaminants and are then brought into direct contact with the test system (BROWN *et al.* 2001). The films act as a reservoir and establish constant exposure levels by partition controlled delivery. Constant exposure levels could be achieved for extended periods (17 d). The reliability of the system was demonstrated by application to the Microtox assay, a luminescence inhibition test (BROWN *et al.* 2001), and to embryo toxicity testing (KIPARISSIS *et al.* 2003). These partitioning driven administration approaches for compensating contaminant losses during exposure constitute a significant advance to the standard exposures in microscale assays. Up to now, however, they are limited to artificial, lab-derived contaminant solutions, and so far cannot directly be used for environmental samples.

One assumption of the partitioning driven administration approaches described above is that a constant aqueous concentration has to be achieved throughout the entire culture vessel in order to ensure even exposure of the biological reporter. Another route to exposure has been presented by BAUMANN *et al.* (2003). They exposed various bioindicators (algae, bacteria, fungi, pollen) directly on contaminated thin-layer chromatographic plates. This system was applied to visualize ecotoxicologically relevant bands after chromatographic separation directly on the plates. This example implied two new avenues to hydrophobic contaminant delivery to

biological reporters and subsequent analysis. Firstly, the sorption of hydrophobic contaminants to solid surfaces can be turned into an advantage in that specific biological reporters adhering to these surfaces can quickly take up these contaminants. With regard to the partitioning driven administration, this would mean that source and sink are brought together as close as possible. Secondly, by having contaminants sorbed to surfaces onto which biological indicators can adhere, a direct link to environmental sampling can be established: the sorbent material could be used as a solid phase for contaminant collection in the field, brought back to the laboratory and used as the surface onto which biological reporters attach and react to provide a measure of toxicity.

Based on these ideas, test substance behavior was investigated in miniaturized test systems using vertebrate cells, namely the fish liver cell line RTL-W1, as an exemplary biological reporter. Using the PAH fluoranthene as a model compound, it was found that more than 90 % of the substance were sorbed from an aqueous solution to polystyrene tissue culture plates within 24 h (fig. 3.1).

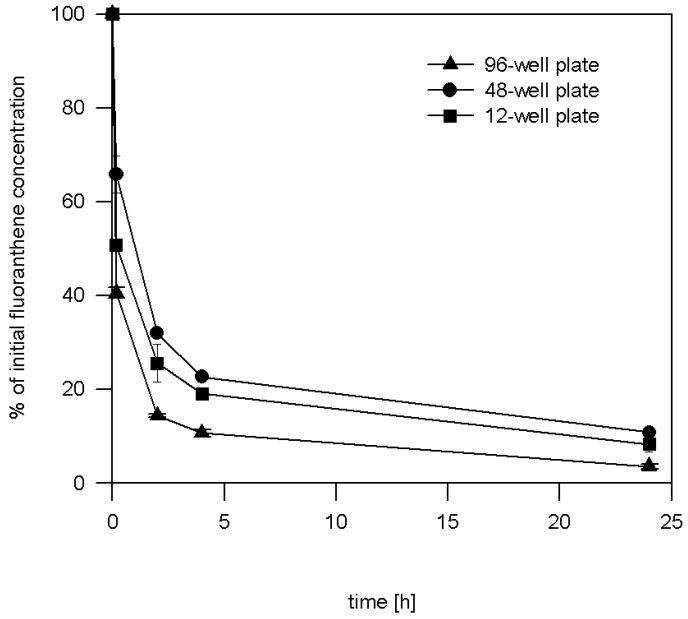

Figure 3.1 Time dependent decrease of aqueous fluoranthene concentrations in three tissue culture plate types made of polystyrene. This figure was kindly provided by Dr. Kristin Schirmer.

Next, the availability of the sorbed fluoranthene and other PAHs to adhering fish cells was assessed. Most vertebrate cell cultures can survive only when growing adhered to suitable cell culture vessel surfaces. Thus, the cells are in close contact to contaminants, when they are adsorbed to the culture surface. Using the induction of EROD activity in RTL-W1 cells as an endpoint of exposure to benzo[k]fluoranthene (BkF), two ways of dosing the BkF were compared. In a standard assay, cells were first grown attached to the bottom of the wells of tissue culture plates and then dosed with the test compound dissolved in DMSO. In parallel, empty wells of tissue culture plates were "coated" with BkF sorbed to the culture surface. Cells

were added to the wells after sorption of the test compound. It can be seen that despite being sorbed to the tissue culture surface, BkF is capable of eliciting EROD activity (fig. 3.2). Although the magnitude of the response curve is higher for the standard assay using dissolved BkF, the EC_{50} values are approximately similar with 10.4 nM for the sorbed and 8.6 nM for the dissolved BkF.

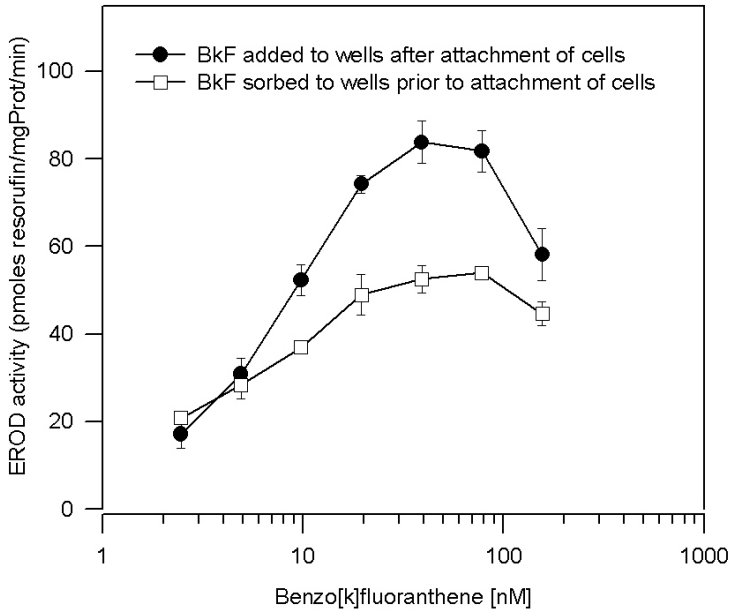

Figure 3.2 Induction of EROD activity in RTL-W1 cells by Benzo[k]fluoranthene (BkF). Cells were exposed to BkF firstly in the standard assay where BkF is added in DMSO solution to attached cells (black circles) and secondly to BkF which was sorbed to the wells prior to cell attachment (open squares). This figure was kindly provided by Dr. Kristin Schirmer.

The measurement of EROD activity in cells adhered onto a PAH coating confirms the finding by BAUMANN *et al.* (2003) of the general bioavailability of compounds sorbed to a solid phase. The idea of the current study is to use the sorptive / bioavailability effects in a positive way. The hypothesis is that contaminants which are sorbed to cell culture vessel surfaces are available to cultured cells, growing in direct contact attached to the contaminant coated surface. The aim of this study was to specifically investigate the compatibility of various materials with fish cell culture bioassays in order to provide a system for a solid phase toxicity assay. This solid phase toxicity assay was supposed to be based on beads which, in future applications, could be applied as solid receiving phases in passive sampling technology (see Chapter 2). Thus, the development of a solid sorbent toxicity assay would allow for the first time the direct coupling of passive sampling to toxicological analysis, thereby the toxicological analysis would proceed without the need for solvent extraction.

3.2 Materials and Methods

The major goal of this part of the thesis was the development of a solvent-free, solid-phase bioassay. For this purpose, in a first step, the compatibility of various sorbent materials with fish cell cultures and potential interferences with fluorescence measurements in bioassays were investigated. In a second step, beads of the materials shown to be compatible with cell culture and bioassays, were coated with PAHs as model contaminants. The ability of the sorbed contaminants to elicit dose dependent effects in cells growing on the bead surface was examined. Results from this test design, using contaminated beads (bead assay), were compared to experiments in a conventional design, where contaminants are dosed to the cells dissolved in organic solvents (standard assay). In a third step, the distribution between compartments in these two bioassay systems (namely cells, medium, plate and sorbent) were investigated, in order to assess the influence of the contaminated beads.

3.2.1 Chemicals and sorbents

16 PAHs were investigated (see table A1, appendix). Benzo[a]anthracene (BaA) (99 % purity), chrysene (Chr) (98 %), benzo[k]fluoranthene (BkF) (99.9 %), and benzo[a]pyrene (BaP) (98 %) were purchased from Sigma Aldrich (Steinheim, Germany). For tests where a mix of the 16 EPA priority polycyclic aromatic hydrocarbons (PAHs) was investigated, a mixture in acetonitrile was obtained from Promochem (Wesel, Germany), containing naphthalene (Nap), acenaphthene (Ace), acenaphthylene (Any), fluorene (Flu), phenanthrene (Phe), anthracene (Ant), fluoranthene (FA), pyrene (Pyr), BaA, Chr, benzo[b]fluoranthene (BbF), BkF, BaP, dibenzo[a,h]anthracene (DBA), benzo[g,h,i]perylene (BghiP), and indeno[1,2,3-cd]pyrene (IndP).

The various sorbents used for the bioassays are listed in table 3.1. They were selected based on their reported ability to sorb PAHs (all except Biosilon) or compatibility with cell culturing (Biosilon only). The sorbents were precleaned in acetone 3 times in an ultrasonic bath for 15 min. Afterwards they were washed with Milli-Q water to remove any acetone residues that could potentially interfere with the fish cell bioassays. This procedure was performed for all materials except for Biosilon because it is a special cell culture product where impurities are not expected.

Table 3.1 Tested sorbent materials

name	material[a]	mesh / bead size	manufacturer
Amberlite 200	PS with polyamine groups	20-50 mesh	Fluka
Amberlite IRA-743	PS with sulfonic acid groups	16-50 mesh	Aldrich
Biosilon	PS	160-300 µm	Nunc
Chromosorb 102	PS-DVB	60/80 mesh	Alltech
Chromosorb 106	cross-linked polystyrene	60/80 mesh	Alltech
melamine resin (MF)	melamine/formaldehyde resin	5 µm	microparticles
melamine resin (MF)	melamine/formaldehyde resin	3-30 µm	Fraunhofer IAP
PDMS beads	PDMS	ca. 3 mm	Gerstel
Silicagel 60	silicon oxide	70-230 mesh	Merck
Tenax TA	PDPO	60/80 mesh	Alltech
Tenax GR	PDPO+graphitized carbon (23%)	60/80 mesh	Alltech
XAD-8	Ethylendimethacrylat	300-1000 µm	Serva

[a] PS = polystyrene, DVB = divinylbenzene, PC = polycarbonate, PDPO = poly-2,6-diphenyl-*p*-phenyleneoxide, PDMS = polydimethylsiloxane

3.2.2 HPLC analysis of PAH solutions and sorbent extracts

To determine the real concentrations of self-prepared PAH solutions instead of relying on nominal values, concentrations were measured using an HPLC System 525 with autosampler HPLC 560 (Bio-Tek Kontron Instruments, Bad Friedrichshall, Germany) and a Jasco FP-1520 fluorescence detector (Groß-Umstadt, Germany). 20 µL sample solution were loaded on a LiChrosphere PAH 250-4 column (Merck, Germany). The mobile phase was a gradient of acetonitrile and Milli-Q water from 60 % acetonitrile to 100 % in the first 15 min, followed by 25 min isocratic elution. Retention times and detection wavelengths are listed in table 3.2. Calculation of sample concentrations was performed using an external five point calibration, which was determined with every sample batch, using dilutions of the commercial standard mixture of 16 PAHs.

In tests with contaminant coated Biosilon, sorbed amounts were validated by extracting 500-1000 mg Biosilon with 2 mL of methanol in an ultrasonic bath for 30 min. The methanolic extracts were measured using the above mentioned HPLC method.

Table 3.2 HPLC program for detection of 15 PAHs (Any was included in the mixtures, but could not be detected by fluorescence measurements)

PAH	retention time t_R	wavelengths excitation/emission	time window
Nap	8.21	280/330	0-12.5
Ace	11.13		
Flu	11.47		
Phe	12.59	246/370	12.5-14.35
Ant	13.73		
FA	14.79	280/450	14.35-15.25
Pyr	15.63	270/390	15.25-20.0
BaA	18.04		
Chr	18.77		
BbF	20.77	255/420	20.0-25.5
BkF	22.16		
BaP	23.45		
DBA	26.01	290/410	25.5-29.2
BghiP	27.57		
IndP	29.40	300/500	29.2-32

3.2.3 Cell cultivation and performance of bioassays

Bioassays were performed using the rainbow trout (*Oncorhynchus mykiss*) liver cell lines R1 (AHNE 1985) and RTL-W1 (LEE *et al.* 1993). Both cell lines were cultured under the same conditions at 18-20 °C in Leibovitz L-15 medium (Gibco, Paisley, United Kingdom) supplemented with 2 % penicillin and streptomycin (Sigma Aldrich, Steinheim, Germany) and 5 % fetal bovine serum (Biochrom AG, Berlin, Germany), which from now on will be referred to as "medium". Cultivation was performed in 75 cm^2 culture flasks (Nunc™ Brand Products, Denmark).

For subculturing, cells were rinsed two times with 2.5 mL Versene (Gibco, Paisley, United Kingdom). Cells were detached from culture flasks using 700 µL trypsin solution (trypsin was from bovine pancreas (12,400 units/mg solid, Sigma Aldrich) and dissolved to 1 % in Hanks' salt solution (Biochrom AG, Berlin, Germany) before use). Enzymatic reaction was stopped by adding of 5 mL medium. Cell suspension was centrifuged at 1,000 rpm for 5 min. Supernatant was removed and the pellet resuspended in 5 mL medium. For normal cell maintenance, cell suspension was split and filled into two new culture flasks, which were filled with 10 mL medium. Cells grew to a confluent layer within seven days.

For the use in bioassays, cells were counted and plated in multiwell plates. Standard 24 well and 96 well tissue culture plates (Falcon, Becton Dickinson, Franklin Lakes, USA), 8 well PolySorp modules (Nunc™ Brand Products, Denmark) or membrane bottom 96 well filter plates (Millipore, Eschborn, Germany) were used depending on the scope of the experiment.

In all cases the experiment ended by the measurement of either cell viability and / or induction of cytochrome CYP1A, measured as 7-ethoxyresorufin-*O*-deethylase (EROD) activity.

3.2.4 EROD and cell viability assays

Cell viability assays were performed using the fluorescent dyes alamarBlue, CFDA-AM (Molecular Probes, MoBiTec, Göttingen Germany), and Neutral Red (Sigma, Taufkirchen, Germany). AlamarBlue is a non-fluorescent dye which is reduced by cellular dehydrogenases, which are localized in the mitochondria membrane and in the cytosol, to a fluorescent product (GONZALEZ AND TARLOFF 2001). The fluorescent product is water soluble and can diffuse out of the cells so that it can be measured fluorometrically without extraction procedures at 530 nm excitation and 595 nm emission (GANASSIN *et al.* 2001). CFDA-AM (5-carboxyfluorescein diacetate acetoxymethyl ester) is an esterase substrate which can be converted by non-specific esterases of living cells from a non-polar, non-fluorescent substance to a polar, fluorescent dye. The substrate rapidly diffuses into the cells whereas the product diffuses slowly out of the cells. The fluorescent product carboxyfluorescein can be detected at 485 nm excitation and 530 nm emission (GANASSIN *et al.* 2001). As alamarBlue and CFDA-AM fluorescent products are non-interfering within fluorescence detection, they can be applied simultaneously (SCHIRMER *et al.* 1997). Test protocols were applied according to (GANASSIN *et al.* 2001) and (SCHIRMER *et al.* 1997). In a second step, after performing the alamarBlue and CFDA-AM assay, a third dye, Neutral Red (3-amino-7-dimehtylamino-2-methylphenanzine hydrochloride), was applied to the same cells. This fluorescent dye is accumulated in the lysosomes of viable cells (BORENFREUND AND PUERNER 1984). If lysosomal membranes in cells are damaged, the accumulation of the dye is inhibited. For fluorescent measurement, the dye has to be fixed and extracted from the cells (DAYEH *et al.* 2003). Detection is performed at 530 nm excitation and 645 nm. Cell viability results are presented as arbitrary fluorescent units (FU). The higher the cell viability or cell number, the higher the fluorescence.

The induction of 7-ethoxyresorufin-*O*-ethylase (EROD) in cell cultures is a measure for the activity of cytochrome CYP1A and can be used for investigating the presence of dioxin-like compounds in general (BOLS *et al.* 1999). Induction of EROD activity was measured according to GANASSIN *et al.* (2001). The substrate 7-ethoxyresorufin is metabolized to resorufin which can be detected fluorometrically (excitation 535 nm / emission 590 nm). Fluorescence was detected directly after addition to live cells of the substrate 7-ethoxyresorufin and 30 min later. Using a linear calibration function, which was determined using a resorufin standard in all used test systems, the activity of the CYP1A can be determined. To normalize the data depending on the cell vitality, the CFDA-AM assay was performed simultaneously (GANASSIN *et al.* 2001). The cell number was determined using fluorescence values from the CFDA-AM test based on an independent calibration. The results were then calculated as amount resorufin produced per minute and cell.

The fluorescence was measured with a multiwell plate reader (Gemini, Molecular Devices, München, Germany) for all used assays.

3.2.5 Coating of sorbent beads

Two methods were applied for coating the sorbent beads with the substances to test. In the first case aqueous solutions of the PAHs were prepared in amber 8 mL glass vials using the stock solution in methanol for single substance tests or in acetonitrile for the mixture of 16 PAHs. The sorbent beads were exposed to the aqueous solution for 4 d on a vortex shaker to provide efficient contact with the solution for sorption to all beads. Prior to use the water was removed using a Pasteur pipette. In the second case a methanolic stock solution of the PAHs was added directly onto the beads and the solvent was allowed to evaporate over night so that the substances remained sorbed onto the dry beads.

3.2.6 Compatibility of sorbent materials with fish cell lines

Firstly, the compatibility of uncontaminated sorbents with fish cell cultures and the bioassays using fluorescent dyes were assessed. The materials were filled dry into the wells of 8 well PolySorp modules using a paper funnel. The amount used varied with the sorbent type and was chosen so that the bottom of the well was covered with a complete layer of beads. 200 µL of either medium or cell suspension (using R1 and RTL-W1 cells) for an initial cell number of 30,000 cells/well were added to the wells. After 24 h of incubation with medium or cell suspension, three cell viability tests were performed within all wells as described below.

PolySorp modules were chosen instead of normal cell culture plates as they do not support cell adherence on their surface. Thus cells which are not attached to the sorbent will get lost during the washing steps in the test protocol and only cells adhering to the sorbents, which remain in the plate, are assessed using the cell viability assays explained below.

3.2.7 Exposure of cells with PAH-coated sorbent materials (bead assay)

For further investigations using contaminant coated sorbents in bioassays a new system for better handling was developed. MultiScreen-BV filter plates (96 well) with a durapore-membrane bottom (low protein binding, pore size 1.2 µm, sterile; Millipore, Eschborn, Germany) were used, as they allow simplified washing steps during the assay performance. The coated sorbents were filled dry or suspended in medium into the wells, RTL-W1 cells were added in suspension. RTL-W1 cells attached directly onto the contaminant coated surface of the sorbent and were incubated usually for 24 h in the bead assay. After exposure the medium had to be removed for bioassay application and depending on the type of assay as described below certain solution exchanges or washing steps had to be done. The filter plates allow this removal of solutions by centrifugation (1,000 rpm for 1 min), whereby the sorbent with the cells remain unaffected in the plate. Thus the disadvantage of sucking solutions from each well separately, which always involves small sorbent losses, can be avoided.

To distinguish between effects caused by the uptake by direct contact to the coated surface and caused by diffusion processes through the medium, tests were performed in 24 well tissue culture plates (Falcon), using additional 10 mm cell culture inserts (Nunc). The inserts allow exchange of dissolved substances between insert and well content through a membrane bottom. Anapore membrane inserts were chosen because the membrane, consisting of aluminum oxide (0.2 µm pore size), has lower sorption potential for the used PAHs than e.g. organic polymer membranes. To look at the indirectly caused effects elicited by contaminants diffusing through the medium to the cells, not adhering to the sorbents' surface, 500 µL RTL-W1 cell suspension were plated directly into 24 well plates (150,000 cells/well). Then cell culture inserts were added to each well, containing 120 mg pretreated Biosilon in 500 µL of medium. To look at the direct contact of cells with contaminant coated Biosilon, 250 µL of a Biosilon suspension (containing 120 mg Biosilon in medium) was filled into the wells using wide orifice pipette tips (Thermo Labsystems, Germany). 250 µL of RTL-W1 cell suspension (containing 150,000 cells) were added to the wells, so that the cells could settle directly on the contaminant coated Biosilon. Cell culture inserts with 500 µL medium were added, so that the same conditions regarding surface and volume ratios were given in both systems. Benzo[a]pyrene coated Biosilon was used as model system in combination with the EROD assay. 3 replicates of a control and 3 BaP concentrations were investigated in the test. After 24 h of incubation the inserts were removed from the plates and the EROD-assay could be performed as described above.

Usually the incubation time was 24 h in the bead assay. Time course studies were performed with an equimolar mixture of four EROD-inducing PAHs (BaA, Chrys, BkF, BaP), to look whether a shift in the effects due to desorption processes from the contaminant coated sorbent can be found over time. Four plates were prepared simultaneously and were measured after 24, 48, 72 and 96 h.

3.2.8 Exposure to PAHs in the standard assay

RTL-W1 cells were plated in 48 or 96 well tissue culture plates with an initial density of 75,000 cells/500 µL or 30,000 cells/200 µL respectively. Cells were incubated for 24 h and were grown to a confluent monolayer in the plates before dosing the samples. The contaminants to test were dissolved either in DMSO or methanol. Various dilution series depending on test system were used. 2.5 µL of standard solution per well were dosed to 48 well plates, and 1 µl to 96 well plates, leading to a final solvent concentration of 0.5 %. Dosing was done using glass capillary pipettes to minimize sorption effects. In all tests untreated cells were compared to a solvent blank. After dosing the cells were incubated for another 24 h with the contaminants.

3.2.9 Distribution of PAHs within bead and standard EROD assay

It is important to know which fraction of the contaminants potentially available in a test system is really taken up into the cells. To look at the distribution of contaminants within the test system, the various involved compartments were extracted. These analyses were performed for two systems: firstly cells exposed in a standard plate assay scenario with contaminants dosed dissolved in solvent and secondly cells exposed in the bead assay on contaminant coated Biosilon in filter plates. For this purpose a 16 PAH standard mixture was diluted in methanol for dosing of the standard plates and Biosilon was coated using methanolic PAH solutions so that the same nominal concentrations per well were reached for both systems. The concentrations of the standard solution and the amounts sorbed to the Biosilon were validated by chemical analysis by HPLC. (960 mg Biosilon were extracted using 2 mL of methanol in an ultrasonic bath in three replicates). Cells were plated and incubated with the cells for 24 h as usual before the compartments were analyzed separately. For each sample the material from 32 wells was pooled and extracted separately. Each concentration was investigated in triplicate.

The procedure for the extraction was the same for both systems in principle: firstly the medium was sucked off using Pasteur pipettes for the standard plate and medium was centrifuged to a new culture plate for the bead assay. Afterwards the cells were separated from the plate or Biosilon respectively by incubation with 100 µL/well trypsin solution for 5 min. Then cells from the standard plate were transferred in suspension to glass tubes, the cells from the bead assay were transferred including the Biosilon beads over a cell strainer (70 µm pore size, Nylon, Falcon) to a glass tube, so that the cells could be washed off with Milli-Q water, separated from the Biosilon beads that remained in the strainer. 2 mL of concentrated hydrochloric acid were added to all cell and medium samples and they were incubated over night. On the following day, the cell and the medium samples were extracted with 2 mL of hexane by vigorous shaking on a vortex mixer for 5 min. Extracting the medium samples, the hexane phase became a turbid emulsion. The samples were centrifuged in glass tubes at 5,000 rpm for 5 min. Afterwards a clear hexane phase could be sucked off and only a thin smeary layer remained on the extracted medium phase. The hexane phases were evaporated under nitrogen and redissolved in methanol in the ultrasonic bath for 15 min. The methanol extract was transferred to HPLC vials. The plates (including the culture plate to which the medium from the bead assay was centrifuged) were filled with 200 µL of hexane per well and were covered with a self-adhesive plate-sealer foil (Nunc) and left over night for extraction. On the following day the hexane was pooled from 32 wells, transferred to glass tubes, evaporated under nitrogen and redissolved in 1.5 mL of methanol in the ultrasonic bath for 15 min. The methanol extract was transferred to HPLC vials. The Biosilon samples were transferred from the cell strainers to glass tubes and were then extracted with 2 mL of methanol in an ultrasonic bath for 30 min. The methanol extract was transferred to HPLC vials. After the transfer of all samples in methanol to the vials they were analyzed using HPLC as described above. The results for each compartment

were calculated as ratio of the total amount used in the test. It could be shown, that the recovery from Milli-Q water was high with acceptable deviations for the higher molecular PAHs (Nap–Pyr median ± standard deviation 34.6 ± 3.5 %, BaA–IndP median 98.5 ± 4.2 %). For the extraction from medium supplemented with fetal bovine serum the recovery is highly variable (Nap–Pyr 63.5 ± 30 %, BaA–IndP 71.1 ± 30 %).

3.3 Results

3.3.1 Compatibility of sorbent materials with fish cell cultures and fluorescent dye bioassays

The compatibility of various sorbent materials with fish cell cultures and the direct use of the materials in bioassays with fluorescent dyes were investigated. Cell viability assays were performed with the materials in medium with and without cells (fig. 3.3).

For all materials except for Amberlite 200 and Amberlite IRA 743, significantly higher fluorescence values were found in the alamarBlue and / or CFDA-AM assay for the cells adhering to the material compared to the material alone. Although melamine resin (MF) seemed to lead to significantly higher fluorescence with cells for the CFDA-AM assay, it was unsuitable for the use in the alamarBlue assay. Cells showed high viability especially on Biosilon. When neutral red, which is normally accumulated in the lysosomes of viable cells, was applied to the beads with and without cells, no significant differences between the cell-containing and the cell-free material was found with fluorescence values sometimes being even higher without cells. PDMS is not at all shown because of severe difficulties in handling, which arose from the elasticity and electrostatic adhesion of the PDMS particles.

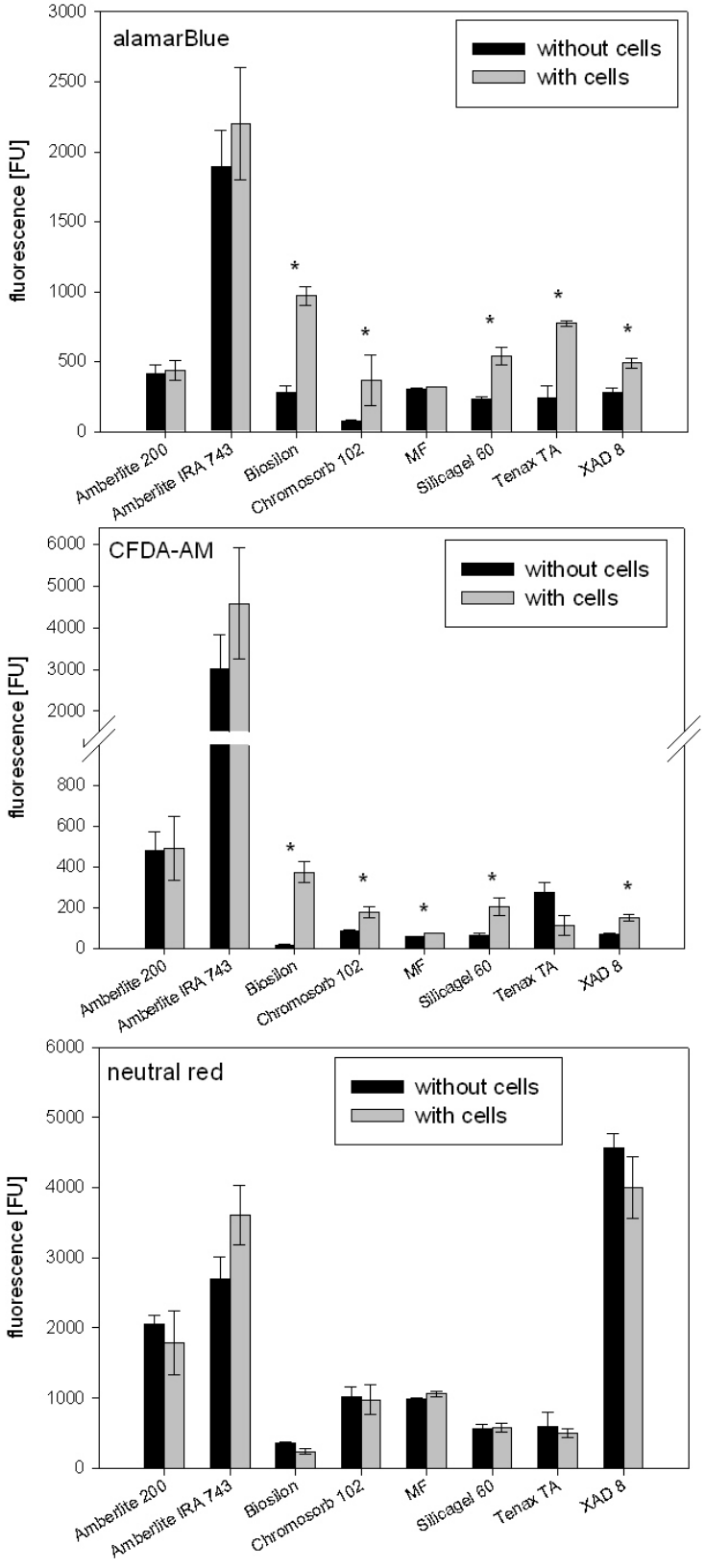

Figure 3.3 Compatibility of sorbent materials with fish cell culture and fluorescent dye bioassays. Asterisks indicate significant differences in fluorescence between material with and without cells (t-test, $p<0.01$).

Further confirmation of the compatibility was derived using various amounts of sorbents and / or different cell densities. For Biosilon, Chromosorb 102, and Tenax TA a linear relationship between fluorescence and cell density was found (presented for Biosilon in fig. 3.4). Thus, cell numbers per well can be calculated from fluorescence values using alamarBlue and CFDA-AM. Differences in fluorescence connected to various amounts of sorbents were detected only for Tenax TA, where the fluorescence increased with increasing sorbent amounts when the same number of cells was added. With all other materials, the fluorescence was unmodified by increasing sorbent amounts.

Based on these results, subsequent experiments were performed with the four materials identified to be compatible with the cultured cells. These materials were Biosilon, melamine resin, Chromosorb, and Tenax.

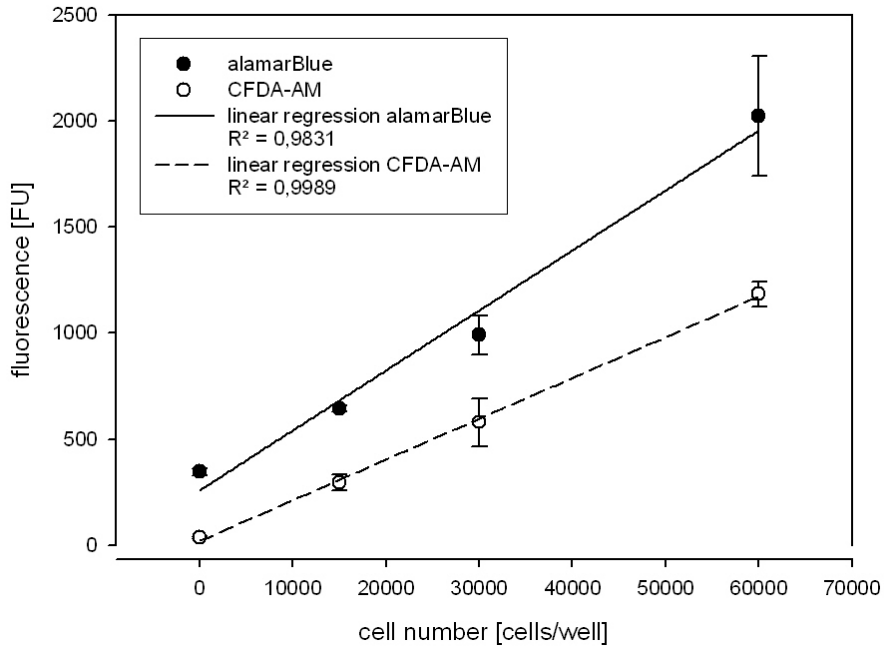

Figure 3.4 Calibration of a standard curve for determination of cell number via fluorescent units in the alamarBlue and CFDA-AM cell viability assay for Biosilon.

3.3.2 EROD induction elicited by PAH contaminated sorbent beads

In a next step the availability of contaminants, sorbed to the various materials, and their ability to elicit effects on adhering cells were assessed. The materials which were found to be compatible with the bioassays were tested, coated with benzo[a]pyrene (BaP), in the EROD-assay. Reproducible, dose-dependent induction of EROD activity was found only for Biosilon (fig. 3.5). Thus, all subsequent experiments focused on Biosilon alone.

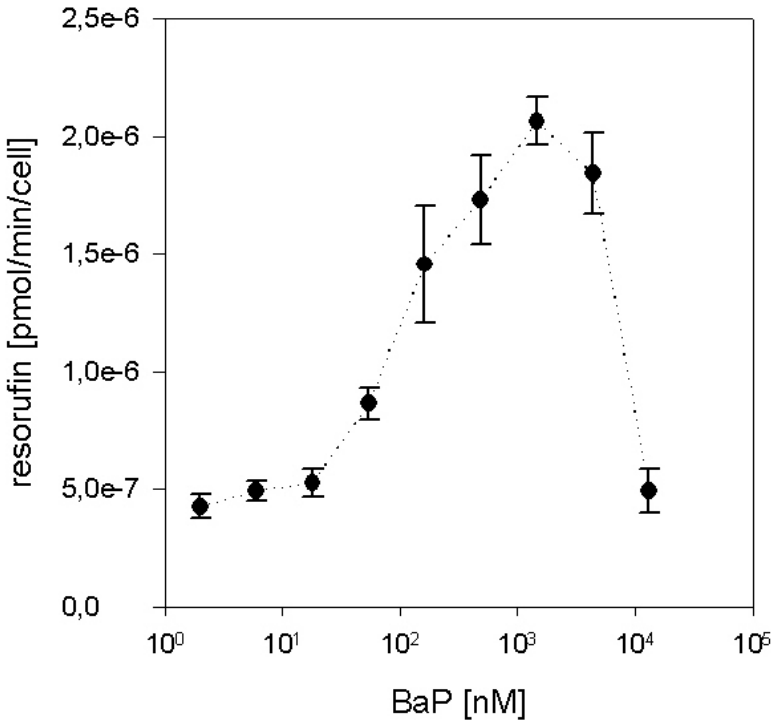

Figure 3.5 EROD induction in RTL-W1 cells grown on BaP contaminated Biosilon. BaP concentrations on the x-axis were calculated using the total amount of BaP sorbed to the Biosilon in the test system divided by the volume of culture medium used in the assay.

3.3.3 Role of sorption in the EROD response

Experiments were performed to establish whether the measured effects are caused by BaP which diffuses through the medium to the cells or by BaP which is taken up directly into the cells by the contact to the coated surface, thus investigating the necessity of cell adherence. It was observed that sorbed BaP is able to induce EROD-activity in both cases, by direct contact or by diffusion through the medium (fig. 3.6). However, cells attached directly on Biosilon were more sensitive. This can be seen by the shift in the induction pattern toward lower concentrations. At the same concentrations, maximum induction is not reached in cells where BaP had to diffuse through the medium in order to reach the cells. These results were confirmed in repeated experiments.

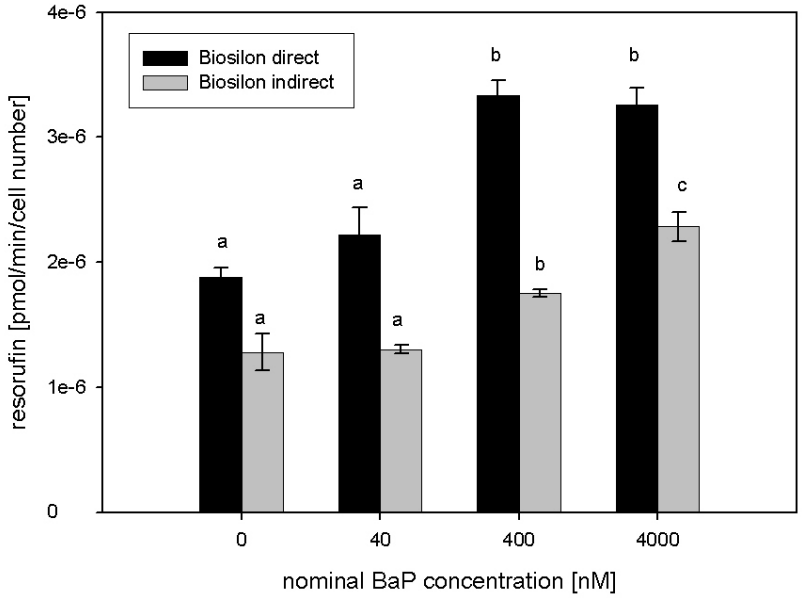

Figure 3.6 EROD induction in RTL-W1 cells elicited by BaP contaminated Biosilon. Cells were grown either directly on Biosilon (black bars) or indirectly by physically separating the Biosilon and the cells so that BaP had to diffuse through the medium in order to enter the cells (gray bars). Bars with identical characters within each data row are not significantly different from each other (Tukey's test, $p<0.05$).

3.3.4 Distribution of PAHs in bead and standard EROD-assay

Chemical analyses of the various compartments in the EROD-test after 24 h of incubation indicated that in the bead as well as in the standard assay the largest portion of the PAHs is present in sorbed form. In the bead assay, the median value of 82.3 % of the total amount remained sorbed on the Biosilon (fig. 3.7). Only 1.2 % of the PAHs were sorbed to the plate, 1.8 % remained in the medium and 1.6 % were detected in the cells. The median total recovered amount was 87 % and similar for each single PAH.

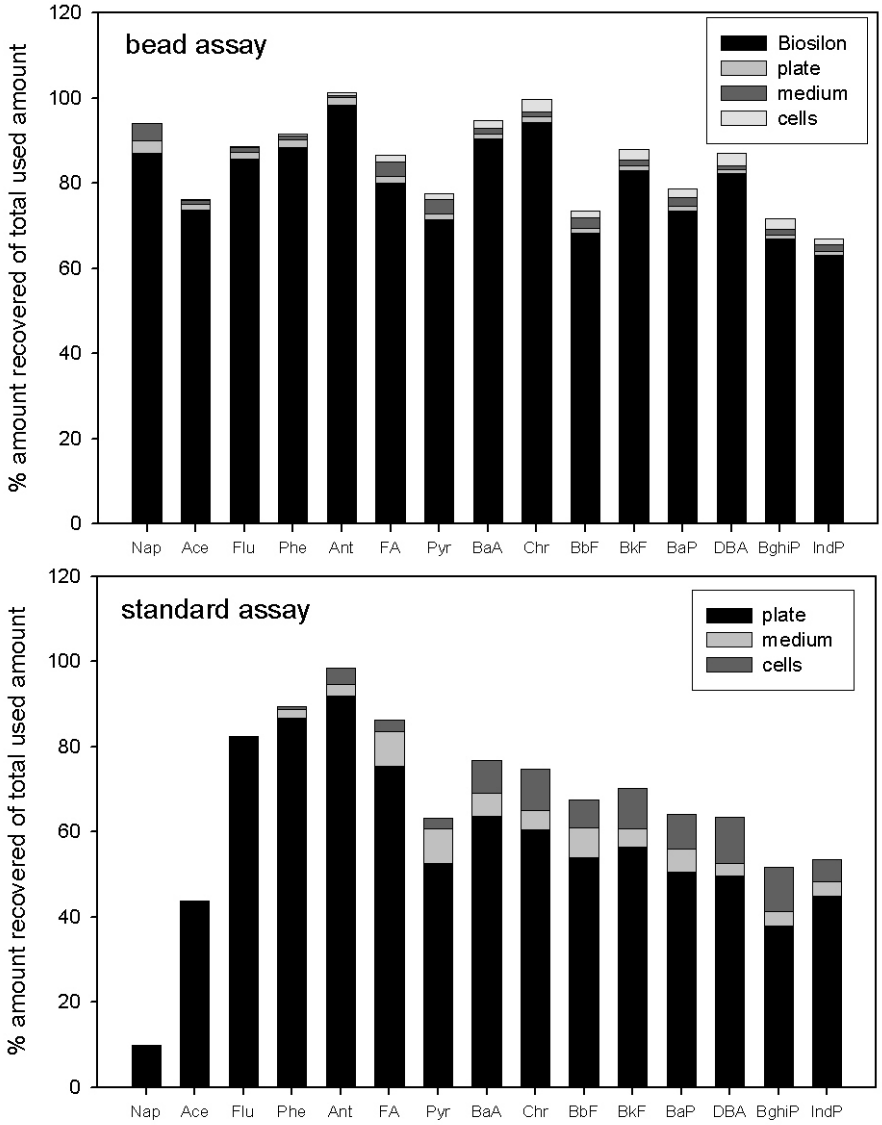

Figure 3.7 Distribution of PAHs between compartments in the EROD-assay with RTL-W1 cells in the bead assay (upper diagram) and the standard assay (lower diagram).

The pattern appeared similar for the standard assay. Here, median ratios of 53.9 % of the total amount were found sorbed to the plate, 4.5 % were remaining in the medium, whereas 7.1 % was found in the cells. The median total recovered amount in the standard assay was 67.4 %, whereby the recoveries of the total amount from all compartments for Nap and Ace were much lower (9.9 % and 43.8 %) and detection limits were reached only for the plate compartment.

The internal exposure, that is the amount taken up into the cells, which is thought to be the portion capable to elicit effects, was 4.5 fold higher in the standard assay as in the bead assay.

3.3.5 Comparison of standard and bead assay

EROD induction curves for the standard assay and the Biosilon bead assay were measured in parallel in order to compare the sensitivity in both systems. Results using BaP as model compound are shown in fig. 3.8.

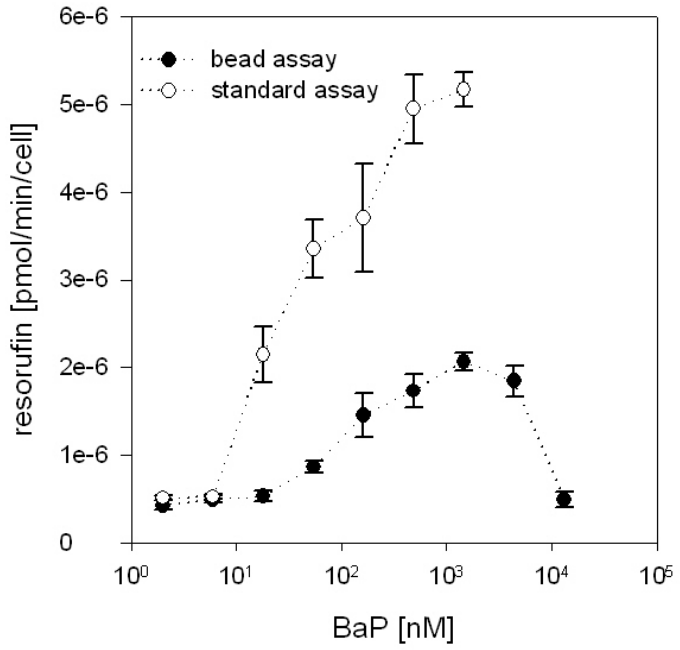

Figure 3.8 EROD induction elicited by BaP in RTL-W1 cells in the standard assay and in the bead assay with contaminated Biosilon. (The bead assay curve corresponds to the curve in fig. 3.5).

Higher magnitudes in EROD induction and a shift to lower concentrations were observed for the standard assay compared to the bead assay (fig. 3.8 & 3.9). For further comparison of the two systems, time courses in EROD induction using a mixture of four EROD inducing compounds were investigated (fig 3.9). For the bead assay, the 24 h induction seemed to be most sensitive. For the subsequent days, induction curves were rather constant in shape over time. In the standard assay, 24 h values were also most sensitive, and the shape of the curve remained similar but with a lower magnitude for 48 and 72 h. After 96 h of exposure, very little EROD induction remained. As it was previously seen in the distribution experiments, the uptake into the cells within 24 h was higher by a factor of 4.5 in the standard assay. Thus 24 h induction curves were also compared with regard to the internal concentrations in the cells (fig 3.10). Higher induction magnitudes were obvious for the standard assay but the differences in the shape of the curves between the two assays were reduced.

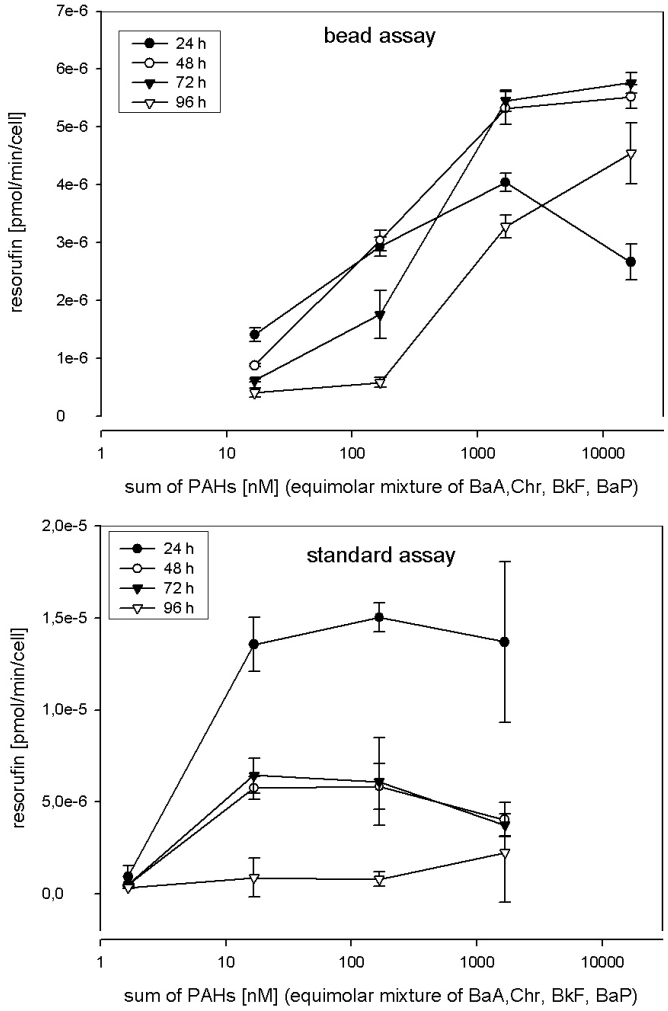

Figure 3.9 Time dependent EROD induction in RTL-W1 cells elicited by a mixture of four EROD inducing PAHs in the standard and the bead assay.

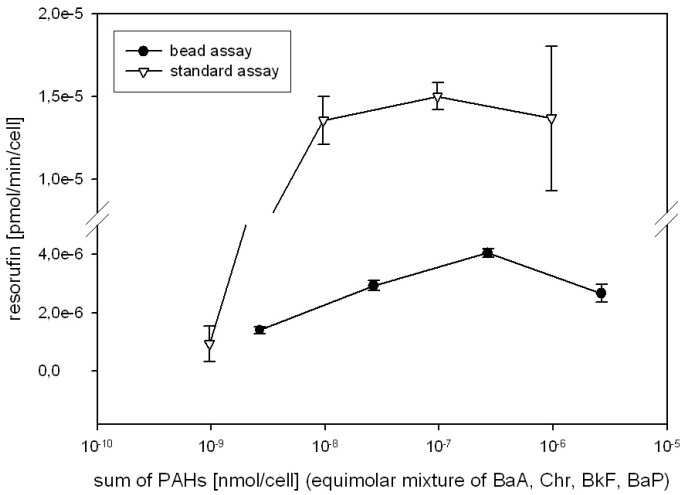

Figure 3.10 Comparison of EROD induction elicited in RTL-W1 cells by a mixture of PAHs within 24 h exposure in the bead and standard assay using internal cell concentrations.

3.4 Discussion

There are various prerequisites for making use of contaminant-coated surfaces in cell bioassays. Firstly, sorbents without contaminants need to be compatible with cells in culture, thus having no toxic effects on the cells and enabling their attachment on the surface. The second prerequisite is the suitability for a simple and reliable handling. Thirdly, sorbed contaminants have to be available to adhering cells in order to enable the detection of dose dependent response in bioassays.

The ability of sorbents to support cell attachment was investigated using three fluorescent dyes. Among them, neutral red was shown to be unsuitable. No significant differences between the material alone and the material plus cells could be found. This might be due to the fact that, in contrast to the other two fluorescent cell viability dyes, neutral red is not metabolized by the cells to a fluorescent product, which is measured. Instead, it is accumulated in the cells in the original form added to the test. After a fixation and extraction step, the neutral red which was accumulated in the cells is normally detected. In use with the sorbents, however, it seems probable that a substantial part of the neutral red was sorbed to the sorbent materials directly so that accumulation in cells and thus cell viability could not be assessed. This hypothesis was supported by the fact that fluorescence increased for the neutral red assay even in absence of cells when increasing amounts of sorbents were used. Thus it is desirable to use either highly water soluble dyes which do not tend to bind to the sorbent materials, or dyes which are detectable as fluorescent metabolites without interferences caused by the parent compound which could still be present in the assay after washing steps.

Based on their compatibility with cell culture, Biosilon, Tenax TA and GR, Chromosorb and melamine resin were further investigated. When these were coated with contaminants and the ability of the contaminants to elicit dose dependent effects on adhering cells was investigated, Biosilon showed to be the only material leading to reproducible results. Biosilon was also the material with the best wettability and simplest handling procedure among the chosen sorbents. For other sorbents, problems in detecting dose dependent EROD induction occurred, which might be attributed to their porous structure and the possibly lower availability of sorbed contaminants to adhering cells.

A linear increase in fluorescent units with increasing cell number on Biosilon was shown in the assays using the fluorescent dyes alamarBlue and CFDA-AM. This indicated the presence of sufficient surface area for cells even when growing during exposure. The linear relationship allows the determination of cell numbers via a calibration using the fluorescent dyes.

The use of increasing amounts of sorbent had no influence when constant cell numbers were used for all materials except for Tenax TA. This may be due to the fact that, in general, low sorbent amounts offer enough surface for the cells to settle whereas Tenax TA does not provide enough space for all cells to attach if smaller amounts of Tenax TA are used. Tenax TA showed

a low wettability and a small portion of the material was floating on top instead of settling to the bottom of cell culture plates in the assays thereby diminishing the surface available for cell attachment.

Regarding the EROD bead assay, the importance of cell adherence to the contaminated Biosilon was shown. Cells which were in direct contact with the Biosilon reacted more sensitively than cells being removed from the exposure source and separated from the contaminated Biosilon through the medium. Therefore it could be confirmed that the closer contact to the exposure source leads to locally enhanced contaminant concentrations, even if the uptake mechanism is not yet known. The influence of the length of diffusion pathway from a contaminant source in small scaled systems was also studied by (MAYER *et al.* 2004), who investigated the transfer of fluoranthene from a source to a disk acting as a sink. When disks were in direct contact, the transfer proceeded much faster than when disks were separated by a 100 µm diffusion boundary layer. Modeling options for a clearer understanding of the uptake processes should be considered in future investigations.

Looking at the distribution of contaminants in the EROD assay, it was observed that Biosilon kept the major part of the hydrophobic substances. Similar to that, those compounds dosed to the cells in solvent solution in the standard assay got sorbed to the plate within 24 h to a substantial amount. In the standard assay, the more volatile compounds, particularly Nap, could only partly be recovered from the system, which was in contrast to the bead assay. This might be explained by losses to the gas phase in the standard assay, whereas the bead assay seemed to be suitable to reduce such losses by sequestering the contaminants in the sorbed state. LEE AND HARDY (1998) reported such stabilization effects for chlorobenzenes sorbed to Tenax TA or Porapak Q, used as the solid receiving phase of a passive sampling device. No obvious losses of the sorbed compounds were observed within several days of storage either at room temperature or 4 °C.

The internal concentration in the cells was higher by a factor of 4.5 in the standard assay than in the bead assay, but for both systems the amount recovered in the cells after 24 h of exposure was small in comparison to the whole amount present in the test system. Thus, nominal exposure concentrations greatly overestimate the concentration required to elicit the observed response, which was also reported by SCHIRMER *et al.* (1997) and HESTERMANN *et al.* (2000). Therefore internal concentrations should be generally taken into account in addition to nominal or measured external exposure concentrations.

Comparing EROD induction curves for the bead and the standard assay, a shift to higher concentrations and a lower induction magnitude was evident for the bead assay. After transforming the concentration axis from external to internal cell concentrations, differences in curve shapes were reduced, even if the standard assay still seemed more sensitive. Many factors influencing the sensitivity of EROD induction assays are reported in the literature, e.g. different media compositions, medium volumes, serum concentrations, or cell numbers (e.g.

HESTERMANN *et al.* 2000; SEGNER *et al.* 2001; DRENTH *et al.* 1998; LONGMAN AND BUEHRING 1986). In the two exposure scenarios investigated here, the same media and volumes were applied. The observed differences might be caused by the different conditions during cell attachment. Whereas cells were incubated for 24 h prior to dosing of PAHs in the standard assay, cells needed to attach to the surface in presence of the contaminants in the bead assay.

3.5 Summary

A bead assay for solvent-free exposure of cultured cells to contaminants was developed. Biosilon was shown to be a suitable material for cell attachment and detection of reproducible dose response curves. The bead assay had obvious advantages as it e.g. enables the reliable testing of volatile compounds and prevents the use of solvents. The most important aspect within this study is that the sorbent material selected and the bead assay developed form the pre-requisite for coupling this technique to passive sampling technology for a holistic approach combining chemical and toxicological analysis.

This chapter has shown Biosilon to be suitable from a biological point of view. To further test its potential use as the receiving phase in passive sampling devices, the chemical properties of Biosilon relating to sampling and chemical analysis were investigated in the following chapter.

Chapter 4

Toximeter development:

evaluating the applicability of the Biosilon filled ceramic tube passive sampler in laboratory experiments

4.1 Introduction

A holistic approach for long-term monitoring of contaminated ground- and surface water sites should include, beyond chemical analysis, investigations on the toxic potential. Up to now, the survey of pollution state is performed mainly based on chemical analysis, whereby the chemical analysis is focused on pollutants known or suspected to be present. Thus toxicologically relevant compounds might be overlooked. It was the aim of the current work to provide a new methodology and technology for the coupling of chemical and biological analysis. The proposed methodology involved a solid material-based, solvent-free bioassay and its combination with chemical analysis. The technology aimed at a passive sampling device which, after time-integrated sampling, allows for the combined chemical-biological analysis approach.

Various passive sampling devices are field validated and commercially available (BOPP *et al.* 2004). However, currently available samplers are generally used to exclusively monitor the sampled contaminants chemically. The only passive sampling devices where a combined chemical-biological monitoring has been accomplished so far are the Semipermeable Membrane Device, SPMD, and the Polar Organic Chemical Integrative Sampler, POCIS (see Chapter 1). Difficulties with high background toxicities of SPMD extracts especially in the Microtox assay have been reported (SABALIUNAS *et al.* 1999). Recently a new approach to overcome this problem has been proposed (LEBO *et al.* 2004), where the receiving phase of the SPMDs is precleaned prior to its use. However, all these pre-cleaning and extraction processes require labor- and solvent-intensive procedures. Furthermore, the bioassays can only be performed using solvents, which may be toxic to the test organisms themselves or may interact with sample constituents (CHAURET *et al.* 1998; YIN *et al.* 2001).

Thus, this study pursued the development of a passive sampling device which enables combined chemical and toxicological analysis, using a solvent free method for biological testing.

The applicability of contaminated Biosilon beads in bioassays was demonstrated in the preceding chapter. In the following, the applicability of Biosilon concerning chemical requirements and the use as the receiving phase in a newly designed passive sampler, the Toximeter, were investigated.

4.1.1 Toximeter design

The Toximeter passive sampler design is based on the Ceramic Dosimeter developed by Grathwohl et al. (GRATHWOHL AND SCHIEDEK 1997; MARTIN *et al.* 2003). The Ceramic Dosimeter layout was chosen for three reasons. Firstly, loose sorbent beads can be applied as a receiving phase. Secondly, it is a robust sampling system whereby its sampling behavior is nearly unaffected by varying hydrodynamic conditions. Thirdly, the Ceramic Dosimeter system has already been successfully applied for groundwater sampling. The Toximeter uses the same ceramic tube, which is 5 cm long and 1 cm in diameter and serves both as a container for the solid sorbent material and as the diffusion barrier. Based on the thickness of the membrane and the small inner pore size (pore size 5 nm), the ceramic tube allows for only diffusive flux to the inner part. The small pore size also prevents microorganisms from entering the sampling device. The idea is to use Biosilon as the sorbent material which, following time-integrated sampling of target analytes, can be divided into two portions. One portion is to be used for a simple solvent extraction to allow for chemical analysis. The other portion serves as material for biological analysis (fig. 4.1).

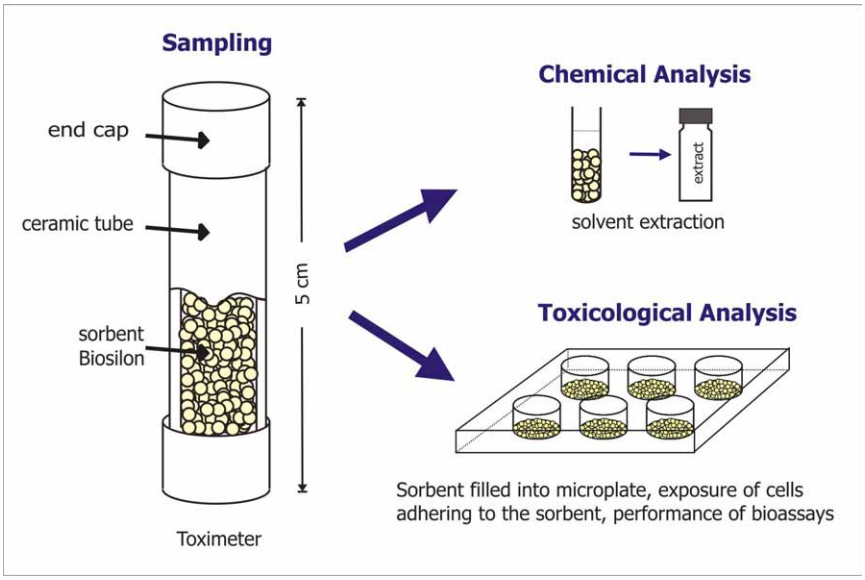

Figure 4.1 Toximeter design and scheme of combined chemical and toxicological analysis

The uptake behavior of the sampler is determined by the ceramic tube. Thus the same calculations used for the Ceramic Dosimeter can be applied. The sampling through the small pore size ceramic tube is controlled by diffusion. The flux of analytes (F [g·cm^{-2}·s^{-1}]) to the receiving phase in the inner part can be described using Fick's first law (equation 1; Grathwohl 1998), where D is the diffusion coefficient [cm^2·s^{-1}] and dC/dz is the concentration gradient.

$$F = D\frac{dC}{dz} \quad (1)$$

For the ceramic tube samplers, the diffusion coefficient has to be adjusted, as it is lower in the porous tube than in water (D_w). Thus the effective diffusion coefficient D_e has to be calculated according to Archie's law (equation 2), whereby Archie's law exponent m of 2.0 was previously applied for PAHs (MARTIN *et al.* 2001). The porosity κ of the ceramic tube is 0.305.

$$D_e = D_w \kappa^m \quad (2)$$

The mass (M [ng]), which is accumulated over time t [s] in the Ceramic Dosimeter or Toximeter, can thus be determined using equation 3 (MARTIN *et al.* 2003),

$$M = F A t = D_e \frac{\Delta C}{\Delta x} A t \quad (3)$$

where A [cm^2] denotes the surface of the ceramic tube, ΔC [ng·mL^{-1}] is the difference in aqueous contaminant concentrations outside and inside the device and Δx [cm] is the thickness of the ceramic tube. It is assumed, that ΔC equals the surrounding aqueous contaminant concentration, as the aqueous concentration in the inner part of the sampler is approaching zero due to the high affinity of the analytes to the receiving phase. Equation 3 enables the prediction of accumulated masses in the Toximeter at a given aqueous contaminant concentration or the calculation of the average contaminant concentrations from the amounts collected in the Toximeter.

The ceramic tube represents a relatively thick diffusion barrier. Therefore the sampling behavior is dominated by diffusion through this barrier alone. This is in contrast to many other sampler types which are based e.g. on organic polymer membranes, where the aqueous boundary layer can greatly influence sampling behavior. The Toximeter is thus independent of hydrodynamic parameters which is of advantage because no labor-intensive calibration steps have to be performed.

4.2 Materials and Methods

The major goal of this part of the thesis was the evaluation of Biosilon as a receiving phase from a chemical point of view as well as the validation of sampling behavior of Biosilon filled Toximeters in the laboratory. Extraction efficiencies for PAH contaminated Biosilon were determined. As well, sorption kinetics of PAHs to Biosilon and partitioning of PAHs in a Biosilon / water system were investigated. Thereafter, Biosilon filled Toximeters were exposed to PAHs in three different exposure scenarios in order to compare directly analyzed aqueous PAH concentrations to Toximeter derived PAH concentrations.

4.2.1 Chemicals and sorbents

Polycyclic aromatic hydrocarbons (PAHs) (see table A1, appendix) were purchased in purity grade 99-99.9 % (if not stated otherwise) from various manufacturers: naphthalene (Nap) from Merck (Darmstadt, Germany); acenaphthene (Ace), fluorene (Flu), phenanthrene (Phe) (98 %), anthracene (Ant), pyrene (Pyr), benzo[a]anthracene (BaA), chrysene (Chr) (98 %), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP) (98 %), and dibenzo[a]anthracene (DBA) (97 %) from Sigma Aldrich (Steinheim, Germany); fluoranthene (FA) (98 %) from Riedel-de Haën (Seelze, Germany); and benzo[b]fluoranthene (BbF), benzo[g,h,i]perylene (BghiP) and indeno[1,2,3-cd]pyrene (IndP) from Promochem (Wesel, Germany). For tests where a mixture of the 16 US EPA priority polycyclic aromatic hydrocarbons (PAHs) was investigated, a mixture in acetonitrile was obtained from Promochem (Wesel, Germany), containing the above mentioned 15 PAHs and acenaphthylene (Any) with a concentration each of 10 ng/µL.

Biosilon, the sorbent material to be tested as the receiving phase in the passive sampling device, was obtained from Nunc (Nunc Brand Products, Denmark). Biosilon consists of polystyrene and is available as sterile, dry, nonporous beads with a diameter of 160-300 µm. It is conventionally used as cell culture carrier, thus guaranteeing absolute purity and enabling its use without pre-treatment (Chapter 3).

4.2.2 Instrumental analysis

PAH analysis was performed using the same HPLC / fluorescence detection method as described in Chapter 3. Acenaphthylene was included in the PAH mixture in all experiments (except for the third Toximeter exposure approach), but was not evaluated because it cannot be detected using fluorescence detection.

4.2.3 Optimization of the extraction protocol for Biosilon

Biosilon is used as solid receiving phase in the Toximeter passive sampling device, developed in this study. To enable combined chemical and toxicological analysis, various extraction protocols for analysis of PAHs sorbed onto Biosilon were investigated. Biosilon beads were exposed to aqueous PAH solutions of different concentrations for 4 d. For first experiments, BaA, Chr, BkF, and BaP were used. Then Biosilon was extracted with 1.5-3 mL of methanol or ethanol under shaking on a vortex shaker for 1 h and 4 h or in an ultrasonic bath for 15-30 min. Normally Biosilon was extracted once, but for testing of the completeness of the extraction, a second extract was firstly analyzed separately and secondly combined with the first extract. This was done in order to compare the total extracted amount after one extraction to the efficiency of the two-fold extraction procedure. The optimized protocol was validated in further experiments using Biosilon coated with a mixture of 16 PAHs.

The resulting standard procedure used 2 mL of methanol in an ultrasonic bath for 30 min for the extraction of up to 1000 mg Biosilon, which was the amount generally used in the final Toximeter setup. In order to determine the total amount of all 16 PAHs sorbed to Biosilon as reference quantity for extraction efficiency calculations, Biosilon was extracted four times and the sum of all extracts was used as the total sorbed amount.

4.2.4 Assessment of the partitioning of PAHs between water and Biosilon

In a first experiment, the kinetics of the sorption of BaP onto Biosilon were examined. Three replicates of an aqueous BaP solution were prepared at a concentration of 3.2 µg/L in Milli-Q water corresponding to about 85 % of the saturation concentration for BaP (3.8 µg/L, MACKAY *et al.* 1992). A total of 9 g of Biosilon were added to each 1 L bottle to provide sufficient material over the sampling period. Three additional bottles containing Milli-Q water and 9 g of Biosilon were used as blank values. Bottles were exposed on a rotary shaker for 1 week at room temperature. Biosilon samples (200 mg) were taken at 8 points in time: 1, 3, 6, 12, 24, 48 h, 3d, and 7d. The actual aqueous BaP concentrations were not determined, instead nominal concentrations were used.

In a second experimental approach, the distribution of 16 PAHs in a water / Biosilon system was investigated. A total of 1000 mg of Biosilon were added to 800 mL of aqueous PAH solution in 1 L amber glass bottles. Seven concentrations were investigated within this experiment for each of the PAHs used in this study (62.5, 125, 250, 500, 1000, 2500, and 5000 ng/L). The solutions were prepared by adding the corresponding amount of the standard mixture in acetonitrile with a glass capillary pipette to 800 mL of autoclaved Milli-Q water. The solutions were shaken overnight before the Biosilon was added. The solutions with Biosilon were incubated at 23 ± 2°C on a rotary shaker in the dark for 10 d. After the 10 d partitioning period, PAH amounts in three compartments were determined. The three compartments were

Biosilon, water and the bottle walls. 500 mL of the solution were carefully decanted, so that no Biosilon was contained in the water sample, and was processed using solid phase extraction (SPE) as described below. For Biosilon analysis, the remaining 300 mL solution were vigorously shaken for resuspending the beads and 200 mL of the suspension were transferred to filter centrifuge tubes (Whatman Vecta Spin 20, 10 µm polypropylene mesh, VWR, Dresden, Germany). The tubes were centrifuged at 5,000 rpm for 5 min in order to dry the Biosilon beads. 200 mg of the dried Biosilon were transferred to 8 mL amber glass tubes and were extracted with 2 mL of methanol in an ultrasonic bath for 30 min as described above. The bottles were emptied and rinsed twice with Milli-Q water in order to remove remaining Biosilon sticking to the walls. After drying the bottles by keeping them upside down for 1 h, they were rinsed with 10 mL of hexane. The hexane extracts were transferred into 1.5 mL methanol after drying on a rotary evaporator and under nitrogen. The methanol extracts of the three compartments were analyzed by HPLC-fluorescence detection as described below. For determination of distribution behavior, data from three individual experiments were pooled. Partition coefficients were determined based on the slope of distribution ratios using the amount sorbed to Biosilon [ng/g] and the amount remaining in water [ng/mL]. Graph Pad software linear regression function was used for examining the significance of correlation and of deviations from linearity (runs test).

4.2.5 Toximeter preparation

All sampler materials, except for the sorbent beads Biosilon, were obtained from imw (Tübingen, Germany). Tubes and caps were precleaned three times in methanol in an ultrasonic bath and stainless steel holders in acetone. After rinsing with Milli-Q water, the materials were sterilized by autoclaving.

The ceramic tubes were filled with 950 mg of Biosilon (Nunc^{TM} Brand Products, Denmark). To enable unhindered diffusion into the inner part of the sampler, the Biosilon-filled samplers were filled with Milli-Q water before capping. The caps used in this study were made of Teflon and pressed onto the two endings of the tube by clamping the Toximeter into a stainless steel holder. In order to ensure the water saturation of the ceramic tube in the filled samplers, they were submerged in water containing bottles and evacuated in an exsiccator for at least 30 min, so that remaining air could be drawn out of the pores. The preparation of the samplers was performed under a sterile flow hood in order to prevent microbial contamination and thus potential degradation of sampled analytes in the inner part. Microorganisms occurring during exposure cannot enter into the sampler due to the small pore size of the ceramic tube.

4.2.6 Processing of the Toximeter after sampling

After exposure, the Toximeters were opened for analysis. They were taken out of the holders; the caps were removed and collected for subsequent analysis. The Biosilon was transferred to filter centrifuge tubes (10 µm polypropylene mesh, Whatman Vecta Spin 3, VWR, Dresden, Germany) by rinsing the inner part of the sampler with 2-3 mL of Milli-Q water. The empty ceramic tubes were kept for subsequent extraction.

Normally it would be sufficient to analyze the Biosilon as the receiving phase alone. In order to investigate the potential accumulation of analytes in the tube and the caps, all parts were extracted in selected experiments.

The Biosilon was dried by centrifugation at 5,000 rpm for 5 min. Afterwards 180 mg were transferred to amber glass vials for later use in bioassays, which were performed within 24 h after termination of sampler exposure. The remaining Biosilon was transferred to amber glass tubes and weighed. Generally not all of the 950 mg were recovered from the ceramic tube and the centrifuge tube. About 20 mg at an average were lost. Thus the amounts for biological analysis were fixed to 180 mg and the weight of the remaining amount of Biosilon was determined prior to chemical analysis so that the total amounts accumulated in the samplers could be calculated. The biological analysis of the Biosilon was performed as described below. The chemical extraction of the Biosilon was performed in an ultrasonic bath for 30 min using 2 mL of methanol.

The ceramic tubes and the Teflon caps were extracted in an ultrasonic bath for 30 min using 15 mL of hexane. The hexane extracts were evaporated and transferred into 1.5 mL of methanol.

The ceramic tubes and caps of the Toximeters were reused in several experiments. Prior to re-use they were pre-extracted three times with methanol. The third extract showed no detectable PAH levels in HPLC analysis. Afterwards the ceramic tubes and caps were rinsed three times with Milli-Q water and autoclaved prior to use.

4.2.7 Processing of water samples

For analysis of PAHs in water samples the contaminants were concentrated using a solid phase extraction (SPE) method. For SPE, Bakerbond SPE Polar Plus C18 extraction columns (500 mg, Mallinckrodt Baker, Griesheim, Germany) were used. The extraction was performed using a Bakerbond vacuum station. The columns were conditioned by adding 10 mL of iso-propanol followed by 50 mL of Milli-Q water containing 10% iso-propanol. Afterwards 800 mL of the water sample (to which 8 mL of iso-propanol were added) were passed through the extraction columns. Then the columns were dried by drawing air through them for 15 min. The subsequent elution was done using two times 5 mL of hexane. The unified hexane eluates were

concentrated using a rotary evaporator to a volume of 0.5-1 mL. The remaining volume was dried under nitrogen and the constituents were redissolved in 1.5 mL methanol in an ultrasonic bath for 15 min.

4.2.8 Performance of bioassays

Bioassays were performed using the rainbow trout (*Oncorhynchus mykiss*) liver cell line RTL-W1 (LEE *et al.* 1993). Cell culturing was performed as described in Chapter 3. Bioassays were carried out in MultiScreen-BV filter plates (96 well) with a durapore-membrane bottom (low protein binding, pore size 1.2 µm, sterile; Millipore, Eschborn, Germany) as described in Chapter 3. After 24 h of exposure on the contaminated Biosilon, the combined EROD/CFDA-AM assay was performed. Each concentration was assessed with 6 replicates, whereby one replicate was without cells to obtain information on background values potentially influencing the measurements.

4.2.9 Laboratory exposure scenarios for calibration of the Toximeter

For all laboratory calibration experiments with the Toximeter, the exposure took place in autoclaved solutions and bottles in order to prevent microbial degradation within the solutions. Three different scenarios were used in an attempt to improve maintenance of constant exposure concentrations over longer periods with the low solubility compounds.

First approach: The uptake of phenanthrene as a model compound was investigated by exposing Toximeters in saturated phenanthrene solutions. The solubility of phenanthrene in water is 1.1 mg/L at 25 °C (MACKAY *et al.* 1992). Thus 20 mg of solid phenanthrene were added to 800 mL of Milli-Q water in order to provide phenanthrene in excess. Bottles were placed over night on an orbital shaker to establish constant saturation concentrations. After shaking, crystals sank to the bottom of the bottles and then Toximeters were added. They were placed vertically in the bottles hanging 1-2 cm above the bottle bottom on a nylon string. The static exposure took place in an incubator at 12 °C (groundwater relevant temperature) for five weeks with three bottles in parallel. Each of the bottles contained five Toximeters.

One sampler from each bottle was collected weekly. Samplers were processed as described below. In parallel to the Toximeters, water samples were taken at each sampling time. A volume of 0.5 mL of phenanthrene solution (containing no visible crystalline phenanthrene) was transferred to a vial containing 0.5 mL methanol. The methanol/water mixture was analyzed directly by HPLC.

Second approach: Toximeters were exposed in a semi-static system to a mixture of 16 PAHs. The aqueous PAH solution was exchanged daily to counteract sorptive losses onto the glass walls and the samplers. Exposure was performed in 1 L amber glass bottles. 800 mL of

autoclaved Milli-Q water were added. Then 40 µL of the 16 PAH stock solution in acetonitrile were added, leading to a nominal concentration of 500 ng/L for each PAH and an acetonitrile ratio of 0.005 % in the solution. This concentration was chosen as a compromise: predicted amounts which were expected to accumulate in the Toximeter should reach the Toximeter detection limit even at the first or second sampling event, but the aqueous concentration should be below the aqueous solubility of the investigated PAHs. This latter point was accomplished for all PAHs except for BghiP and IndP. The predicted sampling rates of the Toximeter are in the 2-4 mL/d range so that the uptake into the receiving phase should not cause a depletion of aqueous concentrations. Every 22-26 h, the PAH solution was removed and new water and stock solution was added to the exposure bottles. The solutions were shaken vigorously for 1 min before the Toximeters were reinserted. The removed water samples were analyzed daily in the first week and weekly for the rest of the exposure period, which was 28 d in total. Initial aqueous PAH concentrations were analyzed for the first preparation at the beginning of the experiment. Three bottles were prepared in parallel, each of them containing four Toximeters, which hung oriented vertically 1-2 cm above the bottle bottom attached to a nylon string. One Toximeter of each bottle was removed every 7 d. The exposure took place on a shaker at 60 rpm. This was done at room temperature (22 ± 2 °C) because exposure at groundwater relevant temperature was technically infeasible.

Third approach: Toximeters were exposed to 16 PAHs in another semi-static system for six weeks. The system used contaminated stir bars to establish and maintain constant PAH concentrations. The method developed by GEROFKE *et al.* (2004) was adjusted to the scenario described here.

A methanolic solution, which was highly concentrated for each of the 16 PAHs, was prepared for contamination of the Teflon coated stir bars (50x8 mm, VWR, Dresden, Germany). Stir bars were submerged into the solution for 7 d without stirring. Then contaminated stir bars were taken out and remaining methanol and crystals were removed by wiping with paper cloth. The bars were subsequently stirred for 3 min in Milli-Q water to remove remaining methanol which might have diffused into the Teflon coating of the bars as well. Stir bars were then placed in 800 mL of Milli-Q water and were allowed to equilibrate for two days under continuous stirring before the samplers were added for one week.

After one week and every week thereafter, samplers were transferred to newly equilibrated bottles which were prepared as described above. Exposure took place in triplicate whereby each bottle initially contained 6 samplers and a single sampler was removed from each bottle after every 7 d for 6 weeks in total. In parallel to the chemical analysis, 180 mg of the Biosilon from the samplers was used for biological analysis in the EROD assay as described in Chapter 3.

4.3 Results

4.3.1 Optimization of the extraction protocol for Biosilon

Extraction efficiencies of two solvents compatible with Biosilon were investigated using BaA, Chr, BkF, and BaP as model compounds. A two-fold extraction with methanol or ethanol showed higher efficiencies for methanol. Recovered amounts (based on the total nominal amount of PAHs in the system) were 62-85 % in the first and 11-16 % in the second extract for methanol whereas they were slightly lower for ethanol with 45-65 % in the first and 6-9 % in the second extract. Further on, all extraction procedures were done using methanol. Unifying the first and second extract showed no higher recovery than for the first extract alone. This might be due to losses in the procedure for concentrating the combined extract in order to reach the volume corresponding to a single extraction, using evaporation under nitrogen and re-solubilization in methanol.

Using various extraction volumes and conditions it could be shown that slightly higher extraction efficiencies under shaking on a vortex microplate shaker can be found when extraction time was increased (from 1 h to 4 h) and / or when extraction volume was increased (from 1.5 to 3 mL). However, the results for extraction for 4 h on a vortex shaker equaled those for 15 min in an ultrasonic bath and additional 15 min solvent contact time, so that this latter time saving method was chosen. Thus extraction of up to 1000 mg Biosilon with 2 mL of methanol in an ultrasonic bath for 30 min was chosen as standard protocol.

Table 4.1 Extraction recoveries for Biosilon (n=4)

	average recovery [%]	standard deviation
Nap	58	10
Ace	77	9
Flu	70	11
Phe	79	7
Ant	77	9
FA	89	4
Pyr	90	3
BaA	90	3
Chr	90	2
BbF	92	1
BkF	91	2
BaP	92	1
DBA	91	2
BghiP	91	2
IndP	91	1

For final determination of extraction efficiencies for all PAHs in the mixture, PAH-coated Biosilon was extracted four times. The third extract showed low levels of Nap - Ant, whereas other compounds were below the quantification limit. No PAHs were detected in the fourth extract. The sum of recovered masses from all extracts for each single PAH was used as reference quantity for extraction efficiency calculations. Average extraction efficiencies were between 77-92 % with lower efficiencies for Nap (58 %) and Flu (70 %) (tab. 4.1).

4.3.2 Assessment of the partitioning of PAHs between water and Biosilon

Sorption kinetics of BaP to Biosilon were investigated over a 7 d period. The sorption process elapsed very fast (fig. 4.2). Within the first 3 h, near equilibrium conditions were achieved. The extract concentration stabilized at a level slightly higher (9 %) than the extract concentration predicted from nominal aqueous BaP concentrations assuming that all of the BaP was sorbed on the Biosilon. This difference might be explained due to uncertainties using nominal aqueous BaP concentrations. No BaP could be detected in Biosilon extract from blank bottles.

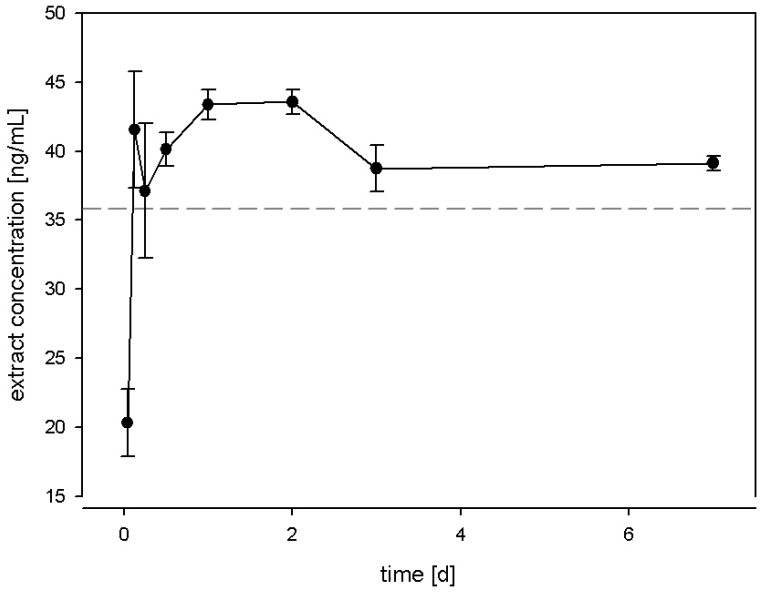

Figure 4.2 Sorption of benzo[a]pyrene (BaP) to Biosilon from aqueous solution over a 7 d period. Error bars represent the standard deviation (n=3). The dashed line represents the extract concentration, which was expected from nominal aqueous BaP concentrations, if all BaP had been sorbed to Biosilon.

In order to assess the suitability of Biosilon as a receiving phase in the passive sampling device, the distribution of PAHs between water and Biosilon was investigated and partition coefficients were determined as a measure of different behavior of the various PAHs towards Biosilon. The ratios were calculated after a 10 d sorption period, for which the establishment of equilibrium can be assumed from the results shown above. Partitioning was evaluated using a mass

balance approach, accounting for the distribution between Biosilon, water and the glass walls of the bottle used for exposure. Nominal and analyzed initial aqueous PAH concentrations were in good agreement for all PAHs except for Nap, Ace, and Flu, where lower amounts than expected were found. These compounds always showed higher deviations in HPLC analyses of solid phase extracts, especially for naphthalene, which likely can be attributed to its volatility. Thus the mass ratios after partitioning were calculated on the basis of the nominal concentrations for all PAHs (fig. 4.3). It can be seen that for the lower K_{ow} PAHs (Nap-Flu), low amounts were sorbed to Biosilon and a large part remained dissolved in the aqueous phase. Between 73 and 78 % of the nominal mass were recovered for Nap and Ace. This can also be explained by lower recovery in SPE extracts for these compounds. For Phe and FA the total recovered mass exceeded 100 %. As the sum consists of three values from individual analyses for the different compartments, this can occur due to variations in chemical analysis procedures. Amounts of higher K_{ow} PAHs (BaA-IndP) sorbed to Biosilon reached values between 80 and 99 % of the total nominal amount. Only small amounts remained in the aqueous solution and a small part was sorbed to the bottles serving as containers for the experiment.

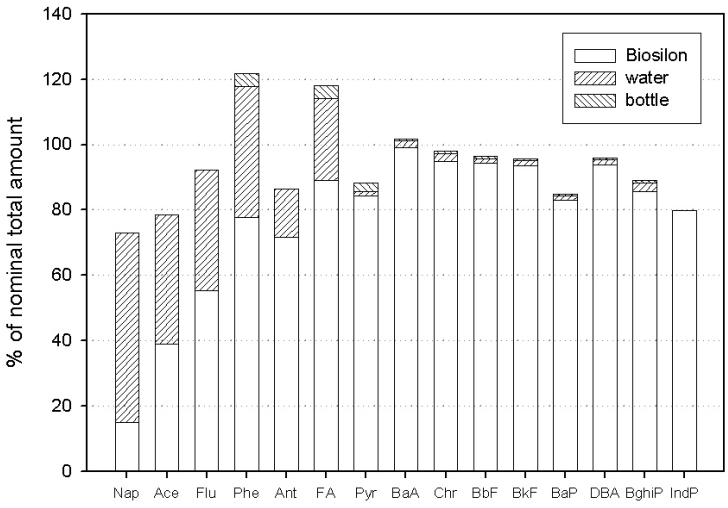

Figure 4.3 Partitioning of PAHs in a water / Biosilon system as mass balance including the bottle as test vessel. One representative example of partitioning is depicted, where 62.5 ng/L was used as initial water exposure concentration. Amounts found in the three compartments after a 10 d partitioning period are presented as ratios of the nominal used amount.

Partition coefficients between Biosilon and water (K_{bw}) were determined using equilibrium concentrations from experiments with various initial PAH concentrations. K_{bw} values were estimated based on the slope derived by linear regression analysis applied to the Biosilon / water distribution curves (fig. 4.4). For these estimations, only initial aqueous PAH concentrations that were below the water solubility limit for each PAH were used, except for IndP, where aqueous concentrations in equilibrium for the lower initial concentrations were below the detection limits.

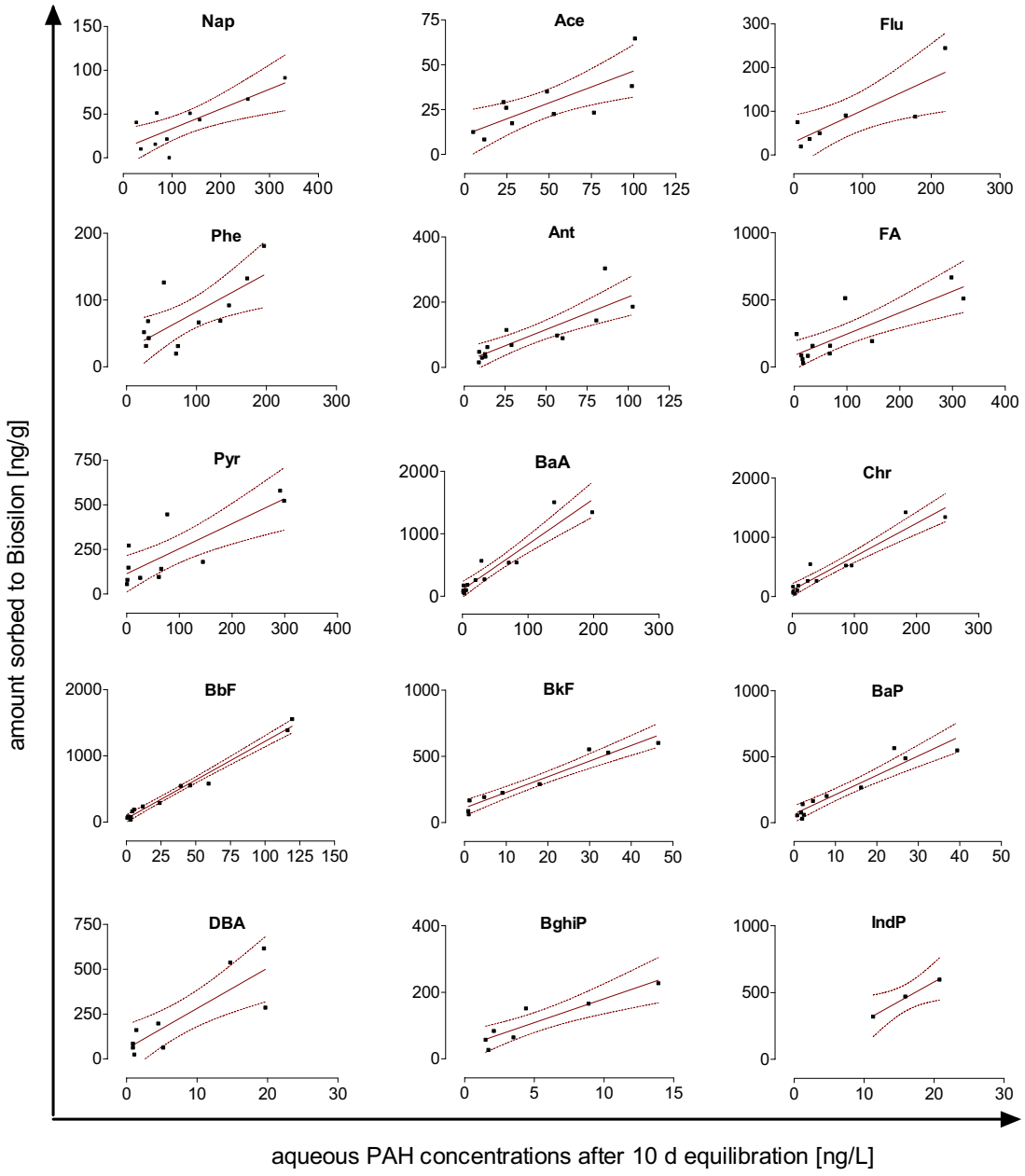

Figure 4.4 Sorption isotherms for each single PAH determined from a Biosilon / water distribution experiment. Black squares depict measured data, straight lines represent the linear regression function and dotted lines indicate the confidence interval (95 %).

Sorption isotherms seemed to deviate from linear correlation for the higher concentrations tested (at nominal initial aqueous concentrations of 5000 ng/L for Nap-BbF, at Ó 1000 ng/L for BkF-DBA, and at Ó 500 ng/L for BghiP and IndP), where the masses sorbed to Biosilon were lower than expected from the initial linear relationship. However, the data density was insufficient for detailed curve shape interpretation at the higher concentrations and thus these values were not included in the analysis (data not shown). Linear regression for the lower concentrations was adjusted using Graph Pad Prism Software. Correlation coefficients were significant for all PAHs as well as no significant nonlinear trend was observed (runs test). For IndP the detection limit was not reached in the water samples for the lower concentrations, so

that only three data points were available for correlation determination. The log K_{bw} values, derived from the slope of the linear regression (tab. 4.2), were found to be linearly correlated with the log K_{ow} (R^2 = 0.9367).

Table 4.2 Partition coefficients Biosilon / water K_{bw}.

	slope[a]	SE (slope) [b]	R² [c]	log K_{bw}	log K_{ow} [d]
Nap	226	60	0.6307	2.35	3.33
Ace	356	101	0.6066	2.55	3.92
Flu	724	214	0.6962	2.86	4
Phe	973	310	0.5034	2.99	4.75
Ant	1989	384	0.7090	3.30	4.54
FA	1590	343	0.6829	3.20	5.22
Pyr	1401	328	0.6701	3.15	5.18
BaA	7249	774	0.8885	3.86	5.91
Chr	5634	526	0.9125	3.75	5.81[e]
BbF	11680	494	0.9807	4.07	5.8
BkF	11800	1100	0.9420	4.07	6
BaP	14710	1640	0.8997	4.17	6.04
DBA	22510	5370	0.7155	4.35	6.75
BghiP	14300	2710	0.8477	4.16	6.5
IndP	29070	2020	0.9952	4.46	6.7[f]

[a] slopes were calculated from sorption isotherms using only the linear range (up to 2500 ng/L for Nap - BbF, up to 1000 ng/L for BkF-DBA, and up to 500 ng/L for BghiP and IndP)

[b] SE = standard error (n = 10-13 for Nap-DBA, n=7 for BghiP, n=3 for IndP)

[c] R² = squared correlation coefficient

[d] literature values for log K_{ow} (source: MACKAY *et al.* 1992) if not stated elsewhere

[e] source: SCHWARZENBACH *et al.* 2003

[f] source: MEYLAN AND HOWARD 1995

4.3.3 Laboratory exposure scenarios for calibration of the Toximeter

The results shown above for chemical analysis as well as for toxicological analysis (see Chapter 3) proved the suitability of Biosilon as a receiving phase in the Toximeter. Thus the sampling behavior of the Toximeter was investigated in three different artificial exposure scenarios. The aim of these experiments was to investigate the ability of the Toximeters to predict average aqueous contaminant concentrations and to look at the PAH accumulation behavior in the Toximeters.

First approach: exposure in saturated phenanthrene solution

In order to provide constant exposure concentrations for Toximeter laboratory exposure experiments, one approach was the use of saturated solutions of phenanthrene as one model PAH. Phenanthrene added in excess should enable the permanent maintenance of saturation concentration even when losses due to sorption to the experiment vessel and uptake into the sampler deprive a partial amount. The solubility of Phe in water at 25 °C is 1.1 mg/L (MACKAY *et al.* 1992). The aqueous solubility at the experimental temperature (12 °C) was estimated to be approximately 0.5 mg/L according to MAY (1978) (cited in SHIU AND MA 2000). The measured concentrations varied around 0.37 ± 0.09 mg/L (n=12). In two cases outliers with concentrations above 1200 µg/L were detected, which were not included in the calculations.
Detectable amounts of Phe were collected in the Toximeters at all sampling times. The uptake into the sampler led to a continuous accumulation of the analyte over the whole exposure period of 34 d (fig. 4.5). The variation among sampler replicates was low with 4 % at the beginning and up to 15 % at the last sampling time. The sampled amounts were below the amounts predicted from the measured aqueous PAH concentrations. Initially nearly 100 % of the expected amount was sampled, but the ratio decreased with progressing sampling time, reaching 64 % on day 34. This can be confirmed if data are compared concerning measured and Toximeter derived aqueous concentrations (fig. 4.6).

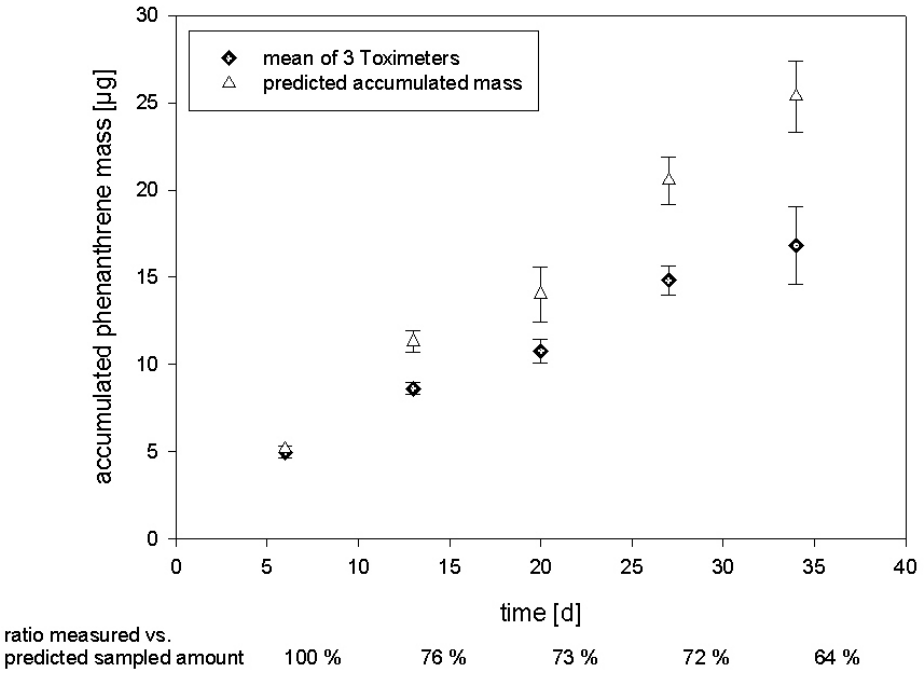

Figure 4.5 Uptake of phenanthrene from a saturated aqueous solution into the Toximeter over a 35 d exposure period. Squares represent the average accumulated masses of 3 individual replicates of Toximeters. Accumulated amounts predicted from aqueous solution concentrations were calculated using equation 3 (n=3, except for day 6, because of 2 outliers). Error bars represent the standard deviation. Per cent values at the bottom of the diagram indicate the average amount recovered in the Toximeters in comparison to the predicted amounts.

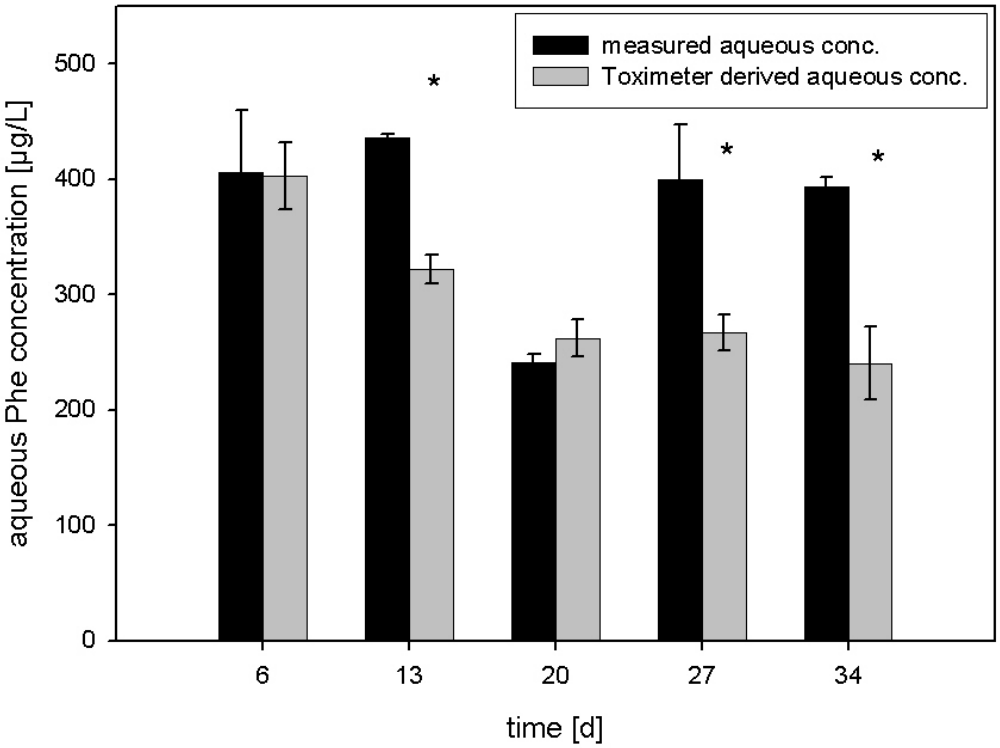

Figure 4.6 Comparison of Toximeter derived and directly determined time averaged aqueous Phe concentrations for the first exposure scenario using a saturated Phe solution. Error bars represent the standard deviation for measured aqueous Phe concentrations (black bars, n=4) and Phe concentrations predicted from Toximeters (gray bars, n=3). Asterisks indicate significant differences between measured and Toximeter derived aqueous Phe concentrations (t-test, $p<0.05$).

Second approach: semi-static, daily PAH solution exchange

In a second approach, the sampling behavior of the Toximeters concerning all 16 used PAHs was investigated. A semi-static approach was used, again to provide constant exposure concentrations, whereby the aqueous PAH solution was exchanged daily. The water samples from solution exchange were analyzed daily in the first week and weekly for the rest of the exposure. An increase in aqueous concentrations could be observed for the lower K_{ow} PAHs (Nap-Pyr) within the first 2-4 d, whereas during the remaining exposure the concentrations varied around 50 % of the nominal aqueous concentrations for Nap-Flu and around 70 % for Phe-Pyr (fig. 4.7). For the higher K_{ow} PAHs (BaA-IndP), concentrations increased over the whole period, which likely is due to sorption effects on the glass vessels and the ongoing saturation of the vessels with every water exchange. All water analysis data were used for comparison between accumulated masses in the Toximeters and expected accumulated masses based on observed aqueous PAH concentrations (fig. 4.8). In the Toximeters, quantifiable amounts were sampled after the first 7 d for Nap-BaP, and after 14 d for the remaining PAHs up to DBA. Detectable quantities were collected for all PAHs except for IndP after the 21 and 28 d exposure.

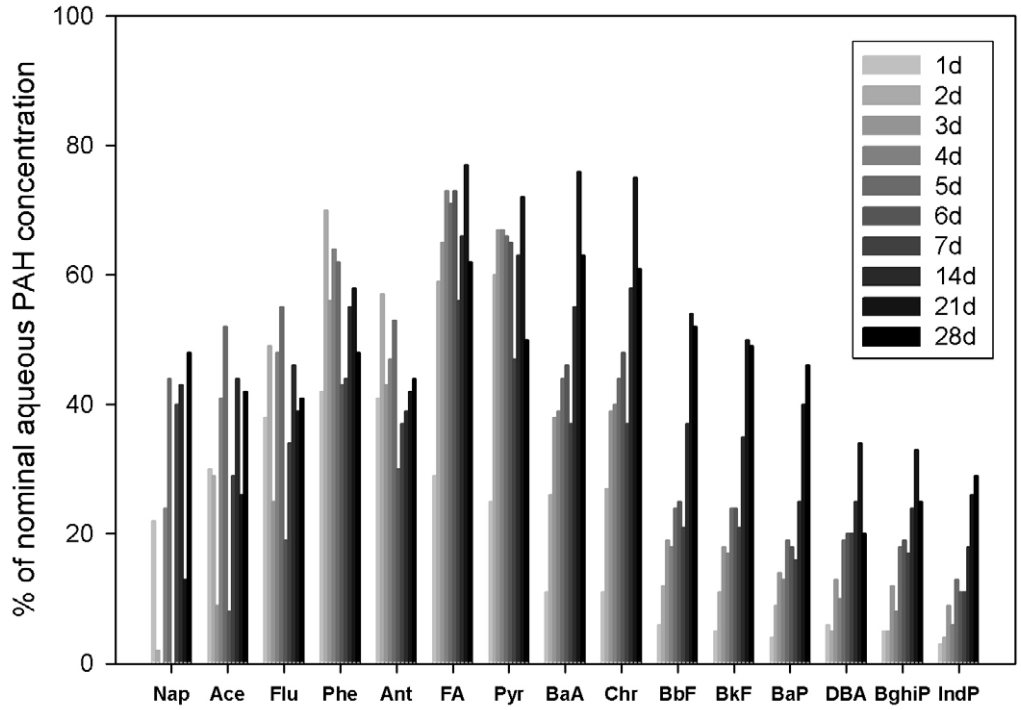

Figure 4.7 Concentrations in water samples from daily water exchange during the semi-static exposure of Toximeters. Samples were analyzed daily within the first week and weekly for the second to fourth week. Whereas aqueous concentrations remained relatively constant for PAHs up to Pyr, significant increases in concentrations were observed over time for the higher molecular weight PAHs.

The quantity of analyte collected increased continuously over the whole exposure period (fig. 4.8) with some exceptions for Nap, Flu, and Phe. Predicted and detected masses were generally in good agreement. Only for BbF-IndP were lower masses detected than expected, whereby the discrepancy between predicted and detected masses increased with increasing hydrophobicity (fig. 4.8). Good accordance was also observed when Toximeter derived aqueous PAH concentrations and average, directly determined aqueous concentrations were compared using the results from the 28 d sampling time (fig. 4.9). A significant underestimation was found only for DBA, and IndP was not detectable in the Toximeter samples over the whole period.

The variability among the Toximeter triplicates was 32 % on average. The higher variability was caused by the higher variability in the aqueous PAH concentrations between the three individual vessels (3-30 %) (fig. 4.9).

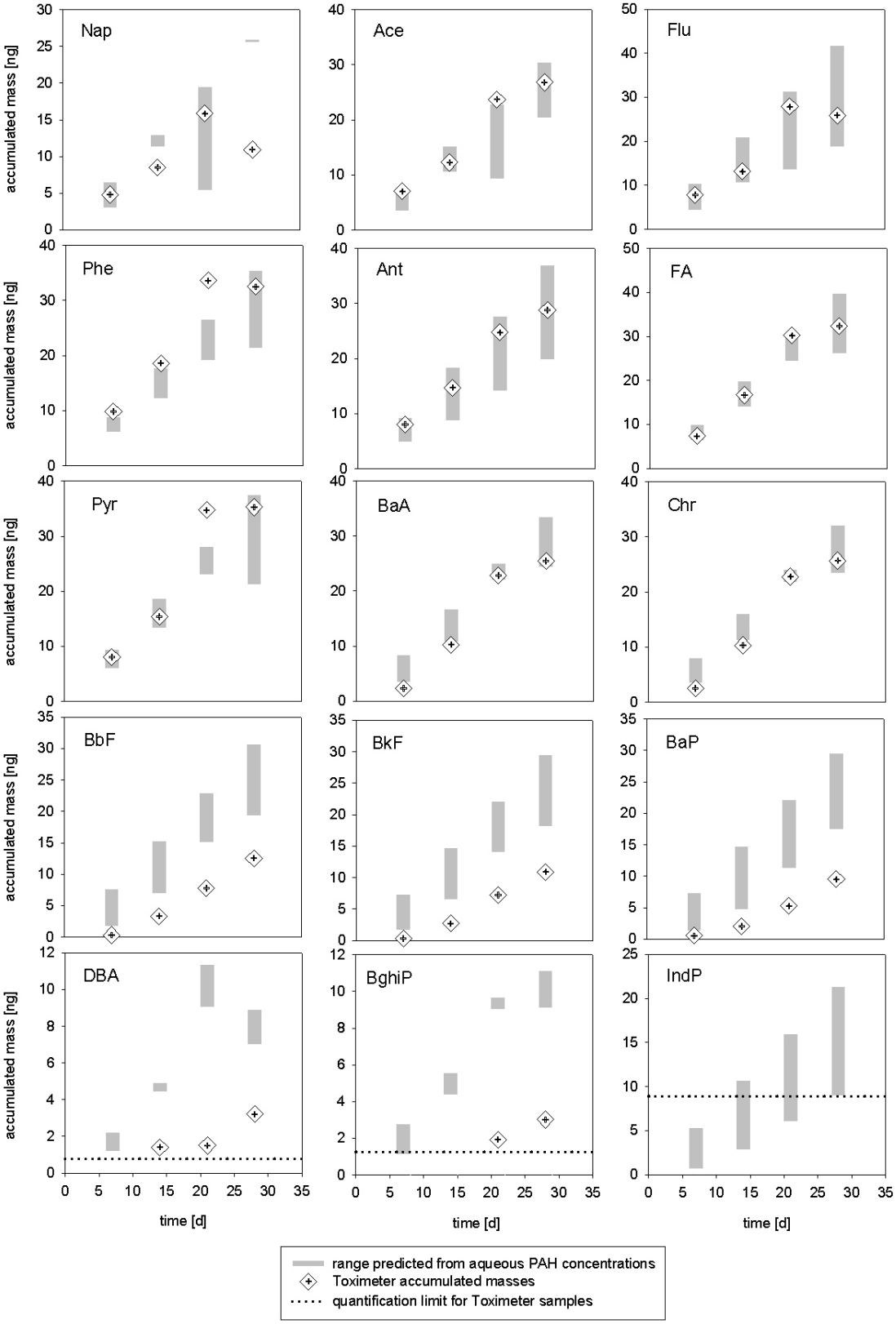

Figure 4.8 Comparison of accumulated amounts in the Toximeters and accumulated amounts predicted from aqueous PAH concentrations for each PAH over the 28 d exposure period in the semi-static exposure scenario. Squares depict the average accumulated mass from three Toximeters. Bars represent the range of accumulated masses predicted from average aqueous concentrations with initial measured concentrations as basis for upper limits and concentrations of exchanged water after 24 h for lower limits. IndP concentrations were below the quantification limit for the Toximeter samples. Acenaphthylene was also included in the PAH mixture, but can not be analyzed using HPLC / fluorescence detection.

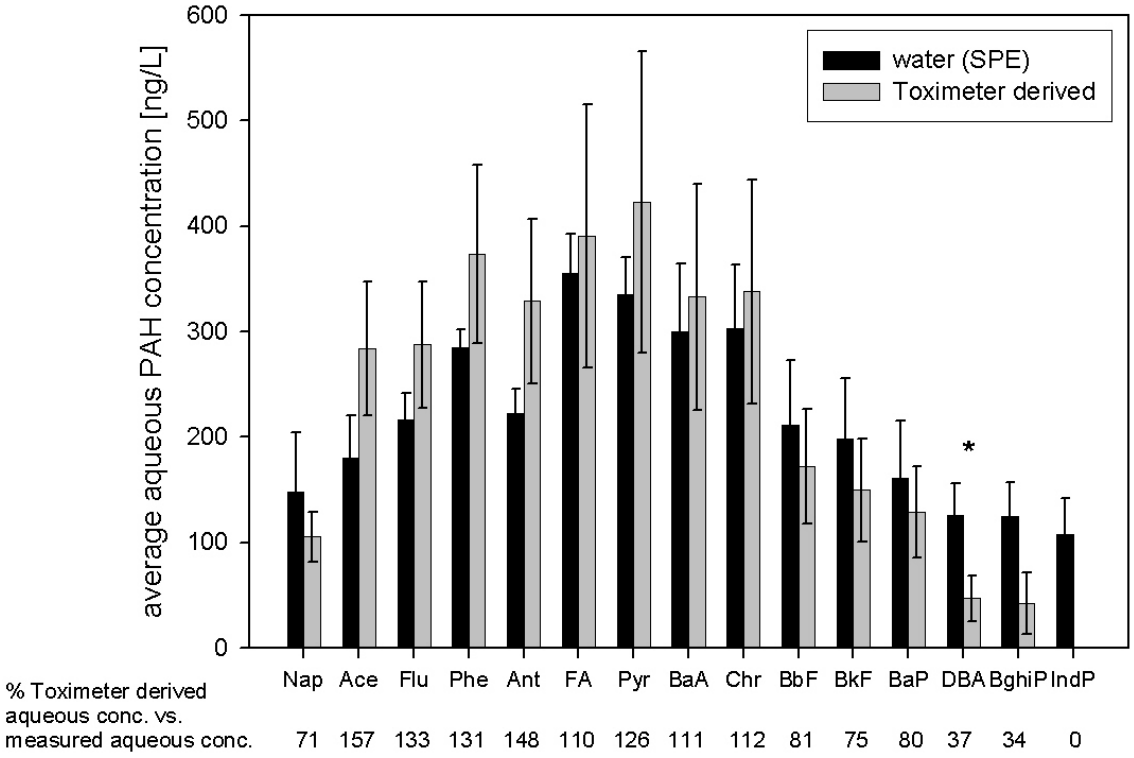

Figure 4.9 Comparison of time averaged directly determined aqueous PAH concentrations, measured during the 4 week exposure, and aqueous concentrations calculated (using equation 3) from accumulated masses in 28 d exposed Toximeters (semistatic exposure scenario). Each bar represents a triplicate of Toximeters or water samples. Error bars represent the standard deviation. Asterisks indicate significant differences between corresponding real and calculated aqueous PAH concentrations (t-test, $p<0.05$). For IndP, no quantifiable amounts were accumulated in the Toximeters over the entire sampling period. Per cent values at the bottom of the diagram indicate the average aqueous concentrations calculated from Toximeter extracts as ratio of the real aqueous PAH concentrations.

Third approach: stir bar approach

The advantage of the third approach over the second one was the more realistic PAH pattern, which was achieved by the use of the contaminated stir bars. While the concentrations for each of the 16 PAHs were similar within the second approach, here in the third approach a concentration distribution with respect to the hydrophobicity of the compounds was achieved by partitioning from the stir bars. Thus the higher K_{ow} PAHs showed much lower concentrations, which better reflects field situations, even when the absolute concentrations for these PAHs were still higher than expected in most field scenarios.

The performance of the contaminated stir bars as procedure to maintain constant aqueous PAH concentrations was examined in preliminary experiments. Deviations between individual bottles / stir bars were around 30 % and over time ranged up to 60 %, which was higher than expected in comparison to the study of GEROFKE *et al.* (2004) where PCBs were investigated.

Nevertheless, because water analyses were performed in parallel to the sampler analysis, the method was suitable for the Toximeter exposure. Water analysis from the control (bottles without Toximeters) and the Toximeter containing bottles showed remarkable differences. Water from the control bottles, which contained only Milli-Q water and a contaminated stir bar, maintained much higher PAH concentrations than water in the bottles containing the Toximeters.

Samplers were exposed in PAH contaminated water using the stir bar method for 42 d. With some exceptions concerning lower K_{ow} PAHs (above all Nap-Ant), increasing amounts of PAHs were accumulated on the Biosilon receiving phase over time (fig. 4.10). Slight decreases at 21 d and 42 d sampling time were observed, but were not statistically different from the preceding time point (Tukey's test, p=0.05). For nearly all substances, the uptake between 14 and 21 d appeared to be stagnant. This was due to very low aqueous exposure concentrations in this third week.

When masses accumulated in the Toximeters were compared to masses predicted from Toximeter bottle aqueous concentrations, a good agreement was observed for the majority of PAHs at most sampling times (fig. 4.10). The Toximeter tended to collect lower masses than expected for Nap, with significant differences for the fourth to the sixth week. For Phe-BaA, good accordance was observed, except for a significantly higher collected mass than predicted for the 7 d sampling time. For BaP a significantly lower mass was detected at day 42. For DBA and BghiP, the sampler quantification limit was reached only at day 42 and for IndP it was not achieved at all (fig. 4.10).

In fig. 4.11, the average aqueous PAH concentrations over the whole exposure period (based on the weekly samples from the Toximeter exposure bottles) were compared to the concentrations, calculated from the Toximeter sampled amounts, which is the common interpretation situation for field deployed samplers. Nap showed a significantly underestimated aqueous concentration from the Toximeter results. For the other PAHs, a good accordance was observed, except for IndP, where the Toximeter quantification limit was not reached.

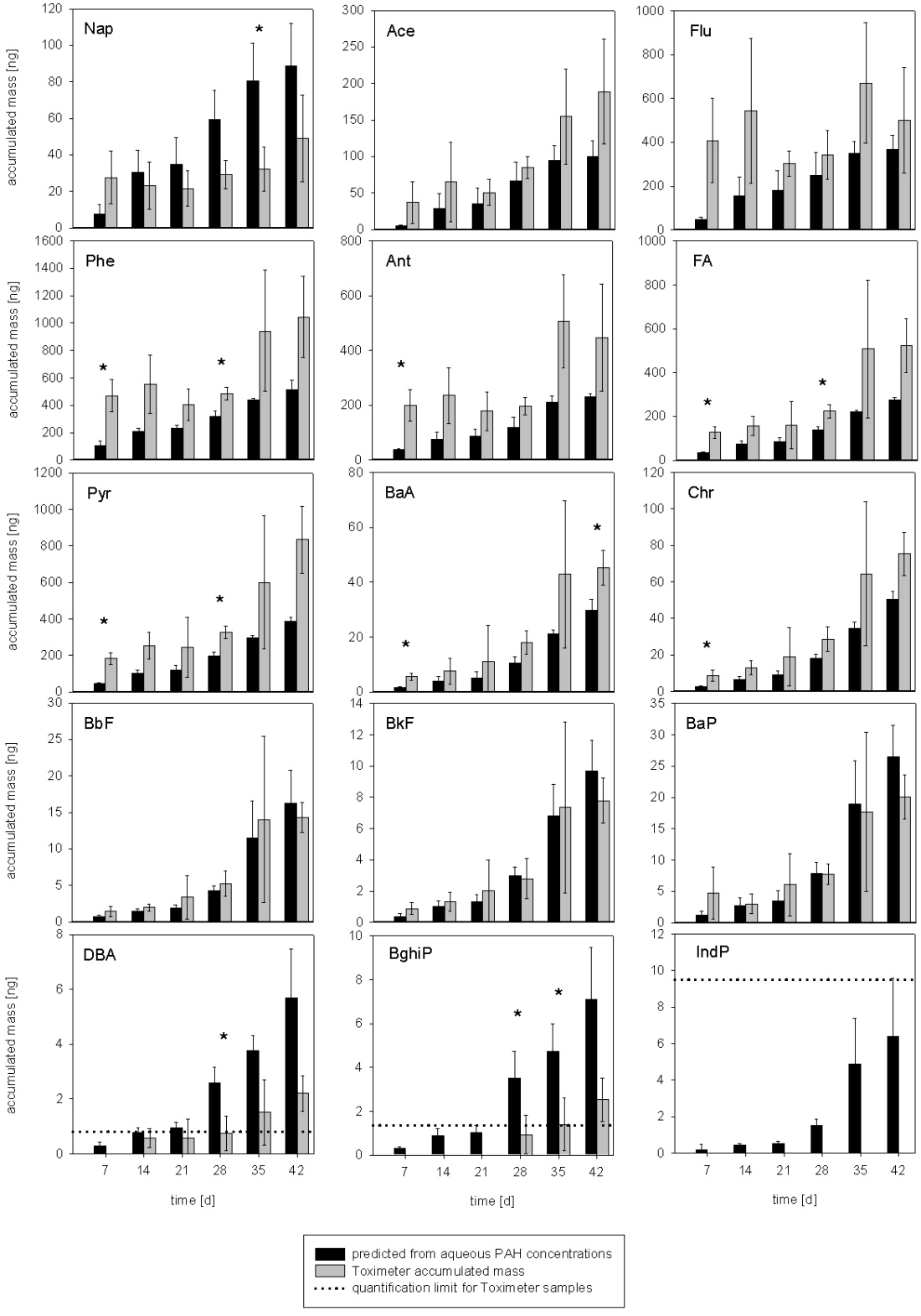

Figure 4.10 Comparison of predicted and measured accumulated amounts in the Toximeters for each PAH over the 42 d exposure period in the contaminated stir-bar exposure scenario. Black bars represent predicted accumulated PAH masses from measured aqueous PAH concentrations in the Toximeter bottles. Gray bars depict the average accumulated masses from three Toximeters. For IndP, no quantifiable amounts were accumulated in the Toximeters. Error bars represent the standard deviation (n=3); asterisks indicate significant differences between corresponding predicted and measured accumulated masses (t-test, $p<0.05$).

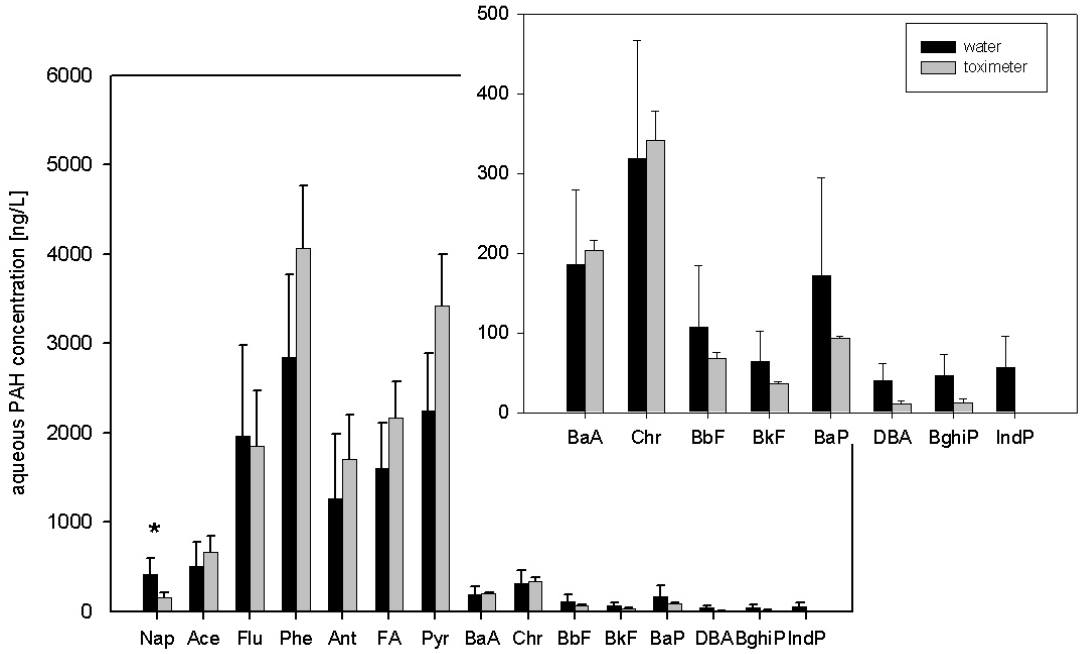

Figure 4.11 Comparison of real and Toximeter-calculated aqueous PAH concentrations. The insert shows a magnification for the lower concentrated PAHs BaA-IndP. Black bars represent the average concentration over the whole exposure time, integrating the concentrations determined at each time point (42 d). Gray bars represent the aqueous PAH concentrations calculated from amounts accumulated in Toximeters after 42 d (using equation 3). Error bars represent the standard deviation (n=3). Asterisks indicate significant differences between corresponding real and calculated aqueous PAH concentrations (t-test, $p<0.05$). IndP did not reach quantifiable amounts in the Toximeters.

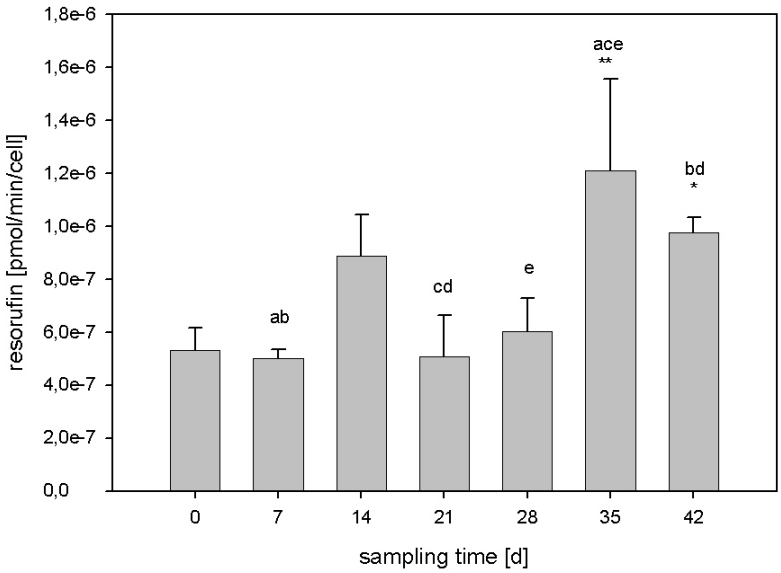

Figure 4.12 EROD induction by PAHs sorbed to Biosilon within the stir-bar exposure scenario. Time point zero represents the control / untreated Biosilon, like it is used in the Toximeters (n=4). For all other points in time, Biosilon from three replicate samplers was used for fish cell exposure. Asterisks indicate significant induction compared to day 0 (Dunnett's test, * $p<0.05$, ** $p<0.01$). Bars labeled with the same characters are significantly differing from each other (Tukey's test, $p<0.05$). Error bars represent the standard deviation.

In parallel to the chemical analysis, Biosilon from the Toximeters after each sampling period was investigated in the EROD assay. For the time points 7-21 d no significant enzyme induction could be found (fig. 4.12). After 35 and 42 d sampling period a slight induction (significant compared to the control, Dunnett's test, $p<0.05$) was observed. Thus, accumulation of EROD inducing compounds to levels detectable in this bioassay was observed, even if the induction magnitude was still small after six weeks of sampling.

Accumulation in caps and tubes

In order to investigate whether tubes and caps acted as sinks for PAHs, tubes and caps were extracted from Toximeters exposed in the first and second exposure scenario. The amounts extracted from both compartments of the sampling device showed high variability. Especially higher PAHs (BaA–IndP) were detected in both the Teflon caps and the ceramic tubes. For the ceramic tubes Nap-Flu were not detectable, whereas Phe-Pyr showed accumulated amounts corresponding to up to 10 % of the amount accumulated on Biosilon in the device. For BaA-IndP, higher amounts up to 10 times the mass sorbed to Biosilon were detected. No tendency over time was identifiable for the tubes. For Nap-Pyr about half of the mass detected in the tubes could be explained by the pores filled with surrounding water. For BaA-IndP only a small part (0.1 – 7 %) could be explained in that way. For the Teflon caps, 10 to 30 % of the amounts collected on Biosilon were detected for Nap-Pyr. For BaA-IndP, amounts up to the 20 times the mass collected in the Toximeter were analyzed. For the caps, a trend of increasing PAH levels over time was observed especially for BaA–IndP, but this trend needs further investigations to be confirmed. There is evidence that the Teflon caps tend to accumulate PAHs with sampling time, which causes difficulties during laboratory exposure experiments due to a limited amount of contaminant. In field situations, however, the accumulation of PAHs in the tube and caps of the sampling device should be a minor problem because of the continuous delivery of sampling medium, provided that the sampler accumulation does not exceed delivery.

4.4 Discussion

4.4.1 Biosilon as a receiving phase for PAHs

Biosilon meets all the basic requirements set forth for the use in the Toximeter. This was shown in Chapter 3 for the compatibility with the biological analysis and in this chapter for its suitability as a sorbent material for PAHs and for simple solvent extraction preceding chemical analysis.

Biosilon beads are made of polystyrene and thus compatible only with a small number of solvents, namely e.g. methanol, ethanol and hexane. When methanol and ethanol were

compared concerning their extraction efficiencies for PAHs on Biosilon, methanol showed higher recoveries. Thus, based on the extraction efficiency and compatibility with HPLC analysis of methanol, a simple extraction method using this solvent was established as standard extraction protocol. This standard procedure worked sufficiently well for all PAHs except for Nap and Flu, which showed lower extraction efficiencies. Especially for Nap this might have been caused by volatilization during sample handling.

Sorption of PAHs onto Biosilon occurred rapidly. Similar results have been reported for sorption of FA to cell culture plates made of polystyrene (SCHIRMER *et al.* 1997) and for sorption of PAHs to Amberlite IRA-743, which is used as the sorbent in the Ceramic Dosimeter for PAH sampling (GRATHWOHL *et al.* 2001). Most of the tested PAHs had a high sorption affinity to Biosilon. The partition coefficients K_{bw} correlated well with K_{ow} values for all compounds. Thus, in the future, K_{bw} values can be estimated for other PAH compounds based on their K_{ow}. For this, a more accurate determination of K_{bw} values might be necessary, using more detailed sorption isotherms, which could be achieved in distribution experiments, applying a range of aqueous PAH concentrations being spaced closely together. For Biosilon, the partition coefficients are between one and two orders of magnitude lower than K_{ow} values. Thus they are lower than for other receiving phases used in passive samplers. For example, for the triolein used in SPMDs, log K_{tw} values for several hydrophobic organic compounds are close to log K_{ow} values (CHIOU 1985). For Amberlite IRA-743, partition coefficients K_p are reported to be 100 000 for Phe and 10 000 for Ace (GRATHWOHL *et al.* 2001), which is also about two orders of magnitude higher than the K_{bw} values. Despite this lower sorption affinity of PAHs to Biosilon, it appears sufficiently effective in providing a sink within the Toximeter for waterborne PAHs. By binding PAHs rapidly, aqueous concentrations of those PAHs in the inner part of the Toximeter can generally be assumed to be kept close to zero. This is particularly true for the less hydrophilic, higher molecular weight PAHs. For the substances Nap-Flu, which tend to sorb to Biosilon to a lesser extent, Biosilon might be a suboptimal choice.

4.4.2 Laboratory exposure scenarios for calibration of the Toximeter

Biosilon was filled into the ceramic tubes and exposed to aqueous PAH solutions in a defined laboratory setting in order to confirm the suitability of the Toximeter design for time-integrated sampling of organic contaminants. The overall goal was to identify if, after time-integrated sampling, PAH concentrations obtained from Biosilon extraction and applied to equation 3 were predictive of the average aqueous concentrations of the PAHs during the sampling period. The underlying approach was to expose the Toximeter to water containing stable aqueous PAH concentrations. In order to keep toxic waste to a minimum, a static or semi-static setting was chosen.

In all laboratory experiments it is a great challenge to develop systems to maintain constant aqueous concentrations for hydrophobic, sparingly soluble PAHs. Therefore three different approaches were chosen for laboratory calibration of the Toximeter. Of these three approaches the first one, which used a saturated Phe solution by adding Phe crystals to water, was the most suitable concerning a constant aqueous concentration, but this approach is not applicable to high molecular PAHs because these do not dissolve sufficiently from crystals. Overall, the greatest difficulty in all three approaches was in determining the true average aqueous PAH concentration for comparison to the concentrations predicted by the Toximeter. Despite yielding the best results regarding a constant aqueous exposure, Phe concentrations still varied up to 25 % over the course of the experiment in the first approach. In the second approach using daily water exchange, BaA-IndP tended to increase over time in their aqueous concentrations. Likely, this is due to a continuous saturation of test vessels and Teflon caps of the Toximeters. This phenomenon is reminiscent of observations made by others using static-renewal water exposure systems (e.g. LANDRUM *et al.* 2003). For the third approach using contaminated stir bars, PAH concentrations varied by up to 60 % in the same exposure bottle. However, larger differences were detected between bottles containing Toximeters and bottles containing only a stir bar. The lower aqueous PAH concentrations in Toximeter containing bottles might have been caused by depletion of aqueous concentrations due to sorption to the Toximeter materials. It was shown in the second approach, where aqueous solutions were exchanged daily, that PAH concentrations strongly decreased within a 24 h period in the presence of Toximeters. Thus the reason for the lower aqueous PAH concentrations in Toximeter containing bottles in contrast to the control bottles without Toximeters in the third approach might have been a kinetic problem. The release of PAHs from the stir bars to the solution was slower than the transfer into the Teflon caps and the tubes of the Toximeters, so that losses were not compensated sufficiently fast. Taken together, the difficulty in obtaining constant aqueous concentrations hampered the derivation of accurate average aqueous concentrations for comparison with the concentrations predicted by the Toximeter. However, it was possible to investigate whether PAH concentrations predicted by the Toximeter were within the range of aqueous concentrations ascertained within the limits of each of the three exposure methods.

In the experiment using Phe-saturated water, the Toximeter appeared to significantly underestimate the true aqueous concentrations. One cause for this underestimation might be the relatively low K_{bw} value for Phe. It is possible that the aqueous Phe concentration in the inner part of the device will not be negligible, which is normally assumed for calculations (see equation 3). If, based on the K_{bw} for Phe, an inner aqueous Phe concentration is assumed and the gradient between the outside and the inside of the Toximeter adjusted accordingly, it can be calculated that an insufficient binding by the Biosilon would result in an uptake of about 10 % less compared to complete binding by the Biosilon and zero aqueous Phe concentration in the inner part of the device. This is still insufficient for explaining the high discrepancy observed between measured and predicted Phe concentrations in the water, however. Another aspect

potentially contributing to underestimation is the inhomogeneous PAH-coating of the sorbent filling in the Toximeter. The Biosilon is tidily packed so that equilibration effects may have occurred probably for the Biosilon adjacent to the ceramic tube leading to longer diffusion pathways to the inner Biosilon beads. Thus, the uptake into the device may have no longer been in the range which can be approximated by the linear relationship assumed by the standard calculations in equation 3. This hypothesis for explaining underestimation was supported by the increasing discrepancy between predicted and measured accumulated masses over time.

Exposure of the Toximeter in the semi-static or the stir-bar approach confirmed a continuous, time-dependent uptake of most PAHs with the exception of Nap and of DBA-IndP, the latter of which remained at or below their level of detection in the Toximeter over the laboratory exposure periods. For both approaches, the Toximeter derived aqueous PAH concentrations well agreed with the actually measured aqueous concentrations for most PAHs with the best fit being found for the PAHs Ace-Chr. In some instances, such as for Flu, Phe and FA in the semi-static exposure scenario on day 28, a stagnation of uptake appeared to occur which was attributed to the same phenomena as discussed above for the first exposure scenario with Phe. Another aspect, which might explain the underestimation of larger PAHs (log K_{ow}>6) by the Toximeter, is the preferential accumulation of these compounds in the Teflon caps and retardation in the ceramic tube, leading to a reduced uptake into the Toximeter.

Taken together, the Toximeter using Biosilon as a receiving phase proofed capable of effectively accumulating PAHs, which was additionally confirmed in the stir-bar approach by the measurement of EROD induction as a biological means of detecting PAHs (see also Chapter 3). In its current design, the Toximeter can be optimally applied for compounds with a log K_{ow} between 4.5 and 6.

4.5 Summary

It can be concluded that the Toximeter, containing Biosilon as a receiving phase, enables the determination of aqueous concentrations for a wide range of PAHs. Limitations must be conceded for Nap and the high molecular weight PAHs DBA-IndP. The fact that caps deprive contaminants from the aqueous solution is an important aspect in laboratory experiments using static or semistatic systems. It should be of lower relevance for field applications, however, due to the large reservoir available for sampling and continuous delivery of new sampling medium. Thus, upon confirming the usefulness of the Toximeter for combined chemical and toxicological long-term monitoring, a field experiment was carried out in order to investigate the sampling behavior of the Toximeter under real field conditions (Chapter 5).

Chapter 5

Toximeter field deployment

5.1 Introduction

The Toximeter is a passive sampling device that links time-integrated sampling of organic contaminants with subsequent chemical and / or biological analysis of sampled analytes. The Toximeter is the first of its kind that was designed specifically for a combined chemical and biological evaluation of passively collected contaminants, thereby the biological analysis is performed without any solvent extraction. The development of the Toximeter was described in Chapters 3 and 4 with respect to Biosilon as a solid receiving phase and a ceramic tube as the diffusion barrier and container for the Biosilon. All these previous steps were done in the laboratory under well defined exposure conditions. Thus, it was the final goal of this thesis to explore the performance of the Toximeter under field conditions.

The behavior of passive sampling devices in the field can vary greatly due to the more complex exposure conditions. Possible factors affecting sampling behavior in water may generally include temperature (diffusion coefficients of the contaminants are temperature dependent), changing water flow regimes and velocities, the formation of biofilms and biodegradation of sampler membranes or sampled analytes (HUCKINS *et al.* 2002). As well, for time-integrative samplers it is important to adjust exposure periods such that contaminant uptake does not reach the non-linear region of the analyte uptake curve because time averaged aqueous concentrations are then difficult to calculate reliably and contaminant concentrations can be underestimated. On the other hand, simplicity and sturdiness are important pre-requisites for a successful implementation of a sampling device in routine environmental monitoring. No previous study has explored the durability and long-term behavior of Biosilon as a receiving phase in a passive sampler in the field. However, the ceramic membrane applied in the Toximeter indeed appears robust toward a number of environmental exposure conditions. Based on its reported use in the Ceramic Dosimeter (MARTIN *et al.* 2003), it did not seem to support microbial growth on its surface during a three-month exposure in groundwater and it is not dependent on water flow velocity. Another advantage of the ceramic tube is its inner small pore-size, which does not allow microorganisms to enter the inner part of the tube. This is important for both the Dosimeter and the Toximeter in terms of preventing biodegradation of sampled contaminants. However, this feature of the ceramic membrane is particularly important in the Toximeter

because the Biosilon needs to be kept free of microorganisms so that it can serve, after sampling, as the growth surface of vertebrate cells in a sterile cell culture environment (Chapter 3).

Thus for field evaluation, 45 Toximeters were deployed in groundwater over a six month period at a strongly PAH contaminated gas works site in Southern Germany in three groundwater wells. The aim was to assess the field applicability of the Toximeter for chemical and biological long term-monitoring of groundwater. In order to provide a comparison to an already field validated system, 45 Ceramic Dosimeters were deployed in parallel. Results from monthly collected passive samplers were compared to results from snap-shot water samples taken every second week.

5.2 Materials and Methods

5.2.1 Sampling site

Sampling was performed at a gas works site in Southern Germany (Baden-Wuerttemberg). Results from previous snap-shot sampling campaigns indicated a high contamination with PAHs but no unusual aquifer attributes. Three different boreholes were chosen for sampling, with two of them being situated within 40 m of one another (boreholes 2 and 3) and the third being maximally 175 m distant (borehole 1). Groundwater flow velocity was about 2.4 m/d (personal communication Dr. Hansjörg Weiß). Groundwater levels were 3.5 and 5.5 m below surface corresponding to 217.6-217.8 m above sea level. Water temperature was determined in parallel to sampling activities because of its influence on contaminant uptake into passive sampling devices.

5.2.2 Passive sampler preparation

Toximeter: Toximeter sampling devices were prepared in exactly the same way as described in Chapter 4. Complete samplers were stored in sterile Milli-Q water in 1 L amber glass bottles at room temperature until field deployment.

Dosimeter: Ceramic Dosimeters were prepared by imw (Innovative Measurement Technologies Weiss, Tübingen, Germany). The diffusion barrier in the Ceramic Dosimeter is the same tube as the one used in the Toximeters (see also Chapter 4). The receiving sorbent material in the Ceramic Dosimeter is Amberlite IRA-743 (Sigma Aldrich, Steinheim, Germany). This is an ion exchange resin on polystyrene basis, which was chosen due to its capacity for binding hydrophobic contaminants and its wettability, which is of importance for use in a water saturated sampling device (MARTIN *et al.* 2001). Ceramic tubes were filled with Amberlite IRA-

743 and Milli-Q water, then closed with Teflon caps and clamped into stainless steel holders. They were submerged in water containing bottles and evacuated in an exsiccator. Prepared Ceramic Dosimeters were stored under Milli-Q water until deployment.

5.2.3 Passive sampler field deployment

Prepared samplers were transported to the field site stored in amber glass bottles filled with Milli-Q water. For each sampling time and borehole, a string containing three Toximeters alternating with three Ceramic Dosimeters was prepared. The samplers were tied to a nylon string using separate nooses. The length of the string and the place where the samplers were attached was chosen such that the samplers could be placed in the middle of the screened portion of each borehole in 6 – 8 m depth. Five strings were hung in each of the 3 boreholes so that triplicates of each sampler type could be collected from each well at five sampling times (1, 2, 3, 4, and 6 months).

Parallel to the deployment of the passive samplers, mini-pressure pumps were installed in the three boreholes in order to allow for regular snap-shot water sampling. The mini-pressure pumps are positive displacement mini gas lift pumps for low flow sampling, which keeps disturbances of the natural groundwater flow regime to a minimum.

5.2.4 Sampling

Snap-shot water sampling

Snap-shot water samples were collected every second week in duplicate. They were taken before passive sampling devices were removed from the well, in order to prevent mixing of water in the borehole. 700 mL of water were sampled, using the mini-pressure pumps, into 1 L amber glass bottles which already contained 10 mL of cyclohexane and 10 µL of internal standard for later extraction. The exact water sample volumes were determined gravimetrically. Water samples were processed directly upon arrival in the laboratory by shaking for 1 h for extraction with the cyclohexane phase. 1.5 mL of the cyclohexane phase was then transferred into GC-vials and directly injected into the GC / MS system within 24 h (see below).

Removal of passive samplers

After 1, 2, 3, 4, and 6 months of exposure, three replicate samplers each of the Toximeter and the Ceramic Dosimeter were removed from the boreholes. Samplers were wrapped in tissue soaked in groundwater and packed in zip plastic bags. They were shipped to the laboratory and extracted within 3 days. Sampling was performed by imw.

5.2.5 Processing of passive sampler after field deployment

Toximeter

Upon arrival after field exposure, Toximeters were stored at 4 °C and processed within 3 d. They were firstly investigated concerning visible changes to the sampler surface. Then they were opened for analysis. Chemical and biological analyses were performed according to the details given in Chapters 3 and 4. Methanol extracts from field deployed Toximeters were split into two portions. One portion was analyzed using HPLC / fluorescence detection at the UFZ. The second portion was analyzed in parallel by GC / MS at the University of Tübingen. For GC / MS, 10 µL of internal standard were added to 1.2 mL of the methanol extract. The internal standard contained 198 µg/mL deuterated PAHs (Nap-D8, Ace-D10, Phe-D10, Chr-D12 and perylene-D12, prepared using PAH Mix 31, Dr. Ehrenstorfer, Augsburg, Germany).

Dosimeter

Analysis of Ceramic Dosimeter samples was performed at the University of Tübingen through imw according to PIEPENBRINK (1998). In brief, Amberlite IRA-743 was transferred from the Ceramic Dosimeters to stainless steel cylinders, coupled to a vacuum manifold. The sorbent was extracted three times with 10 mL of acetone with 10 min contact time for each extraction step. 10 µL of internal standard were added to 5 mL of the combined acetone extract for direct GC / MS analysis. The combined acetone extract contained less than 5 % water from the Ceramic Dosimeter filling. It had no adverse effects on the reproducibility of analytical measurements.

5.2.6 Instrumental analysis

HPLC analysis was performed as described in Chapters 3 and 4. In parallel, samples were analyzed using GC / MS at the University of Tübingen. Thus also 1- and 2-methyl-naphthalene (1-MNap, 2-MNap) and acenaphthylene (Any) could be detected. GC / MS analysis details are described in GRIEBLER *et al.* (2004) and GRATHWOHL *et al.* (2001). In brief, an HP GC 5890 Series II was used in combination with HP MS 5972 A for analyses of water sample, Ceramic Dosimeter and Toximeter extracts. The GC-column for separation of PAHs was a Zebron ZB-5 MS (30 m x 0.25 mm I.D. x 0.25 µm film thickness; Phenomenex, Aschaffenburg, Germany). For quantification of the analysis results, the internal standard with deuterated PAHs was used.

5.3 Results

5.3.1 Visible changes of passive samplers after field exposure

Toximeters and Ceramic Dosimeters derived from borehole 2 showed compelling discolorations, ranging from yellow-reddish to a dark black color. Samplers obtained from boreholes 1 and 3 only showed an occasional slight grayish discoloration (fig. 5.1). Discolorations were visible from the first sampling point (1 month) on. Smeary films or biofilms were not observed for any of the samplers.

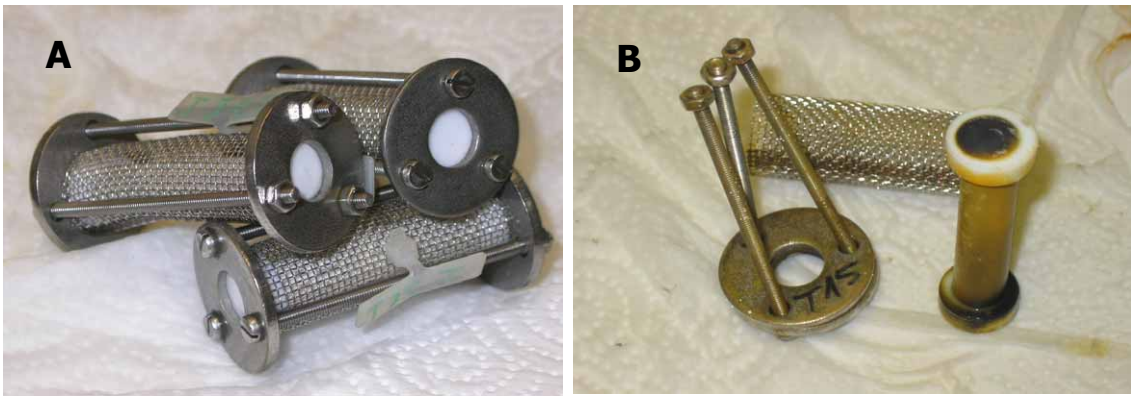

Figure 5.1 Exemplary photographs of Toximeters taken out of borehole 3 (A) and borehole 2 (B) after 3 months of exposure.

5.3.2 Toximeter samples

The aim of applying the Toximeter passive sampler was to provide a simple and cost-effective method in order to firstly predict average aqueous contaminant concentrations over extended exposure periods and secondly investigate the presence of toxicologically relevant compounds. Thus in a first step, Toximeter-derived extracts as well as snap-shot water samples were analyzed chemically. The PAH amounts determined in the Toximeters were then used to predict average aqueous concentrations and compared to the snap-shot sample PAH concentrations actually measured. Finally, Biosilon samples from field exposed Toximeters were subjected to biological analysis, which in turn was used to compare observed versus predicted biological responses based on the PAH content detected by the Toximeter.

Chemical analysis

Chemical analysis of Biosilon from field deployed Toximeters was performed using HPLC / fluorescence detection and GC / MS in parallel. Analysis by fluorescence detection was restricted to identification of compounds based on their retention time. Here, some difficulties arose from overlying peaks in field samples, particularly for Ace and Flu. In contrast, GC / MS analysis allowed a reliable identification of all peaks. Thus, only GC / MS data were used for

further evaluation. This was also preferred because of the comparability to water analysis and Ceramic Dosimeter data which were analyzed in the same GC / MS system.

Prediction of average aqueous PAH concentrations by the Toximeter

Toximeter derived PAH concentrations were compared to aqueous PAH concentrations determined by snap-shot sampling. Calculation of PAH concentrations from Toximeter samples was done according to equation 3 (Chapter 4) using diffusion coefficients calculated at groundwater temperature (13.5 °C) according to Hayduk & Laudie (described in BAUM 1998) using water viscosity from SCHWARZENBACH *et al.* (2003) for the appropriate temperature and molar volume values (LeBas) from MACKAY *et al.* (1992).

For all three boreholes investigated, Toximeters tended to underestimate aqueous PAH concentrations by a median factor of 4 (fig. 5.2). Observations for single compounds (fig. 5.3 - 5.5) showed that Toximeter derived aqueous PAH concentrations were much lower than snap-shot sample determined concentrations for Nap and M-Nap (average factor of 46; range 6-170). For Any-Flu, Toximeter derived concentrations were about a factor of 7 (range 2-20) lower than snap-shot sample concentrations. For Phe-Pyr underestimation significantly lessened (factor 1.9; range 0.9-4), and for BaA and Chr, Toximeter derived concentrations approached true aqueous concentrations (1.4; range 1.2-2.3). A tendency of increasing underestimation over prolonged exposure periods was evident for Nap-Phe, especially in boreholes 2 and 3.

Snap-shot sample aqueous PAH concentrations were fairly constant over time with deviations by a factor of 1.5 – 2, except for Nap and 2-MNap in borehole 2 and 3, where deviations up to a factor of 20 occurred (fig. 5.3 - 5.5). Duplicate water samples, taken sequentially from the same borehole, showed similar results with a median coefficient of variance of 2 % (the maximum deviation found was 14 %). Snap-shot sample PAH concentrations can be found in the appendix, tab. A2-A4.

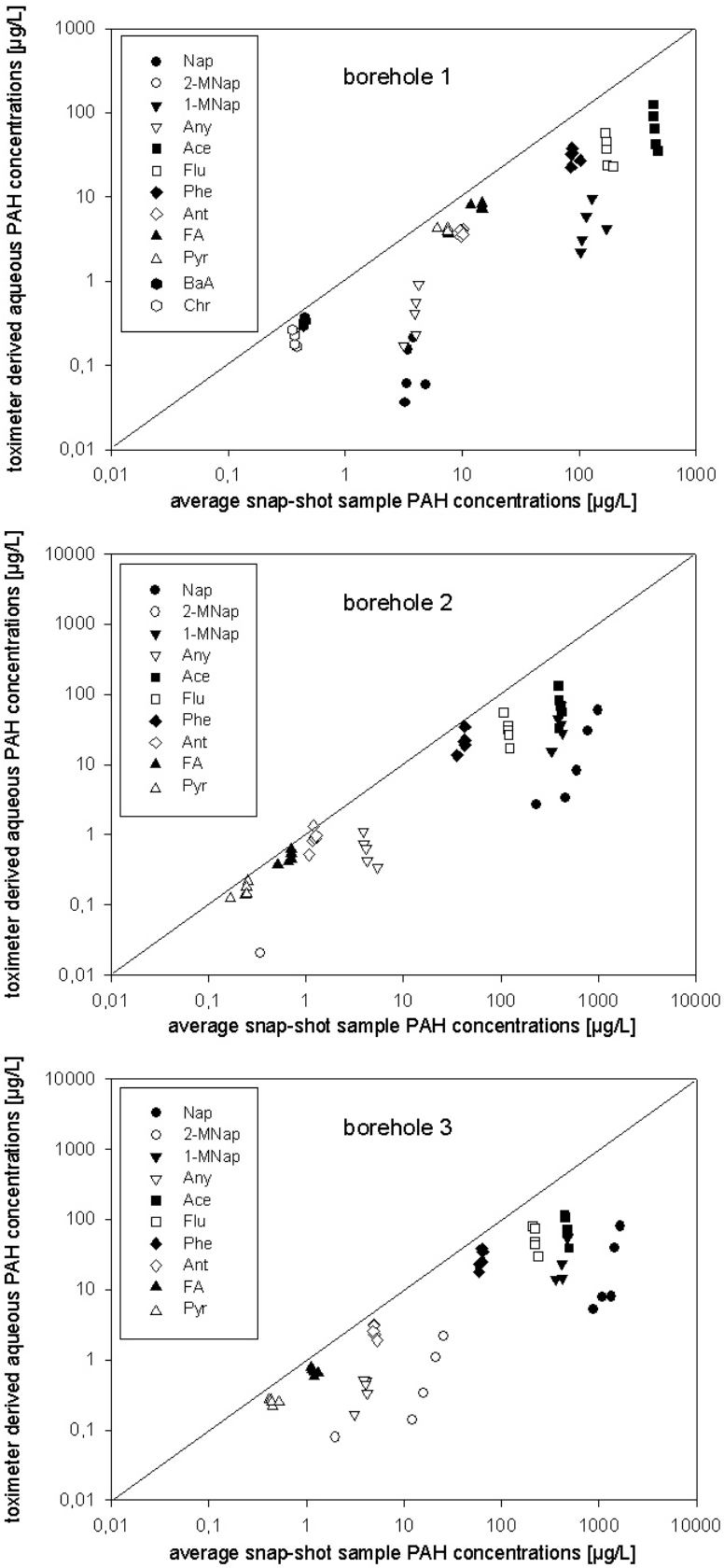

Figure 5.2 Comparison of Toximeter derived and snap-shot sample determined aqueous PAH concentrations. For absolute agreement between the two ways of determining aqueous concentrations, symbols should be located on the diagonal line.

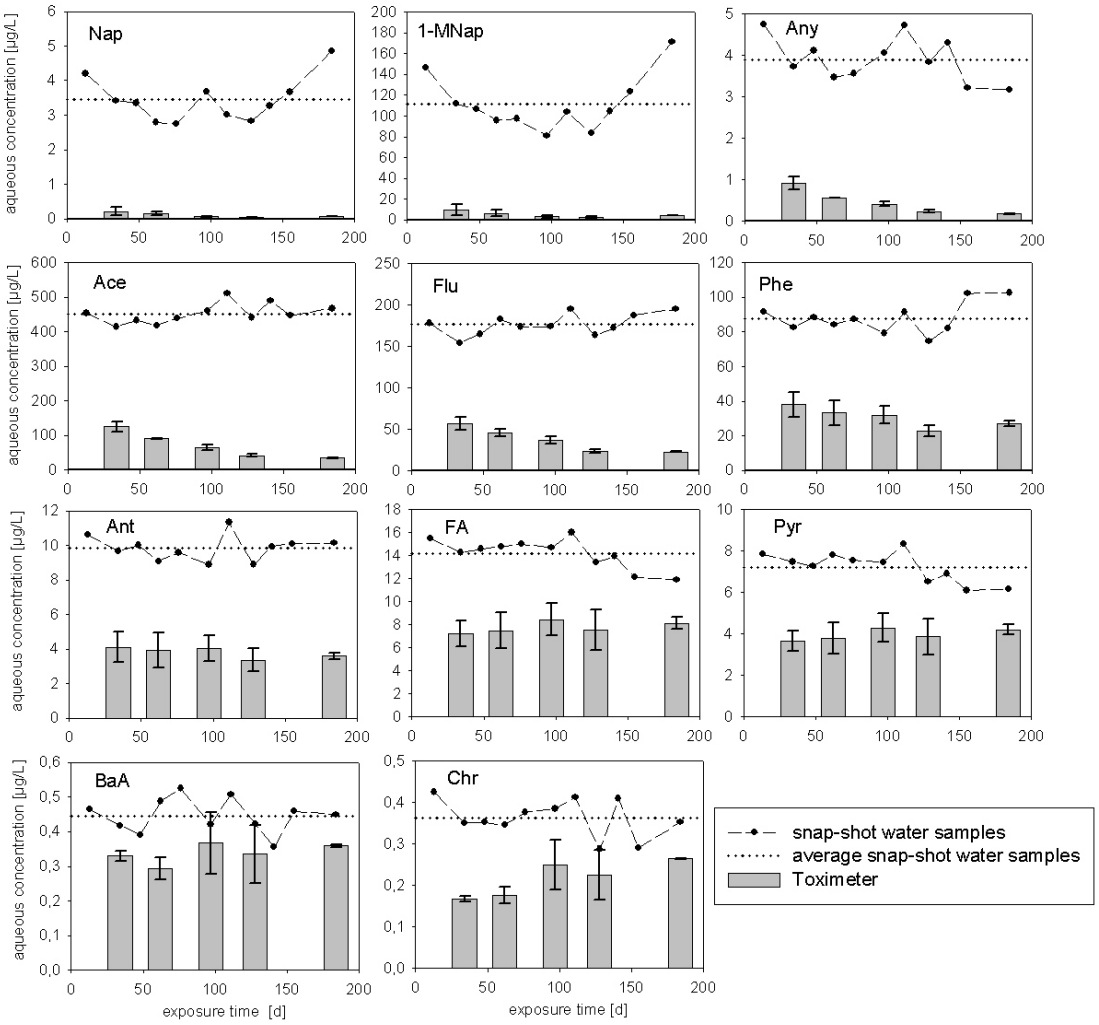

Figure 5.3 Comparison of Toximeter derived average aqueous concentrations and snap-shot sample concentrations for each quantifiable PAH in borehole 1. Toximeter bars represent the mean of three Toximeters with vertical lines indicating the standard deviation. For snap-shot water samples, results from the first sample taken were used, as the deviation between duplicates sample was negligible.

For more detailed interpretation of these results, the accumulation of PAHs in the Toximeter is presented in fig 5.6. For the strongly underestimated PAHs Nap-Any, no continuous, time-dependent accumulation was found. Accumulated masses for these substances declined over time or deviated around a certain mass. With the exception of a few cases, accumulated masses increased continuously for all other PAHs with FA-Chr yielding the most uniform accumulation results. The few exceptions concerned the PAHs Ace-Ant, where seemingly random individual time points did not follow the pattern of a steady accumulation of mass.

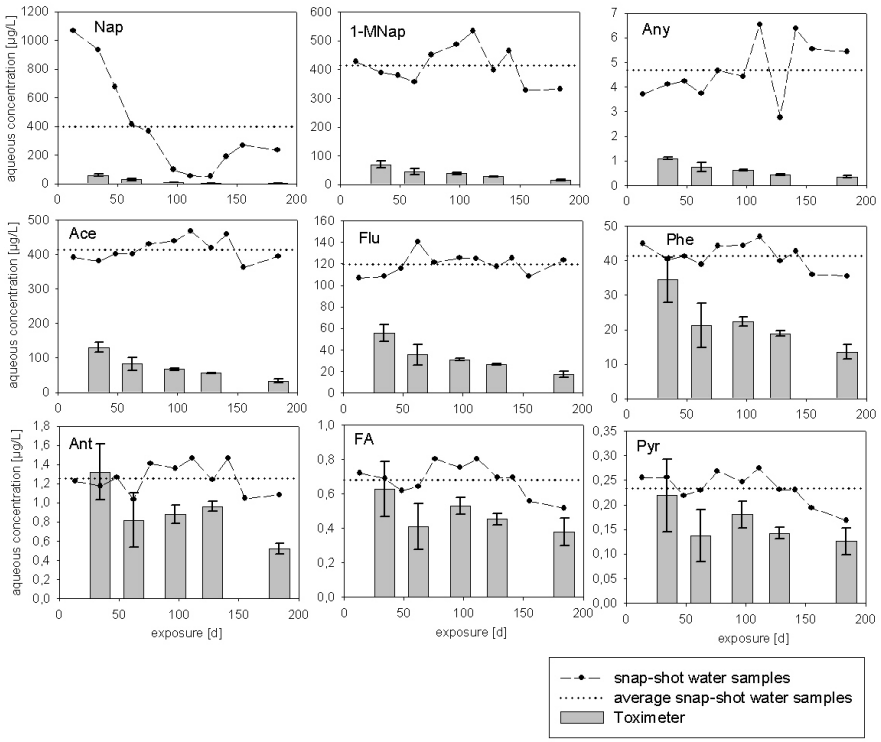

Figure 5.4 Comparison of Toximeter derived average aqueous concentrations and snap-shot sample concentrations for each quantifiable PAH in borehole 2. Toximeter bars represent the mean of three Toximeters with vertical lines indicating the standard deviation. For snap-shot water samples, results from the first sample taken were used, as the deviation between duplicates sample was negligible.

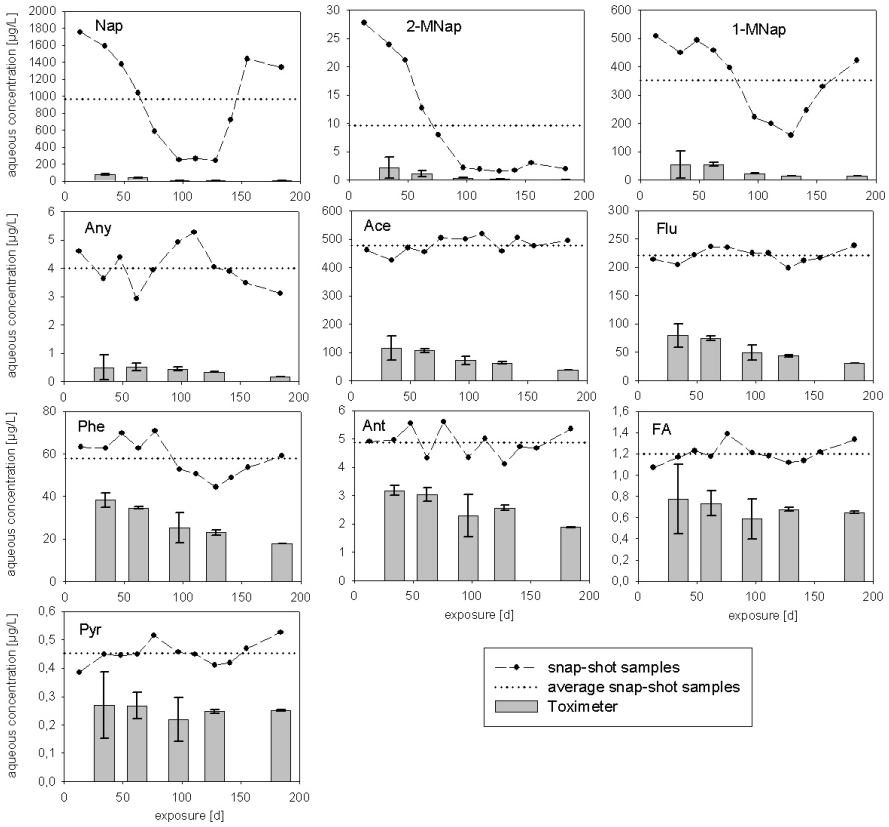

Figure 5.5 Comparison of Toximeter derived average aqueous concentrations and snap-shot sample concentrations for each quantifiable PAH in borehole 3. Toximeter bars represent the mean of three Toximeters with vertical lines indicating the standard deviation. For snap-shot water samples, results from the first sample taken were used, as the deviation between duplicates sample was negligible.

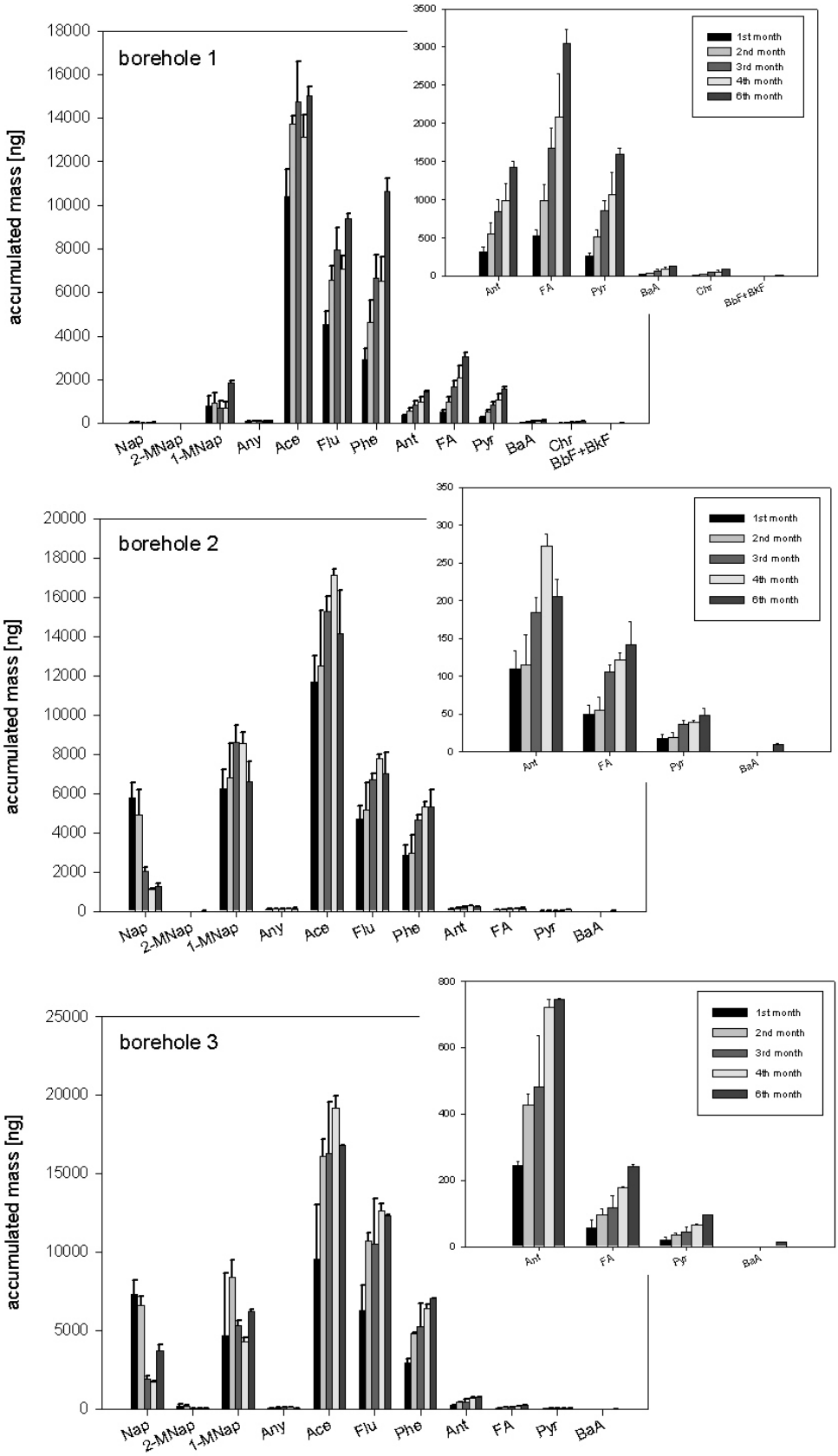

Figure 5.6 Accumulation of PAHs in the Toximeters over exposure time in the three investigated boreholes. Inserts show a magnification for the Ant-BkF results. Error bars represent standard deviations for triplicate Toximeter samples.

Biological analysis

In parallel to the chemical investigations, Biosilon from field exposed Toximeters was investigated biologically in the EROD enzyme assay. The EROD assay detects effects of dioxin-like acting compounds, which include a number of PAHs. Up to about two-fold enzyme induction compared to the control was detected in each borehole (fig. 5.7). A tendency of increasing effects over time, corresponding to increasing accumulated amounts, was generally observed.

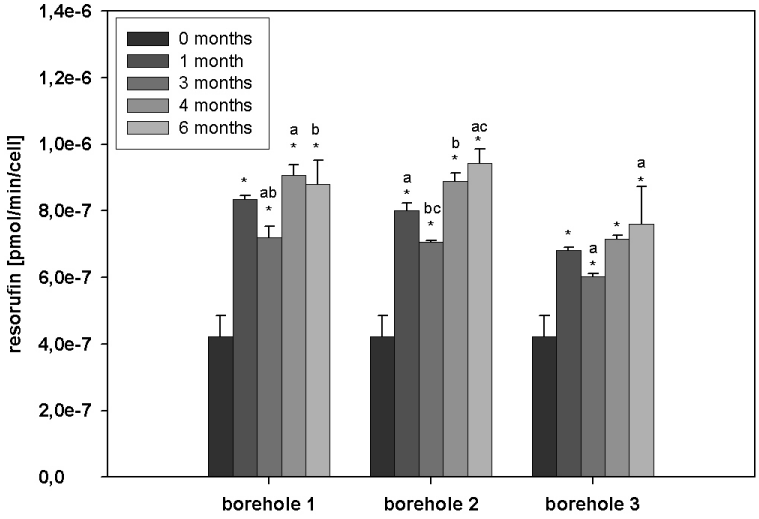

Figure 5.7 EROD induction by contaminated Biosilon from field-exposed Toximeters in the bead assay. Zero months samples represent the control, which contained untreated Biosilon as used for filling the Toximeters. Asterisks indicate a significant induction compared to the control (Dunnett's test, $p<0.05$). Bars with same characters within one group are significantly different from each other (Tukey's test, $p<0.05$). Samples from 2 months Toximeter exposures could not be investigated due to loss.

In order to assess the extent by which the detected PAHs contributed to the measured biological effect, a predicted EROD induction was calculated (tab. 5.1). Not all PAHs are able to elicit EROD induction. From the PAHs detected at the field site, only BaA, Chr, BbF, and BkF are known to be EROD inducing compounds in the fish cell bioassay (BOLS *et al.* 1999). Among them, BkF is the most potent inducer, but the one with the lowest concentration among the detected PAHs. Based on the concentrations detected, toxic equivalency concentrations (TECs) were calculated using the toxic equivalency approach. In this approach, the detected PAH concentrations are multiplied by the so-called toxic equivalency factor (TEF). A TEF describes the ability of a compound to induce EROD induction in comparison to a reference compound, such as BaP. Thus, chemical concentrations can be converted by the TEF to derive the concentration of the reference compound that would have yielded the same biological response. In this study, BaP-TEF values for BaA, Chr, BbF and BkF were derived from the literature (BOLS et al., 1999), assuming that the TEFs would be similar in the bead- compared to the standard EROD assay. The calculated TECs for the individual PAHs were added together and EROD induction predicted, relying on the dose response curve obtained for BaP in the bead assay (see

Chapter 3, fig.3.5). These calculations could be performed only for borehole 1 because the PAHs relevant for EROD induction were undetectable in boreholes 2 and 3. A comparison between the measured and predicted EROD induction revealed that 55 to 80 % of the EROD induction measured were explained by the PAHs detected in borehole 1. However, similar induction effects were observed for the other two boreholes, where no EROD inducing compounds were detected.

Table 5.1 Calculation of predicted EROD induction for Toximeter field samples from borehole 1 and comparison to the actual measured EROD induction

exposure time [d]		34	97	128	184
accumulated mass in Toximeters [ng]	BaA	22	67	85	124
	Chr	11	45	57	91
	BbF-BkF	<LOQ [a]	<LOQ	<LOQ	11
concentrations in the EROD-assay [nM]	BaA	15.3	46.4	59.1	86
	Chr	7.7	31.4	39.5	62.7
	BbF-BkF	-	-	-	7.1
corresponding BaP concentrations [nM] (BaP-TECs)[b]	BaA	2.1	6.5	8.3	12.0
	Chr	1.2	5.0	6.3	10.0
	BbF-BkF	-	-	-	14.5 [c]
EROD induction [10^{-7} pmol resorufin/min/cell]	predicted[d]	4.67	4.93	4.97	7.05
	measured	8.34	7.20	9.07	8.80

[a] <LOQ = below limit of quantification

[b] BaP equivalent concentrations were calculated using TEF_{BaP} values from BOLS *et al.* (1999): TEF_{BaP}(BaA)=0.14, TEF_{BaP}(Chr)=0.16, TEF_{BaP}(BbF)=0.64, TEF_{BaP}(BkF)=3.44; BaP dose response curve for the bead assay was presented in Chapter 3, fig. 3.5.

[c] For calculations, it was assumed that BbF and BkF, which could not individually be detected in the GC method, contributed with equal amounts to their total detected concentration.

[d] predicted based on sum of TECs

5.3.2 Ceramic Dosimeter samples

Ceramic Dosimeters were deployed in parallel to the Toximeters, in order to add a comparable, already validated system. Average aqueous PAH concentrations predicted from the Dosimeter samples were compared to the actually measured snap-shot sample concentrations. A good agreement was found for all detected PAHs, with a slight tendency for underestimation particularly in boreholes 1 and 3 (fig. 5.8). Overall, snap-shot sample PAH concentrations were higher by a median factor of 1.2 for all PAHs. For individual PAHs factors for under or overestimation ranged from 0.3-4, with higher deviations being determined only for Nap and methylated Nap. The PAHs BaA and Chr could be detected only in borehole 1 at only two sampling times (first and sixth month) as well as in only one of the Dosimeter replicates.

Results for single PAHs, such as presented for the Toximeter in fig. 5.3-5.5, are shown in detail in the appendix (fig. A1-A3).

Samples from Ceramic Dosimeters could not be tested in the bead assay for investigation of EROD induction, as the sorbent Amberlite IRA-743 was found to be incompatible with the RTL-W1 cell line as shown in Chapter 3.

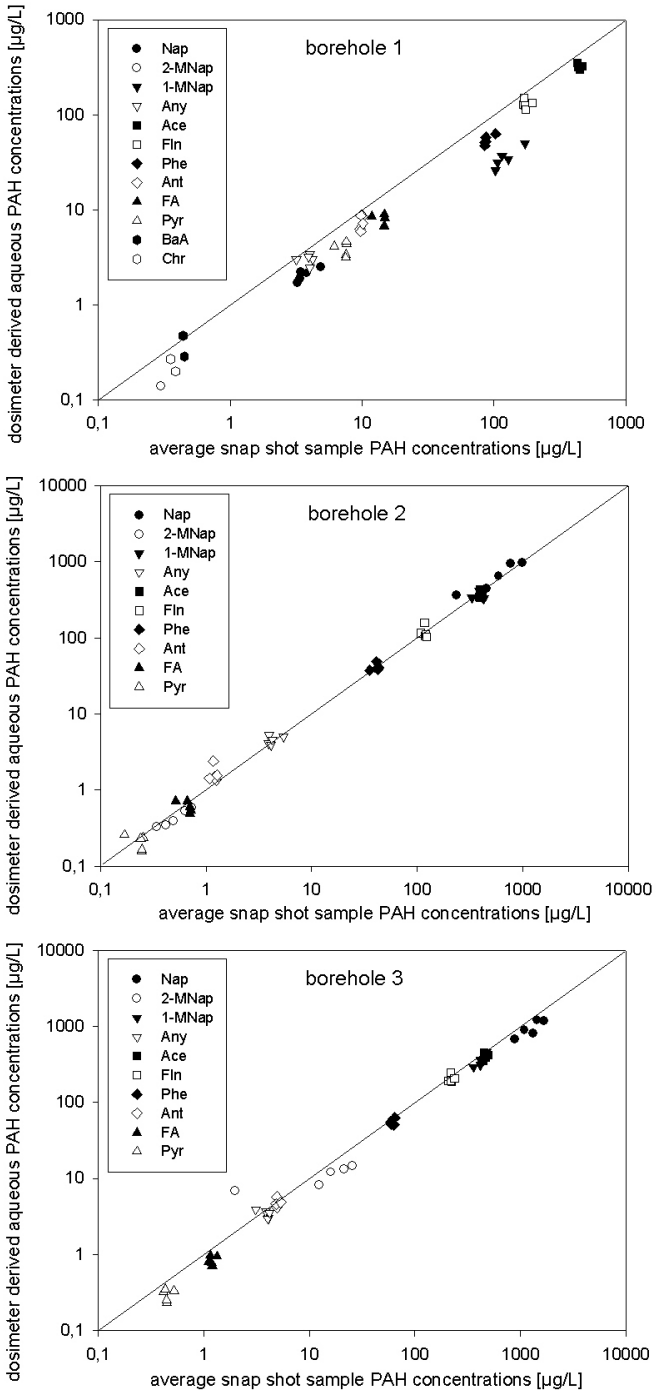

Figure 5.8 Comparison of Ceramic Dosimeter derived and snap-shot sample determined aqueous PAH concentrations. For absolute agreement between the two ways of determining PAH concentrations, symbols should be located on the diagonal line.

5.4 Discussion

The sampling of water under field conditions can always be influenced by unpredictable factors. In the presented field study, a tar oil phase occurred during sampling in borehole 2 which had not been detected in previous snap-shot sampling campaigns. Likely, the tar oil phase had formed due to underground constructional activities at the site, altering the groundwater regime. In spite of these unpredictable exposure conditions, the passive sampling devices showed a normal sampling behavior. Other passive sampling devices are known to be affected by environmental parameters such as microbial deterioration of membranes (SÖDERGREN 1990) or hydrodynamic flow (VRANA AND SCHÜÜRMANN 2002). To account for such influences, the use of Performance Reference Compounds (PRCs) has been recommended, e.g. for Semipermeable Membrane Devices (SPMDs), in order to provide exposure standards for *in situ* calibration (BOOIJ *et al.* 1998; HUCKINS *et al.* 2002). One alternative to PRCs, which precludes the use of samples for toxicological analysis, are labor-intensive calibration steps in the laboratory under various flow regimes although the field situation can never be completely simulated. None of these laborious steps or precautions are required with the ceramic membrane. In fact, the presence of a tar oil phase as observed in the current study is an extreme situation which would likely have led to the disintegration of a number of sampler types. Thus, the present study confirmed the robustness of the ceramic tube membrane in long-term water monitoring under varying and partly extreme exposure conditions.

The Toximeter allowed the prediction of aqueous PAH concentrations within a median factor of four of the actually measured aqueous concentrations without the need for calibration or laborious derivation of groundwater flow conditions and sampling rates. A direct confrontation of snap-shot sample contaminant concentrations versus passive sampler derived concentrations has only rarely been done so far. KINGSTON *et al.* (2000) reported deviations around a factor of 2 (under- as well as overestimation) for sampling of diuron and irgarol 1051 for a solid receiving phase sampler exposed for two weeks in a marine harbor. LUELLEN AND SHEA (2002) deployed SPMDs in Boston Harbor up to 29 d. They reported underestimations compared to water samples by a factor of 2-4 using the SPMD sampling rates which were determined in preceding laboratory calibration experiments. SPMDs deployed in river water for sampling of PAHs and organochlorine pesticides showed average deviations by about a factor of 5 (over- and underestimations) compared to bulk water samples whereby much higher deviations occurred for some compounds at some sampling points (VRANA *et al.* 2001a). Even fewer examples exist for the use of time integrative passive sampling devices in groundwater. In one example, SPMDs have been deployed at a PAH contaminated site and SPMD derived concentrations have been compared to snap-shot water samples taken with three conventional sampling methods (GUSTAVSON AND HARKIN 2000). Deviations between the passive and active sampling results were between 5 to 10-fold when two different ways of calculation were applied. Problems were reported with the presence of free product within one well, where SPMD devices had been

coated by a black viscous material, which was suspected to facilitate transfer of PAHs into the SPMD leading to an overestimation of true aqueous concentrations. Overall, however, underestimation of true contaminant concentrations occurred much more often than overestimation. This was attributed to particle-bound PAHs not available for the SPMDs but included in the analysis of unfiltered water samples (GUSTAVSON AND HARKIN 2000). Another example for passive sampling in groundwater is the study of MARTIN *et al.* (2003). They deployed ceramic dosimeters for three months in groundwater under highly fluctuating aqueous concentration conditions. Dosimeter derived contaminant concentrations for benzene and naphthalene matched very well with results from conventional water samples, with ratios of about 88-115 % of the conventionally determined concentrations.

The aspect of particle-bound PAHs that are not collected by the passive sampler could not explain why aqueous PAH concentrations were underestimated by the Toximeters. This is because the simultaneously deployed Ceramic Dosimeters showed a good agreement between passive sampler-derived and actually measured concentrations, particularly for boreholes 2 and 3. The only case where also the Dosimeter consistently underestimated true aqueous concentrations was for borehole 1. No turbidity was observed in the water samples, but particulate matter was not investigated in this study. However, if particle-bound PAHs are the cause for underestimation, the latter should increase for high molecular weight PAHs due to their higher affinity to particulate matter. Such a trend was not observed. Along these lines, clogging of ceramic membrane pores, e.g. due to the tar oil coating, is unlikely to explain underestimation of actual PAH concentrations in the Toximeter because this should also have impacted on the sampling behavior of the Ceramic Dosimeter, which it apparently did not.

Two possible reasons, already suspected from the laboratory results (Chapter 4), can be advanced to explain the under-prediction of Toximeter-derived aqueous concentrations. The first is the inhomogeneous occupancy of PAHs on Biosilon in the sampling device. Normally, the ceramic tube thickness defines the length of the diffusion pathway in the calculation of Toximeter derived PAH concentrations. However, if Biosilon next to the inner surface of the ceramic tube is approaching partitioning equilibrium, PAHs may have to pass a longer diffusion pathway for getting sorbed, thus also reducing the uptake rates. Secondly, it was observed that especially low K_{ow} PAHs deviated from the truly measured aqueous concentrations. This in turn might be explained by an insufficient capacity of Biosilon to keep the low K_{ow} aqueous PAH concentration in the inner part of the Toximeter close to zero. In that case, the concentration gradient and thus the uptake of the PAHs declines. Thus, the field experiment confirms that the Toximeter works best for PAHs with a log K_{ow} of 5 to 6 but is suboptimal for PAHs with a log K_{ow}<4.5.

Coupling of passive sampling to direct toxicological analysis is a key characteristic of the Toximeter. Up to a two-fold EROD induction compared to the control was detected with Biosilon field samples. However, PAHs with a known EROD inducing ability were detected by the

Toximeter and direct water analysis only in borehole 1. In order to get an impression of the responsibility of the detected PAHs, which are known to be capable of inducing EROD, TEC values and the expected induction were calculated for borehole 1. For TEC calculations, TEF values from standard assay literature data were used. As the transferability of these values to the bead assay was not investigated, the derivation of the quantitative interpretation should be viewed as an approximation only. This is because of the different behavior of test compounds concerning sorption to Biosilon, depending on their chemical properties, as well as between the two assay types. Based on the TEC calculations, the observed biological effects in borehole 1 were partly explained by the detected PAHs. Yet, EROD induction did not show a significant consistent increase with sampling times as detected for the analyzed EROD inducing PAHs. Therefore, the effects cannot clearly be attributed to these compounds. Another aspect is that biological effects were similar for the three boreholes, even if EROD inducing PAHs were not detected in borehole 2 and 3. Thus PAHs, which were not detected in the applied standard chemical analysis, or completely different substance classes might be responsible for the observed effects. For example, contaminants such as indane and indene, known to be present at the field site from previous snap-shot sampling campaigns (personal communication, Dr. Hansjörg Weiß), have been found to possess some EROD inducing potential (SCHIRMER *et al.* 2004). These substances have a log K_{ow} of 2.54-3.46 (EPI suite estimation software, 2000). Although they thus fall into the group of contaminants which will sorb to Biosilon to a lesser extent, a certain amount can be expected to be accumulated in the sampler. The fact that no consistent increase in EROD induction was measured over time again hints at lower hydrophobicity compounds as the responsible chemicals. As these chemicals are less well accumulated by the Biosilon, they might have been approaching equilibrium. Fractionation of Toximeter extracts could help identifying the other EROD inducing compounds at this field site (BRACK *et al.* 1999; KLINKOW AND JEKEL 1999).

5.5 Summary

The present field study showed the applicability of Biosilon filled Toximeters for determination of time weighted average aqueous contaminant concentrations for a range of PAHs even under extreme exposure conditions. These results were obtained without using laboratory calibration data. Results from the current field study agreed well with the observations from laboratory experiments described in Chapter 4. Biological analyses, which were performed in parallel, provided additional information. Effects could only partly be explained by chemical analysis data alone, thus revealing the presence of other relevant compounds which are unknown up to now.

Chapter 6

Concluding remarks and future directions

Coupling chemical analyses with toxicological investigations in long-term monitoring campaigns at contaminated sites is increasingly recognized as an important strategy in order to achieve a holistic site characterization. As well, passive sampling provides a time and cost effective alternative to conventional snap-shot sampling techniques. The Toximeter, developed in this study as a passive sampling device for combined chemical and direct toxicological analysis, aims at combining the advantages of passive sampling and an integrative assessment approach.

The first step in the development of the Toximeter encompassed the establishment of the solvent-free, solid-sorbent bioassay. This development can be discussed in light of two current trends in ecotoxicology. The first trend is the application of test systems which allow the maintenance of a constant exposure concentration (e.g. MAYER *et al.* 1999; BROWN *et al.* 2001). The second trend refers to the interpretation of exposure concentrations: instead of total nominal test concentrations, truly dissolved, free concentrations have recently been suggested as a measure of target dose (e.g. HERINGA 2004), but as well, internal target concentrations can be directly used (SCHIRMER *et al.* 1997; HESTERMANN et al. 2000).

The bead assay developed within this thesis has the potential to aid in advancing both of these trends. The application of the contaminated beads in the bioassay allows the maintenance of constant exposure concentrations due to the large reservoir. In the future, the distribution of contaminants within the test system, which was analyzed here after a 24 h exposure period, should be investigated in a time course in order to derive more detailed information on contaminant distribution in the assay as well as mass transfer mechanisms. Along these lines, the relevance of internal exposure concentrations was confirmed in this study. At first, differences in the sensitivity of the bead and the standard assay (using nominal concentrations) were detected. However, when internal cell concentrations were applied for concentration-response comparison, the shape of the concentration-response curves became much more similar for both approaches. Therefore, the differences observed using nominal doses can be attributed to a major part to the differences in internal exposure concentrations.

Aspects for improving the sensitivity of the new system could be an optimized ratio of Biosilon beads to cell numbers. Longer exposure times were already observed and showed a slight decrease in sensitivity in the EROD assay which might be due to metabolic activity. Thus differences concerning different endpoints are possible and have to be taken into account. Investigations on the influence of the serum concentrations in the test medium might provide new insights with regard to test compound distribution and responsiveness of *in vitro* cell systems.

The second step of this thesis research was the development and validation of the Toximeter sampling device using chemical analysis and the above discussed bead bioassay in the laboratory. The sampling behavior of the Toximeter was investigated in three different exposure scenarios, ranging from static to semi-static. The maintenance of constant aqueous contaminant concentrations was difficult in each of the various approaches and only a range of true PAH concentrations could be defined. Other laboratory exposure systems, such as a flow-through set-up, may more readily provide constant exposure concentrations in order to determine the relationship of true and Toximeter-derived aqueous concentrations. However, Toximeter derived PAH concentrations generally were within this range of true concentrations for most PAHs except for Nap and the higher molecular weight PAHs DBA–IndP. For low K_{ow} compounds like Nap, a lower binding affinity to Biosilon was observed, thus leading to uptake rates lower than expected. For very hydrophobic compounds with log K_{ow}>6, the time to reach the detection limit in the Toximeter was much longer than for the less hydrophobic compounds owing to lower aqueous concentrations and lower diffusion coefficients. Another important aspect for the lower sampled masses of very hydrophobic compounds, at least in the laboratory, appeared to be the loss to the Teflon caps as well as the retardation in the ceramic tube. Decreased sampling rates were also observed for other sampling devices (PETTY *et al.* 2000). Steric hindrance, which might be one reason in reduced uptake, is rather improbable in the Toximeter as the pore size of 5 nm should provide an appropriate diameter for PAH molecular dimensions (HUCKINS *et al.* 1999), but experiments with larger pore size ceramic tubes, which are also commercially available, could help to better understand the phenomena observed.

During field deployment, the third and final step of the Toximeter development within this thesis, the Toximeter was capable of predicting true aqueous PAH concentrations within a median factor of underestimation of four. Underestimation occurred mainly for PAHs with a log K_{ow} <4.5 and for those with a log K_{ow} >6. In order to overcome these limitations, three aspects can be addressed. Firstly, alternative materials for the Toximeter caps, which act as sinks for hydrophobic compounds in the current design, should be looked for. Stainless steel caps might be useful, but still a small disk such as from Teflon not directly exposed to the

water, is needed in order to seal the inner part of the device. Secondly, other sorbent materials could be explored as receiving phases in the Toximeter. It was shown to be difficult to find a material fulfilling all the criteria set forth for use in the Toximeter. Of the ten materials tested in Chapter 3, only one, the Biosilon, met all the requirements. As the properties determining the suitability are now known in more detail, a further search could be initiated, e.g. exploring the use of glass beads. Beyond the search for a potential alternative sorbent material, the compatibility of the prototype Biosilon filled Toximeter with other chemical substance groups aside from PAHs could be explored. Thirdly, improvements could be obtained by also taking variations to the ceramic membrane into account. The ceramic tube as diffusion barrier showed to be advantageous concerning low susceptibility to environmental factors, such as changes in water flow or biofilm formation. The Toximeter field deployment was performed in highly contaminated groundwater. The ceramic tube allows only low diffusive flux to the inner part, thus the Toximeter has low sampling rates and is most suitable in its current design for long-term sampling. However, shorter exposures and application in less polluted environments might be desirable. This could be achieved by larger samplers using longer tubes, by thinner tube walls reducing the diffusion pathway, or by use of a larger pore size of the ceramic tube. Positive effects regarding the undesired retardation of higher K_{ow} compounds in the tube might be achieved in parallel using thinner wall thickness tubes and larger pore sizes. All these parameters have to be carefully investigated in order to find a compromise between higher sampling rates without losing the other advantages, namely the independence of sampling rates from hydrodynamic conditions and the prevention of microbes entering the device.

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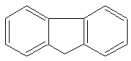
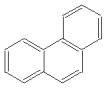
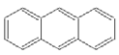
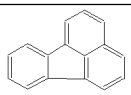
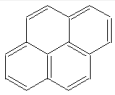
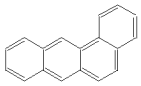
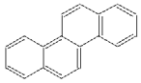
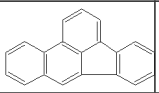
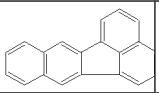
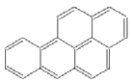
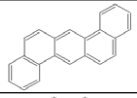
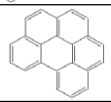
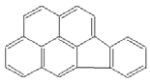
Appendix

Table A1 List of PAHs used in this study and their properties

name	molecular structure	mol. formula	CAS-Number	molecular weight [g/mol]	solubility[a] [mg/L] at 25 °C	solubility [µmol/L] at 25 °C	log K_{ow}[a]	D_w [10^{-6} cm^2/s] at 25 °C[e]
naphthalene (Nap)		$C_{10}H_8$	91-20-3	128.2	31	241.84	3.33	8,24
acenaphthene (Ace)		$C_{12}H_{10}$	83-32-9	154.2	3.8	25.29	3.92	7,50
acenaphthylene (Any)		$C_{12}H_8$	208-96-8	152.2	4.3[b]	28.07	4	7,70
fluorene (Flu)		$C_{13}H_{10}$	86-73-3	166.2	1.9	11.37	4.18	7,14
phenanthrene (Phe)		$C_{14}H_{10}$	85-01-8	178.2	1.1	6.452	4.57	6,91
anthracene (Ant)		$C_{14}H_{10}$	120-12-7	178.2	0.045	2.435	4.54	6,95
fluoranthene (FA)		$C_{16}H_{10}$	206-44-0	202.3	0.26	1.285	5.22	6,56
pyrene (Pyr)		$C_{16}H_{10}$	129-00-0	202.3	0.132	0.667	5.18	6,62
benzo[a]anthracene (BaA)		$C_{18}H_{12}$	56-55-3	228.3	0.011	0.041	5.91	6,07
chrysene (Chr)		$C_{18}H_{12}$	218-01-9	228.3	0.002[b]	0.0088	5.81[b]	6,03
benzo[b]fluoranthene BbF		$C_{20}H_{12}$	205-99-2	252.3	0.0015	0.0059	5.8	5,79
benzo[k]fluoranthene BkF		$C_{20}H_{12}$	207-08-9	252.3	0.0008	0.0032	6	5,79
benzo[a]pyrene BaP		$C_{20}H_{12}$	50-32-8	252.3	0.0038	0.0064	6.04	5,86
dibenzo[a,h] anthracene DBA		$C_{22}H_{14}$	53-70-3	278.35	0.0006	0.0089	6.75	5,42
benzo[g,h,i]perylene BghiP		$C_{22}H_{12}$	191-24-2	276.34	0.00026	0.00094	6.5	5,69
indeno[1,2,3-cd] pyrene IndP		$C_{22}H_{12}$	193-39-5	276.34	0.00019[c]	0.00069	6.7[d]	4,79

[a] from MACKAY *et al.* (1992) if not stated elsewhere
[b] SCHWARZENBACH *et al.* (2003)
[c] PEARLMAN *et al.* (1984)
[d] MEYLAN AND HOWARD (1995)
[e] D_w values were calculated according to Hayduk & Laudie (described in BAUM (1998)); using water viscosity from SCHWARZENBACH *et al.* (2003) for the appropriate temperature and molar volume values (LeBas) from MACKAY *et al.* (1992)

Comparison of Ceramic Dosimeter derived aqueous PAH concentrations and snap-shot sample concentrations from field deployment (Chapter 5)

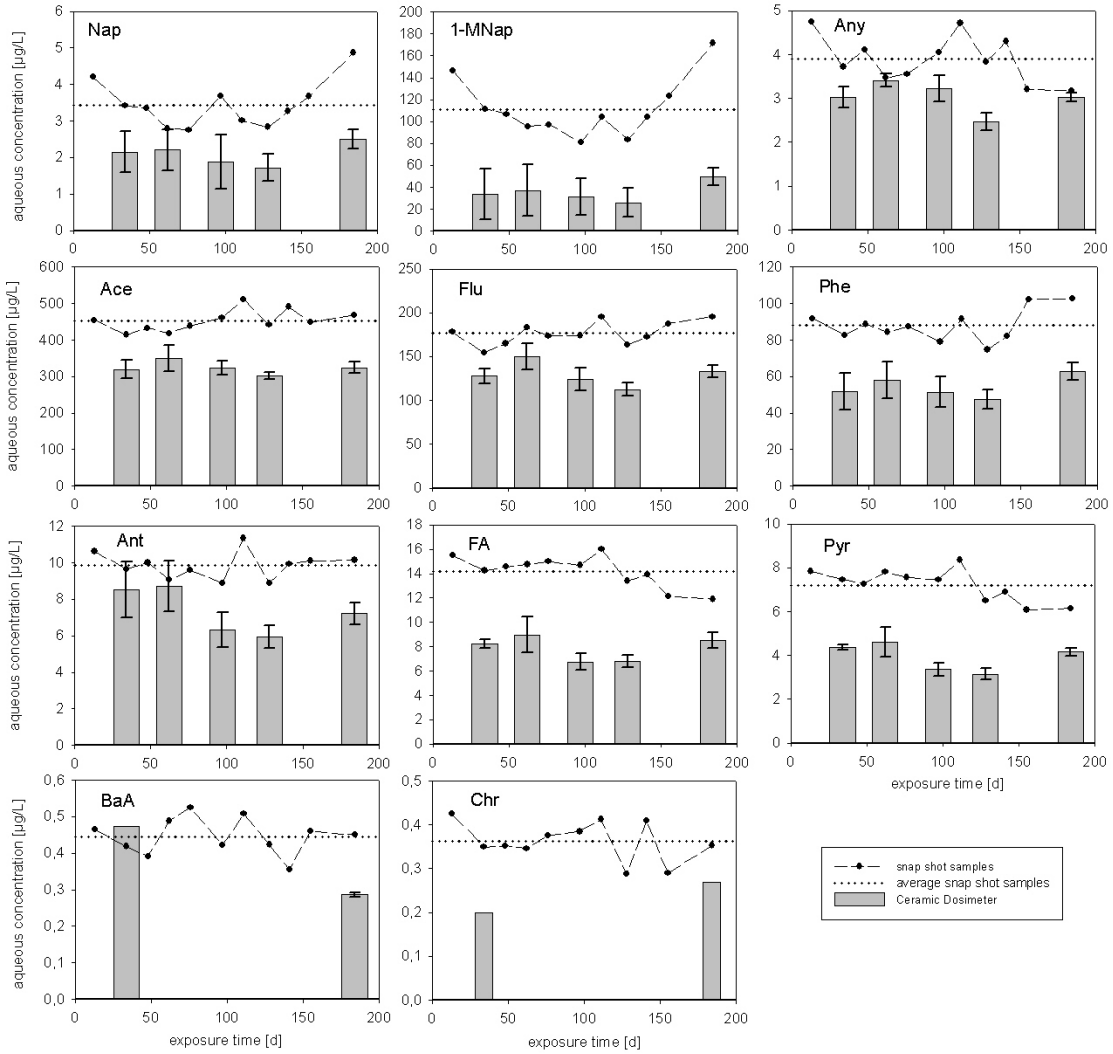


Figure A1 Comparison of Dosimeter derived average aqueous PAH concentrations and snap-shot sample concentrations for each quantifiable PAH in borehole 1. Dosimeter bars represent the mean of three Dosimeters with vertical lines indicating the standard deviation. For snap-shot water samples, results from the first sample taken were used, as the deviation between duplicates sample was negligible. The quantification limit in the Dosimeters was not reached at all time points in all replicates for BaA and Chr.

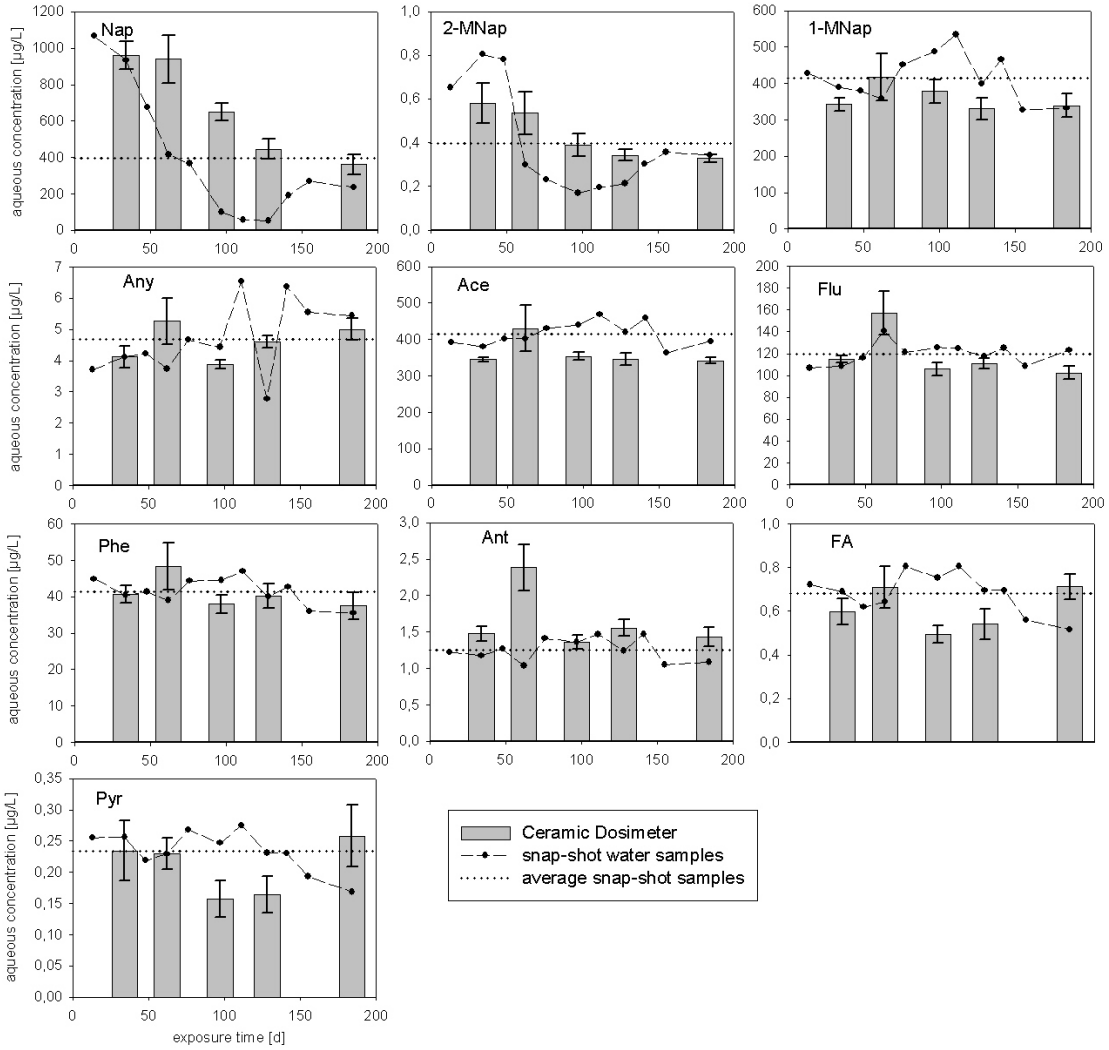


Figure A2 Comparison of Dosimeter derived average aqueous PAH concentrations and snap-shot sample concentrations for each quantifiable PAH in borehole 2. Dosimeter bars represent the mean of three Dosimeters with vertical lines indicating the standard deviation. For snap-shot water samples, results from the first sample taken were used, as the deviation between duplicates sample was negligible.

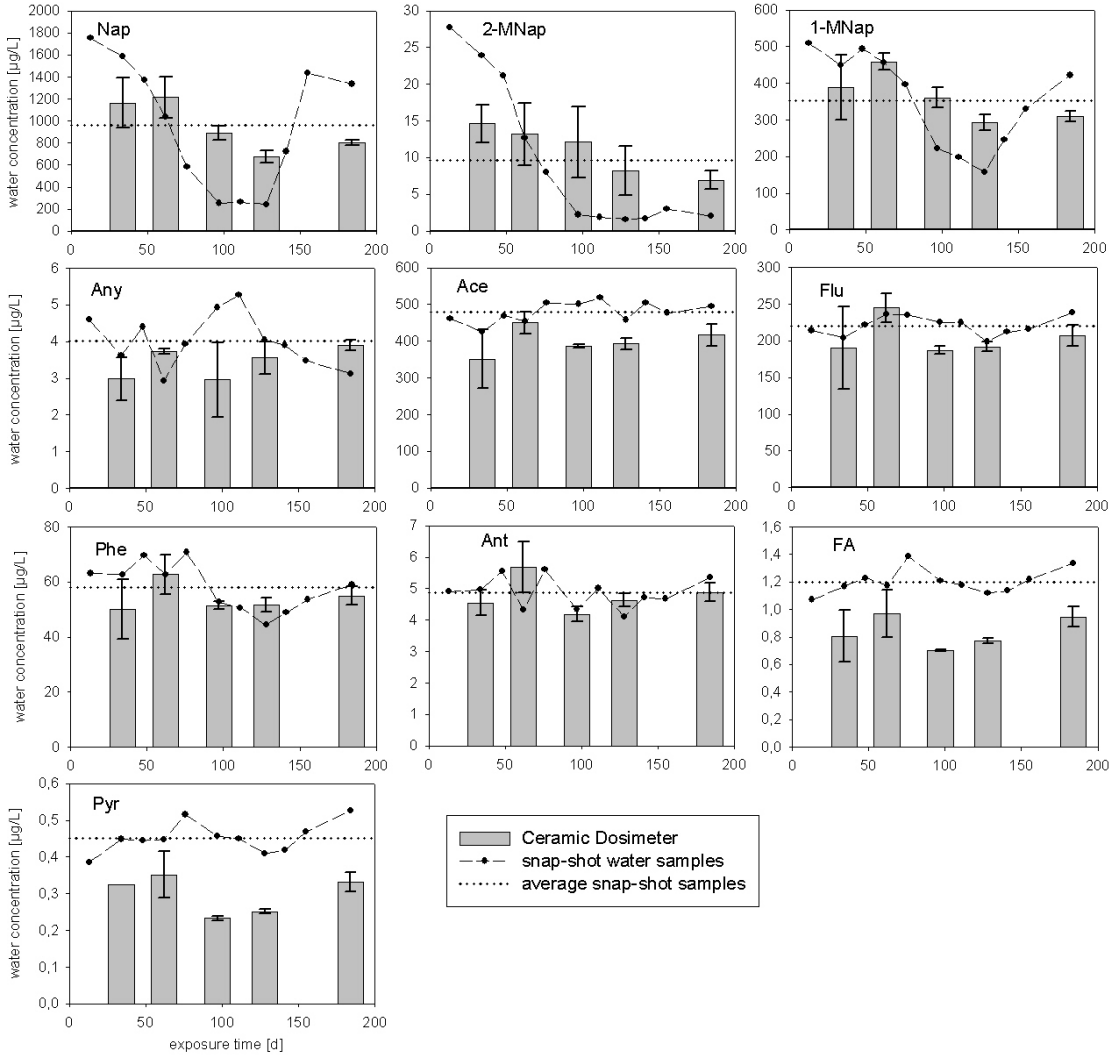


Figure A3 Comparison of Dosimeter derived average aqueous PAH concentrations and snap-shot sample concentrations for each quantifiable PAH in borehole 3. Dosimeter bars represent the mean of three Dosimeters with vertical lines indicating the standard deviation. For snap-shot water samples, results from the first sample taken were used, as the deviation between duplicates sample was negligible.

Snap-shot sample PAH concentrations from Toximeter field deployment (Chapter 5)

Table A2 Snap-shot sample PAH concentrations [µg/L] from borehole 1

	06.10.2003	27.10.2003		10.11.2003		24.11.2003		08.12.2003		29.12.2003		12.01.2004		29.01.2004		11.02.2004		25.02.2004		25.03.2004	
Nap	4.20	3.42	3.00	3.35	4.00	2.79	3.00	2.75	3.00	3.69	4.00	3.01	3.00	2.83	3.00	3.26	3.00	3.66	4.00	4.86	5.00
2-MNap	0.17	0.16	0.20	0.15	0.20	0.17	0.10	0.16	0.20	0.17	0.2	0.21	0.20	0.15	0.20	0.20	0.20	0.24	0.20	0.30	0.30
1-MNap	146	112	112	107	124	95	86	97	104	81	90	104	103	84	90	104	110	123	123	172	170
Any	4.75	3.72	3.71	4.11	4.24	3.47	3.58	3.56	3.79	4.06	4.23	4.72	4.50	3.83	3.81	4.30	4.28	3.20	3.26	3.17	3.15
Ace	454	414	413	433	466	418	411	438	455	461	469	511	497	441	452	490	500	448	445	468	462
Flu	178	154	154	165	180	183	173	173	180	174	185	195	194	163	168	172	174	187	184	195	198
Phe	92	82	78	89	96	84	80	87	92	79	86	91	89	75	77	82	88	102	103	103	104
Ant	10.6	9.6	9.19	10	11.16	9.1	8.54	9.6	10.54	8.9	9.54	11.3	10.97	8.9	9.38	9.90	10.42	10.10	10.25	10.1	10.20
FA	15.5	14.2	14.18	14.6	16.23	14.7	13.33	15	15.82	14.7	16.39	16.0	16.11	13.4	14.48	13.90	14.77	12.10	12.41	11.9	12,18
Pyr	7.83	7.46	7.31	7.26	8.42	7.81	7.10	7.55	8.15	7.45	7.96	8.35	8.00	6.51	6.88	6.89	7.58	6.08	6.04	6.15	6.02
BaA	0.46	0.42	0.41	0.39	0.47	0.49	0.45	0.52	0.48	0.42	0.51	0.51	0.48	0.42	0.50	0.36	0.40	0.46	0.47	0.45	0.46
Chr	0.42	0.35	0.32	0.35	0.39	0.35	0.33	0.38	0.40	0.38	0.40	0.41	0.41	0.29	0.34	0.41	0.43	0.29	0.30	0.35	0.37
Bbf-BkF	0.10	0.03	0.04	0.04	<0.03	<0.03	<0.03	0.15	<0.03	0.11	0.10	0.10	0.09	0.08	0.09	0.08	0.07	<0.03	0.03	0.11	0.10
BaP	<0.05	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
IndP	<0.05	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
DBA	<0.05	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
BghiP	<0.05	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
sum	914	802	796	833	910	819	787	836	873	834	873	946	927	799	826	888	913	897	892	974	972

Table A3 Snap-shot sample PAH concentrations [µg/L] borehole 2

	06.10.2003	27.10.2003		10.11.2003		24.11.2003		08.12.2003		29.12.2003		12.01.2004		29.01.2004		11.02.2004		25.02.2004		25.03.2004	
Nap	1066	933	1014	675	691	415	413	366	364	99	97	56	53	51	49	190	174	268	260	235	251
2-MNap	0.65	0.8	0.9	0.8	0.9	0.3	0.3	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.4	0.4	0.3	0.4
1-MNap	428	389	423	379	400	358	339	452	488	487	479	534	522	398	389	465	490	327	344	332	331
Any	3.71	4.12	4.50	4.23	4.54	3.74	3.61	4.66	4.85	4.43	4.69	6.54	6.36	2.77	2.87	6.38	5.94	5.55	5.61	5.44	6.11
Ace	392	381	396	402	428	402	375	431	465	439	452	468	456	419	419	459	464	363	378	395	395
Flu	107	108	110	116	124	141	127	121	137	126	128	125	126	117	122	125	129	108	113	123	124
Phe	44.9	40	44	41	43	39	36	44	49	44	45	47	46	40	41	43	45	36	38	36	36
Ant	1.23	1.17	1.24	1.27	1.31	1.04	0.89	1.41	1.54	1.36	1.27	1.47	1.62	1.24	1.26	1.47	1.60	1.05	1.25	1.08	1.11
FA	0.72	0.69	0.77	0.62	0.65	0.64	0.56	0.80	0.80	0.75	0.77	0.80	0.81	0.69	0.71	0.70	0.73	0.56	0.60	0.52	0.52
Pyr	0.26	0.26	0.28	0.22	0.22	0.23	0.21	0.27	0.27	0.25	0.26	0.27	0.28	0.23	0.23	0.23	0.25	0.19	0.21	0.17	0.17
BaA	<0.05	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03
Chr	<0.05	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03
Bbf-BkF	<0.05	<0.03	0.14	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03
BaP	<0.05	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
IndP	<0.05	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
DBA	<0.05	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
BghiP	<0.05	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
sum	2044	1860	1994	1621	1694	1360	1296	1422	1510	1201	1208	1240	1212	1031	1025	1292	1312	1111	1141	1127	1146

Table A4 Snap-shot sample PAH concentrations [µg/L] from borehole 3

	06.10.2003	27.10.2003		10.11.2003		24.11.2003		08.12.2003		29.12.2003		12.01.2004		29.01.2004		11.02.2004		25.02.2004		25.03.2004	
Nap	1752	1585	1464	1372	1328	1036	1158	582	578	252	262	264	261	242	214	722	740	1436	1447	1324	1338
2-MNap	27.70	23.90	20.65	21.1	19.17	12.70	14.89	8.00	7.47	2.20	2.31	1.90	1.87	1.60	1.50	1.70	1.67	3.00	2.92	2.20	1.98
1-MNap	509	449	461	493	491	457	506	397	392	221	221	199	198	158	161	246	240	330	331	431	422
Any	4.60	3.63	3.52	4.40	4.25	2.92	2.86	3.93	3.75	4.92	4.98	5.27	5.23	4.04	4.04	3.90	4.12	3.48	3.75	2.78	3.12
Ace	461	425	439	469	479	455	503	504	496	501	531	519	509	457	479	505	523	477	481	497	495
Flu	214	204	205	221	231	236	262	235	226	225	232	225	227	198	198	212	223	216	214	230	238
Phe	63	63	63.6	70	69.4	63	72.8	71	67.3	53	52.6	51	51.3	44	45.6	49	51.60	54	53.1	58	59.0
Ant	4.91	4.97	5.06	5.55	5.74	4.33	5.04	5.61	5.28	4.34	4.51	5.01	5.07	4.11	4.17	4.72	5.09	4.67	4.76	5.01	5.35
FA	1.07	1.17	1.18	1.23	1.30	1.17	1.40	1.39	1.26	1.21	1.28	1.18	1.23	1.12	1.10	1.14	1.25	1.22	1.19	1.26	1.33
Pyr	0.39	0.45	0.45	0.44	0.47	0.45	0.52	0.52	0.46	0.46	0.49	0.45	0.45	0.41	0.40	0.42	0.46	0.47	0.45	0.47	0.53
BaA	<0.05	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03
Chr	<0.05	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03
Bbf-BkF	<0.05	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03
BaP	<0.05	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
IndP	<0.05	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
DBA	<0.05	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
BghiP	<0.05	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
sum	3039	2760	2663	2658	2629	2268	2528	1808	1779	1265	1311	1270	1260	1112	1108	1745	1790	2520	2538	2552	2564

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht habe.

Leipzig, 24.06.2004

- Stephanie Bopp -

Lebenslauf

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Promotion

01.04.2001-30.09.2004	Doktorandin am UFZ - Umweltforschungszentrum Leipzig-Halle GmbH, im Department Zelltoxikologie unter Betreuung von Frau Dr. Kristin Schirmer und Betreuung von Prof. Dr. Michael McLachlan, IOW – Institut für Ostseeforschung Warnemünde, Universität Rostock.
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Studium

14.12.2000	Diplom Geoökologin (univ.)
Nov. 1999 – Nov. 2000	Diplomarbeit Lehrstuhl für Umweltchemie und Ökotoxikologie "Entwicklung eines miniaturisierten Biotestverfahrens zur Toxizitätsabschätzung in Nebelproben"
Nov. 1995 – Dez. 2000	Studium der Geoökologie an der Universität Bayreuth
Hauptfächer	Umweltchemie und Ökotoxikologie
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Schule

1986-1995	Besuch des Gymnasiums Ehingen (Donau), Abschluss Abitur
1982-1986	Besuch der Grundschule in Dächingen und Mundingen