Uptake and effects of particle-bound polycyclic aromatic hydrocarbons (PAH) from sediment suspensions in rainbow trout (*Oncorhynchus mykiss*, Walbaum)

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Markus Brinkmann, B.Sc.
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Referent:	Prof. Dr. Henner Hollert Lehr- und Forschungsgebiet Ökosystemanalyse (ESA) Institut für Umweltforschung der RWTH Aachen
Korreferent:	Prof. Dr. Andreas Schäffer Lehrstuhl für Umweltbiologie und Chemodynamik (UBC) Institut für Umweltforschung der RWTH Aachen

Ich erkläre hiermit, dass ich die vorliegende Arbeit selbständig unter Anleitung verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

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Abstract

In context of the ongoing scientific discussion about the potential ecotoxicological impacts of flood events, it is of vital importance to understand the detailed mechanisms of contaminant uptake from suspended particles and related effects in aquatic biota. As part of the interdisciplinary project Floodsearch II, rainbow trout (*Oncorhynchus mykiss*) were exposed to suspensions of natural sediment from the River Rhine (harbour Ehrenbreitstein). Prior to suspension experiments, the sediment was spiked with the polycyclic aromatic hydrocarbons (PAH) pyrene, phenanthrene, chrysene, and benzo[*a*]pyrene at environmentally relevant concentrations (4.1, 5.0, 3.3 and 8.3 mg kg^{-1} dry weight, respectively). A control treatment without addition of PAHs was also included in the experimental design. The experiment was conducted at an average temperature of 24°C and repeated at 12°C. The nominal concentration of suspended solids was 10 g L^{-1} in both experiments. After 0, 1, 2, 4, 6, 8 and 12 days of exposure, physicochemical parameters, concentrations of PAHs in suspended matter, as well as biomarkers of exposure to the analytes in rainbow trout (biliary PAH metabolites, hepatic 7-ethoxyresorufin-*O*-deethylase (EROD) activity and lipid peroxidation) were measured.

Instrumental chemical analyses revealed that pyrene and phenanthrene concentrations in suspended solids decreased over time, while no significant degradation was observed for chrysene and benzo[*a*]pyrene. Concentrations of biliary PAH metabolites in fish increased slightly in the treatment without addition of PAHs at 24°C, while average levels increased to 166 µg ml^{-1} for 1-hydroxypyrene (control value 4.6 µg ml^{-1}) and 17 µg ml^{-1} for 1-hydroxyphenanthrene (control value 0.1 µg ml^{-1}) in the spiked treatment within two days, followed by a decrease. In the 12°C experiment, uptake of PAHs was slower and maximum metabolite concentrations in bile lower. With a latency of two days, the peak of metabolism in the 24°C experiment was followed by a peak of lipid peroxidation that indicates oxidative stress caused by PAH metabolization. EROD was not significantly induced by the treatments. Furthermore, significant differences were observed between the bioavailability of freshly spiked and field-aged PAH contamination. The set of biomarkers for use in future studies was modified by addition of hepatic gene expression analyses and a modification of the EROD protocol. The results of this study clearly indicate the importance to account for the temporal variability of biomarker responses in sediment suspension experiments that reacted in cascades, thereby deepening the understanding of the underlying processes that led to the observations in Floodsearch.

Zusammenfassung

Im Kontext der aktuellen wissenschaftlichen Diskussion über die möglichen ökotoxikologischen Auswirkungen von Hochwasserereignissen ist es von entscheidender Bedeutung, die Mechanismen der Schadstoff-Aufnahme von suspendierten Partikeln und deren Effekte in aquatischen Organismen zu verstehen. Im Rahmen des interdisziplinären Projekts Floodsearch II wurden Regenbogenforellen (*Oncorhynchus mykiss*) mit Suspensionen eines natürlichen Rheinsedimentes (Ehrenbreitstein bei Koblenz) exponiert, das mit einer Mischung der polyzyklischen aromatischen Kohlenwasserstoffe (PAK) Pyren, Phenanthren, Chrysen und Benzo[*a*]pyren in umweltrelevanten Konzentrationen dotiert wurde. Eine Kontrolle ohne PAK-Zusatz wurde ebenfalls untersucht. Das Experiment wurde bei einer durchschnittlichen Temperatur von 24°C durchgeführt und im Anschluss bei 12°C wiederholt. Die nominale Schwebstoffkonzentration betrug 10 g L^{-1} in beiden Experimenten. Nach 0, 1, 2, 4, 6, 8 und 12 Tagen Exposition wurden physikalisch-chemische Parameter, die Konzentrationen der PAK in Schwebstoffen, sowie Expositionsbiomarker in Regenbogenforellen (PAK-Metabolite in der Gallenflüssigkeit, 7-Ethoxyresorufin-*O*-deethylase (EROD)-Aktivität und Lipidperoxidation in Lebergewebe) untersucht. Eine instrumentelle chemische Analytik ergab, dass die Pyren- und Phenanthren-Konzentrationen im Schwebstoff im Verlauf der Zeit abnahmen, während keine signifikante Dissipation von Chrysen und Benzo[*a*]pyren beobachtet werden konnte. Die Konzentrationen der PAK-Metabolite in Fischgalle waren nach Exposition gegenüber dem undotierten Sediment nur vergleichsweise leicht erhöht. Nach nur zwei Tagen Exposition mit dem dotierten Sediment bei 24°C stiegen die Konzentrationen von 1-Hydroxypyren und 1-Hydroxyphenanthren auf 166 µg ml^{-1} bzw. 17 µg ml^{-1} in der Galle an, gefolgt von einem anschließenden Rückgang. In den Experimenten bei 12°C fand die Aufnahme langsamer und in etwas geringerem Ausmaß statt. Mit einer Latenz von zwei Tagen folgte dem Stoffwechselmaximum bei 24°C eine stark erhöhte Lipidperoxidation. Darüber hinaus wurden drastische Unterschiede in der Bioverfügbarkeit von frisch dotierten und im Freiland gealterten PAK-belasteten Sedimenten gefunden. Die Biomarker-Batterie für weitere Versuche in Floodsearch II konnte erfolgreich durch Genexpressionsanalysen und eine Verbesserung des Protokolls zur Bestimmung der EROD-Aktivität in Lebergewebe erweitert werden.

Die Ergebnisse dieser Studie zeigen deutlich, wie wichtig es aufgrund der zeitlichen Variabilität der Biomarker-Reaktionen in Experimenten mit suspendierten Sedimenten ist, die zugrundeliegenden Mechanismen im Detail zu verstehen.

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1. Introduction

1.1 Toxicity and risk assessment of fluvial sediments

1.1.1 Sediment quality assessment

Sediments are defined as particulate matter with geogenic or biogenic origin that has been deposited at the bottom of aquatic systems (Scheffer & Schachtschabel 2002, Stronkhorst et al. 2004). They mainly consist of minerals, carbonates, quartz sands or organic particles, that might be enclosed by organic or inorganic substances (Power & Chapman 1992). Sediments provide habitats for structurally and functionally diverse communities (Figure 1.1) and constitute important reservoirs for both organic and inorganic substances (Brils 2004, Fenchel 1978). Particularly microorganisms, primarily bacteria, significantly affect biogeochemical cycles of substances in sediments (Ahlf 1995). In particular, benthic organisms are strongly dependent on sediment quality, since many of them ingest sediment particles directly or indirectly as food, or are exposed to pore water (Ahlf 1995, Burton 1991, Höss et al. 1997).

Figure 1.1 Surface of a sediment dominated by the chemoautotrophic sulphur bacteria *Beggiatoa* (filaments) and *Thiovulum* (white spherical cells). Also seen are species of diatoms, euglenoid flagellates, cyanobacteria, ciliates and nematodes. From Fenchel (1992).

Sediments can act as sinks for various contaminants, i.e. heavy metals and non-polar organic compounds that adsorb to suspended particulate matter (SPM), but can also be a secondary source of contamination *via* bioturbation (Power & Chapman 1992), dredging (Koethe 2003)

and during (extreme) flood events (Haag et al. 2001, Hollert et al. 2000, Hollert et al. 2005, Westrich & Förstner 2005). Accordingly, there is general agreement that sediment quality is of major concern to environmental assessment (Haag et al. 2001, Hollert et al. 2008, Westrich & Förstner 2005), resulting in numerous concepts for risk estimation (Ahlf et al. 2002, Burton 1991, 1995, Chapman 1990, 2000, Chapman et al. 2002).

In order to protect the aquatic community, comprehensive methods and approaches for identification and assessment of sediment contamination have been developed since the early 1970s (Brinkmann et al. 2010b). The very first approach was to merely employ chemical analyses. Though being a highly effective analytical tool that is capable of detecting and quantifying both source substances and metabolites, the chemical analysis approach fails to provide information on the actual bioaccessibility and on the biological activity of toxicants (Chapman et al. 2002). Furthermore, chemical analysis does not provide information concerning synergistic and antagonistic potential in sediments. The high number of chemicals in water and sediments also obstructs any complete chemical screening to be done due to time, effort, and cost considerations. About a decade later, *in vitro* bioassay batteries were developed and adapted for the evaluation of sediments, soils and suspended particulate matter (SPM, Brinkmann et al. 2010b).

In order to ensure that the actual bioaccessibility of sediment contaminants is sufficiently considered, bioassays using direct sediment contact exposure were applied in different exposure scenarios (Blaha et al. 2010, Dillon et al. 1994, Duft et al. 2003, Feiler et al. 2009, Höss et al. 2010, Ingersoll et al. 1995, Lee et al. 2004, Re et al. 2009, Ryder et al. 2004, Sae-Ma et al. 1998, Turesson et al. 2007). Since sediment contact assays with whole organism usually provided only acute (mortality) and chronic toxicity data, it has also become necessary to utilize more specific mechanism-based bioassays (e.g. mutagenic, genotoxic, teratogenic, dioxin-like, and estrogen-like responses; for review, see Hollert et al. 2009). In this way, research on sediment toxicity can gain more comprehensive insights concerning the potential ecotoxicological hazard potential of sediments and resulting SPM.

1.1.2 Integrated sediment assessment

For the assessment of aquatic sediments with respect to their possible adverse effects, neither biotests nor chemical-analytical techniques alone are sufficient. Thus, a need for integrated and hierarchical approaches combining chemical, ecotoxicological, and ecological

information has been proposed (Ahlf et al. 2002, Burton 1991, Chapman 2000, Heise & Ahlf 2002). As a consequence, Chapman (1990) introduced the Sediment Quality Triad (SQT) approach which simultaneously investigates sediment chemistry, sediment toxicity, and sediment ecology (e.g. modifications of benthic community structure). These three so called lines-of-evidence are considered as the original components of the SQT and are, furthermore, combined to reach conclusions based on the degree of risk indicated by each measurement and the confidence in each assessment. In the context of ecological risk assessment (ERA) of contaminated sediments, the SQT provides a weight-of-evidence framework (Chapman 2000) comprising a determination related to possible ecological impacts based on multiple lines-of-evidence. It has been proposed to include hydrodynamics as an additional line-of-evidence to assess the environmental impact of contaminated sediments (Chapman & Hollert 2006). Since then, the combination of hydrodynamics and ecotoxicology, as a novel approach, has become a promising field in environmental research (Brinkmann et al. 2010b, Hollert et al. 2008, Wölz et al. 2009).

Another example of a powerful combination of biological testing and instrumental chemical analyses is the concept of bioassay or effect-directed analysis (EDA, Brack 2003). This sequential procedure combines physicochemical fractionation, biological testing and subsequent instrumental chemical analyses (Brack 2003, Hecker & Hollert 2009) that ultimately leads to the identification of the biologically active and, thus, critical substance classes or possibly single substances. Effect-directed analyses possess high potential for the causal analysis of complex environmental impacts - In particular when applying acute as well as mechanism-specific biological endpoints in combination with *in situ* investigations in the context of weight-of-evidence studies. Such approach may provide important inputs and appropriate action plans for managing highly contaminated rivers and streams (Hollert et al. 2009).

1.1.3 Sediment mobility, flood risk assessment and potential impacts of climate change

Fluvial sediments mainly originate from the erosion of terrestrial surfaces or the open channel itself and are transported in coastal direction, ultimately leading to sedimentation in the estuary and the oceans. The sediment bed in rivers can be stable over remarkably long periods. However, increased discharge, e.g. after heavy rainfall, can cause increased bed shear stress and thus the resuspension of sediments, potentially leading to the distribution onto natural floodplains and artificial flood retention areas (Figure 1.2).

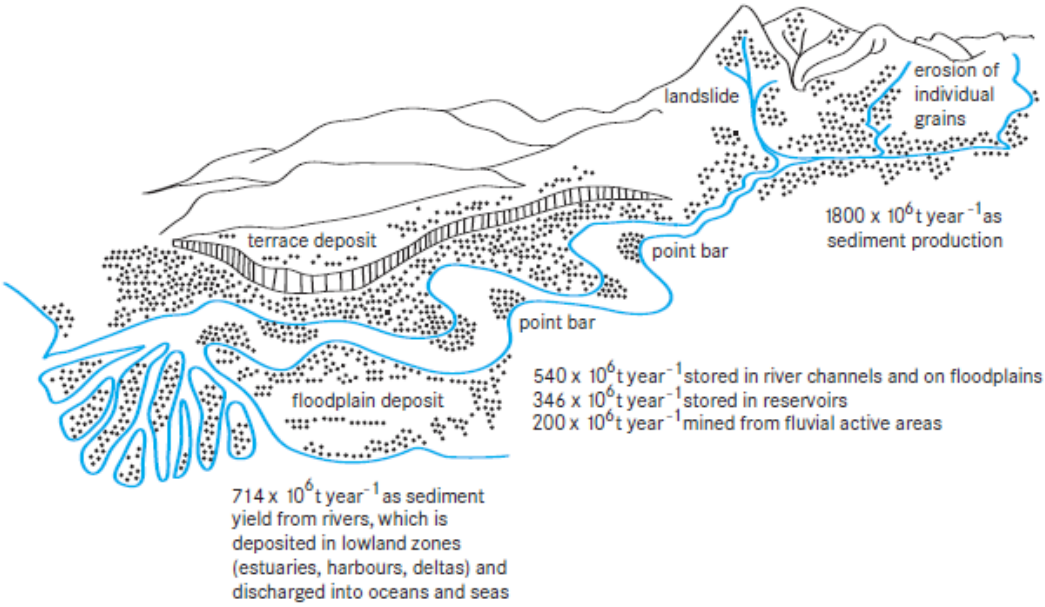

Figure 1.2 Estimated sediment budget for Europe as provided by Owens & Batalla (2003) with modifications after Salomons & Brils (2004).

The various inorganic and organic constituents of sediments, e.g. clay minerals, humic substances, and extracellular polymeric substances (EPS), provide a large number of binding sites for contaminants (Calmano et al. 1993, Gerbersdorf et al. 2011, Gerbersdorf et al. 2009). Consequently, suspended sediment particles may accumulate pollutants from the water column and, thereafter, constitute a secondary source of contamination during dredging or (extreme) flood events (Ahlf et al. 2002, Hollert et al. 2000, Hollert et al. 2003, Wölz et al. 2010). The assessment of sediment stability and the associated uncertainty has meanwhile been identified as an important and emerging aspect (Hollert et al. 2008) that should be further implemented in the European Water Framework Directive (Brinkmann et al. 2010b, Hollert et al. 2007).

In general, globally intensified water cycling (e.g. intensified precipitation events) is expected as an apparent result of global climate change, which will also result in a significantly increased flood risk (Hulme et al. 2002, Wilby et al. 2006). Frequency and intensity of flood events are expected to increase (Ikeda et al. 2005, Kay et al. 2006). Consequently, disastrous flood events such as the 500-year flood at the River Elbe in 2002 would be more frequent in future (Schüttrumpf & Bachmann 2008). In order to manage the impacts of extreme flood events, many countries currently started to allocate additional retention areas, e.g. the „Integrated Rhine Program (IRP)“ along the River Rhine in Baden-Wuerttemberg (Disse &

Engel 2001). Despite building and construction efforts to minimize damages by the flood wave itself, the transported pollutant load remains as an additional concern – especially in light of the conflicting interests between flood retention and, e.g. drinking water production or agriculture (Maier et al. 2005). There are several methods which have been developed by hydraulic engineers to determine the critical bed shears stress for erosion τ (N m^{-2}), i.e. the bottom shear stress at which mass erosion of the sediment layer occurs, e.g. annular flumes (e.g. Spork et al. 1998, Spork et al. 1994), the SETEG flume (Haag et al. 2001, Kern et al. 1999), the microcosms developed by Gust and co-workers (Thomsen & Gust 2000), and the cohesive strength meter (CSM, Tolhurst et al. 1999) for *in situ* or laboratory measurements. To date, however, no standardized protocols exist for conducting exposure experiments with sediment suspensions. Although, many authors have demonstrated that testing of unsuspended sediments is only representative for average flow conditions and does not allow for the prediction of the effects of sediment resuspension (e.g. Aardema & MacGregor 2002, de Carvalho et al. 1998, Ellis et al. 2002, Jürgens et al. 2009, Nendza 2002, Sved & Roberts 1995). Thus, methods have to be developed for conducting exposure experiments with sediment suspensions under realistic conditions in order to derive data for a more comprehensive risk assessment.

Besides the occurrence of extreme flood events, it is alarming that temperatures in German rivers constantly increased during the past decades. In the case of the River Rhine by 3°C, of which a fraction of 2°C is thought to be attributed to the waste heat of power plants and 1°C to climate change (IKSR 2004). The number of days per year with temperatures exceeding 23°C rose from less than 20 in the 1960s to 40-50 in the 1990s and even days with temperature exceeding 25°C have more frequently been observed. Not only the metabolic rate of organisms but also the desorption rate of pollutants from sediments are temperature-dependent. As in the case of dissolved pollutants, it has consequently been shown that sediment toxicity can significantly vary with varying temperatures (e.g. Airas et al. 2008, Heinonen et al. 2002, Honkanen & Kukkonen 2006). Thus it seems plausible to expect that toxicity of sediment-bound contaminants could also be modified as a consequence of climate change.

1.2 Polycyclic aromatic hydrocarbons (PAHs) in the aquatic environment

1.2.1 Origin and traceability

PAHs are a unique class of environmental pollutants which comprises numerous individual substances with two or more fused aromatic hydrocarbon rings (Figure 1.3). They can originate from four different sources: pyrogenic, petrogenic, diagenetic and biogenic, the latter being relatively insignificant (Burgess et al. 2003a).

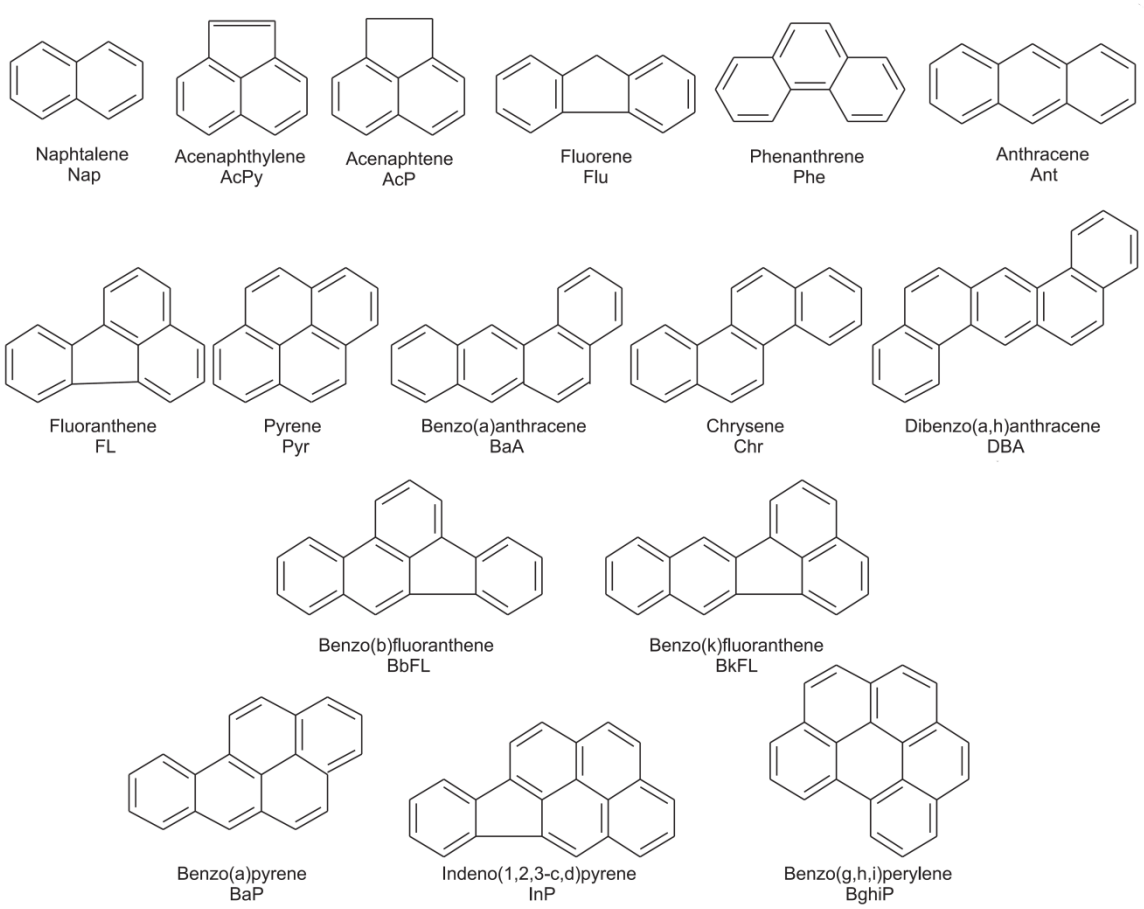

Figure 1.3. The sixteen PAHs originally defined as priority pollutants by the Environmental Protection Agency of the United States of America (EPA-PAHs). From Coelho et al. (2008), modified.

Pyrogenic PAHs originate from the incomplete combustion of organic matter at high temperatures and are usually non-alkylated (Neff 1979), while the mostly alkylated petrogenic PAHs are created at relatively low temperatures (approx. 150°C) over geologic time scales and occur in petroleum and other fossil fuels (Meyers & Ishiwatari 1993). Diagenetic PAHs

originate from biogenic precursors, e.g. terpenes from plants, leading to products such as retene or perylene (Gschwend et al. 1983, Hites Ronald et al. 1980). Due to the many different sources, PAHs are ubiquitously distributed and occur even in remote areas (Burgess et al. 2003a). To identify the potential origin of PAHs from a given polluted site, molecular indices have been developed. A high ratio of alkylated PAHs to non-alkylated PAHs gives evidence for pollution with petroleum derived hydrocarbons, while a high ratio of high molecular weight (HMW) PAHs to low molecular weight (LMW) PAHs is indicative for contamination with pyrogenic PAHs (Mostafa et al. 2009).

1.2.2 Partitioning of PAHs in sediment-water systems

For PAHs as for many other substances, total concentration in sediment does not necessarily reflect the readily available fraction for uptake in aquatic organisms. It has been observed, that either bioaccumulation or toxic effects of PAHs correlate better with the freely dissolved concentration in interstitial water than with the total sediment concentration (Burgess et al. 2003b). The dispersion of PAHs in the aquatic environmental has often been described by the equilibrium partitioning (EqP) model, which can be used to describe the partitioning of substances between two environmental compartments at equilibrium conditions (Chiou et al. 1979). Equation 1.1 gives the most important descriptor of the EqP model, the partitioning coefficient (K_p) (L kg^{-1}), where C_p (µg kg^{-1}) and C_d (µg L^{-1}) are the equilibrium concentrations in the particulate and dissolved phase, respectively.

$$K_p = \frac{C_p}{C_d} \qquad \text{(Equation 1.1)}$$

PAHs are usually rather associated with the organic matter fraction of sediment than with the inorganic particles. Normalizing K_p by the particulate organic carbon concentration (f_{oc}) (kg organic carbon kg^{-1} sediment) thus usually reduces the variability of K_p for one substance between different sediments, resulting in the K_{oc} (L kg^{-1} organic carbon, Equation 1.2) (Burgess et al. 2003b).

$$K_{oc} = \frac{K_p}{f_{oc}} \qquad \text{(Equation 1.2)}$$

Since experimental determination of K_p and K_{oc} is relatively costly and difficult, it seems fortunate that there is a linear relationship for both values with the easily measured octanol-water partitioning coefficient K_{ow} (Karickhoff et al. 1979). The partitioning coefficients, as well as the water solubility of selected PAHs are given in Table 1.1.

Hydrophobic organic pollutants do not remain completely available for partitioning (and uptake) after contamination (Alexander 2000). During the process of sequestration, these chemicals bind to the matrix and form bound residues. This phenomenon (often termed ageing) leads to decreased availability for uptake into organisms (Cornelissen et al. 1998, Menchai et al. 2008, e.g. Puglisi et al. 2009, Reid et al. 2000, Xu et al. 2008) and has thus to be considered when working with spiked sediments and in risk assessment of sediments (Zielke 2011).

Table 1.1 Molecular weight (MW), water solubility (*S*), octanol-water (K_{ow}) and organic carbon normalized (K_{oc}) partitioning coefficients for selected PAHs as given by Burgess et al. (2003b).

PAH	MW	Log *S* (mol L^{-1})	Log K_{ow}	Log K_{oc}
Fluorene	166	-4.94	4.21	3.8
Phenanthrene	178	-5.21	4.57	4.2
Anthracene	178	-6.60	4.53	4.1
Pyrene	202	-6.19	4.92	4.8
Fluoranthene	202	-5.93	5.08	4.8
Chrysene	228	-8.06	5.71	5.4
Benzo[*a*]anthracene	228	-7.32	5.67	5.5
Benzo[*a*]pyrene	252	-7.82	6.11	6.1
Dibenzo[*a,h*]anthracene	278	-8.67	6.71	6.8

1.2.3 Uptake and metabolism of PAHs in fish

Sandvik et al. (1998) systematically compared the distribution patterns of radiolabelled benzo[*a*]pyrene in rainbow trout (*Oncorhynchus mykiss*) following aqueous and dietary exposure, respectively, and found substantial differences between the main target organs (Figure 1.4). After aqueous exposure, intense radiolabelling was observed in the olfactory organ, gills, liver, bile, kidney, urine, intestinal tract and skin. In contrary, following dietary exposure signal was strong in the gastrointestinal tract and bile only. Furthermore, the

systemic availability of benzo[*a*]pyrene (assessed as the total radioactivity) was higher after aqueous exposure. Thus, the authors assumed that either absorption via the gills was more efficient or that bioavailability was reduced by adsorption to feed particles, as well as intestinal metabolism (Sandvik et al. 1998). Apart from aqueous and dietary exposure to PAHs, Namdari & Law (1996) compared the differences between branchial and cutaneous exposure. They found that, similar to many lipophilic substances, both absorption through skin and gills were important processes, the latter being approximately 11-fold faster and therefore more relevant. Similar results were also found for the uptake of other lipophilic organic compounds, with the importance of each uptake pathway varying between substances and fish species (Di Giulio & Hinton 2008).

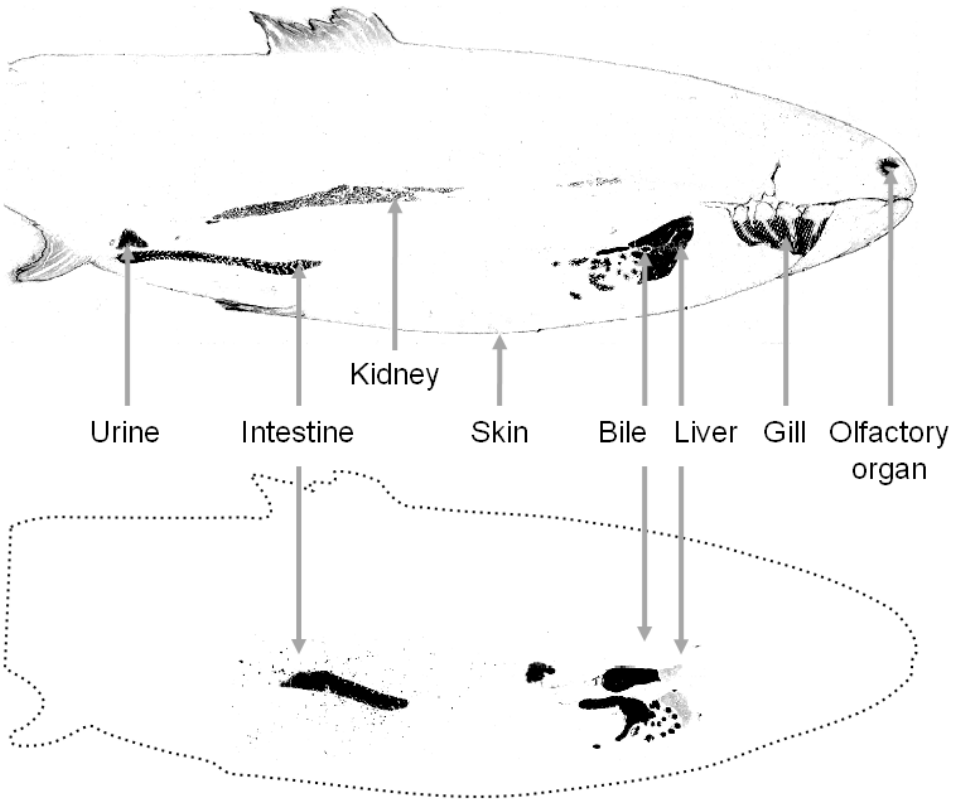

Figure 1.4 Autoradiographic images of rainbow trout 2 days after exposure to ^{3}H-benzo[*a*]pyrene (1.5 mCi kg^{-1}) in water (top) or 2 days after intragastric administration of the same dose (bottom). Black areas correspond to high concentrations of radiolabelled compound. The dotted line represents the anticipated outline of the animal in the bottom figure. After aqueous exposure, strong radiolabelling was observed in the olfactory organ, gills, liver, bile, kidney, urine, intestinal tract and skin. After intragastric administration, radiolabelling was strong in the gastrointestinal tract and bile only. Modified from Sandvik et al. (1998).

Following uptake and distribution of PAHs in fish, they are rapidly metabolized and eliminated from the different tissues (Varanasi et al. 1989). Figure 1.5 shows the complex biotransformation pathway of benzo[*a*]pyrene, which is mediated by a diverse suite of enzymes. *Phase I* reactions (mediated by cytochromes P450, oxidases, dehydrogenases, hydrolases and further enzymes) comprise oxidation, reduction, and hydrolysis, to either expose small hydrophilic groups of a molecule or add such groups to the molecule, thereby increasing its water solubility and facilitating elimination (Andersson & Förlin 1992). In most cases, this process leads to detoxication of PAHs. However, phase I oxidation of some compounds, e.g. benzo[*a*]pyrene, can lead to the formation of highly reactive metabolites (diol epoxides) that can bind DNA leading to mutagenic effects (Figure 1.5). In *phase II* reactions, the xenobiotics are conjugated with bulky endogenous substrates (e.g., glycosides, peptides) by transferases (e.g., glutathione-*S*-transferases, sulfotransferases and UDP-glucuronosyltransferases) to further enhance polarity prior to biliary or urinary excretion (Di Giulio & Hinton 2008).

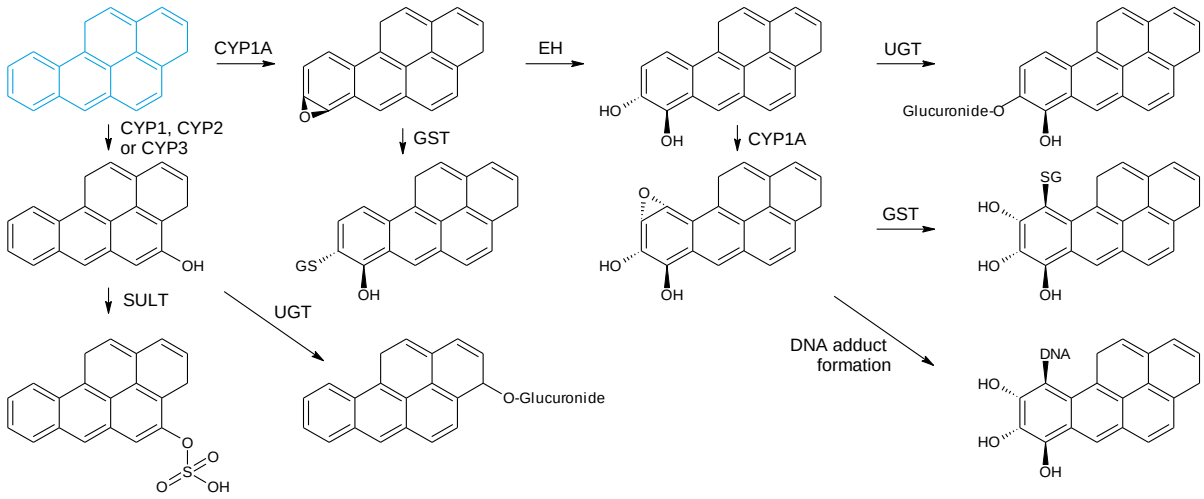

Figure 1.5 Biotransformation pathway of the polycyclic aromatic hydrocarbon benzo[*a*]pyrene (blue). The acronyms CYP, EH, GST, SULT and UGT refer to cytochromes P450, epoxide hydrolases, glutathione-*S*-transferases, sulfotransferases and UDP-glucuronosyltransferases, respectively. Redrawn from Di Giulio & Hinton (2008).

1.2.4 Effects of PAHs in marine and freshwater fish

Unlike many organochlorine chemicals or metals, PAHs are readily metabolized and do not significantly accumulate in fish tissue (see section 1.2.3). Thus, the risk of PAHs to cause adverse effects without leaving any chemical signature is relatively high – a phenomenon that Payne et al. (2003) call "hit and run" potential. PAHs exhibit moderate to high acute and chronic toxicity in fish. Due to reduced emission rates, their comparably low water solubility and high affinity to organic matter in sediment-water systems (cf. Table 1.1), PAHs at present seldom occur at concentrations causing acute toxicity (Tuvikene 1995). Nevertheless, this substance category is still critical to marine and freshwater fish due to sub-lethal effects that have been observed in decades of laboratory and field studies (Chen & White 2004, Payne et al. 2003) and is considered as a priority class of pollutants in context of the European Water Framework Directive (Götz et al. 2009).

Biochemical effects have undoubtedly been the most frequently reported responses of fish to PAH exposure, while most of these were related to mixed-function oxygenase (MFO) enzymes of phase I metabolism reactions (Tuvikene 1995). To a lesser extent, phase II reactions or other biochemical alterations, such as changes in serum enzymes or energy reserves, have been studied (Di Giulio & Hinton 2008). For an in-depth discussion on the use of MFO as biomarkers of exposure to PAHs and other dioxin-like compounds, see section 1.3.2.

Histopathological effects provide strong evidence for the damage PAHs can cause in fish. Neoplasms and other tissue and organ abnormalities, skin and skeletal disorders have been linked to PAH pollution by pulp mills, creosote or coke (Payne et al. 2003). Baumann and Harshberger (1985) found that 30 % of brown bullheads (*Ameiurus nebulosus*) from the Black River in Ohio (historically contaminated from the effluents of a coking plant) had neoplasms and hepatocarcinomas. In the close vicinity of a steel plant in Hamilton Harbor (Lake Ontario) and in the Elizabeth River in Virginia, skin and liver tumors have been linked to PAH pollution (Cairns & Fitzsimons 1988, Vogelbein et al. 1990). Several experimental studies with single PAHs, simple and complex mixtures (e.g. creosote, fossil fuels) or even sediment extracts provide additional support of the hypothesis that there is a causal link between histopathological effects and pollution with polycyclic aromatic hydrocarbons (Payne et al. 2003).

Immunological effects (such as antibody production, alterations of phagocytosis of bacteria by white blood cell and hematological changes), **genetic effects** (such as the formation of

micronuclei and DNA adducts, mutations in DNA and strand breaks, oxidative damage to DNA), **reproductive effects** (such as gonadal alterations, changes in plasma steroid concentrations, reproductive dysfunctions, reduced spawning success), **developmental effects** (reduced growth, teratogenicity) and **behavioral effects** (altered feeding and swimming behavior) of PAHs have been demonstrated. For review, see Payne et al. (2003), Tuvikene (1995) and Chen and White (2004).

1.3 Biomarkers used in the present study

1.3.1 PAH metabolites in fish bile

Most polycyclic aromatic hydrocarbons are rather insoluble in aqueous media and, thus, have to be converted into more water-soluble metabolites prior to excretion via bile liquid (Varanasi et al. 1989). Most fish species have highly efficient biotransformation systems (see section 1.2.3) which facilitate the rapid elimination of lipophilic organic compounds (Andersson & Förlin 1992, Leaver et al. 1992), generally resulting in low residual concentrations of PAHs in fish tissues. Thus, tissue concentrations are not useful as a biomarker of exposure to such readily metabolized substances (Melancon et al. 1992). Because aromatic hydrocarbons and their metabolites are highly fluorescent, however, several analytical techniques can applied to quantify their concentrations in fish bile. Most often, semiquantitative fluorometric methods (e.g. fixed wavelength fluorescence, FF) or quantitative chromatographic methods (e.g. high-performance liquid chromatography with fluorescence detection, HPLC-F) are applied (Beyer et al. 2010). Because of the comparably low demands of FF with respect to instrumentation and costs, this method has been applied in many laboratory and field-monitoring studies on marine (Aas et al. 1998, Insausti et al. 2009, Lima et al. 2008, Neves et al. 2007, Vuorinen et al. 2006), as well as freshwater species (Barra et al. 2001, Hanson & Larsson 2007). HPLC-F and other chromatographic methods are applied when the study design requires more detailed information about the chemical species or quantity (Collier & Varanasi 1991, Haugland et al. 2005, Kammann 2007, Maccubbin et al. 1988, Meador et al. 2008). Comparative studies demonstrated that there was generally good correlation between FF and HPLC-F. Absolute values, however, mostly deviated significantly due to matrix effects (Jonsson et al. 2004). A recent review by Beyer et al. (2010) gives a

comprehensive summary of analytical techniques for the detection of biliary PAH metabolites.

The bile liquid is normally secreted into the gut of fish and subsequently excreted with the feces. During longer periods of starvation, however, the bile remains in the gall bladder and water is extracted to concentrate the liquid (Avery et al. 1992). Directly after feeding, however, water is rapidly excreted into the gall bladder, leading to a very dilute bile fluid (Klaassen & Watkins 1984). Therefore, PAH metabolite concentrations are severely affected by the nutritional status of the investigated fish (Richardson et al. 2004). FF values and metabolite concentrations determined by chromatographic methods are thus often normalized against the concentration of an internal standard that is constantly excreted into bile liquid (and consequently also concentrated during starvation periods) to compensate for the influence of nutritional status on bile concentration. The bile pigment biliverdin (Figure 1.6), a byproduct of heme catabolism, e.g., is one such internal standard often used (Avery et al. 1992). Results on the use of biliverdin for normalization purposes, however, have led to contradictory recommendations (Aas et al. 2000, Ruddock et al. 2003). The authors used the reduction of variability in their metabolite data as the only criterion for normalization quality without taking the underlying mechanisms into account. The biliary biliverdin concentration in fish has been furthermore demonstrated to be affected by the modulating effects of environmental pollutants, such as PAHs, on Heme Oxigenase (HO) activity, which catalyzes the conversion of heme to biliverdin (Jørgensen et al. 1998, van den Hurk 2006).

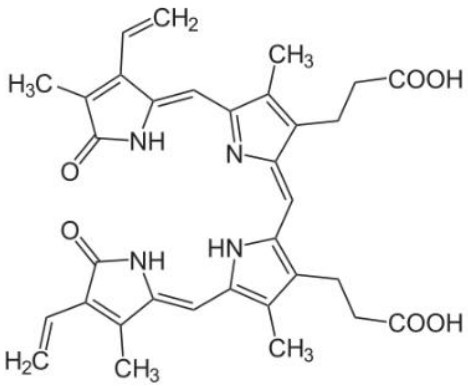

Figure 1.6 Biliverdin is a product of heme metabolism and used to correct biliary metabolite concentrations for the nutritional status of fish.

1.3.2 Ethoxyresorufin-O-deethylase

The cytochromes P450 are a diverse multigene family that has been found in all organisms and is well examined. They cover various functions to metabolize endogenous substances (e.g., steroids and fatty acids) and xenobiotics, respectively (Bernhardt 1996, Whyte et al.

2000). In fish, these proteins are mainly concentrated in the liver, but they are also detectable in the gill and other tissues (Stegeman & Hahn 1994). Cytochrome P450 1A (CYP1A) catalyzes several phase I reactions, such as oxidation, reduction, and hydrolysis, to either expose small hydrophilic groups of a molecule or add such groups to the molecule, thereby increasing its water solubility and facilitating its elimination (Andersson & Förlin 1992). In most cases, this process leads to detoxication of xenobiotics. However, phase I oxidation of some compounds, e.g. the PAH benzo[*a*]pyrene, can lead to the formation of reactive metabolites that can bind DNA leading to mutagenic effects (Huberman et al. 1976).

Induction of CYP1A and other biotransformation enzymes (Figure 1.7) is mediated by the cytosolic aryl hydrocarbon receptor (AhR), a member of the basic helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) family (Pollenz et al. 1996). In the cytosole, the AhR is aggregated with other proteins (Matsumura 1994, Perdew 1992, Whyte et al. 2000). Upon binding of an AhR ligand (e.g., a xenobiotic substance), these proteins are released and the AhR binds to an aryl hydrocarbon nuclear translocation protein (ARNT), resulting in a transcription factor complex that can bind to specific DNA regions, called xenobiotic/dioxin responsive elements (X/DRE). Binding of the heterodimer promotes the transcription of many genes (Ah gene battery), which are subsequently translated into several gene products, including CYP1A and phase II biotransformation enzymes (Nebert et al. 1993).

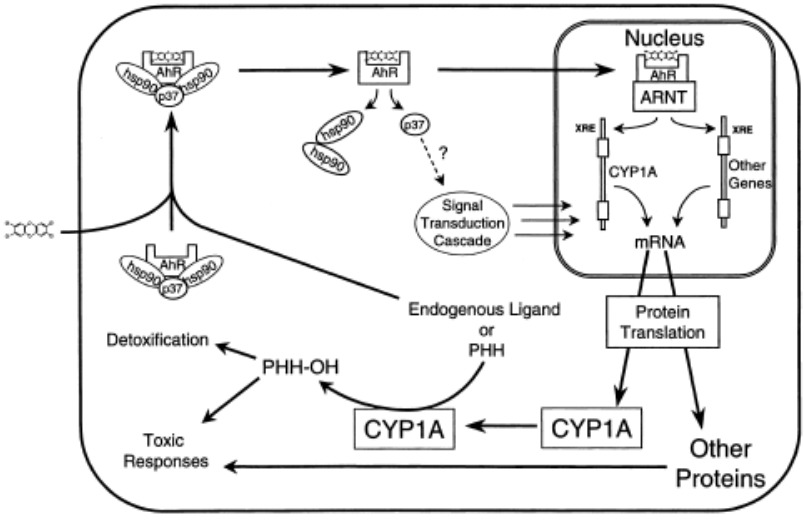

Figure 1.7 Proposed mechanism of AhR-mediated toxicity. Binding of dioxin-like ligands to the AhR results in the formation of a transcription factor complex with an aryl hydrocarbon nuclear translocator protein (ARNT) that binds to specific DNA sequences, xenobiotic/dioxin responsive elements (X/DRE). Binding of the dimer promotes the transcription of many genes (Ah gene battery), which are subsequently translated into several gene products, including the cytochrome P450 1A (CYP1A) subfamily of monooxygenases. (From Whyte et al. 2000)

Due to the inducibility of CYP1A following exposure to Ah receptor agonists this enzyme is a suitable biomarker exposure to planar halogenated hydrocarbons (PHH) and PAHs that has been proposed for biomonitoring purposes over 20 years ago (Payne & Penrose 1975). Meanwhile, many enzyme activities of CYP1A, including EROD, benzo[*a*]pyren hydroxylase (BaPH), and aryl hydrocarbon hydroxylase (AHH), have been studied (Whyte et al. 2000), that can be used as a measure of CYP1A induction. In this study, the EROD activity in liver was measured by quantifying the deethylation of the exogenous substrate 7-ethoxyresorufin to the fluorescent product resorufin (Figure 1.8), as described by Kennedy & Jones (1994).

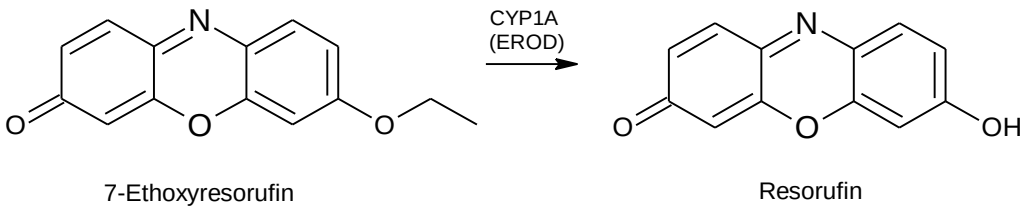

Figure 1.8 The 7-ethoxyresorufin-O-deethylase activity is a catalytic measurement of cytochrome P450-dependent monooxygenase induction (specifically the CYP1A subfamily). The fluorescent product resorufin is quantified by EROD bioassays.

1.3.3 Lipid peroxidation

Eukaryotic cells are dependent on molecular oxygen for the supply of energy via phosphorylation of adenosine diphosphate, thereby coupling oxidation to energy transfer. This process is controlled by the mitochondrial electron transport chain in which oxygen undergoes concerted 4 electron reduction to water. As a consequence, various partly reduced reactive oxygen species (ROS) are generated (Winston & Di Giulio 1991). The hydroxyl radical (OH•) is one of the most potent oxidants known, being capable of reacting with virtually any organic molecule, including cellular macromolecules, e.g. DNA, proteins and lipids (Figure 1.9). Protein degradation, DNA damage, lipid peroxidation and ultimately cell death may result from these reactions (Borg & Schaich 1984). However, the degree of physiological damage caused by oxyradicals is reduced by a number of antioxidant defense mechanisms (Diguiseppi & Fridovich 1984).

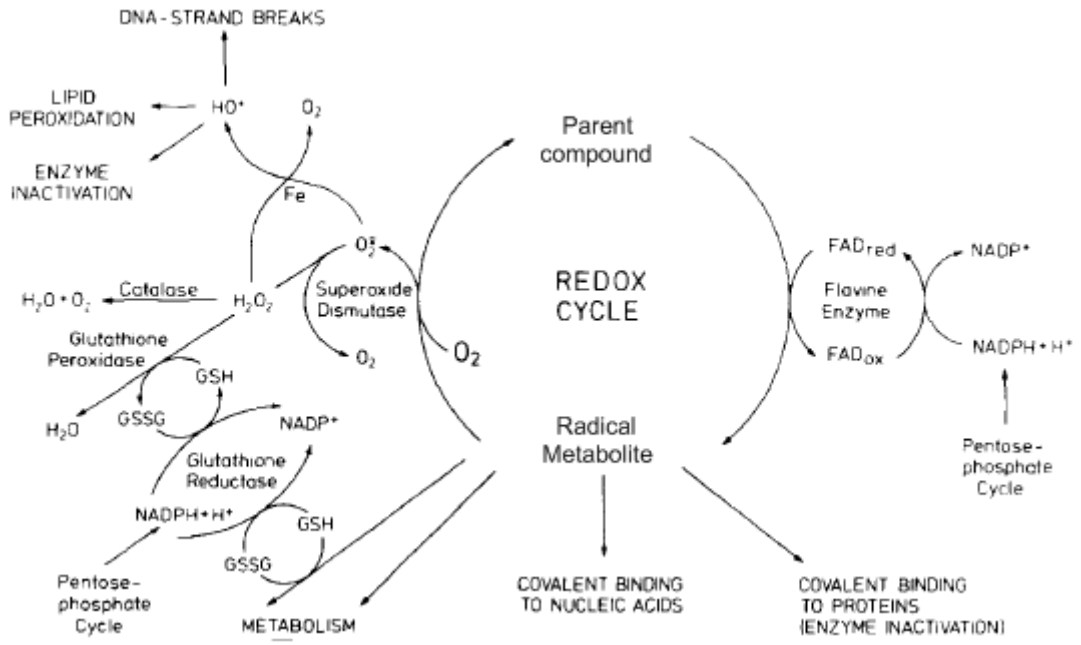

Figure 1.9 Generation of reactive oxygen species (ROS) by redox cycling, as well as key enzymatic antioxidant defences and cellular targets of ROS. From Kappus (1987) with modifications from Di Giulio & Hinton (2008).

Thus, oxidative damage reflects an imbalance between the production of oxidants and the removal of such reactive species. Specially adapted enzymes, such as superoxide dismutase (SOD) that reduces the superoxide anion to hydrogen peroxide, and catalase (CAT) that reduces hydrogen peroxide to water, protect the organism from oxidative damage (Winston & Di Giulio 1991). Many environmental contaminants (e.g. metals, nitroaromatics, quinones, biphenyls) and their metabolites are known to cause toxic effects via xenobiotic-enhanced oxyradical formation (van der Oost et al. 1996, Winston & Di Giulio 1991). Benzo[*a*]pyrene has been shown to be converted to an 1,6-quinone metabolite in fish *via* the precursor 1-hydroxybenzo[*a*]pyrene (Di Giulio & Hinton 2008). Lemaire et al. (1994) reported the generation of hydroxyl radicals by benzo[*a*]pyrene quinones in hepatic microsomes of flounder and perch.

The inductions of antioxidant defence enzymes, as well as oxidative damage itself, have been proposed for biomonitoring purposes. While studies investigating the effects of environmental contaminants on the marker enzymes SOD and catalase are often considered inconclusive (van der Oost et al. 1996), markers for oxidative damage, e.g. lipid peroxidation, are widely used in field studies (e.g. Almroth et al. 2008, Oliveira et al. 2009, Pandey et al. 2008). Figure 1.10 illustrates the formation of malondialdehyde (MDA) as a terminal product of the

lipid peroxidation process that can be used as endpoint for monitoring purposes in various species and tissues.

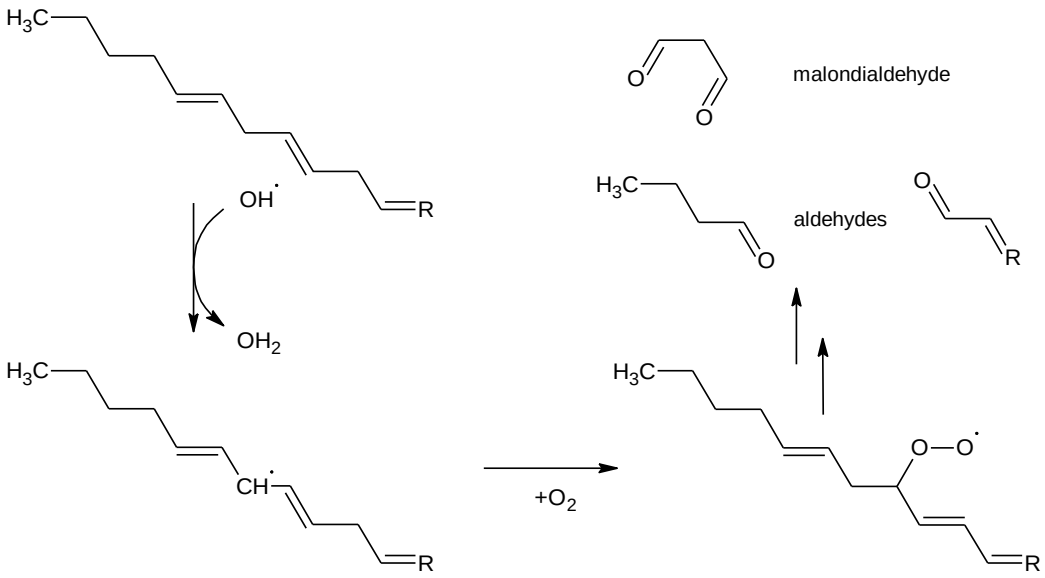

Figure 1.10 The biochemical process of hydroxyl radical-initiated lipid peroxidation. Redrawn and modified from Di Giulio & Hinton (2008).

1.3.4 Real-time RT-PCR measurement of hepatic gene expression

In addition to the investigation of biological effects on the protein, enzyme activity or even metabolic level, the assessment of potential effects of environmental pollutants on the transcript level emerged to a field with impressive research activity during the past years (Hallare et al. 2011). The wide range of biomarker studies applying gene expression analyses on fish species was reviewed in Tom and Auslander (2005) and (Hallare et al. 2011). A large set of modern molecular methods is available to environmental scientists, while the two most frequently used are (1) microarray techniques for multi-gene screenings and (2) quantitative real-time reverse-transcription (RT) PCR that was also applied in the present study. Furthermore, next generation sequencing techniques allow for the rapid determination of the whole transcript. Schirmer et al. (2010) have reviewed the current direction in this rapidly developing field and found a number of knowledge-gaps still to bridge. First, the enormous amounts of data that can be generated need to be appropriately evaluated and statistically judged. Second, aside from the enormous potential, the question remains if a transient expression of certain genes finally leads to an adverse outcome.

1.4 The projects Floodsearch and Floodsearch II

The "Pathfinder" project Floodsearch at RWTH Aachen University (funded by the German Excellence Initiative) has made the first attempt to experimentally link hydrodynamic questions with ecotoxicology to assess the ecological relevance of resuspension events to aquatic ecosystems (Wölz et al. 2009). In this new interdisciplinary approach, rainbow trout had been exposed to artificial sediment (unspiked and spiked with PAHs) under simulated flood-like conditions in an annular flume (Figure 1.11), originally developed for the investigation of erosion and deposition processes (Schweim et al. 2001, Wölz et al. 2009).
Following exposure for 5 days, a set of different biomarkers in the test organisms was investigated (Brinkmann et al. 2010a). The set included measurements on the protein level (Western immunoblot analysis of Cytochrome P450 1A concentrations, 7-ethoxyresorufin-*O*-deethylase activity, glutathione-*S*-transferase activity, catalase activity), as well as biochemical (lipid peroxidation) and cellular alterations (micronucleus formation). Furthermore, concentrations of PAH metabolites in the bile fluid of exposed animals were measured. Experimental results from this initial study supported the assumption that remobilization of sediments during simulated flood events in the annular flume can lead to uptake and biological effects of sediment-bound contaminants. In particular the average micronucleus frequency was significantly elevated after exposure to spiked sediments compared to exposure without sediments and positively correlated with the biliary concentrations of 3-hydroxybenzo[*a*]pyrene, which is a metabolite of the indirect genotoxicant benzo[*a*]pyrene.
The results of the study have shown that relatively short exposure to resuspended sediments during simulated flood events can lead to – potentially adverse – alterations of biological functions in rainbow trout. Thus, the ecological and toxicological impacts of contaminant remobilization during flood events have to be considered highly relevant. Integrated approaches for risk assessment of regularly flooded rivers are urgently required.
This thesis is part of the follow-up project Floodsearch II, which was intended to scientifically broaden the scope of Floodsearch by investigating both spiked and unspiked natural sediment suspensions under different environmental conditions (e.g., pH and temperature) in the annular flume. Furthermore, the dynamics of PAH uptake and biomarker responses in rainbow trout during exposure to contaminated sediment suspensions and a more mechanistic understanding of these processes were in the focus. To this end, this M.Sc. thesis is complemented by the Diploma thesis of Dipl.-Biol. Jochen Kuckelkorn, who conducted

similar exposure experiments with copper-spiked sediment, a B.Sc. thesis by Michael Hennig, who investigated micronuclei and a set of hepatic enzyme activities in samples from this thesis, and the Ph.D. projects of Dipl.-Biol. Sebastian Hudjetz, who works on the fate of PAHs in sediment-water systems and passive sampling techniques, and Dipl.-Ing. Catrina Cofalla, who works on sediment characteristics and stability (Cofalla et al. 2011).

Figure 1.11 Annular flume located at the Institute of Hydraulic Engineering and Water Resources Management, RWTH Aachen University, Germany (Photograph provided by Dipl.-Ing. Catrina Cofalla).

1.5 Aims and objectives

The initial objective of the present study was to:

1. Examine the kinetic processes of uptake, metabolism and effects in rainbow trout exposed to spiked and unspiked natural sediment suspensions to improve the understanding of the proof-of-concept results given by Brinkmann et al. (2010a).
2. To improve the common set of biomarkers used in other studies by either applying new markers (i.e., hepatic gene expression analyses and fixed wavelength fluorescence of bile samples) or optimizing experimental protocols for the determination of biomarkers (i.e., hepatic 7-ethoxyresorufin-*O*-deethylase activity).

More specifically, the following questions were addressed in the present study:

(a) Can rainbow trout be exposed to sediment suspension in simple resuspension tanks and at different temperatures?

Animals within the project Floodsearch were exposed during simulated flood events in an annular flume, i.e. under relatively sophisticated exposure conditions. Apart from the obvious advantages of such realistic exposure conditions, there is one major drawback: The flume cannot be opened during the experiments, thereby limiting the investigation of exposed fish to one point in time. To derive important information about the temporal behavior of the investigated biomarkers, however, it appeared necessary to conduct kinetic experiments. To this end, a simplified exposure setup was chosen, comprising 750 L glass fiber-reinforced plastic containers with a submersible pump to keep particulates in suspension. Furthermore, the use of submersible coolers was evaluated to stabilize and control the temperature during exposure.

(b) Can the dissipation of PAHs from these sediment-water systems be adequately monitored and described?

In context of the project Floodsearch, a methodology for monitoring PAH concentrations during experiments in the annular flume was developed by Dipl.-Biol. Sebastian Hudjetz. Consequently, this methodology was also applied in the current study to monitor the concentration dynamics of the target analytes. Since it was not possible to expose rainbow trout to sediment suspensions under semi-static or flow-through conditions, the exact knowledge of changes in compound concentrations during the experiments was of vital importance.

(c) What are the differences between the bioavailability and uptake kinetics of freshly spiked and naturally aged sediment-bound PAHs?

It is well-known that substantial differences in desorption rates and bioavailability of organic contaminants can be observed between freshly spiked and naturally aged sediments (Reid et al. 2000). For this reason, a direct comparison between a natural sediment with moderate PAH contamination was investigated in exposure experiments either unspiked or spiked with a mixture of additional PAHs to evaluate the comparability for further experiments.

(d) How do the biomarkers of exposure and biological effects respond during the exposure to unspiked and spiked sediment suspensions?

Since it was not possible to open the lid of the annular flume during experiments in context of the project Floodsearch, only rather qualitative information on biomarkers of exposure and biological effects was derived from one point in time before and one after the simulated flood events. In the exposure experiments of this study, the biomarker kinetics were monitored during 12 days to provide more detailed insights in the induction kinetics of the investigated biomarkers.

(e) How does temperature (stress) influence uptake and effects of PAHs from sediment suspensions?

Since degradation, bioavailability and effects of particle-bound pollutants can substantially vary with temperature, exposure experiments were conducted at an average temperature representative for German rivers (12°C) and under nowadays frequently occurring temperature stress conditions (24°C, IKSR 2004) to capture a range of possible consequences of sediment resuspension.

(f) Can the analytical determination of biliary PAH metabolites by means of HPLC-F be substituted by fixed wavelength fluorescence measurements?

Determination of PAH metabolites in fish bile by means of chromatographic methods, e.g. the HPLC-F method applied in the experiments of Floodsearch, is costly and requires sophisticated analytical instrumentation. To evaluate the possibility to replace this method by the less demanding fixed wavelength fluorescence method previously described, both methods were compared using samples from two different experiments.

(g) How can the current method for quantification of hepatic 7-ethoxyresorufin-*O*-deethylase activity be optimized for the determination in S9 fractions?

The method for measuring EROD activity of Kennedy and Jones (1994) applied in Floodsearch was originally designed for use with liver microsomes. In the current experimental design, however, the use of S9 fractions appeared useful because a number of biomarkers can be determined in this subcellular fraction. In order to optimize the original EROD protocol, which can be incorrect or inapplicable due to quenching or auto-fluorescence of S9 samples, a modified approach is presented where the resorufin

fluorescence in incubated samples is not corrected for the background fluorescence of the reagent mixture without samples, but for background fluorescence of the reagent mixture with samples but without enzymatic reaction.

(h) Is the analysis of hepatic gene expression by means of real-time RT-PCR a suitable tool for earlier detection of the effects of sediment-bound contaminants?

In the experiments of Floodsearch it was shown that the more "traditional" biomarkers of exposure to dioxin-like compounds, i.e. EROD and glutathione-*S*-transferase (GST), were not suitable for the detection of exposure to particle-bound PAHs after 5 d exposure in simulated flood events. It was hypothesized that investigation of markers on the transcript level, i.e. gene expression analyses, might be a more applicable tool to earlier detect changes (Tompsett et al. 2009, Zhang et al. 2008). Therefore, preserved liver tissue samples from the Floodsearch project were analyzed for the mRNA expression of a number of genes to verify the applicability of these molecular methods.

2. Materials and methods

2.1 Kinetic study: Uptake and effects of PAHs from sediment suspensions

2.1.1 Experimental design

Juvenile rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) were exposed to suspensions of a natural sediment from the River Rhine (see section 2.1.2), either unspiked or spiked with a mixture of the following PAHs, which were purchased from Sigma-Aldrich (Deisenhofen, Germany): pyrene (purity ≥ 99%, 4.1 mg kg^{-1}), phenanthrene (purity 98%, 5.0 mg kg^{-1}), chrysene (analytical standard, 3.3 mg kg^{-1}), and benzo[*a*]pyrene (purity ≥ 96%, 8.3 mg kg^{-1}). Experiments were conducted in 750 L glass fibre-reinforced plastic containers (Figure 2.1, AGK Kronawitter, Wallersdorf, Germany) in which submersible pumps (rated power 220 W, maximum flow-through 6000 L h^{-1}) were used to constantly suspend the sediments at a nominal concentration of 10 g L^{-1}. Additionally, the tanks were aerated at a rate of 25 L min^{-1}. While temperature was at average 24 °C in the first experiment, it was systematically controlled in the second experiment and kept at an average of 12°C. Tanks were cooled using immersible coolers (Colora Tauchkühler, Germany) controlled by analog plug-in thermostats (UT100, Fuva, Erlangen, Germany).

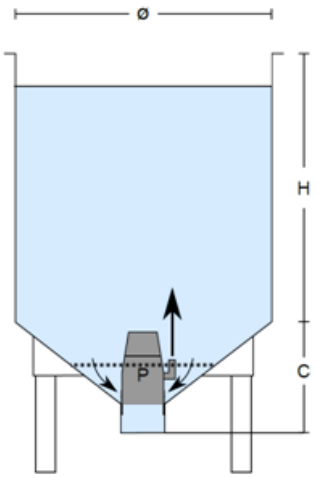

Figure 2.1 Resuspension tanks used for exposure of fish to sediment suspension (P: Pump, Height H: 145 cm, Cone C: 40 cm, Diameter Ø: 80 cm).

In each of the two experiments, (1) physicochemical water parameters, (2) sediment suspensions and (3) fish (n=10) were sampled after 0, 1, 2, 4, 6, 8, and 12 days, respectively. Sediment suspensions were used to determine concentrations of suspended particulate matter (SPM) and PAH in SPM. Several biomarkers (i.e., 7-ethoxyresorufin-*O*-deethylase activity and lipid peroxidation in liver, PAH metabolite concentrations in bile liquid) as well as morphometric indices were assessed in exposed fish.

2.1.2 Rainbow trout

Immature rainbow trout (15-20 g) were purchased from a commercial hatchery (Mohnen Aquaculture, Stolberg, Germany) and allowed to acclimatize to laboratory conditions for at least 2 months prior to the experiments. Fish were reared in lots of 100-150 individuals in 1500 L glass fibre-reinforced plastic tanks at RWTH Aachen University, Institute for Environmental Research, Aachen, Germany. In a recirculating system with a 400 L biofilter and UVC-sterilizer, water was continuously exchanged at a rate of 0.1-0.2 d^{-1} with municipal tap water. Light and dark phases were 12 h each. Fish were fed commercial trout pellets (Ecolife 20, 3 mm, Biomar, Brande, Denmark; crude protein 45 %, crude lipid 28 %, fibre 1.7 %, ash 7.0 %) at a rate of 1-2 % bodyweight per day until experimentation. Fish were used for the present study in accordance to the Animal Welfare Act and with permission of the federal authorities (Landesamt für Natur, Umwelt und Verbraucherschutz NRW), registration number 8.87-50.10.35.08.225.

2.1.3 Sediment sampling and spiking

The sediment used throughout the present study was sampled in April 2010 at the River Rhine (river kilometre 591) close to the fortress Ehrenbreitstein, Koblenz, Germany (+50° 21' 12" N, +7° 36' 27" E) in cooperation with the German Federal Institute of Hydrology (BFG, Koblenz, Germany). A surface sample (approx. 100 kg) was taken using a Van Veen grab (Hydrobios, Kiel, Germany) and subsequently stored at 4°C in darkness prior to experiments. Physicochemical parameters of sediment and water were recorded (Table 2.1).

Table 2.1 Basic physicochemical descriptors of sediment and water sampled at the harbour of Ehrenbreitstein, Germany.

	Temperature / °C	**pH / arbitrary units**	**Conductivity / µS cm^{-1}**	**Dissolved O_2 / mg L^{-1}**	**Redox potential / mV**
Sediment	11.7	7.43	-	-	-275
Water	12.0	7.50	459	11.0	-

The sediment was spiked according to OECD guideline 218 (OECD 218 2004). Briefly, 10 % of the sediment used per experiment were dried at 105 °C and thoroughly crushed. For

spiking of the sediment, PAHs were dissolved in a mixture of 350 ml *n*-hexane and 150 ml acetone, and added to the dried sediment. After complete evaporation of the solvent, water was added to obtain the original water content of natural sediment. The spiked portion (EBR + PAH) was added to the bulk of sediment (90 %), thoroughly mixed, incubated for 7 d at 4°C in darkness, and mixed again prior to the experiments. Unspiked sediments (EBR) were handled as controls and treated as the spiked portion except for the addition of PAHs. After incubation, the sediment mixtures were transferred to the exposure tanks to obtain a nominal suspended matter concentration of 10 g L^{-1}.

2.1.4 Sampling of suspended particulate matter (SPM) and GC-MS-analysis of PAH concentrations

Chemical analyses and sample preparation were performed by Dipl.-Biol. Sebastian Hudjetz, Institute for Environmental Research, Aachen. Sediment suspension samples were taken as 1 L duplicates and centrifuged for 30 min (4500 × g, 4 °C) in a cooling centrifuge (Rotina 420R, Hettich, Tuttlingen, Germany). The supernatant was filtered through 0.7 µm glass fiber filters (MN-GF 1, Macherey & Nagel, Düren, Germany) under vacuum suction. Retained suspended particulate matter and precipitates were pooled and freeze dried (Christ Alpha 1-2, Martin Christ GmbH, Osterode am Harz, Germany). Dried SPM samples were extracted with *n*-hexane (Chromasolv, Sigma-Aldrich) by means of accelerated solvent extraction using a SpeedExtractor® (SpeedExtractor E-916, BÜCHI Labortechnik GmbH, Essen, Germany). The device was operated at the following conditions: two extraction cycles, extraction temperature 100°C, extraction pressure 120 bar. Extracts were reduced close to dryness using a rotary evaporator between 300 and 500 mbar and 40°C (WB 2001; Heidolph, Kehlheim, Germany) and a gentle nitrogen stream. Each extract was redissolved in 1 ml of *n*-hexane p.a. (Sigma-Aldrich) resulting in a sediment equivalent concentration (SEQ) of 10 g dry sediment per 1 ml solvent each. All extracts were stored in the dark at -20°C until further analysis.

A 6890N gas chromatograph system coupled with a G2589A-5973N mass selective detector (MSD) and a 7683 automatic sample injector (all instruments from Agilent technologies, Waldbronn, Germany) was equipped with an Optima 35 MS capillary column (30.0 m × 0.25 mm i.d. and 0.25 µm film thickness, Macherey and Nagel, Düren, Germany) for chromatographic separation. Helium was used as carrier gas at a constant flow rate of 1.1 ml min^{-1}. The system operated at the following conditions: injector temperature 250°C,

injection volume 1 µl in splitless mode; GC-MS transfer line temperature 280°C; ionization by electron impact at 70 eV; oven temperature program: 50°C for 5 min then ramped up at 10°C min^{-1} to 280°C and held for 15 min. The MSD was operated in SIM mode. Data acquisition and processing was performed with the Agilent Technologies MS ChemStation data analysis software and the NIST MS Search Program. Concentration of the PAHs was calculated after PAHs were quantified by means of an external five point calibration curve derived from a standard stock solution (DE-PROM 16, LGC Standards, Wesel, Germany) containing unlabeled EPA-PAHs and expressed as mg per kg dry weight of sediment (dw). The limits of detection (LOD) and quantification (LOQ) were calculated from the chromatograms (ACS Commitee on Environmental improvement 1980) and are given in Table 2.2. Blank measurements were performed for reference.

Table 2.2 Limit of detection (LOD) and limit of quantification (LOQ) of pyrene, phenanthrene, chrysene and benzo[*a*]pyrene in sediment and suspended matter. Data obtained from Dipl.-Biol. Sebastian Hudjetz.

	Pyrene	**Phenanthrene**	**Chrysene**	**Benzo[*a*]pyrene**
LOD / mg kg^{-1}	0.11	0.10	0.17	0.30
LOQ / mg kg^{-1}	0.26	0.23	0.39	0.69

2.1.5 Determination of physicochemical parameters

Temperature, pH, conductivity and dissolved oxygen concentration were determined using adequately calibrated handheld instruments (Hanna, Ann Arbor, USA or WTW, Weilheim, Germany). Total hardness was measured after filtration (see section 2.1.3) using the titrimetric Titriplex B method (Merck, Darmstadt, Germany).

2.1.6 Dissection and tissue preparation

After exposure, fish were individually anesthetized in a 10 L container by adding a saturated solution of ethyl 4-aminobenzoate (benzocaine, Sigma-Aldrich). Subsequently, size and weight were determined for calculation of the index of condition (*K*, Equation 1), the liver somatic index (*LSI*, Equation 2) and the visceral index (*VI*, Equation 3). Subsequently, the

gall bladder was evacuated using a syringe, the bile liquid transferred to 1.5 ml polypropylene vials (Carl Roth, Karlsruhe, Germany), and stored at -20 °C for determination of PAH metabolite concentrations. The liver was rapidly isolated and weighed. The explants were cut into four about equally sized pieces, transferred into sterile 2 ml cryogenic vials (Greiner Bio-One, Frickenhausen, Germany) and quick-frozen in liquid nitrogen. Liver samples were stored at -85 °C until preparation.

$$K = {}^{W}/_{L} \times 100 \qquad \textbf{(Equation 1)}$$

$$LSI = {}^{LW}/_{W} \times 100 \qquad \textbf{(Equation 2)}$$

$$VI = {}^{(W - CW)}/_{W} \qquad \textbf{(Equation 3)}$$

W is the weight of the fish (mg), *LW* the liver weight (mg), *L* the standard length (mm) and *CW* the carcass weight (mg), i.e. the weight of the eviscerated animal.

2.1.7 Determination of 7-ethoxyresorufin-O-deethylase activity

Prior to the measurement of EROD activity, pieces of liver explants were thawed carefully and homogenized in 0.1 M phosphate buffer (EROD buffer, pH 7.4) at a ratio of 1:10 (w/v) for 20 s using an electric disperser (VDI 12, VWR, Darmstadt, Germany). Subsequently, homogenates were transferred to 1.5 ml micro test tubes (Greiner Bio-One) and centrifuged for 15 min (10,000 × g, 4 °C) in a cooling centrifuge (Rotina 420R, Hettich). Supernatant was carefully transferred into fresh 1.5 ml micro test tubes and stored at -80 °C until measurement of enzymatic activity. EROD activity was measured in quadruplicates according to a modification of the methods described by Kennedy & Jones (1994) and Pohl & Fouts (1980).

The pipetting scheme of the assay is illustrated in Figure 2.2. In 96-well microplates (TPP, Trassadingen, Switzerland), 50 µl sample (1:5 dilutions of S9 fractions in EROD buffer, wells A-H, 3-12) and 50 µl of duplicate resorufin standard solutions (0-1 µM in EROD buffer, wells A-G, 1-2) were prepared. In the reference wells without enzymatic reaction, 90 µl acetonitrile were added to wells E-F, 3-12). Subsequently, 120 µl of 2 µM 7-ethoxyresorufin in EROD buffer were added to all wells. Plates were incubated at room temperature for 10 min in darkness prior to addition of 40 µl of 4 mM NADPH in EROD buffer. After incubation at room temperature for 20 min in darkness, the reaction was stopped by adding 90 µl chilled acetonitrile to the wells A-D, 3-12 and the wells A-H, 1-2. After 15 min, the fluorescence of resorufin (excitation: 544 nm, emission: 590 nm) was determined in an Infinite® 200 microplate reader (Tecan, Crailsheim, Germany). To correct for spontaneous substrate conversion, 50 µl EROD buffer (wells H 1 and 2) was treated in the same way as the samples. The specific EROD activity was calculated and expressed as pmol resorufin mg protein^{-1} min^{-1}.

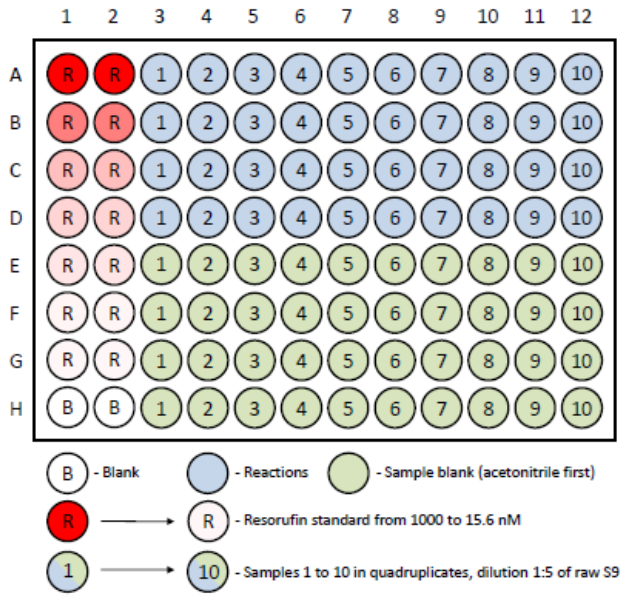

Figure 2.2 Pipetting scheme for the modified EROD assay including reference wells without enzymatic reaction.

2.1.8 Determination of lipid peroxides

Lipid peroxide content in liver tissue was measured according to Ohkawa et al. (1979). Briefly, liver homogenates were prepared in a ratio of 1 g native tissue to 10 ml 1.15 % KCl by means of an electric homogenizer (VDI 12, VWR). Subsequently, 200 µl sample were added 200 µl of 8.1 % sodium dodecyl sulfate (SDS), 1.5 ml of 20 % acetic acid adjusted to pH 3.5, 1.5 ml of 0.8 % thiobarbituric acid (TBA) and 600 µl distilled water in 15 ml polypropylene falcon tubes (Greiner Bio-One). The mixture was heated to 95 °C for 60 min in a water bath. After cooling with tap water, 5 ml of a 15:1 (v/v) mixture of *n*-butanol and

pyridine, and 1 ml distilled water were added and vortexed vigorously for 20 s. After centrifugation (4000 × g, 10 min), 300 µl of the organic layer were transferred to 96-well microplates (TPP) and absorbance at 532 nm was measured using an Infinite® 200 microplate reader (Tecan). Levels of lipid peroxides were expressed as nmol malondialdehyde (MDA) equivalent per g native tissue, which was calculated using 1,1,3,3-tetramethoxypropane as an external standard (Figure 2.3). All measurements were carried out in triplicates.

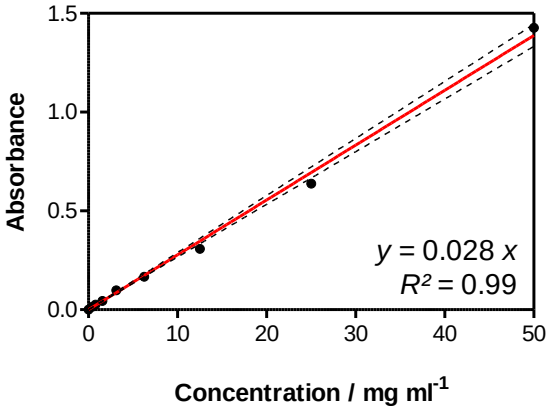

Figure 2.3 Representative standard curve of net absorbance versus concentration of the external standard TMP using the TBA method. The solid line represents the linear regression of data, dashed lines the 95 % confidence interval.

2.1.9 Determination of protein concentrations

Protein concentrations for the calculation of specific enzyme activities were determined in triplicates using the bicinchonic acid (BCA) method privided as a kit (Sigma-Aldrich). The protocol was adapted to microplate measurement, using bovine serum albumin (BSA) as external standard (0.125 – 1.25 mg ml^{-1}). A working solution was prepared by mixing 50 parts reagent A (sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0,1 M sodium hydroxide, pH 11.25) with 1 part reagent B (4 % w/v copper(II) sulfate). In each well of a 96-well microplate (TPP), 200 µl of the working solution were added to 25 µl 1:10 dilutions of liver S9 fractions. After 30 min incubation at 37°C, the extinction at 562 nm was read using an Infinite® 200 microplate

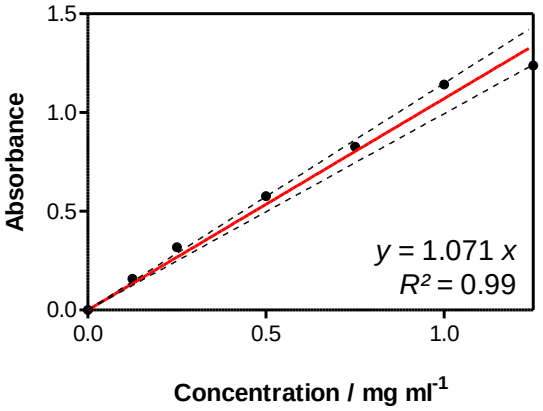

Figure 2.4 Representative standard curve of net absorbance versus concentration of the external standard BSA using the BCA method. The solid line represents the linear regression of data, dashed lines the 95 % confidence interval.

reader (Tecan). Protein concentrations were interpolated from the standard curve (Figure 2.4). A minimum coefficient of determination (R^2) of 0.95 was accepted for standard curves in all used assays for protein quantification.

2.1.10 Treatment of bile samples and HPLC analysis

PAH metabolite and biliverdin concentrations in bile were quantified by Dr. Ulrike Kammann, Johann Heinrich von Thünen-Institut (vTI), Institute of Fisheries Ecology, Hamburg, Germany, according to a modification of the method published by Kammann (2007). Briefly, 25 µl bile fluid was mixed with 95 µl distilled water and 5 µl *β*-glucuronidase/arylsulfatase solution (30/60 U · ml^{-1}) and subsequently incubated for 2 h at 37 °C. After stopping the reaction with 125 µl solution of 5 mg ml^{-1} ascorbic acid in ethanol, the mixture was centrifuged (700 × g, 5 min) and the concentrations of 1-hydroxpyrene, 1-hydroxyphenanthrene and 3-hydroxybenzo[*a*]pyrene were determined by means of HPLC with fluorescence detection (cf. Kammann 2007). The limits of detection (LOD) and quantification (LOQ) are given in Table 2.3. In addition to the absolute bile metabolite concentrations, values relative to the biliverdin concentration were calculated and expressed as ng metabolite ng^{-1} biliverdin.

Table 2.3 Limits of detection (LOD) and limits of quantification (LOQ) for the biliary metabolites 1-hydroxypyrene, 1-hydroxyphenanthrene and 3-hydroxybenzo[*a*]pyrene in undiluted bile samples. Data obtained from Dr. Ulrike Kammann.

	1-hydroxypyrene	**1-hydroxyphenanthrene**	**3-hydroxybenzo[*a*]pyrene**
LOD / ng ml^{-1}	0.65	0.41	4.06
LOQ / ng ml^{-1}	4.10	1.78	12.92

2.2 Optimization of biomarkers for detecting exposure to or effects of PAHs

2.2.1 Fixed wavelength fluorescence (FF) quantification of PAH metabolites in bile liquid

The fixed wavelength fluorescence (FF) of 15 selected bile samples from the 24°C treatments was measured according to the protocol described in Beyer et al. (2010). Briefly, raw bile samples were diluted 1:1000 in a 1:1 (v/v) mixture of methanol (Chromasolv, Sigma-Aldrich) and distilled water. The dilutions were transferred in triplicate to white 96-well microplates (Lumaplate, Perkin Elmer, Rodgau, Germany). The fluorescence of 4-ring PAH metabolites (i.e., 1-hydroxypyrene, excitation wavelength 341 nm, emission wavelength 383 nm, Beyer et al. 2010) was measured using an Infinite® 200 microplate reader (Tecan). The resulting relative fluorescence units (RFU) were correlated with the 1-hydroxypyrene concentrations determined by means of HPLC analysis. To verify the wavelength pair from literature, excitation and emission spectra were recorded for a representative bile sample. To verify the protocol, 1-hydroxypyrene concentrations in bile samples from rainbow trout were determined using the FF and HPLC-F method as part of another project (KonSed, Dipl.-Biol. Sebastian Hudjetz, unpublished data).

2.2.2 Ethoxyresorufin-O-deethylase (EROD) activity: Stern-Volmer-Plot of quenching by liver S9-fractions

The EROD assay in the present study was conducted according to the modified protocol given in section 2.1.5. Quenching of the resorufin emission by liver S9-fractions was assessed in 8 technical replicates by combining 40 µl of liver S9 serial dilutions (0 - 1.29 mg ml^{-1} total protein) with 90 µl acetonitrile, 20 µl resorufin solution (1µM in EROD buffer), and 150 µl EROD buffer in 96-well microplates (TPP). Fluorescence of resorufin (excitation: 544 nm, emission: 590 nm) was determined in an Infinite® 200 microplate reader (Tecan). The F_0/F ratio (i.e., the fluorescence of the mixture without liver S9 divided by the respective fluorescence intensity in mixtures with S9) was calculated and plotted against S9 concentration in the mixture (Stern-Volmer plot).

2.2.3 Real-time PCR measurement of hepatic gene expression

Real-time RT-PCR measurements were performed according to methods previously published by Zhang et al. (2008) at the University of Saskatchewan (Saskatoon, Canada) in context of an internship, which was funded by the Undergraduate Research Opportunities Program (UROP Abroad) of RWTH Aachen University. The presented results, experimental procedures, as well as the exposure conditions in the annular flume (see section 1.4*)* were already published by Brinkmann et al. (2010a) and will not be explained in this place.

Total RNA was extracted from preserved liver tissue of individuals according to manufacturer's protocol with a QIAGEN RNeasy Plus Mini Kit (QIAGEN, Mississauga, Ontario, Canada). RNA concentrations were determined by measuring the absorption at 260 nm using a ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA) and samples were stored at -80°C until processing. First-strand cDNA was synthesized from 1 µg of total RNA using the Superscript III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. Gene expression was quantified by means of real-time Q-RT-PCR using a 96-well Applied Biosystems 7300 real-time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR program included an enzyme activation step at 95°C (10 min), and 40 cycles of 95 °C (15 s) and 60°C (60 s). PCR mixtures sufficient for 200 reactions contained 2 mL of SYBR Green master mix (Applied Biosystems), 200 µL of 10 µM sense/anti-sense gene-specific primers (Table 2.4), and 1.6 mL of nuclease-free distilled water (QIAGEN).

Primers for CYP1A, elongation factor-1 (EF-1) and aryl hydrocarbon receptor-2β (AhR2β) were purchased from Invitrogen. All other primers were obtained from IDT Integrated DNA Technologies (Coralville, IA, USA). A final reaction volume of 20 µL was prepared of 1 µL of diluted cDNA and 19 µL of PCR mixtures. All measurements were conducted in duplicate. Expression of target genes was quantified by use of the comparative cycle threshold method with adjustment of PCR efficiency according to methods reported elsewhere (Simon 2003). The expression level of the target gene was normalized to the reference gene EF-1 to calculate the mean normalized expression (MNE) of the target genes. Levels of gene expression were expressed relative to the average value of the respective control group.

Table 2.4 Primer pair sequences, amplicon sizes, and accession numbers of the investigated genes used in real-time PCR reactions. From Brinkmann et al. (2010a).

Gene	**Primer sequence (5'–3')**	Amplicon size (bp)	GeneBank accession no.
AhRβ	F:TGGCAAATGGACACACATTC R:AGTCTGTTGGGGTTCTGTGG	100	NM_001124252
CYP1A[a]	F:GATGTCAGTGGCAGCTTTGA R:TCCTGGTCATCATGGCTGTA	104	U62796
EL-1α	F:GAGAACCATTGAAAAGTTCGAGAAG R:GCACCCAGGCATACTTGAAAG	71	NM_001124339
GST-P	F:TTCAGGGAGGGGAAGGTATC R:GTTGGTGACAAGCCTTCGTT	101	BQ036247
SOD1[b]	F:TGGTCCTGTGAAGCTGATTG R:TTGTCAGCTCCTGCAGTCAC	201	NM_001124329
UGT[c]	F:ATAAGGACCGTCCCATCGAG R:ATCCAGTTGAGGTCGTGAGC	112	DY802180

[a]This primer pair was previously published by Wiseman and Vijayan (2007)

[b]This primer pair was previously published by Fontagne et al. (2008)

[c]This primer pair was previously published by Mortensen (2007)

2.4 Data analysis

All spreadsheet calculations were performed using Microsoft Excel™ 2007. Graphs were plotted using the software GraphPad Prism 5 (GraphPad, San Diego, USA). Statistical analyses and correlations were conducted using the software Sigma Stat 3.11 (Systat Software, Erkrath, Germany). All datasets that did not pass the Kolmogorov-Smirnov test on Gaussian distribution ($p<0.05$) or the Barlett's test for equal variances ($p<0.05$) were analyzed using nonparametric Kruskal-Wallis ANOVA on ranks ($p\leq0.001$). The datasets passing both tests were analyzed using parametric one-way ANOVA ($p\leq0.001$). The Dunn's method was used to identify significant differences among the treatments. Statistical significance limit throughout all comparisons was set at least to $p\leq0.05$. If not stated differently, values are expressed as mean value ± standard deviation.

3. Results

3.1 Kinetic study: Uptake and effects of PAHs from sediment suspensions

3.1.1 Mortality during the experiments

In this experiment, rainbow trout (*O. mykiss*, Walbaum) were successfully exposed to unspiked and spiked suspensions of sediment from the harbour Ehrenbreitstein. The experimental setup proved suitable to suspend sediments and expose fish to the resulting suspension. In the 12°C experiments, mortality of rainbow trout was low (Table 3.1). In the 24°C experiments, however, fish showed 25.0 % mortality during exposure to the unspiked sediment suspension and 30.6 % following exposure to the spiked sediment.
Thus, no biomarker results are shown for day 12 from animals of the 24°C spiked sediment treatment and only $n = 4$ animals were assessed in the 24°C unspiked treatment.

Table 3.1 Mortality of rainbow trout as detected during the experiments at 12 and 24°C, respectively.

Experimental temperature	Unspiked sediment	Spiked sediment
12°C	0.0 % ($n = 60$)	1.7 % ($n = 60$)
24°C	25.0 % ($n = 72$)	30.6 % ($n = 72$)

3.1.2 Physicochemical parameters during the experiments

While the temperature was constant at 12°C in the experiment with active cooling, temperature increased during four days from 20°C to approximately 24°C in the uncooled experiment (Figure 3.1). The constant temperature in the cooled experiment also stabilized the physicochemical parameters: While the dissolved oxygen concentration was always well above 10.0 mg L^{-1} in the cooled experiments, it decreased to levels as low as 6.5 mg L^{-1} in the uncooled experiment (Figure 3.1). The dissolved oxygen concentration was inversely correlated with temperature in the experiments (Figure 3.2).

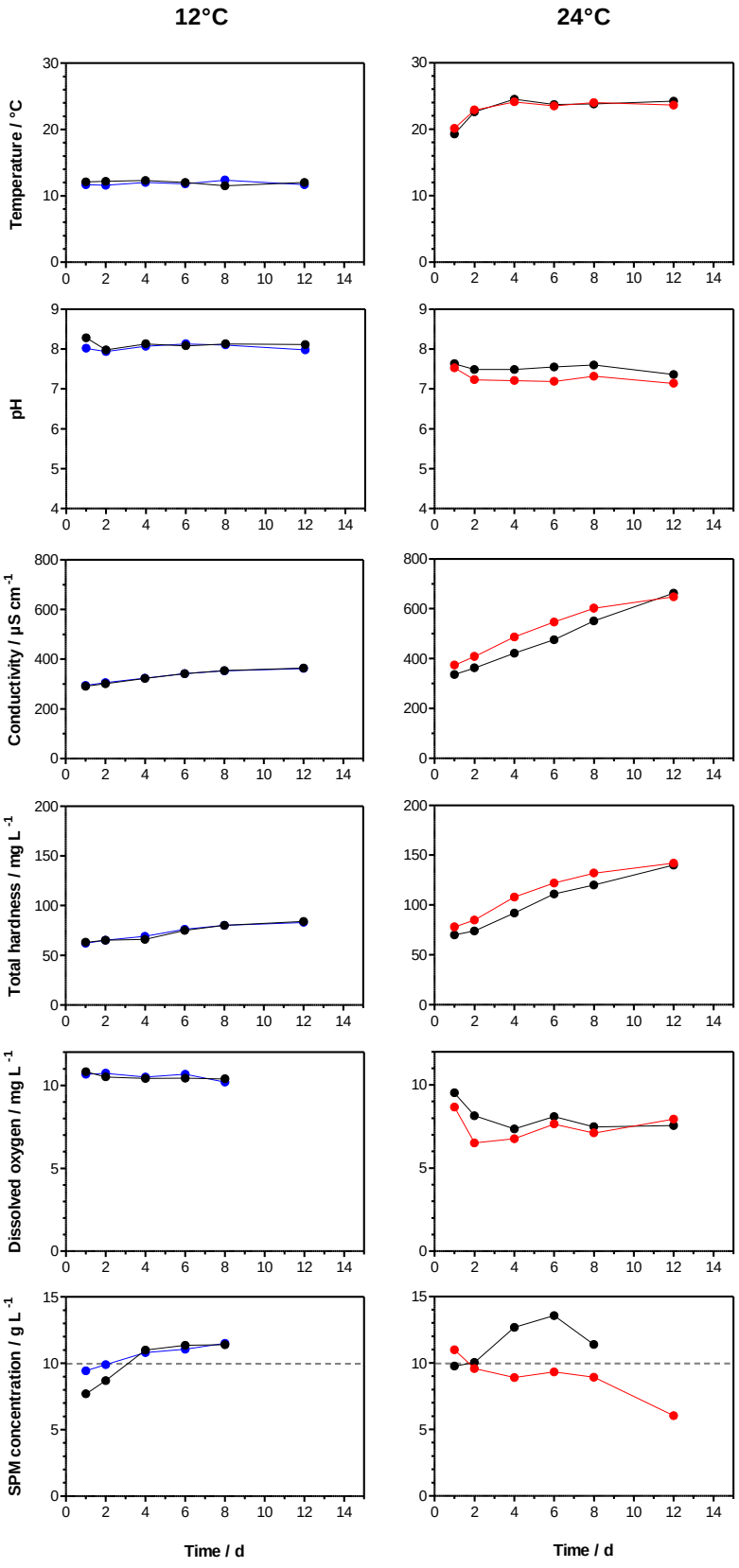

Figure 3.1 Dynamics of physicochemical parameters during the experiments (left: average temperature 12°C, right: average temperature 24°C) in the unspiked (■) and the spiked treatments (●/●).

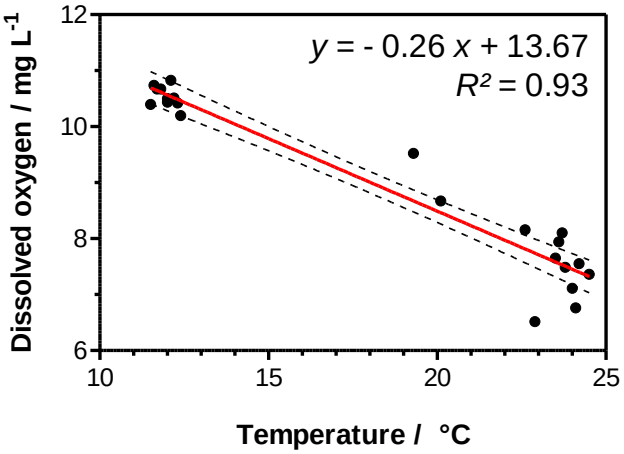

Figure 3.2 Linear regression of dissolved oxygen concentrations and temperature as measured in the cooled and uncooled experiments. Dashed lines represent the 95 % confidence interval.

A stabilizing effect was also observed for the release of carbonate from the sediment, which was assessed as the total water hardness (Figure 3.1). In the cooled experiments, total hardness increased from 63 mg L^{-1} to 84 mg L^{-1} during the exposure period, in the uncooled experiments it almost doubled from 74 to 142 mg L^{-1}. The conductivity as a sum parameter of dissolved ions was positively correlated with the total hardness and changed proportionally in the treatments (Figure 3.3). As shown in Figure 3.1, measured suspended particulate matter (SPM) concentrations deviated substantially from the nominal concentration of 10 g L^{-1} (range: 6.0 to 13.6 g L^{-1}).

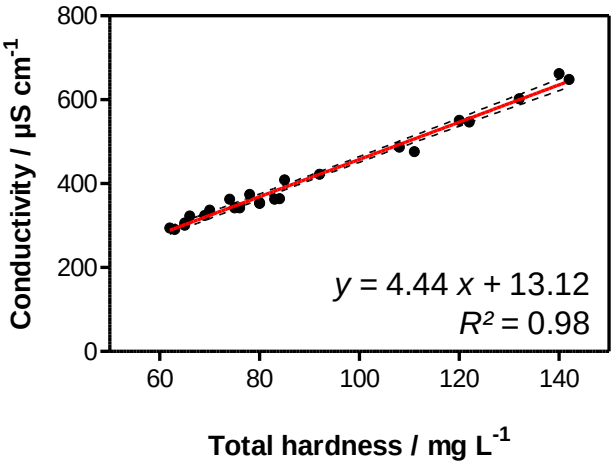

Figure 3.3 Linear regression of conductivity and total hardness as measured in the cooled and uncooled experiments. Dashed lines represent the 95 % confidence interval.

3.1.3 Chemical analyses of PAH concentrations

Concentrations of particle-bound PAHs were assessed by means of GC-MS analysis following lyophilisation and exhaustive extraction using accelerated solvent extraction (ASE) with *n*-hexane as solvent. After the sediment conditioning period of 7 days, i.e. directly prior to addition of sediments to exposure tanks, PAH concentrations in the sediment were determined (C_0), as given in Table 3.2. PAH concentrations in SPM during the experiments were expressed relative to C_0 as the C/C_0 ratio (Figure 3.4). In contrast to chrysene and benzo[*a*]pyrene, where measured concentrations substantially varied over time, a constantly decreasing C/C_0 ratio was observed in the spiked sediments for phenanthrene and pyrene, while the concentrations of all PAHs remained constant in the EBR treatments (Figure 3.4).

Table 3.2 Measured concentrations (C_0) of the PAHs used for spiking prior to the addition of sediments to exposure tanks. Values in italics are below the LOQ. Data obtained from Dipl.-Biol. Sebastian Hudjetz.

Experiment	Phenanthrene	Pyrene	Chrysene	Benzo[*a*]pyrene
Unspiked sediment	0.27 mg kg^{-1} dw	0.51 mg kg^{-1} dw	*0.29 mg kg^{-1} dw*	*0.16 mg kg^{-1} dw*
Spiked sediment	1.88 mg kg^{-1} dw	2.29 mg kg^{-1} dw	1.63 mg kg^{-1} dw	2.32 mg kg^{-1} dw

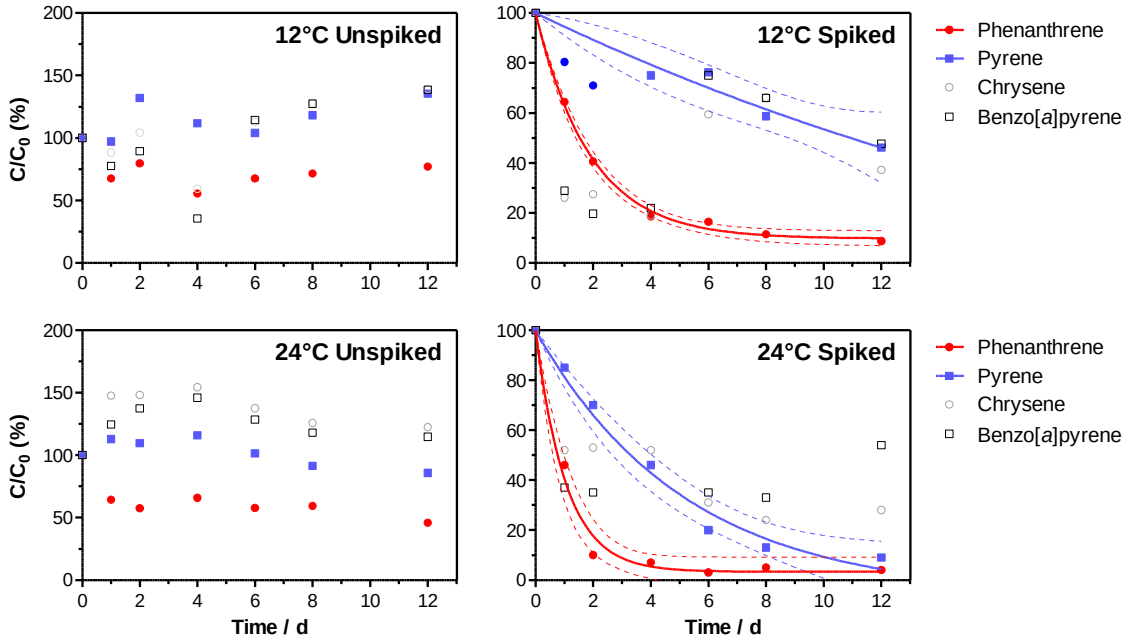

Figure 3.4 PAH concentrations during the experiments at 12 and 24°C in spiked and unspiked treatments were measured by means of GC-MS. Solid lines represent regression with a one-phase exponential decay model, dashed lines the 95 % confidence intervals. Data obtained from Dipl.-Biol. Sebastian Hudjetz.

The data was fitted using a one-phase exponential decay model. The coefficient of determination (R^2) of the fit, as well as the calculated half-lives of pyrene and phenanthrene in the SPM resulting from spiked sediment, is given in Table 3.3.

The three-ring PAH phenanthrene generally had lower half-lives than the four-ring PAH pyrene. With decreasing temperature, the half-lives of the compounds increased by a factor of 1.8 for phenanthrene, and 5.2 for pyrene, respectively.

Table 3.3 Half-lives of pyrene and phenanthrene during the experiments at 12 and 24 °C, respectively. Data were computed with a one-phase exponential decay model.

Experimental temperature	Pyrene		Phenanthrene	
	Mean $t_{1/2}$ / d	**Coefficient of determination R^2**	**Mean $t_{1/2}$ / d**	**Coefficient of determination R^2**
12°C	18.58	0.96	1.32	0.99
24°C	3.58	0.99	0.73	0.99

3.1.4 Morphometric indices

Morphometric indices of exposed fish were determined to assess changes in health and condition during the experiments (Figure 3.5). The condition index (K), i.e. the quotient of weight and volume of fish, showed a constantly decreasing trend in all treatments. However, this decrease was only significant in the 12°C unspiked treatment on day 12 compared to unexposed animals (One-way ANOVA with Dunnet's post-hoc test, $p \leq 0.05$).

The same trend was observed for the visceral index (VI), i.e. the relative weight of intestines compared to the weight of the whole animal, which was significantly decreased already after 4 and 6 days in the unspiked and spiked experiments at 12°C and at day 2 and 4 in the 24°C spiked sediment treatment, respectively (One-way ANOVA on ranks with Dunn's post-hoc test, $p \leq 0.05$). The liver somatic index (LSI) did not show any significant changes during exposure. There were no significant differences of the condition index, the visceral index or the liver somatic index between the experiments with spiked and unspiked sediment.

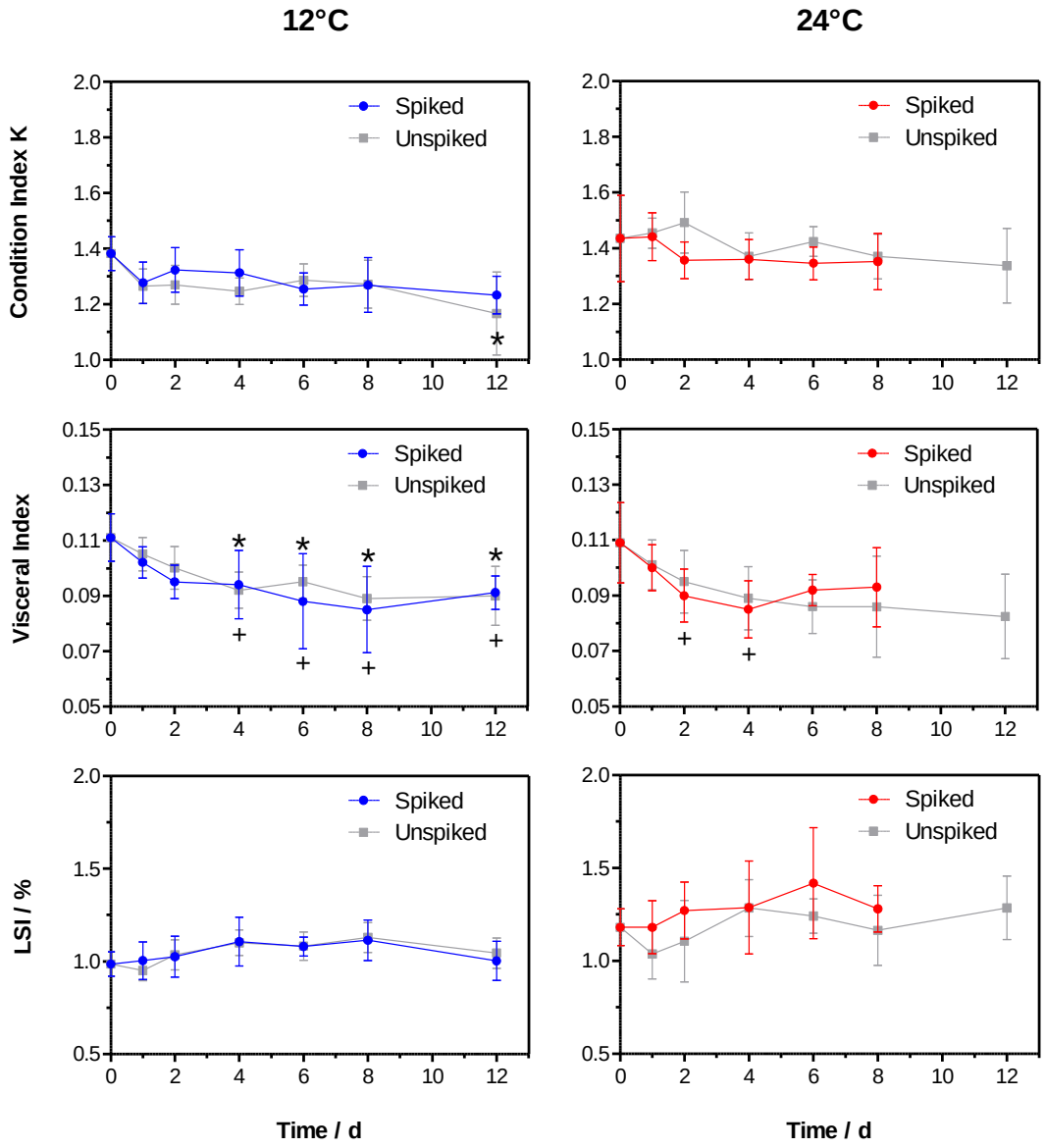

Figure 3.5 Dynamics of morphometric indices during the experiments (left: average temperature 12°C, right: average temperature 24°C) in the unspiked (■) and the spiked treatments (●/●). Symbols represent mean values of n=10 animals, error bars the 95 % confidence intervals. Asterisks denote significant differences between control and unspiked treatments, plus symbols between control and spiked treatments.

3.1.5 Biliary metabolite and biliverdin concentrations

The biliary metabolites 1-hydroxypyrene, 1-hydroxyphenanthren and 3-hydroxybenzo[*a*]pyrene (metabolites of the PAHs pyrene, phenanthrene and benzo[*a*]pyrene, respectively) were measured to demonstrate uptake and metabolism of PAHs from sediment suspensions. It was shown that uptake of substantial amounts of PAHs occurred in all treatments (Figure 3.6), including the unspiked sediment from the harbour

Ehrenbreitstein. Generally, a hyperbolic uptake phase was followed by a decay phase in the spiked treatments. Such elimination phase was not observed in the unspiked treatments. The concentration of each of the metabolites was significantly elevated on every sampling day (confidence interval method, Newman 2008) in the spiked treatments at 12°C and 24°C. The uptake rate (not quantified), however, differed substantially at the two temperatures, where the maximum concentration of 1-hydroxypyrene was reached on day 2 at 24°C and on day 8 at 12°C. The same mode of uptake was observed for 1-hydroxyphenanthrene, where the maximum concentration was reached on day 2 at 24°C and on day 4 at 12°C. The biliary concentration of the benzo[*a*]pyrene metabolite 3-hydroxybenzo[*a*]pyrene constantly increased during exposure at 12°C. No significant increase was observed from day 2 at 24°C.

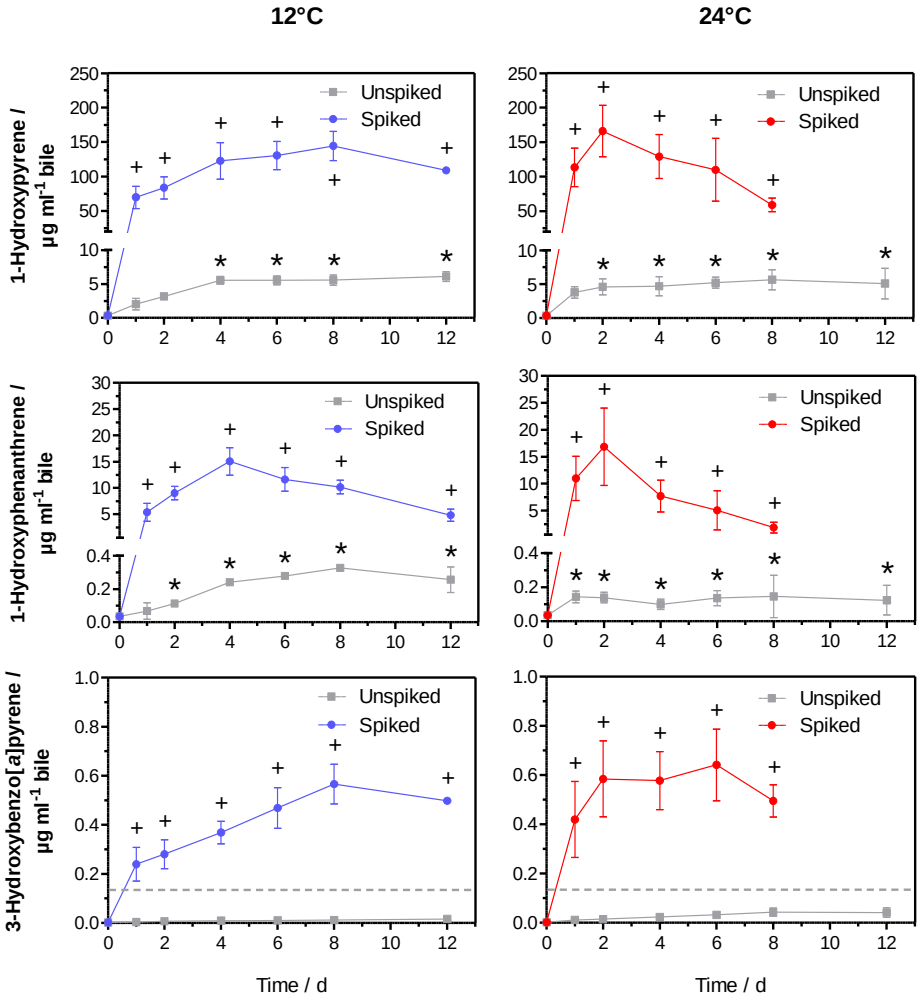

Figure 3.6 Absolute biliary metabolite concentrations during the experiments (left: 12°C, right: 24°C) in the unspiked (■) and the spiked treatments (●/●). Symbols give the mean of n=10 animals, error bars the 95 % confidence intervals. The dashed line marks the LOQ for 3-hydroxybenzo[*a*]pyrene. Asterisks denote significant differences between control and unspiked treatments (Kruskal-Wallis one-way ANOVA on ranks with Dunn's post-hoc test, $p \leq 0.05$), plus symbols between control and spiked treatments. Data obtained from Dr. Ulrike Kammann.

In the unspiked experiments, uptake and metabolism resulted in significantly increased metabolite concentrations after 1 and 2 days for 1-hydroxyphenanthrene and 1-hydroxypyrene, respectively, at 24°C, and after 2 and 4 days at 12°C (Kruskal-Wallis one-way ANOVA on ranks with Dunn's post-hoc test, $p \leq 0.05$). Values for 3-hydroxybenzo[*a*]pyrene were below the LOQ. To correct for the influence of the metabolic rate of the animals, the concentration of biliverdin was measured. In the 12°C treatments, no substantial changes of biliverdin concentrations were observed (Figure 3.7). In the 24°C treatments, however, biliverdin concentrations constantly increased from initial 160 ng ml^{-1} in the untreated control animals to 1209 ng ml^{-1} in the unspiked treatment on day 12. During the first four days of exposure, the biliverdin concentration in bile of fish exposed to spiked sediment was higher compared to fish exposed to unspiked sediment (significant on day 4, Mann-Whitney Rank Sum test, $p \leq 0.05$). Metabolite concentrations were corrected for biliverdin concentrations. After correction, concentration patterns were comparable in the 12°C experiments, but peaks on day one were observed for each of the metabolites at 24°C in the spiked treatments (Figure 3.8).

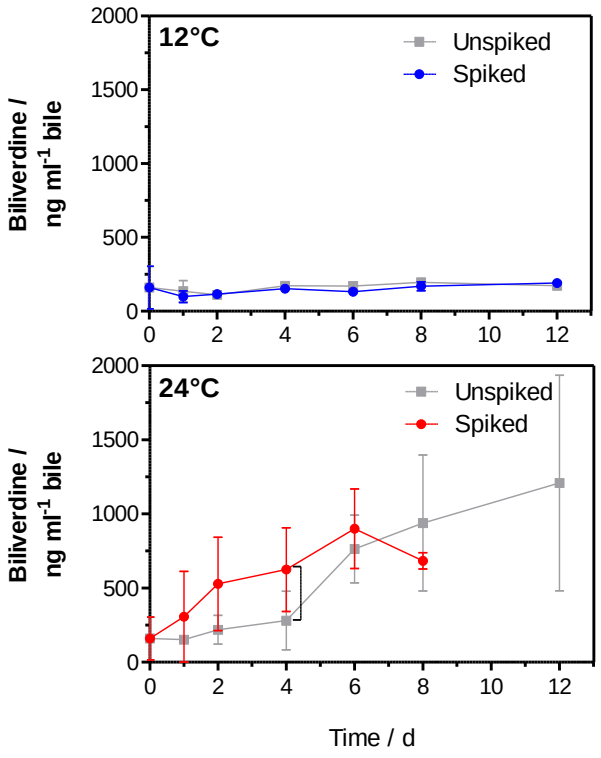

Figure 3.7 Dynamics of biliary biliverdin concentrations during the experiments (top: average temperature 12°C, bottom: average temperature 24°C) in the unspiked (■) and the spiked treatments (●/●). Symbols represent mean values of n=10 animals, error bars the 95 % confidence intervals. Brackets denote significant differences between the experiments (Mann-Whitney Rank Sum test, $p \leq 0.05$). Data obtained from Dr. Ulrike Kammann.

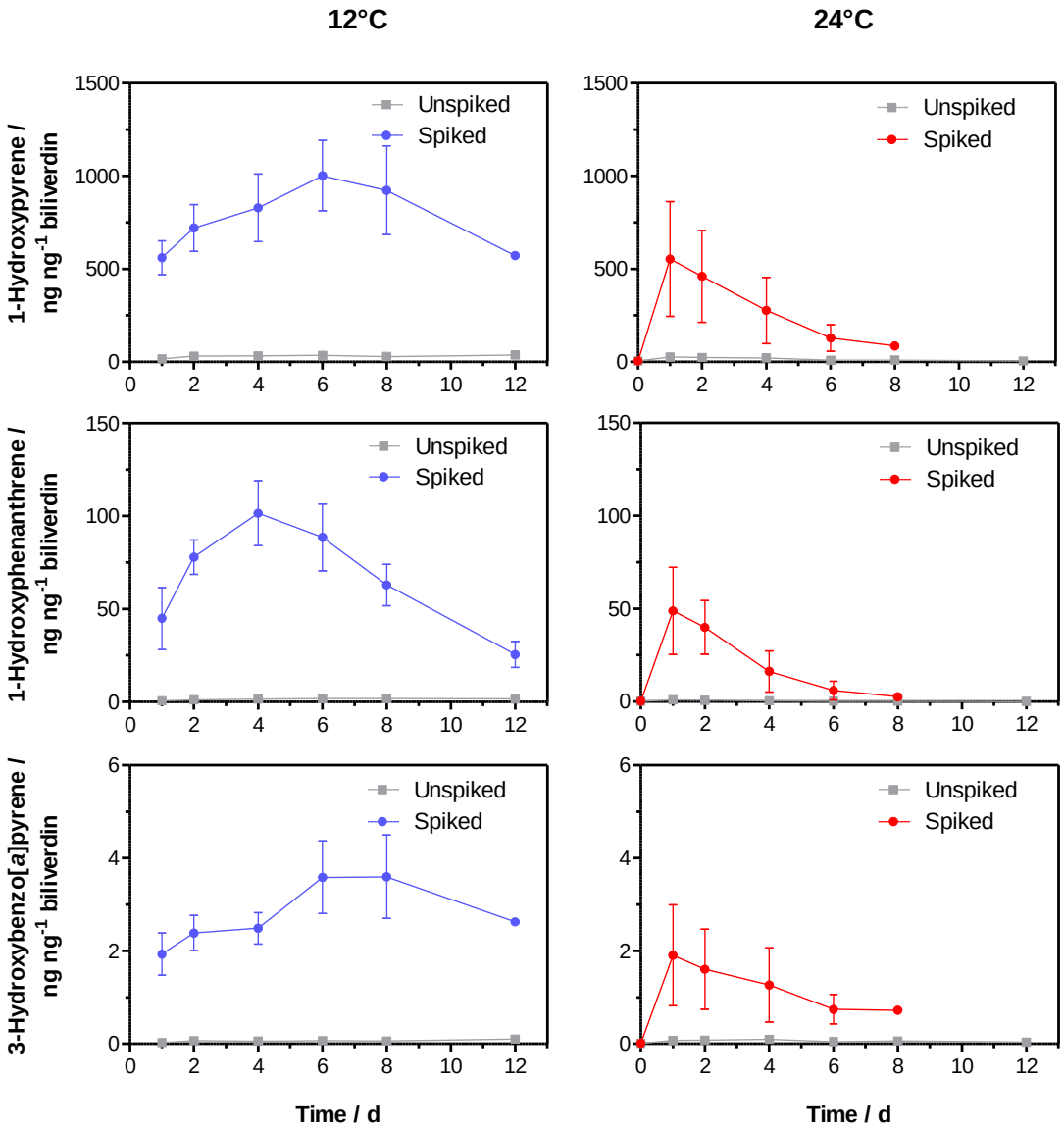

Figure 3.8 Biliary metabolite concentrations normalized to biliverdin during the experiments (left: average temperature 12°C, right: average temperature 24°C) in the unspiked (■) and the spiked treatments (●/●). Symbols represent mean values of n=10 animals, error bars the 95 % confidence intervals. Data obtained from Dr. Ulrike Kammann.

3.1.6 Hepatic EROD activity

The activity of 7-Ethoxyresorufin-*O*-deethylase was measured in hepatic S9 fractions according to the modified method described in section 2.1.5. In the 12°C experiment, the EROD activity was significantly elevated after 8 and 12 days exposure to unspiked sediment (Kruskal-Wallis one-way ANOVA on ranks with Dunn's post-hoc test, $p \leq 0.05$). EROD activity did not significantly change after exposure to the spiked sediment (Figure 3.9). In the

24°C experiment, EROD activity was significantly reduced after 4 days exposure to both the spiked and unspiked sediment (Kruskal-Wallis one-way ANOVA on ranks with Dunn's post-hoc test, $p \leq 0.05$). When treated with the spiked suspension, however, hepatic EROD activity was significantly 1.9-fold higher compared to the unspiked sediment on day one at 24°C (t-test, $p \leq 0.05$).

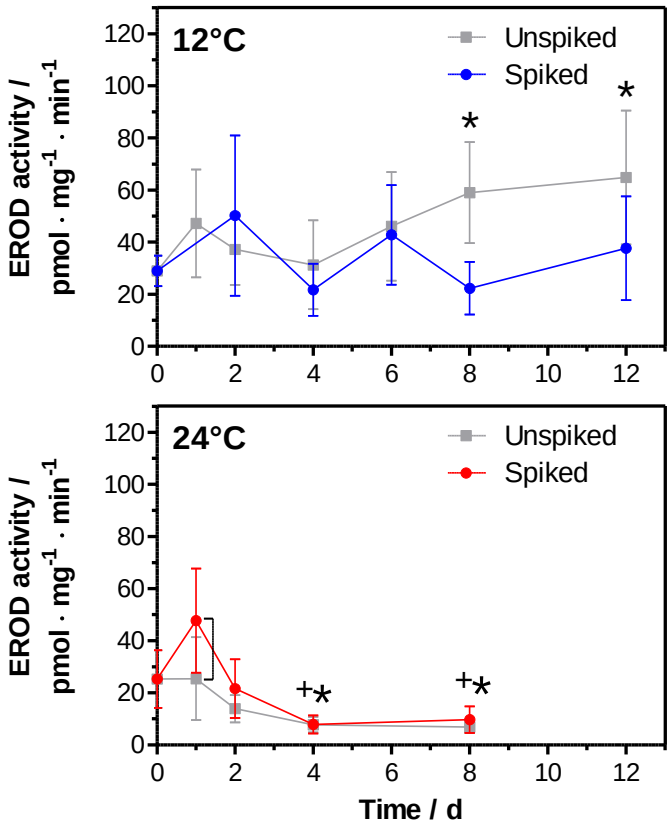

Figure 3.9 Dynamics of hepatic EROD activity during the experiments (top: average temperature 12°C, bottom: average temperature 24°C) in the unspiked (■) and the spiked treatments (●/●). Symbols represent mean values of n=10 animals, error bars the 95 % confidence intervals. Brackets denote significant differences between the experiments (t-test, $p \leq 0.05$), asterisks differences between control and unspiked treatments, and plus symbols between control and spiked treatments (Kruskal-Wallis one-way ANOVA on ranks with Dunn's post-hoc test, $p \leq 0.05$).

3.1.7 Hepatic lipid peroxidation

Hepatic lipid peroxides (LPO) were measured and expressed as malondialdehyde (MDA) equivalents (Figure 3.10). While LPO in animals exposed to unspiked and spiked sediment at

12°C, respectively, did not significantly change over time, there was a constant increase of LPO in the 24°C PAH-spiked treatment during the first four days of exposure (significant, Kruskal-Wallis one-way ANOVA on ranks with Dunn's post-hoc test, $p \leq 0.05$), which subsequently decreased to the level of the other treatments. The average LPO concentration in this treatment was 414.4 ± 271.1 nmol g^{-1} liver tissue on day four, which was approximately 2.7-fold higher compared to the unexposed control fish.

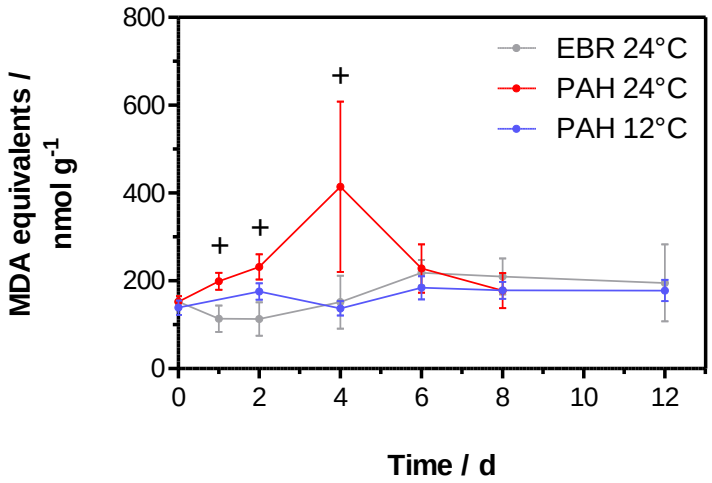

Figure 3.10 Dynamics of hepatic lipid peroxidation during the experiments (top: average temperature 12°C, bottom: average temperature 24°C) in the unspiked (■) and the spiked treatments (○/●). Symbols represent mean values of n=10 animals, error bars the 95 % confidence intervals. Plus symbols denote significant differences between control and spiked treatments (Kruskal-Wallis one-way ANOVA on ranks with Dunn's post-hoc test, $p \leq 0.05$).

3.2 Optimization of biomarkers for detecting exposure to or effects of PAHs

3.2.1 Fixed wavelength fluorescence (FF) quantification of PAH metabolites in bile liquid

A fixed wavelength fluorescence (FF) method was applied to semi-quantitatively determine the concentrations of PAH metabolites in fish bile without the need for chromatographic separation. It was verified by excitation and emission scans that the wavelength pair from literature (excitation wavelength 341 nm, emission wavelength 383 nm) was applicable. While the excitation wavelength exactly matched the literature wavelength (Figure 3.11 A), the emission wavelength in the sample was shifted to 396 nm (Figure 3.11 B).

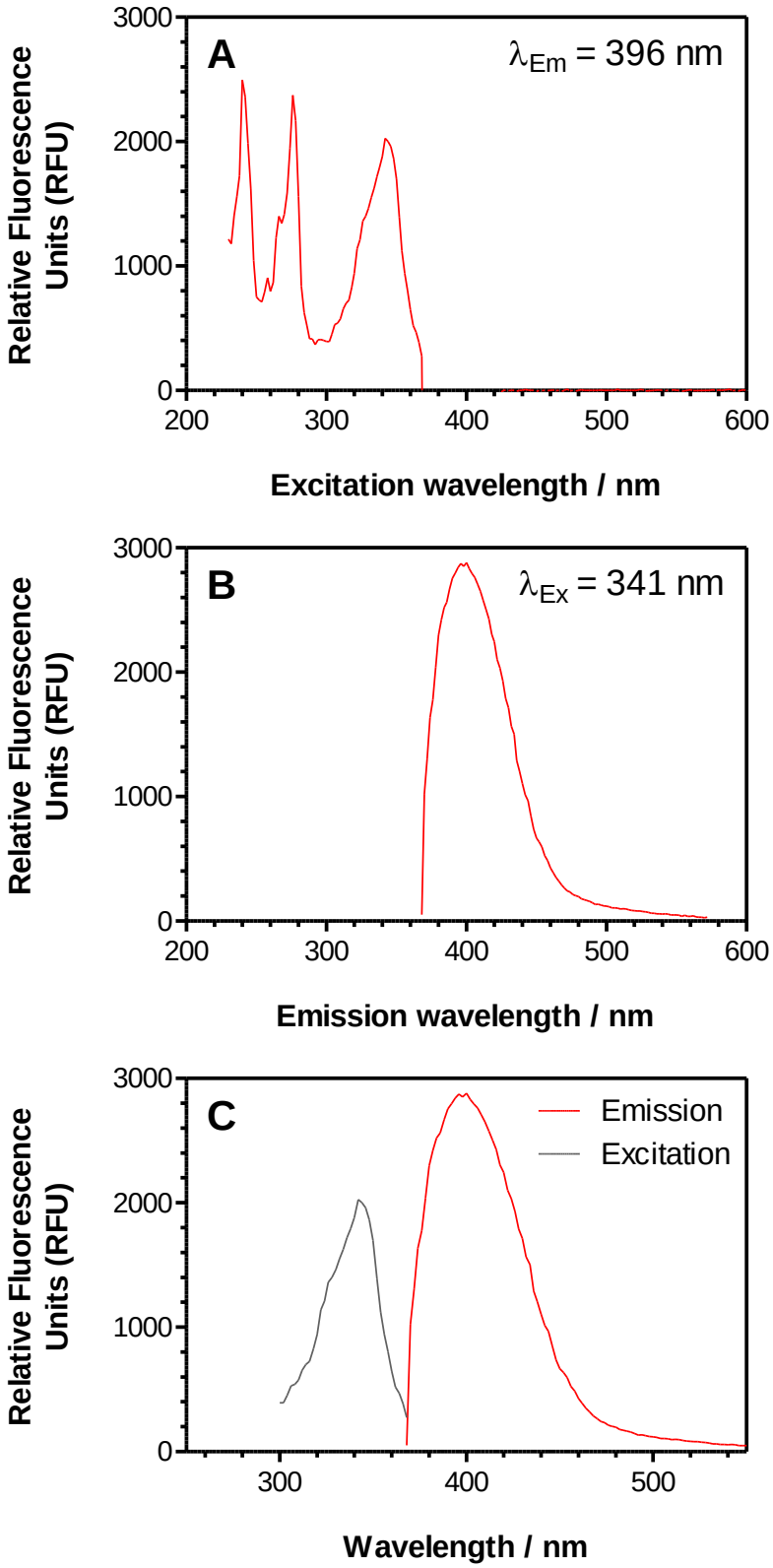

Figure 3.11 Excitation (A) and emission (B) scans of a representative bile sample, as well as a combined plot (C) for verification of the wavelength pair for detection of 4-ring PAHs (Beyer et al. 2010).

Subsequently, the fixed wavelength fluorescence of a random selection of samples was measured and compared with the 1-hydroxypyrene concentrations that were determined by means of HPLC analysis (Figure 3.12). There was a significant linear correlation (Pearson's correlation coefficient 0.99, $p \leq 0.001$) between the two factors. The equation of the regression line is given in Figure 3.12. To verify the protocol, 1-hydroxypyrene concentrations in bile samples from rainbow trout originating from a different project were measured using the FF and HPLC-F method (Figure 3.13). It can be seen that the FF method overestimated the concentration by a factor of approximately 2.6.

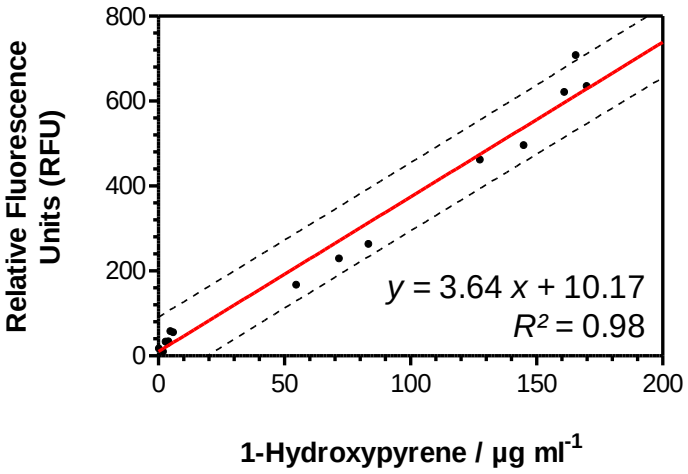

Figure 3.12 Linear regression of 1-hydroxypyrene concentration in bile and Relative Fluorescence Units (RFU) as measured using the fixed wavelength fluorescence (FF) method (excitation 341 nm, emission 383 nm). Dashed lines limit the 95 % prediction interval.

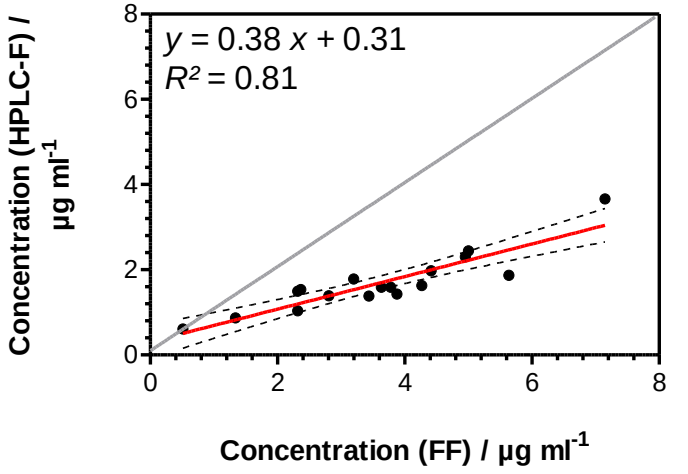

Figure 3.13 Linear regression of 1-hydroxypyrene concentration in bile from quantified using the fixed wavelength fluorescence (FF) method and HPLC-F. The grey line marks the 1:1 reference between measured and predicted data. Dashed lines limit the 95 % confidence interval of the fitted data.

3.2.2 EROD activity: Stern-Volmer plot of fluorescence quenching by liver S9-fractions

To verify the hypothesis that the measurement of EROD activity can be improved by correcting the resorufin emission for quenching or autofluorescence of S9 samples, Stern-Volmer plots were generated (Figure 3.14). There was a significant linear relationship between the S9 concentrations in the assay mixture and the F_0/F ratio, i.e. the fluorescence of the assay mixture without S9 and the fluorescence of the respective sample dilution. Although the quenching effect was comparably low in the S9 concentration range used in the EROD assay (Figure 3.14, shaded plane), it was possible to determine lower EROD activities compared to the common method by Kennedy & Jones (1994).

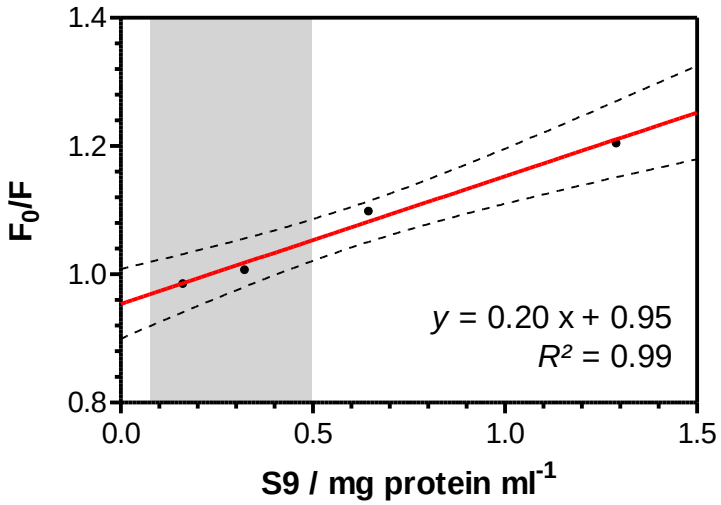

Figure 3.14 Stern-Volmer plot of the F_0/F ratio versus quencher (S9 fraction) concentration. The shaded plane illustrates the total protein concentration range used in the EROD assay. Dashed lines limit the 95 % confidence interval.

3.2.3 Real-time PCR measurement of hepatic gene expression

Prior to the analysis of hepatic gene expression in samples from exposed animals, it was necessary to check for the specificity of the selected primers and to determine primer efficiencies. For each of the selected primer pairs, a linear relationship between the amount of cDNA template (relative copy number) and the cycle threshold (CT), i.e. the cycle at which a certain fluorescence trigger value during real-time PCR is passed, was observed (Figure 3.15).

Primer efficiencies ranged from 1.93 to 2.05, i.e. the amount of DNA doubled during each PCR cycle.

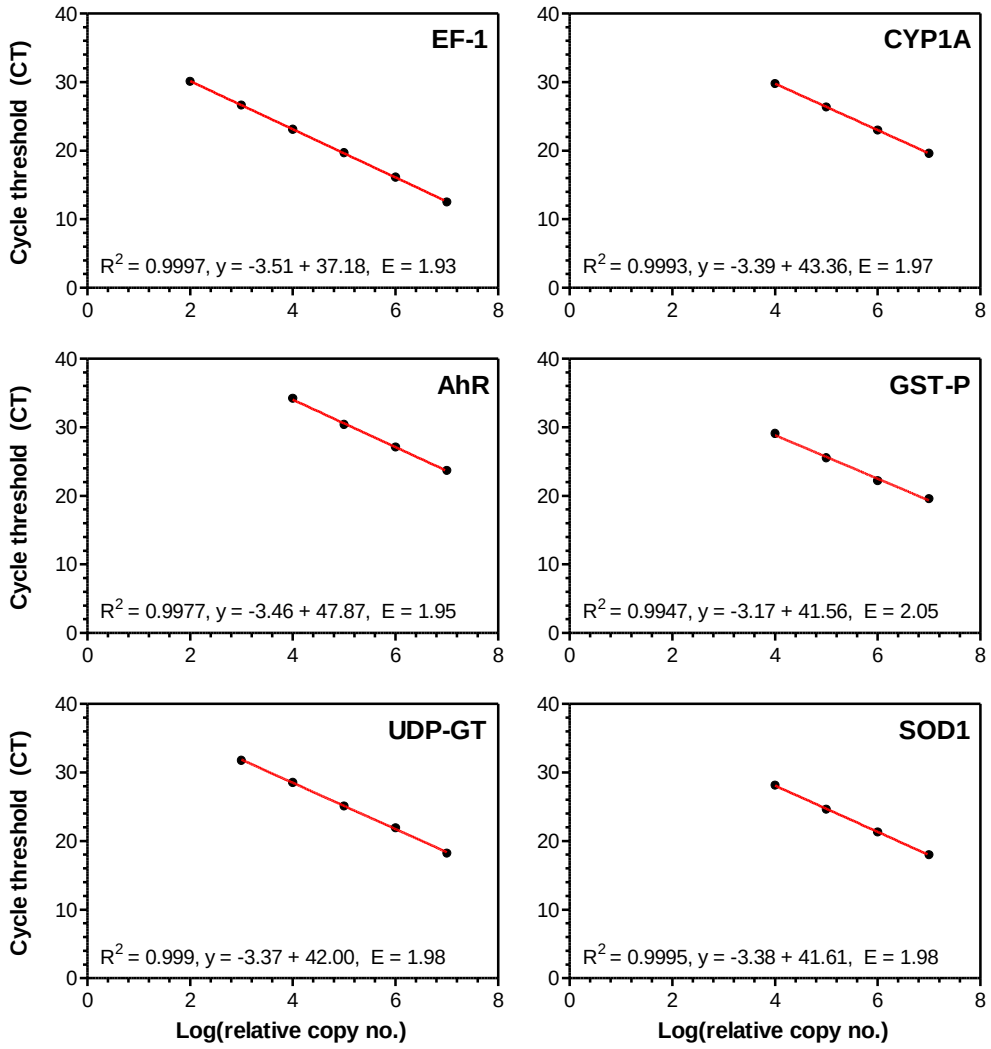

Figure 3.15 PCR efficiencies for primer pairs used in the present study were calculated from linear regression of real-time PCR cycle threshold (CT) values and the relative cDNA copy number, i.e. dilution series of cDNA composite samples. Primer efficiencies (E) are given in the respective plots.

There was a statistically significant ($p \leq 0.05$) 1.8-fold induction of CYP1A mRNA expression in fish that were exposed to spiked sediment during the simulated flood event compared to the flood event in the absence of sediment (Figure 3.16), and 3.4-fold induction compared to the absence of sediment. The expression of AhR2β mRNA was not statistically different after the treatments. UGT and SOD-1 showed very similar expression patterns. The expression of both genes was significantly down-regulated ($p \leq 0.01$) in the presence of polluted sediment compared to the treatment in the absence of sediment. Although the pattern

of the GST-P data was very similar to those of UGT and SOD-1, none of the differences in hepatic expression of GST-P between the treatment groups were statistically significant.

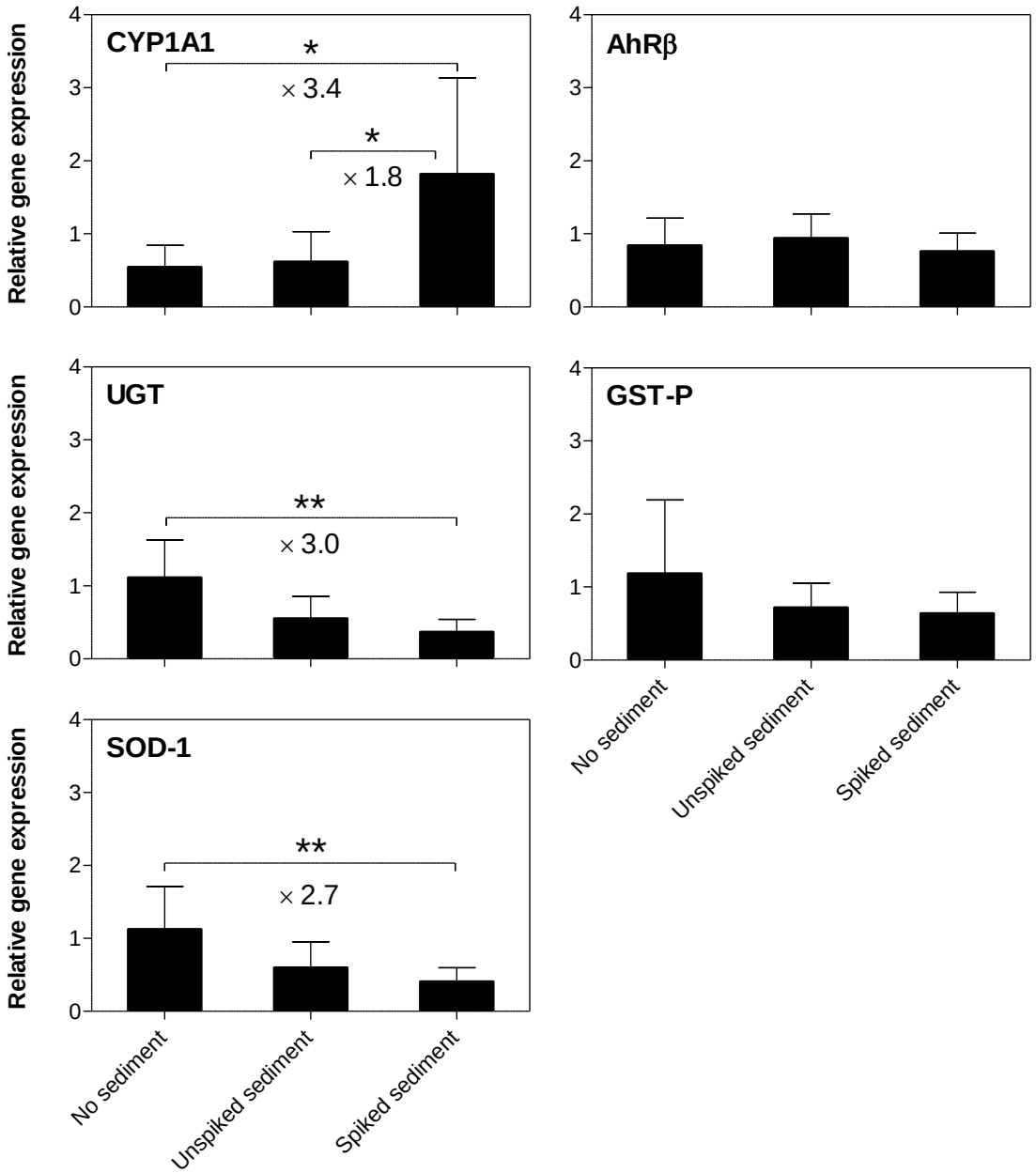

Figure 3.16 Hepatic expression of the genes CYP1A1, AhRβ, GST-P, UGT and SOD-1 of rainbow trout exposed in 5 d simulated flood events without (NO), with unspiked (OECD) and spiked sediments (OECD + PAH), respectively. Each test was conducted with *n*=15 animals. Bars represent the average gene expression relative to the respective control group taken from the maintenance in parallel to the experiment to establish baseline values. Error bars represent the standard deviation. */**Significant alteration compared to the respective control group (Kruskal-Wallis one way ANOVA on ranks and Dunn's method, $p \leq 0.05/0.01$). Data from Brinkmann et al. (2010a).

4. Discussion

4.1 Suitability of the resuspension system to expose rainbow trout to sediment suspension at different temperatures

Animals within the project Floodsearch were exposed during simulated flood events in an annular flume, i.e. under relatively sophisticated exposure conditions. Apart from the obvious advantages of such realistic exposure conditions, there is one major drawback: The flume cannot be opened during the experiments, thereby limiting the investigation of exposed fish to one sample prior to and on sample after exposure. Thus, it was necessary to conduct kinetic exposure experiments with sediment suspensions using a suspension method other than the annular flume.

The sediment suspension system used in the current study was comparably simple and performed only satisfactory with regard to the large differences between the nominal SPM concentration of 10 g L^{-1} and the minimum and maximum measured values of 6.0 and 13.6 g L^{-1}, respectively. More sophisticated methods are available to derive constant SPM concentrations, such as programmable turbidistats (Sinnett & Davis 1983) or serial dilutors (Sved & Roberts 1995). These methods, however, always require flow-through conditions because the losses due to sedimentation are compensated by addition of suspended particles. For larger-scale experiments as conducted in the present study which were necessary to expose the required numbers of animals, this would imply spiking, handling and disposal of several hundred kilograms of sediment.

Temperature was successfully controlled by installing submersible coolers, which in turn led to stabilization of other physicochemical parameters such as dissolved oxygen, total hardness, and pH.

4.2 Dissipation of the PAHs from sediment-water systems

Since it was not possible to expose rainbow trout to sediment suspensions under semi-static or flow-through conditions due to the abovementioned reasons, the exact knowledge of changes in compound concentrations during the experiments was of vital importance.

Xia et al. (2006) found that the overall biodegradation of PAHs in fluvial systems containing high concentrations of SPM (i.e., 4-10 g L^{-1}) was substantially higher compared to systems

with lower or no SPM due to the increased surface of the water-sediment interface at which enhanced microbial degradation takes place. LeBlanc and co-workers (2006) have demonstrated, that the microbial mineralization rate of sediment-bound phenanthrene at 18-20°C was 5 to 10-fold elevated if the sediment was frequently resuspended in comparison to a static sediment bed. The shortest resulting half-life for microbial degradation in this particular study was 100 days, while other researchers found half-lives in the range of 40 days to several months for phenanthrene in undisturbed sediments (Apitz et al. 1999, Heitkamp & Cerniglia 1987). In the present study, significantly shorter half-lives were observed for phenanthrene (Table 3.3), that were comparable to those derived from the investigation of biodegradation in sediment slurries (e.g. Shiaris 1989). Yuan et al. (2001) detected degradation times comparable to those of the 12°C treatments from this study, with half-lives for phenanthrene and pyrene of 0.6 and 13.9 days, and no significant degradation of benzo[*a*]pyrene, in sediment slurries from the Keelung River in Taiwan at 20°C.

Aside from biodegradation, volatilization of sediment-bound PAHs due to the intense aeration of the experimental containers in the present study might also represent an important dissipation pathway for the lower-weight PAHs, i.e. phenanthrene and pyrene (e.g. Ravikrishna et al. 1998, Valsaraj et al. 1997). Due to the large scale of the experiments within this study, however, the exact dissipation pathways of PAHs were not followed in detail.

Another dissipation pathway is uptake and metabolism by the exposed rainbow trout. The estimation of this fraction is particularly important since the extraction of substance by the test organisms should not influence the partitioning of PAHs between sediment and water. Since rainbow trout starved during the experiments, biliary metabolite concentrations represented an integrative measure for the uptake of PAHs from the sediment (Beyer et al. 2010). The gall bladder of fish from the present study contained less than 0.5 ml bile liquid on average (data not shown). Assuming that no bile liquid was excreted during the experiments (since fish were starved), the average extraction of the respective PAHs from the system by fish can be roughly estimated by multiplying the maximum average metabolite concentration in bile with the bile volume and the number of fish per experiment.

The estimated worst-case fractions of phenanthrene and benzo[*a*]pyrene extracted from the system by fish were below 5 % and can be expected to have only neglible influence on the partitioning processes (Table 4.1). Pyrene, however, was extracted to significant extent (up to 35 % in the 24°C spiked sediment treatment). It has previously been demonstrated that pyrene is preferentially metabolized in rainbow trout and thus 1-hydroxypyrene occurs in far greater

concentrations than other metabolites in fish bile (Ruddock et al. 2003). Thus, it cannot be excluded that desorption and partitioning processes of pyrene in the presented experiments were significantly altered by the presence of fish.

Table 4.1 Worst-case fraction of PAHs extracted from sediment-water system by fish were estimated from the biliary metabolites 1-hydroxypyrene, 1-hydroxyphenanthrene and 3-hydroxybenzo[*a*]pyrene.

Temperature	Pyrene		Phenanthrene		Benzo[*a*]pyrene	
	Unspiked	Spiked	Unspiked	Spiked	Unspiked	Spiked
12°C	4.7 %	25.2 %	0.5 %	3.2 %	0.1 %	0.1 %
24°C	6.7 %	35.0 %	0.3 %	4.4 %	0.1 %	0.2 %

All these potential routes of dissipation led to constantly decreasing concentrations of PAHs, especially of those with lower molecular weight, i.e. phenanthrene and pyrene. Apart from the abovementioned drawbacks of the annular flume, new suspended particles are permanently resuspended from the sediment bed, leading to quasi-flow-through conditions which counteract against the dissipation pathways in simulated flood events. While the water-sediment ratio was 100:1 (v/w) in the presented suspension system, the presence of a sediment bed in the annular flume leads to a ratio of 3.3:1 in the annular flume, enabling for the constant resuspension of new contaminated particles.

Several authors have shown, that aging of spiked PAHs can lead to lower desorption rates and lower availability for biodegradation over timescales in the range of months (Fu et al. 1994, Hatzinger & Alexander 1995, Kan et al. 1994, White et al. 1997). This effect was also observed in the current study, where no significant reduction of sediment-bound PAH concentration over time was found in the experiments with field-aged sediments from the harbor Ehrenbreitstein. Since the same processes are key factors affecting the bioavailability of the compounds, it seems likely that the substantial differences observed for uptake and metabolism of the PAHs in fish comparing spiked and unspiked sediment (see 4.3) might be due to differing desorption rates (Reid et al. 2000). However, it should be noted that the biliary concentrations of 1-hydroxypyrene and 1-hydroxyphenanthrene in fish following exposure to the unspiked sediment exceeded the values reported for monitoring campaigns of

different fish species (e.g. Kammann 2007, Ruddock et al. 2003), indicating the high bioavailability of PAHs following resuspension of sediments.

4.3 Differences between the bioavailability and uptake kinetics of freshly spiked and naturally aged sediment-bound PAHs

As already discussed in section 4.2, it is well-known that substantial differences in desorption rates and bioavailability of organic contaminants can be observed with freshly spiked and naturally aged sediments (Reid et al. 2000). For this reason, a direct comparison between a natural sediment with moderate PAH contamination, either unspiked or spiked with a mixture of PAHs was investigated in the present study.

The differences in desorption rates that have been observed with respect to the dissipation of PAHs from the exposure tanks were also apparent in the fraction available for uptake and metabolism by rainbow trout. While the concentration of sediment-bound phenanthrene was 7.0-fold higher in spiked compared to unspiked sediment, the maximum biliary concentration of 1-hydroxyphenanthrene differed 120 and 45-fold (12°C and 24°C, respectively) between spiked and unspiked exposures. The same was observed for pyrene, where the concentration was 4.5-fold higher in spiked compared to unspiked sediment and the 1-hydroxypyrene concentration differed 29 and 24-fold (12°C and 24°C, respectively) comparing unspiked and spiked sediment exposure. The use of spiked sediment increased the bioavailable fraction significantly. In contrast to these results, Oikari et al. (2002) have found that ageing had no significant effect on bioavailability of the PAH retene as assessed by means of EROD activity in rainbow trout fingerlings. The results of the present study, however, indicate that these differences have to be taken into account, especially when comparing effects of spiked and field-aged sediment and in toxicity tests for regulatory purpose (cf. Zielke 2011).

4.4 Dynamics of biomarker responses during the exposure experiments

Since it was not possible to open the lid of the annular flume during experiments in context of the project Floodsearch, only rather qualitative information on biomarkers of exposure and biological effects was derived during the simulated flood events. In the exposure experiments

of this study, the biomarkers were monitored kinetically during 12 days to provide more detailed insights in the induction dynamics of the investigated biomarkers.

In general, uptake and effects of particle-bound PAHs followed a cascade-like pattern in the spiked treatment at 24°C, i.e. a series of peak biomarker reactions (Figure 4.1). Due to the dissipation of pyrene and phenanthrene from the system, the experiment can be subdivided in an uptake and a quasi-depuration phase, in which the biliary metabolite concentrations increased and decreased, respectively. The peak of metabolism on day two was followed by a peak of lipid peroxidation on day four, leading to the assumption that both might be causally related. Preliminary results indicate that a peak of micronuclei in peripheral erythrocytes on day six, i.e. a strong marker for genetic damage with potential effects on the population level (Diekmann et al. 2004a, Diekmann et al. 2004b), follows this peak of oxidative damage (data not shown). Although the induction of the before mentioned biomarkers was only transient, these results imply the potential for a later adverse outcome of exposure to particle-bound PAHs. Thus, an experiment should be conducted to monitor health and performance of fish over a longer period of time, e.g. growth and reproduction, following short exposures to contaminated suspended particles.

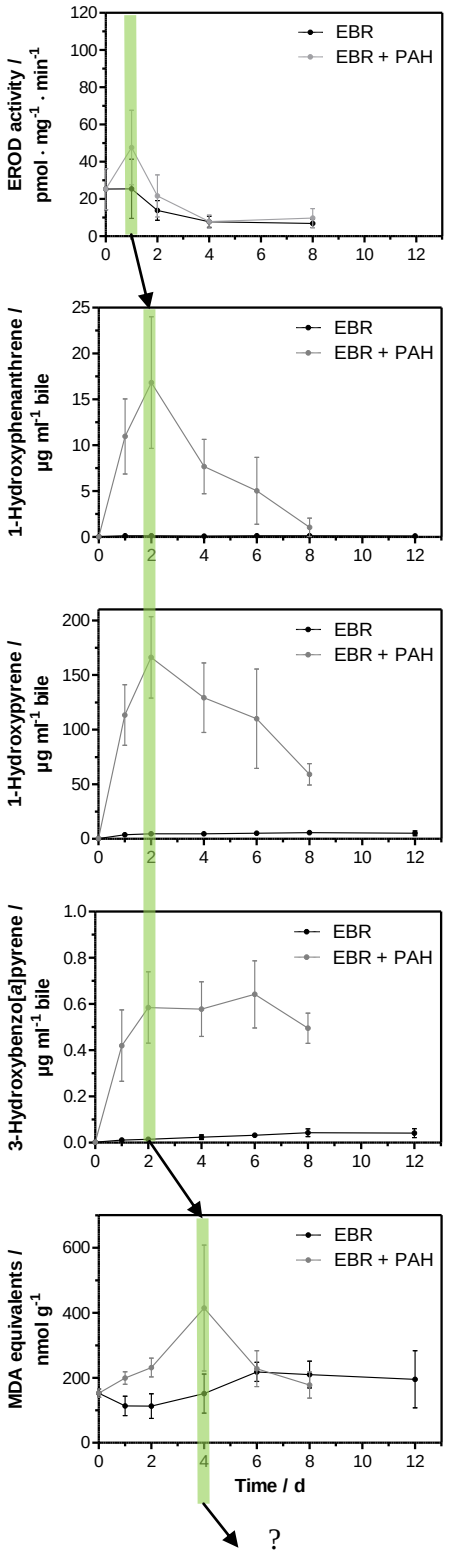

Figure 4.1 Effect-cascade of biomarker reactions in the spiked sediment experiment at 24°C: Following transient EROD induction, peaks in biliary metabolite concentrations and lipid peroxidation were observed.

4.5 Influence of temperature (stress) on uptake and effects of PAHs from sediment suspensions

Since degradation, bioavailability and effects of particle-bound pollutants can substantially vary depending on temperature, exposure experiments were conducted at an average temperature representative for rivers in Central Europe (12°C) and under temperature stress (24°C) to investigate a range of possible consequences of sediment resuspension.

In many studies, critical thermal maxima (CTM) of salmonids were investigated by constantly increasing water temperature and observing behavior, growth and survival. Resulting values ranged from 24 to 29°C, depending on food availability and quality, developmental stage, sex, and other environmental parameters. The comparably high mortality of rainbow trout in the 24°C experiment of the present study (25.0 and 30.6 % for the unspiked and spiked treatment, respectively) can thus be explained by direct effects of temperature. Furthermore, higher temperatures often occur simultaneously with lower oxygen concentrations due to the reduced oxygen solubility (Figure 3.2). Rubin (1998) identified dissolved oxygen concentration as the most critical factor for the survival of rainbow trout during the whole life-cycle. For most salmonid species, dissolved oxygen concentrations below 5-6 mg L^{-1} can result in mortality (Weithman & Haas 1984). Concentrations in the 24°C experiments (average 7.7 mg L^{-1}) were lower compared to the 12°C experiments (average 10.5 mg L^{-1}) but did not fall below these critical values.

Gross metabolism of fish was significantly elevated in animals exposed at 24°C as represented by the elevated excretion rate of biliverdin (cf. Avery et al. 1992), which was also found with regard to uptake and metabolism of PAHs. Although the measured maximum concentrations in bile liquid did not differ much between the two temperatures, PAH uptake was approximately 2-fold faster at 24°C compared to 12°C. Although significant uptake of PAHs from the spiked sediment was observed in the cooled experiment, elevated hepatic lipid peroxidation was only found in the uncooled experiment after 4 days (Figure 3.10). Benzo[*a*]pyrene has been shown to be converted to a 1,6-quinone metabolite in fish *via* the precursor 1-hydroxybenzo[*a*]pyrene (Di Giulio & Hinton 2008). Lemaire et al. (1994) reported the generation of ROS by benzo[*a*]pyrene quinones in hepatic microsomes of flounder and perch, leading to the hypothesis that uptake and metabolism of benzo[*a*]pyrene and other PAHs can finally lead to oxidative damage. Fish appeared to be able to compensate this oxidative damage potential at lower temperature so that LPO was only observed in the spiked 24°C treatment. Similar effects have been observed by other researchers, who

concluded that turnover of lipids was higher at lower temperature, which has a protective function (Grim et al. 2010, Lushchak 2011, Lushchak & Bagnyukova 2006, Parihar & Dubey 1995).

EROD activity was not elevated following exposure to spiked sediment suspensions in the present study – neither at 12 nor at 24°C. Following exposure at 24°C, however, it could be observed that EROD activity was significantly reduced in both treatments by factor 3.2. Whyte et al. (2000) reviewed a large number of datasets on EROD activity in either laboratory or field studies and found a negative correlation with temperature, too.

It can be concluded that the biological effects that were assessed in the present study were only apparent in combination with temperature stress during exposure. These findings support the hypothesis that a combination of chemical exposure and other environmental stressors can lead to enhanced effects in aquatic biota (Holmstrup et al. 2010). Since temperatures of German rivers frequently exceed 25°C during summer as a result of waste heat of power plants and climate change (IKSR 2004), it can be assumed that resuspension of sediments under these conditions could potentially have higher impact on aquatic biota compared to lower temperatures. As in the case of dissolved pollutants, where it has been shown that sediment toxicity can significantly vary with varying temperatures by many authors (e.g. Airas et al. 2008, Heinonen et al. 2002, Honkanen & Kukkonen 2006), the results of this study indicate that enhanced toxicity of sediment-bound contaminants has to be expected as a consequence of climate change as an additional threat to the net increase of sediment resuspension and sedimentation, which is accompanied by many negative side-effects, such as 50 % reduction of appropriate water reservoirs within the next few decades (Spreafico & Bruk 2004).

4.6 Suitability of fixed wavelength fluorescence measurements for the determination of bile metabolites

Determination of PAH metabolites in fish bile by means of chromatographic methods, e.g. the HPLC-F method applied in the experiments of Floodsearch and Floodsearch II, is costly and requires sophisticated analytical instrumentation. To evaluate the possibility to replace this method by the less demanding fixed wavelength fluorescence method previously described, both methods were compared using samples from two different experiments.

Most studies applying fixed wavelength fluorescence calibrate the arbitrary fluorescence units of the respective instrument against either (a) an external standard solution of hydroxy PAHs, (b) bile liquid of fish exposed to single PAHs or (c) against an external PAH standard with subsequent expression as PAH equivalent units (Beyer et al. 2010). Each of the methods has major drawbacks: When using external standards, matrix effects due to "internal filter effects" (e.g., collisional quenching or resonance energy transfer) are not taken into account. Exact quantification of distinct metabolites using bile liquid from animals exposed to single PAHs is, however, limited by the large number of different conjugated and non-conjugated metabolites present which fluoresce at similar wavelength pairs (Aas et al. 1998, Aas et al. 2000, Ariese et al. 2005).

Thus, the performance of the fixed wavelength fluorescence method was evaluated by direct calibration of the sample fluorescence versus the analytically determined metabolite concentration by means of HPLC-F in the present study (Figure 3.12). Using a set of validation samples from another project (KonSed, Dipl.-Biol. Sebastian Hudjetz, 2011), the predicted concentrations of 1-hydroxypyrene by the abovementioned method were compared with the concentrations measured by HPLC-F (Figure 3.13). A good correlation between the predicted and measured concentrations was found, however with a systematic overestimation of biliary 1-hydroxypyrene concentrations in the validation samples by a factor of approximately 2.6. This finding might also be attributed to internal filter effects in the calibration dataset. Thus, the FF method seems appropriate for studies in which only qualitative information is needed and for comparisons of samples within one experiment with comparable exposure conditions. For future measurements within the project Floodsearch II, however, the chromatographic method seems to be the more suitable one due to the more detailed information provided.

4.7 Optimization of the current method for quantification of hepatic 7-ethoxyresorufin-*O*-deethylase activity for determination in S9 fractions

The method of measuring EROD activity according to Kennedy and Jones (1994) applied in Floodsearch was originally designed for use with liver microsomes. In order to optimize the original EROD protocol, a modified approach was presented where the resorufin fluorescence in incubated samples is corrected for the background fluorescence of the samples (without enzymatic reaction). Such correction was also applied by Pohl & Fouts (1980), who

performed the measurement of EROD activity in cuvettes. Thus, the combination of both protocols seemed useful to correct for inter-sample differences due to quenching or autofluorescence. As can be seen in particular for samples with comparably low EROD activity, the number of valid measurements was significantly increased compared to the commonly used correction method (data not shown). On the one hand, this method has the potential to provide accurate quantification of EROD activity even when it is not possible to perform the measurements with microsomal fractions, e.g. if an ultracentrifuge is not available in on-site laboratories or on research vessels. On the other hand, the measurement following this protocol increases the amounts of the reagents used and the costs for investigation of each sample by factor two.

4.8 Suitability of hepatic gene expression by means of real-time RT-PCR as a tool for earlier detection of the effects of sediment-bound contaminants

In the experiments of the predecessor project Floodsearch it was shown that the more "common" biomarkers of exposure to dioxin-like compounds, i.e. EROD and glutathione-*S*-transferase (GST), were not suitable for the detection of exposure to particle-bound PAHs. Thus, it was hypothesized by Brinkmann et al. (2010a) that investigation of markers on the transcript level, i.e. gene expression analyses, might be a more applicable tool to earlier detect changes (as already demonstrated before by Tompsett et al. 2009, Zhang et al. 2008). Therefore, preserved liver tissue samples from the Floodsearch project were analyzed for the mRNA expression of a number of genes to verify the applicability of these molecular methods. The PAHs that were used to spike sediments are known to be moderately potent AhR agonists *in vitro* (Barron et al. 2004), and have been shown to cause significant induction of biotransformation enzymes in rainbow trout (Fragoso et al. 2006, Jonsson et al. 2006, Oikari et al. 2002, Ramachandran et al. 2006). Surprisingly, the activity of neither the phase I biotransformation enzyme EROD, nor the phase II enzyme GST was altered by exposure to the PAH spiked sediments during the 5 d simulated flood events of Floodsearch. Furthermore, none of the examined treatment groups showed detectable levels of CYP1A protein. The expression of genes belonging to the AhR-gene battery, however, was significantly altered after exposure to polluted sediments, indicating a higher sensitivity compared to the traditional biomarkers on the protein and enzyme level (CYP1A protein content, EROD activity). The presence of biliary metabolites of the three PAHs clearly demonstrated

bioavailability, substantial uptake and metabolic transformation. Thus, it was hypothesized by Brinkmann et al. (2010a) that (a) AhR-mediated biotransformation enzymes were not highly inducible under the exposure scenario in the annular flume or (b) exposure time of 5 d was not sufficient. Several authors showed that EROD activity and CYP1A expression in fish were significantly influenced by developmental stage and age (Cantrell et al. 1996, Peters & Livingstone 1995), with the early life-stages mostly showing higher activity and inducibility. Furthermore, temperature, pH and other environmental parameters, as well as inhibitors can significantly influence EROD activity in fish (for review, see Whyte et al. 2000). From the data of the exposure experiment within this thesis, however, it can be seen that EROD activity was not significantly increased at any time following exposure to either unspiked or spiked sediment from Ehrenbreitstein. Thus, it would be interesting to follow the dynamics of hepatic gene expression during exposure to the suspensions.

5. Conclusions and Outlook

Within the current thesis, the set of biomarkers used for sediment resuspension studies with rainbow trout was successfully extended. The fixed wavelength fluorescence method was shown to be a useful tool to detect biliary PAH metabolites during resuspension experiments, but less precise than the chromatographic determination by means of HPLC-F. Thus, the latter method will be applied in further experiments.

Biomarkers responded in a cascade-like pattern. Thus, sediment resuspension experiments in the annular flume must be accompanied by selected suspension exposures to improve the understanding of the underlying processes. The current method for conducting these exposures should be modified due to potential alterations of the sediment-water partitioning of PAHs by fish. Field-aged PAH contaminated sediments should be preferred over the use of spiked sediments for mechanistic studies, since the bioavailable fractions differed significantly. Temperature was shown to be an environmental stressor that can potentially lead to increased effects caused by particle-bound contaminants in flood events. Availability of field-aged PAH contamination in the sediment from the harbour Ehrenbreitstein was comparably high, leading to the hypothesis that even moderately contaminated sediments can pose a risk to aquatic biota during resuspension and flood events

In future, the here developed set of biomarkers will be applied for further experiments in the annular flume. It will be necessary to demonstrate the relevance of the measured biomarker responses with respect to adverse effects by investigating more markers (e.g. histopathology, reproductive endpoints, and growth) after a longer post-exposure incubation period. The findings of the kinetic experiments will be completed with gene expression analyses in the preserved liver samples of this study in order to get insights in potential differences between transcript expression of genes and enzyme activities following exposure to sediment suspensions. Furthermore, the relevance of the exposure scenario should be verified by systematic behavioural studies since it seems likely that fish would, e.g. show avoidance of waters with high turbidity during flood events.

6. Literature

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Zielke H 2011: Time-related alterations and other confounding factors in direct sediment contact tests, PhD thesis, RWTH Aachen University Aachen

7. Scientific publications related to this thesis

Journal publications

Cofalla, C., Roger, S., Frings, R.M., Schüttrumpf, H., **Brinkmann, M.**, Hudjetz, S., Wölz, J., Hollert, H., Gerbersdorf, S.U., Hecker, M., Kammann, U., Lennartz, G., Schäffer, A. (2011) Eine Methode zur Bestimmung der Wechselwirkungen zwischen Sedimentdynamik und Ökotoxikologie. *Hydrologie und Wasserwirtschaft* 11: 227-230.

Gerbersdorf, S.U., Hollert, H., **Brinkmann, M.,** Wieprecht, S., Schüttrumpf, H., Manz, W. (2011) Anthropogenic pollutants affect ecosystem services of freshwater sediments: the need for a "triad plus x" approach. *Journal of Soils and Sediments*, DOI: 10.1007/s11368-011-0373-0

Brinkmann, M., Hallare, A.V., Hudjetz, S., Keiter, S., Seiler, T.-B., Wölz, J., Gerbersdorf, S.U., Cofalla, C., Roger, S., Schüttrumpf, H., Hollert, H. (2010) Toxicity and risk assessment of fluvial sediments and suspended matter: a short overview of past and recent developments in sediment toxixity assessment. *Umweltwissenschaften und Schadstoffforschung* 22: 651-655.

Brinkmann, M., Hudjetz, S., Cofalla, C., Roger, S., Kammann, U., Zhang, X., Wiseman, S., Giesy, J., Hecker, M., Schüttrumpf, H., Wölz, J., Hollert, H. (2010) A combined hydraulic and toxicological approach to assess re-suspended sediments during simulated flood events. Part I–multiple biomarkers in rainbow trout. *Journal of Soils and Sediments* 10:1347–1361

Wölz, J., Cofalla, C., Hudjetz, S., Roger, S., **Brinkmann, M**., Schmidt, B., Schäffer, A., Kammann, U., Lennartz, G., Hecker, M., Schüttrumpf, H. & Hollert, H. (2008) In search for the ecological and toxicological relevance of sediment re-mobilisation and transport during flood events. *Journal of Soils and Sediments* 9(1): 1-5.

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Brinkmann, M., Hudjetz, S., Cofalla, C., Roger, S., Kammann, U., Schüttrumpf, H., Hollert, H. (2011) Uptake and effects of particle-bound polycyclic aromatic hydrocarbons (PAH) from spiked sediment suspensions in rainbow trout (*Oncorhynchus mykiss*, Walbaum). Proceedings, 21th SETAC Europe Annual Meeting 2011, Milan, Italy.

Kuckelkorn, J., **Brinkmann, M.,** Hudjetz, S., Cofalla, C., Roger, S., Buchinger, S., Reifferscheid, G., Erdinger, L., Zimmer, H., Schüttrumpf, H., Hollert, H. (2011) Assessing the toxicity of metals in suspended sediments with different biomarkers. Proceedings, 21th SETAC Europe Annual Meeting 2011, Milan, Italy.

Gottselig, N., Bluhm, K., **Brinkmann, M.,** Hudjetz, S., Peddinghaus, S., Schulze, M., Heimann, W., Schulz, R., Hollert, H. (2010) Mutagenic activity in sediments and fish samples of the quarry pond Karlskopf of River Rhine: linking effects from the laboratory to the field. Aquatic Toxicity Workshop, October 3-6 2010, Toronto, Canada.

Kuckelkorn, J., **Brinkmann, M.**, Hudjetz, S., Cofalla, C., Roger, S., Buchinger, S., Reifferscheid, G., Erdinger, L., Schüttrumpf, H., Hollert, H. (2010) Bewertung der Metalltoxizität in resuspendierten Sedimenten anhand der Quantifizierung von Metallothionein als Biomarker. Proceedings, SETAC German Language Branch Annual Meeting 2010, Dessau, Germany.

Brinkmann, M., Hudjetz, S., Cofalla, C., Roger, S., Schüttrumpf, H., Hollert, H. (2010) The interdisciplinary project FLOODSEARCH II – Flood risk assessment between sediment dynamics, contamination and climate change. Proceedings, 20th SETAC Europe Annual Meeting 2010, Sevilee, Spain.

Hudjetz, S., **Brinkmann, M**., Cofalla, C., Roger, S., Schmidt, B., Schäffer, A., Schüttrumpf, H., Hollert, H., Wölz, J. (2010) Searching for the (eco)toxicological relevance of sediment re-mobilisation and transport during flood events in rivers. Part A: instrumental chemical analysis and effect-directed analysis (EDA). Proceedings, 20th SETAC Europe Annual Meeting 2010, Seville, Spain.

Platform presentations

Cofalla, C., Hudjetz, S., Roger, S., **Brinkmann, M.,** Eisenbeis, P., Hollert, H., Schüttrumpf, H. (2011) Remobilization of particle-bound contaminants from re-suspended sediments and their impact on aquatic organisms. Proceedings 7th International SedNet Conference, April 2011, Venice, Italy.

Heger, S., Bluhm, K., **Brinkmann, M.**, Winkens, K., Schneider, A., Wollenweber, M., Maletz, S., Wölz, J. Agler, M., Angenent, L., Seiler, T.-B., Hollert, H. (2011) What's up inside the reactor - biotests for risk assessment of biofuel fermentation. Proceedings, 2nd SETAC Europe YES-Meeting 2011, Aachen, Germany.

Cofalla, C., Roger, S., Hudjetz, S., **Brinkmann, M.**, Schüttrumpf, H., Hollert, H. (2010) Floodsearch - Hydrotoxic Investigations of Contaminated Sediment in an Annular flume. Proceedings, 32th PIANC Congress, Liverpool, United Kingdom, 11.-14.05.2010 ISBN: 978-2-87223-179-9.

Brinkmann, M. Hudjetz, S., Cofalla, C., Roger, S., Kamman, U., Giesy, J., Hecker, M., Schüttrumpf, H., Wölz, J., Hollert, H. (2010) FLOODSEARCH Part B: A multiple biomarker approach to assess the toxicity of resuspended sediments during simulated flood events. Proceedings, 20th SETAC Europe Annual Meeting 2010, Seville, Spain.

Brinkmann, M., Hudjetz, S., Wölz, J., Cofalla, C., Roger, S., Schüttrumpf, H., Hollert, H. (2009) Risikobewertung und Toxizität von Sedimenten und Schwebstoffen in Fließgewässern. Seminarreihe "Regionale Wasserwirtschaft in Theorie und Praxis" 2009, Trier, Germany.

Cofalla, C., **Brinkmann, M.**, Roger, S., Hudjetz, S., Kammann, U., Zhang, X., Giesy, J., Hecker, M., Wölz, J., Hollert, H., Schüttrumpf, H. (2010) Hydrotoxische Untersuchungen schadstoffbehafteter Sedimente im Kreisgerinne, Proceedings: 40. Internationales Wasserbau-Symposium Aachen, Aachen.

Brinkmann, M., Hudjetz, S., Cofalla, C., Roger, S., Kammann, U., Giesy, J., Hecker, M., Schüttrumpf, H., Wölz, J., Hollert, H. (2009) Searching for the (eco)toxicological relevance of sediment remobilisation and transport during flood events in rivers. Part B: CYP1A1, micronucleus test, gene expression analysis and PAH metabolites in bile. Proceedings, SETAC German Language Branch Annual Meeting 2009, Weihenstephan, Germany.

8. Curriculum vitae

Markus Brinkmann
Kronenberg 49
52074 Aachen

Telefon: +49 (0) 241/ 80-26686
Fax: +49 (0) 241/ 80-22182
E-Mail: markus.brinkmann@bio5.rwth-aachen.de

Ausbildung

Juli 2009 bis August 2011	Biologiestudium (M. Sc.) an der RWTH Aachen University
24. Juli 2009	Bachelorprüfung Biologie (Note 1,7)
Juni bis August 2009	Forschungsaufenthalt in Saskatoon, Kanada (University of Saskatchewan) im Undergraduate Research Opportunities Programme (UROP) der RWTH Aachen
Juli 2008 bis Januar 2009	ERASMUS-Semester in Stockholm, Schweden (Universität Stockholm) und Forschungsaufenthalt am ITM (Institut für angewandte Umweltwissenschaft), Abteilung für ökotoxikologische Testmethoden (Magnus Breitholtz)
Oktober 2007 bis Juli 2009	Biologiestudium (B. Sc.) an der RWTH Aachen University
Oktober 2006 bis Oktober 2007	Biologiestudium (B. Sc.) an der Ruprecht-Karls-Universität, Heidelberg

Studienbegleitende Tätigkeiten

Bis Februar/März 2011	Organisation und Durchführung des zweiten SETAC Europe Young Environmental Scientists Meeting (YES-Meeting) in Aachen.
Seit September 2009	Wissenschaftliche Hilfskraft im Lehr- und Forschungsgebiet Ökosystemanalyse (Prof. Dr. Henner Hollert) am Institut für Umweltforschung, RWTH Aachen University.
Oktober 2007 bis Juli 2009	Studentischer Mitarbeiter im Lehr- und Forschungsgebiet Ökosystemanalyse (Prof. Dr. Henner Hollert) am Institut für Umweltforschung, RWTH Aachen University.
Februar bis Oktober 2007	Studentische Hilfskraft am Institut für Zoologie, Abteilung Aquatische Toxikologie (Prof. Dr. Thomas Braunbeck), Arbeitsgruppe Dr. Henner Hollert.

Mitgliedschaften

Seit Mai 2011	Mitglied des Editorial Board von Environmental Sciences Europe (ESEU)
Mai 2009 bis Mai 2011	Vorsitzender SETAC Europe Student Advisory Council (SAC) und non-voting Member im SETAC Europe Council (SEC)
Seit Mai 2008	SETAC Europe Student Advisory Council (SAC), gewähltes Mitglied
Seit März 2005	Society of Environmental Toxicology and Chemistry (SETAC)

Session Chair

SETAC Europe YES-Meeting 2011 in Aachen	Session "Omics and biomarkers: Linking sub-organismic responses to ecologically relevant effects."
SETAC Europe YES-Meeting 2009 in Landau	Session "Fate of pollutants in the environment."

Tagungsteilnahmen

2007	SETAC Europe Jahrestagung in Porto, Portugal
2007	SETAC German Language Branch, Leipzig, Deutschland
2008	SETAC Europe Jahrestagung in Warschau, Polen
2009	SETAC Europe Young Environmental Scientists Meeting, Landau, Deutschland
2009	SETAC Europe Jahrestagung in Göteborg, Schweden
2009	SETAC German Language Branch, Weihenstephan, Deutschland
2009	Regionale Wasserwirtschaft in Theorie und Praxis, Trier, Deutschland
2009	SETAC North America, New Orleans, USA
2010	SETAC Europe Jahrestagung in Sevilla, Spanien
2011	SETAC Europe Young Environmental Scientists Meeting, Aachen, Deutschland
2011	SETAC Europe Jahrestagung in Mailand, Italien

Abschlussarbeiten

Brinkmann, M. (2009) A multiple biomarker approach to assess the toxicity of resuspended sediments. Department of Ecosystem Analysis, Institute for Environmental Research, RWTH Aachen University.

Brinkmann, M. (2008) Effects of STP effluents on development and reproduction of the harpacticoid copepod *Nitocra spinipes* - Are matrix population models adequate tools to evaluate data from lifecycle studies? Institute for Applied Environmental Science (ITM), Stockholm University.

Vorträge

Cofalla, C., Hudjetz, S., Roger, S., **Brinkmann, M.,** Eisenbeis, P., Hollert, H., Schüttrumpf, H. (2011) Remobilization of particle-bound contaminants from re-suspended sediments and their impact on aquatic organisms. Proceedings 7th International SedNet Conference, April 2011, Venice Italy.

Heger, S., Bluhm, K., **Brinkmann, M.**, Winkens, K., Schneider, A., Wollenweber, M., Maletz, S., Wölz, J. Agler, M., Angenent, L., Seiler, T.-B., Hollert, H. (2011) What's up inside the reactor - biotests for risk assessment of biofuel fermentation. Proceedings, 2nd SETAC Europe YES-Meeting 2011, Aachen, Germany.

Cofalla, C., Roger, S., Hudjetz, S., **Brinkmann, M.**, Schüttrumpf, H., Hollert, H. (2010) Floodsearch - Hydrotoxic Investigations of Contaminated Sediment in an Annular flume. Proceedings, 32th PIANC Congress, Liverpool, United Kingdom, 11.-14.05.2010 ISBN: 978-2-87223-179-9.

Brinkmann, M. Hudjetz, S., Cofalla, C., Roger, S., Kamman, U., Giesy, J., Hecker, M., Schüttrumpf, H., Wölz, J., Hollert, H. (2010) FLOODSEARCH Part B: A multiple biomarker approach to assess the toxicity of resuspended sediments during simulated flood events. Proceedings, 20th SETAC Europe Annual Meeting 2010, Seville, Spain.

Brinkmann, M., Hudjetz, S., Wölz, J., Cofalla, C., Roger, S., Schüttrumpf, H., Hollert, H. (2009) Risikobewertung und Toxizität von Sedimenten und Schwebstoffen in Fließgewässern. Seminarreihe "Regionale Wasserwirtschaft in Theorie und Praxis" 2009, Trier, Germany.

Cofalla, C., **Brinkmann, M.**, Roger, S., Hudjetz, S., Kammann, U., Zhang, X., Giesy, J., Hecker, M., Wölz, J., Hollert, H., Schüttrumpf, H. (2010) Hydrotoxische Untersuchungen schadstoffbehafteter Sedimente im Kreisgerinne, Proceedings: 40. Internationales Wasserbau-Symposium Aachen, Aachen

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Seiler, T.-B., Schulze, T., Streck G., Schwab, K., Zielke, H., **Brinkmann, M.**, Bernecker, C., Brack, W., Braunbeck, T. & Hollert, H. (2008) Evaluation of the leaching power of passive membrane dialysis compared to conventional extraction procedures. Proceedings, 18th SETAC Europe Annual Meeting 2008, Warsaw, Poland.

Poster

Hudjetz, S., **Brinkmann, M.,** Cofalla, C., Roger, S., Kammann, U., Hecker, M., Schmidt, B., Schüttrumpf, H., Wölz, J., Hollert, H. (2011) A multiple biomarker approach to assess the toxicity of re-suspended sediments during simulated flood events – a feasibility study. Proceedings, 7th International SedNet Conference, April 2011, Venice Italy.

Brinkmann, M., Hudjetz, S., Cofalla, C., Roger, S., Kammann, U., Schüttrumpf, H., Hollert, H. (2011) Uptake and effects of particle-bound polycyclic aromatic hydrocarbons (PAH) from spiked sediment suspensions in rainbow trout (*Oncorhynchus mykiss*, Walbaum). Proceedings, 21th SETAC Europe Annual Meeting 2011, Milan, Italy.

Lundström, E., Breitholtz, M., **Brinkmann, M**., Preuss, T.G. (2011) Modelling harpacticoid copepod populations; a comparison of matrix and individual based modelling. Proceedings, 21th SETAC Europe Annual Meeting 2011, Milan, Italy.

Kuckelkorn, J., **Brinkmann, M.,** Hudjetz, S., Cofalla, C., Roger, S., Buchinger, S., Reifferscheid, G., Erdinger, L., Zimmer, H., Schüttrumpf, H., Hollert, H. (2011) Assessing the toxicity of metals in suspended sediments with different biomarkers. Proceedings, 21th SETAC Europe Annual Meeting 2011, Milan, Italy.

Heger, S., Bluhm, K., **Brinkmann, M.**, Winkens, K., Schneider, A., Wollenweber, M., Maletz, S., Wölz, J. Agler, M., Angenent, L., Seiler, T.-B., Hollert, H. (2011) What's up inside the reactor - biotests for risk assessment of biofuel fermentation. Proceedings, 21th SETAC Europe Annual Meeting 2011, Milan, Italy.

Gottselig, N., Bluhm, K., **Brinkmann, M.,** Hudjetz, S., Peddinghaus, S., Schulze, M., Heimann, W., Schulz, R., Hollert, H. (2010) Mutagenic activity in sediments and fish samples of the quarry pond Karlskopf of River Rhine: linking effects from the laboratory to the field. Aquatic Toxicity Workshop, October 3-6 2010, Toronto, Canada.

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Brinkmann, M., Hinger, G., Bluhm, K., Sagner, A., Takner, H., Eisenträger, A., Braunbeck, T., Engwall, M. Thiem, A., Hollert, H. (2010) Heterocyclic aromatic compounds are Ah receptor agonists *in vitro*. Proceedings, 20th SETAC Europe Annual Meeting 2010, Seville, Spain.

Brinkmann, M., Hudjetz, S., Cofalla, C., Roger, S., Schüttrumpf, H., Hollert, H. (2010) The interdisciplinary project FLOODSEARCH II – Flood risk assessment between sediment dynamics, contamination and climate change. Proceedings, 20th SETAC Europe Annual Meeting 2010, Sevilee, Spain.

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In Vorbereitung

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