A multiple biomarker approach to assess the toxicity of resuspended sediments

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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 the past decade, an increasing number of extreme weather events have been observed around the globe, ultimately resulting in the resuspension of contaminated sediments in rivers. Recently, several studies have identified the relevance of hydrodynamics to environmental risk assessment of regularly flooded rivers. Thus, it is necessary to understand and predict possible ecotoxicological consequences of contaminant remobilization caused by flood and run-off events, and new scientific approaches are required. The combination of hydrodynamics and ecotoxicology has become an emerging field in environmental research.

In the present study, methodologies of hydraulic engineering and ecotoxicology were combined in a new interdisciplinary approach to assess the risks associated with sediment resuspension. Rainbow trout (*Oncorhynchus mykiss*) were exposed to unspiked artificial sediments and to sediment that was spiked with polycyclic aromatic hydrocarbons (PAH) under simulated flood conditions in an annular flume, a facility that can be used to study erosion and sedimentation processes. A set of different biomarkers was used to verify the hypothesis that resuspension of sediments can lead to adverse effects in aquatic biota. Hepatic activities of the enzymes 7-ethoxyresorufin-*O*-deethylase (EROD), glutathione-*S*-transferase (GST) and catalase (CAT), lipid peroxidation in liver homogenates, PAH metabolites in bile fluid and micronucleus formation in blood cells were assessed.

Quantification of biliary metabolites has shown to be a suitable marker for substantial uptake and metabolic transformation of PAHs during the simulated flood event. Exposure to contaminated sediments caused a significant induction of micronuclei in peripheral erythrocytes, where the micronucleus frequency in this treatments was positively correlated with the biliary concentration of 1-hydroxybenzo[*a*]pyrene, a metabolite of the genotoxic PAH benzo[*a*]pyrene. The flood event with unspiked artificial sediment caused significant hepatic lipid peroxidation that was, surprisingly, significantly lowered in the presence of spiked sediment. Results from the enzymatic markers, however, were inconclusive.

The presented study has shown that relatively short exposure during simulated flood events to resuspended sediments can lead to adverse effects in rainbow trout. Thus, the ecological and toxicological impact of contaminant remobilization during floods has to be considered highly relevant and integrated approaches for risk assessment of regularly flooded rivers are urgently required.

Zusammenfassung

Während der letzten zehn Jahre wurde eine weltweit steigende Zahl extremer Wetterereignisse beobachtet, die zur Resuspension kontaminierter Sedimente in Fließgewässern führen können. Die Relevanz hydrodynamischer Untersuchungen für die Bewertung der Umweltfolgen von Hochwässern in regulär überfluteten Flusssystemen wurde bereits von mehreren Studien identifiziert. Daher ist es notwendig, mögliche ökotoxikologische Konsequenzen der Remobilisierung von Schadstoffen während extremer Abflussereignisse zu verstehen und vorhersagen zu können – innovative wissenschaftliche Ansätze sind dringend erforderlich. Die Kombination von Methoden aus Hydrodynamik und Ökotoxikologie nimmt einen steigenden Stellenwert ein.

Zur Abschätzung der Risiken, die mit der Resuspension kontaminierter Sedimente einhergehen, wurden in dieser Studie Methoden des Wasserbau-Ingenieurwesens und der Ökotoxikologie in einem interdisziplinären Ansatz miteinander kombiniert. Regenbogenforellen (*Onchorhynchus mykiss*) wurden in einem Kreisgerinne, einem Gerät zur Untersuchung von Erosions- und Sedimentationsprozessen – unter simulierten Hochwasserverhältnissen gegenüber Kunstsedimenten exponiert, die mit Polyzyklischen Aromatischen Kohlenwasserstoffen (PAK) dotiert wurden. Eine Batterie verschiedener Biomarker wurde untersucht um die Hypothese zu verifizieren, dass die Resuspension kontaminierter Sedimenten zu adversen Effekten in aquatischen Organismen führen kann. Die hepatischen Enzymaktivitäten von 7-Ethoxyresorufin-*O*-Deethylase (EROD), Glutathion-*S*-Transferase (GST) und Katalase (CAT), sowie die Lipidperoxidation in Leberhomogenaten, Konzentrationen von PAK-Metaboliten in Gallenflüssigkeit und die Induktion von Mikronuklei in peripheren Erythrozyten wurden quantifiziert.

Die Messung von Gallenmetaboliten konnte als ein geeigneter Marker für die erhebliche Aufnahme und metabolische Transformation von PAKs während des simulierten Hochwassers identifiziert werden. Die Exposition gegenüber kontaminierten Sedimenten führte zu einer signifikanten Induktion der Mikrokernrate, die positiv mit der Konzentration von 1-Hydroxybenzo[*a*]pyren in der Gallenflüssigkeit korrelierte – einem Metabolit des genotoxischen PAK Benzo[*a*]pyren. Weiterhin verursachte das simulierte Hochwasserereignis erheblichen oxidativen Stress (Lipidperoxidation), der überraschenderweise in der PAK-exponierten Gruppe signifikant verringert war. Die Untersuchungen der Enzymaktivitäten ergaben keine eindeutigen Ergebnisse.

Die vorliegende Studie konnte zeigen, dass relativ kurze Exposition gegenüber resuspendierten Sedimenten während simulierter Hochwasserereignisse adverse Effekte in Regenbogenforellen verursachen kann. Die ökologischen und toxikologischen Auswirkungen der Remobilisierung von Schadstoffen während extremer Hochwasserereignisse sollten unbedingt bei der Hochwasserfolgenbewertung berücksichtigt werden. Integrierte Ansätze für die Risikobewertung regulär überfluteter Fließgewässer sind daher dringend notwendig.

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1. Introduction

1.1 Sediments

Sediments are particulate matter with geogenic or biogenic origin that has been deposited at the bottom of aquatic systems (Scheffer & Schachtschnabel 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, e.g. iron oxides (Power & Chapman 1992). Sediments provide habitats for structurally and functionally diverse communities and constitute important reservoirs for both organic and inorganic substances (Brils 2004). Particularly microorganisms, primarily bacteria, significantly affect biogeochemical cycles of substances in sediments (Ahlf 1995). Mainly benthic organisms are strongly dependent on sediment quality, since many of them ingest sediment particles directly or indirectly as food (Ahlf 1995, Burton 1991, Höss et al. 1997).

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 & Forstner 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 & Forstner 2005), resulting in numerous concepts for risk estimation (Ahlf et al. 2002, Burton 1991, 1995, Chapman 1990, 2000, Chapman et al. 2002). So far, most sediment assessment studies focused on the development of suitable bioanalytical methods and the evaluation of their potential to characterise sediment-bound contaminants (Brack et al. 2007a, Brack et al. 2007b, Hollert et al. 2000, Hollert et al. 2003, Rao et al. 1990, Wölz et al. 2008). The role of sediment resuspension, e.g. during flood events, and possible ecotoxicological effects of remobilised particle-bound contaminants to aquatic organisms have scarcely been investigated (for review, see Hollert et al. 2008).

One of the major concerns in this context is the occurrence of increasingly frequent and intense flood events that is discussed as one potential consequence of climate change (Wölz et al. 2009). Netzband et al. (2007) have identified that flood events in four river basins (Danube River, Rio Duoro, Humbe River, and Elbe River) have to be considered to adequately manage sediment quality. In the past decade, an increasing number of extreme weather events have been observed around the globe, resulting in 500-year floods such as what occurred in 2002 in the River Elbe, Germany (Schüttrumpf & Bachmann 2008), that led to the remobilisation of highly contaminated sediment layers. Some studies suggested potential adverse effects of SPM to aquatic organisms. In this context, Einsporn et al. (2005) reported histological alterations in livers of flounders (*Platichthys flesus* L.) and digestive glands of blue mussels (*Mytilus edulis*) collected from the Elbe estuary and the Wadden Sea 5 months after the 2002 flood event. In comparison to earlier long-term studies conducted at the same sampling sites, a significant impairment of the function of certain cell organelles, i.e. lysosomes, was found, which are involved in the detoxication and elimination of contaminants in the fish liver. Additionally, a long-term study investigating trends in 7-ethoxyresorufin-*O*-deethylase (EROD) activity in livers of dab (*Limanda limanda*) from the German Bight (North Sea) between 1995 and 2003 (Kammann et al. 2005) reported significantly elevated EROD activities in fall 2002. It was hypothesized that such an increase may be related to the remobilisation of dioxin-like compounds during the Elbe River flood event in 2002 (Hollert et al. 2008). Thus, it is necessary to understand and predict possible ecotoxicological consequences of contaminant remobilization caused by flood and run-off events. New scientific approaches are required for the assessment of regularly flooded rivers – the combination of hydrodynamics and ecotoxicology has become an emerging field in environmental research (Hollert et al. 2008). Recently, it has been proposed to include hydrodynamics as an

additional Line-of-evidence in Weight-of-evidence approaches to assess the environmental impact of sediments (Chapman & Hollert 2006).
In this context, biomarkers (i.e., EROD activity) have shown to be useful tools to evaluate effects of particle-bound contaminants in-field. In this study, a multiple biomarker approach was thus used to investigate exposure to organic contaminants that were remobilised during a simulated flood event.

1.2 Biomarkers

Ecotoxicological responses can be assessed at different levels of organization (Figure 1.1). On the one hand, effects at high levels of organization, e.g. the community or ecosystem level, are of high ecological relevance, but specificity, in terms of identification of single stressors, may be poor. On the other hand, effects at lower levels of organization, e.g. the molecular or cellular level, may be highly specific to certain (chemical) stressors but the ecological relevance can be questioned (Hanson 2008, Walker et al. 2006).

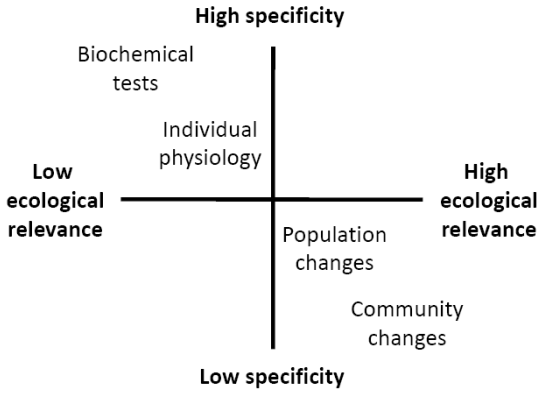

Figure 1.1 Scheme highlighting the conflict between specificity and ecological relevance in biological effects monitoring. Modified from Addison (1996).

There are several definitions of the term biomarker, most being relatively wide by means of including both the sub-individual and the population or ecosystem level (McCarty & Munkittrick 1996, Peakall 1994). Here, biomarkers are defined based on the suggestions of van Gestel and van Brummelen (1996), who distinguished between different levels of organization. Accordingly, *biomarkers* provide measures below the individual level, *bioindicators* are organisms that describe the environmental status by their presence or absence and *ecological indicators* are descriptors of the structure and function of the ecosystem.
Biomarkers are often subdivided into *biomarkers of exposure*, that demonstrate exposure of an organism to a certain chemical, and *biomarkers of toxic effect*, that give information on the degree of adverse effects (Walker et al. 2006). The biological markers used in the present study, EROD, glutathione-*S*-transferase (GST) and catalase (CAT) activity, lipid peroxidation, and metabolites of polycyclic aromatic hydrocarbons (PAH) in bile, are typically referred to as biomarkers of exposure. Thus, these biomarkers were used to demonstrate bioavailability of contaminants in the experiments. In the case of micronucleus formation in peripheral erythrocytes, however, this subdivision is misleading. Exposure to mutagenic substances may cause formation of micronuclei, giving a measure of exposure to such substances, although the loss of genetic material is obviously an adverse effect that has implications on the population level.

1.2.1 Phase I biotransformation: 7-ethoxyresorufin-*O*-deethylase (EROD)

The cytochromes P450 are a diverse multigene family that has been found in all organisms thus far 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). Biotransformation of xenobiotics to excretable products in the liver can be subdivided in phase I and phase II reactions. 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 to DNA, thereby increasing mutagenic effects (Huberman et al. 1976).

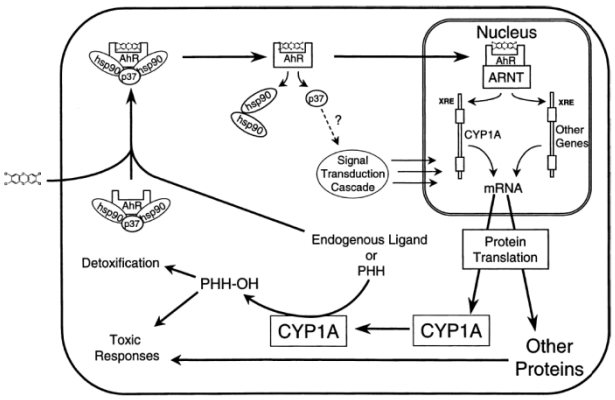

Figure 1.2 Proposed mechanism of AhR-mediated toxicity. Binding of dioxin-like ligands to the AhR results in the formation of a transcription factor complex with an aryl hydrocarbon nuclear translocator protein (ARNT) that binds to specific DNA sequences, xenobiotic/dioxin responsive elements (X/DRE). 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)

Induction of CYP1A and other biotransformation enzymes (Figure 1.2) are mediated by the cytosolic aryl hydrocarbon receptor (AhR), that is 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 a dioxin-like 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).

The inducibility after exposure to dioxin-like compounds renders CYP1A a suitable biomarker of 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 1.3), as described by Kennedy & Jones (1994).

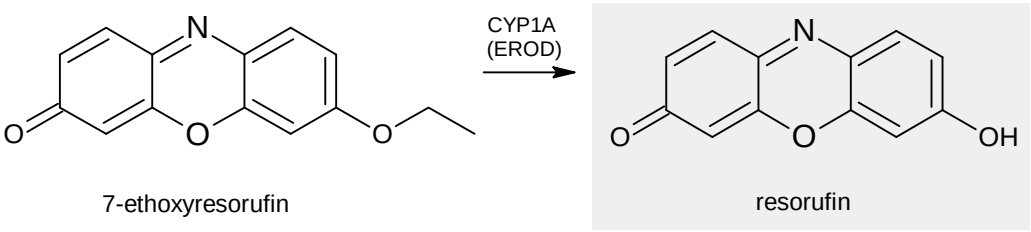

Figure 1.3 The 7-ethoxyresorufin-*O*-deethylase activity is a catalytic measurement of cytochrome P450-dependent monooxygenase induction (specifically the CYP1A subfamily). The fluorescent product resorufin is quantified by EROD bioassays.

1.2.2 Phase II biotransformation: glutathione-*S*-transferase (GST)

Glutathione-*S*-transferases (GST) have been found in the cytosole of many cell types and tissues, respectively, especially in the liver of vertebrates (Walker et al. 2006). They catalyze, among diverse physiological reactions, the conjugation of xenobiotics with the tripeptide glutathione (GSH) in phase II of the biotransformation process and, thereby, play an important role in the detoxication and excretion of such substances (Leaver et al. 1992). The cellular levels of such glutathione-*S*-transferases are controlled via the same AhR mediated pathway as CYP1A (Figure 1.4). Thus, the specific GST activity has been used as a biomarker of exposure to lipophilic organic compounds in several laboratory and field studies for several electrophilic substances (Frasco & Guilhermino 2002).

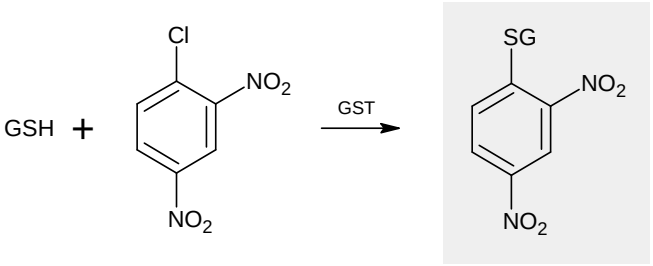

Figure 1.4 Glutathione-*S*-transferases catalyze the reaction of the –SH group of glutathione with potential alkylating agents, thereby neutralizing their electrophilic sites and increasing the water-solubility of the product. The reaction product is quantified by GST bioassays.

1.2.3 Oxidative stress: catalase (CAT) and lipid peroxidation

Eukaryotic cells are dependent on molecular oxygen (O_2) for the supply of energy via phosphorylation of ADP (adenosine diphosphate), thereby coupling oxidation to energy transfer. This process is controlled by the mitochondrial electron transport chain in which oxygen undergoes a concerted 4 electron reduction to water (Figure 1.5). As a consequence, various partly reduced reactive oxygen species (oxyradicals) are generated (Winston & Di Giulio 1991). The hydroxyl radical, especially, is among the most potent oxidants known, being capable of reacting with virtually any organic molecule, including cellular macromolecules, e.g. DNA, proteins and lipids. Protein degradation, DNA damage, lipid peroxidation (Figure 1.6) and ultimately cell death may result from these reactions (Borg & Schaich 1984). Nevertheless, the degree of physiological damage caused by oxyradicals is limited by a number of antioxidant defense mechanisms (Diguiseppi & Fridovich 1984). 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

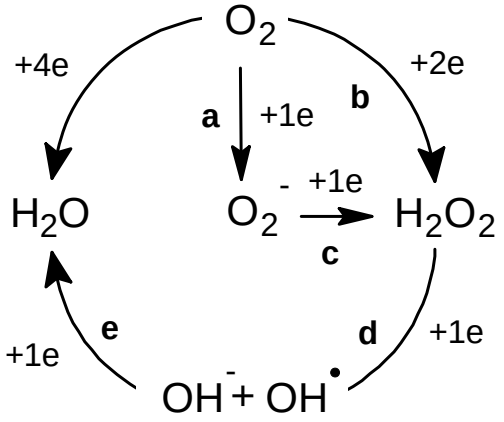

Figure 1.5 Electron transport reactions during oxygen reduction metabolism. Oxygen can be directly reduced to water. Furthermore, hydrogen peroxide can be formed from molecular oxygen by direct 2 electron reduction (b) or in two separate steps, thereby forming superoxide anion as intermediate (a, c). The potently oxidizing hydroxyl radical is generated *via* 1 electron reduction of hydrogen peroxide (d) and can be further reduced to water (d). Step c is catalyzed by the enzyme SOD, step d and e by catalase. Modified from Winston & Di Giulio (1991).

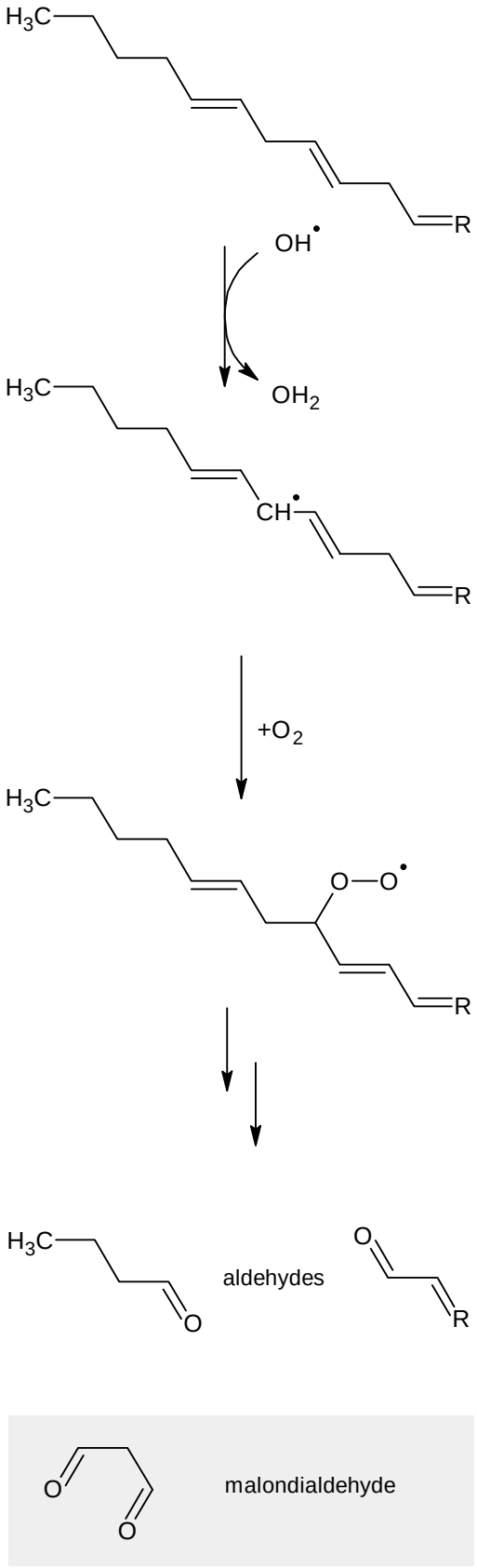

Figure 1.6 Basic mechanism of the peroxidation of unsaturated fatty acids. The product malondialdehyde is quantified by lipid peroxidation bioassays.

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 1.6 illustrates the formation of malondialdehyde (MDA) as a terminal product of the lipid peroxidation process that can be used for monitoring purposes in various species and tissues.

1.2.4 Genotoxicity: micronucleus formation

Micronucleus formation, i.e. chromosomal fractions or complete acentric chromosomes that are not incorporated into the main nucleus after mitosis (Heddle et al. 1991), has been used to assess genotoxicity of environmental contaminants in several laboratory experiments and field-studies. Mostly, erythrocytes from blood samples are used for the micronucleus test *in vivo*, since a high number of cells is concentrated in a small volume and sample preparation is simple (Udroiu 2006). The tissues producing erythrocytes are comprised in the hemopoietical organs. In fish, the cephalic kidney is the main tissue producing erythrocytes. While micronucleated cells from those organs demonstrate a genotoxic event that occurred during the cell cycle, peripheral blood cells reflect events that occurred during the average lifetime of circulating erythrocytes, since those undergo no further division (Schlegel & MacGregor 1982). Thus, assessment of the

micronucleus frequency in peripheral blood samples is particularly applicable to investigate the effects of chronic exposure (Udroiu 2006).

1.3 Model organism: rainbow trout

The salmonid rainbow trout (*Oncorhynchus mykiss*) is widely used for ecotoxicological studies, although it is not considered a standard test-organism. The database ETOX of the German Federal Environment Agency (UBA) contains >1000 datasets of toxicity tests for several substances and many different end-points in rainbow trout, including acute, chronic and reproductive toxicity and enzyme responses (UBA 2009). Especially, the endpoints investigated in the present study are covered by various investigations that were performed using *O. mykiss*. Furthermore, there are several advantages of using this organism. First, they are accessible from most commercial hatcheries in large quantities. Second, they are a representative species for mid-latitude rivers and streams and have an appropriate size to derive sufficient sample volumes to measure multiple biomarkers. Therefore, rainbow trout are to prefer over smaller or even tropic species, e.g. Zebrafish (*Danio rerio*) or Japanese Medaka (*Oryzias latipes*).

Figure 1.7 Rainbow trout (*O. mykiss*) are aquatic model organisms that have been widely used for toxicological studies.

1.4 Aims and objectives of the present study

The initial objective of the present study was to establish a suitable test system for direct expose of organisms, i.e. rainbow trout, to simulated flood-like conditions in the laboratory, thereby directly demonstrating possible adverse effects of particle-bound contaminants. Thus, methodologies of ecotoxicology and hydraulic engineering were combined by means of exposing the animals in an annular flume (Figure 2.2), a facility that can be used to study erosion and sedimentation processes.
In this particular study, rainbow trout were exposed to artificial sediment (OECD 218 2004) that was spiked with the PAHs pyrene, phenanthrene, chrysene and benzo[*a*]pyren during a 5 d simulated flood event in the annular flume. The following questions were addressed by this proof-of-concept study:

(a) Which technical requirements have to be met to permit constant experimental conditions for the exposure of rainbow trout in the annular flume?

The annular flume was originally designed to perform studies on sediment transport, erosion and sedimentation processes. Experiments with living animal were not planned. Thus, the original setup was extended. A flow-through cooling unit and an aeration system were installed to permit an optimal content of dissolved oxygen. Furthermore, dissolved oxygen, pH, turbidity and temperature were monitored on-line. This first investigation was mainly intended to test the new setup for its suitability for coupled hydro-toxicological experiments.

(b) Are artificial formulated sediments (OECD 218 2004) suitable for use in the annular flume?

In this proof-of-concept study, spiked artificial sediments were used to expose the test-organisms to particle-bound contaminants, which are intended to represent a standardized substrate for laboratory testing. Nevertheless, such sediments have not been used in the annular flume prior to this study – no data concerning erodibility and sediment dynamics was available.

(c) Are rainbow trout suitable test organisms for use in coupled hydro-toxicological investigation? Which endpoints can be identified for further studies?

This study was also intended to elucidate if rainbow trout constitute a suitable test species for investigations in the annular flume. A prerequisite for the applicability of this test-system is that the influence of the animals on the sediment transport processes during the experiments is negligible. Furthermore, it is necessary to be able to meet the requirements of the organisms to environmental parameters, such as pH, temperature and dissolved oxygen.
For the determination of suitable endpoints for further studies, a number of biomarkers were investigated to demonstrate either exposure to or adverse effects of sediment-bound contaminants, including biochemical markers (EROD, GST, and CAT activity, lipid peroxidation), chemical analysis of metabolites in bile (1-hydroxypyrene, 1-hydroxyphenanthrene, and 1-hydroxybenzo[*a*]pyrene) and the micronucleus test with peripheral erythrocytes, a definite marker for chromosome damage.

(d) How can coupled hydro-toxicological studies aid to understand the impact of sediment resuspension and remobilization of contaminants during (extreme) flood events on ecosystem quality?

There is a gap of knowledge concerning possible ecotoxicological consequences of contaminant remobilization caused by flood and run-off events (Hollert et al. 2008). Recently, it has been proposed to include hydrodynamics as an additional Line-of-evidence in Weight-of-evidence approaches to assess the environmental impact of sediments (Chapman & Hollert 2006). The combination of methods from hydraulic engineering and ecotoxicology, as used in this study, could help to derive important information on the impact of flood events on biota and ecosystem health and thus, by improving the understanding of processes in-field, lead to the development of suitable models for the prediction of the relation between sediment dynamics and adverse effects.

2. Materials and methods

2.1 Experimental design

In the present study, the toxicity and hazard potential of resuspended sediments to aquatic biota was assessed combining methods of hydraulic engineering and ecotoxicology. Rainbow trout (*Oncorhynchus mykiss*) were exposed over 5 d to simulated flood events according to DIN 4049-3 (1994) in an annular flume at the Institute for Hydraulic Engineering and Water Resources Management (RWTH Aachen University, Aachen, Germany). In two experiments, unspiked artificial sediment (OECD 218 2004) as well as sediment that was spiked with a mixture of the polycyclic aromatic hydrocarbons (PAH) pyrene (4.0 mg kg^{-1}), phenanthrene (5.0 mg kg^{-1}), chrysene (2.8 mg kg^{-1}), and benzo[*a*]pyrene (8.0 mg kg^{-1}) were used. PAH concentrations were chosen according to previously determined sediment concentrations in the field, such as shown in German rivers and streams by Keiter et al. (2008). Control groups of fish were assessed in parallel to the experimental groups. Each test was conducted with n=15 animals. Subsequently, a set of different biomarkers was investigated using different tissues and endpoints (Table 2.1). The conceptual framework of the present study is given in Figure 2.1. The physico-chemical properties of sediments and suspended matter in the annular flume were investigated in a different parallel study to extensively describe the remobilization processes that may lead to adverse effects.

Table 2.1 Biomarkers investigated and methodology of analysis.

Biomarker	Tissue	Reference
Micronucleus formation	Blood	Huber *et al*., 1983
Lipid peroxidation	Liver (S9)[1]	Ohkawa *et al.*, 1978
7-Ethoxyresorufin-*O*-deethylase	Liver (S9)[1]	Kennedy & Jones, 1994
Glutathione-*S*-transferase (GST)	Liver (S9)[1]	Habig *et al*., 1974
Catalase (CAT)	Liver (S9)[1]	Baudhuin *et al*., 1964

[1]*S9: supernatant after 15 min centrifugation at 9000 × g*

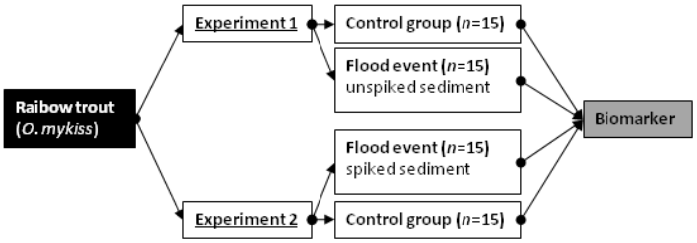

Figure 2.1 Conceptual framework of the present study.

2.2 Annular flume

The annular flume at the Institute for Hydraulic Engineering and Water Resources Management (Figure 2.2), as described by Schweim et al. (2001), is a circular channel that was designed to experimentally investigate erosion and deposition processes. The channel is covered with a lid that can be adjusted to different flow depths, always touching the water surface. Flume and lid are inversely rotated so that an endless flow in tangential direction is generated by the relative velocity between flume and lid. The resulting bottom shear stress τ is mainly dependent on this relative velocity.
Due to centrifugal forces, a secondary flow is generated that may disturb consistent conditions for erosion and deposition. Thus, the flume was extensively calibrated by means of Laser Doppler Velocimetry (LDV) measurements to meet the requirement of a uniform distribution of bottom shear stresses (Spork et al. 1998). A resulting optimal ratio of rotational speeds between lid (ω_l) and flume

Figure 2.2 Annular flume at the Institute for Hydraulic Engineering and Water Resources Management, Aachen, Germany.

(ω_f) of -1.6 was determined (i.e., the lid is rotating 1.6-fold faster that the flume and in opposite direction).

The setup used in the present study consisted of a channel of 0.25 m width and a mean diameter of 3.25 m. The maximum bed shear stress of the DIN 4049-3 hydrograph was set to 0.3 N m^{-2}. The annular flume was placed in a climatic chamber to permit consistent experimental conditions. Additionally, the water was cooled by a flow-through cooling unit (Titan 500, Aquamedic, Bissendorf, Germany) and aerated. Turbidity, as well as temperature, pH and dissolved oxygen, were continuously recorded (13.2 ± 0.9 °C, pH 7.9 ± 0.3, O_2 9.3 ± 3.0 mg L^{-1} during exposure to unspiked sediments; 12.7 ± 0.2 °C, pH 7.9 ± 0.1, O_2 8.5 ± 0.6 mg L^{-1} during exposure to spiked sediments; data: C. Cofalla).

2.3 Chemicals

The following PAHs were selected in the present study (Sigma-Aldrich, Deisenhofen, Germany): benzo[*a*]pyrene (≥96 %), chrysene (Supelco, analytical standard), phenanthrene (98 %), and pyrene (≥99 %). If not stated differently, all chemicals were purchased from Sigma-Aldrich (Deisenhofen, Germany).

2.4 Sediment preparation and spiking

The artificial sediments used in the present study were prepared and spiked according to international guidelines (OECD 218 2004). Finely ground and air-dried Lithuanian *Sphagnum* moss peat (Klasmann-Deilmann GmbH, Geeste, Germany) was mixed with approximately 7.5 parts (w/v) water, adjusted to pH 5.75 ± 0.25 with calcium carbonate (Sigma-Aldrich) and the suspension gently stirred for 48 h. Subsequently, the pre-treated peat (5 % dw) was united with 20 % dw kaolin clay (Erbslöh Lohrheim GmbH, Lohrheim, Germany) and 75 % dw quartz sand (Quarzwerke GmbH, Frechen, Germany) in a cement mixer. Water and calcium carbonate were added to obtain a final water content of 42-44 % and pH 7.0 ± 0.5. Subsequently, sediments were conditioned for 7 d prior to erosion experiments or spiking.

Ten percent of the readily conditioned artificial sediment (i.e., 12 kg) were dried at 105 °C and thoroughly crushed. The used PAHs were dissolved in a mixture of 3.5 L hexane and 1.5 L acetone and added to the dried sediment. After complete evaporation of the solvent, water was added to obtain the original water content. The spiked portion was united with the remaining sediment, incubated for 7 d, and thoroughly mixed prior to erosion experiments. The sediment mixtures were transferred to the annual flume and smoothened to obtain an even 4 cm sediment layer. Water was added to a final depth of 20 cm. As described by Spork et al. (1994), significant changes of the critical bed shear stress for erosion and the density profile may not be expected after a consolidation time of 3 d, which was therefore also assumed in the present study.

2.5 Fish

Immature rainbow trout, *O. mykiss* (15-20 g), were purchased from a commercial hatchery (Mohnen Aquaculture, Stolberg, Germany) and allowed to acclimatize to laboratory conditions for at least 6 months prior to the experiments. Fish were reared in lots of 20-30 individuals in 300 L plastic tanks at RWTH Aachen University, Aachen, Germany. In a flow-through system, water (15 ± 2 °C; pH 7.8 ± 0.24; NH_3 < 0.1 mg L^{-1}) was continuously exchanged at a rate of 3-4 d^{-1} with dechlorinated 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. The fish used in the experiments were grown to a mean weight of 101 ± 34 g and a mean length of 190 ± 22 mm. Fish were used for the present study in accordance to the Animal Welfare Act and with permission of the federal authorities, Aachen, Germany, registration number 8.87-50.10.35.08.225.

2.6 Tissue preparation

After exposure, fish were individually anesthetized in a 10 L container by adding a saturated solution of ethyl 4-aminobenzoate (benzocaine). Subsequently, size and weight were determined for calculation of the coefficient of condition (*K*, Equation 1) and the liver somatic index (*LSI*, Equation 2). Peripheral blood samples were taken with heparinized syringes from the caudal vein. Two smears per individual were immediately prepared on microscope slides that were previously cleaned with 99 % ethanol (Merck, Darmstadt, Germany). After drying, samples were fixed in methanol (Merck) for at least 1 min and stored at room temperature until determination of micronucleus frequencies. 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.

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

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

W = weight of the fish (mg), *LW* = liver weight (mg), *L* = standard length (mm).

2.7 Preparation of homogenates and liver subcellular fractions

All steps in the preparation of tissue homogenates were carried out on ice. For measurement of 7-ethoxyresorufin-*O*-deethylase (EROD) activity, pieces of liver explants were thawed carefully and homogenized for 20 s using an electric disperser (VDI 12, VWR, Darmstadt, Germany) in 1.5 ml of chilled solution of 0.15 M KCl and 1 mM EDTA in phosphate buffer (0.1 M, pH 7.4). Subsequently, homogenates were transferred to 1.5 ml micro test tubes (Greiner Bio-One) and centrifuged for 15 min (10000 × g, 4 °C) in a cooling centrifuge (Rotina 420R, Hettich, Tuttlingen, Germany). Supernatant was carefully transferred to fresh 1.5 ml micro test tubes and stored at 0 °C until measurement of EROD activity on the same day. For measurement of glutathione-*S*-transferase (GST) and catalase

(CAT) activity, homogenates were prepared in a ratio of 1 g native tissue to 9 ml chilled homogenization buffer (12.5 ml of 2 M sucrose, 25 ml of 20 mM MOPS pH 7.4, 10 ml of 10 mM EDTA in ethanol, 0.2 ml of 0.1 M phenylmethylsulfonylfluoride in isopropanol, 13 mg ε-aminocaproic acid, 0.3 M β-mercaptoethanol, and 20 µl dithiothreitol in a final volume of 100 ml distilled water) by means of an electric homogenizer (VDI 12, VWR) and subsequently centrifuged for 15 min (9000 × g, 4 °C). The supernatant was carefully transferred to 1.5 ml micro test tubes and stored at -85 °C until measurement of enzymatic activity.

2.8 Determination of 7-ethoxyresorufin-O-deethylase activity

EROD activity was measured in triplicates according to the method described by Kennedy and Jones (1994). In 96-well microtitre plates (TPP, Trassadingen, Switzerland), 50 µl sample (raw centrifugate or, if necessary, dilutions in 0.1 M phosphate buffer containing 0.15 M KCl and 1 mM EDTA) and 50 µl of resorufin (0-10 µM) and bovine serum albumin (BSA, 0-10 mg ml^{-1}) standard solutions in HEPES-Cortland buffer (pH 8.0) were prepared. Subsequently, 120 µl of 2 µM 7-ethoxyresorufin in HEPES-Cortland buffer were added to all wells. Plates was incubated at room temperature for 10 min in darkness prior to addition of 40 µl of 4.2 µM NADPH in HEPES-Cortland buffer. After incubation at room temperature for 10 min in darkness, the reaction was stopped with 90 µl of 150 µg L^{-1} chilled fluorescamine in acetonitrile. After 10 min, the fluorescence of both resorufin (excitation: 544 nm, emission: 590 nm) and fluorescamine (excitation: 340 nm, emission: 460 nm) was determined in an Infinite® 200 microplate reader (Tecan, Crailsheim, Germany). To correct for spontaneous substrate conversion, 50 µl homogenization buffer 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}.

2.9 Determination of glutathione-S-transferase activity

Activity of GST was determined in triplicates by the method of Habig et al. (1974), adapted to microplate measurement. A solution of 11.4 mM reduced glutathione (GSH) in phosphate buffer (0.1 M, pH 6.5) and a solution of 25 mM 1-chloro-2,4-dinitrobenzene (CDNB) in ethanol were freshly prepared. In 96-well microtitre plates (TPP), 20 µl sample were mixed with 250 µl phosphate buffer (0.1 M, pH 6.5) and 10 µl CDNB solution. The reaction was started by adding 25 µl GSH solution and extinction at 340 nm was recorded at 25 °C for 5 min (20 s intervals) in an Infinite® 200 microplate reader (Tecan). To correct for spontaneous substrate conversion, 20 µl homogenization buffer was treated in the same way as the samples. For calculation of CDNB concentrations with the Lambert-Beer law, the molar extinction coefficient of 9.6 mM^{-1} cm^{-1} was used. The specific GST activity was expressed as nmol CDNB mg $protein^{-1}$ min^{-1}.

2.10 Determination of catalase activity (CAT)

CAT activity was determined in triplicates according to Baudhuin et al. (1964), adapted to microplate measurement. The reaction mixture, consisting of 10 ml imidazole buffer (10 mM, pH 7.2), 100 mg bovine serum albumin (BSA), and 35 µl of 30 % hydrogen peroxide (H_2O_2) in a total volume of 100 ml distilled water, was freshly prepared and stored at 0 °C in a light-tight bottle. In 1.5 ml micro test tubes (Greiner Bio-One), 4 µl sample were added to 50 µl Triton X-100. The reaction was started by adding 500 µl reaction mixture. After incubation for 15 min at 0 °C, the reaction was stopped with 500 µl Titanium(IV) oxysulfate - sulfuric acid solution (Sigma). After 10 min, 250 µl of this mixture

were transferred to 96-well microtitre plates (TPP) and the concentration of the remaining hydrogen peroxide was determined photometrically as the yellow peroxy titanium sulfate at 414 nm in an Infinite® 200 microplate reader (Tecan), using the molar extinction coefficient of 19.1 μM^{-1} cm^{-1}. To correct for spontaneous substrate conversion, 4 µl homogenization buffer was treated in the same way as the samples. The specific CAT activity was expressed as nmol H_2O_2 mg $protein^{-1}$ min^{-1}.

2.11 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 9 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 microtitre plates (TPP) and absorbance (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. All measurements were carried out in triplicates.

2.12 Determination of protein concentrations

Protein concentrations for the calculation of specific enzyme activities were determined in triplicates according to the Bradford method (Bradford 1976), adapted to microplate measurement, using bovine serum albumin (BSA) as external standard. The Bradford reagent was added in a ratio of 1:30 to 5 µl sample. After 30 min incubation at room temperature, extinction at 595 nm was read using an Infinite® 200 microplate reader (Tecan).

2.13 Micronucleus assay

Previously prepared smears of peripheral blood samples were stained by adding 12 µl of a 0.2 µm MCE membrane filtered (Millipore Millex, Schwalbach, Germany), 0.004 % acridine orange solution (w/v) in phosphate buffered saline (PBS). For each individual fish, 4000 erythrocytes fixed on two separate smears were examined using an epifluorescence microscope at 1000 × magnification. The following scoring criteria were used for identification of micronuclei: a) cells with oval appearance and intact cytoplasm, b) oval nuclei with intact nuclear membrane, c) micronuclei less than or equal to one third the size of the main nuclei, d) micronuclei clearly separated from the main nuclei (Huber et al. 1983, Titenko-Holland et al. 1998). Results were recorded as micronucleated cells relative to the total number of cells counted.

2.14 Quantification of PAH metabolites in bile

PAH metabolites in bile were quantified according to Kammann (2007). Briefly, 25 µl bile fluid was mixed with 95 µl distilled water and 5 µl *β*-glucuronidase/arylsulfatase solution (30/60 $U \cdot ml^{-1}$), 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 1-hydroxybenzo[*a*]pyrene were determined by means of HPLC with fluorescence detection (cf. Kammann 2007).

2.15 Data analysis

All spreadsheet calculations were performed using Microsoft Excel™ 2007. All graphs were plotted using the software GraphPad Prism 5 (GraphPad, San Diego, USA). Statistical analyses and comparisons 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 Holm-Sidak or Dunn's method was used to identify significant differences among the treatments. When differences between control groups and treatments were significant, induction factors relative to the mean of the respective control group were calculated for each treatment. Induction factors were compared using unpaired t-test. Welch's correction was applied if data did not pass the F-test for equal variances. Statistical significance limit throughout all comparisons was set at least to $p\leq0.05$. If not stated differently, all values are expressed as mean value ± standard deviation.

3. Results

Within this proof-of-concept study, the annular flume has been shown to be an applicable tool to conduct hydro-toxicological studies with artificial sediment according to OECD 218. The technical requirements to permit consistent experimental conditions for exposure of rainbow trout within the annular flume (i.e., climatic chamber, cooling unit, and aeration) were successfully implemented into the existing experimental setup. In the present study, rainbow trout prove to be a suitable model organism. Significant effects of the technical installations on the exposure scenario and, in particular, the fish could not be observed. Fish were vital after exposure in the flood scenario, which is a prerequisite to permit the investigation of sub-lethal biomarkers. A set of different suitable biochemical and histological markers was identified for application in coupled hydro-toxicological investigations. A summary of the results of all investigated biomarkers is given in Table 3.1.

Table 3.1 Summarized results of the biomarker investigation: biometric indices, lipid peroxidation in crude liver homogenate, 7-ethoxyresorufin-*O*-deethylase (EROD), Glutathione-*S*-Transferase (GST) and catalase (CAT) activity in liver S9 fractions, and micronucleus formation in peripheral blood samples, respectively, were quantified. Values are given as mean value ± standard deviation.

	Treatment			
	Control I (*n*=15)	Unspiked sediment (*n*=15)	Control II (*n*=15)	Spiked sediment (*n*=15)
Biometric indices				
Coefficient of condition (K)	1.43 ± 0.08^{a}	1.42 ± 0.12^{a}	1.43 ± 0.14^{a}	1.41 ± 0.11^{a}
Liver somatic index (LSI)	1.08 ± 0.11^{a}	1.06 ± 0.19^{a}	1.04 ± 0.10^{a}	1.02 ± 0.14^{a}
Crude liver homogenate				
Lipid peroxidation / nmol g^{-1}	75.22 ± 11.42^{a}	125.80 ± 25.23^{b}	86.76 ± 22.02^{a}	105.20 ± 18.49^{c}
Liver homogenate (S9)				
EROD activity / pmol mg^{-1} min^{-1}	11.66 ± 6.93^{a}	n.d.	14.65 ± 7.26^{a}	15.49 ± 11.88^{a}
GST activity / pmol mg^{-1} min^{-1}	15.51 ± 4.13^{a}	14.67 ± 2.47^{a}	8.78 ± 2.61^{b}	9.47 ± 2.81^{b}
CAT activity / nmol mg^{-1} min^{-1}	561.60 ± 250.90^{a}	326.50 ± 75.14^{a}	153.80 ± 74.73^{b}	76.98 ± 48.61^{b}
Peripheral blood				
Micronucleus formation / ‰	1.40 ± 0.66^{a}	3.08 ± 2.04^{a}	0.53 ± 0.30^{b}	2.27 ± 1.12^{c}

n.d.: not determined.

$^{a, b, c}$*: Treatment groups sharing the same letter do not differ significantly (determined using one way ANOVA and Holm-Sidak method, p≤0.05, and one way ANOVA on ranks with Dunn's method, p≤0.05, respectively).*

3.1 Coefficient of condition (K) and liver somatic index (LSI)

The fish used in the experiments were grown to a mean weight of 101 ± 34 g and a mean length of 190 ± 22 mm. Size and weight of the animals were comparable between the control groups and the treatments (one way ANOVA, p>0.001), resulting in a mean index of condition K of 1.42 ± 0.11 (Figure 3.1 A). Furthermore, the liver somatic index (Figure 3.1 B) was calculated for the animals from the control and treatment groups, respectively, where no significant differences could be detected

either (LSI 1.05 ± 0.14 %, one way ANOVA, p>0.001). Thus, the assumption was made that differences between the treatments were more likely an effect of the presence or absence of the model contaminants than of physiologically inhomogeneous experimental groups.

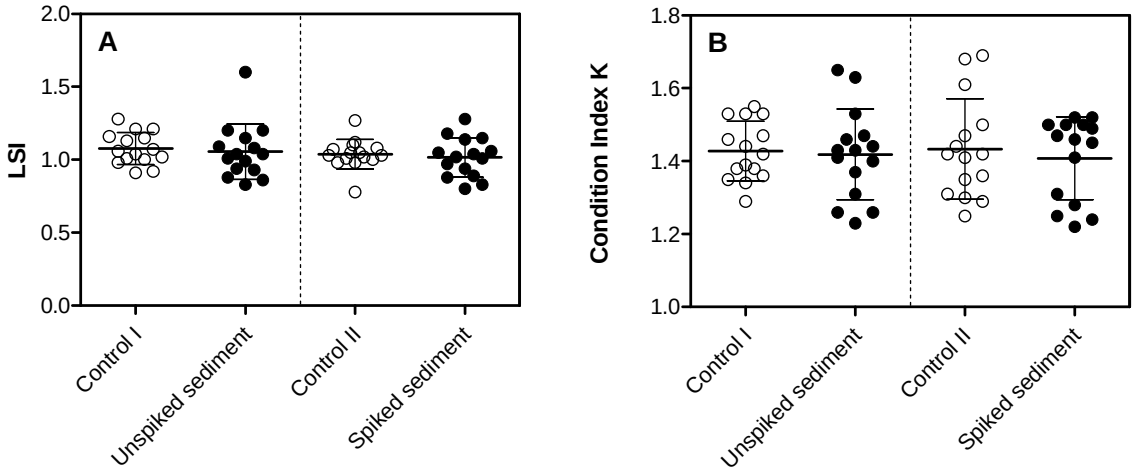

Figure 3.1 Liver somatic index (*LSI*) and index of condition (*K*) of rainbow trout exposed in 5 d simulated flood events to unspiked and spiked sediments, respectively. Control groups were taken from the maintenance in parallel to the treatments. Each test was conducted with n=15 animals. Horizontal lines represent the mean value, error bars the standard deviation.

3.2 7-Ethoxyresorufin-O-deethylase activity

The specific EROD activity as a marker enzyme for phase I biotransformation was determined by quantifying the deethylation of exogenous 7-ethoxresorufin in S9 fractions of trout liver homogenates (Figure 3.2). Measurements for fish exposed to unspiked sediment in a 5 d simulated flood event could not be conducted because the used dilution of the centrifugate (20-fold) resulted in resorufin concentrations below the detection limit of the method. For all other treatment groups, the dilution was adapted (4-fold) and valid specific enzyme activities were derived.
Regarding the fish that were exposed to spiked artificial sediment, the EROD activity was not significantly altered compared to the control group (one way ANOVA, p>0.001), although the animals with the highest EROD activity (approx. 45 pmol mg^{-1} min^{-1}) originated from this group (Figure 3.2). The average specific EROD activity throughout all tested groups was 14.1 ± 9.2 pmol mg^{-1} min^{-1}.

3.3 Glutathione-S-transferase activity

The specific GST activity was assessed by quantification of the conversion of CDNB in S9 fractions of liver homogenates (Figure 3.3). There was a substantially and significantly lower (Kruskal-Wallis one way ANOVA on ranks with Dunn's method, p≤0.01) GST activity in the group exposed to spiked sediment (9.5 ± 2.8 nmol mg^{-1} min^{-1}) within the simulated flood event compared to the unspiked sediment (14.7 ± 2.5 nmol mg^{-1} min^{-1}). Nevertheless, comparison of the treatments with the respective control groups indicated no evidence of significant enzyme activity alteration by treatments (Kruskal-Wallis one way ANOVA on ranks with Dunn's method, p≤0.05).

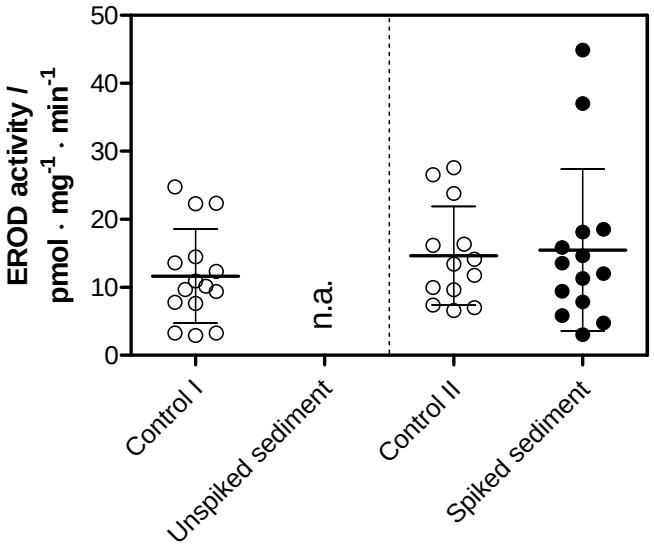

Figure 3.2 EROD activity in the liver of rainbow trout exposed in 5 d simulated flood events to unspiked and spiked sediments, respectively. Control groups were taken from the maintenance in parallel to the treatments. Each test was conducted with *n*=15 animals. Dots represent mean values of triplicate measurements. Horizontal lines represent the mean value, error bars the standard deviation. *N.a.: not assessed.*

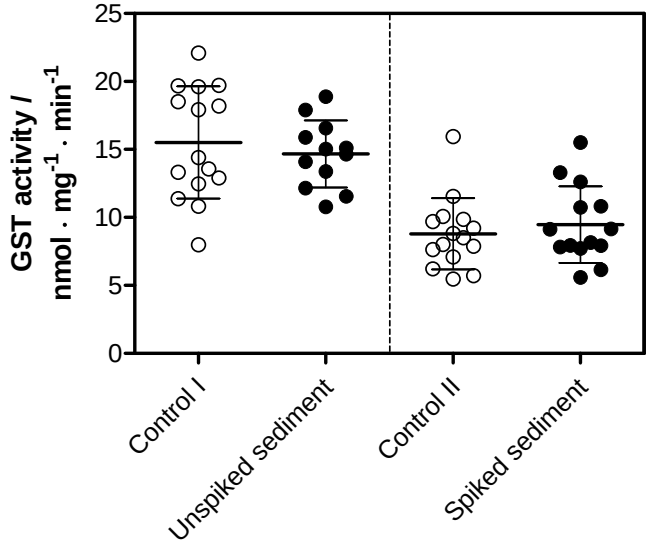

Figure 3.3 GST activity in the liver of rainbow trout exposed in 5 d simulated flood events to unspiked and spiked sediments, respectively. Control groups were taken from the maintenance in parallel to the treatments. Each test was conducted with *n*=15 animals. Dots represent mean values of triplicate measurements. Horizontal lines represent the mean value, error bars the standard deviation.

3.4 Catalase activity

The specific catalase enzyme activity was determined indirectly by quantification of the residual hydrogen peroxide concentration after incubation with S9 fractions of fish liver homogenates (Figure 3.4). Although there were significant differences between both control groups and both treatments, respectively, (Kruskal-Wallis one way ANOVA on ranks with Dunn's method, $p \leq 0.05$), the experimental groups exposed in the flood events did not differ significantly from the respective control group.

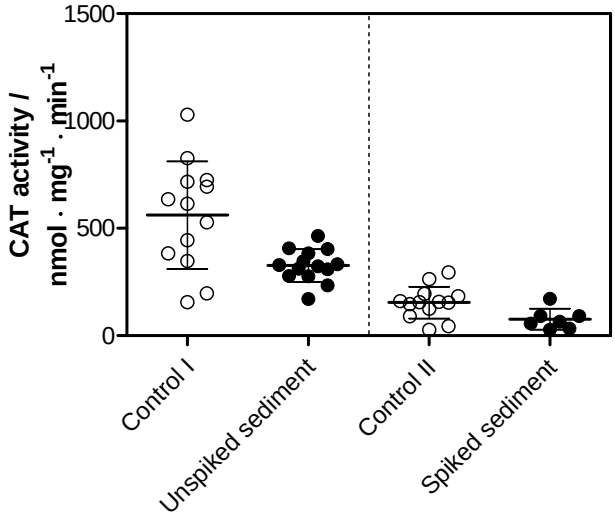

Figure 3.4 CAT activity in the liver of rainbow trout exposed in 5 d simulated flood events to unspiked and spiked sediments, respectively. Control groups were taken from the maintenance in parallel to the treatments. Each test was conducted with n=15 animals. Dots represent mean values of triplicate measurements. Horizontal lines represent the mean value, error bars the standard deviation.

3.5 Lipid peroxidation

Lipid peroxidation was determined by quantification of the degradation product MDA in crude liver homogenates (Figure 3.5). There was no significant difference between both control groups (one way ANOVA with Holm-Sidak method, $p \leq 0.05$), where the average MDA concentration was 81.0 ± 18.1 nmol g^{-1}. Exposure to unspiked artificial sediment during the simulated flood event resulted in an average MDA concentration of 125.8 ± 25.2 nmol g^{-1}, while exposure to spiked sediment led to an average concentration of 105.2 ± 18.5 nmol g^{-1}. Even though there was a significant difference between the treatments (one way ANOVA with Holm-Sidak method, $p \leq 0.05$) with regard to the absolute values, induction factors relative to the median of the respective control group were calculated to permit precise quantification of this difference. While the presence of unspiked sediment during the simulated flood event led to a 1.7-fold induction of lipid peroxidation, induction was only 1.2-fold (significant; t-test, $p \leq 0.001$) when fish were exposed to spiked sediment in the flood scenario (Figure 3.5).

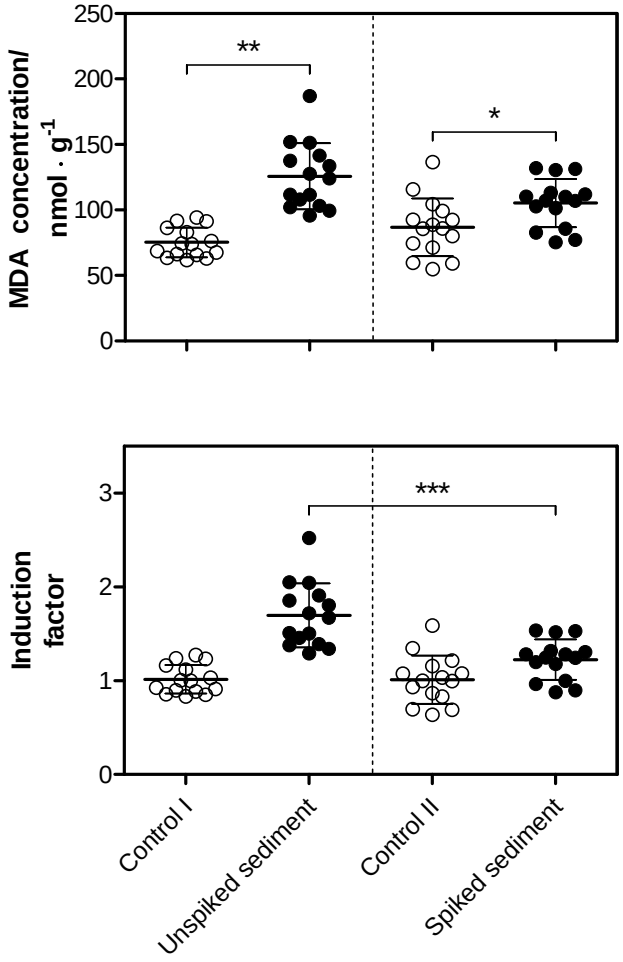

Figure 3.5 Lipid peroxidation, measured as MDA concentration, in the liver of rainbow trout exposed in 5 d simulated flood events to unspiked and spiked sediments, respectively, expressed as absolute values and induction factors relative to the median of the control group. Control groups were taken from the maintenance in parallel to the treatments. Each test was conducted with n=15 animals. Dots represent mean values of triplicate measurements. Horizontal lines represent the mean value, error bars the standard deviation. **/**/***Significant alteration ($p \leq 0.05/0.01/0.001$)*

3.6 Micronucleus formation

The micronucleus frequency in 4000 erythrocytes per exposed fish was determined using fluorescence microscopy (Figure 3.6). Frequencies differed significantly between both control groups (Kruskal-Wallis one way ANOVA on ranks and Dunn's method, $p \leq 0.05$). Thus, induction factors relative to the median of the respective control groups were calculated for both treatments. Although exposure to the unspiked sediment led to 2.2-fold increased micronucleus frequencies, this induction was not significant in consequence of elevated variances. Even though absolute values were lower for the treatment with spiked sediments compared to the unspiked sediments, the respective induction was 4.3-fold (significant; Kruskal-Wallis one way ANOVA on ranks and Dunn's method, $p \leq 0.01$).

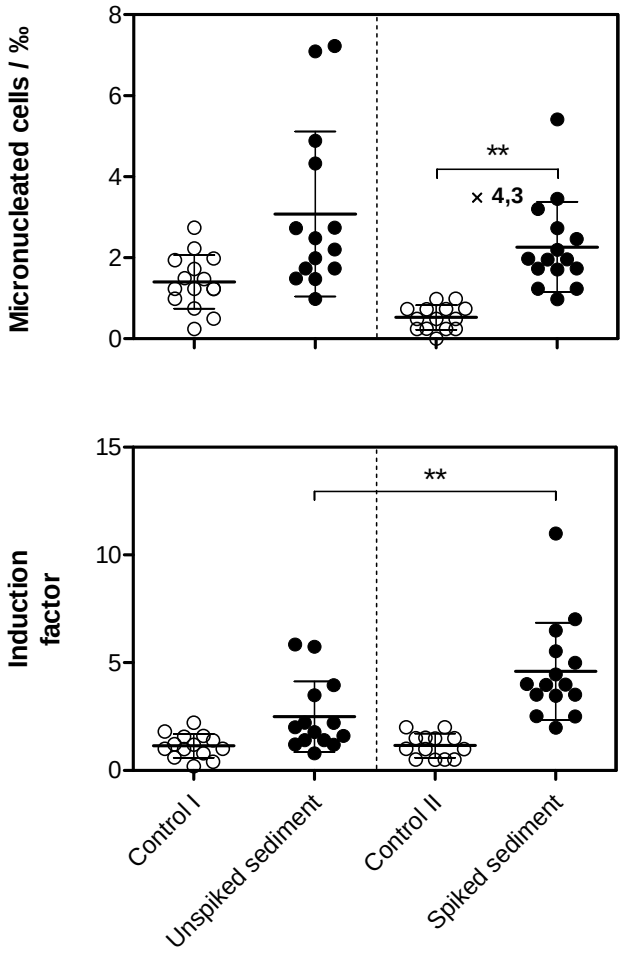

Figure 3.6 Micronucleus frequency in peripheral erythrocytes of rainbow trout exposed in 5 d simulated flood events to unspiked and spiked sediments, respectively, expressed as absolute values and induction factors relative to the median of the control group. Control groups were taken from the maintenance in parallel to the treatments. Each test was conducted with n=15 animals. Dots represent the proportion of micronucleated cells in 4000 erythrocytes of each animal. Horizontal lines represent the mean value, error bars the standard deviation. ***Significant alteration ($p \leq 0.01$)*

3.7 PAH metabolites in bile

The concentrations of the PAH metabolites 1-hydroxypyrene, 1-hydroxyphenanthrene and 1-hydroxybenzo[*a*]pyrene were quantified in co-operation with Dr. Ulrike Kammann (vTI, Hamburg) in bile liquid by means of HPLC with fluorescence detection (Figure 3.7). Except for 1-hydroxybenzo[*a*]pyrene, where metabolite concentrations in both control groups and the group exposed to unspiked sediments could not be reliably quantified, all measured concentrations were well above the limits of quantification. The concentration of 1-hydroxypyrene in bile of animals exposed to spiked sediments was 4596-fold higher compared to animals exposed to unspiked sediments (significant, Kruskal-Wallis one way ANOVA on ranks and Dunn's method, $p \leq 0.001$), while the concentrations of 1-hydroxyphenanthrene and 1-hydroxybenzo[*a*]pyrene were increased 514 and 250-fold, respectively (significant, Kruskal-Wallis one way ANOVA on ranks and Dunn's method, $p \leq 0.001$). While exposure to unspiked sediments did not result in elevated concentrations of the metabolites 1-hydroxypyren and 1-hydroxybenzo[*a*]pyrene, the average concentration of 1-

hydroxyphenanthrene was significantly 4.4-fold higher compared to the respective control group (Kruskal-Wallis one way ANOVA on ranks and Dunn's method, $p \leq 0.001$), indicating a possible contamination of the artificial sediment (Figure 3.7).

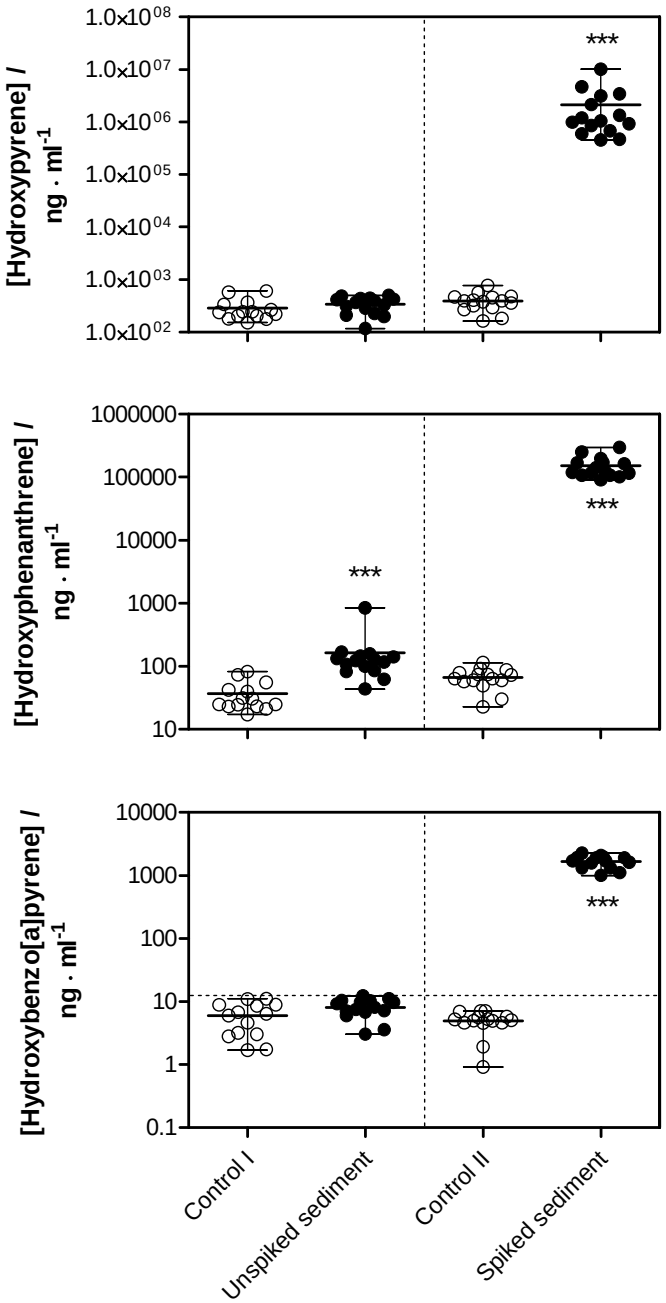

Figure 3.7 Concentrations of the PAH metabolites 1-hydroxypyrene, 1-hydroxyphenanthrene and 1-hydroxybenzo[*a*]pyrene in bile of rainbow trout exposed in 5 d simulated flood events to unspiked and spiked sediments, respectively. Control groups were taken from the maintenance in parallel to the treatments. Each test was conducted with n=15 animals. Dots represent the concentration of the respective metabolite in individual animals. Horizontal lines represent the mean value, error bars the range. The horizontal dashed line indicates the quantification limit for hydroxybenzo[*a*]pyrene. ****Significant alteration compared to the respective control group (Kruskal-Wallis one way ANOVA on ranks and Dunn's method, p≤0.001).*

To elucidate a possible correlation between mutagenicity, measured as the proportion of micronucleated erythrocytes in peripheral blood samples of fish exposed to spiked sediment, and the concentration of the mutagenic metabolite 1-hydroxybenzo[*a*]pyrene in bile after exposure, a correlation analysis was performed (Figure 3.8). The proportion of micronucleated erythrocytes was found to exhibit a positive correlation with the concentration of hydroxybenzo[*a*]pyrene (Spearman's rank correlation coefficient, r=0.64, p=0.01), while there was no such correlation with the concentrations of the other bile metabolites.

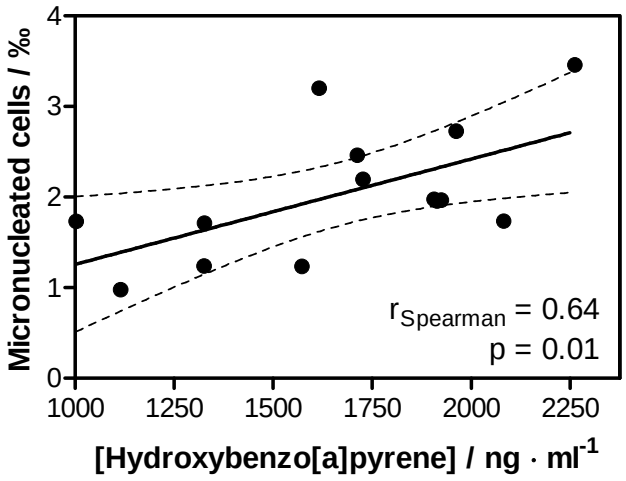

Figure 3.8 Linear regression of 1-hydroxybenzo[*a*]pyrene concentrations in bile and the proportion of micronucleated cells of rainbow trout exposed in a 5 d simulated flood event with sediment that was spiked with PAHs. The tests were conducted with n=15 animals. The dashed curves represent the 95 % confidence limits. The proportion of micronucleated cells was positively correlated with the concentration of 1- hydroxybenzo[*a*]pyrene (Spearman's rank correlation coefficient, r=0.64, p=0.01).

4. Discussion

4.1 Applicability of the annular flume for coupled hydro-toxicological studies

A prerequisite for the applicability of the annular flume as a test-system for coupled hydro-toxicological studies is to provide the technical requirements to control for environmental parameters that are critical to a balanced physiology of the test-organisms, such as pH, temperature and dissolved oxygen. The annular flume was originally designed to perform studies on sediment transport, erosion and sedimentation processes (Spork et al. 1998, Spork et al. 1994). Experiments with whole animals have not been addressed in earlier studies. For the present study, the original setup was extended by installing a flow-through cooling unit and an aeration system to permit an optimal content of dissolved oxygen. The central question in this context was if the additional installations were sufficient to provide an adequate environment for exposure of rainbow trout in the annular flume. All animals were vital after exposure to both unspiked and spiked sediment in the simulated flood event (Figure 4.1) and showed no alterations of the index of condition *K* or the liver somatic index *LSI*. Thus, exposure to particle-bound contaminants in the annular flume seems principally possible. However, an application for further studies requires (1) the investigation of the contribution of individual stress parameters to adverse effects, e.g. turbidity, current and contaminants – individually and in different combinations by means of a systematic sensitivity analysis – and (2) the verification of the current methodology with different test-species.

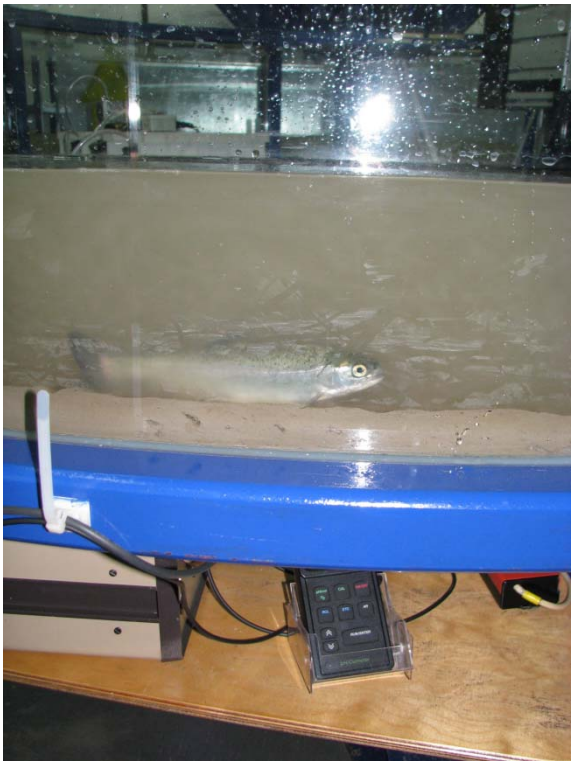

Figure 4.1 Rainbow trout on the sediment bed within the annular flume.
Picture: Catrina Cofalla

4.2 Suitability of artificial formulated sediments for use in the annular flume

In this proof-of-concept study, spiked artificial sediments according to OECD 218 (2004) were used to expose the test-organisms to particle-bound contaminants, which were intended to represent a standardized substrate for laboratory testing. Although Hudjetz and co-workers (2009) have shown that artificial sediments were principally erodible in the annular flume (Figure 4.2), it is questionable whether the test design allows to extrapolate to the field situation. Recently, it has been shown that the similarity of microbial communities from artificial and natural sediments was less than 40 %, where different

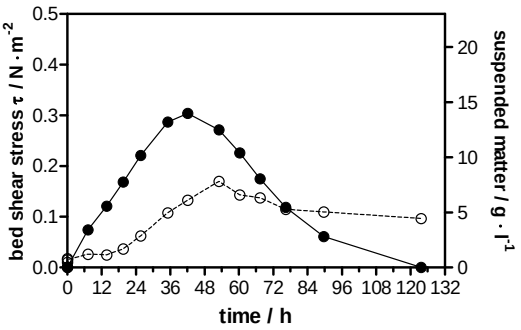

Figure 4.2 Interrelation between bed shear stress τ (•) and the concentration of suspended particulate matter (◦) during the simulated 5 d flood event. From Hudjetz et al. (2009).

operational taxonomic units appeared to dominate the artificial and natural sediment, respectively (Goedkoop et al. 2005). Thus, there is an elevated risk of over- or underestimation of the toxicity and bioavailability of contaminants that are bound to formulated sediments compared to natural sediments. Furthermore, Gerbersdorf and co-workers (2008) have shown that sedimentological parameters, e.g. the critical shear stress for erosion, are largely dependent on colloidal and bound extracellular polymeric substances (EPS) that strongly correlate with the microbial biomass and community of sediments. To derive a scientifically defensible basis for the development of models to predict the adverse effects of remobilization processes in-field, it is necessary to accurately emulate the biological and physico-chemical properties of natural sediments. Thus, additional experiments with natural sediments, either contaminated or spiked, have to be performed.

4.3 Identification of suitable biomarkers for use in coupled hydro-toxicological studies

A number of biomarkers were investigated to demonstrate either exposure to or adverse effects of sediment-bound contaminants and to identify suitable endpoints for further studies, including biochemical markers, i.e. 7-ethoxyresorufin-*O*-deethylase (EROD), glutathione-*S*-transferase (GST), and catalase (CAT) activity, as well as lipid peroxidation, chemical analysis of metabolites in bile (1-hydroxypyrene, 1-hydroxyphenanthrene, and 1-hydroxybenzo[*a*]pyrene) and the micronucleus test with peripheral erythrocytes. Within this investigation, it was possible to establish the methods for measurement of all biomarkers that were selected. In this context, rainbow trout prove to be a suitable test-species to conduct multiple biomarker studies. The amounts of tissue that were obtained were sufficient to measure each marker in all exposed animals, giving the opportunity to compare the different markers within and across the individuals. Nevertheless, the enzymatic biomarkers showed no alterations following exposure to particle-bound contaminants. Accordingly, 5 d exposure time in simulated flood events may not be sufficient to detect effects on the protein level. The investigation of markers on the transcript level, i.e. gene expression analyses, might be a suitable tool to earlier detect changes (Tompsett et al. 2009, Zhang et al. 2008).

4.3.1 Biotransformation enzymes

The PAHs that were investigated in the present study are known to be moderately potent Ah receptor agonists *in vitro* (Barron et al. 2004) and have been shown to cause significant induction of biotransformation enzymes in rainbow trout in several studies (Fragoso et al. 2006b, Jonsson et al. 2006b, Oikari et al. 2002, Ramachandran et al. 2006). In contrast, the activity of the phase I biotransformation enzyme EROD and GST, an enzyme catalyzing reactions of phase II, was not altered after exposure to either unspiked or spiked sediments within the 5 d simulated flood events in the present study. The mean activity throughout all tested groups (14.1 ± 9.2 pmol mg^{-1} min^{-1}) was in good accordance with previous studies (Fragoso et al. 2006a, Jonsson et al. 2006a). Since biliary metabolites of the three PAHs pyrene, phenanthrene and benzo[*a*]pyrene clearly demonstrated bioavailability, uptake and metabolic transformation (see 4.3.4) it is most likely that compounds and metabolites reached the receptor site as well. Thus, it may be assumed that the biotransformation enzymes were not well inducible in immature trout. Accordingly, several authors have found that the developmental stage of most fish species tremendously influenced EROD activity (Cantrell et al. 1996, Peters & Livingstone 1995), while the early life-stages mostly showed higher activity and inducibility. Thus, several authors have used juvenile rainbow trout for laboratory experiments. While Fragoso et al. (2006a) used fingerlings of 2-3 g, Ramachandran and co-workers (2006) have studied juvenile rainbow trout (8-10 weeks), where significant inductions of CYP1A were detected following

exposure to waterborne contaminants. However, it was not possible to identify a suitable stage other than juvenile trout for conducting coupled hydro-toxicological studies prior to the experiments. Furthermore, temperature, pH and other environmental parameters can influence EROD activity significantly (for review see Whyte et al. 2000). Furthermore, EROD activity may have been reduced by high concentrations of the respective contaminant. Depending on the uptake kinetics of the PAHs during the simulated flood event, exposure time might have been too short to detect effects on the enzyme level. Most authors, however, reported significant induction of CYP1A already after 6-96 h exposure (Fragoso et al. 2006a, Jonsson et al. 2006a, Ramachandran et al. 2006). In the present study, however, biotransformation enzymes did not constitute suitable biomarkers of exposure to particle-bound PAH contamination. In this context, gene expression analyses might allow for earlier detection of PAH uptake and Ah receptor binding and could therefore be used to measure short-term exposure during 5 d simulated flood events (Aardema & MacGregor 2002).

4.3.2 Oxidative stress

The level of lipid peroxidation throughout both control groups, expressed as malondialdehyde equivalent concentration, was 81.0 ± 18.1 nmol g^{-1} in the current study. Arnold and co-workers (1995) reported significantly higher concentrations in rainbow trout of 300 g weight (6.99 ± 1.72 µmol g^{-1}), while Bay et al. (2002) found lower levels of lipid peroxides in 24-day old larval rainbow trout (1.8 ± 0.13 nmol g^{-1}). The levels of catalase in the experimental groups exposed to unspiked and spiked artificial sediments in the flood events did not differ significantly from the respective control groups, demonstrating that there were no alterations of the levels of this anti-oxidant defence enzyme by the treatments. Salaberria et al. (2009) found CAT activities of 1.03 ± 0.21 µmol min^{-1} mg^{-1} in control animals that were in good accordance to the values found in the control groups of the present study (0.6 ± 0.3 and 0.2 ± 0.8 µmol min^{-1} mg^{-1}, respectively).
However, the levels of lipid peroxides in the liver of exposed animals were significantly elevated in both treatments compared to the control groups. Since oxidative damage reflects an imbalance between the production of oxidants and the removal of such reactive species by protective enzymes, these results clearly indicate a higher production of oxyradicals due to increased respiration as a consequence of an increased swimming activity. Furthermore, Lemaire et al. (1994) reported the generation of hydroxyl radicals by benzo[*a*]pyrene quinones in hepatic microsomes of flounder and perch. Controversially, the oxidative damage, measured as lipid peroxidation, was significantly lower in the treatment group exposed to spiked sediments during the flood event compared to the treatment group exposed to unspiked sediments. Since only catalase was measured as a marker for anti-oxidant enzymes, it may be assumed that other protective proteins were induced by the investigated xenobiotics. Accordingly inconclusive results have been shown by other studies investigating *in vivo* effects on rainbow trout (cf. Arnold et al. 1995, van der Oost et al. 1996). In contrast, Hollert & Braunbeck (1997) found significant correlation between catalase activity and lipid peroxidation in the permanent fish cell-line RTG-2 exposed to suspended particulate matter during a flood event in the Neckar River *in vitro*.

4.3.3 Mutagenicity

The proportion of micronucleated cells within the control groups (1.40 ± 0.66 and 0.53 ± 0.30 ‰, respectively) was in good accordance to previously described values for rainbow trout by Strunjak-Perovic et al. (1.80 ± 1.57 ‰, 2003) and Schultz et al. (approx. 1 ‰, 1993). However, the micronucleus frequencies differed significantly between both control groups, indicating that this

particular biomarker shows relatively high variation even in unexposed animals. In comparison to other studies, the proportion of micronuclei in the treatment group exposed to the spiked sediment was relatively low (2.27 ± 1.12 ‰). Rocha et al. (2009) as well as Schultz et al. (1993) reported maximum average micronucleus frequencies around 6 ‰ in a field-study investigating fish from the highly contaminated Tiête River in Brazil and a laboratory experiment, respectively. Exposure to unspiked sediments during the simulated flood event caused elevated proportions of micronucleated erythrocytes (2.2-fold, not significant). This might be in addition to the increased lipid peroxidation a result of higher respiration due to the current within the flume. Aniagu et al. (2006) have reported comparable results, where chub (*Leuciscus cephalus*) showed significantly elevated oxidative DNA damage when subjected to exhaustive exercise in a swimming experiment.
Nonetheless, the induction of micronuclei in animals exposed to spiked sediments during the simulated flood event was significantly 2-fold higher, thereby indicating a genotoxic potential of the particle-bound PAHs. The micronucleus test is in particular highly ecologically relevant since it is a definitive marker for the irreparable loss of genetic material (Heddle et al. 1991). Several studies have shown that the micronucleus test constitutes an applicable and sensitive biomarker for the identification of genotoxic and mutagenic effects in the field (Al-Sabti & Harding 1990, Al-Sabti & Metcalfe 1995, Balch et al. 1995). Braunbeck et al. (2009) have hypothesized a possible connection between the fish decline in the Neckar River and the presence of genotoxic compounds. Böttcher and co-workers (2009) found a correlation between *in vitro* and *in situ* mutagenicity tests in the upper Danube River where severe fish declines have been reported, while Keiter et al. (2008) measured relatively high PAH concentrations in sediments (Σ 19 PAHs = 26.3 mg kg^{-1}) at the same sampling sites. These studies indicate that genotoxicity is highly relevant to German fish populations and might be related to sediment contamination. Especially with regard to the relatively short exposure time to contaminated suspended matter in the present study, the implication of hydrodynamics in environmental risk assessment of regularly flooded rivers is of extremely high concern.

4.3.4 PAH metabolites in bile

Many aquatic species rapidly metabolize and excrete PAHs (Meador et al. 1995). Thus, quantification of PAH metabolites in bile has been shown to be a very sensitive biomarker of exposure to PAH contamination (Kammann 2007). The most important metabolite in fish bile is 1-hydroxypyren that can contribute up to 76 % of the sum of PAH metabolites. The metabolites of phenanthrene, chrysene and benzo[*a*]pyrene are detected at significantly lower concentrations (Ruddock et al. 2003).
This pattern was also observed in the present investigation. The concentrations used for spiking with the respective substance were in descending order benzo[*a*]pyrene > phenanthrene > pyrene > chrysene, with 8.0, 5.0, 4.0, and 2.8 mg kg^{-1}, respectively. Contrarily, the concentrations of bile metabolites after 5 d exposure to spiked sediment during the simulated flood event resulted in the exact opposite distribution, where 1-hydroxypyrene, 1-hydoxyphenanthrene and 1-hydroxybenzo[*a*]pyrene were measured at concentrations of 2150.00 ± 2553.00, 151.64 ± 59.23, and 1.68 ± 0.37 µg ml^{-1}. These observations could be explained by the different affinity of the parent compounds as it was observed by Ruddock et al. (2003). Additionally, the very high differences between the bile metabolite concentrations might reflect a disparity with regard to bioavailability and uptake of the PAHs.
Within the last years, several laboratory studies have investigated the concentrations of PAH metabolites in bile after exposure to PAHs (Collier & Varanasi 1991, Kammann 2007, van Schanke et al. 2001). Van Schanke et al. (2001) measured the levels of 1-hydroxybenzo[*a*]pyrene in the bile fluid of dab 3 d after oral administration of 10 mg kg^{-1} body weight benzo[*a*]pyrene, resulting in a concentration of 0.81 µg ml^{-1}. Collier & Varanasi (1991) measured a maximum of 1.02 µg ml^{-1} 28 d

after a single intraperitoneal injection of 5.0 mg kg^{-1} body weight benzo[*a*]pyrene. With respect to these studies, comparably high uptake of particle-bound PAHs to rainbow trout was observed in the present work – even during short-term exposure within the 5 d simulated flood event. Furthermore, it was possible to positively correlate the elevated micronucleus frequency in exposed trout to the biliary 1-hydroxybenzo[*a*]pyrene concentration. Benzo[*a*]pyrene is known to cause genotoxicity in laboratory experiments (Metcalfe 1988) and to contribute to the genotoxic effects in field studies (e.g. Barbee et al. 2008). The results from the present work clearly demonstrated an adverse genotoxic effect with implications on the population level.

4.3.5 Possible modifications of the current set of biomarkers and endpoints

The biomarkers that were investigated in the present study were selected for their relatively high specificity in terms of PAH contamination. For future studies, it is intended to test natural and naturally contaminated sediments in the annular flume, which can contain complex mixtures of various contaminants. Thus, it is necessary to modify the set of biomarkers: levels of metallothioneins should be included to display exposure to metal contamination (Wisniewska et al. 1970). Histological investigation of ultrastructural changes (Arnold et al. 1996b, a) and markers for endocrine disrupting compounds (EDC), e.g. vitellogenin (Jones et al. 2000), should also be evaluated.
Furthermore, bioaccumulative substances might also be quantified in different tissues of the exposed animals to experimentally confirm the hypothesis that short-term exposure to particle-bound contaminants during flood events might lead to an increased body burden. Within the discussion concerning the practical implementation of the EU Water Framework Directive (WFD), an annex to 2000/60/EC came into force, which claims that concentrations of priority substances in sediments and tissues must not increase (EC 2006). Thus, investigation of the interdependency between biota and sediments with regard to bioaccumulation during remobilization events is of extremely high concern.
Secondly, genomic biomarkers, e.g. gene expression analyses using quantitative real-time polymerase chain reaction (Q-RT-PCR), could improve the sensitivity of analyses evaluating the short-term impact of remobilization events. Those markers display the first initial and thus fast response to uptake of xenobiotics (Aardema & MacGregor 2002, Waters & Fostel 2004). Recent studies in rodents also suggested that genomic analyses may facilitate the understanding of the effects of complex mixtures (Aardema & MacGregor 2002, Hamadeh et al. 2002). Furthermore, gene expression analyses allow for the determination of the specific modes of action (MOA) of certain chemicals in fish and may also permit the identification of key toxicants in natural sediments (Tompsett et al. 2009, Zhang et al. 2008). Thus, several studies have recently been conducted to study the effects of xenobiotics on gene expression profiles in fish (for review see Steinberg et al. 2008).

4.4 Potential and limitations of the current approach

It was hypothesized that the combination of methods from hydraulic engineering and ecotoxicology may assist to derive important information on the impact of flood events on biota and ecosystem health and thus improve the understanding of processes in-field. In the present proof-of concept study, it was experimentally supported that remobilization during simulated flood events in the annular flume can lead to uptake and adverse effects of sediments-bound contaminants. This new approach has been shown to be applicable to successfully conduct hydro-toxicological studies with rainbow trout. However, technical modifications of the annular flume (e.g., automatic feeding) and an increase of the dimensions (1) to permit even better environmental conditions and (2) to reduce the influence of the exposed organisms on the physico-chemical processes would be desirable. Furthermore, it will be

necessary to systematically control environmental variables as pH and temperature for application of the annular flume for further studies, especially in the context of climate change.

5. Conclusions & Outlook

This study has shown the suitability of the annular flume to investigate the adverse effects caused by the remobilization of sediment-bound contaminants. Unspiked and spiked artificial sediments were successfully used for this proof-of-concept. However, it is questionable if those formulated sediments can be transferred to natural conditions and the field situation. A set of biomarkers was investigated to identify suitable markers for exposure to particle-bound contaminants in rainbow trout. Specific activities of the enzymes 7-ethoxyresorufin-*O*-deethylase (EROD), glutathione-*S*-transferase (GST) and catalase (CAT) were no sensitive biomarkers. In contrast, lipid peroxidation, micronucleus formation in peripheral erythrocytes and biliary PAH metabolites were shown to be sensitive and conclusive markers of adverse effects. In this context, metabolic transformation of benzo[*a*]pyrene could clearly be related to genotoxicity in the micronucleus test. Thus, adverse effects with implications on the population level have to be anticipated.

To investigate whether gene expression analyzes could facilitate the interpretation of the presented data and constitute an applicable biomarker for coupled hydro-toxicological studies, quantitative real-time polymerase chain reaction (Q-RT-PCR) will be applied to liver samples from this experiment. In a next step, the annular flume will be used to test natural and naturally contaminated sediments to test if the current approach is transferable to more realistic conditions. Thus, additional biomarkers, i.e. metallothioneins, vitellogenin and histological markers, will be added to the battery of biomarkers to reflect a broader range of contaminants. In the future, the experimental setup will be improved by increasing the dimensions of the annual flume and enabling the systematic control of environmental variables, such as pH and temperature. Furthermore, an extensive sensitivity analysis will lead to a comprehensive quantification of the individual contribution of each stressor (turbidity, current, contamination) to derive a complete database for model development. In the last instance, this work is intended to improve the understanding of the interrelation between biota and particles during flood events, thereby facilitating the environmental risk assessment and management of regularly flooded rivers and, ultimately, the marine environment as well.

The presented study has shown that relatively short exposure during simulated flood events to resuspended sediments can lead to adverse effects in rainbow trout. Thus, the ecological and toxicological impact of contaminant remobilization during floods has to be considered highly relevant (Einsporn et al. 2005, Netzband et al. 2007, Wölz et al. 2009) and integrated approaches for risk assessment of regularly flooded rivers are urgently required.

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