# Evaluation of an interdisciplinary method to assess the hydrotoxicological relevance of contaminated sediments after resuspension events

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*To Silvana and Ferdinand*

# Abstract

There is a consensus within the scientific community that sediments act as a long term sink for a variety of organic and inorganic pollutants which, however, can re-enter the water column upon resuspension of deposited material under certain hydraulic conditions such as flood events. In the last years, interdisciplinary research has become increasingly important in response to new challenges related to the implementation of the European Water Framework Directive (WFD). This directive commits European Union member states to achieve a good ecological and chemical status in European river catchments. Furthermore, the importance of sediments as a secondary long-term source for pollutants and, hence, as an important factor for water quality has been integrated into the WFD. Within the implementation of the WFD, it is important to understand the potential short and long term impact of suspended particulate matter (SPM)-associated contaminants on aquatic organisms as well as the related uptake mechanisms for a sound risk assessment. Increasingly frequent flood events usually cause remobilization of contaminated sediment layers in rivers and, thus, are of high relevance for a sound understanding of associated ecotoxicological impacts.

The Floodsearch project framework, performed in close collaboration between the Institute for Environmental Research and the Institute of Hydraulic Engineering and Water Resources Management (both RWTH Aachen University), aimed to establish a novel approach combining methods of hydrodynamic engineering and ecotoxicological assessment to enable the investigation of the potential risks associated with such remobilization events. This new approach, developed by an interdisciplinary team was entitled the hydrotoxicological approach by Cofalla (2015). It can be used as a tool with practical relevance to investigate and assess contaminated sediments and their influence on aquatic organisms upon resuspension. The present PhD thesis consisting of three individual studies aims at the evaluation of the hydrotoxicological approach and has a focus on the ecotoxicological aspects without neglecting the engineering side.

In the first proof-of-concept study, hydraulic engineering and ecotoxicology were combined in a new interdisciplinary approach to assess the toxicity of resuspended polluted sediments after a simulated flood event. Specifically, the objective of this study was to bridge the gap between the physical resuspension of pollutants and resulting toxicological impacts on aquatic organisms. Formulated sediment was prepared according to OECD guideline 218 and spiked with a mixture of four polycyclic aromatic hydrocarbons (PAHs). Rainbow trout (*Oncorhynchus mykiss*) were exposed to resuspended sediments. A suite of different molecular, biochemical and histological markers was used to verify the hypothesis that resuspension of sediments can lead to the remobilization of PAHs and subsequently to effects in aquatic organisms. The experiments were carried out in an annular flume designed to investigate the transport behavior of fine-grained sediments. Several physicochemical and sedimentological parameters as well as exposure concentrations of contaminants were measured to characterize environmental conditions and erosion behavior of sediments. During the elaboration of a biomarker battery comprising biomarkers with suitable endpoints, rainbow trout proved to be a suitable test species (a) being easy to rear, (b) being sufficiently robust for exposure experiments and (c) providing sufficient amounts of sample material for bioassays and chemical analysis. The micronucleus frequency was significantly 4.3-fold elevated after exposure and biliary metabolite concentration was shown to be the most sensitive marker of PAH exposure. The original setup of the annular flume was successfully modified including a flow-through cooling unit and an aeration system to maintain suitable and stable environmental conditions for the test animals. Flood events were successfully simulated, resulting in the resuspension of formulated sediment, and different erosion behaviors of sediments during the simulated flood events were observed and could be characterized. This proof-of-concept study clearly demonstrated the feasibility of a combined hydrotoxicological approach in support of the investigation of the potential ecotoxicological relevance of sediment resuspension events and showed that sediment remobilization during short simulated flood events in the annular flume can lead to uptake and effects of sediment-bound pollutants in rainbow trout.

In the second study, preliminary tests were carried out in resuspension containers to provide a broader knowledge base for subsequent experiments in the annular flume. The main purpose of those

experiments were (a) the assessment of the dissipation of PAHs from sediment/water systems and of the subsequent differences between desorption and bioavailability of spiked contaminants, and its implications for the following experiments in the annular flume, (b) kinetic monitoring of the chosen biomarkers to provide detailed insights in their dynamics as a function of extended exposure to suspended matter and (c) the assessment of a combination of chemical exposure and another environmental stressor, temperature, in aquatic biota. Due to recent changes in climate, temperatures of German rivers frequently exceed 25 °C during summer. Effects of resuspension of sediments on biota under elevated temperature regimes are likely to differ from those under lower temperature regimes. To elucidate this differential response of aquatic vertebrates, rainbow trout were exposed to suspensions of sediment from the Rhine River that was spiked with a mixture of PAH. The experiments were conducted under two different temperature regimes (24 °C or 12 °C) and physicochemical parameters, including concentration of PAHs in SPM, and biomarkers in fish such as biliary PAH metabolites were measured over the course of a 12 d study. Concentrations of pyrene and phenanthrene decreased over time, while no decrease was observed for chrysene and benzo[*a*]pyrene. The biomarker cascades, more specifically the temporal dynamics of biomarker reactions, did not only show quantitative differences (i.e. different induction intensity or rate of biomarker responses) at the two temperatures but also qualitative differences, i.e. different biomarker responses were observed. In bile of fish exposed to PAHs spiked sediment, concentrations of 1-hydroxypyrene and 1-hydroxyphenanthrene increased significantly during the first two days, and then decreased. At 12 °C, the uptake of PAHs was slower and maximum metabolite concentrations in bile were lower than in fish exposed at 24 °C. Following a latency of two days, the concentration of PAH metabolites in bile of fish exposed at 24 °C was followed by a peak in lipid peroxidation. PAHs spiked into sediments under laboratory conditions were significantly more bioavailable than the PAHs that were already present in un-spiked field-collected sediments.

In the final study described in this thesis, findings from both preceding studies were joined to refine the hydrotoxicological approach and to re-evaluate the new interdisciplinary method. To elucidate the effects of sediment-bound organic pollutants, such as PAHs, rainbow trout were exposed to three resuspended natural sediments with different contamination levels. Physicochemical parameters including dissolved oxygen concentration, pH and temperature, total PAH concentration in sediments and SPM as well as different biomarkers of exposure in fish such as 7-ethoxyresorufin-*O*-deethylase (EROD) activity and biliary PAH metabolites were measured following 7 d exposure within an annular flume. Concentrations of PAHs in SPM remained constant and represented the different contamination level in the un-suspended sediments. Significant differences in bile metabolite concentrations as well as in EROD induction compared to control experiments (untreated animals and animals that were exposed in the annular flume without sediment) were observed for all exposure scenarios. The ratio between 1-hydroxypyrene in bile from fish exposed to the three different contamination levels was 1.0 : 3.6 : 10.7 and correlated well with (1) the ratio of pyrene concentrations in corresponding sediments which was 1.0 : 3.1 : 12.7 and (2) with the ratio of particle-bound pyrene in SPM which was 1.0 : 2.7 : 11.7. In contrast, hepatic lipid peroxidation and micronuclei formation represented the different contamination levels less conclusively. The results of this study clearly demonstrated that firmly-bound PAH from aged sediments can become bioaccessible upon resuspension under flood-like conditions and are readily absorbed by aquatic organisms such as rainbow trout. Associated short term effects were clearly documented and possible adverse long-term impacts due to genotoxicity are likely to follow.

The present thesis demonstrates that the interdisciplinary approach created an innovative research tool to assess the hydrotoxicological relevance of contaminated sediments after resuspension events. However, there are certain drawbacks and limitations of this innovative approach such as the influence of larger animals on erosion characteristics within the annular flume and the dependency on vertebrate test animals. In a next step and with future method optimizations, however, those shortcomings can be resolved and the hydrotoxicological approach can be used to the full extent as a tool with practical relevance to investigate and assess contaminated sediments and their influence on aquatic organisms upon resuspension.

# Zusammenfassung

Sedimente können eine Senke für organische und anorganische Schadstoffe darstellen, welche jedoch nach Resuspension, beispielweise bei Hochwasserereignissen, wieder in den Wasserkörper gelangen können. Im Kontext der Implementierung der Europäischen Wasserrahmenrichtline (WRRL), die Europäische Mitgliedsstaaten verpflichtet einen guten ökologischen und chemischen Zustand in Europäischen Flusseinzugsgebieten zu erreichen, gewann interdisziplinäre Forschung in den letzten Jahren vermehrt an Bedeutung. Sedimente wurden als sekundäre, langfristige Schadstoffquelle und somit als wichtiger Faktor für die Wasserqualität in der WRRL verankert. Die Umsetzung der WRRL und die damit einhergehende Risikobewertung setzen ein fundiertes Verständnis der potentiellen kurz- und langfristigen Auswirkungen von Schwebstoff-gebundenen Schadstoffen auf aquatische Lebewesen sowie derer Aufnahmewege voraus. Zudem ist ein fundiertes Wissen über immer häufiger auftretende Hochwässer, die im Allgemeinen eine Remobilisierung von schadstoffbehafteten Sedimentschichten in Flüssen bewirken, von entscheidender Wichtigkeit für eine Bewertung damit verbundener ökotoxikologischer Risiken. Das Floodsearch Projekt-Framework, das in enger Kooperation zwischen dem Institut für Umweltforschung und dem Institut für Wasserbau und Wasserwirtschaft (beide RWTH Aachen University) durchgeführt wurde, zielte darauf ab, unter Kombination von ökotoxikologischen und wasserbaulichen bzw. wasserwirtschaftlichen Methoden einen neuen Ansatz zu entwickeln, um das potentielle Risiko solcher Remobilisierungsereignisse zu untersuchen. Dieser neue, interdisziplinäre Ansatz wurde von Cofalla (2015) als Hydrotoxikologie benannt und kann als praktisches Bewertungswerkzeug genutzt werden, um resuspendierte, kontaminierte Sedimente und deren Einfluss auf aquatische Organismen zu untersuchen und zu bewerten. Die vorliegende Doktorarbeit, die aus drei einzelnen Studien besteht, zielt darauf ab, den hydrotoxikologischen Ansatz mit Schwerpunkt auf der Ökotoxikologie zu evaluieren ohne die ingenieurwissenschaftliche Seite außer Acht zu lassen.

In der ersten Machbarkeitsstudie wurden Wasserbau und Ökotoxikologie zu einem neuen interdisziplinären Ansatz kombiniert, um die Toxizität von resuspendierten kontaminierten Sedimenten bei einem simulierten Hochwasserereignis zu bewerten. Das Ziel dieser Studie war es, die Wissenslücke zwischen der physikalischen Resuspension von Schadstoffen und den resultierenden toxikologischen Auswirkungen auf aquatische Organismen zu schließen. Kunstsediment wurde gemäß OECD Richtlinie 218 hergestellt und mit einer Mischung aus vier polyzyklischen aromatischen Kohlenwasserstoffen (PAK) dotiert. Regenbogenforellen (*Oncorhynchus mykiss*) wurden gegenüber resuspendierten Sedimenten exponiert. Eine Reihe verschiedener molekularer, biochemischer und histologischer Biomarker wurde genutzt, um die Hypothese zu verifizieren, dass die Resuspension von schadstoff-behafteten Sedimenten zu Effekten in aquatischen Organismen führen kann. Die Experimente fanden in einem Kreisgerinne statt, das ursprünglich zur Untersuchung des Transportverhaltens feinkörniger Sedimente genutzt wurde. Mehrere physikochemische und sedimentologische Parameter, sowie Schadstoffkonzentrationen wurden bestimmt, um die vorherrschenden Umweltbedingungen und das Erosionsverhalten der Sedimente zu bestimmen. Während der Entwicklung eines Biomarker-Setups mit entsprechenden Endpunkten erwies sich die Regenbogenforelle als geeignete Testspezies, da sie (a) einfach zu halten, (b) ausreichend robust für Expositionsexperimente ist und (c) ausreichend Probenmaterial für Biotests bietet. Die Mikrokernrate war nach der Exposition signifikant um das 4.3-fache erhöht und die Konzentration an Gallenmetaboliten erwies sich als der sensitivste Biomarker für die PAK Exposition. Der ursprüngliche Versuchsaufbau des Kreisgerinnes wurde erfolgreich um z.B. eine Durchflusskühlung und ein Belüftungssystem erweitert und schuf stabile Umweltbedingungen für die Versuchsorganismen. Hochwasserereignisse konnten erfolgreich nachgestellt werden und führten zu Resuspension der Kunstsedimente, deren Erosionsverhalten charakterisiert werden konnte. Diese Studie konnte deutlich die Machbarkeit eines kombinierten hydrotoxikologischen Ansatzes zeigen, der klassische Untersuchungen potentieller ökotoxikologischer Folgen von Sedimentresuspensionen methodisch unterstützt. Zudem wurde gezeigt, dass eine solche Resuspension während kurzer simulierter Hochwasserereignisse zur Aufnahme von sediment-gebundenen Schadstoffen und entsprechenden Effekten in Regenbogenforellen führen kann.

In der zweiten Studie wurden Vorversuche in Resuspensionsbecken durchgeführt, um eine breitere Wissensbasis für nachfolgende Versuche im Kreisgerinne zu schaffen, indem (a) die Dissipation von PAK im Sediment/Wassersystem und die nachfolgenden Unterschiede in der Desorption und Bioverfügbarkeit dotierter Schadstoffe, sowie deren Konsequenz für die nachfolgenden Experimente im Kreisgerinne bewertet

wurde, (b) ein kinetisches Monitoring ausgewählter Biomarker durchgeführt wurde, um Einblicke in deren Dynamik in Relation zu Expositionszeit und zu Schwebstoffkonzentration zu erlangen, und (c) die Auswirkung der Kombination von chemischer Exposition und dem zusätzlichen Stressor Temperatur auf Fische bewertet wurde. Aufgrund jüngster klimatischer Veränderungen übersteigen die Wassertemperaturen deutscher Flüsse im Sommer häufig 25 °C. Resuspensionseffekte von Sedimenten auf aquatische Biota unterscheiden sich unter erhöhten Temperaturbedingungen aller Wahrscheinlichkeit nach von denen unter niedrigeren Temperaturen. Um diese Hypothese zu verifizieren, wurden Regenbogenforellen gegenüber mit PAK dotierten Sedimenten aus dem Rhein unter zwei unterschiedlichen Temperaturbedingungen exponiert. Physikochemische Parameter, u.a. PAK Konzentration in Schwebstoffen, und Biomarker in den Fischen, wie beispielsweise PAK Metabolite, wurden über den Verlauf der 12-tägigen Studie bestimmt. Schwebstoffkonzentrationen von Pyren und Phenanthrene wurden geringer, während die von Chrysen und Benzo[*a*]pyren während der Versuchsdauer nicht abnahmen. Die Biomarker Kaskade, bzw. die zeitliche Dynamik der Biomarker Antworten, wies nicht nur quantitative Unterschiede (unterschiedliche Induktionsintensität bzw. Rate der Biomarker Antworten) bei den beiden unterschiedlichen Temperaturen auf, sondern auch qualitative Unterschiede, d.h. unterschiedliche Biomarker-Antworten. In der Gallenflüssigkeit exponierter Fische stiegen die Konzentrationen von 1-Hydroxypyren und 1-Hydroxyphenanthren während der ersten zwei Tage signifikant an und nahmen dann ab. Bei 12 °C war die PAK-Aufnahme langsamer und die Maximalkonzentration niedriger als bei 24 °C. Nach einer Verzögerung von zwei Tagen folgte auf das Gallenmetabolitmaximum bei 24 °C ein Maximum der Lipidperoxidation. PAK, die unter Laborbedingungen dotiert wurden, waren signifikant höher bioverfügbar als die PAK, die bereits in den natürlichen Sedimenten vorhanden waren.

In der letzten Studie dieser Doktorarbeit wurden Erkenntnisse aus beiden vorherigen Studien kombiniert, um den hydrotoxikologischen Ansatz zu optimieren und diese neue, interdisziplinäre Methode zu evaluieren. Um die Effekte Sediment-gebundener PAK, zu bewerten, wurden Regenbogenforellen gegenüber drei resuspendierten, natürlichen Sedimenten mit unterschiedlichen Belastungsleveln exponiert. Physikochemische Parameter, wie bspw. $O_2$-Konzentration, pH-Wert und Temperatur, PAK-Konzentrationen in Sedimenten und Schwebstoffen, sowie verschiedene Expositionsbiomarker, wie 7-ethoxyresorufin-*O*-deethylase (EROD) Aktivität und PAK-Gallenmetabolite wurden im Anschluss an eine 7-tägige Exposition im Kreisgerinne gemessen. Die PAK-Konzentrationen in den Schwebstoffen blieben konstant und spiegelten die unterschiedlichen Belastungslevel in den Ausgangssedimenten wieder. Signifikante Unterschiede in der Konzentration von Gallenmetaboliten und der EROD Induktion wurden bei allen Expositionsszenarien im Vergleich zu den Kontrollexperimenten (unbehandelte Tiere im Kreisgerinne ohne Sediment) beobachtet. Das Verhältnis von 1-Hydroxypyren in der Gallenflüssigkeit von Fischen, die gegenüber drei unterschiedlichen Belastungssituationen exponiert wurden, lag bei 1.0 : 3.6 : 10.7 und korrelierte gut mit (1) dem Verhältnis von Pyrenkonzentrationen in den dazugehörigen Sedimenten mit 1.0 : 3.1 : 12.7 und (2) dem Verhältnis des Partikel-gebundenen Pyrens in den Schwebstoffen mit 1.0 : 2.7 : 11.7. Dahingegen spiegelten die hepatische Lipidperoxidation und die Mikrokernrate die unterschiedlichen Belastungssituationen weniger eindeutig wieder. Die Ergebnisse dieser Studie zeigen deutlich, dass fest gebundene PAK aus Altsedimenten bei Resuspensionsereignissen unter hochwasser-artigen Bedingungen wieder biozugänglich werden und von aquatischen Organismen wie bspw. Regenbogenforellen absorbiert werden können. Dazugehörige kurzfristige Effekte konnten dokumentiert werden und mögliche Langzeiteffekte aufgrund der nachgewiesenen Genotoxizität sind wahrscheinlich.

Die vorliegende Arbeit zeigt deutlich, dass der hier präsentierte interdisziplinäre Ansatz ein innovatives Forschungswerkzeug darstellt, um die hydrotoxikologische Relevanz von kontaminierten Sedimenten unter Resuspensionbedingungen zu bewerten. Allerdings weist dieser innovative Ansatz zurzeit noch einige Schwächen und Limitierungen auf, wie bspw. der Einfluss größerer Versuchstiere auf das Erosionsverhalten im Kreisgerinne oder die Abhängigkeit von Wirbeltieren als Versuchstiere. In einem nächsten Schritt und mit zukünftigen Optimierungen können diese Defizite überwunden und der hydrotoxikologische Ansatz kann als ein Werkzeug mit praktischer Relevanz genutzt werden, um kontaminierte Sedimente und deren Einfluss auf aquatische Organismen während Resuspensionsereignissen zu untersuchen und zu bewerten.

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# Table of contents

# List of tables

# List of figures

Chapter 1

# Introduction

Parts of this chapter have been previously published in the following peer-reviewed articles:

Cofalla, C. & **Hudjetz, S.**, Roger, S., Brinkmann, M., Frings, R., Wölz, J., Schmidt, B., Schäffer, A., Kammann, U., Hecker, M., Hollert, H., Schüttrumpf, H. (2012) A combined hydraulic and toxicological approach to assess re-suspended sediments during simulated flood events – part II: an interdisciplinary experimental methodology. *Journal of Soils and Sediments* 12, 429-442

**Hudjetz S**., Herrmann H., Cofalla C., Brinkmann M., Kammann U., Schäffer A., Schüttrumpf H., Hollert H. (2014): An attempt to assess the relevance of flood events – biomarker response of rainbow trout exposed to resuspended natural sediments in an annular flume. *Environmental Science and Pollution Research* 21, 13744-13757

## 1.1 **Sediments and suspended particulate matter**

Sediments play an integral role regarding hydrological, geomorphological and ecological processes within aquatic systems such as rivers, lakes and coastal zones (Westrich & Förster 2007) and are a mixture of inorganic and organic material deposited by water, wind or glaciers (Scheffer & Schachtschabel 2002). In fluvial systems, inorganic sediment components originate primarily from the weathering of rocks and the mobilization and erosion of soils or other loose material present in the respective drainage basins. Furthermore adjacent trees, aquatic macrophytes and fish contribute to the organic sediment components. According to Bridge (2003) those sediment sources as well as the sediment quality are controlled by multiple factors such as the nature of the exposed rocks and soil types, the amount and intensity of precipitation, annual temperature changes, the presence of vegetation and slope angle. Therefore, sediment yield, both regarding quality and quantity, is strongly correlated to the topography and climate of the drainage basin in question and varies in time and space. Furthermore changes in human land use such as deforestation, agricultural activity and urbanization have increased many erosion processes and therefore also sediment supply to rivers (Owens & Batalla 2003). According to Syvitski et al. (2005) humans increase on the one hand the river transport of sediment following soil erosion and on the other hand decrease the flux of sediment to the coastal zone through sediment retention in reservoirs. Both anthropogenic influenced processes have major impacts on river systems as well as on coastal estuaries in terms of total sediment budget, which in turn influences e.g. river morphology, nutrient cycling and distribution of pollutants.

Depending on various factors such as river geometry, the water discharge of a river system or more precisely the bed shear stress situation determines the transport capacity and finally the quantity of sediments being transported downstream from the source area to the outlet of the river system. During peak discharges such as at times of excessive rain falls or melting of snow sediment transport rates are highest. In areas of low shear stress, sediment begins to settle on the riverbed or - in case of a flood event - on river floodplains or even in urban areas close to rivers. At the coastal outlet of river systems, sediments often form widely branched estuaries.

Sediments and their movements are an integral part of the morphological and ecological processes of aquatic ecosystems, which include for example nutrient cycling by interaction with the overlaying waters, transfer of nutrient and pollutants from source to outlet and creation and sustenance of coastal landforms (Owens 2007). Furthermore, they play a key role in providing

habitats for a various aquatic and benthic species, which are highly dependent on sediment quality (Ahlf 1995, Burton 1995, Höss et al. 1997). Many of those species play an important role as food source for ecologically and economically important aquatic organisms such as crabs, shrimp and fish (Chapman 1990, Swartz et al. 1986, Swartz et al. 1985). Therefore, changes of the benthic community structures affect the whole aquatic ecosystem in both negative and positive ways. Furthermore, especially benthic microorganisms such as bacteria and microalgae, but also the meso- and macrofauna play an important role in the stability of sediments (Ciutat et al. 2006, Gerbersdorf et al. 2008, Graf & Rosenberg 1997, Widdows et al. 2000). Major mechanisms of sediment destabilization and stabilization, respectively, seem to be bioturbation by the meso- and macrofauna (Meadows et al. 1990, Riemann & Schrage 1978) as well as the secretion of extracellular polymeric substances by bacteria and microalgae (EPS, de Brouwer et al. 2000, de Brouwer et al. 2005). EPS consist mostly of carbohydrates, proteins and a multitude of other organic compounds (Decho 1990, 2000) and accomplish a variety of functions due to of their glue-like structure and their high adsorption capacities (Flemming & Wingender 2001b, a). These functions include: concentration of extracellular enzymes, adhesion of organisms to surfaces (Costerton et al. 1978) and agglutination of sediment particles, absorption of organic and inorganic matter, as well as pollutants (Dohse & Lion 1994, Pal & Paul 2008) which influences their bioavailability and degradation. For river Elbe as well as for river Neckar sediments with grain sizes < 2 mm, it has been observed that the higher the EPS content, the higher is the critical bed shear stress and, therefore, the overall sediment stability (Gerbersdorf et al. 2009, Gerbersdorf et al. 2008, Le Hir et al. 2007). It is thus apparent that EPS have a major impact on the dynamics of sediments as well as on the associated contaminants (Gerbersdorf et al. 2011).

## 1.2 **Sediment contamination and remobilization**

Sediments are sinks for a variety of contaminants including heavy metals, polychlorinated biphenyls (PCBs), dioxins and polycyclic aromatic hydrocarbons (PAHs). According to Chapman (1990) exposure to sediment-bound contaminants can result in harmful effects to benthic and aquatic life including lethality, impacts on reproductive functions and cancer in aquatic organisms. Many of the lipophilic pollutants associated with sediments also tend to bioaccumulate along the food chain, which can ultimately result in exposure of humans through consumption of fish and seafood from contaminated areas (Barhoumi et al. 2016, Dickman & Leung 1998, Fremy & Bordet 2002, Smith & Gangolli 2002, Zhao et al. 2014).

During undisturbed conditions bioavailability of sediment-bound contaminants may be limited but it is known to increase during anthropogenic or natural hydrodynamic events like dredging (Koethe 2003) or floods (Haag et al. 2001, Hollert et al. 2000, Westrich & Forstner 2005, Wölz et al. 2008). One of the major concerns regarding the remobilization of sediments is the more frequent occurrence of extreme weather events such as heavy rainfall. These result in increasingly frequent and strong flood events and are described as one of the potential consequences of global climate change (Hunt 2002, Ikeda et al. 2005, Kay et al. 2006, Najjar et al. 2010, Palmer et al. 2008). During the past decade, numerous extreme weather events occurred around the globe and led e.g. to the 500-year flood in the River Elbe, Germany in 2002 (Schüttrumpf & Bachmann 2008). During this flood event layers of highly contaminated legacy sediment were remobilized and transported downstream (Stachel et al. 2005). Given the increasing likelihood of such extreme flood events, there is a growing need to develop scientific approaches that will help to further our understanding and allow predicting their possible toxicological and ecotoxicological impacts.

## 1.3 **Sediment risk assessment**

### 1.3.1 **Current state of research**

Today, most approaches to assess the toxicity of sediments focus on a combination of targeted chemical analysis of priority pollutants, biotests, and the measurement of *in situ* parameters such as benthic species composition. The combination of these lines of evidence, the so called "sediment quality triad" (SQT), has proved to be a very promising approach (SQT, Brack et al. 2005, Chapman 1990, Hollert et al. 2002, Long & Chapman 1985). However, SQT approaches do not consider dynamic changes, such as flood events or dredging operations, which can lead to remobilization of sediment layers, ultimately influencing the bioavailability of sediment-bound contaminants. Consequently, it has been suggested in recent years to integrate hydraulic engineering (hydrodynamics) in weight-of-evidence approaches as an additional line-of-evidence to provide a more realistic assessment of the environmental impact of contaminated sediments (Chapman & Hollert 2006, Gerbersdorf et al. 2011). Consequently, the combination of hydrodynamic and ecotoxicological methods into a new interdisciplinary approach has emerged as a novel field in environmental research (Brinkmann et al. 2010b, Cantwell et al. 2008, Feng et al. 2007, Hollert et al. 2007, Wölz et al. 2009b, Yang et al. 2008b).

### 1.3.2 **The hydrotoxicological approach**

The combination of two scientific fields, i.e. ecotoxicology and civil engineering research, and their specialized knowledge in ecotoxicological and hydrodynamic as well as sedimentological methods lead to the development of a new interdisciplinary approach. This new approach, developed by an interdisciplinary team (Brinkmann et al. 2010a, Brinkmann et al. 2013, Cofalla et al. 2012, Hudjetz et al. 2014, Schüttrumpf et al. 2011, Wölz et al. 2009b), and named the hydrotoxicological strategy was thoroughly described by Cofalla (2015). It can be used as a tool with practical relevance to investigate and assess contaminated sediments and their influence on aquatic organisms upon resuspension.

In contrast to the previous common approaches, which are based on the separate evaluation of sediment erosion probabilities and ecotoxicological risk assessment, the hydrotoxicological approach relies on the experimental combination of those scientific paths. According to Cofalla (2015), five parameter groups are assessed during the first step of the hydrotoxicological approach. A subsequent statistical examination of generated data identifies useful intersections and a concluding third step allows assessing ecotoxicological risks linked with resuspension probabilities (Figure 1.1).

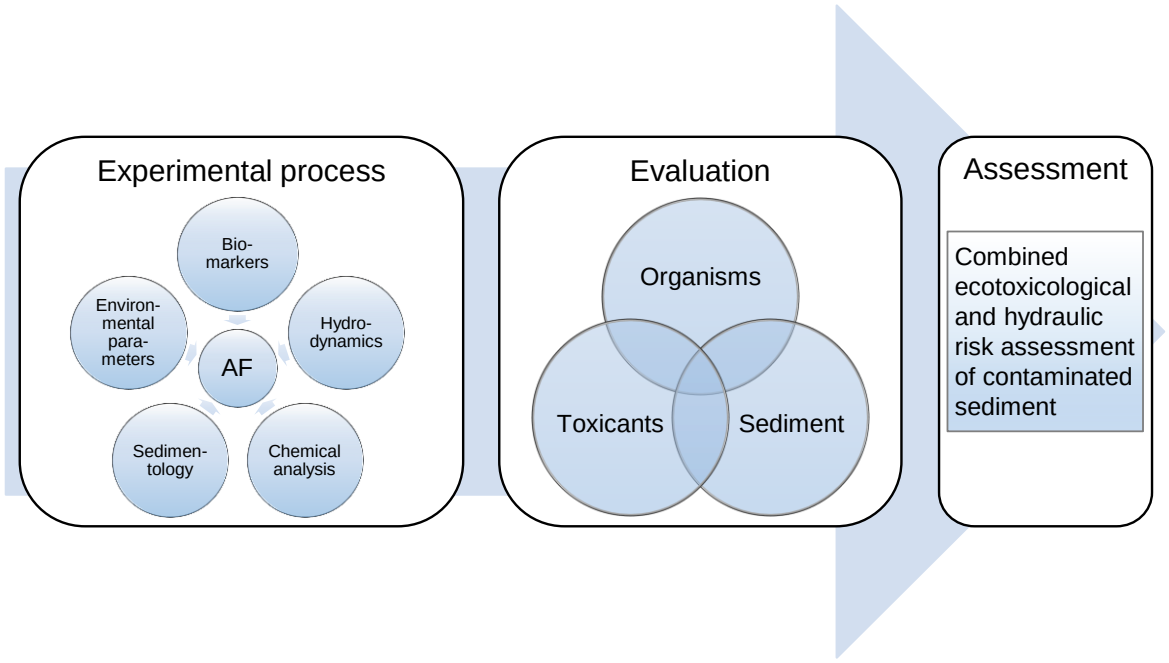


Figure 1.1: Process scheme of the hydrotoxicological approach (modified according to Cofalla 2015); AF: Annular flume.

The present PhD thesis focuses on the evaluation of the hydrotoxicological approach and has a focus on the ecotoxicological aspects, whereas the PhD thesis of Cofalla (2015) detailed the

method development as well as hydraulic investigations and finally gives a broader overview on the parameter interrelation.

## 1.4 **Regulatory aspects**

It has been widely accepted that sediment-bound substances are of global major concern for the water quality in aquatic systems and the health of aquatic ecosystems (Campos et al. 2016, Chapman et al. 2013, Gerbersdorf et al. 2007, Haag et al. 2001, Hilscherova et al. 2007, Hollert et al. 2000, Hollert et al. 2007, Hollert et al. 2003a, Keiter et al. 2006, Kosmehl et al. 2007, Rao et al. 1990, Schweim et al. 2001, Weber et al. 2008). Therefore, they have to be considered when assessing the risks linked with their possible remobilization. Consequently, a large variety of risk assessment concepts for sediments has been developed (Ahlf et al. 2002, Burton 1991, Burton 1995, Chapman 1990, 2000, 2002, Chapman & Hollert 2006, Chapman et al. 2013, Hollert et al. 2009). They include chemical analysis, *in vitro* biotests and the assessment of *in situ* effects on benthic biocoenoses. Effect directed analysis (EDA) allows for the identification of unknown toxic substances (Brack 2003, Brack et al. 2016) and passive samplers can be used to assess the bioavailability of xenobiotics (Greenberg et al. 2014, Lydy et al. 2014). Furthermore, hydrodynamics and physical stress can be integrated in the assessment (Burton & Johnston 2010, Westrich & Forstner 2005). Coupled with ecotoxicology a new research area emerged during the last years (Cofalla et al. 2011, Droppo et al. 2016, Gerbersdorf & Wieprecht 2015, Hudjetz et al. 2014, Schüttrumpf et al. 2011, Wölz et al. 2009b).

Nevertheless, the role of sediments as a secondary source of pollutants has at first been neglected by the European Union (EU) when it comes to water quality assessment as defined by the EU water framework directive (WFD, Directive 2000/60/EC, Heise & Foerstner 2006). The WFD aims at achieving both a good ecological and good surface water chemical status in European river basin districts and marine waters up to one nautical mile from shore by the year 2027, using an integrated approach of emission and pollutant standards. In a later amendment (Directive 2008/105/EC), however, the relevance of sediments has been acknowledged and is now integrated in the WFD (article 2 (35) and article 16 (7 and 8)). Threshold concentrations of 33 priority pollutants, ranging from pesticides and metals to polycyclic aromatic hydrocarbons, which must not be exceeded in sediments and biota, are listed in this daughter directive in Annex II (Directive 2008/105/EC). This Directive on Environmental Quality Standards (EQS directive) therefore sets environmental quality standards for different

substances of concern in surface waters. Furthermore, there is the possibility to apply those EQS for sediment and biota, instead of water. In a recent amendment of both directives (Directive 2013/39/EU) of the European commission this list of 33 priority pollutants is supplemented by further 12 substances including pharmaceuticals and e.g. stricter EQS for a number of substances are established. It is now possible to derive the chemical status of a surface or underground water from the concentration of a priority substance and the associated EQS value (Wernersson et al. 2015).

One important aspect in the risk assessment of sediments is to assess particle bound substances, which are released from sediments into the water column during flood events and other resuspension events which lead to sediment relocation (Hollert et al. 2007, Noack et al. 2015, Westrich & Förster 2007). To date, SPM is neither mentioned explicitly in the WFD (CEC 2000) nor in the EC EQS Directive (CEC 2008) although it has a substantially influence on water quality and bioaccessibility, i.e. possible transfer of pollutants to aquatic organisms. However, environmental quality standards (EQS) for organic substances in surface waters comprise now both dissolved fractions and fractions adsorbed to SPM. This can lead to analytical inaccuracies or even problems, specifically in regard to hydrophobic compounds strongly absorbed to particles (90 % or more) when e.g. the annual average concentration is assessed (Werres et al. 2009). In a guidance document by a drafting group comprised of experts from EU member states, it was proposed to surrogate analysis of whole water sample by analysis of the SPM fraction (chapter 5.3 and 6.2, CEC 2009) and a subsequent conversion to the concentration in the whole water to overcome this problem (Schubert et al. 2012).

Increased SPM and associated contaminants as a result of e.g. flood or dredging events are already mentioned in the Directive on Environmental Quality Standards as “losses from pollution accumulated in sediments” (EQS Directive, CEC 2008), however EQS for suspended particulate matter should be implemented within the WFD to account for the importance of SPM in risk assessment. Besides direct impacts of SPM-bound contaminants on aquatic organisms, transport and deposition of contaminated SPM may endanger the goal to achieve a good chemical and ecological status even at remote or downstream sites (Förstner et al. 2004, Hilscherova et al. 2007).

## 1.5 **Hydrodynamic principles**

In this chapter, the basic principles of water flow, of sediment entrainment as well as of sediment transport are introduced. The complex interaction between fluid flow and erodible material governs the morphology and the sediment movement within the fluvial system (Knighton 1998). To understand the principles of sediment entrainment and transport in streams as well as in laboratory experiments the first part of this chapter covers the basics of flow in open channels. The second part then introduces to the basics of sediment entrainment and transport. The chapter concludes with the presentation of the actual hydraulic test stand, the annular flume, which was used within the project.

### 1.5.1 **Mechanics of flow**

Three fundamental physical principles govern fluid dynamics (Wendt 2008):

- mass is conserved
- energy is conserved
- Newton's second law: $F = m \times a$

*F: force; m: mass; a: acceleration*

Two principal forces control water flow in an open channel: (1) force of gravity and (2) a resisting force. The driving force of water flow is gravity acting in a downslope direction at an acceleration of g * sin α (gravitational acceleration: g = 9.81 $m*s^{-2}$ and angle of slope: α). The resisting force originates from friction between the flowing water and the bed surface and from friction within the water body which can also be described as molecular viscosity (Knighton 1998). Flow conditions can be classified into various types according to different criteria, which will be described in the next chapters.

## 1.5.2 **Flow classification**

### 1.5.2.1. **Uniform and non-uniform, steady and non-steady flow**

When water discharge in a stream is constant in space and time, but the cross sectional area varies downstream, the equation of continuity (equation 1.1) can be used to describe the relation between discharge, flow velocity and cross sectional area in terms of mass conservation:

$$Q = v_1 \times A_1 = v_2 \times A_2 \qquad \text{(Eq. 1.1)}$$

*Q: discharge; v: velocity of flow; a: cross sectional area*

Discharge *Q* is therefore defined as cross sectional area *a* multiplied by mean flow velocity *v*. Figure 1.2 shows two different types of flows, which can be described as (1) uniform and (2) non-uniform. Uniform flows do not vary in velocity and cross sectional area along stream, whereas non-uniform flows vary in velocity and cross sectional area and are typical for natural flow conditions (Bridge 2003).

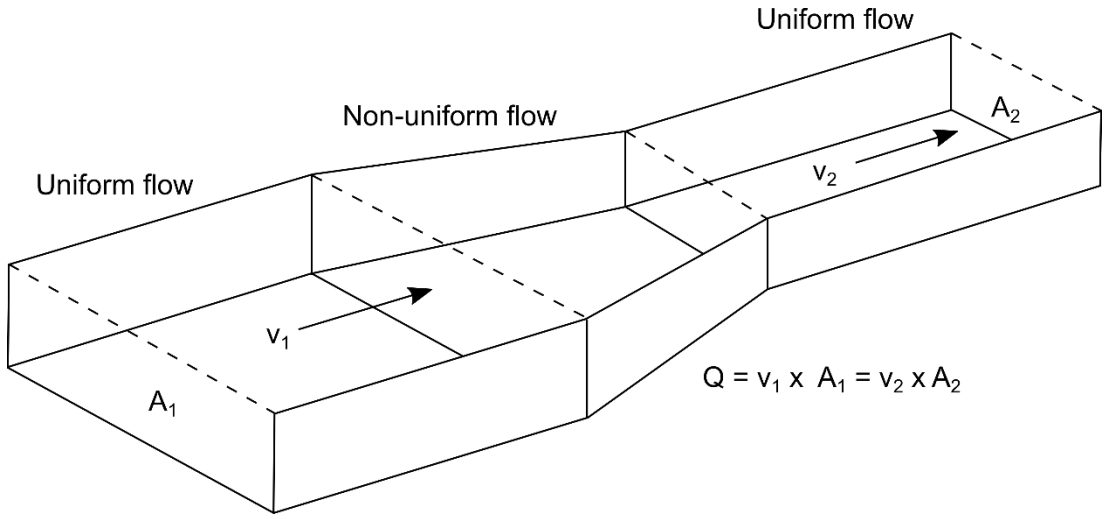


Figure 1.2: Uniform and non-uniform flow (redrawn and modified after Bridge 2003).

Steady flow does not vary in velocity over time, whereas non-steady flow varies with velocity over time. Turbulent flow (see next chapter) is always unsteady by definition.

### 1.5.2.2. **Laminar and turbulent flow**

Water movement can occur as laminar and turbulent flow and depends on different conditions such as flow velocity and water depth or hydraulic radius *R* (Figure 1.3), which is defined as cross sectional area *a* divided by the wetted perimeter *P*.

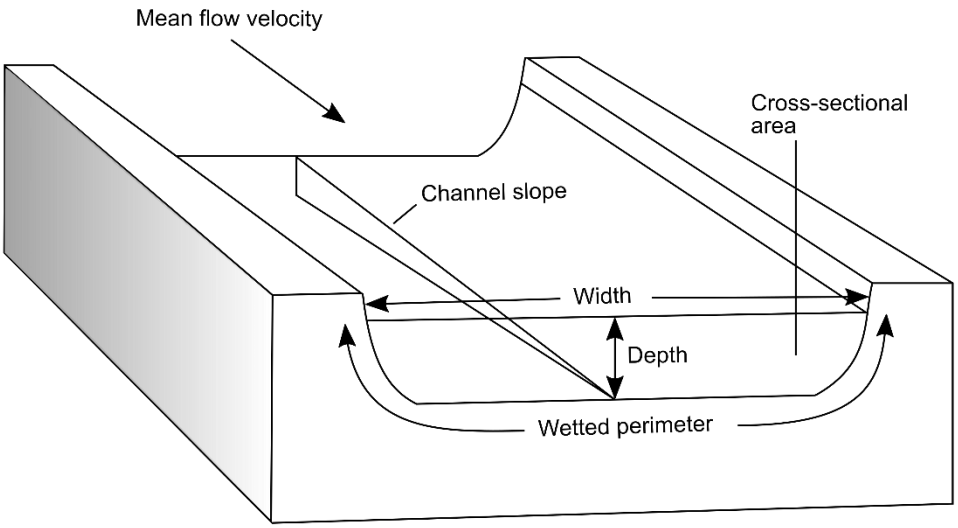


Figure 1.3: Properties of a river cross-section relevant to flow characteristics (redrawn and modified after Summerfield 1991).

The Reynolds number (*Re*, equation 1.2) is used to define those conditions and is basically dimensionless measure of flow rate (Summerfield 1991):

$$Re = \frac{v \times R}{\upsilon} \qquad \text{(Eq. 1.2)}$$

*Re: Reynolds number; v: velocity of flow; R: hydraulic radius (cross-sectional area / wetted perimeter); ν: kinematic viscosity*

At Reynolds numbers < 500 laminar flow occurs in stream channel, between Reynolds numbers of 500 and 2000 components of laminar and turbulent flow exist and above Reynolds numbers of 2000 flow is turbulent. In laminar flow, each fluid element follows a specific path, which can be described as very thin flow “layers”, and does not mix with adjacent flow layers. The layer nearest to the boundary has no forward velocity but each succeeding layer has an increasing forward velocity, which results in a parabolic velocity profile (Figure 1.4).

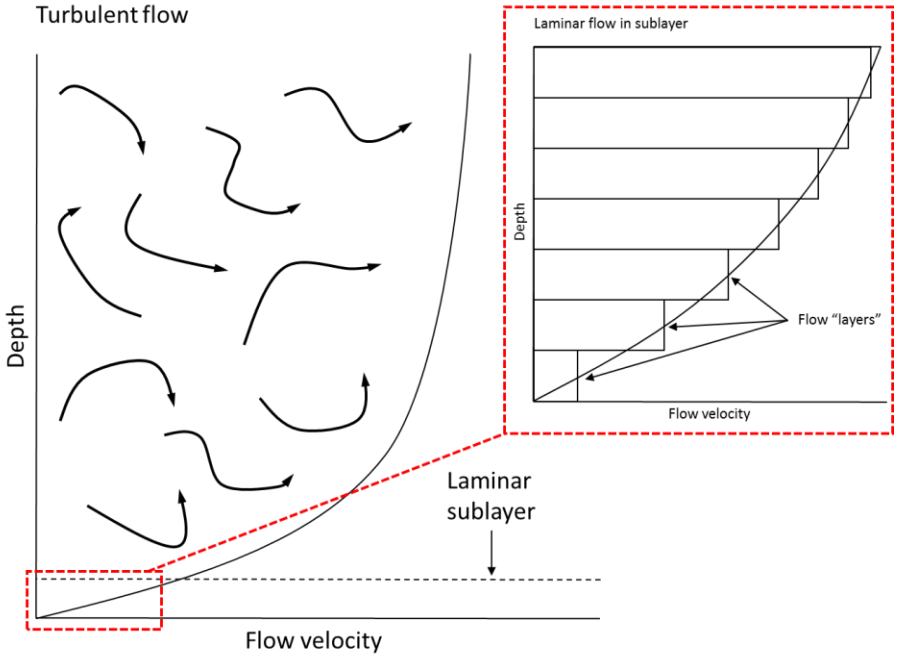


Figure 1.4: Velocity profiles for laminar and turbulent flow conditions (redrawn and modified after Summerfield 1991).

In turbulent flow, which is the predominant type of flow in open channels, the previous parallel streamlines disappear, fluid elements follow irregular paths and a constant exchange in eddies between adjacent layers occurs. Figure 1.4 shows a velocity profile that is common for most open channels: near the bottom, a laminar sublayer exists where the velocity increases in a near linear way. Above the sublayer, flow is turbulent and velocity increases in a logarithmic way.

### 1.5.2.3. **Subcritical and supercritical flow**

In streams, three important different flow regimes can be found: subcritical flow, critical flow and supercritical flow. The ratio of the prevailing mean flow velocity (v) and the propagation velocity of a surface wave defines these flow regimes and can be expressed as the Froude number *F*:

$$F = \frac{v}{\sqrt{g \times d}} \tag{3}$$

*F: Froude number; v: velocity of flow; g acceleration of gravity; d: depth of flow*

According to Summerfield (1991) different discharge conditions can be accomplished by changes in both depth and velocity of flow and therefore result in either a slow and deep, subcritical flow or a rapid and shallow, supercritical flow. Transition between subcritical and

supercritical flow are characterized by a change in velocity of flow and are called either a hydraulic jump (increase in water depth, transition from supercritical to subcritical) or a hydraulic drop (decrease in water depth, transition from subcritical to supercritical). Both transitions often result from sudden changes in bed geometry.

#### 1.5.2.4. **Velocity of flow**

Velocity of flow is a variable and sensitive property of open channel flow because it depends on a variety of other factors and furthermore varies with distance from the streambed, across the stream, along the river and with time (Knighton 1998). Erosion, transportation and deposition processes are strongly influenced by flow velocity. Mean velocity at a cross section is normally used to define the initiation of erosion. On the other hand, flow velocity is strongly influenced by flow resistance, which is a determinant parameter in the interaction between the fluid flow and the channel boundary, and consists of several components such as boundary resistance, channel resistance and free surface resistance (Bathurst 1993). Kington (1998) states that suspended sediment and vegetation might also have a considerable influence.

Empirical equations have been developed to estimate flow velocity based on different parameters such as hydraulic radius, channel slope and different coefficients. Two of the most commonly used equations are the Chezy equation:

$$v = C \times \sqrt{R \times s} \qquad \text{(Eq. 1.4)}$$

*C: Chezy coefficient; v: velocity of flow; R: hydraulic radius; s: channel gradient*

and the Manning-Strickler equation, which is more widely applied (Summerfield 1991):

$$v = \frac{R^{2/3} \times s^{1/2}}{n} \qquad \text{(Eq. 1.5)}$$

n: Manning roughness coefficient = $1/k_{st}$ ($k_{st}$: Strickler coefficient) ; v: velocity of flow; R: hydraulic radius; s: channel gradient

Both the Manning roughness and the Chezy coefficient can be estimated from tables after classifying the stream in question.

### 1.5.3 **Sediment entrainment and transport**

The relationship between gravitational force and resisting force ultimately determines the ability of flowing water to erode and transport particles and therefore the magnitude of sediment erosion and transport (Knighton 1998). In the following sections, principles of sediment entrainment and transport as well as influence of various important parameters such as grain size will be presented shortly.

#### 1.5.3.1. **Sediment entrainment**

When exposed to flowing water, submerged material, e.g. silt, sand or gravel, begins to move depending on its physical properties such as shape, size and arrangement (Knighton 1998). Grain size is the most important parameter to classify submerged material and, besides density, primarily influences its erodibility (Table 1.1). A further distinction can be made between non-cohesive material, e.g. sand and material with larger grain sizes, and cohesive material in the silt and clay range.

Table 1.1: Grain size classification according to Wentworth (1922). Depicted are class names and associated size ranges in mm as well as a classification in non-cohesive and cohesive sediment.

| Class name | Size range [mm] | |
|---|---|---|
| Boulders | ≥ 256 | Non-cohesive |
| Cobbles | 64 – 256 | |
| Gravel | 2 – 64 | |
| Sand | 0.062 – 2 | |
| Silt | 0.004 – 0.062 | Cohesive |
| Clay | ≤ 0.004 | |

For the latter, erosion depends mostly on cohesive bonds between particles rather than on properties such as grain size and density. Those cohesive bonds originate on one part from electro-chemical forces e.g. depending on mineralogy, size and arrangement of the material, and (Summerfield 1991) on the other part on the content of microorganisms, that excrete EPS that leads to an agglutination of sediment particles and influences erosion stability (Gerbersdorf et al. 2009, Gerbersdorf et al. 2008, Le Hir et al. 2007).

The initial beginning of movement of a solid particle in water is called entrainment and takes place when with increasing flow velocity, fluid forces (lift and drag forces, Figure 1.5) acting on this particle exceed resisting forces from immersed weight and constraining effects (Summerfield 1991).

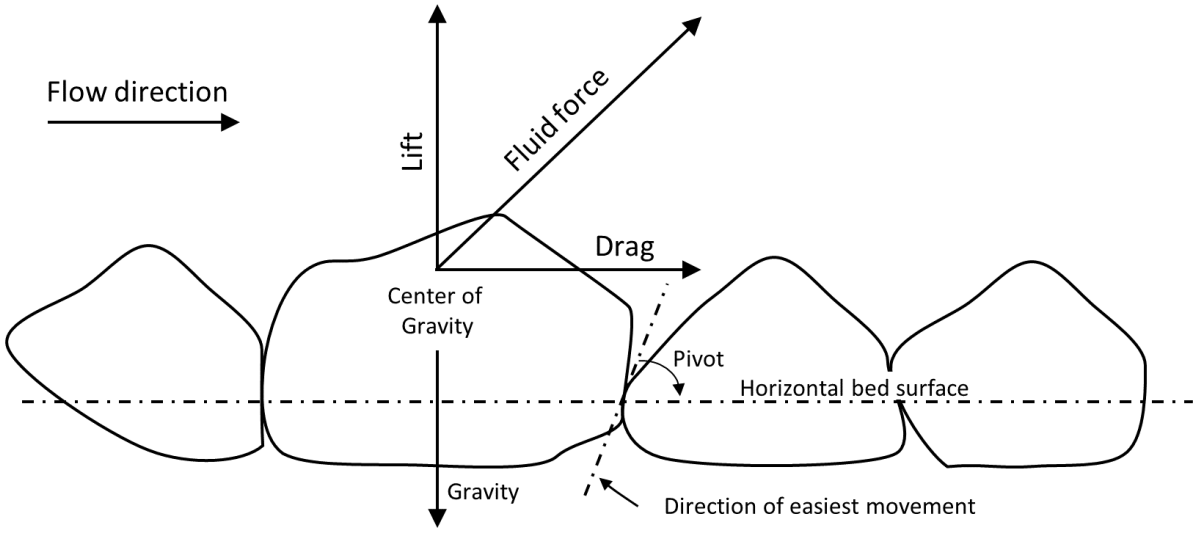


Figure 1.5: Forces acting on sediment particles (redrawn and modified after Summerfield 1991).

There are two important approaches to predict beginning or threshold of sediment entrainment and transport: (1) critical velocity and (2) critical bed shear stress. Both approaches rely on empiric, experimental data and offer diagrams to predict sediment entrainment under various conditions.

The approach after Hjulström (1935) uses mean velocity to define conditions when sediment entrainment of specific particle sizes takes place. Using a Hjulström diagram, entrainment of material with a specific grain size at a specific flow velocity can be predicted. However, it neglects influence of channel roughness and nature of the laminar sublayer or near-bed velocity.

The approach after Shields (1936) uses the bed shear stress necessary to initiate sediment movement to predict entrainment. For sediment particles the mean boundary bed shear stress

$$\tau_0 = \rho_w \times g \times R \times S \tag{6}$$

*$\tau_0$: bed shear stress; $\rho_w$: density of water; g acceleration of gravity; R: hydraulic radius; S: channel slope*

must exceed the critical bed shear stress $\tau_{crit}$ to initiate initial movement of particles. Furthermore, the mean shear stress is at its maximum at the bed and decreases in a linear fashion to reach zero at the water surface.

Shields refines this basic principle and relates a dimensionless critical shear stress (or Shields parameter θ) to a particle Reynolds number Re* and provides an empirical diagram where sediment entrainment of different grain sizes under various fluvial conditions can be read out (Figure 1.6).

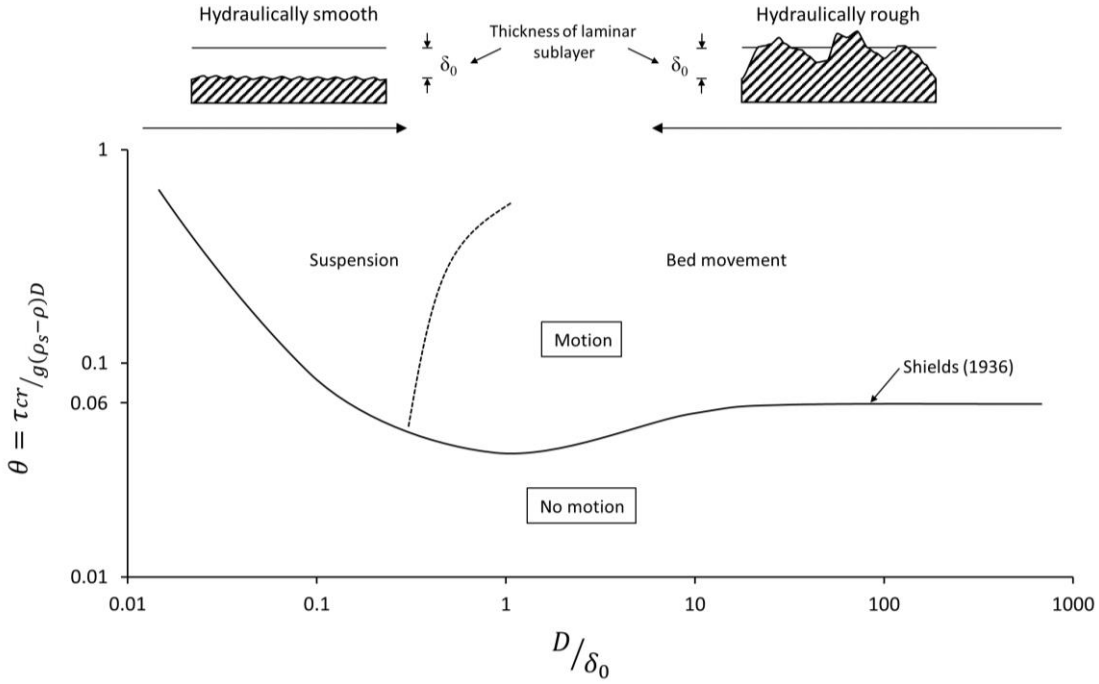


Figure 1.6: Shields diagram (redrawn and modified after Knighton 1998). A dimensionless critical shear stress (or Shields parameter θ) is related to a particle Reynolds number (expressed as $D/\delta_0$). The diagram differentiates conditions when sediment motion happens from conditions when no motion happens.

Previous elucidations generally apply to non-cohesive material, whereas entrainment of cohesive material is more complex and shows different erosion characteristics. In contrast to non-cohesive material, resistance properties of cohesive material depend on the state and history of consolidation (Bridge 2003). Entrainment also takes place when the bed shear stress exceeds critical shear stress but instead of particles of specific grain sizes who start to move, whole surface areas collapse and begin to move.

### 1.5.3.2. **Sediment transport**

In fluvial systems, material can be transported as dissolved load, suspended load and bed load. Dissolved load originates from weathering of bedrock and consists of material transported in solution. Suspended load in e.g. lowland river consists of grains smaller than 0.1 mm, e.g. fine sand, silt and clay, and travels with the flow at approximately the same speed. It is held in suspension by upward-directed fluid stress or fluid turbulence under turbulent flow regime

acting against its tendency to settle on the bed (Summerfield 1991). The finest fraction is called wash load and stays in suspension as long as some flow exists. Settling movement or settling velocity $V_g$ for non-cohesive material under laminar settling conditions can be described with the Stokes' Law of Settling. Cohesive material shows a different settling behavior due to adhesive processes such as flocculation (Droppo et al. 2015). Material with a diameter greater than 0.1 mm (gravel and sand) comprises the bed load and moves downstream by rolling, sliding and saltation (Summerfield 1991) at speeds slower than the surrounding fluid, whereas saltation is the most common form of bed load transport.

The concentration of suspended load can be measured by taking water samples and determining the amount of solid material. When multiplied with discharge data, an estimation of suspended load transport for a specific sampling locating can be made (Summerfield 1991). Furthermore, suspended load can be estimated by different equations. Determination of bed load is much more difficult because sampling devices often interfere with the channel bed and are more complex to operate. However, bed load can be estimated e.g. with the Meyer-Peter and Müller (1948) equation and other bed load equations.

### 1.5.4 **Annular flume**

The erodibility of sediments and the response of organisms to such events (Nowell & Jumars 1987) can be investigated with a variety of laboratory flumes of different sizes and shapes, such as straight open-channel flumes, U-shaped flumes and annular flumes. According to Spork (1997), small-scale laboratory flumes however neglect the influence of flow on sediment grain properties and settling velocity due to the 1-dimensional-nature of feasible investigation. It is hence advisable to use larger-scale instruments that allow to investigate 3-dimensional erosion characteristics under the influence of realistic flow and turbulence conditions (Spork 1997). Large straight open-channel flumes and annular flumes both meet those pre-conditions; however, annular flumes provide several advantages over straight flumes and have long been used for testing erosion and sedimentation properties of sediments and soil (Fukuda & Lick 1980, Maa 1989, Nowell & Jumars 1987, Partheniades et al. 1966, Schweim et al. 2001). Besides other advantages, Spork (1997) lists in his work the following, which also apply to this study:

(1) It is possible to generate an endless flow without pumps, which allows to investigate erosion and sedimentation without breaking down suspended or flocculated sediment and which do not interfere with test animals (Widdows et al. 1998).
(2) Sediment does not settle in unwanted portions of the annular flume (e.g. pipes) as is the case in straight flumes.
(3) Within the annular flume, a steady, endless flow exists. Furthermore, inlet and outlet sections resulting in flow interferences do not exist.

Nevertheless, one major disadvantage exists: due to the circular shape and the rotary motion secondary currents develop (Spork 1997). An adequate and necessary calibration of the flume helps to minimize those currents: the appropriate ratio between opposite rotation of the ring and flume, however, significantly decreases secondary currents (figure 1.7).

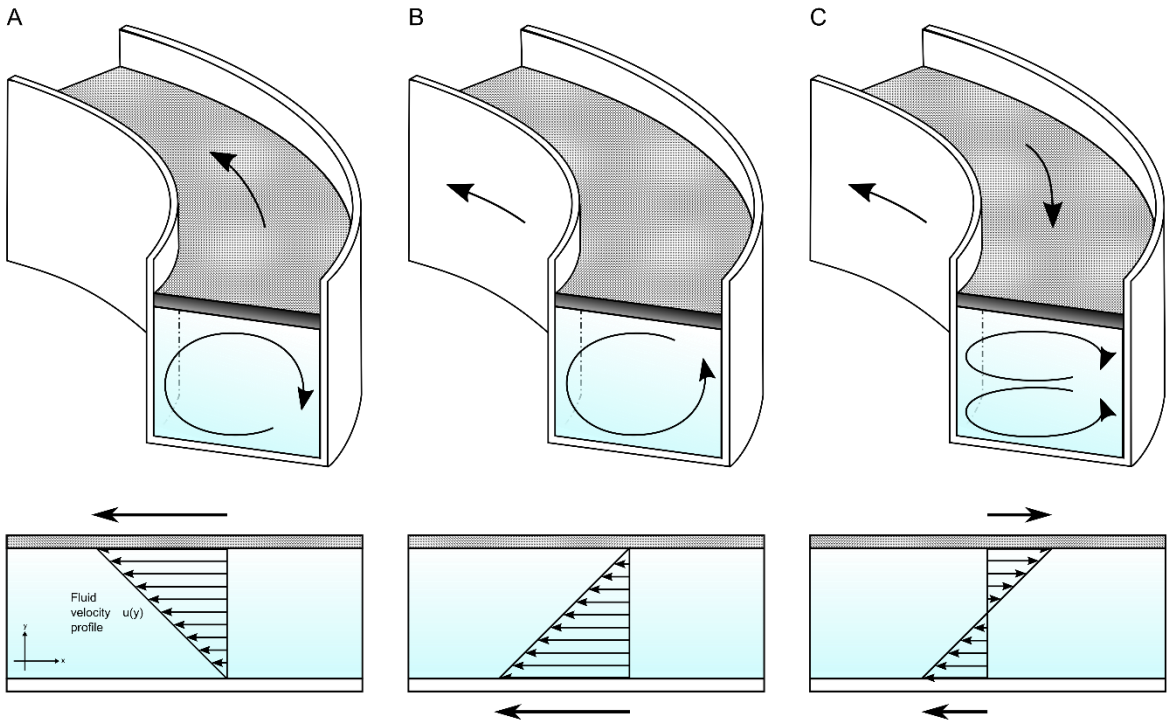


Figure 1.7: Circular section of an annular flume: (A) lid movement in one direction and channel remaining stationary results in a clockwise secondary current, (B) lid remaining stationary and channel moving in one direction results in a counter-clockwise secondary current, (C) opposite rotation of lid and flume results in two secondary currents moving in opposite directions; drawing adopted and modified according to Spork (1997).

Opposite rotation of lid and flume results in minimized secondary currents on the bottom of the flume and creates an endless flow (with an adjustable channel velocity) in tangential direction and can be described as a simplified COUETTE flow (Figure 1.7.C). The resulting bottom shear stress $\tau$ depends mainly on the velocity ratio $\omega_l/\omega_f$ of the planes relative to each other. Further

information and a detailed hydraulic background regarding this topic can be found in Spork (1997).

For hydrotoxicological resuspension experiments within this study, a specific annular flume with a moving circular channel and a moving lid was chosen as instrument (Figure 1.8). The annular flume consists of a circular glass channel (width 0.25 m, radius of circle 1.5 m) and a coaxial acrylic glass lid. Channel and lid rotated in opposite directions producing an endless flow (Cofalla et al. 2012, Spork et al. 1994, Spork et al. 1995). To ensure a homogeneous distribution of the bed shear stress over the channel width at a mean water depth of 325 mm, a speed ratio between lid and flume of $\omega_l/\omega_f$ = -2.0 was used.

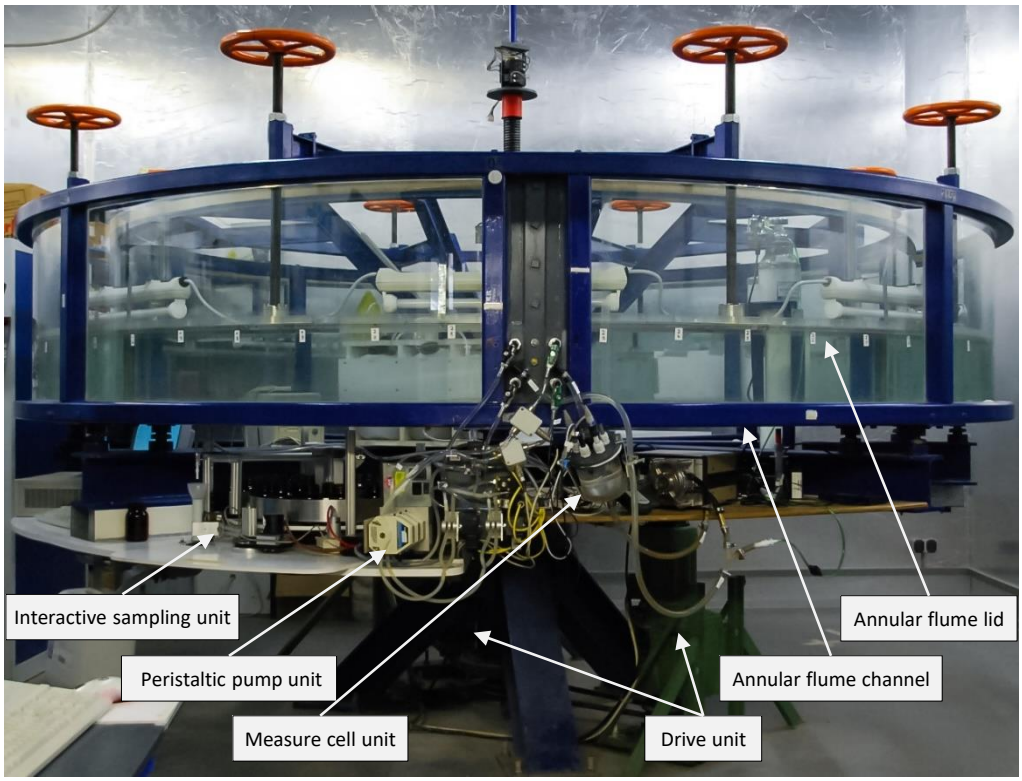


Figure 1.8: Annular flume with instrumentation and sampling mechanisms used for the hydrotoxicological experiments within this study; photo by Catrina Cofalla, adopted and modified accordingly.

To ensure constant conditions, the annular flume is positioned in a climate chamber. Automated sampling mechanisms and measurement equipment allow the sampling of water and sediment as well as to observe a variety of physicochemical parameters (e.g. pH value, $O_2$ saturation and temperature) during experiments.

## 1.6 **PAHs**

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic substances characterized by two or more fused aromatic rings consisting only of carbon and hydrogen, hence carrying neither substituents nor having heteroatoms. With increasing molecular weight, their water solubility and vapor pressure decreases while the boiling point increases (Harvey 1997, table 1.2). Low molecular weight PAHs (LMW PAHs) with less than three fused aromatic rings are considered to be acutely toxic (Sims & Overcash 1983). Due to their slightly higher water solubility and more accessible chemical structure, they are more likely to be degraded by microorganisms than high molecular weight PAHs (HMW PAHs) with four or more fused aromatic rings and a very low water solubility. HMW PAHs are considered to be genotoxic (Nylund et al. 1992, Phillips 1983) and are more recalcitrant to microbial degradation (Haritash & Kaushik 2009, Shuttleworth & Cerniglia 1995).

Table 1.2: Physicochemical properties of selected PAHs (adapted from Sims & Overcash 1983).

| Name | No. of rings | Bp[a] [°C] | Vp[b] [Pa at 25 °C] | Log $K_{ow}$[c] | Sol[d] [mg $l^{-1}$] |
|---|---|---|---|---|---|
| Naphthalene | 2 | 218 | 11 | 3.37 | 30 |
| Phenanthrene | 3 | 340 | $2.0 \times 10^{-2}$ | 4.46 | 1.29 |
| Chrysene | 4 | 448 | $1.1 \times 10^{-5}$ | 5.61 | 0.002 |
| Benzo[*a*]pyrene | 5 | 496 | $2.6 \times 10^{-7}$ | 6.04 | 0.0038 |
| Benzo[*g,h,i*]perylene | 6 | 500 | $6.0 \times 10^{-8}$ | 7.23 | 0.0003 |

*[a] Bp: boiling point, [b] Vp: vapor pressure, [c] log $K_{ow}$: logarithm of the octanol:water partitioning coefficient, [d] Sol: aqueous solubility*

PAHs primary originate from the incomplete combustion of organic materials during human activities, such as industrial processing of coal and crude oil, vehicle traffic, heating and cooking (Lima et al. 2005). However, numerous natural processes like forest or grassland fires, volcanic activity and carbonization during the creation of coal and oil (Blumer & Youngblood 1975, Yamanaka et al. 1999, Zolotov & Shock 2000) contribute to the human emissions. As a result, PAHs are widespread in literally all environmental compartments and the US Environmental Protection Agency (EPA) already described 16 important PAHs early in the 1980´s as organic priority pollutants (Figure 1.9, EPA Method 610 1984) and are now considered a priority class of pollutants by the European Water Framework Directive.

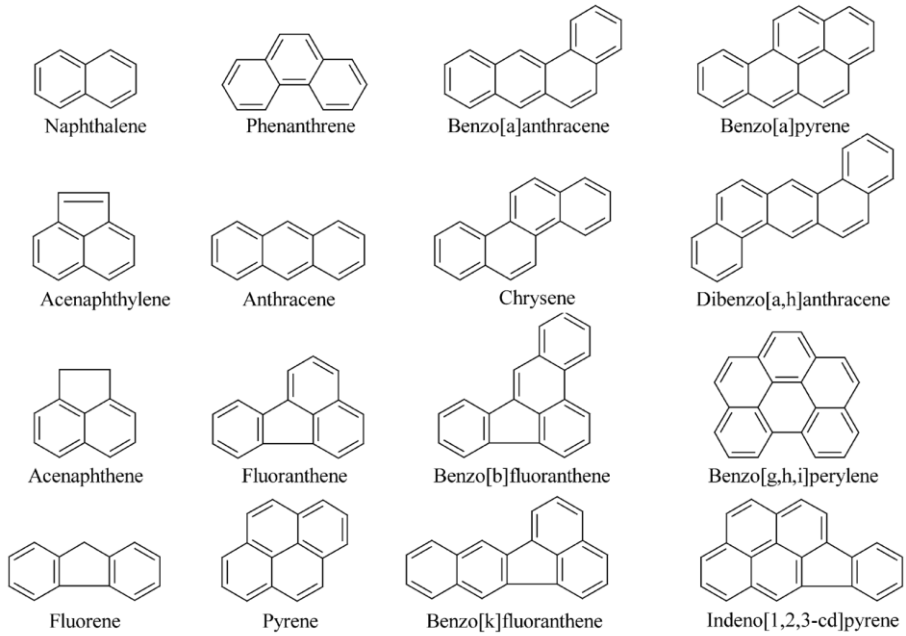


Figure 1.9: Molecular structure of the 16 PAHs described by the US Environmental Protection Agency (from Fähnrich et al. 2003).

Due to their high lipophilicity, PAHs are virtually insoluble in water and adsorb on sediments, as well as on suspended particulate matter (SPM) and soil (Colombo et al. 2006, Delle Site 2001, Karickhoff et al. 1979, Laflamme & Hites 1978, Means et al. 1980). This behavior leads to reduced bioavailability and consequently biodegradation (Scow & Johnson 1997).In consequence, PAHS accumulate in sediments, SPM and soils (Marschner 1999, Wilcke 2000, Yang et al. 2008a). Uptake of PAHs in aquatic organisms such as fish may occur through food or directly through the skin or gills. In contrast to the persistence of PAHs in various environmental compartments, their bioaccumulation in vertebrate aquatic organisms is comparatively low and biotransformation processes cause a quick excretion of PAHs. This leads to a phenomenon called “hit and run” by Payne et al. (2003) and describes the fact that PAHs may cause toxic effects without leaving a chemical signature. Enzymes belonging to a diverse multigene family (CYP1A) mediate toxicity as well as detoxification of PAHs (Billiard et al. 2002, Hankinson 1995) and are induced by the aryl hydrocarbon receptor (AhR). Details regarding the function and the induction pathway can be found in chapter 1.8.1.2. Aquatic organisms with poor biotransformation capabilities such as mussels therefore accumulate PAHs, are less prone to toxic effects and high levels of PAHs were reported for such organisms (Aas et al. 2001). Besides the AhR mediated toxicity, other AhR independent toxicity i.e. the disruption of cardiovascular function and morphogenesis caused by low-molecular-weight PAHs during critical stages of fish development was described by Incardona et al. (2005). In a

later work Incardona et al. (2011) demonstrated, that PAHs of different molecular weight have distinct toxicokinetic pathways in fish embryos and negatively affect to the embryonic cardiovascular system through different toxic mechanisms.

In various studies liver alterations and tumors were linked with environmental PAH occurrence (Baumann & Harshbarger 1995, Mahmood et al. 2014, Malins et al. 1985, Varanasi et al. 1987). Whereas other studies reported critical effects of PAHs exposure in fish with ecological relevance such as delayed growth (Heintz et al. 2000), developmental malformation during early life stages, e.g. skeletal, craniofacial, and eye defects (Colavecchia et al. 2007). Incardona et al. (2015) recently documented reduced survival of juvenile salmon and herring caused by abnormal hearts and reduced cardiorespiratory function, both key determinant of individual survival and population recruitment, after exposure to trace levels of crude oil during embryo life stages. The latter finding further underlines the importance of the long-term toxicity of PAHs present at trace levels for fish populations exposed to e.g. oil spills.

Therefore, the persistence in aquatic systems and the acute as well as long-term toxicity of PAHs, which includes cytotoxicity, mutagenic, carcinogenic and (anti)estrogenic effects (Bispo et al. 1999, Grimmer 1985, Jacob 1996, 2008, Santodonato 1997, Xue & Warshawsky 2005), have adverse toxicological impacts on aquatic and as well as on terrestrial organisms (Bellas et al. 2008, Sverdrup et al. 2002).

## 1.7 **Sampling and Chemical analysis**

In this chapter, the principles of sampling, sample preparation and chemical analysis of environmental samples will be introduced. After a short introduction, the analytical process, including sampling and sample preparation techniques and different analytical methods are presented. The chapter concludes with a short summary of the analytical techniques used within this project.

### 1.7.1 **Analytical approach**

In environmental analysis, the major challenge lies in the complexity of environmental samples, who normally comprise a multitude of xenobiotic and natural substances within different matrices such as water, sediment, soil or biota material. In contrast to ecotoxicological test methods, who provide integrative information on the toxicological effects of complex samples,

chemical analysis always relies on the identification and quantification of single substances or substance groups, e.g. PAHs or PCB. During the last four decades, analytical chemistry has made tremendous improvement regarding both sensitivity and resolution of methods and analytical equipment (Epstein 2003, He & Toh 2006, Holčapek et al. 2012) and currently moves towards a more sustainable green analytical chemistry (Gałuszka et al. 2013) to e.g. reduce the use of toxic reagents. Today limits of detections as low 1 ppt (parts per trillion, e.g. 1 ng/L) are achievable. This offers the opportunity to detect and analyze substances of concern such as pharmaceuticals and hormones, which show adverse effects on aquatic organisms even if present at ultra-low concentrations. Figure 1.10 illustrates a common analytical workflow, which was also followed during chemical studies within the present study.

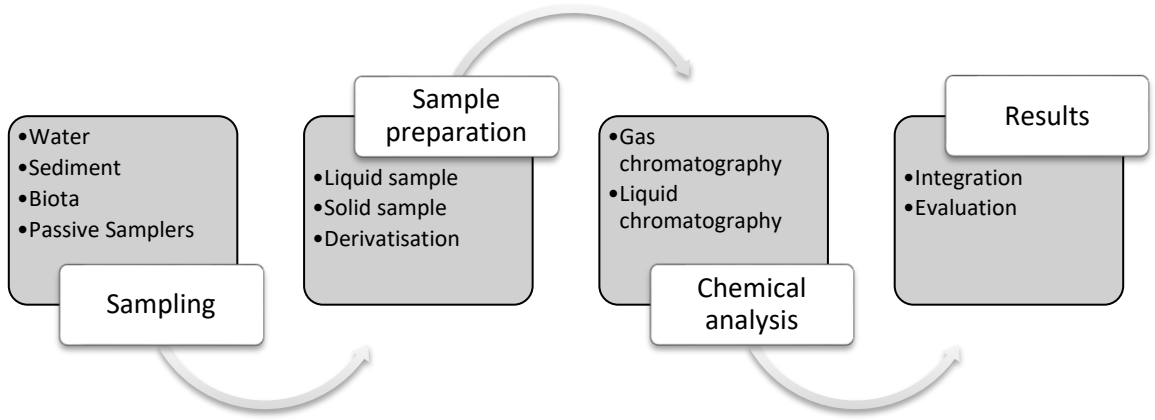


Figure 1.10: Scheme of an analytical process: from sampling to qualitative and quantitative results.

### 1.7.2 **Sampling**

Sampling is, besides conceptual works, the first step in any chemical or ecotoxicological environmental study and should be undertaken with care and preparation as its quality determines the outcome of the whole study. Samples must be representative of the environmental matrix being assessed within a given study to give useful insights to the actual contamination state of the matrix sampled. Depending on the sampling matrix, e.g soil, sediment, water or suspended particulate matter, different sampling techniques can be used to collect samples. These techniques include simple surface sampling with a spade or grab sampling device, e.g. a van Veen grab sampler, as well as core sampling to sample soil or sediment depth profiles. Water can be sampled by immersion of a sample bottle by hand or with

automated systems including pumps, which function as time-integrating samplers and collect consecutive samples to gain a sample that covers a specific time period.

Furthermore, a wide range of passive samplers is used as time integrating sampling devices for sediment, water and air sampling. In contrast to the grab sampling approach presented before, those integrating passive samplers allow to analyze substances present in ultra-low quantities, which would be near or even below the level of quantifications in a conventional grab sample. These passive sampler include e.g. semipermeable membrane devices (SPMD) and polar organic chemical integrated samplers (POCIS). Estimated water or sediment concentrations can be predicted with specific mathematical models that describe the interaction between the analyte, the sampling matrix and the passive sample. In addition, biota such as plants and animals have been more commonly used as environmental samplers in numerous studies. These include e.g. caging studies with fish species and studies with bivalve molluscs (Burton Jr et al. 2005, Gale et al. 1997, Vincze et al. 2015). Often ecotoxicological *in vivo* biomarkers and histological changes are assessed simultaneously in such studies and provide additional information on effects of environmental contaminants on different trophic levels (Vincze et al. 2015).

After the sampling process proper handling and storage of samples to insure, e.g. its original chemical composition, furthermore determines the quality and the representativeness of the analytical results.

### 1.7.3 **Sample preparation**

Before injection of a sample into an analytical device several preparation steps depending on the sample matrix are normally necessary. According to Domini et al. (2005), all sample preparation methods are governed by three principle objectives: (1) sample conditioning, which aims at adapting the physical or chemical state of the sample to the instrumental requirements, e.g. transfer of analytes from solid to liquid matrix; (2) removal of interfering species, i.e. clean up of the sample by separation and extraction techniques; (3) additional operations such as e.g. dilution, concentration or derivatization. Subsequent steps of sample preparation, depending on the type of sample matrix, achieve those objectives. A routine sample preparation protocol includes a drying process for solid sample material, liquid sample extraction methods such as e.g. liquid-liquid extraction (LLE) or solid-phase extraction (SPE), solid sample extraction methods such as e.g. Soxhlet extraction or pressurized liquid extraction (PLE) and finally clean-

up methods and if necessary derivatization methods. A comprehensive overview of those sample preparation techniques can be found in e.g. Domini et al. (2005) and Seiler et al. (2008).

### 1.7.4 **Analytical methods**

Depending on the target compound of interest, different analytical methods are available today, whereas chromatography is one of the most important methods in chemical analysis (Górecki 2005). All chromatographic techniques aim at the separation of analyte components by distributing them between two physical different phases. One of which is a stationary phase while the other, the mobile phase, moves in a definite direction. The stationary phase consists of a solid, a gelatinous or a liquid material, whereas the mobile phase constitutes a fluid such as a liquid (HPLC), a gas (GC) or a supercritical fluid (supercritical-fluid chromatography (SFC), Dominguez et al. 2001, Ettre 1993). In liquid, i.e. HPLC, and gas chromatography, packed and open tubular columns are used as stationary phases whereas different organic solvents and gases are used as mobile phases and are forced at elevated pressure through the columns. A chemical compound, which is subjected to a chromatographic process, partitions between the stationary and the mobile phase depending on its partition coefficient and is separated according to the fraction of time the compound resides in the stationary phase relative to its total transit time through the column (Poster et al. 2006), which is called retention time. Results of chromatographic analysis are presented as chromatograms that plot the intensity of the detector signal as a function of time. Identification and quantification of analytes relies therefore on distinct retention times and furthermore on specific information gained from detector output such as ultra violet spectra or mass spectra derived from analytical standards.

Common detectors coupled to high performance liquid chromatographic instruments are for example ultraviolet/visible light (UV/Vis) absorbance detectors and fluorescence (FLD) detectors, or flame ionization detectors and mass spectrometry (MS) combined with gas chromatography. The most frequent used analytical technique for chemical identification is gas chromatography coupled with mass spectrometry (GC/MS) due to high resolving power and sensitivity. Poster el al. (2006) give a detailed review of the possibilities and limitations of gas chromatography regarding analysis of environmental samples containing PAHs. However, it is also possible to combine liquid chromatography with mass spectrometry (LC-MS). Due to the fact, that LC-MS has become more and more reliable and especially less expensive, significant progress has been made in the last decade applying this technique to a wide range of toxicants including more hydrophilic ones (Bobeldijk et al. 2001, Grung et al. 2007, Petrovic & Barcelo

2006, Pico & Barcelo 2008). Today LC coupled to high resolution mass spectrometry is used as a reliable tool to cope with the analytical challenges new emerging pollutants such as e.g. endocrine disrupting substances and (veterinary) drug residues, which are often present in nanogram to microgram per litre concentration range, pose (Gros et al. 2012, Hernandez et al. 2014, Petrovic et al. 2010). Furthermore, high resolution LC-MS allows the identifications of unknown pollutants and substances of concern (Krauss et al. 2010). For further reading and a comprehensive overview of standard chromatographic techniques as well as detection in chromatography please refer to e.g. Górecki (2005) and Siouffi (2005).

Chromatographic analysis and characterization of PAHs and their metabolites in different environmental compartments have been an important scientific focus for decades (Dijkmans et al. 2014, Grimmer 1985, Poster et al. 2006, Shuttleworth & Cerniglia 1995). This ongoing research comprises major long-term monitoring programs, for example the Arctic Monitoring and Assessment Program (AMAP, Dahle et al. 2006) focusing on PAHs in marine biota and sediment as well as numerous research programs focusing on fresh water species such as the European eel (Nagel et al. 2012) or the European eelpout (Kammann & Gercken 2010). Furthermore, frequent measurements of PAHs for air-quality assessment, in biological tissues for health effect monitoring and in food, e.g. in edible oils and barbecued food, for safety reasons are common practices nowadays (Algarra et al. 2005, Bostrom et al. 2002, Dennis et al. 1983, Dost & Ideli 2012, Kazerouni et al. 2001).

Measurements of PAHs in environmental matrices such as sediments require sophisticated analytical chemical procedures, mostly because of the high complexity of environmental samples (Poster et al. 2006). Typically, sediment or soil samples require drying and extracting by different techniques before chemical analysis can be initiated. Furthermore, sample extracts always contain interfering and target component mixtures consisting of compounds with a wide range of polarities, volatilities, molecular sizes and chemical characteristics (Poster et al. 2006). Specific separation and detection techniques described earlier address this variety. In the identification and quantification of PAHs in environmental samples mainly chromatographic methods, such as high performance liquid chromatography (HPLC), either coupled with ultraviolet-visible (UV/Vis), a fluorescent (FLD) or a mass spectrometric (MS) detector, and gas chromatography coupled with different detectors (de Boer & Law 2003) play a critical role (Poster et al. 2006). The analysis of PAH metabolites in bile fluid is covered in chapter 1.8.2.4, for further readings please refer to Beyer et al. (2010).

## 1.8 **Biotesting**

Four decades ago only chemical chromatographic methods where used to identify contaminants and their metabolites in organisms and environmental samples, however these methods failed to assess the total toxic potential including synergistic and antagonistic effects (Chapman et al. 2002a). Furthermore, they neglected bioavailability of pollutants that may be present in different binding states. To overcome those limitations of solely chemical approaches, in the 80's of the 20$^{th}$ century a variety of biological test systems were developed and standardized and allowed to assess possible toxic effects of different matrices such as sediments, sediment extracts, particulate matter and water samples (Burton 1991). Those biological tests include *in vivo* and in vitro tests at different organization levels, from molecular to organism and even population level. Especially the combination of different test systems into biotest batteries is a most promising approach. This chapter shortly introduces the most important bioassay that have been used during this study. For further reading, please refer to reviews by e.g. Hallare et al. (2011) or Kammann et al. (2012). Wernersson et al. (2015) recently published a highly important technical report, which presents and summarizes e.g. *in vivo* and *in vivo* bioassays, biomarkers and other state of the art of aquatic effect-based monitoring tools. Furthermore, the authors describe how these tools can help EU Member States under the scope of the EU WFD to make monitoring programs more efficient.

### 1.8.1 ***In vitro*** **bioassays**

As described by e.g. Heise et al. (2000) chromatographic chemical analyses such as GC-MS or HPLC neither can determine all compounds present in a given environmental sample nor assess associated adverse effects in various organisms. As a viable alternative a broad spectrum of biological test systems, or bioassays, can be used to evaluate the ecotoxicological hazard of a variety of different sample matrices such as contaminated sediments or soil, liquid samples or individual substances. Bioassays provide integrative parameters for exposure to and effects of substances on the specific biological system based on responses of specific biomarkers.

Furthermore, results of those bioassays investigations are intercomparable when using the same protocol and when performed in laboratories under standardized conditions (Heise et al. 2000). Ecotoxicological experiments determine specific endpoints out of a broad range of possible effects, which requires different approaches. In consequence, a multitude of bioassays is used to address baseline toxicity (Vanleeuwen et al. 1992, Verhaar et al. 1995) or mechanism specific

effects (Ahlf et al. 2002). Popular assays include the Neutral Red retention with RTL-W1 cells for determining acute cytotoxicity (Borenfreund & Puerner 1984), the EROD assay to determine dioxin-like activity (Eichbaum et al. 2013, Schiwy et al. 2015a) and the Ames assay (Reifferscheid et al. 2012) which is used to determine the mutagenic potential of samples. Apart from these bioassays, which are based on molecular mechanisms, other test systems like the embryotoxicity test with *Danio rerio* (Hollert et al. 2003b), the algal growth inhibition test or the *Daphnia* magna acute immobilization test (OECD 2004a, 2011) are examples for bioassays which cover different organizational levels affected by toxic contamination (i.e. cells or whole organisms).

#### 1.8.1.1. **Cytotoxicity – Neutral Red retention assay**

Mixtures of toxicants present in environmental matrices can cause a variety of changes in cell metabolism and structure, which may lead to cell death. This so called baseline toxicity, i.e. non-mechanism specific toxicity, can be assessed with acute cytotoxicity tests, which are widely applied in ecotoxicology (Segner 1998). While several endpoints can be used to determine cytotoxicity , the most common is the neutral red retention assay (Borenfreund & Puerner 1984) with the permanent fish cell line RLT-W1 and allows the determination of the acute cytotoxic potential of samples. However, this assay does not allow identifying the effect pathway or identifying responsible toxicants. Instead, information regarding appropriate dilutions for subsequent bioassays with specific sub-lethal endpoints, such as the EROD assay (see chapter 1.8.1.2) to assess the dioxin-like activity, is provided (Hollert et al. 2002).

After exposure to a sample and a staining step with neutral red followed by a washing step, neutral red uptake and incorporation by viable cells is measured photometrically. Total cell viability i.e. amount of undamaged damaged cells compared to a negative and positive control can directly be associated with neutral red concentration.

Within this study however, cytotoxicity was assessed by determining total protein concentration, which can be related to cell viability or cell growth, during the EROD assay. Whole protein was determined through fluorescamine associated to the amino acids of proteins and forming a fluorescent complex (Brunstrom & Halldin 1998, Kennedy & Jones 1994). Samples that showed cytotoxicity i.e. reduced fluorescamine concentrations during the first EROD replicate were diluted according to the highest inducing dilution of the first replicate.

### 1.8.1.2. **EROD assay**

The dioxin-like potential of sample extracts is often determined as the 7-ethoxyresorufin-*O*-deethylase (EROD) activity (Safe 1986) in the cell line RTL-W1 from the rainbow trout liver (Lee et al. 1993). In this bioassay, the induction of the cytochrome P450 1 A complex (CYP1A) is measured as the specific EROD enzyme complex activity in cultivated cells via degradation of the artificial substrate 7-ethoxyresorufin to resorufin (Behrens et al. 1998, figure 1.11).

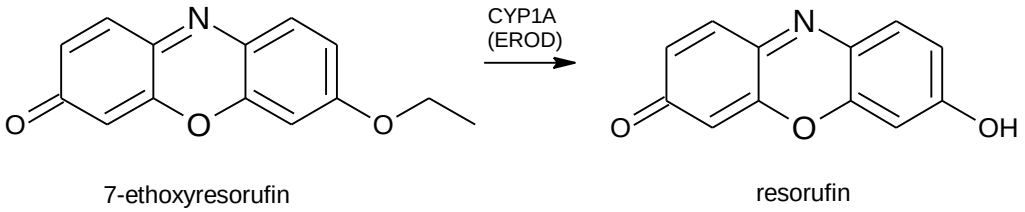


Figure 1.11: Deethylation of 7-ethoxyresorufin to resorufin by the EROD enzyme activity of the CYP1A complex.

The EROD is a phase I enzyme which is part of the cytochrome P450-dependent biotransformation system (CYP1A, Eichbaum et al. 2013, Fent 2007, Hahn et al. 1994). It is induced by dioxin-like acting compounds, which can be characterized as hydrophobic, aromatic compounds with a planar structure fitting the binding sites (Brack & Schirmer 2003, Hilscherova et al. 2000). The induction pathway is mediated by the aryl hydrocarbon receptor (AhR). This ligand-dependent transcription factor binds to specific dioxin response elements on the DNA, thus promoting the expression of CYP1A genes, including that for EROD (Goksoyr & Husoy 1998, Hilscherova et al. 2000, Safe 1995).

These phase I enzymes cover various functions for metabolizing endogenous substances (e.g. steroids and fatty acids) and xenobiotics (Bernhardt 1996, Whyte et al. 2000). Several phase I reactions, such as oxidation, reduction, and hydrolysis are catalyzed by cytochrome P450 1A (CYP1A). To increase the water solubility of a specific xenobiotic molecule and facilitating its elimination small hydrophilic groups are either exposed or added (Andersson & Förlin 1992a) and, in most cases, leads to detoxication of xenobiotics. However, phase I oxidation of some compounds, e.g. of PAHs - such as benzo[*a*]pyrene, can lead to the formation of reactive metabolites. These diol epoxides can bind to DNA, and, thus, exhibit mutagenic effectiveness (Huberman et al. 1976). During phase II metabolism phase I products, e.g. hydroxylated derivates and epoxides, are further transformed into highly water-soluble conjugates such as sulfates and glucuronides to allow facilitated excretion (Beyer et al. 2010, Lech & Vodicnik 1984).

The cytosolic aryl hydrocarbon receptor (AhR) mediates the induction of CYP1A and other biotransformation enzymes (figure 1.12, Pollenz et al. 1996).

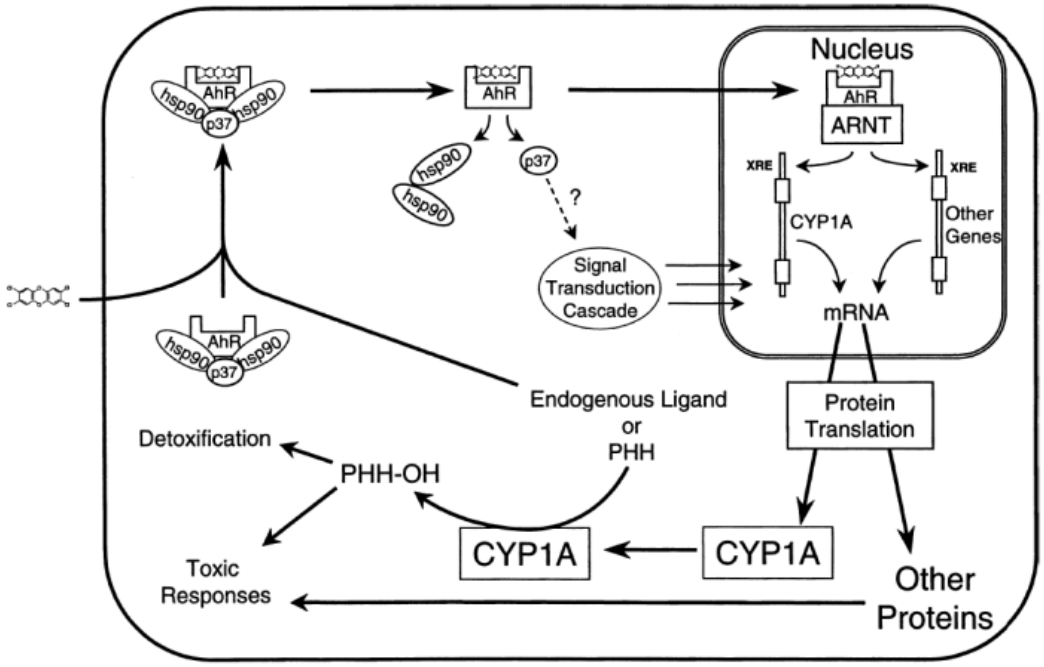


Figure 1.12: Mechanism of AhR-mediated toxicity after binding of dioxin-like ligands to the aryl hydrocarbon receptor. As a result, a transcription factor complex with an aryl hydrocarbon nuclear translocator protein (ARNT) is formed and binds to specific DNA sequences (xenobiotic/dioxin responsive elements (X/DRE)). Binding promotes gene transcription of the Ah gene battery and subsequent translation into several gene products, including the cytochrome P450 1A (CYP1A) subfamily of monooxygenases (from Whyte et al. 2000).

In the cytosole, the AhR exists deactivated and aggregated with specific protein factors (Matsumura 1994, Perdew 1992, Whyte et al. 2000). The binding of a dioxin-like ligand (e.g. a xenobiotic substance) releases these proteins and the AhR combines with the aryl hydrocarbon nuclear translocation protein (ARNT), resulting in a transcription factor complex that can bind to specific DNA regions and is called xenobiotic/dioxin responsive elements (X/DRE). The transcription of various genes (Ah gene battery) is promoted by the binding of the heterodimer, which are subsequently translated into several gene products, including CYP1A and phase II biotransformation enzymes (Nebert et al. 1993b). The close relation of the hazardous potential of PAHs and other dioxin-like xenobiotics to their interaction with the intracellular AhR and the subsequent induction of CYP1A and other biotransformation enzymes are e.g. used in the EROD bioassay to assess their dioxin-like toxicity.

### 1.8.2 *In vivo* biomarkers

In contrast to *in vitro* or laboratory bioassays, where the potential impact of environmental samples, such as sediment extracts are assessed, *in vivo* biomarkers allow to assess effects and exposure of benthic or aquatic organisms to contaminant mixtures in e.g. sediments (Martin-Diaz et al. 2004).

In the mid 90s Van Gestel & Van Brummelen (1996) defined the term biomarker as "any biological response to an environmental chemical at the below-individual level, measured inside an organism or in its products (urine, faeces, hairs, feathers, etc.), indicating a departure from the normal status, that cannot be detected from the intact organism." By doing so, the authors clearly limited the term biomarker to measurements on the biochemical, histological, morphological and physiological level to solely assess the health of an organism and to exclude behavioral effects.

#### 1.8.2.1. Micronucleus assay

Exposure to genotoxic compounds, such as aflatoxins, chlorinated hydrocarbons and PAH metabolites (Al-Sabti & Metcalfe 1995, Bolognesi & Hayashi 2011), may cause irregularities during the mitosis of cells. Chromosomal fractions occur that are not incorporated into the main nucleus resulting finally in the formation of micronuclei in daughter cells (Figure 1.13)

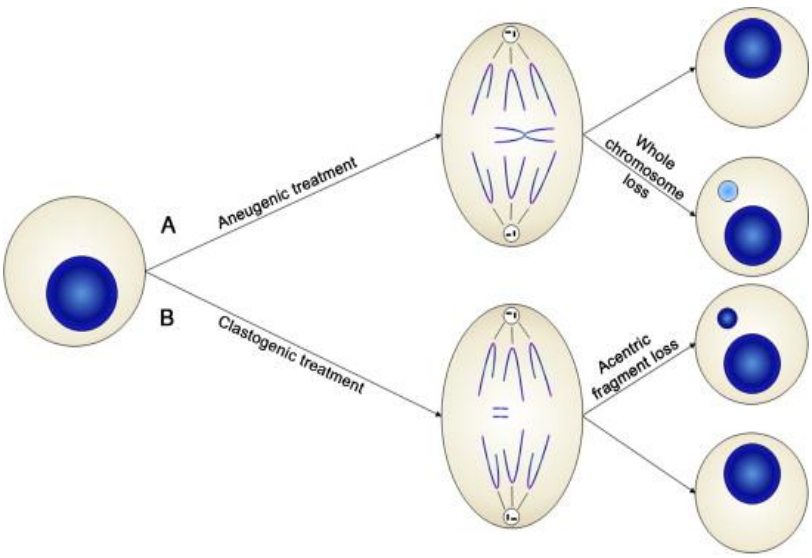


Figure 1.13: Proposed mechanism of micronucleus formation after exposure to genotoxic substances. A: aneugenic substances cause spindle apparatus malfunctions and lead to whole chromosome losses during the anaphase. B: clastogenic substances act as DNA adducts and lead to acentric fragment losses during the anaphase. Both pathways result in cytosolic micronuclei in daughter cells (modified according to Terradas et al. 2010).

The formation of micronuclei has been widely used to assess the genotoxicity of environmental samples in various field studies (e.g. Buschini et al. 2004, de Lemos et al. 2008, Minissi et al. 1996, Rocha et al. 2009) as well as laboratory based experiments (review e.g. Bolognesi & Hayashi 2011). Water samples in *in vivo* studies are most commonly analyzed for genotoxicity using micronuclei in peripheral fish erythrocytes (Al-Sabti & Metcalfe 1995). These cells are highly concentrated in the blood exhibiting a uniform shape and well-defined cell nucleus, so that they are easily identified during analysis. Furthermore, sample preparation is simple. The induction rate is determined after microscopic quantification of stained peripheral blood smears (Hooftman & Deraat 1982, Udroiu 2006).

Induction of micronuclei in different cell types occurs to a limited extent spontaneous, whereas genotoxic substance significantly increase the induction rate. As described by Schmid (1975) and by Al-Sabti & Metcalfe (1995), this increase can be used as an ecotoxicological endpoint for DNA damage on chromosomal level. The chromosomal fractions in micronuclei are lost for further generations, leading to an irreparable genetic damage. The micronucleus assay can therefore be considered as an important bioassay for genotoxic effects (Braunbeck et al. 2009).

#### 1.8.2.2. **Hepatic EROD activity**

The assessment of the hepatic EROD activity in fish is a well-established *in vivo* biomarker of exposure and a highly sensitive indicator of xenobiotic exposure to planar halogenated and polychlorinated hydrocarbons (Stegeman & Hahn 1994, Whyte et al. 2000). The principles of the EROD enzyme functions were already described in detail in chapter 1.8.1.2. In short, after an oxidative deethylation of the artificial substrate 7-Ethoxy-resorufin, the resulting product resorufin is determined via fluorescence detection (Burke & Mayer, 1974). In contrast to the laboratory EROD bioassay with the RTL-W1 fish cell line, where the dioxin-like potential of sample extracts or substances is determined, the measurement of EROD activity in hepatic cells from exposed animals aims at assessing the level of exposure to and uptake of AhR agonists. Elevated *in vivo* EROD activity indicates changes on biochemical level that may lead to adverse effects on higher levels of biological organization. According to Whyte et al. (2000) early life stages of fish are particularly sensitive to AhR ligands, while fish embryos are most sensitive (Cantrell et al. 1996, Schiwy et al. 2015b). Adverse effects include alteration of reproductive parameters in adult fish (Munkittrick et al. 1992), oxidative stress after AhR agonist exposure (Nebert et al. 1993a), imbalance in oxygen homeostasis (Whyte et al. 2000), genotoxicity after formation of reactive PAH metabolites (Varanasi et al. 1987). Further effects comprise

reproductive effects, morphological and histopathological changes, such as kidney lesions and increased liver somatic index, i.e. ratio of liver weight to whole body weight, and mortality (for review see Whyte et al. 2000).

The hepatic EROD activity, which is determined in this study, is a sensitive biomarker to detect the exposition and level of exposure to AhR-antagonists at an early stage of toxicity (Hallare et al. 2011). However, there are a wide range of biotic and abiotic factors, such as hormone and reproduction status, age, or seasonal influences, which may influence EROD activity in either direction (overview Whyte et al. 2000). This has to be kept in mind, when interpreting laboratory and field studies, such as in this project, utilizing this biomarker (Andersson & Förlin 1992a).

#### 1.8.2.3. **Lipid peroxidation**

Oxidative stress in cells is a result of imbalance between the production of reactive oxygen species (ROS) and free radicals and the removal of these species by enzymes such as superoxide dismutase or catalase, which act as a cell oxidative protection system (Gutteridge 1995). Reactive oxygen species in eukaryotic cells originate from processes associated with the energy metabolism. Molecular oxygen acts as the final electron acceptor and is reduced to water by the mitochondrial electron transport chain (Figure 1.14). According to Winston & Di Guilio (1991) various partly reduced ROS are generated during this process, the hydroxyl radical (OH•) being one of the most potent oxidants. Furthermore, xenobiotic-induced malfunctions of the CYP450 enzyme systems by a wide range of environmental contaminants (e.g. metals, biphenyls) may lead to an increased production of free oxygen radicals (van der Oost et al. 1996, Winston & Di Giulio 1991). These highly reactive radicals interact with cell components such as amino acids, lipids and proteins leading to damaged cell structures and function. As described by various authors (Gutteridge 1995, Livingstone 2001, Winston & Di Giulio 1991), free radicals therefore inhibit enzymatic activities, modify DNA sequences and damage membranes.

Various methods can be used as biomarkers to quantify oxidative stress. On the one hand, activities of enzymes involved in oxidative system, such as catalase (CAT) and superoxide dismutase (SOD) can be determined. On the other hand, it is possible to quantify oxidative effects of free radicals directly and to use them as biochemical indication. Van der Oost et al. (2003) describe lipid oxidation and DNA oxidation as suitable indicators.

In this study, lipid peroxidation was used to determine levels/effects of oxidative stress. Lipid peroxidation is a biochemical chain reaction, which is triggered by a free radical reaction with polyunsaturated fatty acids to lipid radicals (Gutteridge 1995). The subsequent processes generate highly reactive degradation products of fatty acids such as malondialdehyd (MDA), which may in turn lead to peroxidative damage of (cell) membranes and organelles and may cause cell death (Esterbauer et al. 1991). The degradation product MDA is most commonly determined photometrically in order to quantify lipid peroxidation (Ohkawa et al. 1979, Valavanidis et al. 2006).

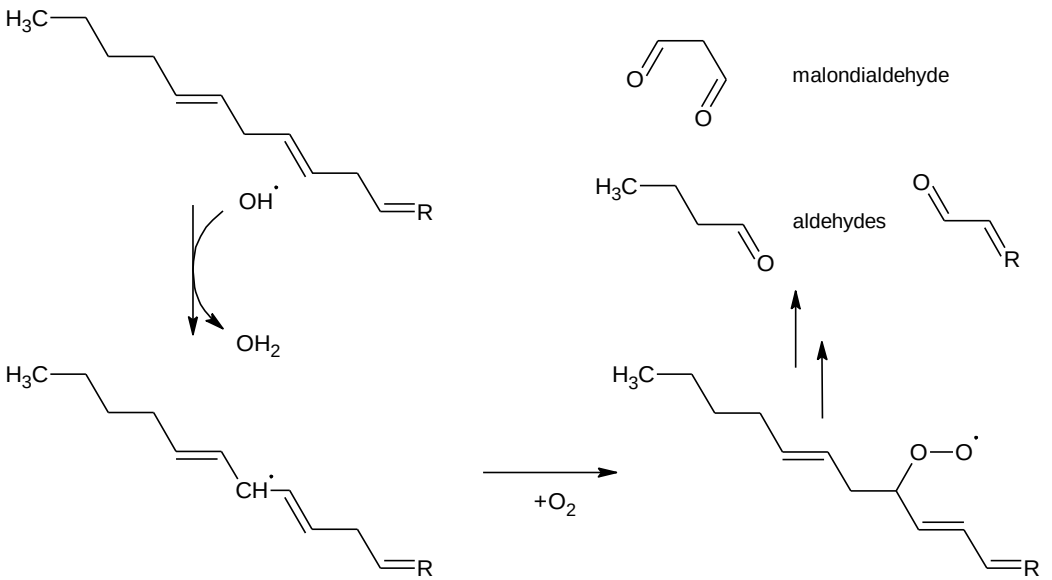


Figure 1.14: The biochemical process of hydroxyl radical-initiated lipid peroxidation. Redrawn and modified from Di Giulio & Hinton (2008) by Brinkmann (2010a).

Carney Almroth et al. (2008) and Brinkmann et al. (2010a) showed a significant correlation between increased reactive oxygen species ROS and MDA levels (also determined in this study) and oxidative damage in rainbow trout exposed to sewage water.

### 1.8.2.4. **Bile metabolites**

With increasing molecular weight, water solubility of PAHs decreases (Harvey 1997) leading to a very low water solubility of more toxic HMW PAHs. In order to be able to excrete PAHs via bile liquid, fish have to convert these xenobiotics into more water-soluble metabolites with various phase I and phase II biotransformation systems (see chapter 1.8.1.2), which are known to be highly effective in those aquatic organisms (Andersson & Förlin 1992b, Leaver et al. 1992). This effective biotransformation leads to low residual concentrations in fish tissue. For example, Budzinski et al. (2004) observed strongly elevated levels of PAH metabolites in soles (*Solea solea*) after an oil spill following an tanker wreckage in 1999, whereas no increase in

PAH levels in tissue of fish could be detected. Therefore and in contrast to other xenobiotics such as PCBs, which bioaccumulate in tissue, direct assessment of PAH bioaccumulation in fish tissue often leads to an underestimation of exposure and is therefore not useful as a biomarker of exposure (Melancon et al. 1992).

As alternative, PAH metabolites in bile fluid are used as an indirect biomarker of exposure to PAHs (Kammann 2007a, Kammann & Gercken 2010, Kammann et al. 2012). The integrated PAH-metabolite concentration in bile fluid after biotransformation reflects the current level of exposure (Aas et al. 2001, Collier & Varanasi 1991, Johnson et al. 2007, Krahn et al. 1984). PAH metabolites are nowadays used in various limnic and marine ecotoxicological studies as standard biomarkers of exposure (Aas et al. 2001, Budzinski et al. 2004, Kammann 2007a, Krahn et al. 1986, Nagel et al. 2012, Tomy et al. 2014).

As early as four decades ago, Lee et al. (1972) described uptake and biotransformation of PAH compounds in fish. According to Lee et al. (1972), PAH metabolism occurs primarily in liver tissue. The major part of the metabolites is excreted with the bile fluid directly from the liver into the bile bladder, whereas urine is the second excretion pathway. Depending on the nutritional condition of the fish, the concentration of PAH metabolites in the bile may vary: During lack of food, bile fluid remains in the bile bladder and is concentrated (Avery et al. 1992, Beyer et al. 1997, Richardson et al. 2004). Directly after food intake, water excretion into the bile bladder strongly dilutes the bile fluid (Klaassen & Watkins 1984). This severe influence of the nutritional condition can be compensated by evaluating the concentration of biliverdine, the green bile dye and a byproduct of heme catabolism. It is assessed and used to normalize PAH metabolite concentration in bile fluid, however, associated benefits and/or disadvantages of this normalization method are still discussed within the scientific community (Avery et al. 1992, Richardson et al. 2004, Kammann 2007).

After extraction of bile fluid from exposed fish (Fig 1.15) and an optional hydrolyzation step to transform conjugated secondary metabolites into hydroxylated primary metabolites different analytical methods can be applied to identify and quantify bile metabolites. According to Ruddock et al. (2003), 1-hydroxypyrene is the main metabolite in fish bile (up to 76% of the sum of PAH metabolites), whereas 1-hydroxyphenanthrene, 1-hydroxychrysene and metabolites of benzo(*a*)pyrene are present in considerably lower concentrations (Kammann 2007a).

A recent review by Beyer et al. (2010) gives a comprehensive summary of analytical methods for the detection and quantification of biliary PAH metabolites: The most common methods, such as fixed or synchronous spectrometry (Figure 1.15) or quantitative chromatographic analysis with fluorescence detection, rely on the fluorescent nature of aromatic hydrocarbons and the characteristic fluorescence emission of different PAHs can be used to identify specific compounds. Other techniques include GC-MS (Fernandes et al. 2008), Shpol'skii spectroscopy (Ariese et al. 1993) and Supercritical fluid chromatography (Moyano et al. 1997).

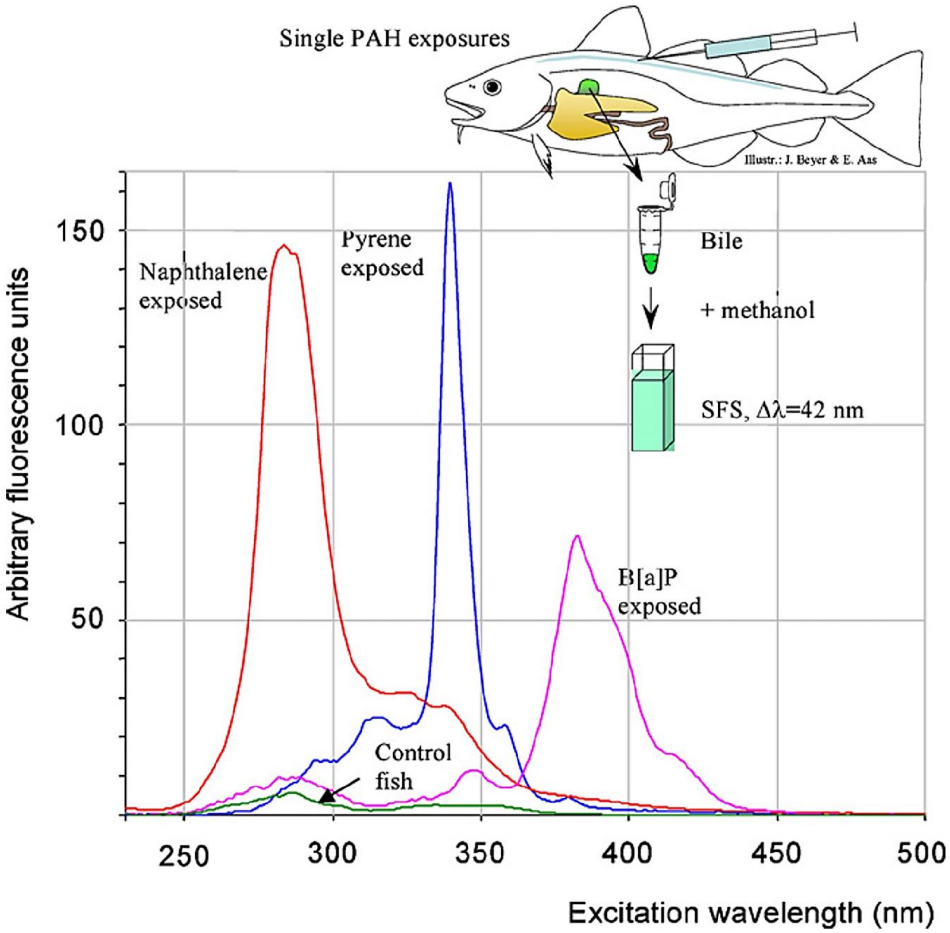


Figure 1.15: Chromatogram obtained after synchronous fluorescence spectrometry of non-hydrolyzed bile from fish after different PAH injection experiments and unexposed control. Image from Beyer et al. (2010).

In this study, PAH metabolite concentration in fish bile assessed with HPLC-FLD (high-performance liquid chromatography with fluorescence detection) is used as a biomarker of exposure to assess total exposure to PAH contaminated sediment during remobilization events.

## 1.9 **Aims and objectives**

In this chapter, the aims and objectives of this study will be introduced. After presenting the superordinate aims of the whole study, the individual goals of the sub studies involved are detailed.

### 1.9.1 **Superior aims of the thesis**

The present PhD thesis aims at the evaluation of the hydrotoxicological approach with a focus on the ecotoxicological aspects without neglecting the engineering side. It evaluates the approach at its different states of development consisting of (1) the proof-of-concept study that intended to establish a new experimental setup and methodology assessing potential effects of contaminated sediment upon resuspension, (2) preliminary investigations regarding the influence of temperature as an additional stressor during resuspension events and (3) the application of the hydrotoxicological approach under more natural conditions with a refined test stand and an updated ecotoxicological test setup.

### 1.9.2 **Research objectives**

The PhD thesis was designed with three research objectives in mind. The first objective was the interdisciplinary development of a combined hydraulic and toxicological approach to assess resuspended contaminated sediments during simulated flood events, whereas the focus of this PhD thesis lay on the development and implementation of a suitable biomarker battery i.e. on the toxicological side of method development. This was realized during the proof-of-concept study under simplified conditions by using formulated, e.g. not natural, sediment. The second objective was the assessment of a potential influence of temperature stress that may become relevant during the ongoing climate change and was realized with a specifically designed, separate test stand. The third objective was the evaluation of the approach developed during the proof-of-concept study under natural conditions, e.g. with natural sediment from different streams, with a refined test stand.

*Objective I: A proof-of-concept study*

The proof-of-concept study consisted of two parts: (1) a multiple biomarker approach to assess the impact of resuspended sediment on an aquatic model organism during a simulated flood

event (Brinkmann et al. 2010a), and (2) sedimentological and hydrodynamic investigations of a simulated flood event in an annular flume (Cofalla et al. 2012).

**Chapter 3** covers the first part of proof-of-concept study where a multiple biomarker approach was used to investigate exposure to organic pollutants remobilized during a simulated flood event. The objective was to establish a suitable test system for direct exposure of rainbow trout (*Oncorhynchus mykiss*) to simulated flood-like conditions in the laboratory and assess possible adverse effects of particle-bound pollutants after resuspension. Thus, methodologies of ecotoxicology and hydraulic engineering were combined by exposing rainbow trout to artificial sediment spiked with a mixture of PAHs during a 5 d simulated flood event. To determine suitable endpoints for further studies, of the suitability of a set of biomarkers at different levels of biological organization to demonstrate either exposure to or effects of sediment-bound pollutants was assessed. Furthermore, the suitability of rainbow trout as a test species for investigations in the annular flume was evaluated.

**Chapter 4** covers the second part of the proof-of-concept study, which focused on a number of instrumental, hydraulic and chemical-analytical objectives. Those aimed at (1) providing and an adequate and stable environment for test animals (2) simulating and investigating a flood event in an annular flume (3) modifying the preparation and spiking process of a OECD sediment guideline for large scale experiments and (4) evaluating the effectiveness of spiking artificial sediment with selected PAHs by means of chemical-analytical and bioassay driven analysis.

*Objective II: preliminary investigations and influence of temperature stress*

**Chapter 5** covers the preliminary investigations regarding e.g. the influence of temperature stress on the toxicology of PAHs in rainbow trout and aimed at investigating the influence changes in temperature regime have on biomarker cascades during exposure to particle-bound PAHs. In a specifically designed, separate test stand, experiments were conducted to elucidate the time- and temperature-dependency of the toxicological properties of resuspended sediments on rainbow trout which were exposed to suspensions of sediments. In contrast to the proof-of-concept approach described in chapter 3 and 4, natural sediments, either without treatment or spiked with a mixture of PAHs representative of those occurring in sediments of German rivers. To investigate the influence of temperature on uptake and effects of pollutants bound to particles during a climate change scenario, the experiment was conducted at two average

temperatures, 12 °C or 24 °C. Concentrations of PAHs in suspended particulate matter (SPM), as well as uptake and biotransformation, as determined by concentrations of PAH metabolites in bile, were quantified. Several functional responses of rainbow trout that occurred during resuspension of pollutants bound to particulates were also measured.

*Objective III: Evaluation of the hydrotoxicological approach with natural sediments*

**Chapter 6** covers the evaluation of the hydrotoxicological approach under natural conditions, e.g. with natural sediments from different streams, with a refined test stand. Effects of resuspended sediments on rainbow trout in semi-natural environments under changing bed shear stress regimes in an annular flume were elucidated. The annular flume was used to generate rising bed shear stress levels resulting in increasing concentrations of suspended particulate matter originating from the respective sediment bed. Sediments from two different German rivers with different pollution characteristics were and concentrations of PAHs in suspended particulate matter and sediments, as well as uptake and metabolism, as determined by concentrations of PAH metabolites in bile, were quantified. Furthermore, a set of different biomarkers in rainbow trout, which had proven in objective II to give reliable answers on functional changes during resuspension of particle-associated pollutants, was measured.

**Chapter 7** finally demonstrates the limitations as well as the logical progression of this approach in a concluding chapter. This includes future test stand modifications and optimizations, the use of more sophisticated passive sampling methods to further reduce the number of test animals and a desirable validation of laboratory studies with specifically designed field studies as well as implications of the outcome of the Floodsearch projects for integrated interdisciplinary future flood risk.

Chapter 2

# The Floodsearch Idea: Concept Overview

Parts of this chapter have been previously published in the following peer-reviewed articles:

Wölz J., Cofalla C., **Hudjetz S.**, Roger S., Brinkmann M., Schmidt B., Schäffer A., Kammann U., Lennartz G., Hecker M., Schüttrumpf H., Hollert H. (2009b): In search for the ecological and toxicological relevance of sediment re-mobilisation and transport during flood events. *Journal of Soils and Sediments* 9, 1-5

## 2.1 **Concept overview**

This chapter presents the Floodsearch concept idea and shortly the two underlying scientific research projects that this thesis is based on. The first part specifies the Floodsearch concept idea. In the second part of the chapter, the general background of the projects is presented. In the third and fourth part, the aims of the proof-of-concept project "Floodsearch I" and the follow-up project "Floodsearch II" are described.

## 2.2 **The Floodsearch concept**

The Floodsearch concept is based on the idea of combining the traditionally separated disciplines ecotoxicology and hydraulic engineering to commonly investigate remobilization processes of particulate bound contaminants in a new interdisciplinary approach and to assess the associated risks for aquatic organisms and ecosystems. The long-term objective is the development of a standardized test setup for contaminated sediments and ultimately a decision support tool for stakeholders.

In response to increasing concerns about the potential toxicological impacts of (extreme) flood events, scientists from several disciplines joined to develop a new research approach and initiated an interdisciplinary research project named Floodsearch. This new research approach aims to assess the risks associated with the remobilization of particulate bound contaminants often observed after severe flood events. Impacts of extreme flood events and aspects of remobilization of sediment-bound toxic compounds are characterized and evaluated in controlled experiments fusing flood simulation technologies with the assessment of biological effects. The overall goal is to establish a novel and more realistic approach of flood event testing, which can be applied to a number of different questions and species. This interdisciplinary methodology was entitled hydrotoxicological approach (see chapter 1.3.2, Cofalla 2015). Ultimately, the hydrotoxicological approach will assist to further our understanding of the potential biological risks associated with increasingly frequent extreme flood events, e.g. as a consequence of climate change, by bridging the gap between the physical (re-)mobilization of contaminants and resulting toxicological impacts on aquatic organisms.

As a first step, a proof-of-concept project was initiated to develop and assess the novel interdisciplinary approach (Floodsearch I, Figure 2.1). As a second step, a follow-up project was then initiated to refine the approach and transfer the knowledge gained in the first project

from artificial conditions to more natural conditions (Floodsearch II, Figure 2.1). Furthermore, an additional investigation was initiated to specifically assess temperature influence as a consequence of climate change on the uptake of contaminants from resuspended sediments under static conditions.

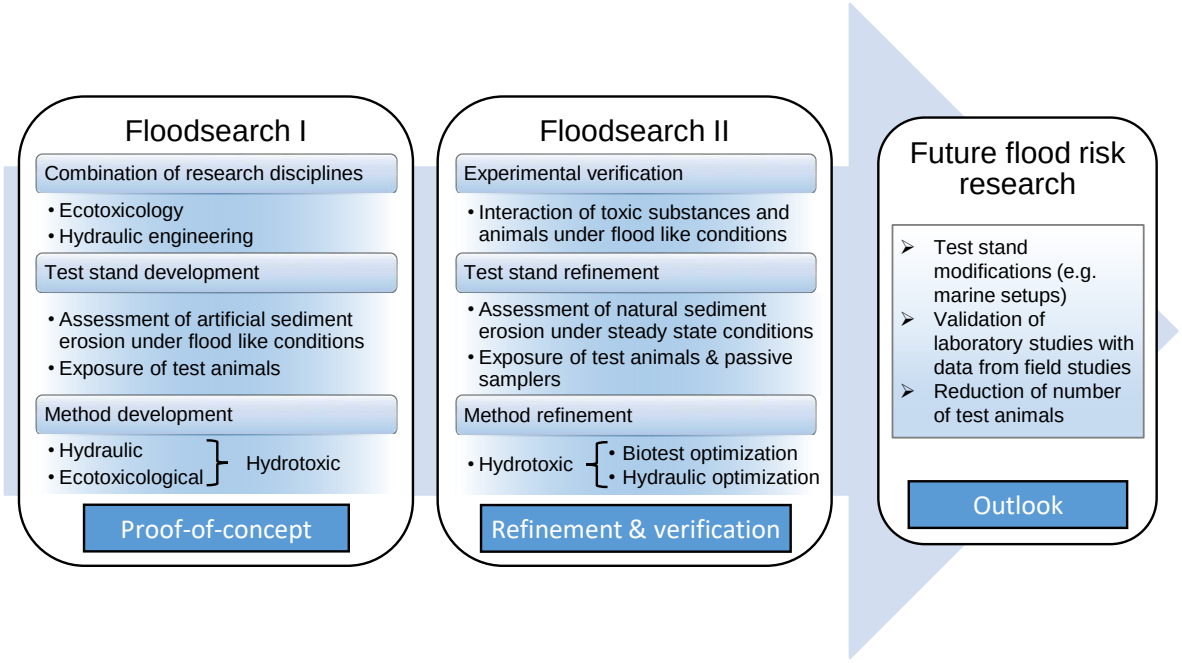


Figure 2.1: The Floodsearch concept consists of a proof-of-concept study and a follow-up study that refined and verified the method developed during the preceding study.

The future development of the Floodsearch concept (Figure 2.1), i.e. the hydrotoxicological approach, including future test stand modifications and optimizations, use of more sophisticated passive sampling methods to further reduce the number of test animals and a desirable validation of laboratory studies with specifically designed field studies as well as implication of the outcome of the Floodsearch projects for integrated interdisciplinary future flood risk research are discussed in chapter 7.

## 2.3 **Background**

There is general agreement that sediment-bound substances are of major importance for the fate and effects of trace contaminants as well as water quality in aquatic systems. Sediments can act as sinks for various pollutants but could also become a contamination source under certain circumstances such as dredging or flood events that result in remobilization of particulates (Ahlf et al. 2002, Gerbersdorf et al. 2007, Hollert et al. 2000, Hollert et al. 2003a, Netzband et al. 2002). For a long time, however, water quality assessment as defined by the EU water

framework directive (WFD) neglected the role of sediments as a secondary source of pollutants (Heise & Foerstner 2006). A decade ago, the draft of a daughter directive (COM(2006)397/F1) of the EU-WFD, which aimed at regulating sediment monitoring issues for member states, the EU-WFD has acknowledged the relevance of sediments. Article 2(2) lists ascertained threshold concentrations of 33 priority pollutants (Annex I, A and B), which have to be met for sediments as well as biota (Förstner 2007). Recently, this daughter directive and the WFD were further amended (Directive 2013/39/EU) and the European commission supplemented this list of 33 priority pollutants by further 12 substances (see chapter 1.4).

With a delay of over one decade after the first geochemical studies have been conducted to assess the contribution of sediments to water quality, the assessment of biological effects of particle-bound pollutants has become a major topic in international water research (Burton 1991, Giesy & Hoke 1989, Keiter et al. 2006, Power & Chapman 1992). To date, most sediment assessment studies have focused on the development of suitable bio-analytical methods and the evaluation of their potential to characterize sediment-bound contaminants. In contrast, the role of sediment (re-)mobilization in floods and possible ecotoxicological effects of contaminants bound to suspended material on aquatic organisms has scarcely been investigated (for review see Hollert et al. 2007).

One of the major concerns with regard to the remobilization of sediments is the occurrence of increasingly frequent and strong flood events that are currently discussed as one of the potential consequences of global climate change. The past decade has witnessed an increasing number of extreme weather events around the globe resulting in 500-year floods such as occurred in 2002 in the River Elbe, Germany (Schüttrumpf & Bachmann 2008) and events of remobilization of highly contaminated old sediments. Given the increasing likelihood of such extreme events, there is a rising necessity for the development of scientific approaches for the assessment of regularly flooded rivers to aid in understanding and predicting their possible toxicological and ecotoxicological consequences. Specifically, the fusion of hydrodynamic research and modeling with ecotoxicological investigations is evolving into an emerging field of research. Recently, it has been shown that hydrodynamic aspects can be included as an additional line-of-evidence in weight-of-evidence studies assessing the impact of sediments (Chapman & Hollert 2006). During recent years, various studies elucidated the potential ecotoxicological risk of particulate matter remobilized during flood events using acute toxicity assays (Maier et al. 2006, Oetken et al. 2005, Westrich & Förstner 2007), cell-based methods

(Brack et al. 2007a, Hollert et al. 2000, Hollert et al. 2003a) as well as mechanism-specific assays (e.g. dioxin-like potencies and mutagenicity; (Brack et al. 2007a, Hollert et al. 2003a, Rao et al. 1990, Wölz et al. 2008). However, all of these studies were conducted in the laboratory and/or using organic extracts as worst case scenarios and surrogates for the hazard potential of these SPM. It remains still unclear what the true environmental impact of the short "pulse" exposure, which is characteristic for flood events, on aquatic species to particle-bound toxic compounds is.

Some studies suggested potential adverse effects of flood-related SPM to aquatic organisms (Schulze et al. 2015, Wölz et al. 2008). Furtheremore, 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 data from long-term studies conducted at the same sampling sites, a significant impairment of the function of cell organelles (lysosomes), which are involved in the detoxification and elimination of pollutants in the fish liver, was found. In addition, a long term study investigating trends in 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 remobilization of dioxin-like pollutants during the River Elbe flood event (Hollert et al. 2007).

## 2.4 **Floodsearch I**

The proof-of-concept project Floodsearch I experimentally linked hydrodynamic questions with ecotoxicology to assess the ecological relevance of resuspension events to aquatic organisms (Wölz et al. 2009b).

### *Aims of the Joint Research Project Floodsearch I*

Floodsearch I aimed to combine traditionally separated disciplines utilized in water research in order to derive a realistic assessment of the risks associated with the erosion of old sediment layers, the transport of these sediments in the water column and the bioavailability and hazard potential of contaminants contained in these sediments to aquatic organisms. The combination of hydrodynamic and ecotoxicological technologies in this proof-of-concept approach aimed at providing (i) more detailed information on the erosion and transport mechanisms of polluted

sediments, (ii) first insights into the effects of flood events on biota under natural conditions, and (iii) establishing a novel methodology for the evaluation of the impact of sediment remobilization on water quality that are in accordance with challenges posed to scientists by global climate change and the resulting increasing likelihood of more severe flood events. Specifically, it aimed to characterize the remobilization of contaminated sediments and the resulting toxicity to aquatic organisms such as fish.

Thus the primary objectives of Floodsearch I were defined as follows:

- Characterization of remobilized particle-bound contaminants in the water layer under simulated extreme flood conditions in an annular flume.
- Assessment of the toxicological relevance of these resuspended contaminants using a combination of *in vitro* and *in vivo* test systems. In brief, an array of different molecular, biochemical and histological endpoints were evaluated on their applicability. These included the induction of metabolic pathways (e.g. CYP1A1 or formation of PAH metabolites in the bile), genotoxicity (e.g. micronuclei formation) in blood cells as biomarkers for *in situ*-mutagenicity), alteration of enzyme activities such as activity of glutathione-S-transferase (GST) and activity of catalase (CAT) and determination of lipid peroxide concentrations in liver as a measure for oxidative damage. Furthermore immunoblot analyses of CYP1A protein content and real-time RT-PCR measurements of hepatic gene expression were performed.
- Characterization of sediment resistance towards shear stress on surfaces in the context of extreme flood events as well as the characterization and evaluation of sediment transport by analyzing aspects and parameters of erosion and remobilization (Schweim et al. 2001). The annular flume was deemed particularly suitable for sediment research because there is no disturbance of the investigated processes and phenomena by water circulation and pumps. In fact, the flow field is well-known, the flow continues indefinitely, and boundary conditions are manageable.

## 2.5 **Floodsearch II**

As a sequel to the successful proof-of-concept project Floodsearch I, the project Floodsearch II aimed at widening the profoundness and complexity of the sedimentological, ecotoxicological and hydraulic investigations as well as refining the novel research methodology, the

hydrotoxicological approach, by proofing the interaction between bound contaminants and exposed test animals.

The proof-of-concept project was successful in establishing a new interdisciplinary experimental research methodology, which allows to combine traditionally separated disciplines, ecotoxicology and hydraulic engineering, to commonly investigate remobilization processes of particulate bound contaminants. However, the Floodsearch I study was designed as a proof-of-concept study with clear inherent limitations such as the use of artificial sediment and a limited instrumentation of the test stand. Furthermore, a multitude of optimization concepts concerning both, the test stand including the hydraulic and sedimentological methods as well as the ecotoxicological methods, arose during the study.

Therefore, the subsequent project Floodsearch II was initialized, on the one hand, to refine the test conditions to be more natural with regard to temperature, pH and the nature of sediments and, on the other hand, to select and further refine ecotoxicological biomarker methods that proved reliable and showed promising results during the proof-of-concept study. In order to be used as a standardized test procedure, the hydrotoxicological test design was therefore refined both regarding the hydraulic and the ecotoxicological methods. This effort aimed to verify and to deepen the understanding of interactions between sediment erosion, sediment bound particles and test animals and ultimately provide a decision support tool for stakeholders.

### *Aims of the Joint Research Project Floodsearch II*

As a central research question, the experimental verification of an interaction of toxic substances (PAHs) from both spiked and unspiked natural sediment suspensions with exposed animals under different environmental conditions (pH and temperature) under flood like conditions was defined. In contrast to the approach used in the preceding Floodsearch I proof-of-concept project, a stepwise increase of the bed shear stress was chosen over a DIN hydrograph. This allowed studying the erosional behavior during steady states for defined bed shear stresses.

In addition to the biomarker battery that proofed to deliver reliable results during the method development in the proof-of-concept study (see chapter 3) different new methods to assess contaminant behavior and interactions with test animals were assessed as well as existing methods were improved. These included the analysis of metallothionein concentration in fish liver as biomarker of exposure to metals with a newly established high-performance liquid

chromatography with fluorescence detection (HPLC-FLD) method and passive sampling techniques with silicone passive samplers. The main goal of this new sampling approach within the experimental design was to realize a reliable concept to assess the amount of freely available PAHs to aquatic animals, which would allow a reduction of animal testing during future projects. 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.

Chapter 3

# A combined hydraulic and toxicological approach to assess resuspended sediments during simulated flood events – Part I: multiple biomarkers in rainbow trout

# A combined hydraulic and toxicological approach to assess resuspended sediments during simulated flood events – Part I: multiple biomarkers in rainbow trout

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## 3.1 **Abstract**

**Purpose** One of the central issues related to global changes in weather is the increasing occurrence of flood events that can result in the resuspension of contaminated sediments in rivers. Here we report on a proof-of-concept study combining hydraulic engineering and ecotoxicology in a new interdisciplinary approach to assess the toxicity of re suspended polluted sediments after a simulated flood event.

**Materials and methods** Rainbow trout (*Oncorhynchus mykiss*) were exposed for 5 days under simulated flood conditions in an annular flume with artificial sediments that were spiked with a mixture of polycyclic aromatic hydrocarbons (PAH) at environmentally relevant concentrations. Specifically, the objective of this study was to bridge the gap between the physical resuspension of pollutants and resulting toxicological impacts on aquatic organisms. A suite of different molecular, biochemical and histological markers was used to verify the hypothesis that resuspension of sediments can lead to remobilization of PAHs and subsequently to effects in aquatic organisms.

**Results and discussion** The micronucleus frequency was significantly 4.3-fold elevated after exposure. There was no significant indication of Aryl hydrocarbon receptor signaling (no EROD induction or increased CYP1A protein content, only slight induction of CYP1A gene expression). Biliary metabolite concentration was shown to be the most sensitive marker of PAH exposure. Results for other biomarkers (glutathione-S-transferase, catalase and lipid peroxidation) were inconclusive.

**Conclusions** In combination with chemical analyses of suspended matter, the presented approach will be used to improve our understanding of the re mobilization of pollutants from sediments in support of environmental risk assessment.

## 3.2 **Introduction**

There is general agreement that sediment pollution poses a threat to the health of aquatic ecosystems (Haag et al. 2001, Hollert et al. 2007, Westrich & Forstner 2005). Indeed, numerous concepts for risk estimation in these systems have been developed (Ahlf et al. 2002, Burton 1991, Burton 1995, Chapman 1990, 2000, Chapman et al. 2002b). To date, most sediment assessments have focused on development of suitable bioanalytical methods and the evaluation of their potential to characterize sediment-bound contaminants (Brack et al. 2007a, Brack et al.

2007b, Hollert et al. 2000, Hollert et al. 2003a, Rao et al. 1990, Wölz et al. 2008). The role of sediment resuspension, e.g., during flood events, and possible ecotoxicological effects of remobilized particle-bound contaminants to aquatic organisms have scarcely been investigated (Baborowski et al. 2005, Hollert et al. 2007, Ockenfeld et al. 2005, Strauch et al. 2005).

A major ecotoxicological concern that is discussed in the context of climate change is the increasing frequency and intensity of flood events. Indeed, flooding has been shown to result in the remobilization of pollutants from sediments or flood plain soils (Wölz et al. 2009b). In the past decade, an increasing number of extreme weather events have been observed around the globe (Schüttrumpf & Bachmann 2008), that have resulted in floods such as the 500-year flood event that occurred in 2002 in the River Elbe, Germany (Schüttrumpf & Bachmann 2008). The magnitude of this flood event increased awareness of the risks posed by flood events and has resulted in many efforts aimed at increasing our understanding of the potential ecotoxicological risks associated with flood events (e.g., Huber et al. 2009; Pahlow et al. 2009).

Increased bioavailability and potential adverse effects of particle-bound compounds from these resuspended sediments to aquatic organisms have been suggested. Histological alterations in livers of flounders (*Platichthys flesus*) and digestive glands of blue mussels (*Mytilus edulis*) collected from the Elbe estuary and the Wadden Sea have been observed 5 months after the 2002 flood event (Einsporn et al. 2005). In comparison to earlier long-term studies conducted at the same sampling sites, a significant impairment of cellular function has been observed after the flood. 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 might be related to the remobilization of dioxin-like compounds during the Elbe River flood event (Hollert et al. 2007).

As a consequence of the potential toxicological risks associated with flood events, Netzband et al. (2007) recommended that for the adequate management of sediment quality in four river basins (Danube River, Rio Duoro, Humbe River, and Elbe River) such events have to be considered. Thus, novel scientific approaches are required to understand and predict possible ecotoxicological consequences of pollutant remobilization caused by floods. 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 polluted sediments (Chapman & Hollert 2006). One such approach is the combination of hydrodynamics and ecotoxicology,

which has become an emerging field in environmental research (Hollert et al. 2007, Wölz et al. 2009b). Recent studies have identified the importance of investigating the partitioning processes of particle-bound pollutants during resuspension events (Cantwell et al. 2008). Furthermore, it has been shown that desorption from particles was the main source of dissolved polycyclic aromatic hydrocarbons (PAH) during simulated resuspension of Yangtze River sediments using a particle entrainment simulator. (Feng et al. 2007, Yang et al. 2008b).

In this proof-of-concept study, a multiple biomarker approach was used to investigate exposure to organic pollutants that were remobilized during a simulated flood event according to DIN 4049-3 (1994a), 2.2.52. The initial objective was to establish a suitable test system for direct exposure of rainbow trout (*Oncorhynchus mykiss*) to simulated flood-like conditions in the laboratory, and thereby, demonstrate possible effects of particle-bound pollutants after resuspension. Thus, methodologies of ecotoxicology and hydraulic engineering were combined by means of exposing animals in an annular flume (Figure 3.1), a facility that can be used to study erosion and sedimentation processes (Schweim et al. 2001).

Figure 3.1: Annular flume at the Institute for Hydraulic Engineering and Water Resources Management, Aachen, Germany; Picture: C. Cofalla.

Rainbow trout were exposed to artificial sediment (OECD 2004b) that was spiked with a mixture of PAHs during a 5 d simulated flood event. Furthermore, this study was also intended to elucidate if rainbow trout constitute a suitable test species for investigations in the annular

flume. For the determination of suitable endpoints for further studies, a number of biomarkers at different levels of biological organization were investigated to demonstrate either exposure to or effects of sediment-bound pollutants.

## 3.3 **Materials and methods**

### 3.3.1 **Experimental design**

Rainbow trout were exposed over 5 d to simulated flood events according to the DIN 4049-3 (1994a) hydrograph in an annular flume (Figure 3.1) at the Institute for Hydraulic Engineering and Water Resources Management (RWTH Aachen University, Aachen, Germany). In this study, a flood event was defined according to DIN 4049-3 (1994), 2.2.43, as an increased current in surface waters that lead to a flood. Thus, the only systematically altered variable during flood simulation was the rotational speed of the annular flume and, thereby, bed shear stress and turbidity. Artificial sediments (OECD 218, 2004) were used as substratum in the annular flume. The exposure experiment was conducted with sediment that was spiked with a mixture of the following polycyclic aromatic hydrocarbons (PAH), 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 $kg^{-1}$). Two additional experiments either with unspiked sediment or without sediment, respectively, served as references without PAHs. PAH concentrations were chosen according to previously determined concentrations in sediments of German rivers and streams (e.g. Hollert et al. 2009, Keiter et al. 2008). Control groups of fish were taken from a maintenance stock and assessed in parallel to the experimental animals to establish untreated baseline values for the biological endpoints investigated in this study. After exposure, a set of biomarkers was investigated. The set included biochemical markers (7-ethoxyresorufin-*O*-deethylase, glutathione-*S*-transferase, and catalase activity, lipid peroxidation), gene expression analyses using quantitative real-time RT-PCR, determination of Cytochrome P450 1A1 (CYP1A1) protein content, chemical analysis of PAH metabolites in bile (1-hydroxypyrene, 1-hydroxyphenanthrene, and 3-hydroxybenzo[*a*]pyrene) and the micronucleus test with peripheral erythrocytes. Each test was conducted with $n$=15 animals.

### 3.3.2 **Annular flume**

The annular flume is a circular channel that was designed to experimentally investigate erosion and deposition processes, as described by Schweim et al. (2001). 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. Temperature, pH and dissolved oxygen, which were continuously recorded, had the following values (mean ± SD): 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. Turbidity data as a function of bed shear stress was measured in parallel and is reported in Cofalla et al. (2010a, 2012). Chemical analyses of PAH concentrations in sediment and suspended matter is reported in Hudjetz et al. (2009, 2010).

### 3.3.3 **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 and the suspension gently stirred for 48 h. Subsequently, the pre-treated peat (5% dry weight (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 were dried at 105°C and thoroughly crushed. PAHs were dissolved in a mixture of 3.5 L hexane and 1.5 L acetone, added to the dried sediment, and thoroughly mixed. After complete evaporation of the solvents, 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 annular 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 in 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.

### 3.3.4 **Fish**

Immature rainbow trout 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 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±0.2; $NH_3$ <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 the start of the experiment. The fish used in the experiments were grown to a mean weight of 110±34 g and a mean length of 194±20 mm. All experiments were conducted in accordance with the Animal Welfare Act and with permission of the federal authorities, Aachen, Germany, registration number 8.87-50.10.35.08.225.

### 3.3.5 **Tissue preparation**

After exposure, fish were individually anesthetized in a 10 L container by adding a saturated solution of ethyl 4-aminobenzoate (benzocaine). Size and weight were determined for calculation of the coefficient of condition (*K*) and the liver somatic index (*LSI*). Peripheral blood samples were taken from the caudal veins using heparinized syringes. 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 entire liver was rapidly removed 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 or submerged in RNAlater (Ambion, Austin, Texas) solution. Liver samples in RNAlater were stored at –20°C. Samples for measurement of biochemical markers were stored at –85°C until analysis.

### 3.3.6 **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 phosphate buffer (0.1 M, pH 7.4) containing 0.15 M KCl and 1 mM EDTA. 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 refrigerated centrifuge (Rotina 420R, Hettich, Tuttlingen, Germany). Next, the supernatant (S9 fraction) 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) activities, 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 $\varepsilon$-aminocaproic acid, 0.3 M $\beta$-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 (9,000 × 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.

### 3.3.7 **Determination of 7-ethoxyresorufin-*O*-deethylase activity (EROD)**

EROD activity was measured in triplicates according to the method described by Kennedy and Jones (1994). In 96-well plates (TPP, Trassadingen, Switzerland), 50 µL of S9, as well as 50 µL serial external standard dilutions of resorufin (0–10 µM) and bovine serum albumin (BSA, 0–10 mg $mL^{-1}$) 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 were 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 $protein^{-1}$ $min^{-1}$. Since the presence of PAHs did not significantly induce EROD activity compared to the

respective control group, it was only measured for the experimental group exposed to PAH spiked sediment, the respective control group, and another control group to estimate interassay variability.

### 3.3.8 **Determination of glutathione-S-transferase activity (GST)**

Activity of GST was determined in triplicates according to the method of Habig et al. (1974), adapted to 96 well microplate measurement. Fresh solutions 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 prepared. In 96-well microplates (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. CDNB concentrations were calculated according to the Lambert-Beer law, and a molar extinction coefficient of 9.6 $mM^{-1}$ $cm^{-1}$ was used. The specific GST activity was expressed as nmol CDNB mg $protein^{-1}$ $min^{-1}$.

### 3.3.9 **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 on ice in a light-tight bottle. In 1.5 mL micro test tubes (Greiner Bio-One), 4 µL sample was added to 50 µL Triton X-100 and 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)-oxysulphate - sulphuric 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 sulphate at 414 nm in an Infinite® 200 microplate reader (Tecan), using a molar extinction coefficient of 19.1 $\mu M^{-1}$ $cm^{-1}$. 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 $protein^{-1}$ $min^{-1}$.

### 3.3.10 **Determination of lipid peroxide concentrations**

Lipid peroxide content in liver was measured according to the methods of 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 combined with 200 µL of 8.1% sodium dodecyl sulphate (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 (approx. 10°C), 5 mL of a 15:1 (v/v) mixture of *n*-butanol and pyridine, and 1 mL distilled water were added and the sample was vortexed vigorously for 20 s. After centrifugation (4,000 × g, 10 min), 300 µL of the organic layer was 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, using 1,1,3,3-tetramethoxypropane (TMP) as an external standard. All measurements were conducted in triplicate.

### 3.3.11 **Immunoblot analysis of CYP1A**

Immunoblot analyses of CYP1A protein content were performed according to methods previously published by Wiseman and Vijayan (2007). Briefly, S9 fractions of hepatic homogenates (40 µg total protein) were separated on a 8% SDS-PAGE set at 150 V for 1 h using 1X TGS (250 mM Tris, 1.92 M glycine, 1% SDS) and transferred onto a 0.45 µM nitrocellulose membrane (BioRad) using Trans-blot SD semi-dry electrophoretic transfer cell (BioRad). A 5% solution of non-fat dry milk in 1X TTBS (2 mM Tris, 30 mM NaCl, 0.01% Tween 20, pH 7.5) was used as a blocking agent (1 h at room temperature) and for diluting antibodies. The blots were incubated with primary CYP1A antibodies for 1 h at room temperature followed by 1 h incubation with the appropriate secondary antibody. Membranes were washed after incubation in either primary (2 × 15 min washes in TTBS) or secondary antibodies (2 × 15 min in TTBS followed by 1 × 5 min in TBS, 2 mM Tris, 30 mM NaCl, pH 7.5). Band detection was carried out with BCIP-NBT substrate for CYP1A. Images were captured with VersaDoc Imaging System (Alpha Innotech, San Leandro, CA, USA).

### 3.3.12 **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). Total RNA was extracted from preserved liver tissue of individual animals 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, DE, 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 3.1), and 1.6 mL of nuclease-free distilled water (QIAGEN). Primers for CYP1A1, 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 made up with 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 3.1: Primer pair sequences, amplicon sizes, and accession numbers of the investigated genes used in real-time PCR

| Gene | Primer sequence (5'–3') | | Amplicon size (bp) | GeneBank accession no. |
|---|---|---|---|---|
| | Forward | Reverse | | |
| AhRβ | TGGCAAATGGACACACATTC | AGTCTGTTGGGGTTCTGTGG | 100 | NM_001124252 |
| CYP1A1[a] | GATGTCAGTGGCAGCTTTGA | TCCTGGTCATCATGGCTGTA | 104 | U62796 |
| EL-1α | GAGAACCATTGAAAAGTTCGAGAAG | GCACCCAGGCATACTTGAAAG | 71 | NM_001124339 |
| GST-P | TTCAGGGAGGGGAAGGTATC | GTTGGTGACAAGCCTTCGTT | 101 | BQ036247 |
| SOD1[b] | TGGTCCTGTGAAGCTGATTG | TTGTCAGCTCCTGCAGTCAC | 201 | NM_001124329 |
| UGT[c] | ATAAGGACCGTCCCATCGAG | 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)

### 3.3.13 **Determination of protein concentrations**

Protein concentrations for the calculation of specific enzyme activities (except for EROD) were determined in triplicates according to the Bradford method (Bradford 1976), adapted to microplate measurement, using bovine serum albumin (BSA) as external standard. 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). Protein concentrations for immunoblotting were measured according to manufacturer's protocol using the Bicinchoninic Acid Kit (Sigma-Aldrich) and BSA as the external standard. A minimum coefficient of determination ($R^2$) of 0.95 was accepted for standard curves in all used assays for protein quantification.

### 3.3.14 **Micronucleus assay**

The proportion of micronucleated cells in peripheral erythrocytes was determined according to methods published by Rocha et al. (2009). 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.

### 3.3.15 **Treatment of bile samples and HPLC analysis**

PAH metabolites in bile samples were determined by a modified version of the method described by Kammann (2007a), basing on Krahn et al. (1984). A volume of 25 µL bile was mixed with 95 µL water to which 5 µL of ß-glucuronidase/arylsulfatase solution (30/60 U $mL^{-1}$) were added. The resultant solution was incubated for 2 h at 37°C on a heated shaker. The reaction was stopped by addition of 125 µL ethanol containing 5 mg/mL ascorbic acid. The final solution represents a tenfold dilution of the bile sample and was centrifuged (5 min, 700 × g). The clear supernatant was used for HPLC analysis immediately. The concentrations of 1-hydroxypyrene, 1-hydroxyphenanthrene and 3-hydroxybenzo[*a*]pyrene were determined in a 50 µL aliquot using a LaChrom HPLC system (Merck Hitachi) comprising a quaternary pump (L-7100), an auto sampler (L-7200) and a fluorescence detector (L-7480). Standard solutions were diluted in acetonitrile containing 5 mg $mL^{-1}$ ascorbic acid and stored in the dark. In difference to Kammann (2007a), samples were chromatographed with a flow of 0.55mL/min on a Nucleosil 100-3 C18 (3 x 125 mm) reverse phase column equipped with a guard column. The initial mobile phase was acetonitrile/0.1% trifluoroacetic acid 50/50 (v/v). After 10 min the solvent composition progressively changed to 60% actonitrile over 4 min and changed afterwards to 100% acetonitrile over 2 min. The excitation/emission wavelength pairs for 1-hydroxypyrene, 1-hydroxyphenanthrene and 3-hydroxybenzo[*a*]pyrene were 346/384, 256/380 and 380/430 nm respectively. The UV-absorption measurement was performed in 1:20 diluted bile fluids by means of a microplate reader (FLUOstar OPTIMA, BMG Labtech, Offenburg, Germany) at 380nm.

Every sample was hydrolyzed and subjected to HPLC analysis twice. The limit of detection (LD) and the limit of quantification (LQ) were calculated from a standard curve according to DIN 32645 (1994) with a confidence level of 99%. Considering the dilution of the sample during sample preparation a LD of 0.7 and a LQ of 4.5 ng $mL^{-1}$ bile were determined for 1-hydroxypyrene. For 1-hydroxyphenantrene (3-benzo[*a*]pyrene) a LD of 0.1 (4.1) and a LQ of 0.3 (12.9) ng $mL^{-1}$ were calculated. Most of individual results for 3-hydroxybenzo[*a*]pyrene in bile of control fish were found to be below the LQ. A fish bile sample as laboratory reference

material was included in every sample batch to monitor the stability of the method by measuring 1-hydroxypyrene (variation coefficient of results 15%). Calibrations consisting of five standard concentrations were repeated daily with every sample batch.

### 3.3.16 **Data analysis**

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 ($p \leq 0.001$). The datasets passing both tests were analyzed using parametric one-way ANOVA ($p \leq 0.001$). 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 or median of the respective control group were calculated for each treatment. Statistical significance limit throughout all comparisons was set at least to $p \leq 0.05$. If not stated differently, all values are expressed as mean value ± standard deviation. Statistical analyses and comparisons were conducted using the software Sigma Stat 3.11 (Systat Software, Erkrath, Germany). All graphs were plotted using GraphPad Prism 5 software (GraphPad, San Diego, USA).

## 3.4 **Results**

During exposure experiments with rainbow trout in the annular flume (unspiked, spiked and no sediments, respectively) untreated control animals from the maintenance were examined (control 1, 2 and 3, respectively) to establish untreated baseline values. This was particularly useful to reduce inter-experimental variation since average size and weight, as well as LSI and K differed significantly between some of the groups (Table 2). Thus, it was possible to calculate induction factors for the investigated biomarkers relative to the control animals that were reared under comparable conditions. Turbidity data as a function of bed shear stress was measured in parallel (data not shown) and is reported in Cofalla et al. (2010a, 2012). Chemical analyses of PAH concentrations in sediment and suspended matter (data not shown) is reported in Hudjetz et al. (2009, 2010).

### 3.4.1 **Biochemical markers**

EROD activity after exposure to particle-bound PAHs during the 5 d simulated flood event was not significantly different from that of the control group, although the animals with the greatest

EROD activity (approx. 45 pmol $mg^{-1}$ $min^{-1}$) originated from this group. Similarly, no significant alteration of GST enzyme activity was detected in PAH exposed trout (Table 2). Although there were statistically significant differences of CAT activity between the different control groups, CAT activity was not significantly different in the trout exposed to the flood events relative to the respective control groups. There were significant differences of lipid peroxidation between the respective control groups (data not shown).

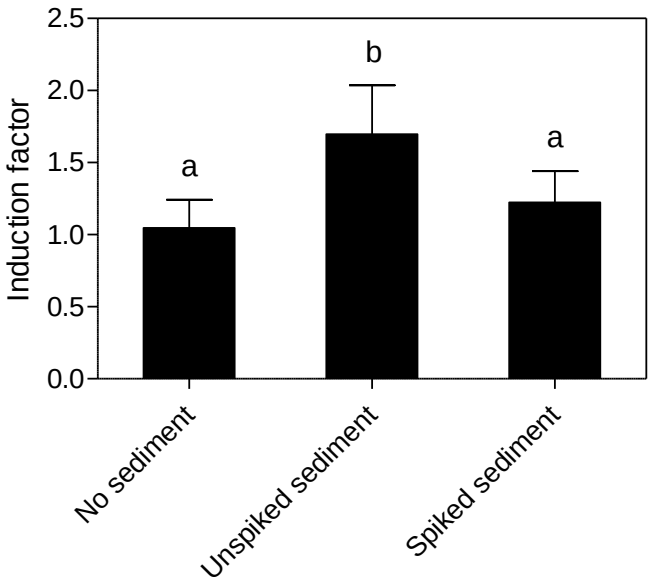


Figure 3.2: Lipid peroxidation, measured as MDA equivalent concentration in the liver of rainbow trout, exposed in 5 d simulated flood events without, with unspiked and spiked sediments, respectively. Values are expressed as induction factors relative to the median of the respective control group taken from the maintenance in parallel to the treatments to establish untreated baseline values. Each test was conducted with n=15 animals. Bars represent the mean value, error bars the standard deviation. a, b: Treatment groups sharing the same letter do not differ significantly (Kruskal-Wallis one way ANOVA on ranks with Dunn's method, $p \leq 0.01$).

Exposure to unspiked artificial sediment during the simulated flood event resulted in an average MDA concentration of 125.8 ± 25.2 nmol $g^{-1}$ (1.7-fold induction), while exposure to spiked sediment led to an average concentration of 105.2 ± 18.5 nmol $g^{-1}$ (1.2-fold induction). There was no effect in the absence of sediment (Figure 3.2).

Table 3.2: Summarized results of the biomarker investigation: biometric indices, 7-ethoxyresorufin-*O*-deethylase (EROD), Glutathione-S-Transferase (GST) and catalase (CAT) activity in liver S9 fractions were quantified. Values are given as mean value ± standard deviation. Animals from untreated control groups were taken from the maintenance to establish baseline values. Animals from control 1 were investigated within the same week as animals exposed to unspiked sediment, control 2 during exposure to spiked sediments and control 3 during the simulated flood event without sediments.

| | **Treatment** | | | | | |
|---|---|---|---|---|---|---|
| | Control 1 (*n*=15) | Unspiked sediment (*n*=15) | Control 2 (*n*=15) | Spiked sediment (*n*=15) | Control 3 (*n*=15) | No 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] | 1.57 ± 0.13[b] | 1.51 ± 0.08[a,b] |
| Liver somatic index (LSI) | 1.08 ± 0.11[a] | 1.06 ± 0.19[a] | 1.04 ± 0.10[a] | 1.02 ± 0.14[a] | 1.11 ± 0.18[b] | 0.85 ± 0.12[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] | n.d. | n.d. |
| GST activity / pmol $mg^{-1}$ $min^{-1}$ | 15.51 ± 4.13[a,c] | 14.67 ± 2.47[a,c] | 8.78 ± 2.61[a] | 9.47 ± 2.81[a] | 34.03 ± 7.33[b,c] | 43.21 ± 11.53[b] |
| CAT activity / nmol $mg^{-1}$ $min^{-1}$ | 561.6 ± 250.90[a] | 326.5 ± 75.14[a,c] | 153.8 ± 74.73[b,c] | 76.98 ± 48.61[b] | 370.4 ± 196.5[a] | 481.1 ± 153.6[a] |

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, $p \leq 0.05$, and one way ANOVA on ranks with Dunn's method, $p \leq 0.05$, respectively)

### 3.4.2 **CYP1A immunoblot analysis and hepatic gene expression**

None of the examined treatment groups exhibited detectable amounts of CYP1A protein (data not shown). There was a statistically significant ($p \leq 0.05$) 1.8-fold induction of CYP1A1 mRNA expression in fish that were exposed to polluted sediment during the simulated flood event compared to the flood event in the absence of sediment (Figure 3.3). 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 to approx. 0.4-fold ($p \leq 0.01$) 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.

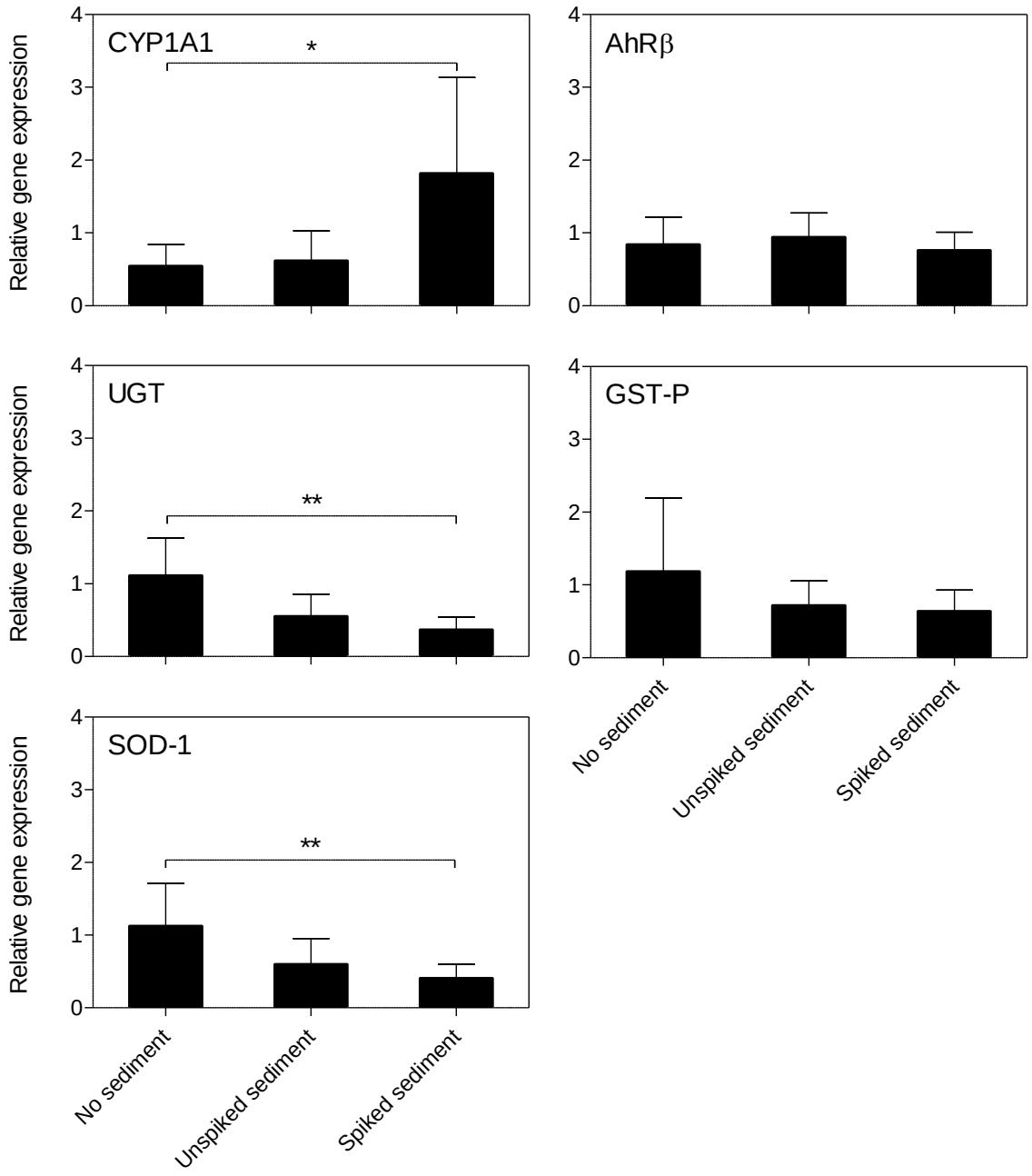


Figure 3.3 Hepatic expression of the genes CYP1A1, AhRβ, GST-P, UGT and SOD-1 of rainbow trout exposed in 5 d simulated flood events without, with unspiked and spiked sediments, respectively. Each test was conducted with n=15 animals. Bars represent the average gene expression relative to the respective 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, $p \leq 0.05/0.01$).

### 3.4.3 **Micronucleus formation**

The number of micronuclei in 4000 erythrocytes per exposed fish was determined using fluorescence microscopy (Figure 3.4). Frequencies differed significantly between the control

groups ($p \leq 0.05$, data not shown). Thus, induction factors of the experimental groups relative to the median of the respective control groups were calculated for the treatments. There was no induction of micronuclei in the absence of sediment relative to the respective control group. Although the micronucleus frequency after exposure to the unspiked sediment was 2.2-fold greater compared to the experiment without sediment, this induction was not significant. After exposure to the spiked sediment the induction factor was significantly 4.3-fold greater ($p \leq 0.001$).

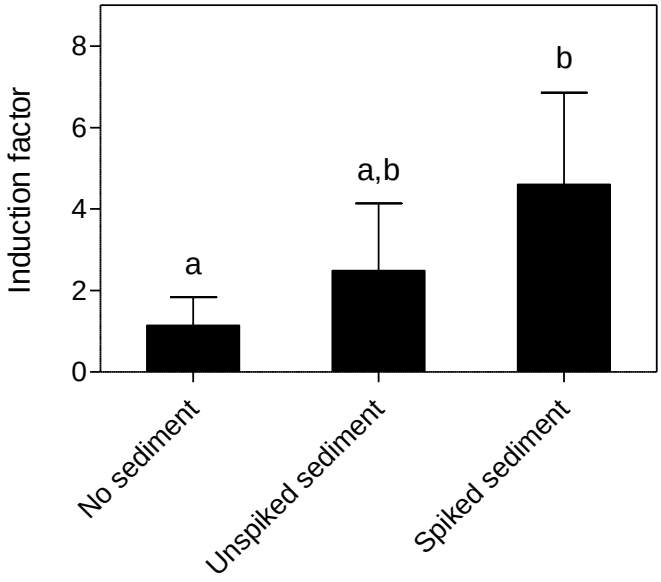


Figure 3.4: Micronucleus frequency in peripheral erythrocytes of rainbow trout exposed in 5 d simulated flood events without, with unspiked and spiked sediments, respectively, expressed as induction factors relative to the median of the respective control group taken from the maintenance in parallel to the treatment to establish untreated baseline values. Each test was conducted with n=15 animals. Bars represent the average proportion of micronucleated cells in 4000 erythrocytes of each animal, error bars the standard deviation. a, b: Treatment groups sharing the same letter do not differ significantly (Kruskal-Wallis one way ANOVA on ranks with Dunn's method, $p \leq 0.01$).

### 3.4.4 **PAH metabolites in bile**

Except for concentrations of 3-hydroxybenzo[*a*]pyrene in the control groups and unspiked treatments, all measured metabolite levels were well above the limits of quantification (Figure 3.5). The concentration of 1-hydroxypyrene in bile of animals exposed to spiked sediments was significantly greater (4596-fold) compared to animals exposed to unspiked sediments ($p \leq 0.001$), and the concentrations of 1-hydroxyphenanthrene and 3-hydroxybenzo[*a*]pyrene were significantly increased by 514 and 250-fold, respectively ($p \leq 0.001$). While exposure to

unspiked sediments did not result in elevated concentrations of the metabolites 1-hydroxypyren and 3-hydroxybenzo[*a*]pyrene, the average concentration of 1-hydroxyphenanthrene was significantly greater (4.4-fold) compared to the respective control group ($p \leq 0.001$).

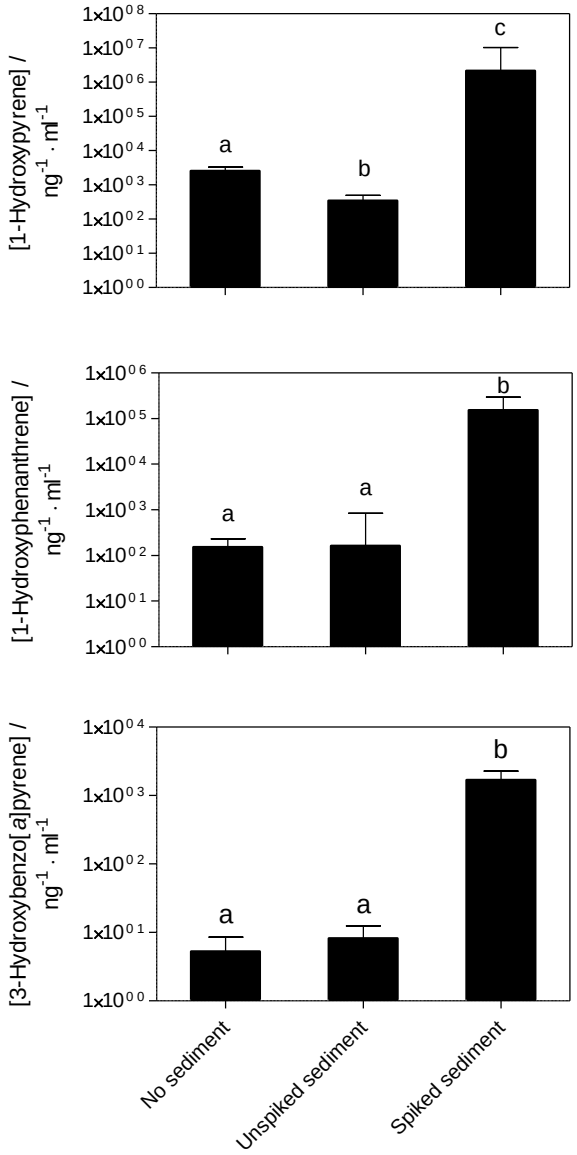


Figure 3.5: Concentrations of the PAH metabolites 1-hydroxypyrene, 1-hydroxyphenanthrene and 3-hydroxybenzo[a]pyrene in bile of rainbow trout exposed in 5 d simulated flood events without, with unspiked and spiked sediments, respectively. Each test was conducted with n=15 animals. Bars represent the average concentration of the respective metabolite in individual animals, error bars the range. a,b,c: Treatment groups sharing the same letter do not differ significantly (Kruskal-Wallis one way ANOVA on ranks and Dunn's method, $p \leq 0.001$).

The proportion of micronucleated erythrocytes was found to exhibit a weak but significant positive correlation with the concentration of 3-hydroxybenzo[*a*]pyrene (Spearman's rank correlation coefficient, $r$=0.64, $p$=0.01).

No such correlations with the concentrations of other bile metabolites were observed (Figure 3.6).

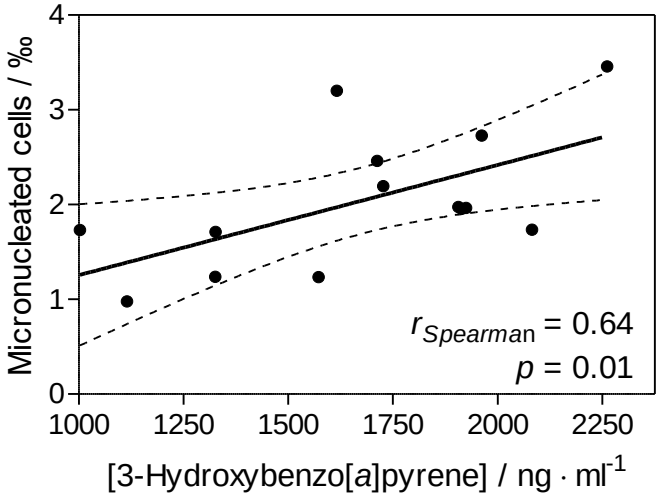


Figure 3.6: Linear regression of 3-hydroxybenzo[a]pyrene concentrations in bile and the proportion of micronucleated cells of rainbow trout exposed in a 5 d simulated flood event with PAH spiked sediment. The tests were conducted with n=15 animals. The dashed curves represent the 95% confidence limits. The proportion of micronucleated cells was positively correlated with the concentration of 3-hydroxybenzo[a]pyrene (Spearman's rank correlation coefficient, r=0.64, p=0.01).

## 3.5 **Discussion**

### 3.5.1 **Identification of suitable biomarkers for coupled hydrotoxicological studies**

A number of biomarkers were investigated to demonstrate either exposure to or effects of sediment-bound PAHs 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, mRNA abundance of selected genes, determination of CYP1A1 protein content, PAH metabolites in bile and the micronucleus test with peripheral erythrocytes. In this context, rainbow trout proved 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 pollutants. Accordingly, a 5 d exposure time in simulated flood events may not be sufficient to detect effects at the protein and enzyme level, respectively, and may not be sufficient for the maximum effect to develop regarding the other markers. However, real-time PCR analysis of changes in

gene expression proved to be a useful method of detecting physiological responses to PAH exposure (e.g., 1.8-fold induction of CYP1A1 mRNA) under this exposure scenario.

Many aquatic species rapidly metabolize and excrete PAHs (Meador et al. 1995a). Thus, quantification of PAH metabolites in bile has been shown to be a very sensitive biomarker of exposure to PAH pollution (Kammann 2007a). The most abundant metabolite in fish bile, 1-hydroxypyrene, can contribute up to 76% of the sum of PAH metabolites. Compared to 1-hydroxypyrene, the metabolites of phenanthrene, chrysene and benzo[*a*]pyrene are detected at significantly lesser concentrations (Ruddock et al. 2003). This pattern was also observed in the present study. The concentrations used for spiking with the respective substance were in descending order benzo[*a*]pyrene > phenanthrene > pyrene > chrysene. In contrast, the concentrations of bile metabolites following the 5 d exposure to spiked sediment resulted in exactly the opposite distribution, where 1-hydroxypyrene, 1-hydoxyphenanthrene and 3-hydroxybenzo[*a*]pyrene were measured at concentrations of 2150.00 ± 2553.00, 151.64 ± 59.23, and 1.68 ± 0.37 µg $mL^{-1}$, respectively. These observations could be explained by different bioavailability of the parent compounds due to sorption to the sediment particles as hypothesized by Ruddock et al. (2003). Within the last years, several laboratory studies have investigated the concentrations of PAH metabolites in bile after exposure to PAHs. Oral administration of 10 mg $kg^{-1}$ body weight benzo[*a*]pyrene resulted in a concentration of 0.81 µg $mL^{-1}$ hydroxybenzo[*a*]pyrene in the bile fluid of dab (van Schanke et al. 2001), while other researchers 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 in *Parophrys vetulus* (Collier & Varanasi 1991). Furthermore, it was possible to positively correlate the elevated micronucleus frequency in exposed trout to the biliary 3-hydroxybenzo[*a*]pyrene concentration. Benzo[*a*]pyrene is known to cause genotoxicity in laboratory experiments (Metcalfe 1988) and to contribute to the genotoxic potential in field studies (e.g. Barbee et al. 2008a). However, it is not possible to directly extrapolate these findings to the field. Aging can significantly alter the bioavailability of sediment-bound contaminants (Alexander 2000). The results from the present work clearly demonstrated a genotoxic effect after relatively short exposure to resuspended sediments during the simulated flood event.

The PAHs that were used in the present study are known to be moderately potent AhR agonists *in vitro* (Barron et al. 2004b), and have been shown to cause significant induction of biotransformation enzymes in rainbow trout (Fragoso et al. 2006, Jonsson et al. 2006a, 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. The mean activity in all tested groups (14.1 ± 9.2 pmol $mg^{-1}$ $min^{-1}$) was in good accordance with control groups from previous studies (Fragoso et al. 2006, Jonsson et al. 2006a). 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 moderately altered after exposure to polluted sediments, indicating a higher sensitivity compared to biomarkers on the protein level. The presence of biliary metabolites of the three PAHs pyrene, phenanthrene and benzo[*a*]pyrene clearly demonstrated bioavailability, substantial uptake and metabolic transformation. Thus, it can be assumed that (a) AhR-mediated biotransformation enzymes were not highly inducible under the current exposure scenario or (b) exposure time of 5 d was not sufficient. Accordingly, several authors have found that the developmental stage of most fish species influenced EROD activity and CYP1A expression (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). In this study, however, no CYP1A protein was detected after exposure to the treatments and the previously mentioned factors affecting EROD activity are unlikely to have influenced the measurements. Thus, it may be assumed that exposure time was not sufficient to induce detectable amounts of CYP1A protein and EROD activity, respectively.

The level of lipid peroxidation throughout the control groups was significantly lower compared to values (6990 ± 1720 nmol $g^{-1}$) reported by Arnold and co-workers (1995). CAT activity 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 in the levels of this anti-oxidant defence enzyme by any of the treatments. CAT activities in control animals of 1.03 ± 0.21 μmol $min^{-1}$ $mg^{-1}$ similar to values from the current study have been reported by Salaberria et al. (2009). However, the levels of lipid peroxides in the liver of exposed animals were significantly elevated compared to the control groups. Oxidative damage reflects an imbalance between the production of oxidants and the removal of such reactive species by protective enzymes. Thus, these results indicate a higher production of oxyradicals due to increased respiration, e.g. as a consequence of the increased swimming activity in the simulated flood events. Oxidative damage, measured as lipid peroxidation, was

significantly less in the treatment group exposed to spiked sediments during the flood event compared to the treatment group exposed to unspiked sediments. However, the only measured marker for anti-oxidant enzymes (CAT) did not differ between these experimental groups.

The proportion of micronucleated erythrocytes in the control groups (1.40 ± 0.66, 0.53 ± 0.30‰, and 0.97 ± 0.67‰) were in general agreement with previously described levels for rainbow trout by Strunjak-Perovic et al. (1.80 ± 1.57 ‰, 2003) and Schultz et al. (approx. 1 ‰, 1993). However, the proportion of micronuclei in the treatment group exposed to the spiked sediment was relatively low (2.27 ± 1.12 ‰) compared to maximum inductions from other field and laboratory studies in different species (6 ‰, Rocha et al. 2009, Schultz et al. 1993). Exposure to unspiked sediments during the simulated flood event caused a slight but not significant induction of micronucleated erythrocytes compared to the control group from the maintenance stock. Induction of micronucleated erythrocytes could result from increased respiration resulting from swimming against the current within the flume. Accordingly, Chub (*Leuciscus cephalus*) showed significantly elevated oxidative DNA damage when subjected to exhaustive exercise in a swimming experiment (Aniagu et al. 2006). Nonetheless, micronuclei were significantly induced in animals exposed to spiked sediment compared to the treatment without sediment (4-fold), thereby indicating 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).

The biomarkers investigated in the present study were selected for their relatively high specificity as indicators of PAH exposure. The goal of future studies is to test non-polluted and polluted natural sediments in the annular flume, which can contain complex mixtures of various contaminants. Thus, it is necessary to modify the set of biomarkers to be able to capture a broader range of different types of biological effects. Among others, these should include endpoints indicative of the exposure to metals, such as metallothioneins (Wisniewska et al. 1970), and endocrine disruptors, such as vitellogenin or markers of steroidogenesis (Jones et al. 2000), respectively. Furthermore, histological investigation of ultrastructural changes should be assessed as indicators of tissue damage or alterations (Arnold et al. 1996b, a, Grund et al. 2009). In addition, 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 pollutants during flood events might lead to an increased body burden. As part of the discussions 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 (CEC 2006). Thus, investigation of the interdependency between biota and sediments with regard to bioaccumulation during remobilization events is of great relevance (Förstner 2009).

### 3.5.2 **Suitability of artificial formulated sediments for use in the annular flume**

In this proof-of-concept study, spiked artificial sediments that were prepared according to OECD 218 (2004) were used to expose rainbow trout to particle-bound pollutants, which were intended to represent a standardized substrate for laboratory testing. For physicochemical sediment characteristics, as well as turbidity as a function of bed shear stress (data not shown), see Cofalla et al. (2010a, 2012). For measured sediment PAH concentrations, see Hudjetz et al. (2009, 2010). Although artificial sediments were principally erodible in the annular flume, it is questionable whether the test design allows extrapolating 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 operational taxonomic units appeared to dominate the artificial and natural sediment, respectively (Goedkoop et al. 2005). In addition, 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. Furthermore, it cannot be assumed that substantial aging of sediment-bound pollutants occurred within 7 d of the sediment conditioning step. Thus, results of the current study might not be directly transferrable to the same concentrations in environmentally aged sediments due to greater bioavailability of the PAHs (Alexander 2000). To derive a scientifically defensible basis for the development of models to predict the effects of remobilisation processes in-field, it is necessary to accurately emulate the biological and physicochemical properties of natural sediments. Thus, additional experiments with natural sediments, either polluted or spiked, must be performed.

### 3.5.3 **Applicability of the annular flume for coupled hydrotoxicological studies**

A pre-requisite for the applicability of the annular flume as a test-system for coupled hydrotoxicological studies is the ability to control for environmental parameters that are critical to a balanced physiology of the used 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 living animals have not been addressed in earlier studies. For the present study, the original setup was therefore extended by installing a flow-through cooling unit and an aeration system to allow the user control of water temperature and dissolved oxygen. All animals survived exposure to both unspiked and spiked sediments in the simulated flood event, enabling the exposure to particle-bound pollutants in the annular flume and investigation of sublethal effects. However, application of the annular flume test system in future ecotoxicological studies should include (1) investigation of the contribution of the different stress parameters such as turbidity, current and pollutants on the assessment endpoints (individually and in different combinations by means of a systematic sensitivity analysis), and (2) the verification of the current methodology with different test-species.

### 3.5.4 **Potential and limitations of the current approach**

It was hypothesized that the combination of hydraulic engineering and ecotoxicology may assist in our understanding of the impact of flood events on biota and ecosystem health. In the present proof-of concept study, it was experimentally demonstrated that sediment remobilization during simulated flood events in the annular flume can lead to uptake and effects of sediments-bound pollutants. This novel approach has been shown to be applicable to successfully conduct hydrotoxicological studies with rainbow trout. However, technical modifications of the annular flume (e.g., automatic feeding) and an increase of the dimensions to (1) enhance simulation of environmental conditions, and (2) reduce the influence of the exposed organisms on the physicochemical processes would be desirable. Furthermore, it will be necessary to systematically control environmental variables, such as pH, temperature and other physicochemical water characteristics for application of the annular flume in further studies, especially in the context of climate change.

The presented study clearly demonstrates that relatively short exposures to resuspended sediments during simulated flood events can lead to alterations of biological functions in rainbow trout. Thus, the ecological and toxicological impact of pollutant re-mobilization during floods has to be considered highly relevant and integrated approaches for risk assessment of regularly flooded rivers are urgently required.

### 3.5.5 **Acknowledgements**

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Chapter 4

# A combined hydraulic and toxicological approach to assess resuspended sediments during simulated flood events – Part II: an interdisciplinary experimental methodology

# A combined hydraulic and toxicological approach to assess resuspended sediments during simulated flood events – Part II: an interdisciplinary experimental methodology

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## 4.1 **Abstract**

**Purpose:** Flood events are expected to increase both in intensity and frequency due to climate change in the near future. From an environmental toxicology perspective there is concern that such flood events could lead to the remobilization of contaminated sediment layers in rivers. The aim of this pilot study was to establish a novel and interdisciplinary framework combining methods of hydrodynamic engineering and ecotoxicological assessment to enable investigation of the potential risks associated with such remobilization events.

**Materials and methods:** Formulated sediment was prepared according to OECD guideline 218 and spiked with a mixture of four polycyclic aromatic hydrocarbons (PAHs) (phenanthrene, chrysene, pyrene, benzo[*a*]pyrene) at concentrations of 3.3–8.3 mg $kg^{-1}$ dry weight. Rainbow trout (*Oncorhynchus mykiss*) were exposed as test animals to resuspended sediments in three out of five experiments. The experiments were carried out in an annular flume designed to investigate transport behavior of fine-grained sediments. Several physicochemical (e.g. pH) and sedimentological (e.g. turbidity) parameters were measured to characterize environmental conditions and erosion behavior of sediments. Furthermore, exposure concentrations were measured by means of an *in vitro* assay (7-ethoxyresorufin-*O*-deethylase (EROD) RTL-W1 cell assay) and chemical analysis.

**Results and discussion:** Preparation and spiking of large amounts of formulated sediments were feasible but not practical. Successful spiking could be confirmed by the bioanalytical methods with the spiked sediments showing significantly elevated EROD induction compared to control sediments. Conditions within the annular flume remained stable throughout all experiments and were adequate to support rainbow trout. Flood events were successfully simulated, resulting in resuspension of formulated sediment. Different erosion behaviors of sediments during the simulated flood events were observed and could be associated with changes in microbial composition of sediments due to differences in storage conditions. Therefore, maintaining constant storage conditions of formulated sediments is crucial to enable consistency and comparability among erosion experiments.

**Conclusions:** This study clearly demonstrated the feasibility of a combined hydrotoxicological approach in support of the investigation of the potential ecotoxicological relevance of sediment resuspension events. However, based on the results presented here it is recommended to include additional physicochemical parameters, such as redox potential and conductivity, and to extend

the experimental setup to natural sediments and different aquatic organisms. Future studies will use natural sediments containing representative microbial communities and extracellular polymeric substances to enable extrapolation from the annular flume to conditions in natural flowing waters.

## 4.2 **Introduction**

Sediments are sinks for a variety of contaminants including heavy metals, polychlorinated biphenyls (PCBs), dioxins and polycyclic aromatic hydrocarbons (PAHs). Exposure to sediment-bound contaminants can result in harmful effects to benthic and aquatic life including lethality, impacts on reproductive functions and cancer in aquatic organisms (Chapman 1990). Many of the pollutants associated with sediments also tend to bioaccumulate along the food chain, which can ultimately result in exposure of humans through consumption of fish and seafood from contaminated areas (Dickman & Leung 1998, Fremy & Bordet 2002, Smith & Gangolli 2002).

Bioavailability of sediment-bound contaminants may be limited during undisturbed conditions but may increase during anthropogenic or natural hydrodynamic events like dredging (Koethe 2003) or floods (Haag et al. 2001, Hollert et al. 2000, Westrich & Forstner 2005, Wölz et al. 2008). One of the major concerns regarding the remobilization of sediments is the occurrence of extreme weather events such as heavy rain fall. These result in increasingly frequent and strong flood events that are one of the potential consequences of global climate change (Hunt 2002, Ikeda et al. 2005, Kay et al. 2006, Najjar et al. 2010, Palmer et al. 2008). During the past decade, an increasing number of extreme weather events occurred around the globe. One such event led to the 500-year flood that occurred in the River Elbe, Germany in 2002 (Schüttrumpf & Bachmann 2008), and which resulted in the remobilization of layers of highly contaminated legacy sediment. Given the increasing likelihood of such extreme events, there is a growing need for the development of scientific approaches that will help to further our understanding and allow prediction of their possible toxicological and ecotoxicological impacts.

Today, most approaches to assess the toxicity of sediments focus on a combination of targeted chemical analysis of priority pollutants, biotests, and the measurement of *in situ* parameters such as benthic species composition. The combination of these lines of evidence, the so called “sediment quality triad” (SQT), has proved to be a very promising approach (SQT, Brack et al. 2005, Chapman 1990, Hollert et al. 2002, Long & Chapman 1985). However, SQT approaches

do not consider dynamic changes, such as flood events or dredging operations, which can lead to remobilization of sediment layers, ultimately influencing the bioavailability of sediment-bound contaminants. As a consequence, in recent years it has been suggested to integrate hydraulic engineering (hydrodynamics) in weight-of-evidence approaches as an additional line-of-evidence to provide a more realistic assessment of the environmental impact of contaminated sediments (Chapman & Hollert 2006, Gerbersdorf et al. 2011). Consequently, the combination of hydrodynamic and ecotoxicological methods into a new interdisciplinary approach has emerged as a novel field in environmental research (Brinkmann et al. 2010b, Cantwell et al. 2008, Feng et al. 2007, Hollert et al. 2007, Wölz et al. 2009b, Yang et al. 2008b).

While the necessity to combine these different approaches has been recognized, to date there is still a lack of experimental methods to address current research needs. A variety of laboratory flumes, such as straight open-channel, U-shaped and annular flumes, have been developed to investigate the erodibility of sediments and the response of organisms to such events (Jonsson et al. 2006b, Nowell & Jumars 1987). Within the framework of the interdisciplinary proof-of-concept study (FLOODSEARCH, Schüttrumpf et al. 2011, Wölz et al. 2009b) an annular flume was chosen as the test system because it provides several advantages over straight flumes and has long been established for testing cohesive sediment properties (e.g. Maa et al. 1998, Nowell & Jumars 1987, Partheniades et al. 1966, Schweim et al. 2001). One of the major advantages of annular flumes is the ability to generate an infinite flow without the need of pumps which break down suspended or flocculated sediment and interfere with the use of test animals (Widdows et al. 1998). Furthermore, sediment does not settle in unwanted sections of the annular flume (e.g., pipes) as is the case in straight flumes.

A pre-requisite for the use of annular flumes as a test system for coupled ecotoxicological and erosion studies is the availability of a standardized test material. An artificial sediment based on the OECD test guideline 218 (OECD 218, 2004) was chosen for the experiments in this study. Even though this OECD sediment is commonly used for small scale laboratory experiments (c.f. Höss et al. 2010), there were no data available concerning the feasibility of large scale preparation of this sediment. The formulated sediment was spiked with a mixture of four different PAHs which are known to be moderately potent Ah-receptor agonists *in vitro* (Barron et al. 2004a) and were found in elevated concentrations in fluvial sediments from the Danube River (Keiter et al. 2008, 2006). Furthermore, they were previously shown to cause significant induction of biotransformation enzymes in rainbow trout (*Oncorhynchus mykiss*)

(Fragoso et al. 2006, Jonsson et al. 2006a, Oikari et al. 2002, Ramachandran et al. 2006). Additionally, assimilation and biotransformation can be quantified by analyzing biliary metabolites (Kammann 2007a).

Sediment resistance towards shear stress in the context of flood events and sediment transport was characterized during experiments in the annular flume. Both were evaluated by analyzing aspects and parameters of erosion and remobilization. The initial objective of the research was to establish a suitable test system for direct exposure of rainbow trout (*Oncorhynchus mykiss)* to simulated flood-like conditions in the laboratory, and thereby, demonstrate possible effects of particle-bound pollutants after resuspension (Brinkmann et al. 2010a). Thus, rainbow trout were selected as the test species due to their prominence in the literature, their good availability and easy maintenance. In order to test the hypothesis that resuspended contaminated sediments may lead to adverse effects in aquatic organisms, the activities of different hepatic enzymes, specific metabolites in bile fluid and micronucleus formation in blood cells were assessed in a parallel study by Brinkmann et al. (2010a).

The proof-of-concept study consisted of two parts: (1) a multiple biomarker approach to assess the impact of resuspended sediment on an aquatic model organism during a simulated flood event (Brinkmann et al. 2010a), and (2) sedimentological and hydrodynamic investigations of a simulated flood event in an annular flume (this paper).

The objectives of the present study were to: (i) provide and control an adequate and stable environment for test animals; (ii) simulate and investigate a flood event in an annular flume; (iii) modify the preparation and spiking process of OECD 218 sediment for large scale experiments; and (iv) evaluate the effectiveness of spiking artificial sediment with selected PAHs by means of chemical-analytical and bioassay driven analysis.

## 4.3 **Materials and methods**

### 4.3.1 **Chemicals**

Four different polycyclic aromatic hydrocarbons (PAHs) were chosen for the present study: phenanthrene (98% purity), chrysene (analytical standard), pyrene (≥ 99% purity) and benzo[*a*]pyrene (≥ 99% purity), all purchased from Sigma Aldrich (Deisenhofen, Germany).

### 4.3.2 **Formulated sediment**

The sediments were prepared based on OECD guideline 218 (OECD 218, 2004) using the following ingredients (Table 4.1).

Table 4.1: Composition of formulated sediment prepared for erosion and toxicity experiments in the annular flume.

| Constituent | Characteristics | % of sediment dry weight |
|---|---|---|
| Peat | Lithuanian *Sphagnum* moss peat (Klasmann-Deilmann GmbH, Geeste, Germany), finely ground and air dried | 5.0 |
| Quartz sand | Quartz sand with grain size 50–200 µm (Quarzwerke GmbH, Frechen, Germany) | 74.5 |
| Kaolinite clay | Kaolin clay (Erbslöh Lohrheim GmbH, Lohrheim, Germany) | 19.9 |
| Calcium carbonate | $CaCO_3$, powder, chemically pure (Sigma-Aldrich) | 0.6 |
| Water | Tap water, pH 7.8–9.3, conductivity 150–300 µS $cm^{-1}$ | 42–44 |

Lithuanian *Sphagnum* moss peat was air dried and finely ground to a particle size ≤1 mm using a laboratory-scale grinding device (Jupiter, Wernau, Germany) and then mixed with approximately 7.5 parts (w/v) water. The pH of this suspension was adjusted to pH 5.75 ± 0.25 with calcium carbonate and then gently stirred for 48 h to allow the pH to stabilize and to support the development of a stable microbial component. The conditioned peat suspension was mixed with quartz sand and kaolin clay in a cement mixer (Lescha S230, AltradLescha GmbH, Burgau, Germany). Afterwards, sediments were adjusted to a final water content of 42–44% and a pH of 7.0 ± 0.5 through the addition of water and calcium carbonate. According to the guideline, sediment was stored for seven days in the dark at room temperature prior to spiking procedures.

To spike the sediment with PAHs, 10% of the conditioned sediment was dried at 105°C over night and thoroughly ground to a fine powder. The PAHs were dissolved in a mixture consisting of 3.5 L *n*-hexane and 1.5 L acetone and subsequently added to the dried sediment. Target concentrations per kilogram dry weight of PAHs in prepared sediment were as follows: phenanthrene 5.0 mg, pyrene 4.1 mg, chrysene 3.3 mg, benzo[*a*]pyrene 8.3 mg. The sum of total PAHs (20.7 mg $kg^{-1}$ dry weight) is within the range found in moderately to heavily contaminated fluvial sediments (Keiter et al. 2006, Wölz et al. 2008). According to the adapted ATV (Abwassertechnische Vereinigung e.V., ATV-M362-1 1997) classification scheme (Ahlf et al. 2002), spiked sediments prepared for this study can be classified as class IV (heavily contaminated) sediments. Solvents were allowed to evaporate for 72 h, and then water was

added to restore the original composition of the sediment. The treated sediment aliquot was then combined with the remaining sediment and thoroughly homogenized in a cement mixer. Prepared sediments were then conditioned at room temperature (18–22°C) for two different time periods in the dark. Due to the high proportion of sand (74.5% dw), the mean grain size (0.178 mm) was higher than for natural fluvial sediments. Sediment for experiments 1, 2 and 3 was stored for seven days, whereas sediment for experiment 4 was stored for 32 days to evaluate the influence of storage time on sediment cohesiveness and erosion behaviour. Installation of sediments in the annular flume was carried out manually (placed bed) and proved to be suitable for conducting large-scale experiments.

### 4.3.3 **Experimental setup**

Experiments were conducted in the annular flume that is located at the Institute for Hydraulic Engineering and Water Resources Management (RWTH Aachen University, Germany). The annular flume was originally designed to investigate the transport behaviour of cohesive sediments (Spork 1997). The flume consists of a circular glass channel (width 0.25 m, radius of circle 1.5 m) and a coaxial hanging Plexiglas lid. Channel and lid rotate in opposite directions producing an endless flow that resembles a COUETTE-current (Spork et al. 1994, Spork et al. 1995). The flow velocity in the annular flume was controlled by the rotation rate. To ensure an equally distributed shear stress over the channel width, a mean water depth of 175 mm and a rotational speed ratio of lid to channel of –1.6 were applied (Spork 1997). The lid was positioned on top of the water surface to generate the desired flow.

In order to characterize the transport behaviour of (un-)contaminated sediment, it was necessary to measure turbidity and take suspended matter samples during erosion and deposition processes. The samples were needed to calibrate measurements of turbidity. Here, we followed an integrated approach and performed a simulated flood event with continuously changing bed shear stress. In contrast to a standard erosion experiment that allows a detailed characterization of each sediment layer, this kind of remobilization allowed a rough estimate of fundamental erosional characteristics. In order to evaluate the new interdisciplinary method it was important to link all influencing parameters in their most natural way and define compromises to fulfil the aims of the interdisciplinary work.

### 4.3.4 **Experimental program**

Five experiments were conducted in the annular flume (Table 4.2).

Table 4.2 Experimental program.

| Experiment | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Sediment storage time | 2 weeks | 2 weeks | 2 weeks | 4 weeks | - |
| Sediment consolidation time | 3 days | 3 days | 3 days | 3 days | - |
| PAH concentration | - | - | 20.73 mg $kg^{-1}$ | 20.73 mg $kg^{-1}$ | - |
| Number of test animals | - | 15 | 15 | - | 15 |
| Max. flood intensity [$\tau$] | 0.40 N $m^{-2}$ | 0.30 N $m^{-2}$ | 0.30 N $m^{-2}$ | 0.40 N $m^{-2}$ | 0.40 N $m^{-2}$ |

Sediments prepared for each experiment - either unspiked (experiments 1 and 2) or PAH-spiked (experiments 3 and 4) - were inserted into the annular flume and smoothed manually to create a homogenous sediment layer of 4 cm (Figure 4.1). Subsequently, water was carefully added to a height of 195 mm above the sediment surface avoiding sediment disturbance. Experiment 5 served as control experiment and was conducted without sediment. After introduction of sediments and a consolidation time of three days (Schweim 2005), rainbow trout were added to the annular flume and exposed for five days under simulated flood conditions. Details on fish maintenance and biomarker studies are provided in Brinkmann et al. (2010a).

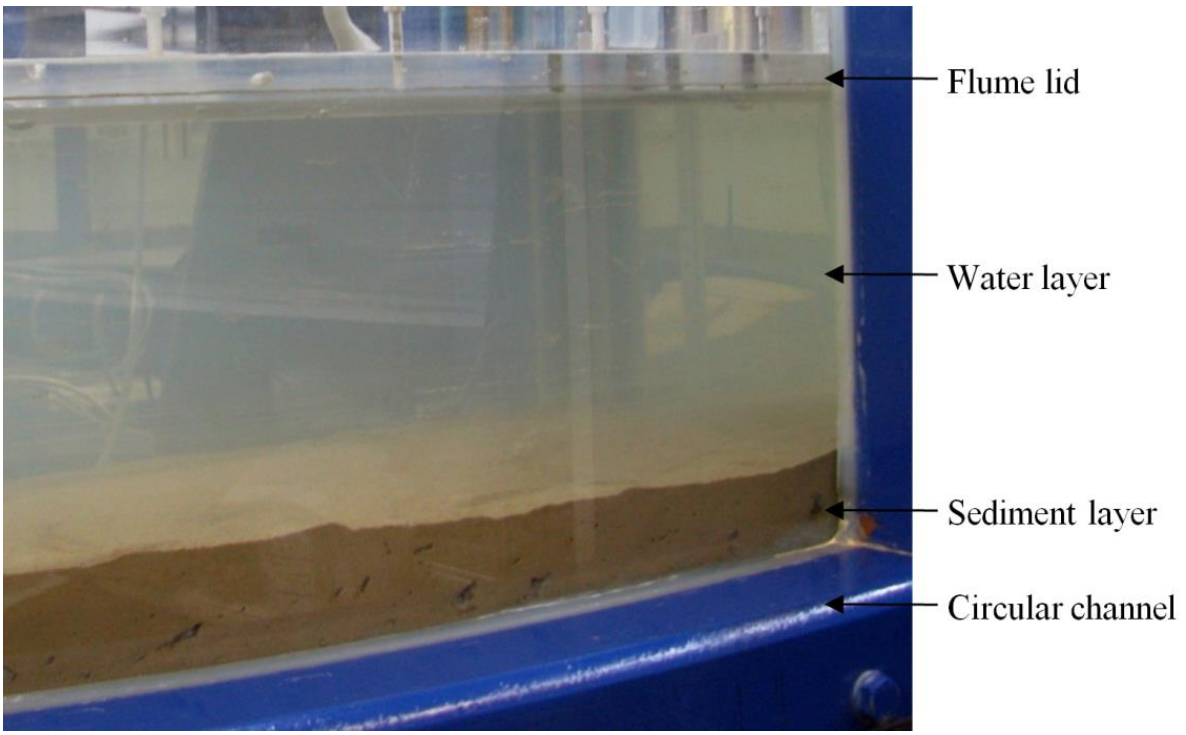


Figure 4.1 Installed sediment during consolidation in the annular flume. Approx. 4 cm sediment layer and 17.5 cm of water layer.

### 4.3.5 **Hydrograph**

The annular flume was operated to simulate bed shear stress variations according to a hydrograph based on the German standard 4049-3 (DIN 4049-3, 1994b). The shape of the hydrograph was adapted to meet operational requirements of the annular flume. Natural flood events were characterized by variation of discharge over time followed by a variation of water levels resulting in varying bed shear stress levels. As flow depth cannot be adjusted in the annular flume, the rotational speed of channel and top lid were changed in accordance with the shape of the flood curve to increase the flow velocity and the bed shear stress leading to resuspension of sediment. The flood curve used was a theoretical derivation of a natural flood event and did not represent all characteristics, including naturally occurring shear stress variations. It is characterized by a steep increase and a smoother decrease of the bed shear stress over time (Figure 4.2). Flood duration of five days was chosen as a compromise between suitable fish exposure time and maximum continuous operation time of the annular flume. The decreasing part of the flood started around hour 35. The increasing and the decreasing part of the simulated flood event were evaluated separately.

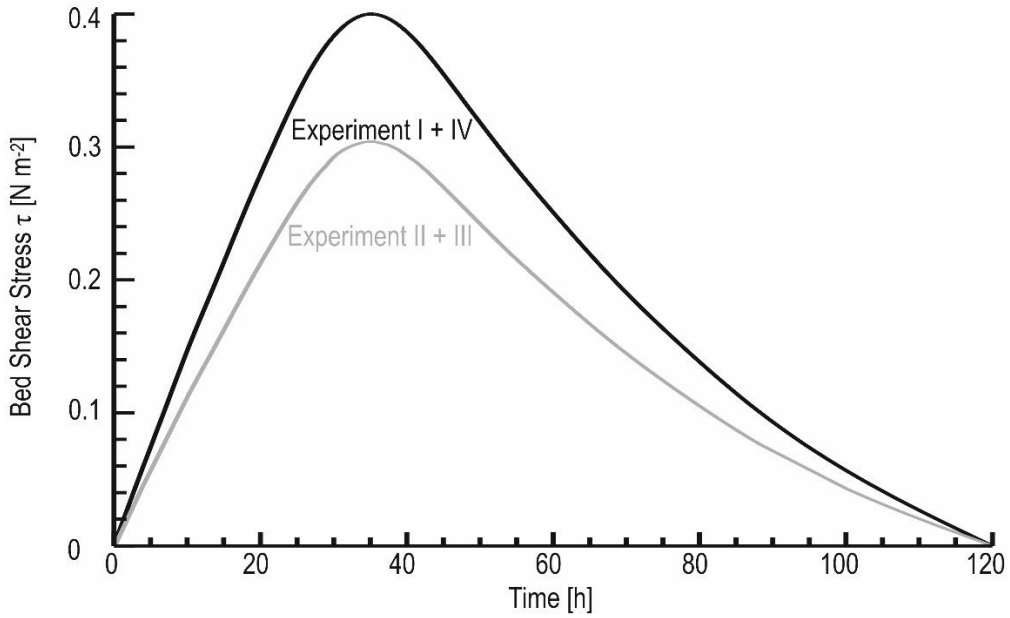


Figure 4.2 Simulated flood curves according to DIN 4049-3 (DIN 4049-3, 1994b).

Two different flood curves were simulated. During experiments 1, 4 and 5 the maximum bed shear stress was $\tau$ =0.40 N $m^{-2}$ and for experiments 2 and 3 the maximum bed shear stress was $\tau$ = 0.30 N $m^{-2}$ (see Figure 4.2). Pre-tests were conducted to measure the flow velocity at different rates of rotations with Laser Doppler Velocimetry (LDV). The logarithmic law of the

wall with a correction of wall-friction was used to determine the applied bed shear stress that results from measured flow in the near-bed region (Spork 1997).

### 4.3.6 **Instrumentation**

Prior to initiation of the experiments, the annular flume was modified to enable optimum test conditions for rainbow trout (water temperature of approx. 12°C, dissolved oxygen (DO) concentrations close to saturation, pH 7-8 (Eddy & Underhill 1974)) to be maintained. The water was cooled using a flow-through cooling device (Teco TR 20, Ravenna, Italy) and aerated with an air stone. Six measurement ports were used to measure temperature, pH and DO continuously throughout the experiments. In order to monitor the resuspension of the sediment, turbidity of the test solution was determined using a method developed by the Institute of Hydraulic Engineering. Specifically, the suspension was pumped through a glass pipe equipped with a light source (diode) and an opposite sensor, which measures light transition through the suspension. Turbidity is inversely proportional to the light intensity at the sensor. This measuring technique allows the measurement of a wide variety of suspended sediment concentration. Multiple suspended solid samples (250 ml each) of varying concentrations were used to calibrate the turbidity instrument.

Samples of suspension were taken in intervals of $\tau_i = \tau_{i-1}+\Delta\tau$ with $\Delta\tau = 0.5$ N m$^{-2}$. The first sample was taken at $\tau_0 = 0.0$ N m$^{-2}$. Samples were taken 95 mm above the flume bed.

### 4.3.7 **Extraction and sample preparation**

Aliquots of the differently treated sediments (from experiments 1 and 2, both samples were taken before the experiments started) were transferred to round-bottom flasks (Schott, Mainz, Germany), shock-frozen using liquid nitrogen and immediately thereafter freeze-dried (Christ Alpha 1-2, Martin Christ GmbH, Osterode am Harz, Germany) for 48 to 72 h. Dried sediments were homogenized and 20 g portions were extracted by means of Soxhlet extraction with acetone (Sigma Aldrich) at 8–10 cycles per hour for 14 h. Each extract was reduced to a volume of approximately 5 ml. After concentrating extracts close to dryness under a gentle nitrogen stream (Hollert et al. 2000), each extract was dissolved in 2 ml of *n*-hexane p.a. (Sigma Aldrich) and split into two aliquots of 1 ml each. One aliquot was concentrated again close to dryness and dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich) for use in the EROD bioassay with the permanent cell line RTL-W1, while the other aliquot was analyzed for PAHs. The

resulting sediment equivalent concentration (SEQ), of each extract was 10 g dry sediment per 1 ml solvent. All extracts were stored at -20°C until further analysis. An empty extraction thimble that was subjected to the same extraction procedures as described above was included as a process control.

### 4.3.8 **EROD bioassay**

Induction of EROD (7-ethoxyresorufin-*O*-deethylase) was determined with the permanent cell line RTL-W1 according to Behrens et al. (1998) with modifications (Gustavsson et al. 2004, Seiler 2004). RTL-W1 cells (Lee et al. 1993) were obtained from Drs. Niels C. Bols and Lucy Lee, University of Waterloo, Canada. Dioxin-like activities of sediment extracts were expressed as biological toxicity equivalency concentrations of TCDD (bio-TEQs, equation 4.1) to enable comparison of $EC_{25}$ values among different samples (Keiter et al. 2008, Wölz et al. 2008). Fixed effect level-based bio-TEQs were calculated according to Engwall et al. (1996) and Brack et al. (2000). For the calculation of bio-TEQ values of the sediments of experiments 1 and 3, three and six independent replicates were used, respectively.

$$Bio - TEQ\left[\frac{pg}{g}\right] = \frac{TCDD_{25}\left[\frac{pg}{ml}\right]}{EC_{25}\left[\frac{g}{ml}\right]} \qquad \text{(Eq. 4.1)}$$

### 4.3.9 **Chemical 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 280°C; injection volume 1 µl in splitless mode; GC-MS transfer line temperature 320°C; ionization by electron impact at 70 eV; oven temperature program: 50°C (5 min) ramped up at 10°C $min^{-1}$ to 280 °C and held for 5 min, then increased at 10°C $min^{-1}$ to 320°C and held for 6 min. The MSD was operated in scan mode. Data acquisition and processing was performed with the Agilent Technologies MS ChemStation data analysis software and the NIST MS Search Program. Recovery of the PAHs was calculated after PAHs were quantified by means of an external six point calibration curve derived from a standard stock solution (DE-PROM 16, LGC Standards,

Wesel, Germany) containing unlabelled EPA-PAHs. Calibration curves were then fitted linearly using GraphPad Prism 5.0 where the coefficients of determination ($r^2$) for the different calibration curves ranged from 0.98 to 0.99.

Based on measured contaminant concentrations, chemical toxicity equivalency concentrations (chem-TEQs) were calculated and then compared to bio-TEQ values as derived with the EROD bioassay. These TEQs were generated by multiplying measured compound concentrations with corresponding REP values as determined by Bols et al. (1999), specifically for RTL-W1 cells and each PAH investigated.

Limits of detections (LODs) and quantifications (LOQs) were derived from calibration curves (Hubaux & Vos 1970) and calculated according to DIN 32645 using an Excel spreadsheet and were between 1 and 2.6 ng $\mu l^{-1}$ injected and 4 and 10.4 ng $\mu l^{-1}$ injected, respectively (see Table 4.4).

## 4.4 **Results**

### 4.4.1 **Physicochemical parameters**

All five experiments were successful concerning stable physicochemical parameters. Results for experiment 5 are not given due to technical problems during saving processes which resulted in the loss of raw data. DO, temperature and pH were relatively constant throughout all experiments (Table 4.3).

Table 4.3 Results of physicochemical parameters.

| Experiment | DO [mg $l^{-1}$] | Temperature [°C] | pH value [-] |
|---|---|---|---|
| 1 | 8.5 ± 0.89 | 12.6 ± 0.72 | 7.64 ± 0.67 |
| 2 | 9.3 ± 2.9 | 13.2 ± 1.12 | 7.93 ± 0.72 |
| 3 | 8.5 ± 0.59 | 12.7 ± 0.66 | 7.92 ± 0.62 |
| 4 | 10.0 ± 0.11 | 12.6 ± 0.68 | 8.20 ± 0.66 |

Average temperature across all four experiments was 12.8 ±0.28°C (mean ± SD). Average DO and pH values were between 8.5 and 10.0 mg $L^{-1}$, and between pH 7.64 and 8.2, respectively. In all experiments, the DO concentration was close to saturation. Greatest values for DO and pH were measured in experiment 4. In experiment 1 and 2 the measuring cell plugged resulting in the outliers observed at the 20 and 40 hour interval after the beginning of the experiment and does not represent the conditions within the annular flume (Figure 4.3).

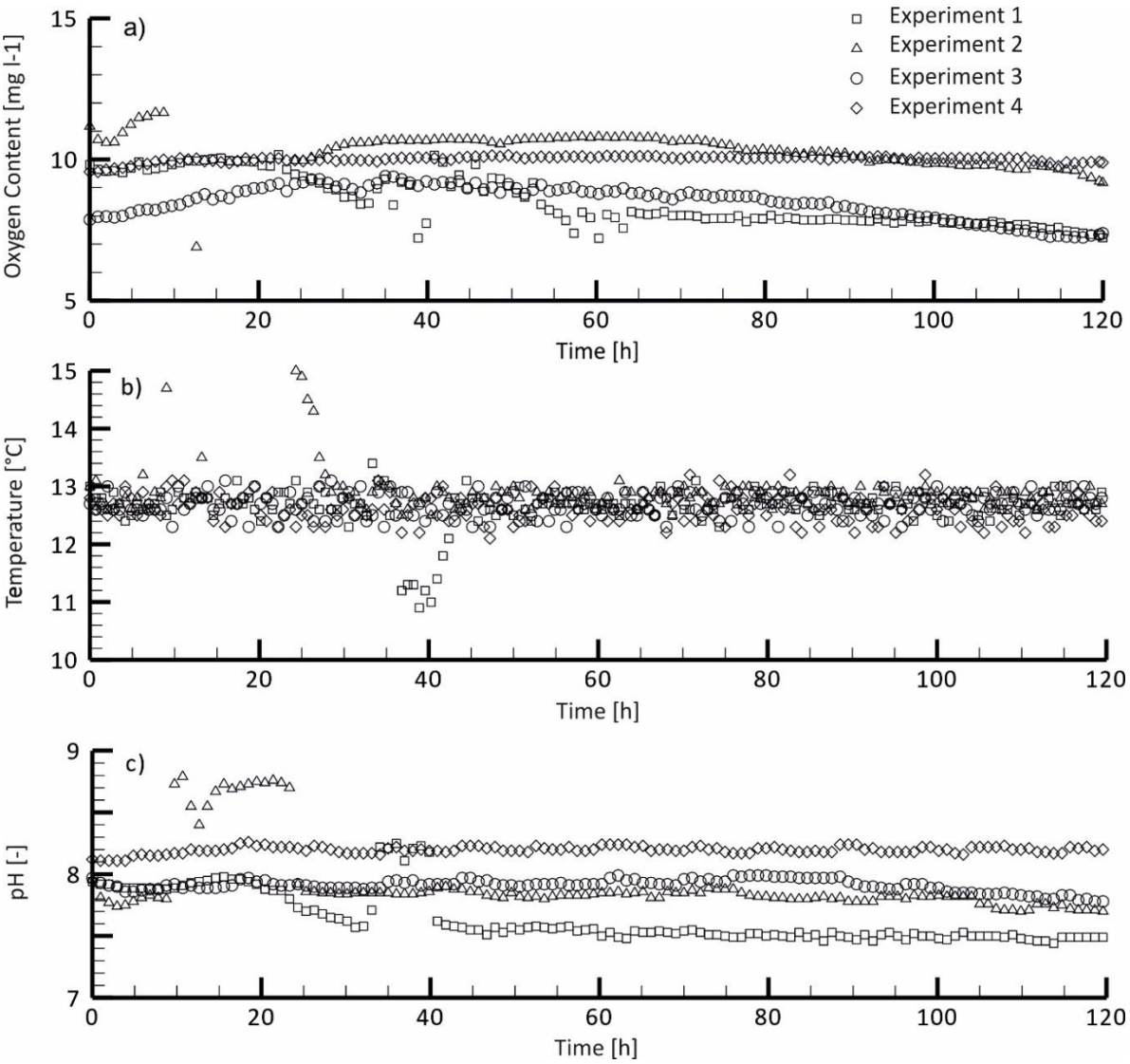


Figure 4.3 Physicochemical parameters during the course of experiments 1, 2, 3 and 4 (a, dissolved oxygen; b, temperature; c, pH value). Dots in each plot present the individual measured values.

### 4.4.2 **Sedimentological parameters**

With the exception of experiment 4, there were significant changes in sedimentological parameters as a function of increasing shear stress in all experiments (Figure 4.4 a and b). Sediments were erodible in experiments 1, 2 and 3, and the critical shear stress in these experiments occurred at $\tau$ values between 0.15 and 0.25 N $m^{-2}$(Cofalla et al. 2010b). The changes of suspended sediment during increasing bed shear stresses are described with the given equations for experiments 1, 2 and 3. The initial concentrations of suspended sediment (C) in experiments 2 and 3 were = 0.4 g $l^{-1}$. The resuspended sediment concentration in experiment 4 stayed below C = 0.4 g $l^{-1}$ during the entire time. The measured increase of suspended sediment was negligible in this experiment. Hence, the critical shear stress of mass

erosion was not reached during the course of experiment 4. The sediment concentration at the end of the experiments decreased to values between 4.03 g $l^{-1}$ (in experiment 3) and 7.80 g $l^{-1}$ (in experiment 1).

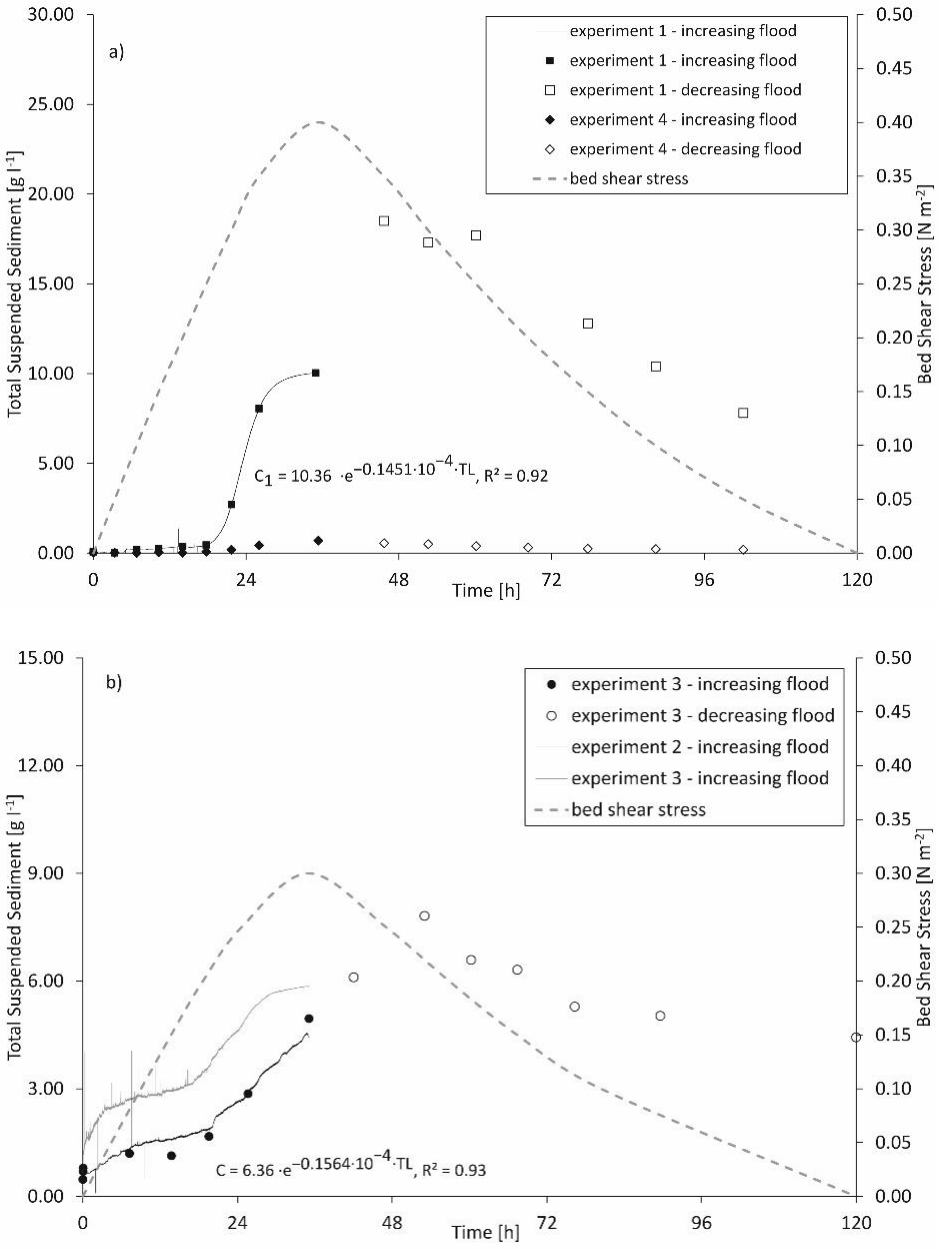


Figure 4.4 Development of suspended sediment concentration and bed shear stress over: a) experiments 1 and 4 (performed without fish); and b) experiments 2 and 3 (performed with fish).

### 4.4.3 **Preparation of formulated sediment**

The process of sediment preparation was complicated by the procedures listed below. According to OECD guideline 218, peat was dried for several days followed by a grinding process which required a suitable grinding device. The available laboratory-grade device was,

however, undersized and prolonged the whole grinding process. The watering and homogenizing process required a specially designed stirring device. For safety reasons, the whole procedure of spiking such large volumes of sediment had to be carried out with great care (experiments 3 and 4).

### 4.4.4 **Recovery of PAHs**

Chemical analysis revealed measurable concentrations but somewhat low recovery rates of PAHs in the spiked sediment, while concentrations of all PAH congeners in the control sediment were below the LOD (Table 4.4). Average PAH recovery was 65% of nominal concentrations. Highest recovery rates, between 63 and 74%, were determined for benzo[*a*]pyrene and phenanthrene, respectively. Least recovery occurred for chrysene (52%) and pyrene (57%).

Table 4.4 Recovery of PAHs used for spiking the formulated sediment in experiment 3. Note: PAH concentrations in control sediments (experiment 1) were below the LODs of individual PAHs.

| Substance | LOD [µg $g^{-1}$dw] | LOQ [µg $g^{-1}$dw] | Spiked concentration [mg $kg^{-1}$] | Mean measured concentration [mg $kg^{-1}$]; n = 2 | Recovery [%] |
|---|---|---|---|---|---|
| Phenanthrene | 0.18 | 0.71 | 5.0 | 3.1 | 63 |
| Pyrene | 0.1 | 0.4 | 4.1 | 2.4 | 57 |
| Chrysene | 0.14 | 0.55 | 3.3 | 1.7 | 52 |
| Benzo[*a*]pyrene | 0.26 | 1.04 | 8.3 | 6.2 | 74 |
| Sum PAH/ Mean recovery | - | - | 20.7 | 13.4 | 65 |

### 4.4.5 **Bio-TEQ and chem-TEQ values**

Dioxin-like potentials (bio-TEQs) determined in bioassays differed significantly (t-test, $p \leq 0.05$) between the reference (experiment 1) and PAH spiked sediment (experiment 3, Figure 4.5). Bio-TEQs for the reference sediment were less than 100 pg $g^{-1}$ sediment dw. Biological analysis of PAH spiked sediment samples revealed a mean bio-TEQ of 855 pg $g^{-1}$ sediment (see Figure 4.5). For the sediment from experiment 1, a chem-TEQ of 8.51 pg $g^{-1}$ sediment dw was calculated based on determined LOD values. Chemical analysis revealed a mean chem-TEQ of 1826 pg $g^{-1}$ sediment dw for the sediment from experiment 2 (see Figure 4.5). Chem-TEQs from experiments 1 and 3 differed significantly (t-test, $p \leq 0.05$).

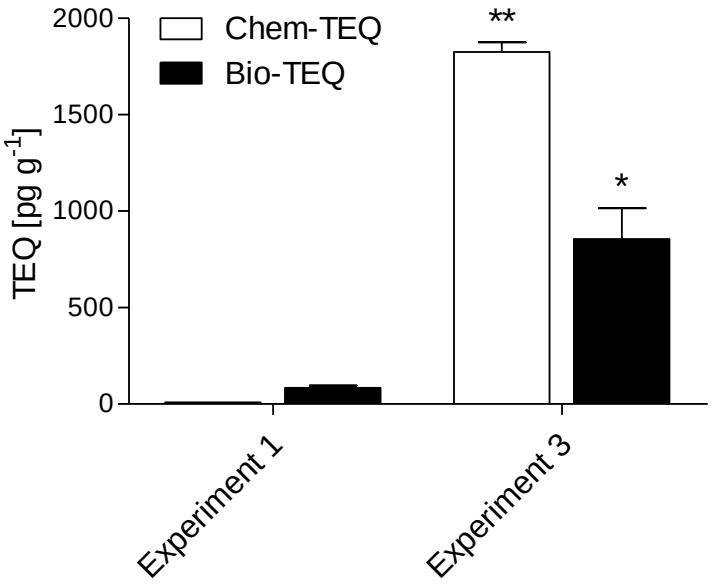


Figure 4.5 Comparison of bio-TEQ and chem-TEQ values calculated for sediment samples of experiment 1 and 3 (unspiked and spiked, respectively); bars represent mean values with standard deviations; * significant difference of bio-TEQs (t-test, $p \leq 0.05$), ** significant difference of chem-TEQs (t-test, $p \leq 0.05$).

## 4.5 **Discussion**

This study demonstrated that the modified annular flume is generally suitable for conducting coupled hydraulic and ecotoxicological investigations in support of the assessment of potential impacts to fish of the resuspension of contaminated sediments during flood events. However, this proof-of-concept study revealed a number of limitations associated with this interdisciplinary approach that are described below.

### 4.5.1 **Formulated sediment**

A prerequisite for the use of the annular flume as a test system for coupled eco-toxicological and erosion studies is the availability of large volumes of natural sediments of constant quality in terms of grain-size composition, geologic and anthropogenic baseline contamination and comparable content of extracellular polymeric substances (EPS). Alternatively, controlled studies can be conducted using formulated sediments, which offer certain benefits over natural sediments, such as the absence of indigenous fauna and contaminants as well as a known composition (Goedkoop et al. 2005). In contrast, formulated sediments have limitations such as lower biological activity, different redox conditions and simplified organic matter composition compared to natural sediments (Suedel & Rodgers 1994, Verrhiest et al. 2002). For the proof-of-concept study presented here we used artificial sediment to avoid introducing

additional variations due to inhomogeneous, natural sediment in order to verify the newly developed experimental methodology. However, the mean grain size of the artificial sediment was higher (0.178 mm) compared to natural fine-grained fluvial sediments which often have lower mean grain sizes due to the high proportion of clay and silt. For example, sediment from the River Rhine, which will be used in future studies, has a mean grain size of 0.0062 mm. Pre-tests showed a strong segregation of the artificial sediment components in the order (bottom to top) sand-peat-kaolinite. In order to achieve a homogenous sediment layer we had to install a placed bed, although the erosional behaviour of this differs from settled beds (Mehta & Partheniades 1982). A settled bed represents a more realistic sediment structure. Natural uncontaminated sediments with a clay and silt content of 80-90% will be used in future experiments.

This study revealed that sediment storage conditions are of high relevance and can significantly influence erosion characteristics. Sediments that were stored for extend periods of 32 days (experiment 4) showed a completely different consistency which led to a higher erosional resistance compared to sediment stored for 7 days (experiments 1, 2 and 3).

### 4.5.2 **Provision of stable environmental conditions**

One of the basic requirements of the methodology was the establishment of a stable environment in the annular flume that was suitable for the test species (rainbow trout). This study clearly demonstrated that the environmental conditions in the annular flume were such that they did not affect survival or behaviour during a five day experiment (Brinkmann et al. 2010a). The modified flume enabled maintenance of constant temperatures in all the experiments conducted. However, there were slight differences in DO and pH, which were likely related to the suspension of sediments and pollutants. Former investigations showed that water quality parameters such as pH and redox conditions changed in the water column when cohesive sediments were suspended. Due to oxidative processes that occur during suspension of anoxic sediment layers, redox-potential and pH-value can increase and decrease, respectively. These changes can be of toxicological relevance because the variation of redox- and pH-values has been shown to influence the release or bioavailability of former sediment-bound toxic substances (Calmano et al. 1992, Calmano et al. 1993, Cantwell et al. 2002, Koelling 1986, Salomons et al. 1987, Stumm 1992, Zoumis et al. 2001), e.g. a decrease of the pH value increases the freely dissolved heavy metal concentration. Hence, for a greater understanding of the environmental conditions in the annular flume, and to be able to identify

variations due to sediment suspension, additional parameters (such as conductivity and redox potential) should be measured in future experiments.

### 4.5.3 **Resuspension of sediments**

The study demonstrated that it was possible to erode artificial OECD-sediment in the annular flume and roughly characterize its transport behaviour relative to natural sediments. In order to erode the sediment bed in the annular flume, a simulated flood event was chosen. A flood event is one way to undertake the integrated approach that combines all influencing parameters during contaminated sediment transport, in a natural way. The chosen study design was successful in enabling determination of sediment transport and biological effects of contaminated sediments on fish in the same experiment. These results will inform further combinatory experiments that will test eroding and ecotoxicological properties of natural sediments from a settled bed in the annular flume.

The maximum bed shear stress of the simulated flood event was determined in pre-tests. In the first experiment, with a maximum $\tau$ of 0.4 N $m^{-2}$, technical problems were encountered (e.g. plugging of pipes, abrasion of pumps) preventing the reliable measurement of physicochemical parameters. Hence, we reduced the bed shear stress in experiments 2 and 3 to a maximum $\tau$ = 0.3 N $m^{-2}$. Another limitation of the chosen experimental design was that the simulated flood did not allow a detailed characterization of the transport processes, for example erosional progress over sediment depth. However, it was possible to define the beginning of erosion and observe deposition over the course of the study in order to successfully test the combination of biological and hydrological methodologies. The maximum bed shear stress chosen in the present study caused considerable erosion of the formulated sediment. Suspended particulate matter concentrations achieved during the experiments were comparable to concentrations measured during flood events in small European streams. For example, Grasso et al. (2007) reported maximum concentration between 5.3 and 9.3 g $l^{-1}$ during high discharge events, whereas Oeurng et al. (2010) reported concentrations up to 15.74 g $l^{-1}$. However, critical bed shear stress, as well as resulting suspended particulate matter load, during flood events are dependent on a multitude of factors such as sediment composition and resulting sediment stability, topography and vegetation cover of the catchment area, as well as flood magnitude and therefore individual rainfall events (e.g. Dieckmann et al. 1985, Lenzi & Marchi 2000, Lopez-Tarazon et al. 2009, Oeurng et al. 2010, Rovira & Batalla 2006).

More detailed experiments will be undertaken in a follow-up study. Specifically, a natural deposit sediment bed will be used in the annular flume and stressed with a stepwise increasing bed shear stress as described by Mehta et al. (1982).

### 4.5.4 **Recovery of PAHs**

There were differences of up to 31% among recovery of the four PAHs used for spiking. The highest recovery rate (74%) was observed for benzo[*a*]pyrene. Phenanthrene, the only low molecular weight PAH, showed greater recovery (63%) than chrysene and pyrene (52% and 57%, respectively). These findings are in accordance with the literature, as different PAH congeners undergo different degradation processes such as adsorption, volatilization, photolysis and microbial degradation; the latter of which was likely to have had the greatest impact on the observed PAH degradation (Haritash & Kaushik 2009). Microbial degradation has been shown to be highly effective for low molecular weight PAHs (Banerjee et al. 1995, Kastner & Mahro 1996, Shuttleworth & Cerniglia 1995), while high molecular PAHs are generally more recalcitrant against such degradation (Erickson et al. 1993, Juhasz & Naidu 2000, Park et al. 1990, Shuttleworth & Cerniglia 1995), especially during short incubation times as tested in this study. Furthermore, in mixtures of low and high molecular weight PAHs, low molecular weight PAHs are degraded preferably. However, there are a number of other potential factors, such as adsorption of PAHs to plastic surfaces during sample storage in PE jars and volatilization as well as photolysis during sediment preparation, storage and during the experiment course that could have led to the observed reduction of recovery rates. Despite these differences in spiking efficiency, the spiking procedure was suitable to obtain PAH concentrations in the formulated sediment comparable to PAH concentrations found in natural fluvial sediments. For example, Hilscherova et al. (2007) reported mean 16-EPA - PAH concentration between 7.6 and 23.7 mg $kg^{-1}$ for sediments of Czech Republic rivers, and Feiler et al. (2009) found mean 16-EPA-PAH concentrations between 8.4 and 15.0 mg $kg^{-1}$ in contaminated sediments collected from the River Elbe and some of its tributaries.

### 4.5.5 **EROD induction potential**

Extracts of spiked sediments caused significantly increased EROD induction in the *in vitro* assay using the permanent cell line RTL-W1 compared to control sediments. The spiked sediment of experiment 3 had a moderate mean bio-TEQ value (855 pg $g^{-1}$ SEQ) and EROD inducing potential compared to findings of Wölz et al. ((2008), who determined a bio-TEQ

value of 1900 pg $g^{-1}$ for River Elbe sediments and bio-TEQ values between 1160 and 6640 pg $g^{-1}$ in suspended particulate matter samples from the River Rhine. Hollert et al. (2002) reported an EROD induction potential of 1767 pg $g^{-1}$ for sediments from a small creek (Forellenbach) in Baden-Württemberg, Germany, which is heavily contaminated with PAHs. Another study, which investigated the contamination of Baltic Sea sediment cores (Wölz et al. 2009a), revealed very high dioxin-like activities with a highest bio-TEQ of 8920 pg $g^{-1}$at a heavily polluted former dumping site. Heimann et al. (2011) reported bio-TEQ values between 3620 and 7920 pg $g^{-1}$ for sediments from Upper Rhine oxbow lakes. Hence, AhR-mediated activities of spiked sediment extracts were within the range of activities that were found in other studies investigating contaminated sediments in German river systems with comparable contamination. Thus, the spiked sediment was suitable as a standard substitute for natural contaminated sediment to perform studies addressing basic mechanisms of resuspension and uptake in fish, as shown by Brinkmann et al. (2010a).

Reference sediment (experiment 1) showed low but clear EROD inducing potential. Humic acids, which are the main compounds of dissolved organic matter from soils and sediments (Steinberg et al. 2003), were previously shown to induce EROD activity (Bittner et al. 2006). Therefore, it can be assumed that the low bio-TEQ values (< 100 pg $g^{-1}$) are attributed to humic acids originating from the peat portion of the formulated sediment. This hypothesis is further supported by the chemical analysis that was performed with sediment and SPM extracts of experiment 1, revealing no PAH contamination of the ingredients of the formulated sediment (data not shown). Calculated chem-TEQ values correlated well with the corresponding bio-TEQs. However, chem-TEQs for experiment 3 were 2-fold higher than the corresponding bio-TEQs. This can be attributed to the lipophilic nature of PAHs which tend to adsorb, for example, on the plastic surfaces of 96-well plates during the EROD bioassay, thus lowering actual concentration and therefore the potential EROD inducing potential. Several studies support this hypothesis by documenting that the use of microtiter plates made of polystyrene can result in underestimation of the toxic potential of chemicals with specific properties, mainly volatility and lipophilicity, because the concentration of the investigated chemicals does not stay constant over the time of exposure (Pery et al. 2001, Riedl & Altenburger 2007, Schreiber et al. 2008).

While this study revealed significant and marked induction of *in vitro* EROD activity as a result of the exposure to spiked sediments, no such induction was observed in a parallel study that investigated EROD induction in trout exposed in the annular flume to the same sediments

(Brinkmann et al. 2010). These differences may be attributed to the anticipated rapid metabolization and elimination of low PAH concentrations in fish (Kammann 2007a, Meador et al. 1995b). Furthermore, *in vitro* EROD induction potential was assessed using sediment extracts obtained by Soxhlet extraction with organic solvents, thus representing the total extractable fraction of PAHs. In contrast, the EROD induction in fish liver, being an *in vivo* measurement, accounts only for the bioavailable fraction of the PAHs; either dissolved in water or readily desorbed from SPM at the gills or intestines. To further elucidate the bioavailability of PAHs during resuspension events, additional investigations would be necessary. Specifically, it is recommended to use extraction methods that more realistically resemble uptake of contaminants by an organism such as hydroxypropyl-β-cyclodextrin (HPCD, Reid et al. 2000b), Tenax (Cornelissen et al. 1997, Zielke et al. 2010) or methanol extraction (Barriuso et al. 2004), as well as passive sampling techniques such as semipermeable membrane devices (SPMD, Huckins et al. 1990).

## 4.6 **Conclusions and outlook**

This project successfully combined hydraulic and ecotoxicological methodologies in a single experimental setup. The newly developed approach allows the investigation of hydrodynamic events, such as erosion and deposition behaviour of sediment during floods, in combination with a variety of ecotoxicological endpoints within a stable and controllable environment.

However, there were a number of limitations that require further optimization of the current approach. The test setup should be optimized to reduce the influence of exposed fish on sediment stability, and therefore, to reduce sediment disturbance at low bed shear stress levels by, for example, increasing the water depth. Furthermore, the preparation of great amounts of artificial sediment was generally feasible but not practical. The use of industrial grade equipment for sediment preparation is advised in order to reduce preparation time.

As a next step we plan to substitute formulated material with natural reference material, such as moderately contaminated sediment from, for example, a sampling location in the River Rhine at Ehrenbreitstein close to Koblenz, to assess the erosion behaviour and associated remobilization of particle-bound toxicants in natural environments. This will help to extrapolate studies within the annular flume to conditions in natural flowing waters.

In conclusion, the proof-of-concept hydrotoxicological study within the annular flume represents a novel and promising approach to deepen the understanding of remobilization characteristics of contaminated natural sediments and associated effects on aquatic organisms in the field, thus helping to establish a more realistic approach to sediment management and sediment risk assessment. Based on this approach, it will be possible to develop new process models, interdisciplinary evaluation methods and environmental criteria to combine different facets like flood risk, sediment dynamics and ecotoxicological sediment quality.

## 4.7 **Acknowledgements**

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Chapter 5

# How flood events affect rainbow trout: Evidence of a biomarker cascade in rainbow trout after exposure to PAH contaminated sediment suspensions

# How flood events affect rainbow trout: Evidence of a biomarker cascade in rainbow trout after exposure to PAH contaminated sediment suspensions

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## 5.1 **Abstract**

Increasing frequency and intensity of flood events are major concerns in the context of climate change. In addition to the direct hydrological implications of such events, potential ecotoxicological impacts are of increasing interest. Here it is vital to understand mechanisms of contaminant uptake from suspended particulate matter (SPM) and related effects in aquatic biota under realistic conditions. However, little is known about these processes. Due to recent changes in climate, during summer temperatures of German rivers frequently exceed 25 °C. Effects of resuspension of sediments on biota under elevated temperature regimes are likely to differ from those under lower temperature regimes. To elucidate this differential response of aquatic vertebrates, rainbow trout were exposed to suspensions of sediment from the Rhine River that was spiked with a mixture of polycyclic aromatic hydrocarbons (PAH). The experiments were conducted under two different temperature regimes (24 °C or 12 °C). Physicochemical parameters, including concentration of PAHs in SPM, and biomarkers in fish (biliary PAH metabolites, 7 ethoxyresorufin O-deethylase activity, lipid peroxidation, gene expression and micronuclei) were measured over the course of a 12 d study. Concentrations of pyrene and phenanthrene decreased over time, while no decrease was observed for chrysene and benzo[a]pyrene. The biomarker cascades, more specifically the temporal dynamics of biomarker reactions, did not only show quantitative differences (i.e. different induction intensity or rate of biomarker responses) at the two temperatures but also qualitative differences, i.e. different biomarker responses were observed. A slight significant increase of biliary metabolites in fish was observed in un-spiked sediment at 24 °C. In bile of fish exposed to PAH spiked sediment concentrations of 1 hydroxypyrene and 1 hydroxyphenanthrene increased significantly during the first two days, and then decreased. At 12 °C uptake of PAHs was slower and maximum metabolite concentrations in bile were lower than in fish exposed at 24 °C. Following a latency of two days, concentration of PAH metabolites in bile of fish exposed at 24 °C was followed by a peak in lipid peroxidation. PAHs spiked into sediments under laboratory conditions were significantly more bioavailable than the PAHs that were already present in un-spiked field-collected sediments.

**Keywords:** Sediment resuspension • Temperature stress • Micronuclei • Lipid peroxidation

## 5.2 . Introduction

As a potential implication of climate change, the number of extreme weather events such as droughts, storms or heavy rain falls which often lead to flood events has been increasing globally (Solomon et al. 2007). As a consequence, it has been predicted that both frequency and intensity of floods will increase in the coming decades (Ikeda et al. 2005, Kay et al. 2006). Not only are there direct hydrological implications of floods that pose a risk to humans and ecosystems, but there will also be the risk of resuspension of historically polluted sediments in their river basins. Under non-flood conditions, sediments are usually a sink for both inorganic and organic contaminants. However, upon resuspension, these sediments can constitute a secondary source of pollutants, which consequently become available for uptake into aquatic organisms (Ahlf et al. 2002, Hollert et al. 2003a, Wölz et al. 2010b) and – among other factors – cause that quality goals of the European Water Framework Directive (WFD) might not be met in many river basins (Wilby et al. 2006). To date, the complexity of the various interactions between sediments, pollutants and biota has been little investigated (Ciarelli et al. 1999, Ciutat et al. 2006, Gerbersdorf et al. 2011, Hyötyläinen et al. 2002, Reible et al. 1996, Roberts 2012).

Besides the occurrence of extreme floods, temperatures in rivers have been constantly increasing during the past few decades. In the case of the Rhine River, Germany, the annual average temperature has risen by 3 °C, of which 2 °C is likely caused by waste heat of power plants and 1 °C to climate change (IKSR 2004). The number of days per year with temperatures exceeding 23 °C has increased from less than 20 in the 1960s to 40-50 in the 1990s (IKSR 2004). Days with temperatures exceeding 25 °C are also more frequent.

Metabolic rates of poikilothermic organisms as well as desorption of pollutants from sediments are temperature-dependent (Noyes et al. 2009). It has been shown that the toxicity of sediments and bioaccumulation of pollutants can be temperature-dependent (e.g. Airas et al. 2008, Heinonen et al. 2002, Honkanen & Kukkonen 2006, Ng & Gray 2011). Additionally, besides the influence of temperature itself (Hari et al. 2006) effects of environmental stressors, such as ammonia or acidification, were more severe under temperature stress (Morgan et al. 2001). Thus, it is plausible to expect that toxicity and resulting effects of sediment-bound contaminants remobilized during flood events could also be affected as a consequence of climate change.

It has been proposed to include hydrodynamics, i.e. transport processes of water in fluvial systems, and sediment mobility as a more realistic approach to assess potential effects of

contaminated sediments (Brinkmann et al. 2010b, Chapman & Hollert 2006, Hollert et al. 2007, Wölz et al. 2009b). Until recently, however, toxicologists lacked appropriate laboratory methods to systematically investigate fates and effects of sediment-bound pollutants under flood-like conditions. A first attempt to provide such methodologies was made by the interdisciplinary project "Floodsearch", which was funded by the German Excellence Initiative at RWTH Aachen University, Germany (Wölz et al. 2009b). In this study, rainbow trout (*Oncorhynchus mykiss*) were exposed to artificial sediments that had been spiked with polycyclic aromatic hydrocarbons (PAH). These exposures were made during simulated 5 d flood events in an annular flume, i.e. a circular channel that is typically used for erosion and sedimentation studies. A set of different biomarkers was investigated after exposure and the hypothesis that resuspension of sediments can result in uptake of particle-bound contaminants and effects in aquatic biota was verified (Brinkmann et al. 2010a, Cofalla et al. 2012, Schüttrumpf et al. 2011). Apart from transient changes in several biomarkers, the frequency of micronuclei in peripheral erythrocytes - a definitive marker for genetic damage with potential to cause population-level adverse effects (Diekmann et al. 2004a, Diekmann et al. 2004b) - was significantly elevated after exposure to the contaminated sediment. The annular flume provides opportunities for testing of dynamics of contaminants in sediments and their accumulation into and effects on aquatic organisms (Cofalla et al. 2011). However, there is a major drawback of this method, which is that since contact of the instrument lid to the surface of the water is required for generation of the flow in the simulated flood event, animals, and samples of water and sediments cannot be taken during the simulation. Thus, additional experiments had to be conducted to be able to characterize the temporal trends of biomarker induction in exposed animals. It is of vital importance to biomarker experiments to fully understand the underlying physiological mechanisms and influencing factors (Forbes et al. 2006, Kammann et al. 2012).

The aim of the present study was to investigate the influence changes in temperature regime have on biomarker cascades during exposure to particle-bound PAHs. Here we report the results of experiments conducted to elucidate the time- and temperature-dependency of the toxicological properties of resuspended sediments on rainbow trout, which were exposed to suspensions of sediments. Sediment was used as collected from the environment or spiked with a mixture of PAHs representative of those occurring in sediments of rivers in Germany. These included: pyrene (PYR), phenanthrene (PHE), chrysene (CHR) and benzo[*a*]pyrene (BAP). To account for the influence of temperature on uptake and effects of pollutants bound to particles, the experiment was conducted at two average temperatures, 12 °C or 24 °C. The temperature

of 12 °C was chosen since optimal growth of rainbow trout was observed at 12-14 °C according to Johnson et al. (1987). The temperature of 24 °C was chosen to represent temperature stress. It is just below the IULT (incipient upper lethal temperature), i.e. the temperature at which animals do not tolerate further temperature increase, according to Bjornn & Reiser (1991). Concentrations of PAHs in suspended particulate matter (SPM), as well as uptake and biotransformation, as determined by concentrations of PAH metabolites in bile, were quantified. Several functional responses of rainbow trout that occurred during resuspension of pollutants bound to particulates were also measured. Expression of CYP1A (Cytochrome P450 1A) mRNA in liver was used as a measure of stimulation of phase I biotransformation of xenobiotics mediated by the aryl hydrocarbon receptor (AhR) on the transcript level. The mixed function mono-oxygenase (MFO) enzyme activity 7-ethoxyresorufin *O*-deethylase (EROD) was used as a measure of CYP1A activity. Glutathione-*S*-transferase (GST) and UDP glucuronyltransferase (UDPGT) mRNA expression were assessed as indicators of induction of phase II conjugation of xenobiotics. Expression of Caspase 3 mRNA was used as a measure of apoptosis (programmed cell-death). Lipid peroxidation (LPO) was used as a measure of oxidative stress. Formation of micronuclei (MN) in peripheral erythrocytes was used as a measure of genotoxicity. Biliverdin concentration in bile was measured to estimate gross energy metabolism.

## 5.3 **Materials and methods**

### 5.3.1 **Experimental design**

Juvenile rainbow trout were exposed to suspensions of a sediment from the Rhine River, either un-spiked or spiked with a mixture of the following PAHs (nominal concentrations in mg $kg^{-1}$ dw are given in brackets), purchased from Sigma-Aldrich (Deisenhofen, Germany): PYR (purity ≥ 99%, 4.1 mg $kg^{-1}$), PHE (purity 98%, 5.0 mg $kg^{-1}$), CHR (analytical standard, 3.3 mg $kg^{-1}$), and BAP (purity ≥ 96%, 8.3 mg $kg^{-1}$). Experiments were conducted in 750 L glass fiber-reinforced plastic containers (Figure 5.1) purchased from AGK Kronawitter (Wallersdorf, Germany). Submersible pumps (maximum flow-through 6000 L $h^{-1}$) were used to constantly suspend the sediments at a nominal concentration of 10 g $L^{-1}$. Tanks were aerated at a rate of 25 L $min^{-1}$. In the first experiment the mean temperature was 23.8 ± 0.5 °C, while in the second experiment the mean temperature was 11.9 ± 0.3 °C. Tanks were cooled using submersible coolers (Colora Tauchkühler, Lorch, Germany), which were controlled by analog

plug-in thermostats (UT100, Fuva, Erlangen, Germany). In each of the two experiments physicochemical water parameters and concentrations of PAHs (and metabolites, respectively) were measured, after 0 (i.e. untreated control animals), 1, 2, 4, 6, 8, or 12 d, in suspended sediments and fish ($n$=10 per sampling point, i.e. 60 individuals per treatment, 10 for each of the two untreated controls, 260 individuals in total). Due to mortality, no animals for biomarker analysis were available for day 12 in the 24 °C spiked sediment treatment and only $n$=4 animals were assessed in the 24 °C un-spiked treatment. In the same animals, biomarkers of exposure or effect were determined.

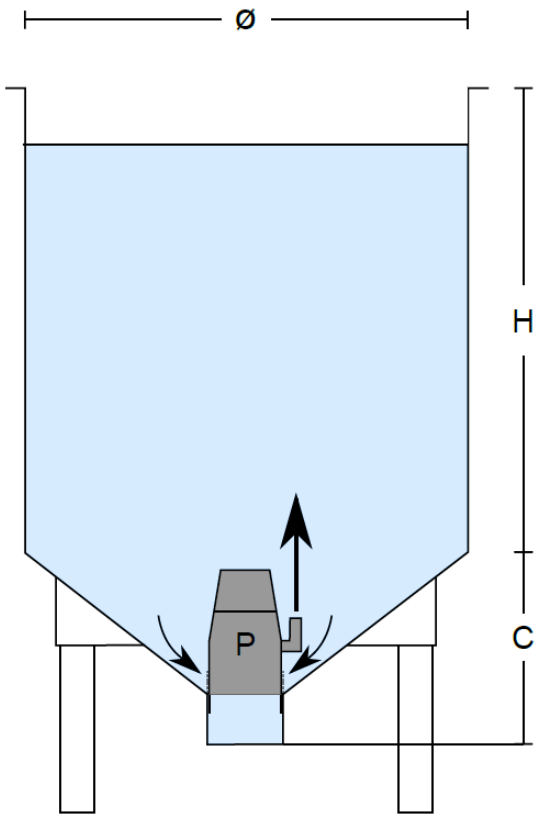


Figure 5.1 Resuspension tanks used for exposure of fish to sediment suspension (P: Pump, Height H: 145 cm, Cone C: 40 cm, Diameter Ø: 80 cm).

The temperature was held constant at 11.9 ± 0.3 °C in the experiment with active cooling, temperature increased during four days from 20 °C to 23.8 ± 0.5 °C in the uncooled experiment. Dissolved oxygen concentrations were 10.5 ± 0.2 mg $L^{-1}$ in the 12 °C experiments. In contrast, concentrations of dissolved oxygen decreased to concentrations as low as 6.5 mg $L^{-1}$ in the uncooled experiment during the first four days of the experiment and then averaged at 7.6 ± 0.4 mg $L^{-1}$. In the 12 °C experiments, total hardness increased from 63 mg $L^{-1}$ to 84 mg $L^{-1}$ over the exposure period. In the uncooled experiments hardness almost doubled from

74 to 142 mg $L^{-1}$. SPM concentrations deviated from the nominal concentration of 10 g $L^{-1}$ and ranged between 6.0 and 13.6 g $L^{-1}$.

### 5.3.2 **Experimental fish**

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 use in experiments. Fish were reared in groups 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 full replacements per day 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) at a rate of 1-2 % bodyweight per day until experimentation. The final weight and length of used fishes was 58 ± 23 g and 161 ± 19 mm, respectively.

All experiments were conducted in accordance with the Animal Welfare Act and with permission of the federal authorities (Landesamt für Natur, Umwelt und Verbraucherschutz NRW, Germany), registration number 8.87-50.10.35.08.225.

### 5.3.3 **Sediment sampling and spiking**

The sediment was collected in April 2010 from the Rhine River (river kilometre 591) close to the fortress Ehrenbreitstein in the vicinity of Koblenz, Germany (+50° 21' 12" N, +7° 36' 27" E). Samples were collected in cooperation with the German Federal Institute of Hydrology (BFG, Koblenz, Germany). This location was chosen as it is known to be moderately contaminated and to be representative based on particle size and organic carbon content (Heininger et al. 2007). A surface sample (approx. 100 kg ww) was taken by use of 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 directly recorded (Table 5.1).

Table 5.1 Basic physicochemical parameters of sediment and water sampled at the harbour of Ehrenbreitstein, Germany.

| | Temperature / °C | pH | 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 | - |

Aliquots of 7.5 kg dw of the sediment were spiked with PAHs according to OECD guideline 218 (OECD 218 2004). Briefly, 10 % of the sediment (i.e. 750 g dw) that was used in each experiment were dried overnight at 105 °C and thoroughly homogenised. PAHs were dissolved in a mixture of 350 ml *n*-hexane and 150 ml acetone, and added to the dried sediment. After evaporation of the solvent, distilled water was added to reconstitute the original water content of the sediment. The spiked portion was added to the bulk of sediment (90 %), thoroughly homogenised using an electric mixer, incubated in darkness at 4 °C for 7 d, and mixed again prior to the experiments. Un-spiked sediments served were investigated for reference and treated identically to the spiked sediments except for the addition of PAHs. After incubation, the sediments were transferred to the exposure tanks to obtain a nominal suspended matter concentration of 10 g $L^{-1}$.

### 5.3.4 **Sampling of sediment suspensions and quantification of PAHs**

Sediment suspension samples were taken as 1 L duplicates per sampling event 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. Retained suspended particulate matter and precipitates were pooled and lyophilized (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 pressurized liquid extraction (PLE) by use of a SpeedExtractor® (SpeedExtractor E-916, BÜCHI Labortechnik GmbH, Essen, Germany). The device was operated under 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 stream of nitrogen. Each extract was re-dissolved in 1 ml of *n*-hexane p.a. (Sigma-Aldrich) which resulted in a concentration equivalent to 5 g dry sediment (SEQ) per 1 ml solvent each. 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), which 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) was used for chromatographic separation of PAHs. 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. Concentrations of PAHs were calculated after PAHs were quantified in duplicate by means of an external five point calibration curve derived from a standard stock solution (DE-PROM 16, LGC Standards, Wesel, Germany) that contained unlabeled EPA-PAHs, and expressed as mg $kg^{-1}$ dw. Limits of detection (LOD) and quantification (LOQ) were calculated from chromatograms (ACS Commitee on Environmental improvement 1980) and recovery rates were estimated for unlabeled external standards using pre-cleaned quartz sand (BÜCHI Labortechnik GmbH, Table 5.2). A one-phase exponential decay model (equation 5.1) was used for calculation of the half-lives of PYR and PHE in the spiked sediment treatments (equation 5.2) with GraphPad Prism 5 (GraphPad, San Diego, USA).

$$y = (y_0 - Plateau) \cdot e^{-k \cdot x} + Plateau \qquad \text{(Eq. 5.1)}$$

$$Half - life = {}^{\ln(2)}/_{k} \qquad \text{(Eq. 5.2)}$$

*Plateau* is the PAH concentration at infinite time, $y_0$ is the PAH concentration at time zero and *k* the rate constant.

Table 5.2 Limit of detection (LOD), limit of quantification (LOQ) and recoveries for PYR, PHE, CHR and BAP in sediment and suspended matter.

| | **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 |
| Recovery / % | 95 % | 101 % | 103 % | 109 % |

### 5.3.5 **Determination of physicochemical parameters**

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

### 5.3.6 **Animal 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) and then exsanguinated. Subsequently, length and mass were determined for calculation of condition index (*K*, equation 5.3), liver somatic index (*LSI*, equation 5.4), and visceral index (*VI*, equation 5.5).

$$K = {}^{W}/_{L^3} \times 100 \qquad \text{(Eq. 5.3)}$$

$$LSI = {}^{LW}/_{W} \times 100 \qquad \text{(Eq. 5.4)}$$

$$VI = {}^{(W - CW)}/_{W} \qquad \text{(Eq. 5.5)}$$

$W$ 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.

The gall bladder was evacuated by use of a syringe, and bile transferred to 1.5 ml polypropylene vials (Carl Roth, Karlsruhe, Germany), and stored at -20 °C until determination of PAH metabolite concentrations. The liver was rapidly isolated and mass determined. Explants of liver were cut into four equally sized pieces, transferred into sterile 2 ml cryogenic vials (Greiner Bio-One, Frickenhausen, Germany) and snap-frozen in liquid nitrogen. Samples of liver were stored at -85 °C until preparation.

### 5.3.7 **Analysis of biomarkers**

Lipid peroxidation, micronuclei in peripheral erythrocytes, as well as concentrations of biliary PAH metabolites (selected major phase I metabolites and their phase II conjugates) and biliverdin in bile were determined following the methods of Ohkawa et al. (1979), Rocha et al. (2009), and Kammann (2007a), respectively, according to previously published protocols (Brinkmann et al. 2010a). Prior to measurement of EROD activity, pieces of liver explants were

thawed carefully and homogenized in 0.1 M phosphate buffer (pH 7.4) at a ratio of 1:10 (w/v) for 20 s using an electric disperser (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, Tuttlingen, Germany). Supernatant was carefully transferred into fresh 1.5 ml micro test tubes and stored at -80 °C until measurement of enzymatic activity. EROD activity in liver was measured following the protocol published in Brinkmann et al. (2010a) according to a combination of the methods described by Kennedy & Jones (1994) and Pohl & Fouts (1980). Slight modifications were made to correct for fluorescence quenching of the sample: In addition to the buffer blank, a sample reference was included in which the whole reaction mixture including S9 was mixed. Other than in the reaction wells, acetonitrile was added first to precipitate proteins. Concentrations of protein for the calculation of specific enzyme activities were determined in triplicates by the bicinchonic acid (BCA) method provided as a kit (Sigma-Aldrich). A minimum coefficient of determination ($r^2$) of 0.95 was accepted for standard curves in all used assays for protein quantification. Hepatic gene expression was determined via reverse-transcription quantitative real-time PCR according to Zhang et al. (2008) following the protocol published by Brinkmann et al. (2010a). Briefly, 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 5.3), and 1.6 mL of nuclease-free distilled water (QIAGEN). PCR products of mixed cDNA samples using previously published gene-specific primers (Table 5.3) were separated on 1 % agarose gels with a Gene Ruler DNA size standard (Thermo Scientific) to confirm single PCR amplicons with the desired length. Melting curve analyses were performed during real-time PCR to ensure target specificity and single peak amplification, respectively. For complete method descriptions, please refer to the supplemental material.

Table 5.3 Primer pair sequences, amplicon sizes, and accession numbers of the investigated genes used in real-time PCR reactions. Modified from Brinkmann et al. (2010a).

| **Gene** | **Primer sequence (5'–3')** | Amplicon size (bp) | GeneBank accession no. |
|---|---|---|---|
| AhRβ | F:TGGCAAATGGACACACATTC<br>R:AGTCTGTTGGGGTTCTGTGG | 100 | NM_001124252 |
| CYP1A[a] | F:GATGTCAGTGGCAGCTTTGA<br>R:TCCTGGTCATCATGGCTGTA | 104 | U62796 |
| EL-1α | F:GAGAACCATTGAAAAGTTCGAGAAG<br>R:GCACCCAGGCATACTTGAAAG | 71 | NM_001124339 |
| GST-P | F:TTCAGGGAGGGGAAGGTATC<br>R:GTTGGTGACAAGCCTTCGTT | 101 | BQ036247 |
| SOD1[b] | F:TGGTCCTGTGAAGCTGATTG<br>R:TTGTCAGCTCCTGCAGTCAC | 201 | NM_001124329 |
| UGT[c] | F:ATAAGGACCGTCCCATCGAG<br>R:ATCCAGTTGAGGTCGTGAGC | 112 | DY802180 |
| Caspase 3*β*[d] | F: GGAGAACAGGAATGAGTTCT<br>R: TCACGTTGTACCCCAAACGT | 87 | FR751081 |

[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)

[d]This primer pair was previously published by Bobe et al. (2004)

### 5.3.8 **Data analysis**

Calculations were performed in spreadsheets by use of Microsoft Excel™ 2007. Graphs were plotted by use of the software GraphPad Prism 5. Statistical analyses and correlations were conducted by use of 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 Barlett's test for equal variances ($p<0.05$) were analyzed by use of nonparametric Kruskal-Wallis ANOVA on ranks ($p\leq0.001$). The datasets passing both tests were analyzed by use of parametric one-way ANOVA ($p\leq0.001$). Dunn's method was used as the multiple range test to identify significant differences among treatments. Since most of the data did not fulfil the criteria for two-way or multiple-way ANOVA, this method was not applied in the present study for comparability purposes. For selected comparisons between un-spiked and spiked sediment treatment groups, one-tailed parametric Student's t-tests were used if data were normally distributed and of equal variance. If one of the criteria for the parametric test was not met, non-

parametric Mann-Whitney Rank Sum tests were performed. The probability of Type I error (α) was set to $p \leq 0.05$. In the text, values are expressed as mean value ± standard deviation, unless indicated.

## 5.4 **Results**

### 5.4.1 **Fish mortality**

When exposure was conducted at 12 °C, mortality of rainbow trout was small in both the un-spiked and spiked sediment treatments with 0 and 2 %, respectively. However, when conducted at 24 °C, mortalities of 25 % and 31 % were observed when fish were exposed to un-spiked and spiked suspended sediment, respectively. Due to the high mortality, no animals for biomarker analysis were available for day 12 in the 24 °C spiked sediment treatment and only $n$=4 animals were assessed in the 24 °C un-spiked treatment.

### 5.4.2 **Chemical analyses of PAH concentrations**

After the sediment-conditioning period of 7 d, directly prior to addition of sediments to exposure tanks, concentrations of PAHs in un-spiked sediments from the harbour Ehrenbreitstein (EBR) were significantly lower than nominal concentrations ($C_0$, Table 5.4).

Table 5.4 Nominal and measured concentrations (C0) of the PAHs used for spiking prior to the addition of sediments to exposure tanks. Values in italic typesetting are below the LOQ.

| Experiment | | Phenanthrene mg kg$^{-1}$ dw | Pyrene mg kg$^{-1}$ dw | Chrysene mg kg$^{-1}$ dw | Benzo[*a*] pyrene mg kg$^{-1}$ dw |
|---|---|---|---|---|---|
| Un-spiked sediment | Measured | 0.27 | 0.51 | *0.29* | *0.16* |
| Spiked sediment | Nominal | 5.00 | 4.10 | 3.30 | 8.30 |
| | Measured | 1.88 | 2.29 | 1.63 | 2.32 |

PAH concentrations in SPM during the experiments were expressed relative to $C_0$ as the $C/C_0$ ratio (Figure 5.2). In contrast to CHR and BAP, where measured concentrations did not show clear trends as a function of exposure time, a constantly decreasing $C/C_0$ ratio was observed in the spiked sediments for PHE and PYR. In the un-spiked treatments, concentrations of all PAHs remained constant (Figure 5.2).

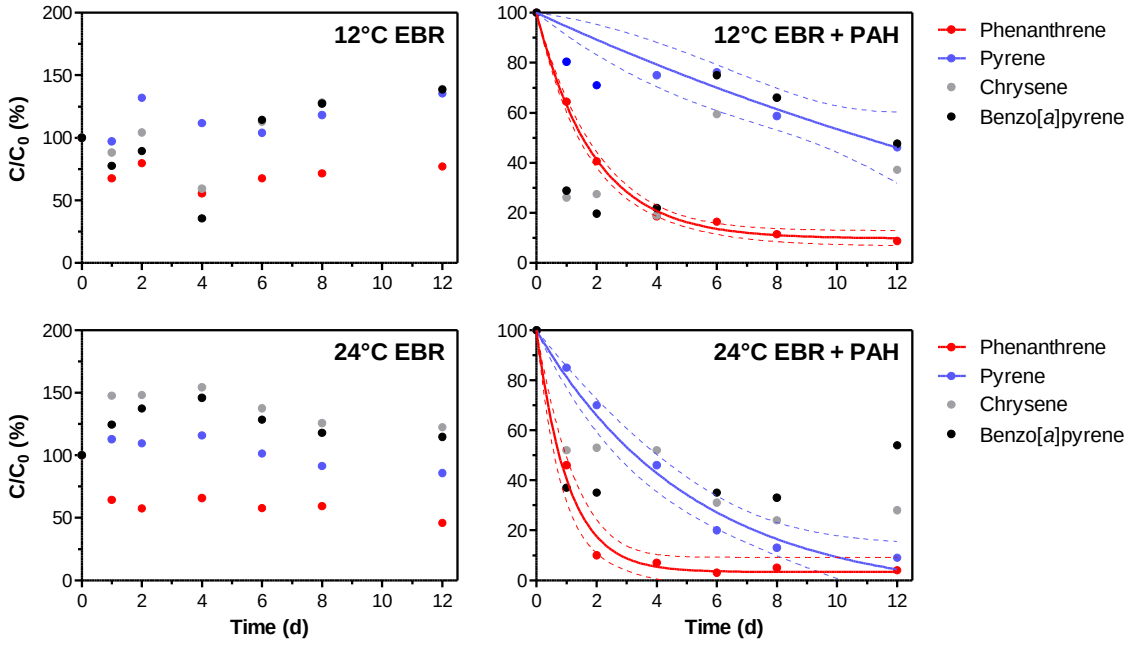


Figure 5.2 PAH concentration changes in SPM relative to the initial concentration in the sediment (as given in Table 5.4) during the experiments at 12 and 24 °C in spiked and un-spiked treatments were measured by means of GC-MS. Solid lines represent regression with a one-phase exponential decay model, dashed lines the 95 % confidence intervals.

The data were fitted using a one-phase exponential decay model. The calculated half-lives of PYR and PHE in the SPM resulting from spiked sediment were significantly shorter in exposure chambers where the temperature was at 24 °C compared to those where the temperature was 12 °C (Table 5.5). The three-ring substance PHE generally had shorter half-lives than the four-ring substance PYR. Half-lives of PHE and PYR were 1.8- and 5.2-fold greater, respectively, in chambers held at the greater temperature.

Table 5.5 Half-lives of PYR and PHE during the experiments with spiked sediments 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 |

### 5.4.3 **Morphometric indices**

Morphometric indices of exposed fish determined to assess changes in health and condition during the experiments, such as condition index (*K*) were negatively proportional to exposure time. However, the relationship for *K* was statistically significant in only the 12 °C un-spiked treatment on day 12 compared to un-exposed animals (One-way ANOVA with Dunnet's post-hoc test, $p \leq 0.05$). The visceral index (*VI*) was significantly lower than the initial values after 4 and 6 days in the un-spiked and spiked experiments conducted at 12 °C, respectively, and on the days 2 and 4 in the spiked experiment conducted at 24 °C (One-way ANOVA on ranks with Dunn's post-hoc test, $p \leq 0.05$). The liver somatic index (*LSI*) was not significantly different from the initial control values in fish exposed to either concentration of PAHs at either temperature during the course of the exposure. There were no significant differences in *K*, VI or LSI between spiked and un-spiked sediments.

### 5.4.4 **Biological analysis**

Significant concentrations of PAH metabolites, i.e. the sum of original hydroxylated PAHs and those after deconjugation of phase II products with β-glucuronidase/arylsulfatase, were observed in bile of fish from all treatments, including the un-spiked sediment from the harbour Ehrenbreitstein (Figure 5.3). Concentrations of PAH metabolites in bile of fishes in the spiked treatments described a hyperbolic uptake phase followed by a depuration phase that was more pronounced at 24 °C. No such depuration phase was observed in the un-spiked treatments. In the PAH spiked treatment group concentrations of metabolites were significantly greater than the control (confidence interval method, Newman 2008) at 12 °C and 24 °C. When fish were exposed to spiked sediment, concentrations of PAH metabolites increased over the first days, and then decreased later in the experiment. Dynamics of concentrations of PAH metabolites in bile were markedly different between the two temperatures. The maximum concentration of 1-hydroxy-PYR was detected on day 8 at 12 °C and on day 2 at 24 °C. A similar pattern of accumulation was observed for 1-hydroxy-PHE, where the maximum concentration was detected on day 4 at 12 °C and on day 2 at 24 °C. Concentration of 3-hydroxy-BAP constantly increased in bile until day 8 at 12 °C. At 24 °C, the concentration of this metabolite was significantly elevated already at day 2, with no further increase.

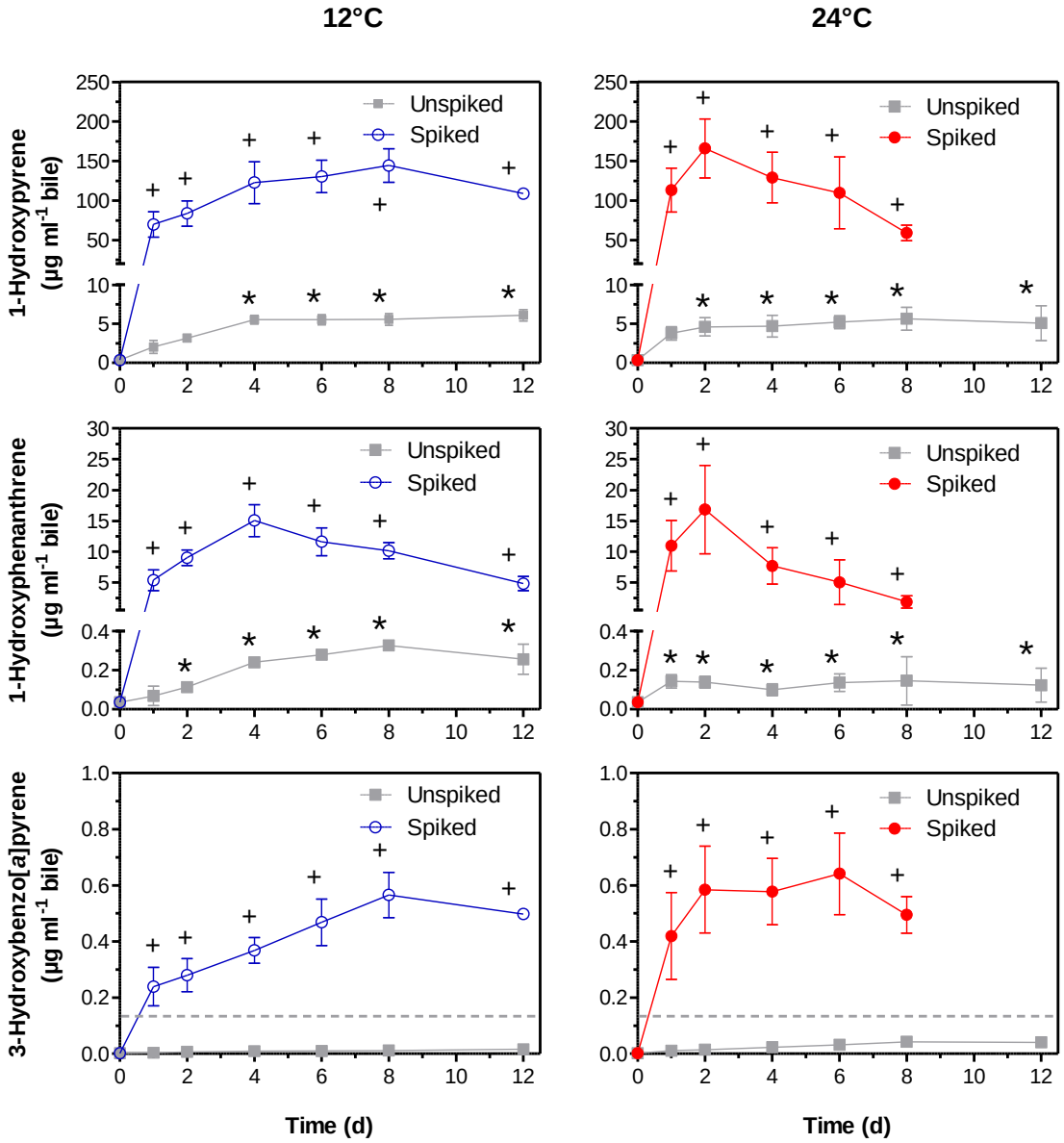


Figure 5.3 Absolute biliary metabolite concentrations (following treatment with β-glucuronidase/arylsulfatase) during the experiments (left: 12 °C, right: 24 °C) in the un-spiked (■) and the spiked treatments (○/●). Symbols give the mean of n=10 animals, error bars the 95 % confidence intervals. The dashed line marks the LOQ for 3-hydroxybenzo[a]pyrene. Asterisks denote significant differences between control and un-spiked 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.

In the un-spiked sediment experiments, uptake and biotransformation at 12 and 24 °C resulted in significantly increased 1-hydroxy-PHE concentrations compared to initial values after 2 and 1 d (2.5 and 3.5-fold), respectively, and significantly greater 1-hydroxy-PYR concentrations (18.7 and 15.3-fold) compared to initial values were measured after 4 and 2 d, respectively (Kruskal-Wallis one-way ANOVA on ranks with Dunn's post-hoc test, $p \leq 0.05$). Concentrations of 3-hydroxy-BAP were lower than the LOQ in bile of fish exposed to un-spiked experiment. To estimate effects of metabolic rates of fish, concentrations of biliverdin were

measured. When fish were exposed at 12 °C there was no statistically significant difference in concentrations of biliverdin between those exposed to spiked or un-spiked sediments (Figure 5.4). In fish exposed at 24 °C, concentrations of biliverdin increased from 160 ng $ml^{-1}$ in the untreated fish to 1209 ng $ml^{-1}$ in the un-spiked treatment on day 12. During the first 4 d of exposure, concentrations of biliverdin in bile of fish exposed to spiked sediment at 24 °C were significantly greater than those in bile of fish exposed to un-spiked sediment (significant on day 4, Mann-Whitney Rank Sum test, $p \leq 0.05$).

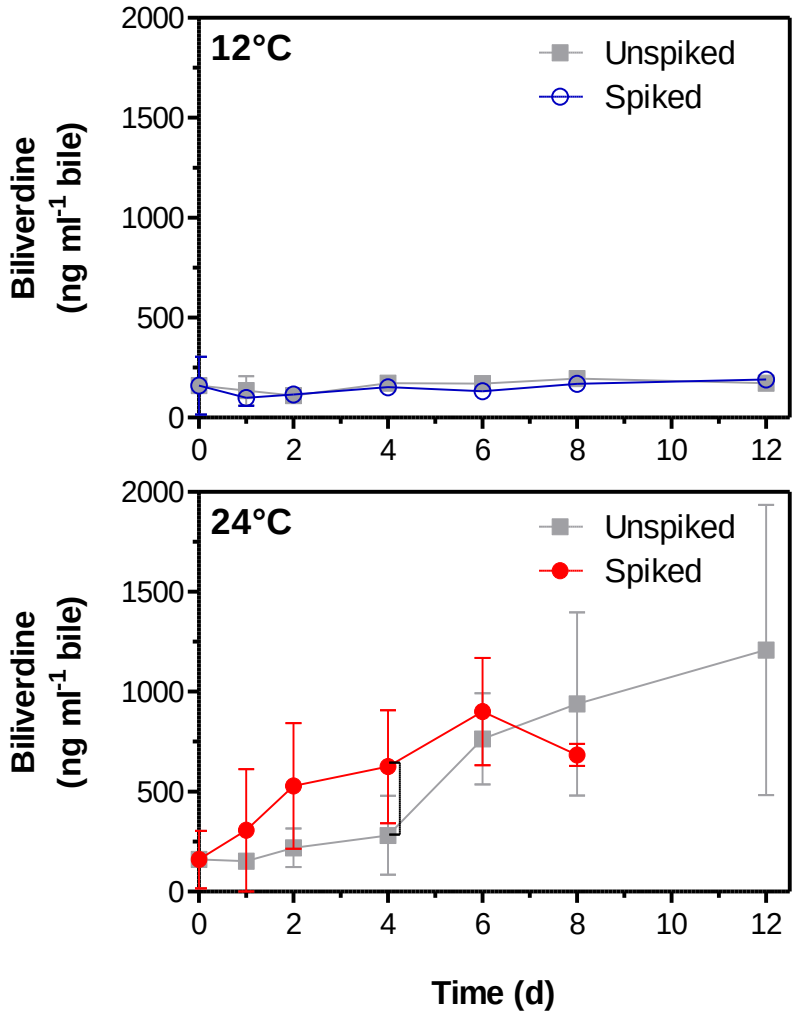


Figure 5.4 Dynamics of biliary biliverdin concentrations during the experiments (top: average temperature 12 °C, bottom: average temperature 24 °C) in the un-spiked (■) and the spiked treatments (○/●). Symbols represent mean values of $n$=10 animals, error bars the 95 % confidence intervals. Brackets denote significant differences between the experiments (Mann-Whitney Rank Sum test, $p \leq 0.05$).

With the exception of the experiment conducted at 12 °C with spiked sediment, significant differences in EROD activities in trout livers were observed as a function of exposure time (Figure 5.5). In the 12 °C experiment with un-spiked sediment, EROD activity was significantly

higher than that of untreated animals after 8 and 12 days exposure (Kruskal-Wallis one-way ANOVA on ranks with Dunn's post-hoc test, $p \leq 0.05$). When exposed at 24 °C, EROD activity was significantly lower after 4 days exposure to both the spiked and un-spiked sediment compared to untreated animals (Kruskal-Wallis one-way ANOVA on ranks with Dunn's post-hoc test, $p \leq 0.05$). When exposed to the suspension of spiked sediment at 24 °C, EROD activity in livers of fish was significantly 1.9-fold greater than that in livers of fish exposed to un-spiked sediment on day one (t-test, $p \leq 0.05$). Expression of mRNA of CYP1A, GST, UDPGT, and Caspase 3 in liver was not significantly up-regulated relative to that of untreated controls after 1, 4 or 8 days, respectively, in any treatment (data not shown).

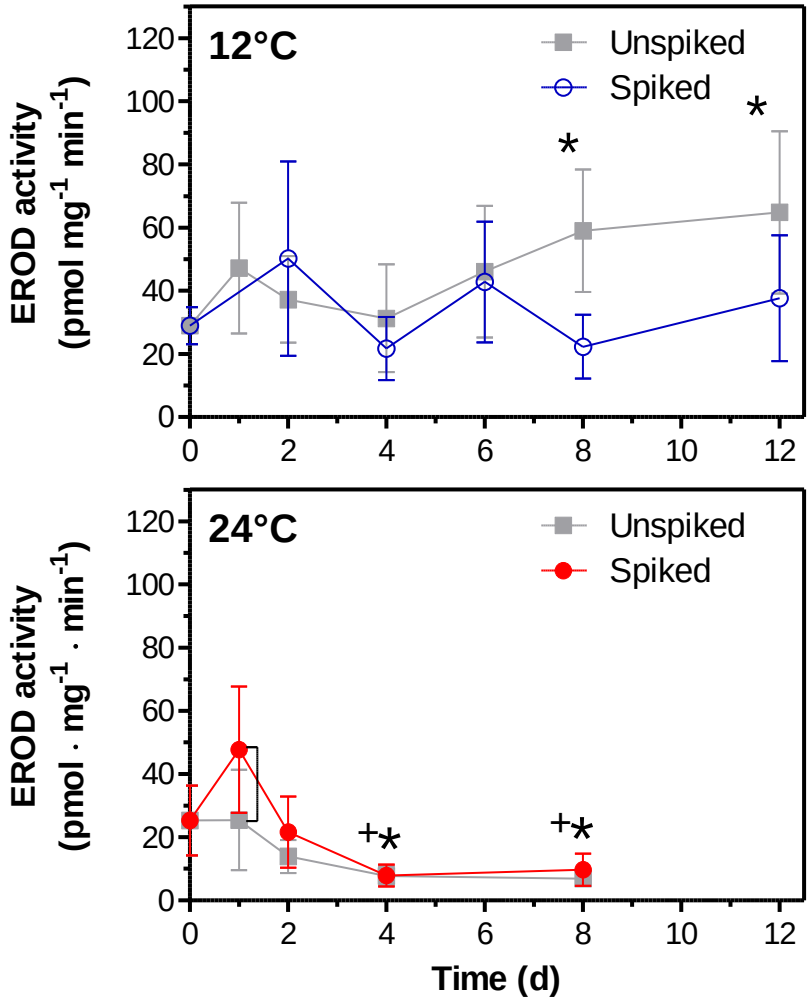


Figure 5.5 Dynamics of hepatic EROD activity during the experiments (top: average temperature 12 °C, bottom: average temperature 24 °C) in the un-spiked (■) and the spiked treatments (○/●). Symbols represent mean values of $n$=10 animals, error bars the 95 % confidence intervals. Brackets denote significant differences between the experiments (t-test, $p \leq 0.05$), asterisks differences between control and un-spiked treatments, and plus symbols between control and spiked treatments (Kruskal-Wallis one-way ANOVA on ranks with Dunn's post-hoc test, $p \leq 0.05$).

There were no statistically significant differences in LPO measured as equivalent concentrations of malondialdehyde (MDA) as a function of exposure time with the exception of the 24 °C PAH-spiked experiment (Figure 5.6). In fish from this treatment group a constant increase of LPO was observed during the first 4 d of exposure (significant, Kruskal-Wallis one-way ANOVA on ranks with Dunn's post-hoc test, $p \leq 0.05$), and which then subsequently decreased to concentrations measured in fish from the other treatment groups. The average LPO concentration in this treatment was $414 \pm 271$ nmol $g^{-1}$ liver on day four, which was approximately 2.7-fold greater than that of unexposed control fish at this temperature.

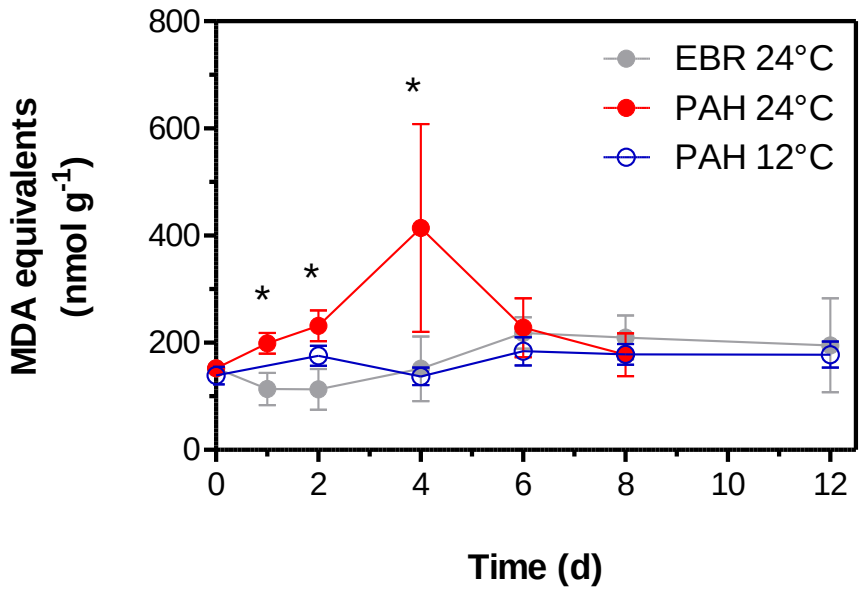


Figure 5.6 Dynamics of hepatic lipid peroxidation during the experiments in the un-spiked (■) and the spiked treatments (○/●). Symbols represent mean values of *n*=10 animals, error bars the 95 % confidence intervals. Plus symbols denote significant differences between control and spiked treatments (Kruskal-Wallis one-way ANOVA on ranks with Dunn's post-hoc test, $p \leq 0.05$).

Micronuclei in peripheral erythrocytes showed a generally increasing trend during exposure of fish in all treatments and at both temperatures. However, significant differences between the spiked and un-spiked treatments were only observed in the 12 °C group on days 6 and 12 (2.2 and 2.1-fold, respectively; Figure 5.7).

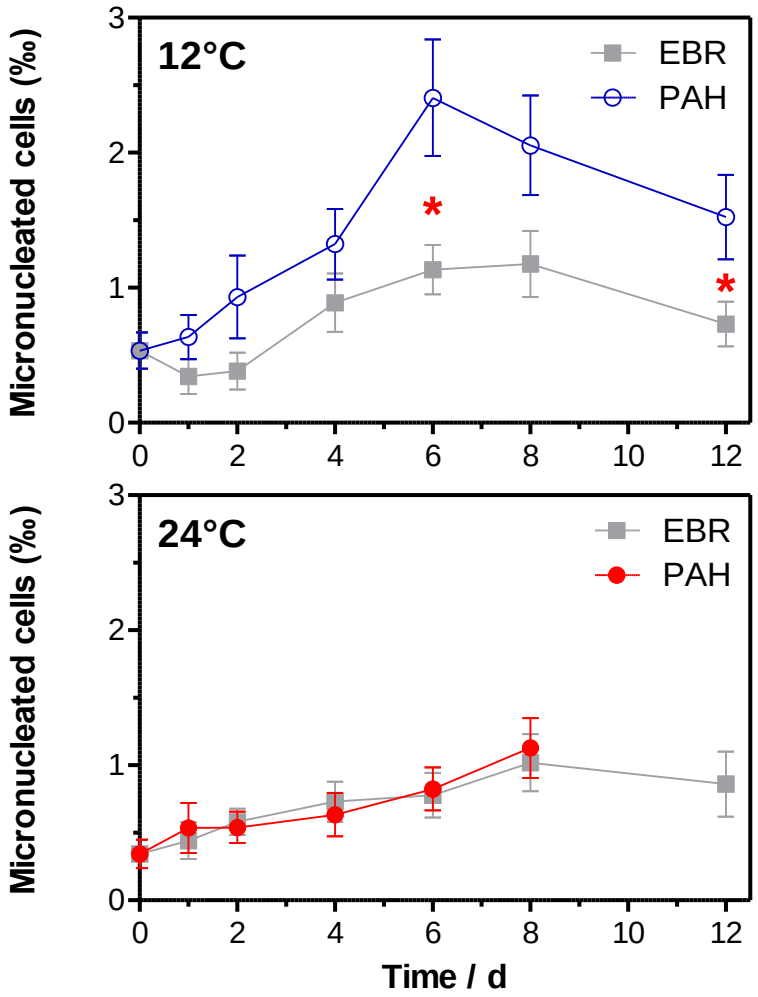


Figure 5.7 Dynamics of micronuclei in peripheral erythrocytes during the experiments (top: average temperature 12 °C, bottom: average temperature 24 °C) in the un-spiked (■) and the spiked treatments (○/●). Symbols represent mean values of $n$=10 animals, error bars the SEM. Brackets denote significant differences between the experiments (t-test, $p \leq 0.05$).

## 5.5 **Discussion**

### 5.5.1 **Dissipation of the PAHs from sediment-water systems**

Since rainbow trout were exposed to suspensions of sediments at constant SPM concentrations, changes in PAH levels during the experiments could have affected the results. The rate of biodegradation of PAHs in fluvial systems containing greater concentrations of SPM (i.e., 4-10 g $L^{-1}$) was found to be greater than that in systems with lower concentrations of SPM by Xia *et al.* (2006). Most likely, this effect is driven by the greater surface area at the water-sediment interface at which microbial degradation takes place. Microbial mineralization of phenanthrene

was 5 to 10-fold greater if the sediment was frequently resuspended compared to a un-disturbed sediment bed (LeBlanc et al. 2006). The shortest half-life for microbial degradation in this particular study was 100 d, while other researchers found half-lives in the range of 40 d to several months for phenanthrene (Apitz et al. 1999, Heitkamp & Cerniglia 1987). In the present study, significantly shorter half-lives were observed (Table 5.5), that were comparable to those determined in sediment slurries (e.g. Shiaris 1989). Volatilization of sediment-bound PAHs due to the intense aeration of the experimental containers, as well as uptake and biotransformation by exposed fish might also represent an important dissipation pathway for the lower weight PAHs, such as PHE and PYR (e.g. Ravikrishna et al. 1998, Valsaraj et al. 1997). These potential routes of dissipation were likely to have led to constantly decreasing concentrations of PAHs, especially of those with lower molecular weight. Since only the concentration of parent PAHs was determined, it cannot be excluded that transformation products with different toxicity and behavior were formed.

In contrast to the experiments performed in this study, new suspended particles are permanently resuspended from the sediment bed in the annular flume that was used in the predecessor project. This results in quasi-flow-through conditions, which minimizes dissipation during the simulated floods. Aging of spiked PAHs can lead to lower rates of desorption and less biodegradation (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 affect bioavailability, it seems likely that the differences observed for uptake of the PAHs in fish comparing spiked and un-spiked sediment were due to differing desorption rates (Reid et al. 2000a).

### 5.5.2 **Differences between spiked and naturally aged sediment-bound PAHs**

Substantial differences in desorption and subsequent bioavailability of organic contaminants can occur between spiked and naturally aged sediments (Reid et al. 2000a). Such differences in desorption of PAHs were apparent in the fraction available for uptake by rainbow trout during this study. While the concentration of sediment-bound PHE was 7.0-fold greater in spiked than in un-spiked sediments (i.e. with naturally aged PAH contamination), the maximum biliary concentration of 1-hydroxy-PHE was 120 and 45-fold greater in fish exposed to spiked relative to un-spiked sediment at 12 °C or 24 °C, respectively. The same effect was observed for PYR. The use of spiked sediment increased the bioavailable fraction significantly.

### 5.5.3 **Dynamics of biomarker responses during exposure: biomarker cascades**

Since no sampling was possible during simulated floods in the project Floodsearch, only qualitative information on biomarkers could be derived (Brinkmann et al. 2010a, Wölz et al. 2009b). In the exposure experiments of the present study, biomarkers were monitored kinetically to provide detailed insights in their dynamics as a function of extended exposure to suspended matter. Uptake and effects of PAHs followed a cascade-like pattern in the spiked treatment groups, as indicated by a series of peak biomarker responses (Figure 5.8). Due to dissipation of PYR and PHE from the system, the experiment can be subdivided in an uptake and a quasi-depuration phase, in which the concentrations of biliary PAH metabolites increased and decreased, respectively. The peak concentrations of metabolites observed on day two was followed by a peak of lipid peroxidation on day four at 24 °C. Production of micronuclei, a marker for genetic damage that can be predictive of potential population level effects (Diekmann et al. 2004b), was observed on day 6 in the 12 °C treatment (Figure 5.7). EROD activity was significantly induced in the un-spiked treatment group compared to untreated control animals after 8 and 12 d at 12 °C (Kruskal-Wallis one-way ANOVA on ranks with Dunn's post-hoc test, $p \leq 0.05$), but not compared to the spiked sediment treatment (Mann-Whitney Rank Sum test, $p \leq 0.05$). Exposure time or concentration at 12 °C might not have been sufficient to induce EROD since the PAHs used for spiking are considered weak to moderate AhR agonists (Barron et al. 2004b). At 24 °C, however, a significant induction of EROD activity comparing animals from the spiked and un-spiked treatments on day one (t-test, $p \leq 0.05$) was followed by a decrease, which was most probably non-specific due to temperature stress (cf. Whyte et al. 2000). Although induction of biomarkers was transient, potential long-term adverse effects of exposure to particle-bound PAHs cannot be excluded. Thus, an experiment to monitor health and performance of fish over a longer period of time following short exposures to contaminated SPM should be conducted to answer the question if the observed biomarker cascade is followed by a potentially adverse effect (question mark, Figure 5.8).

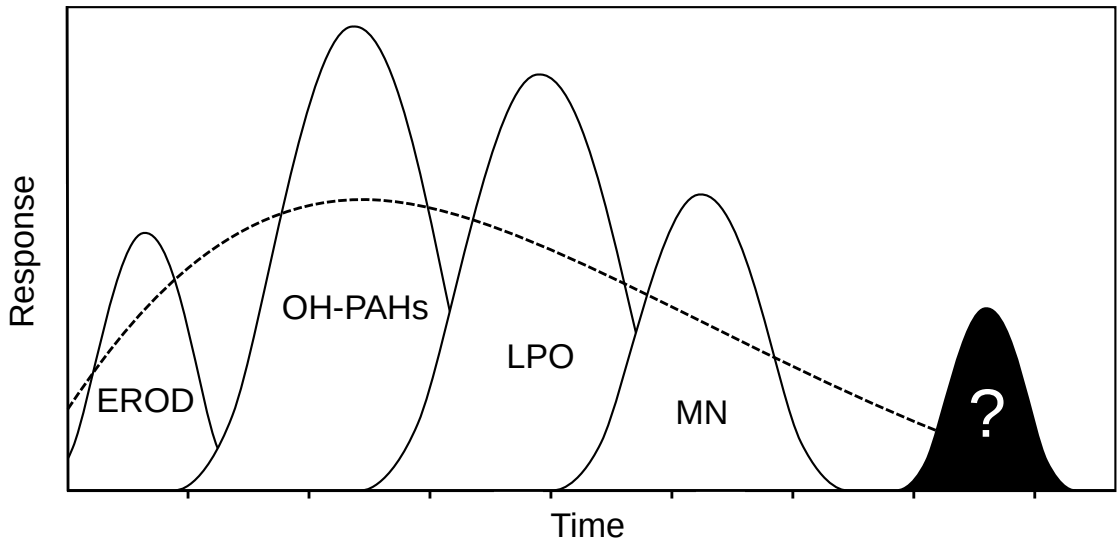


Figure 5.8 Conceptual model of the observed biomarker dynamics, in which distinct peak responses follow each other in a cascade-like pattern (combination of experiments at 12 and 24 °C): 7-ethoxyresorufin-*O*-deethylase (EROD, 24 °C), hydroxylated PAH metabolites (OH-PAHs, 12 and 24 °C), lipid peroxides (LPO, 24 °C) and micronuclei (MN, 12 °C). Biomarkers with small peaks were significantly altered only at one temperature following exposure to PAH spiked sediment suspensions, the biomarker with a high peak, i.e. biliary metabolites were altered at both temperatures. The black peak represents a possible adverse effect that might follow the observed biomarker cascade; the dashed line represents a hypothesized integrated stress level.

### 5.5.4 **Influence of temperature (stress) on the biomarker cascade**

Since degradation, bioavailability and effects of particle-bound pollutants can 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 some studies, critical thermal maxima for salmonid fish species were investigated by constantly increasing water temperature. The reported temperatures with significant effects on growth and survival of rainbow trout ranged from 24 to 27 °C, depending on other environmental parameters (Myrick & Cech 2004). The greater mortality of rainbow trout in the 24 °C experiment of the present study (25 and 31 % for the un-spiked and spiked treatment, respectively) could thus be explained by direct effects of temperature.

Gross energy metabolism of fish was significantly greater at 24 °C as represented by the elevated excretion rate of biliverdin (cf. Avery et al. 1992), which was similar to rates of uptake and biotransformation of PAHs. Although maximum concentrations of PAH metabolites in bile

did not differ much between the two temperatures, PAH uptake was approximately 2-fold faster at 24 °C compared to that at 12 °C. Significant uptake of PAHs from the spiked sediment was observed at 12 °C, but elevated hepatic lipid peroxidation was only found at 24°C after 4 d (Figure 5.6). BAP has been previously shown to be converted to a 1,6-quinone metabolite in fish *via* the precursor 1-hydroxy-BAP (Di Giulio & Hinton 2008), which was reported to cause oxidative stress (Lemaire et al. 1994). Please note that only the metabolite 3-hydroxy-BAP was measured in the present study. Seubert & Kennedy (2000), however, have shown that 1-hydroxy- and 3-hydroxy-BAP co-occur among other metabolites in the investigated species. Rainbow trout appeared to compensate for oxidative stress at lower temperature so that an increased level of LPO was only observed in the spiked 24 °C treatment. Similar effects have been observed by other researchers who concluded that the rate of turnover of lipids was more rapid at lower temperatures, and which has a protective function (Grim et al. 2010, Lushchak 2011, Lushchak & Bagnyukova 2006, Parihar & Dubey 1995). Significant differences between the spiked and un-spiked treatments concerning micronuclei in peripheral erythrocytes have only been observed at 12 °C.

It can be concluded that some effects of PAHs on fish observed in the present study were only apparent in combination with temperature stress during exposure, while others were only apparent at 12 °C. The biomarker cascades did not only show quantitative differences (i.e. different induction intensity or rate of biomarker responses) at the two temperatures but also qualitative differences, i.e. different biomarker responses were observed. The correlation between gross energy metabolism and some of the biomarkers (i.e., biliary metabolites and LPO) supports 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 dissipated heat from power plants and due to 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 (e.g. Airas et al. 2008, Heinonen et al. 2002, Honkanen & Kukkonen 2006).

## 5.6 **Acknowledgements**

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Chapter 6

# An attempt to assess the relevance of flood events - Biomarker response of rainbow trout exposed to resuspended natural sediments in an annular flume

# An attempt to assess the relevance of flood events – biomarker response of rainbow trout exposed to resuspended natural sediments in an annular flume

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## 6.1 **Abstract**

There is a consensus within the scientific community that sediments act as a long term sink for a variety of organic and inorganic pollutants which, however, can re-enter the water column upon resuspension of deposited material under certain hydraulic conditions such as flood events. Within the implementation of the European water framework directive (WFD) it is important to understand the potential short and long term impact of suspended particulate matter (SPM)-associated contaminants on aquatic organisms as well as the related uptake mechanisms for a sound risk assessment.

To elucidate the effects of sediment-bound organic pollutants, such as polycyclic aromatic hydrocarbons (PAHs), rainbow trout (*Oncorhynchus mykiss*) were exposed to three resuspended natural sediments with different contamination levels. Physicochemical parameters including dissolved oxygen concentration, pH and temperature, total PAH concentration in sediments and SPM as well as different biomarkers of exposure in fish (7-ethoxyresorufin *O*-deethylase activity, biliary PAH metabolites, micronuclei and lipid peroxidation) were measured following 7 d exposure within an annular flume, a device to assess erosion and deposition processes of cohesive sediment.

Concentrations of PAHs in SPM remained constant and represented the different contamination level in the un-suspended sediments. Significant differences in bile metabolite concentrations as well as in EROD induction compared to control experiments (untreated animals and animals that were exposed in the annular flume without sediment) were observed for all exposure scenarios. The ratio between 1-hydroxypyrene in bile from fish exposed to the three different contamination levels was 1.0 : 3.6 : 10.7 and correlated well with (1) the ratio of pyrene concentrations in corresponding sediments which was 1.0 : 3.1 : 12.7 and (2) with the ratio of particle-bound pyrene in SPM which was 1.0 : 2.7 : 11.7. In contrast, hepatic lipid peroxidation and micronuclei formation represented the different contamination levels less conclusive.

The results of this study clearly demonstrate that firmly-bound PAH from aged sediments can become bioaccessible upon resuspension under flood-like conditions and are readily absorbed by aquatic organisms such as rainbow trout. Associated short term effects were clearly documented and possible adverse long-term impacts due to genotoxicity are likely to follow.

**Keywords:** Multiple biomarkers • Rainbow trout • Sediment • Resuspension • SPM • Annular flume • PAH

## 6.2 **Introduction**

It is widely accepted that sediments act as sinks for a variety of organic and inorganic pollutants under normal flow, i.e. undisturbed conditions (Power & Chapman 1992). However, under certain flow regimes or under anthropogenic influences, e.g. flood events or dredging operations, these historically polluted sediments in river basins can constitute a secondary source of a wide range of organic and inorganic pollutants, such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and heavy metals. Several studies demonstrated that in consequence those pollutants can also become bioaccessible by a range of aquatic organisms (Ahlf et al. 2002, Hollert et al. 2003a, Wölz et al. 2010b). Until to date, there is still a deficiency in understanding the various and complex interactions between sediments, contaminants and aquatic biota (Gerbersdorf et al. 2011, Roberts 2012).

PAHs have been in the focus of research for almost four decades (Benner et al. 1989, Blumer & Youngblood 1975, Gustafsson et al. 1997, Haritash & Kaushik 2009). They are a group of ubiquitously distributed organic pollutants with two or more fused benzene rings which result from transformation and incomplete combustion processes of organic material and result from both natural as well as anthropogenic sources. In aquatic systems PAHs mostly originate from anthropogenic combustion processes (Hansen 2003, Lima et al. 2005, Neff et al. 2005, Wild & Jones 1995) and tend to adsorb quickly to organic sediment and suspended particulate matter (SPM) particles. Bioacessability mainly depends on organic content of those compartments and declines with increasing content of total organic carbon (Karickhoff et al. 1979, Means et al. 1980). Uptake in fish and other aquatic organisms occurs through the food chain or during exposure to contaminated sediment or water (Neff 1985) and according to McElroy (1989) depends on several factors, such as physicochemical properties of the PAHs, environmental variables and biological factors. In contrast to other organic pollutants such as PCBs, PAHs do not substantially bioaccumulate in fish which is related to high metabolic biotransformation rates which mostly lead to a detoxification of parent PAHs to hydrophilic conjugated metabolites (van der Oost et al. 2003). Individual toxicity of PAHs depends on their ring structure, and is more distinct in higher molecular substances. Acute toxicity in fish is less pronounced whereas higher molecular PAHs and their intermediate metabolic products show high chronic toxicity (Eisler 1987, Neff 1979, Tuvikene 1995). Those sublethal responses include genotoxic, carcinogenic and immunotoxic effects as well as changes in metabolism. Tuvikene (1995) and Krahn et al. (1986) reported significant correlations between hepatic

lessions and total PAH concentrations in marine fish. Thus, PAHs have long been rated as priority pollutants by several environmental agencies such as the Environmental Protection Agency (EPA, 1984) as well as by other governmental agencies such as the European Parliament and the council which list several PAHs as priority pollutants in the Water Framework Directive (WFD, CEC 2000). This directive commits states of the European Union to achieve a good chemical and ecological status in European river catchments until the year 2015 (CEC, 2000). In a recent amendment to the original directive, the relevance of sediments as a secondary long-term source for contaminants and, consequently, as a critical factor for surface water quality has been integrated into the WFD (CEC, 2008). Furthermore, in a recently adopted legislative proposal (CEC, 2013) the European commission added twelve further substances to the list of priority substances as well as setting biota standards for several substances including PAHs. Among other factors – legacy contaminated sediments may endanger the accomplishment of quality goals defined by the WFD in many river basins (Wilby et al. 2006).

For an increasingly sound understanding of associated ecotoxicological impacts interdisciplinary research has become more and more important in the last few years especially in response to new challenges emerging from the implementation of the European Water Framework Directive. Different authors proposed to integrate hydrodynamics and sediment mobility into existing assessment strategies to obtain a more realistic approach to evaluate potential effects of contaminated sediments (Brinkmann et al. 2010b, Chapman & Hollert 2006, Hollert et al. 2007, Wölz et al. 2009b). The interdisciplinary project “Floodsearch“, which was funded by the German Excellence Initiative at RWTH Aachen University, Germany (Schüttrumpf et al. 2011, Wölz et al. 2009b) provided a methodology to investigate the fate and effects of sediment-bound pollutants under flood-like conditions.

Here we report the results of experiments conducted within an annular flume, which had the goal to elucidate the effects of resuspended sediments on rainbow trout in semi-natural environments under changing bed shear stress regimes. The annular flume was used to generate rising bed shear stress levels, which resulted in increasing concentrations of suspended particulate matter originating from the respective sediment bed. Sediments from two different rivers with different pollution characteristics were either used as collected from the environment or mixed in a 1:1 ratio to simulate a sediment with moderate contamination. Concentrations of PAHs in suspended particulate matter and sediments, as well as uptake and metabolism, as

determined by concentrations of PAH metabolites in bile, were quantified. A set of different biomarkers in rainbow trout, which had previously proven to give reliable answers on functional changes during resuspension of particle-associated pollutants (Brinkmann et al. 2013) was measured. To determine the processes mediated by the aryl hydrocarbon receptor (AhR) the hepatic activity of 7-ethoxyresorufin *O*-deethylase (EROD) was determined. Oxidative stress was assessed by measuring lipid peroxide levels (LPO). Micronuclei formation (MN) in peripheral erythrocytes was used to determine genotoxicity.

## 6.3 **Materials and methods**

### 6.3.1 **Experimental design**

Juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed to suspensions of sediments from the rivers Rhine and Moselle under varying bed shear stress regimes. Experiments were conducted in an annular flume (Figure 6.1), which consisted of a circular glass channel (width 0.25 m, radius of circle 1.5 m) and a coaxial acrylic glass lid. Channel and lid rotated in opposite directions producing an endless flow (Cofalla et al. 2012, Spork et al. 1994, Spork et al. 1995). To ensure a homogeneous distribution of the bed shear stress over the channel width at a mean water depth of 325 mm, a speed ratio between lid and flume of $\omega_l/\omega_f = -2.0$ was used. The flume was aerated with a diaphragm pump, while the ambient temperature was kept constant at 14.6 ± 0.2°C and a light-dark period of 12 h was realized.

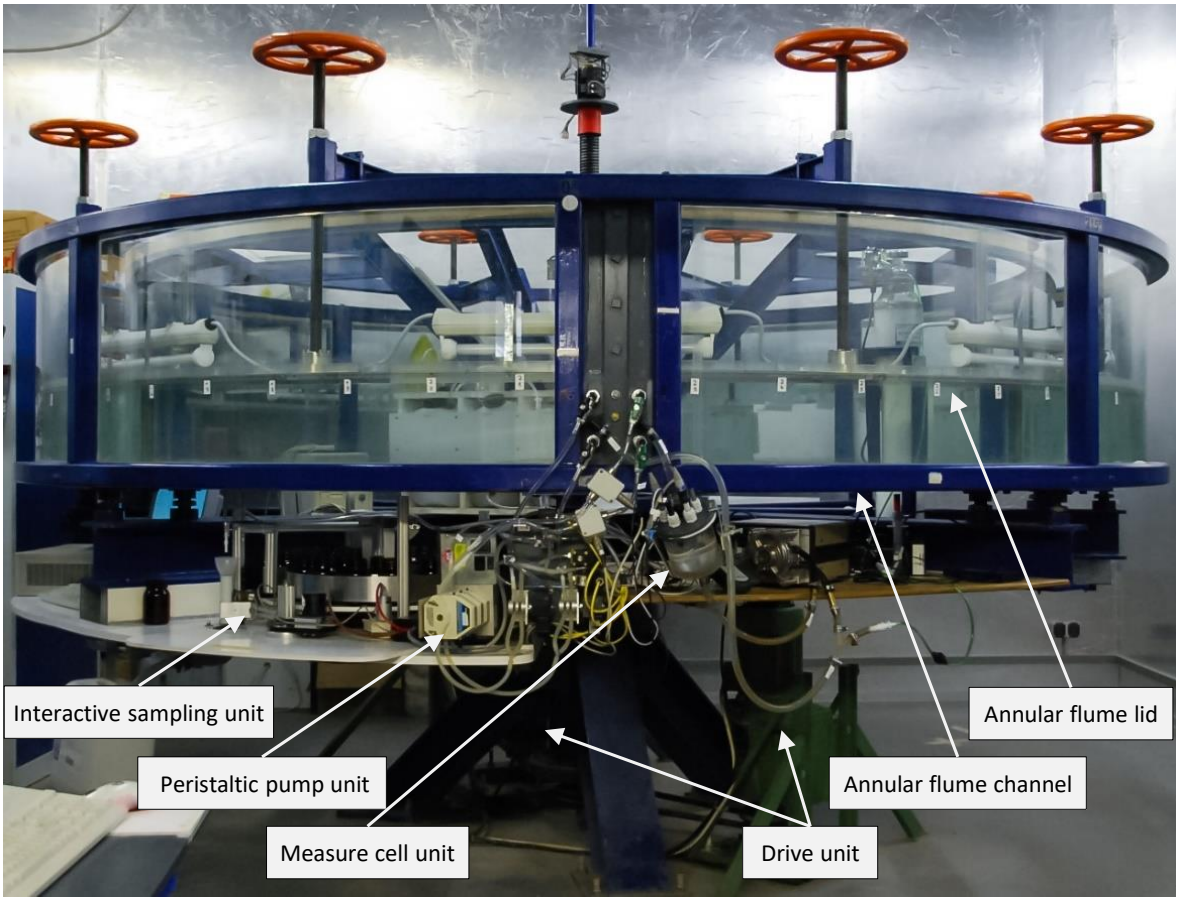


Figure 6.1 Annular flume with sampling and measurement equipment at the Institute for Hydraulic Engineering and Water Resources Management, Aachen, Germany.

A total of four experiments, which varied regarding sediment contamination, were conducted (Table 6.1). In each of the experiments physicochemical water parameters were logged at an interval of 60 s and water samples for determination of SPM withdrawn at specific time intervals. At the beginning and end of each experiment sediments, and at the end of each experiment fish ($n$=10 per sampling point, 40 individuals in total) were sampled. Concentrations of PAHs in SPM and sediment samples as well as biomarkers of exposure or effect in fish were determined.

Table 6.1 Overview of controls (C1, C2) and experiments (A, A/B, C) conducted in the annular flume.

| **Controls and experiments within the annular flume** | |
|---|---|
| C1 | Untreated control |
| C2 | Annular flume without sediment |
| A | Sediment A from the Rhine |
| A/B | Sediment mixture A/B (1:1) |
| B | Sediment B from the Moselle |

### 6.3.2 **Experimental fish**

Immature rainbow trout (15-20 g, *Oncorhynchus mykiss*) were purchased from a commercial hatchery (Mohnen Aquaculture, Stolberg, Germany) and allowed to acclimatize to laboratory conditions for at least two months prior to use in experiments. Fish were reared in groups of 100-150 individuals in 1500 L glass fibre-reinforced plastic tanks. In a recirculating system with a 400 L biofilter and UVC-sterilizer, water was continuously exchanged at a rate of 0.1 - 0.2 full replacements per day 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) at a rate of 1-2 % bodyweight per day until experimentation. The final weight and length of used fishes was 145.2 ± 73.0 g and 214.7 ± 31.3 mm, respectively.

All experiments were conducted in accordance with the Animal Welfare Act and with permission of the federal authorities (Landesamt für Natur, Umwelt und Verbraucherschutz NRW, Germany), registration number 8.87-50.10.35.08.225.

### 6.3.3 **Sediment sampling and preparation**

Sediment A was collected in April 2011 from the Rhine (river kilometre 591) close to the fortress Ehrenbreitstein in the city of Koblenz, Germany (+50° 21' 12" N, +7° 36' 27" E). This first location was chosen as it is known to be moderately contaminated and to be representative for a wide range of cohesive fluvial sediments based on particle size and organic carbon content (Feiler et al. 2009, Höss et al. 2010). Sediment B was collected in June 2012 from the Moselle near the village Stadtbredimus, Luxembourg (+49° 33' 54" N, +6° 22' 8" E). This second location was chosen as it is known to be mainly contaminated with PAHs as well as other petroleum-derived hydrocarbons and to be comparable to sediment A in terms of particle size and organic carbon content (Feiler et al. 2009).Surface samples were taken by use of a swimming dredge and subsequently stored at 4 °C in darkness prior to experiments. Physicochemical parameters of sediments and water were directly recorded (Table 6.2). Both sediments were collected in cooperation with the German Federal Institute of Hydrology (BFG, Koblenz, Germany).

A third sediment was prepared by mixing sediment A and B at a ratio of 1:1 directly prior to corresponding experiments to achieve a sediment with an intermediate contamination level compared to the moderately contaminated sediment from the Rhine and the heavily contaminated sediment from the Moselle.

Table 6.2 Physicochemical parameter of sediments and overlaying waters from the Rhine (Sediment A) and the Moselle (Sediment B) at time of sampling.

| | **Sediment A** | | **Sediment B** | |
|---|---|---|---|---|
| **Parameter** | Sediment | Water | Sediment | Water |
| Temperature [°C] | 11.7 | 12.0 | 18.8 | 20.3 |
| pH [-] | 7.43 | 7.50 | 7.36 | 9.00 |
| Redox potential [mV] | -275.0 | - | 84.8 | - |
| Conductivity [µS $cm^{-1}$] | - | 459 | - | 1531 |
| Dissolved $O_2$ [mg $L^{-1}$] | - | 11.0 | - | 9.1 |

### 6.3.4 **Sediment handling and erosion program**

Prior to all experiments sediments were sieved through a 6.3 mm sieve, mixed with tap water and transferred to the annular flume. Subsequently, water was added to a height of 325 mm above the channel bed. In order to achieve a homogenous sediment layer the settled bed approach as described by Mehta and Partheniades (1982) was used to create an even sediment layer with a total high of 40 mm with a natural sediment structure. After a consolidation period of seven days rainbow trout were added to the annular flume and exposed for seven days under increasing bed shear conditions. Due to technical problems, Experiment B was terminated after an exposure period of only five days.

A standard erosion program that allowed a detailed characterization of each sediment layer as described by Mehta et al. (1982) was used to resuspend sediments during each experiment. The bed shear stress τ was increased step-wise by 0.05 N $m^{-2}$ to a maximum of 0,4 N $m^{-2}$ during seven days, which corresponds to a maximum flow velocity of 0,38 m $s^{-1}$ (Cofalla, 2011).

### 6.3.5 **Sampling of sediments and SPM and quantification of PAHs**

Sediment samples were taken before and after each experiment and stored in the dark at 4 °C until further treatment. Sediment suspension samples were taken automatically as 250 mL duplicates per sampling event and subsequently filtered through 0.7 µm glass fiber filters (MN-GF 1, Macherey & Nagel, Düren, Germany) under vacuum. Sediments and retained SPM were lyophilized (Christ Alpha 1-2, Martin Christ GmbH, Osterode am Harz, Germany) and stored in the dark until further treatment.

Dried sediment and SPM samples were analyzed for concentrations of 16 EPA-PAHs (EPA 1984) according to DIN ISO 18287 (2006), with slight modifications. All SPM samples as well as sediment samples were analyzed by gas chromatography mass spectrometry (GC-MS) after accelerated solvent extraction. Glass fiber filters were extracted as blanks and treated in the same way as the samples. The limit of quantification (limit of detection) for SPM samples was 0.02 (0.007) µg $g^{-1}$ dry weight, whereas the limit of quantification (limit of detection) for sediment samples was 0.50 (0.17) µg $g^{-1}$ dry weight.

### 6.3.6 **Determination of physicochemical parameters**

Temperature, pH, conductivity, redox potential and dissolved oxygen concentration were determined and logged automatically at intervals of 60 s by the use of calibrated instruments (SenTix, WTW, Weilheim and Ahlborn Mess- und Regelungstechnik, Holzkirchen, Germany), which were situated in a flow-through cell attached to the annular flume. Total hardness was measured after filtration using the titrimetric Titriplex B method (Merck, Darmstadt, Germany).

### 6.3.7 **Analysis of biomarkers**

After exposure, fish were individually anesthetized in a solution of benzocaine in tap water (Sigma-Aldrich) and then exsanguinated. Subsequently, length and mass were determined for calculation of condition index (*K*), liver somatic index (*LSI*), and visceral index (*VI*).

Peripheral blood samples from the caudal vein were taken with heparinized syringes. For each individual, two smears were prepared on separate microscopic glass slides that were previously cleaned with 99 % ethanol (Merck, Darmstadt, Germany). Smears were air-dried and the slides subsequently fixed in methanol (Merck) for 1 min, then stored at room temperature until determination of micronucleus frequencies. A gallbladder bile sample was taken by use of a syringe and transferred to 1.5 ml polypropylene vials (Carl Roth, Karlsruhe, Germany). Bile samples were then stored at -20 °C until determination of PAH metabolite concentrations. The liver was isolated, weighed, cut into four about equally sized pieces, transferred into sterile 2 ml cryogenic vials (Greiner Bio-One, Frickenhausen, Germany) and subsequently frozen in liquid nitrogen. Until determination of 7-ethoxyresorufin *O*-deethylase (EROD) activity, liver samples were stored at -85 °C.

EROD activity in liver cells, lipid peroxidation, micronuclei in peripheral erythrocytes, as well as concentrations of biliary PAH metabolites were determined following methods described by

Brinkmann et al. (2013) and Kammann (2007a), respectively. Details regarding the individual protocols and the equations to calculate the indices can be found in the supplemental material.

### 6.3.8 **Data analysis**

Calculations were performed in spreadsheets using Microsoft Excel™ 2013. Graphs were plotted and statistical analyses and correlations were conducted by either the use of the software GraphPad Prism 5 (GraphPad, San Diego, USA) or the software SigmaStat 3.11 (Systat Software, Erkrath, Germany). All datasets that did not show equal variances ($p \leq 0.05$) according to Bartlett's test were log-transformed (y' = ln(y)) before statistical analysis. All datasets that passed the Shapiro-Wilk normality test or the D'Agostino & Pearson omnibus normality test on Gaussian distribution ($p \leq 0.05$) and were subsequently analyzed using the parametric one-way ANOVA ($p \leq 0.05$). Tukey's method was then used as the multiple range test to identify significant differences among experiments. The probability of Type I error (α) was set to $p \leq 0.05$. Datasets that did not pass the normality tests were analysed with Kruskal-Wallis one-way ANOVA on ranks with Dunn's post-hoc test. Unless indicated, values are expressed as mean value ± standard deviation. Outliers were detected using the Grubb's test provided as an online calculator by GraphPad (San Diego).

## 6.4 **Results**

### 6.4.1 **Physicochemical parameters**

Water temperature during all experiments ranged between 14.47 ± 0.10 °C and 14.75 ± 0.07 °C (Experiment A and Control C2). Dissolved oxygen concentrations (DO) ranged from 4.43 ± 0.52 to 5.55 ± 0.61 mg $L^{-1}$ (Experiment A/B and Experiment A) in exposure experiments and were lower than in the control experiment where DO was 6.47 ± 0.19 mg $L^{-1}$. In Experiment B dissolved oxygen concentration decreased during the exposure period and reached values of 2.6 mg $L^{-1}$ at the end of the experiment. Total hardness as well as conductivity increased from values between 1.15 and 1.52 mmol $L^{-1}$ and between 300 and 370 µS $cm^{-1}$ (Experiment B and Experiment A), respectively, over the exposure period to values ranging between 1.63 and 2.16 mmol $L^{-1}$ and between 440 and 490 µS $cm^{-1}$ (Experiment B and Experiment A/B), respectively. Redox potential increased during all exposure experiments from low values at the beginning ranging from -0.40 to 21 mV to values between 243 and 271 mV (Experiment A and Experiment A/B), whereas redox potential in Control C2 increased from 137 mV to 247 mV.

The pH value decreased slightly in all experiments during the second half of the exposure duration and was on average lower in exposure experiments (pH 7.44 ± 0.04 - 7.64 ± 0.11, Experiment A and Experiment B) than in the control experiment C2 (pH 7.92 ± 0.06).

### 6.4.2 **Suspended particulate matter**

In all experiments SPM concentrations increased consistently from low values at the beginning of each experiment to higher values at the end of each experiment and closely followed the increase of bed shear stress.

In exposure experiments with fish final SPM concentrations ranged from 18.01 and 20.96 g $L^{-1}$ for A and A/B, respectively. At the end of Experiment B, SPM concentration reached a value of 10.27 g $L^{-1}$ (Figure 6.2).

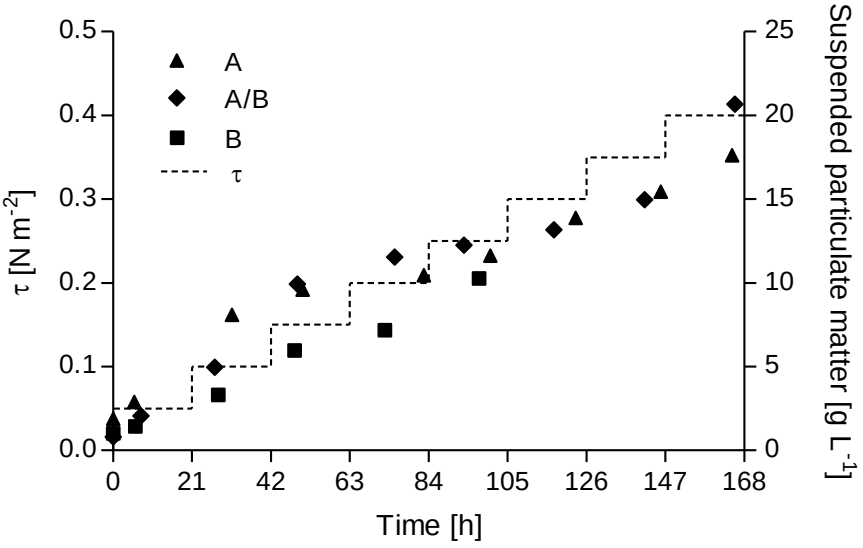


Figure 6.2 Bed shear stress τ (dashed line) and SPM concentration (symbols) during the exposure experiments in the annular flume.

### 6.4.3 **Chemical analyses of PAH concentrations in sediments and SPM**

*Sediment* After the consolidation period of 7 d, directly prior to addition of rainbow trout to the annular flume, as well as after the end of each experiment, concentrations of 16-EPA-PAHs in sediment samples were determined (Table 6.3). Concentration of 16 EPA-PAH was highest in sediment B at the beginning of the exposure experiment with 98.26 mg $kg^{-1}$ dw compared to both other sediment which showed concentrations of 23.63 and 7.94 mg $kg^{-1}$ dw (sediment A/B and B). At the end of all experiments total PAH concentrations were generally lower than in the beginning by a factor of 4, 2 and 1.5 in sediment B, A/B and A, respectively.

Most abundant PAHs in all sediments were four or five ring PAHs like FLA, PYR and BBF with percentages ranging from 10.5 to 20.4 %. BAP was also present in all sediments with percentages between 6.1 and 8.9 % of total PAHs (Σ-PAHs).

Table 6.3 Content of 16 EPA-PAHs in sediments at the beginning and end of each experiment.

| **Experiment** | | **A** | | **A/B** | | **B** | |
|---|---|---|---|---|---|---|---|
| | | **Start** | **End** | **Start** | **End** | **Start** | **End** |
| | | mg $kg^{-1}$ dw | | mg $kg^{-1}$ dw | | mg $kg^{-1}$ dw | |
| Naphthaline | NAP | 0.07 | 0.04 | 0.10 | 0.04 | 0.28 | 0.04 |
| Acenaphthylene | ACY | 0.16 | 0.08 | 0.52 | 0.39 | 2.80 | 0.71 |
| Acenaphthene | ACE | 0.03 | 0.02 | 0.33 | 0.13 | 1.90 | 0.25 |
| Fluorene | FLU | 0.08 | 0.04 | 0.58 | 0.21 | 4.40 | 0.69 |
| Phenanthrene | PHE | 0.50 | 0.27 | 1.40 | 1.00 | 7.70 | 1.40 |
| Anthracene | ANT | 0.28 | 0.13 | 1.00 | 0.65 | 5.40 | 1.30 |
| Fluoranthene | FLA | 1.50 | 0.92 | 4.70 | 2.10 | 22.00 | 4.30 |
| Pyrene | PYR | 1.10 | 0.69 | 3.40 | 1.50 | 14.00 | 3.00 |
| Chrysene | CHR | 0.62 | 0.40 | 1.80 | 0.75 | 6.70 | 1.70 |
| Benzo[*a*]anthracene | BAA | 0.62 | 0.40 | 2.00 | 0.87 | 7.90 | 1.90 |
| Benzo[*b*]fluoranthene | BBF | 1.00 | 0.70 | 2.50 | 1.20 | 8.30 | 2.30 |
| Benzo[*k*]fluoranthene | BKF | 0.32 | 0.22 | 0.89 | 0.44 | 3.00 | 0.81 |
| Benzo[*a*]pyrene | BAP | 0.64 | 0.45 | 1.90 | 1.00 | 6.00 | 1.80 |
| Benzo[*ghi*]perylene | BPE | 0.45 | 0.30 | 1.10 | 0.80 | 3.40 | 0.94 |
| Indeno[*1,2,3-cd*]pyrene | IND | 0.43 | 0.31 | 1.10 | 0.78 | 3.50 | 0.97 |
| Dibenzo[*a,h*]anthracene | DBA | 0.14 | 0.087 | 0.31 | 0.21 | 0.98 | 0.28 |
| **Sum PAHs** | | **7.94** | **5.05** | **23.63** | **12.07** | **98.26** | **22.39** |

*Suspended particulate matter* PAH concentrations in SPM from all exposure experiments were higher at the beginning of each experiment and decreased slightly during the exposure period. Mean concentrations were 7.30 ± 0.97 mg $kg^{-1}$ dw, 17.26 ± 2.21 mg $kg^{-1}$ dw and 77.63 ± 3.15 mg $kg^{-1}$ dw for Experiment A, A/B and B, respectively (Figure 6.3).

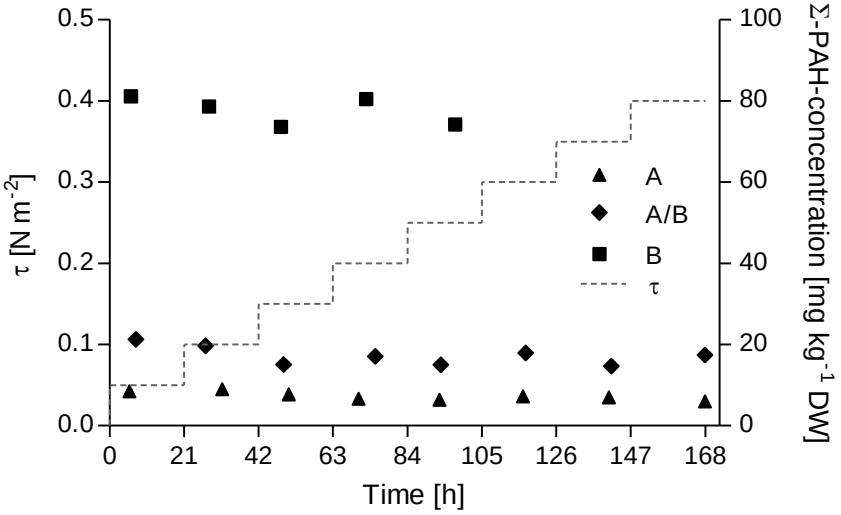


Figure 6.3 PAH concentrations in SPM during the exposure experiments in the annular flume displayed as sum of 16-EPA PAHs. The dashed line represents the progression of the bed shear stress (τ).

### 6.4.4 **Biomarker analysis**

*Morphometric indices* were evaluated to assess changes in condition and health during exposure and control experiments. The condition index (*K*) was significantly higher in Experiment B (*K* 1.50 ± 0.08) compared to C1, C2 and Experiment A (*K* 1.29 ± 0.11, 1.26 ± 0.07 and 1.32 ± 0.08, respectively). Between Experiment A/B (*K* 1.49 ± 0.33) and C2 (*K* 1.26 ± 0.07) the condition index was significantly different (one-way ANOVA with Tukey's post-hoc test, $p \leq 0.05$). The visceral index (*VI*) was significantly higher in C1 (*VI* 13.50 ± 1.04) compared to all other experiments (*VI* 9.31 – 11.77) as well as in Experiment A/B (*VI* 11.77 ± 1.64) compared to C2 (*VI* 9.31 ± 0.75, one-way ANOVA with Tukey's post-hoc test, $p \leq 0.05$). The liver somatic index (*LSI*) of the untreated Control C1 (LSI 1.2 ± 0.37) was significantly higher compared to exposure experiments (LSI 0.83 ± 0.16 – 0.90 ± 0.13) and the Control C2 (LSI 0.78 ± 0.09).

*EROD activity* Significant differences in EROD activities in trout livers were observed compared with Control C1 (Figure 6.4). In all experiments with sediment, EROD activity was significantly higher than that of untreated animals from both the untreated Control C1 and Control C2 ($p \leq 0.05$) with activities in livers of fish from 11.63 ± 4.27 pmol $mg^{-1}$ $min^{-1}$, 37.27 ± 14.39 and 34.81 ± 10.24 pmol $mg^{-1}$ $min^{-1}$ for experiments with sediment from the Rhine, sediment mixture and sediment from the Moselle, respectively. EROD activity of Experiment A/B and B did not differ significantly.

Compared to the Control C2, induction factors for Experiments A, A/B and B were calculated with values ranging from 14.9, 47.6 and 44.5, respectively.

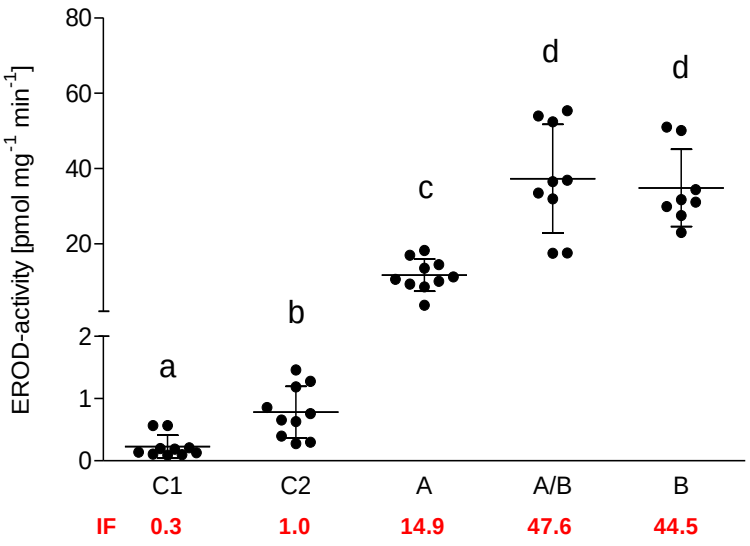


Figure 6.4 EROD activities in trout livers after exposure experiments in the annular flume in pmol $mg^{-1}$ $min^{-1}$. Symbols represent individual animals, bars the mean value and error bars the standard deviation. Experiments sharing the same letter did not differ significantly (one-way ANOVA with Tukey's post-hoc test with log-transformed data, $p \leq 0.05$). IF – calculated induction factors based on mean EROD activity of Control C2.

*Lipid peroxidation* Between both control experiments C1 and C2 were not statistically significant differences in LPO measured as equivalent concentrations of malondialdehyde (MDA, Figure 6.5). In fish from sediment experiments LPO was increased, reflected by induction factors ranging from 1.16 to 1.39. The average MDA concentration was 361.20 ± 62.22 and 351.14 ± 99.25 mmol MDA $g^{-1}$ for C1 and C2. Significant differences in MDA concentrations were observed between two sediment experiments (A and B) compared to both control experiments ($p \leq 0.05$, Figure 6.5). Average MDA concentrations in sediment treatments were 486.50 ± 89.71, 407.73 ± 49.91 and 470.90 ± 77.10 mmol MDA $g^{-1}$for Experiments A, A/B and B.

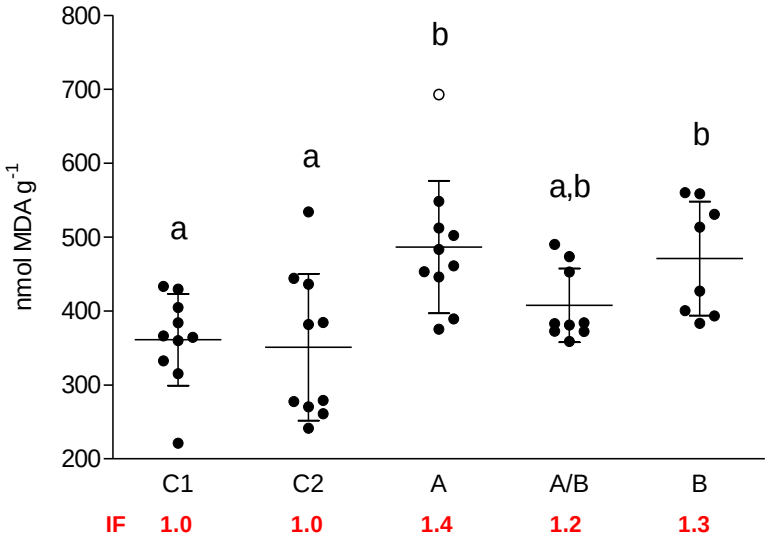


Figure 6.5 Hepatic lipid peroxidation in trout livers after exposure experiments in the annular flume in nmol malondialdehyde (MDA)-equivalent $g^{-1}$ liver. Symbols represent individual animals, bars the mean value and error bars the standard deviation. Experiments sharing the same letter do not differ significantly (one-way ANOVA with Tukey's post-hoc test, $p \leq 0.05$). Outliers according to Grubbs' test ($p \leq 0.05$) are shown as circles. IF – calculated induction factors based on mean MDA content of Control C2.

*PAH metabolites* Significant concentrations of 1-hydroxypyrene were observed in bile of fish from all experiments, including the Control C2 ($p \leq 0.05$, Figure 6.6). After exposure to sediments, high concentrations of the PAH metabolite could be detected. Concentrations of 1-hydroxypyrene in bile from fish exposed in Experiment B were significantly higher with an average of 502.57 ± 110.83 µg $ml^{-1}$ compared to the Control C2 (0.97 ± 0.38 µg $ml^{-1}$) and Experiment A (46.47 ± 7,34 µg $ml^{-1}$; $p \leq 0.05$). In Experiment A/B 1-hydroxypyrene concentrations reached 168.00 ± 31.91 µg $ml^{-1}$ and were significantly higher than those found in C2 ($p \leq 0.05$). Compared to the Control C2 induction factors increased from Experiment A to A/B to B with values ranging from 48, 173 and 519.

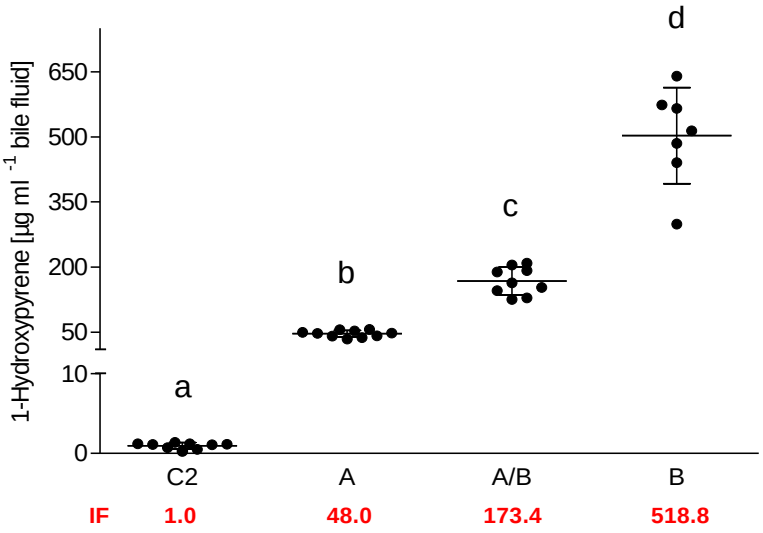


Figure 6.6 Absolute concentrations of 1-hydroxypyrene in trout bile fluid after exposure experiments in the annular flume in µg ml$^{-1}$. Symbols represent individual animals, bars the mean value and error bars the standard deviation. Experiments sharing the same letter do not differ significantly (one-way ANOVA with Tukey's post-hoc test after log-transformation, $p \leq 0.05$). IF – calculated induction factors based on mean 1-hydroxypyrene content of Control C2.

*Micronuclei* in peripheral erythrocytes from exposed fish showed a generally increasing trend during exposure in all experiment within the annular flume. Micronuclei rate was highest in Experiment B (0.77 ± 0.28 ‰) followed by A/B (0.71 ± 0.29 ‰) and A (0.57 ± 0.37 ‰). However, significant differences between the annular flume experiments and control experiment C2 were only observed for Experiment B, resulting in an induction factor of 2.24 ((p < 0.05; Figure 6.7).

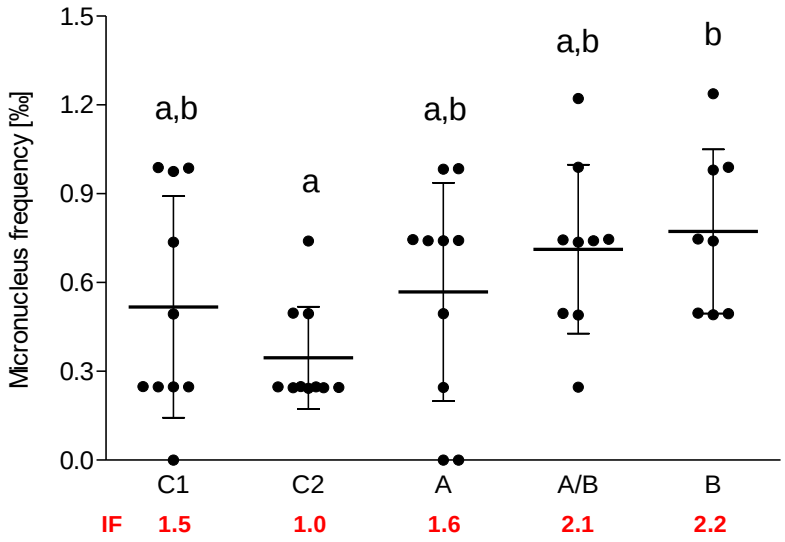


Figure 6.7 Micronuclei rate in peripheral erythrocytes of trout after exposure experiments in the annular flume. Symbols represent individual animals, bars the mean value and error bars the standard deviation. Experiments sharing the same letter do not differ significantly (One-Way ANOVA with Tukey Post-hoc test, $p < 0.05$). IF – calculated induction factors based on mean micronuclei rate of Control C2.

## 6.5 **Discussion**

### 6.5.1 **Behavior of physicochemical parameters**

*Water temperature* is one of the most important environmental parameters for salmonid species as due to their poikilothermic nature, it influences all physiological processes such as growth, metabolism and reproduction. Furthermore, suboptimal temperature regimes can cause severe stress or even lethality (Hokanson et al. 1977, Myrick & Cech 2004). During all experiments water temperature was well near the optimal range for rainbow trout or the fundamental thermal niche as defined by Christie and Regier (1988). According to Bear et al. (2007) this niche lies between 10.1 and 14.1° C for rainbow trout. In thermal preference tests McMahon et al. (2008) reported a preference temperature for rainbow trout of 14.8° C when tested in a gradient of 11-17° C. Therefore, negative influence of physiological functions on experiments outcome, especially on biomarker response, due to temperature stress during exposure experiments can be ruled out.

*Dissolved oxygen* (DO) concentration is directly and negatively correlated to water temperature, as with increasing temperature oxygen levels decrease. Another important factor influencing DO is microbiological induced oxygen depletion which e.g. takes place when sediments are

resuspended and reducing compounds such as detritus are released from the sediment. Flood events and dredging operations often lead to lowered DO concentrations caused by oxygen depletion and reduced biogenic $O_2$ input (Ryan 1991). During all experiments average to low DO concentrations were observed. During the experiment with sediment from the Moselle notable oxygen depletion resulting in temporal DO concentrations below 3 mg $L^{-1}$ was observed which was attributed to the higher content of organic matter and associated microbial processes compared to the sediment from the River Rhine. These finding are well in line with data reported by other studies which found DO concentrations between 2 and 4.3 mg $L^{-1}$ during a flood event at the River Elbe (BFG 2008) and a flood event at the River Oder (IKSO 1999).

*Total Hardness and conductivity* increased slightly throughout the exposure period in all experiments and was positively correlated with SPM content ($r_{spearman}$ 0,89 - 0.99). Increase in total hardness is a result of an increase in ion release, mainly calcium and magnesium, from suspended solids. However, changes in total hardness have primarily impacts on metal toxicity (Bradley & Sprague 1985, Hansen et al. 2002) whereas impact on bioavailability of organic contaminants is less pronounced (Akkanen & Kukkonen 2001).

*Redox potential* was low at the start of each exposure experiment reflecting near anoxic conditions after the consolidation phase. Sediment disturbance and begin of aeration during the exposure period led to an increase of redox potential due to the oxidation of initially reduced sediment compounds as well as a slight decrease in pH. Delaune and Smith (1985) reported a similar behavior of both redox potential and pH during their resuspension experiments and concluded that the extent of pH decrease is related to the sulphide concentration and oxidation state in the sediment. Redox potential and pH influence sediment-contaminant complexes and play important role in the mobility of sediment associated contaminants. Changes of those parameters result in increased desorption of contaminants from suspended sediments such as metals (Petersen et al. 1997, Simpson et al. 1998) whereas desorption of hydrophobic organic contaminants depends mainly on the solubility of the contaminant and its distribution/partitioning coefficient ($K_d$) as well as the concentration differences between the resuspended sediment and the water column (Goossens & Zwolsman 1996, Latimer et al. 1999, Roberts 2012).

### 6.5.2 **Suspended particulate matter**

SPM content was strongly related to bed shear stress in all exposure experiments ($r^2$ = 0.95 - 0.97, $p < 0.0005$, data not shown), which demonstrates the ability of the annular flume to steadily resuspend natural sediments by a stepwise increase of bed shear stress. Ranging between 10.27 and 20.96 g $l^{-1}$ maximum SPM concentrations during exposure experiments were within values reported by other authors for SPM load in rivers. Eyrolle et al. (2012) found SPM concentrations of 3.67 g $l^{-1}$ during a flood event in the river Rhone, whereas Xia et al. (2006) documented 28 g $l^{-1}$ SPM in the Yellow River in China. However, there are substantial differences in SPM load in different river systems depending on e.g. discharge (normal flow or flood conditions), relief types and river basin size as the review by Meybeck et al. (2003) who assessed discharge-weighted total suspended solids at 60 global river sampling stations demonstrates. Elevated SPM concentrations were also reported during dredging events by e.g. Quick et al. (2011) who reported 1.5 g $l^{-1}$. The high SPM concentrations within our study are therefore worst case scenario values which would be expected during severe flood events.

### 6.5.3 **Chemical analyses of PAH concentrations in sediments and SPM sediments**

Sum of 16-EPA-PAH in sediments and SPM increased from A to A/B to B resulting in a contamination gradient between individual experiments. After exposure periods, however, total PAH concentrations in sediments were by a factor of 1.6, 2 and 4.4 (Experiment A, A/B and B) lower than initial values. In contrast relative PAH concentration of SPM per kg dw remained stable during each of the exposure experiments, while total PAH load per liter increased during the exposure periods due to rising SPM concentrations. The decrease in sediment PAH concentration as well as the fact that PAH concentrations remained stable in SPM may be explained by a change in grain size distribution of the sediment during the resuspension event. Contaminants were most likely bound to smaller particle and were resuspended more easily than larger particles which lead to a decrease of PAH concentrations in the remaining sediment. Other elimination processes in aquatic systems, such as microbial degradation (Haritash & Kaushik 2009), adsorption, volatilization (e.g. Ravikrishna et al. 1998, Valsaraj et al. 1997) and photolysis of PAHs may have attributed to the reduction but cannot comprehensively explain these differences. Total PAH concentration in sediment A was in accordance with previously reported findings for the Rhine where 3.8 mg $kg^{-1}$ dw were found by (Feiler et al. 2013) or where 2.16 mg $kg^{-1}$were reported by Heimann et al. (2011). Wölz et al. (2010a) reported slightly lower concentrations of PAH with 1.92 mg $kg^{-1}$ in SPM sampled during a flood event

in the Rhine. Total PAH concentration in sediment B was however by a factor of 4 higher than stated by Feiler et al. (2013) and more comparable to highly contaminated sites like e.g., Athabasca river, Canada, with ∑-PAH values of up to 34.7 mg $kg^{-1}$ dw (Headley et al. 2001), Oder river with ∑-PAH values of up to 146.4 mg $kg^{-1}$ dw (Witt & Gründler 2005) or Hai River with ∑-PAH values of up to 255.3 mg $kg^{-1}$ dw (Jiang et al. 2007).

### 6.5.4 **Biomarker responses after exposure to natural sediments**

*PAH uptake path and metabolites* As demonstrated by various studies there is a clear relation between PAH contamination in sediments or overlying waters and PAH metabolites in bile fluid of exposed fish (e.g. Johnson-Restrepo et al. 2008, Jung et al. 2011, Yang & Baumann 2006). The most prominent metabolite is 1-hydroxypyrene (1-OH-PYR) or its conjugated precursor which is often used to assess total PAH exposure (Ruddock et al. 2003). In this study significant amounts of 1-OH-PYR, with an increasing gradient from the moderately contaminated sediment A towards the most contaminated sediment B, were found in fish bile after exposure experiments. The ratio between 1-OH-PYR in bile from fish exposed to sediment A, A/B and B was 1 : 3.6 : 10,7 and correlated well with (1) the ratio of particle bound pyrene in corresponding sediments which was 1 : 3.1 : 12.7 and (2) with the ratio of particle bound pyrene in SPM which was 1 : 2.7 : 11.7 (Figure 6.8).

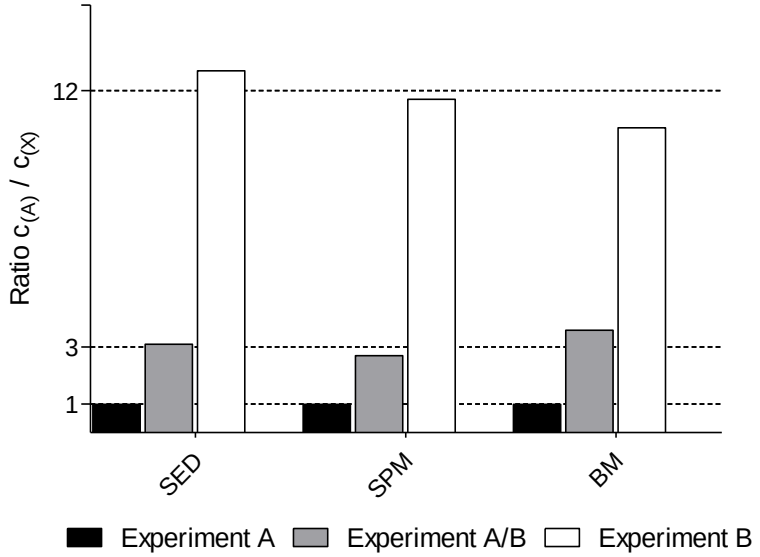


Figure 6.8 Ratios of pyrene or 1-hydroxypyrene in different compartments during exposure experiments in the annular flume. All ratios were normalized to corresponding content of pyrene in Experiment A. SED – sediment compartment; SPM – suspended particulate matter compartment; - BM – bile metabolite 1-hydroxypyrene in bile of exposed fish.

Possible uptake paths of PAHs are through the gills, the skin or the gastro-intestinal tract, whereas the most prominent uptake route for waterborne PAHs seems to be through the gills (De Voogt et al. 1991, Kennedy & Law 1990, Lee et al. 1972, Yang et al. 2000). According to Nichols et al. (1996) dermal absorption could account for less than 10 % of initial intake in trout and varies with the contaminant, the species and the life stage of exposed fish. However, dermal absorption may play a much larger role for fish living in direct contact with sediment, i.e. benthic species. Uptake of various organic chemicals from food is well documented in literature (Balk et al. 1984, Grung et al. 2009, Kelly et al. 2004) and accounts according to Randall et al. (1998) only for a small proportion of body burden and is depended on feeding rates. Other authors suggest that dietary uptake predominates at higher log $K_{ow}$, whereas aqueous uptake plays a significant role at lower log $K_{ow}$ (Qiao et al. 2000). In the present study exposed fish were not fed throughout the exposure period, but significant amounts of ingested sediment were found in guts of exposed animals (Figure 6.9).

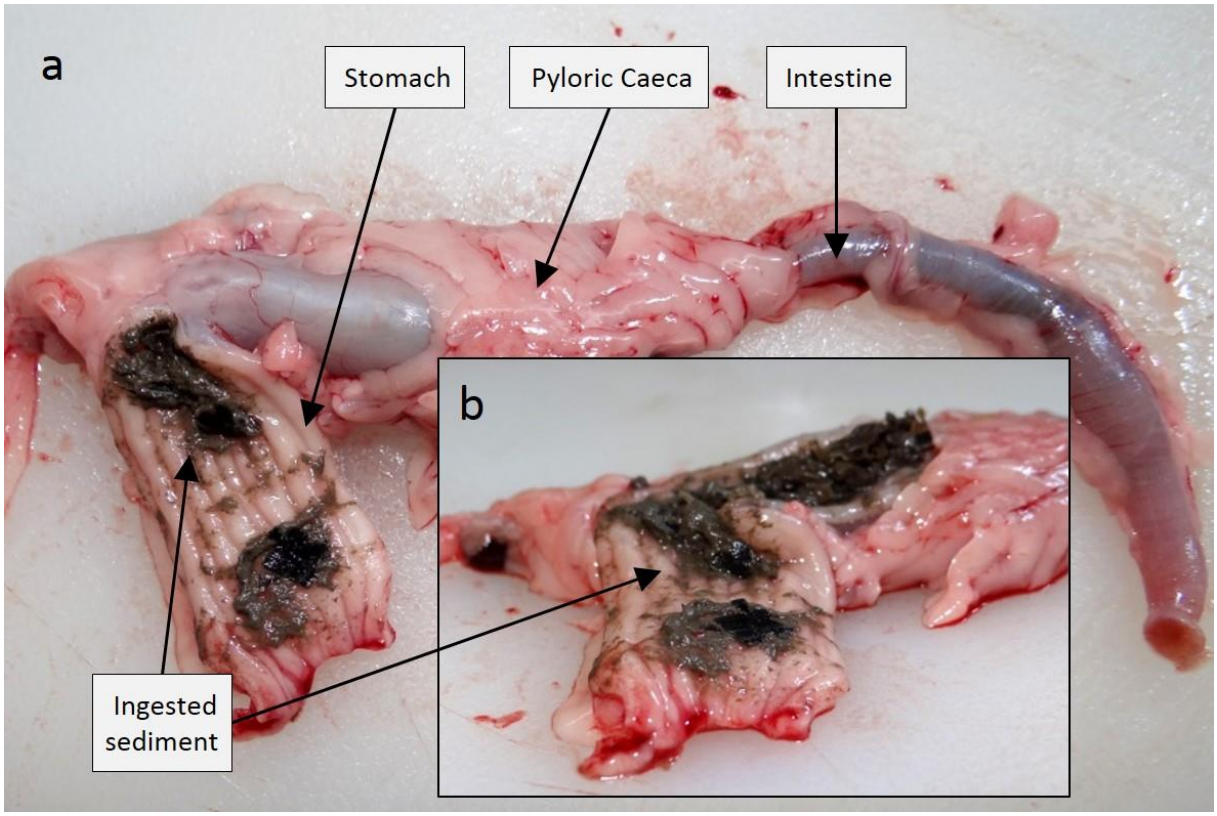


Figure 6.9 Sediment particles in gastro-intestinal tract of exposed rainbow trout after dissection. A - Dissection of stomach with sediment found inside. B – Dissection of stomach and first part of the intestine with sediment found inside.

Those findings are supported by other authors, who also reported the presence of sediment in gastro-intestinal tracts of fish (Malins et al. 1985, Schrap & Opperhuizen 1990, Varanasi et al. 1987). However there exist only few studies regarding the significance of ingested sediment for

pollutant uptake. Kolok et al. (1996) did not found increased body concentrations of Benzo[*a*]pyrene and hydroxy- Benzo[*a*]pyrene after sediment ingestion. In contrast Moermond et al. (2004) presented a modeling approach which identified up to around 20 % of total contaminant uptake being related to sediment ingestion and absorption after being solubilized by digestive fluids. Furthermore, it is well documented for benthic invertebrates that sediment ingestion is a significant uptake route for hydrophobic compounds (Weston et al. 2000). Therefore we would not rule out the possibility that sediment ingestion contributed to total PAH burden during exposure experiments.

We conclude that the bioavailable fraction is closely related to the particle bound pyrene. In contrast, Brinkmann et al. (2013) showed in their study where PAH spiked sediments were used instead of sediments with a natural and aged PAH contamination, that the bioavailable fraction was significantly higher which was reflected by much higher distribution rations: in spiked sediment 1-hydroxypyrene was increased by a factor of 120 compared to unspiked sediment. However, concentrations of biotransformation products following exposure to sediment from the Rhine (Sediment A in the present study), i.e. bile metabolites of pyrene, were lower compared to our findings. This can be attributed to the static exposure conditions used in the study by Brinkmann et al. (2013) as respiratory volume, general metabolism and therefore also biotransformation rates are increased under physical exercise (Stevens & Randall 1967, Wood & Munger 1994) as well as under hypoxic conditions. McKim and Goeden (1982) reported an increased total pollutant uptake with increasing ventilation volume and decreasing DO concentration in water. Therefore, we strongly assume that the combination of physical exercise, SPM induced stress and reduced DO concentration resulting in a higher ventilation volume led to an increased contaminant uptake and biotransformation compared to static exposure conditions.

Compared to field studies where PAH metabolites in eel bile were found in the range of 5 µg $ml^{-1}$ (Nagel et al. 2012, Ruddock et al. 2003), 1-PYR-OH contents of exposed rainbow trout were notably higher with values between 46 and 502 µg $ml^{-1}$. These findings strongly support the hypothesis that resuspension of PAH contaminated sediments in case of flood or dredging events leads to an elevated amount of readily bioavailable PAHs as well as to an increased uptake and metabolism in fish.

*EROD activity* in livers of exposed rainbow trout showed a similar but less pronounced gradient following the contamination level as found for bile metabolites. The ratio between EROD

activities in livers from fish exposed to sediment A, A/B and B was 1 : 3.2 : 3 which even implies a slight reduction in EROD activity in livers exposed to the most contaminated sediment. Possible explanations for this finding are inhibitory effects of different EROD inhibiting substances like PAHs (Willett et al. 2001) and PCBs (Wassenberg et al. 2002) found in sediment from the Moselle (Feiler et al. 2009). Exposure time is another important factor in the determination of EROD activity as in most fish species induction occurs within the first 48 h with a maximal induction reached after less than seven days (Whyte et al. 2000). The maximal induction is often followed by a sharp decrease in activity, as shown by Munkittrick et al. (1995) who found a 20 to 40 fold increase after 7 or 14 days, respectively, followed by a sharp decline. Induction factors found in our study were well in line with those by Munkittrick et al. (1995) and Viganò et al. (1993). However, the shorter exposure duration during Experiment B (5 d) should be taken into account as EROD activity may not have reached its maximal induction. Overall the EROD activity reflected well the different contamination scenarios and proved to be a reliable biomarker for the assessment of PAH contaminated sediments as also demonstrated by Van Veld et al. (1990) and Addison et al. (1994) who assessed EROD induction in fish exposed to PAH contaminated environments.

*Micronuclei* induction follows an ascending trend, from Experiment A through A/B to Experiment B. However, significant differences compared to control experiment C2 could only be detected for Experiment B with an induction factor of 2.2. No significant differences between Experiment B and A/B as well as A could be detected, although PAH contamination of SPM as well as amount of biotransformation products of absorbed PAH were significantly higher during Experiment B. Especially metabolites of Benzo[*a*]pyrene, which were found in elevated concentrations in both SPM and sediment of Experiment B, are known to have genotoxic effects (Al-Sabti & Metcalfe 1995). Besides contamination level, formation of micronuclei is depended on exposure time (Das & Nanda 1986), whereas the maximal micronucleus rate is reached after six to seven days (Brinkmann et al. 2013, De Flora et al. 1993). This implies that the shorter exposure time of five days during Experiment B may have led to a lower micronucleus rate compared to the regular exposure time of seven days.

In contrast to other studies inductions factors which ranged in our study from 1.6 to 2.2 were lower: Brinkmann et al (2013) found induction factors between 4.3 and 4.5 for PAH-spiked sediments, whereas De Flora et al. (1993) showed an induction factor of 3.3 for fish exposed to contaminated water.

Nevertheless, micronuclei were significantly induced in fish exposed to sediment from the Moselle used in Experiment B which denotes the genotoxic potential of particle-associated contaminants, mostly PAHs. In the scope of ecological relevance the micronucleus test is of particular interest because it is a reliable biomarker for the irremediable loss of genetic information (Heddle et al. 1991). Relations between genotoxic effects and associated impacts on population as well as ecosystem level are well documented by e.g. White et al. (1999) and Diekmann et al. (2004b).

*Lipid peroxidation* is a reaction to increased levels of oxy-radicals and reflects oxidative damage of cell compounds (Carney Almroth et al. 2008). We found a significant increase in Experiments A and B compared to Control C2, which indicates oxidative stress caused by the exposure scenarios. Those findings are comparable to previous studies by Carney Almroth (2008) who found 1.8 fold increased lipid peroxidation rates in fish caged in a polluted river and Brinkmann et al. (2010a) who found 1.2 to 1.7 fold increased LPO rates. However, in contrast to EROD activity and bile metabolites no gradient linked to contaminant concentration could be determined. Several factors may have influenced the formation of antioxidative capacities. The most important factor was presumingly the effect of lower $O_2$ concentrations during both experiments with highest contamination levels (i.e. Experiment A/B and B). A common adaption of fish exposed to low concentrations of DO are increased antioxidative capacities as described e.g. by Hermes-Lima and Zenteno-Savin (2002) or by Martinez-Alvarez et al. (2005). Those increased antioxdative mechanisms may have compensated oxidative stress caused by readily available contaminants.

### 6.5.5 **Implications for sediment management**

The Directive on Environmental Quality Standards (EQS Directive, CEC 2008) sets environmental quality standards (EQS) for different substances of concern in surface waters. Furthermore, there is the possibility to apply those EQS for sediment and biota, instead of water. To date SPM is not mentioned explicitly in the WFD (CEC 2000) or in the EC EQS Directive (CEC 2008) although it has a substantially influence on water quality and bioaccessibility, i.e. possible transfer of pollutants to aquatic organisms. However, environmental quality standards (EQS) for organic substances in surface waters comprise at the moment both dissolved and fractions to adsorbed SPM. This can lead to analytical inaccuracies or even problems, specifically in regard to hydrophobic compounds strongly absorbed to particles (90 % or more) when e.g. the annual average concentration is assessed (Werres et al. 2009). In a guidance

document by a drafting group comprised of experts from EU member states, it was proposed to surrogate analysis of whole water sample by analysis of the SPM fraction (chapter 5.3 and 6.2, CEC 2009) and a subsequent conversion to the concentration in the whole water to overcome this problem (Schubert et al. 2012).

Increased SPM and associated contaminants as a result of e.g. flood or dredging events are already mentioned in the Directive on Environmental Quality Standards as "losses from pollution accumulated in sediments" (EQS Directive, CEC 2008), however EQS for suspended particulate matter should be implemented within the WFD to account for the importance of SPM in risk assessment. Besides direct impacts of SPM-bound contaminants on aquatic organisms, transport and deposition of contaminated SPM may endanger the goal to achieve a good chemical and ecological status even at remote or downstream sites (Förstner et al. 2004, Hilscherova et al. 2007).

## 6.6 **Conclusion**

Our results clearly show (1) the impact of increased SPM contents on the bioaccessibility of organic contaminants even after short resuspension events and (2) the associated adverse effects on aquatic organisms. Assessed biomarkers of exposure (EROD, LPO, bile metabolites and micronuclei) reflected the different contamination levels in sediments by increasing responses. Especially bile metabolites were a good evidence for increased bioavailability of SPM-bound contaminants and elevated EROD activates indicated immediate sublethal effects. Whereas increased micronuclei frequencies in exposed rainbow trout suggest potential long term effects of SPM associated contaminants. Although uptake trough gills seems to be the major uptake route for contaminants, evidence for a possible uptake through the gastro-intestinal tract was found.

The annular flume proved to be a good and reliable experimental device to assure near realistic exposure conditions for rainbow trout to contaminated natural sediments under various flow conditions. While similar findings have also been reported by other authors for different resuspension scenarios (Goossens & Zwolsman 1996, Latimer et al. 1999, Roberts 2012), our interdisciplinary study was carried out under near realistic exposure conditions taking e.g. physical stress and lowered DO concentrations into account. We assume that the combination of these factors led to an increased contaminant uptake and biotransformation compared to static exposure conditions. Furthermore, we think that our interdisciplinary approach, i.e. the

combination of ecotoxicology and civil engineering knowledge in the field of hydraulics and sediment dynamics is a clear advantage when trying to assess sediment resuspension events under more realistic conditions and ought to be considered as a useful tool for future studies as well as in sediment risk assessment.

Within the implementation of the water framework directive it is important to understand the potential short- and long-term impact of suspended particulate matter-bound contaminants on aquatic organisms as well as the associated uptake mechanisms for a sound risk assessment. Consequently, we propose a higher emphasis of SPM and associated contaminants in the context of an integrated river basin management within the water framework directive.

## 6.7 **Acknowledgements**

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## 6.8 **Supplemental material**

### 6.8.1 **Animal dissection and tissue preparation**

After exposure, fish were individually anesthetized in a solution of benzocaine in tap water (Sigma-Aldrich) and then exsanguinated. Subsequently, length and mass were determined for calculation of condition index (*K*, equation 6.1), liver somatic index (*LSI*, equation 6.2), and visceral index (*VI*, equation 6.3).

$$K = {W_F}/{L^3} \times 100 \qquad \text{(Eq. 6.1)}$$

$$LSI = {W_L}/{W_F} \times 100 \qquad \text{(Eq. 6.2)}$$

$$VI = {(W_F - W_C)}/{W_F} \qquad \text{(Eq. 6.3)}$$

$W_F$ is the weight of the fish in mg, $W_L$ the liver weight in mg, $L$ the standard length in mm and $W_C$ the carcass weight in mg, i.e. the weight of the eviscerated animal.

Peripheral blood samples from the caudal vein were taken with heparinized syringes. For each individual, two smears were prepared on separate microscopic glass slides that were previously cleaned with 99 % ethanol (Merck, Darmstadt, Germany). Smears were air-dried and the slides subsequently fixed in methanol (Merck) for 1 min, then stored at room temperature until determination of micronucleus frequencies. A gallbladder bile sample was taken by use of a syringe and transferred to 1.5 ml polypropylene vials (Carl Roth, Karlsruhe, Germany). Bile samples were then stored at -20 °C until determination of PAH metabolite concentrations. The liver was isolated, weighed, cut into four about equally sized pieces, transferred into sterile 2 ml cryogenic vials (Greiner Bio-One, Frickenhausen, Germany) and subsequently frozen in liquid nitrogen. Until determination of 7-ethoxyresorufin *O*-deethylase (EROD) activity, liver samples were stored at -85 °C.

### 6.8.2 **Treatment of bile samples and determination of biliary PAH metabolites**

Concentrations of biliary 1-hydroxypyrene and biliverdin were quantified according to a modification of the method published by Kammann (2007b). Briefly, 25 µl of the 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. The reaction was stopped with 125 µl solution of 5 mg $ml^{-1}$ ascorbic acid in ethanol and the mixture centrifuged (700 × g, 5 min). The concentration of 1-hydroxpyrene was determined by means of high-performance liquid chromatography (HPLC) with fluorescence detection (cf. Kammann 2007b).

### 6.8.3 **Determination of EROD activity**

Pieces of liver explants were thawed carefully and homogenized by use of an electric disperser (VDI 12, VWR, Darmstadt, Germany) at a ratio of 1:10 (w/v) in homogenization buffer (pH 7.4) according to Bonacci et al. (2003), containing 50 mM dipotassium hydrogenphosphate, 0.75 mM sucrose, 1 mM ethylendiamine tetraacetic acid, 0.5 mM dithiothreitol and 0.4 mM phenylmethylsulfonyl fluoride. Subsequently, homogenates were transferred into 1.5 mL micro test tubes (Greiner Bio-One) and centrifuged for 20 min (9000 × g, 4 °C) in a cooling centrifuge (Rotina 420R, Hettich, Tuttlingen, Germany). All steps were carried out on ice. The supernatants (S9 fractions) were carefully transferred to fresh 1.5 mL micro test tubes and stored on ice until measurement of EROD activity and protein concentrations on the same day.

EROD activity was measured in duplicate according to the method described by Burke & Meyer (1974) adopted by Maria et al. (2005). In a semi-micro quartz cuvette (Hellma, Müllheim, Germany), 1 mL solution of 7-ethoxyresorufin (Sigma-Aldrich, Deisenhofen, Germany) in Tris-HCl buffer (0.1 M Tris, 0.15 M potassium chloride, pH 7.4) were mixed with 100 µL of the S9 fractions by repeated inversion of the cuvette. Directly prior to the measurement, the reaction was initiated by addition of 10 µL 10 mM NADPH (Sigma-Aldrich) in Tris-HCl buffer. If the activity was too high, S9 fractions were diluted at a ratio of 1:10 (v/v) with homogenization buffer. Fluorescence of the reaction mixture was recorded in 10 s intervals for 5 min (excitation: 530 nm, emission: 585 nm). To correct for spontaneous substrate conversion, blank measurements containing 100 µL homogenization buffer were treated as the samples. A serial dilution series of resorufin in Tris-HCl buffer was used as external standard.

Protein concentrations for the calculation of specific enzyme activities were determined in triplicates using the bicinchonic acid (BCA) method provided as kit (Sigma-Aldrich). Bovine serum albumin (BSA) was used as external standard (0.125 – 1.25 mg $mL^{-1}$). In 96-well microplates (TPP, Trasadingen, Switzerland), 200 µl of the working solution were added to 25 µl 1:10 dilutions of liver S9 fractions. The extinction at 562 nm was read after 30 min incubation at 37°C, using an Infinite 200 microplate reader (Tecan, Crailsheim, Germany). Protein concentrations were interpolated from the obtained standard curves. The specific EROD activity was then calculated from the relative fluorescence units and the protein concentrations and expressed as pmol resorufin generated per mg protein and minute.

### 6.8.4 **Determination of micronuclei in peripheral erythrocytes**

The proportion of micronucleated cells in peripheral erythrocytes was determined according to methods published in Rocha et al. (2009). The previously prepared smears were stained by adding a few drops of a 0.2 µm membrane filtered (Millipore Millex, Schwalbach, Germany), 0.004 % acridine orange solution (w/v) in phosphate buffered saline (PBS, Sigma-Aldirch). After 3 min incubation, the staining solution was discarded, the slides rinsed with distilled water and air-dried (Ueda et al. 1992). For each individual fish, 4000 erythrocytes were examined on the two separate smears using an epifluorescence microscope at 1000 × magnification (Nikon Instruments, Düsseldorf, Germany). 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). In order to avoid experimenter's bias, the slides were coded. Results were expressed as micronucleated cells relative to the total number of cells counted.

Chapter 7

# Conclusion and future perspective

Parts of this chapter have been previously published in the following peer-reviewed article:

Brinkmann, M. & **Hudjetz, S.**, Kuckelkorn, J., Yu, T., Cofalla, C., Roger, S., Kammann, U., Schüttrumpf, H., Hollert, H. (2013) How flood events affect rainbow trout: Evidence of a biomarker cascade in rainbow trout after exposure to PAH contaminated sediment suspensions. *Aquatic Toxicology* 128-129: 13-24.

**Hudjetz S**., Herrmann H., Cofalla C., Brinkmann M., Kammann U., Schäffer A., Schüttrumpf H., Hollert H. (2014): An attempt to assess the relevance of flood events – biomarker response of rainbow trout exposed to resuspended natural sediments in an annular flume. *Environmental Science and Pollution Research* 21, 13744-13757

## 7.1 **Conclusion and outlook**

The preceding chapters have illustrated the progress of the interdisciplinary method development in the proof-of-concept phase, an additional supplemental study and, finally, the evaluation in the third study phase. It was highlighted that knowledge from different, normally separated research areas were successfully fused to create an innovative research tool to assess the hydrotoxicological relevance of contaminated sediments after resuspension events. However, there are certain drawbacks and further method improvements should be undertaken to optimize the interdisciplinary approach.

Within this chapter, comprehensive conclusions from each study objective (Objective I, II and III, see chapter 1.9) will be detailed and critically evaluated, before future perspectives in this research area are discussed and an outlook is given.

## 7.2 **Objective I – proof-of-concept study**

The proof-of-concept study was designed to determine the possibility to interdisciplinary combine techniques from ecotoxicology, hydraulic engineering and water management to assess effects of sediment resuspension events on aquatic organisms. To achieve this goal (a) a suitable ecotoxicological test battery had to be elaborated, comprising a suitable test-species and an appropriate biomarker battery as well as chemical analyses, and (b) a suitable hydraulic test stand had to be developed including an appropriate experimental exposure program, suitable sediments and representative model contaminants.

*Ecotoxicological contribution*

During the elaboration of a biomarker battery comprising biomarkers with suitable endpoints, rainbow trout proved to be a suitable test species (a) being easy to rear, (b) being sufficiently robust for exposure experiments and (c) providing sufficient amounts of sample material for bioassays and chemical analysis. Among the biomarkers chosen for assessment, the enzymatic biomarkers 7-ethoxyresorufin-*O*-deethylase (EROD), glutathione-*S*-transferase (GST), and catalase (CAT) activity, the content of CYP1A protein, as well as quantification of lipid peroxidation in combination with results of CAT assessment showed no significant response. (For details see chapter 3). However, results of of lipid peroxidation and CAT assessment indicated a higher production of oxyradicals due to increased respiration, e.g. as a consequence of the increased swimming activity in the exposure events.

Real-time PCR analysis of alterations of gene expression of e.g. CYP1A1 gene successfully detected responses to contaminant exposure. Furthermore, quantification of PAH metabolites proved to be a sensitive biomarker to detect exposure to PAH contamination. Quantification of micronucleated peripheral erythrocytes, as a biomarker of irreparable loss of genetic material with a high ecological relevance, proved to be suitable and showed significant difference between control and exposure groups.

*Hydraulic engineering contribution*

The most important requirement to be able to conduct the interdisciplinary experiments was the establishment of a suitable test stand allowing, on the one hand, the realization of hydraulic boundary conditions and, on the other hand, the exposure of aquatic organisms such as fish. The original setup of the annular flume was successfully modified including a flow-through cooling unit and an aeration system to maintain suitable and stable environmental conditions regarding pH, temperature and dissolved oxygen, for aquatic test organisms. Zero mortality of test animals during all experiments clearly showed the excellent practical implementation of those measures.

In order to be able to erode sediments in the annular flume under controlled conditions, a suitable test program was developed to simulate a flood event. The bed shear stress was altered according to a DIN hydrograph (DIN 4049-3, 1994b) resulting in suspended particulate matter concentrations comparable to other studies. Thereby, the erosion of artificial sediments from a placed bed could be induced and its transport behavior could be characterized. The critical bed shear stress was characterized and suspended particulate matter concentrations were analyzed. Physicochemical parameters logged throughout all experiments contributed to the understanding of erosion processes.

As a further highly important requirement, large quantities of artificial OECD sediment, which was chosen to avoid introducing additional variations due to inhomogeneous natural sediment, of constant quality were successfully produced and spiked with PAHs as model contaminants. PAHs were chosen due to their ubiquitous presence in most aquatic ecosystems and their dioxin like activity. Spiking of large quantities of artificial sediment proved to be a challenge and required large amounts of solvents and resulting long evaporation times. Nevertheless, spiking was successful and provided sediments with PAH contaminations comparable to concentrations found in the field. Throughout the study, the use of artificial sediment revealed several flaws,

such as a high mean grain size and the tendency to show a strong segregation behavior of the artificial sediment components. In the light of a proof-of-concept study, however, the use of artificial sediment was a suitable approach to assess the newly developed hydrotoxicological method. It was possible to define the beginning of sediment erosion and observe deposition of suspended particulate matter, whose concentration was comparable with amounts found during flood events in European streams (e.g. Eyrolle et al. 2012). It should be kept in mind that the here presented test design likely does not allow extrapolating erosion behavior to the field situation as e.g. microbial communities, which strongly correlate with colloidal and bound extracellular polymeric substances (EPS) and therefore sedimentological parameters, highly differ between artificial and natural sediments (Goedkoop et al. 2005).

*Interdisciplinary outcome – achievement of objective I*

The proof-of-concept study Floodsearch I was based on the hypothesis that a combination of experimental knowledge and different scientific approaches of hydraulic engineering and ecotoxicology may assist in understanding principle processes and resulting impacts of flood events involving contaminated legacy sediments on biota and ecosystem health. The hydrotoxicological approach, of which a first experimental design was successfully developed during Floodsearch I, clearly proved to be applicable to successfully conduct hydrotoxicological studies with aquatic organisms in an annular flume. Furthermore, it showed that sediment remobilization during short simulated flood events in the annular flume can lead to uptake and effects of sediments-bound pollutants in rainbow trout.

Besides the practical implementation of the hydrotoxicological approach in experimental studies, technical and experimentally processes had to be communicated and elucidated between scientists from the two different disciplines, e.g. the appropriate storage of sediment and suspended particulate matter samples or the scientific basis of sediment erosion and sedimentation.

The outcome of the proof-of-concept project initiated planning for the follow-up project Floodsearch II during the last phase of the proof-of-concept study and a series of improvements of the hydrotoxicological approach regarding technical modifications and experimental optimizations were initiated (Figure 6.1 in chapter 6).

## 7.3 Objective II – preliminary investigations and influence of temperature stress

Starting from findings following the proof-of-concept part of the study, preliminary tests were carried out to provide a broader knowledge base for subsequent experiments in the annular flume. Sediments, either native contaminated or spiked with key pollutants were resuspended using submerged pumps in 750 L glass fiber reinforced plastic tanks. Rainbow trout were exposed to the resulting suspensions.

The main purpose of those experiments were (a) the assessment of the dissipation of PAHs from sediment/water systems and of the subsequent differences between desorption and bioavailability of spiked contaminants, and its implications for the following experiments in the annular flume, (b) kinetic monitoring of the chosen biomarkers to provide detailed insights in their dynamics as a function of extended exposure to suspended matter and (c) the assessment of a combination of chemical exposure and another environmental stressor, temperature, in aquatic biota.

*Dissipation of PAHs from sediment/water systems*

PAHs are known to dissipate from sediment/water systems due to several mechanisms such as microbial mineralization, volatilization and UV degradation (LeBlanc et al. 2006, Shuttleworth & Cerniglia 1995, Valsaraj et al. 1997). In contrast to both, the preceding experiments and the experiments planed in the subsequent project Floodsearch II, rainbow trout were here exposed to suspensions of sediments at constant SPM concentrations. Sediments were constantly resuspended in the tanks using an aeration system, which probably led to an enhanced microbial degradation and volatilization (for details see chapter 5). Especially in fluvial systems containing greater concentrations of SPM (i.e., 4-10 $g\,L^{-1}$), which correlate to target concentrations in the annular flume as well as to target concentrations in the resuspension experiments, biodegradation was found to be greater than in systems with lower concentrations of SPM most likely due to a greater surface area at the water-sediment interface favoring microbial degradation (Xia et al. 2006). In addition, frequent resuspension enhances microbial mineralization compared to that in an un-disturbed sediment bed, (LeBlanc et al. 2006). As a result, half-lives of PAHs from spiked sediments in the present study were by about one order of magnitude significantly shorter than half-lives observed for natural contaminated sediments by other authors (Apitz et al. 1999, Heitkamp & Cerniglia 1987). Furthermore, uptake and

biotransformation by exposed fish might have represented an important dissipation process. These potential routes of dissipation were likely to have led to constantly decreasing concentrations of PAHs, especially of those with lower molecular weight. Therefore changes in PAH levels during the experiments with spiked sediments probably have affected the results. However, no significant reduction of sediment-bound PAH concentration over time was found in the experiments with field-aged sediments.

Furthermore, a substantial increase of the bioavailable PAH fraction was observed for spiked sediments in contrast to un-spiked but naturally contaminated sediments resulting in a highly increased concentration of biliary metabolites in exposed fish. Those results regarding differences in desorption and subsequent bioavailability of organic contaminants between spiked and naturally aged sediments correlate well with findings by e.g. (Reid et al. 2000a) and had consequences for the subsequent planning of the follow-up project Floodsearch II.

In contrast to the experiments performed in this study, the resuspension method in annular flume studies differs significantly, as particles are permanently resuspended from the sediment bed in the annular flume resulting in quasi-flow-through conditions, which likely minimizes the PAH dissipation. Furthermore, experimental conditions differ regarding aeration of the sediment suspension, which is less pronounced, and the absence of an open surface of the flume. However, strong dissipation of spiked PAHs during the present study suggests that the use of naturally contaminated and aged sediments for the experiments in the annular flume is advisable as aging of PAHs can lead to less biodegradation and more realistic desorption rates (Fu et al. 1994, Hatzinger & Alexander 1995, Kan et al. 1994, White et al. 1997).

*Kinetic monitoring of biomarkers*

Since no sampling was possible during simulated floods in the project Floodsearch, only qualitative information on biomarkers could be derived (Brinkmann et al. 2010a, Wölz et al. 2009b). In the exposure experiments of the present part of the thesis, biomarkers were monitored kinetically to provide detailed insights in their dynamics as a function of extended exposure to suspended matter. Uptake and effects of PAHs followed a cascade-like pattern in the spiked treatment groups, as indicated by a series of peak biomarker responses. Those responses included increasing and then decreasing concentrations of biliary PAH metabolites resulting in a peak concentration of metabolites on day two. The other biomarkers, including lipid peroxidation, micronuclei production and EROD activity, gave more ambiguous results

with small peaks significantly altered only at one temperature following exposure to PAH spiked sediment suspensions. Reasons for this smaller biomarker response might be explained with insufficient exposure time or concentration as well as the weak to moderate AhR induction potential of the PAHs used for spiking. At the 24°C scenario, a significant induction of EROD activity was followed by a decrease, which was most probably non-specific due to temperature stress (cf. Whyte et al. 2000). Although the induction of biomarkers was transient, potential long-term adverse effects of exposure to particle-bound contaminants cannot be excluded. Whereby the question remains, if potential adverse longtime effects will follow the biomarker cascade that was observed in the present study after short exposures of fish to contaminated SPM. A monitoring of health and performance of fish over a longer period of time following exposure experiments, e.g. in resuspension tanks or the annular flume, could give insights on this important research question.

*Chemical exposure and temperature stress*

Depending on temperature conditions, bioavailability, degradation and effects of particle-bound pollutants may show variations (e.g. Airas et al. 2008, Heinonen et al. 2002, Honkanen & Kukkonen 2006, Ng & Gray 2011). Therefore the influence of temperature stress as a potential additional stressor during sediment resuspension was assessed with exposure experiments at an average temperature representative for rivers in Central Europe (12 °C) and under temperature stress (24 °C) conditions. The goal was to further widen the scope of the overall research topic of this thesis, effects of sediment-bound contaminants on aquatic organisms, by implementing this additional stressor.

As an immediate effect of temperature stress, a significantly greater mortality of fish in the 24 °C experiment of the present study could be observed. These results correlate well with findings e.g. Myrick & Cech (2004) who reported significant effects of temperature on growth and survival of rainbow trout between 24 to 27 °C, depending on other environmental parameters. Another direct effect of temperature stress impacted gross energy metabolism of fish, which was significantly greater at 24 °C as represented by the elevated excretion rate of biliverdin (cf. Avery et al. 1992). Among biomarker responses to temperature stress, PAH uptake was approximately 2-fold faster at 24 °C compared to that at 12 °C, this, however, did not result in higher concentrations of PAH metabolites in bile between the two temperatures. Elevated hepatic lipid peroxidation was only found at the spiked 24°C treatment and might have been a response to a 1,6-quinone metabolite arising from the biotransformation of BAP (Di

Giulio & Hinton 2008) and which was reported to cause oxidative stress (Lemaire et al. 1994). In contrast to the spiked 24 °C treatment, fish appeared to compensate for oxidative stress at lower temperature so that no elevated level of LPO could be detected. This finding is supported by studies by e.g. Grim et al. (2010) or Lushchak (2011) who concluded that the rate of turnover of lipids was more rapid at lower temperatures, and which has a protective function. Rates of micronuclei in peripheral erythrocytes only differed significantly between the spiked and un-spiked sediment in the 12 °C treatment.

Thus, only in combination with temperature stress, some effects of PAHs on fish were induced, whereas other effects only were apparent without temperature stress. The biomarker cascades did not only show quantitative differences (i.e. different induction intensity or rate of biomarker responses) at the two temperatures but also qualitative differences, i.e. different biomarker responses were observed. The correlation between gross energy metabolism and some of the biomarkers (i.e., biliary metabolites and LPO) supports 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 months as a result of dissipated heat from power plants and due to climate change (IKSR 2004), it can be assumed that resuspension of sediments and associated contaminants under these conditions could potentially have higher impacts on aquatic biota compared to lower temperatures (e.g. Airas et al. 2008, Heinonen et al. 2002, Honkanen & Kukkonen 2006).

*Consequences for future experiments within the annular flume*

Findings from this preliminary study were used to support the planning of future experiments involving the annular flume. The most prominent finding concerned the choice of sediment. Spiked sediments showed a strong dissipation of spiked PAHs during the course of the experiments, and thus, the use of natural contaminated and aged sediments for the experiments in the annular flume was strongly suggested to achieve a more realistic approach. Furthermore, the influence of temperature stress might be a feasible additional parameter for studies within the annular flume, but would require to modify the existing setup, i.e. the climatic chamber containing the annular flume.

## 7.4 **Objective III – evaluation of the hydrotoxicological approach**

After the successful proof-of-concept study, which led to the development of the hydrotoxicological approach (see chapter 3), and the preliminary experiments within the resuspension tanks, which allowed to formulate the hypothesis of a biomarker cascade following exposure to contaminated SPM (see chapter 5), concluding experiments within the annular flume were conducted that joined findings from both preceding research parts and aimed at evaluating the new interdisciplinary method with an refined approach.

*Method improvements*

During this last part of the present thesis, a total of 11 experiments with a runtime of 14 days each were conducted within the annular flume. In contrast to the first proof-of-concept study, experiments were divided into two experimental groups (a) sedimentological experiments to assess the erosional behavior of the three sediment types, and (b) ecotoxicological experiments to assess the impact of resuspended sediments on juvenile rainbow trout. This proofed to be a feasible approach and a clear improvement of the proof-of-concept approach, where those two experimental setups where still combined, and allowed to study the erosion behavior of all three sediment types under undisturbed conditions. To evaluate the effects of different contamination levels of freshwater sediments on fish, the following natural sediments were chosen and used during the study: sediment A as a moderately contaminated sediment originating from the river Rhine, sediment B as a heavily contaminated sediment originating from the River Moselle, and sediment A/B as a mixture consisting of 50 % sediment A and 50 % sediment B serving as a sediment with an intermediate contamination level. Furthermore, in favor of a natural deposit sediment bed and a stepwise increase of the bed shear stress, which allowed studying the erosional behavior during a steady state for defined shear stresses as described by Mehta et al. (1982), the original sediment handling and resuspension concept was discarded (cf. chapter 4.3.5).

Sediment samples were taken at the beginning and the end of each experiment, whereas suspended particulate matter (SPM) samples were taken in duplicates at each bed shear stress level. Samples were analyzed to determine (a) the total content of SPM over time by gravimetric analysis, (b) the total content of EPA-PAHs over time, and (c) the organic content of sediment samples by loss on ignition. Those additional analyses gave a much more comprehensive picture of the associated contaminant dynamics over time and were supported by a widened

range of physicochemical parameters (redox potential, conductivity, dissolved oxygen, pH), which were logged during each experiment.

*Experimental outcome and evaluation of the refined approach*

Experimental outcome concerning a) physicochemical parameters, b) improved chemical analyses and c) the adjusted biotest battery are discussed and evaluated in the following passages.

a) Physicochemical parameters

Frequent monitoring of important physicochemical parameters and the possibility to control the room temperature and humidity in the improved climatic chamber, where the annular flume was situated, or improved aeration of the annular flume allowed for an optimized process control during the experiments. Water temperature as one of the most important environmental parameters for the poikilothermic salmonid species was well near the optimal range for rainbow trout during all experiments, and negative influence of physiological functions on experiments outcome due to temperature stress could be ruled out. Dissolved oxygen concentrations varied between experiments and were at average to low levels and comparable to other studies on flood events with high SPM concentrations, where lowered DO concentrations were caused by oxygen depletion and reduced biogenic $O_2$ input (e.g. Ryan 1991). Nonetheless, improved aeration of the annular flume prevented oxygen levels to fall in regions that would have been critical for the exposed fish. Total hardness levels increased slightly during all experiments and were positively correlated with SPM content. However, increased hardness levels presumably did not have an impact on bioavailability of PAHs (cf. Akkanen & Kukkonen 2001) and thus on associated effects assessed by the biomarker battery. Redox potential and pH increased from initial low values to higher values at the end of the experiments due to increasing sediment disturbance and aeration. Both play a role in sediment-contaminant complexes and mediate the mobility of specific sediment associated contaminants such as metals, whereas desorption of organic contaminants depends primarily on physicochemical properties of the contaminant such as solubility and its distribution/partitioning coefficient ($K_d$) as well as the concentration differences between the resuspended sediment and the water column (Goossens & Zwolsman 1996, Latimer et al. 1999, Roberts 2012). An influence of redox potential and pH especially on sediment stability as well as on organic contaminant behavior could not be ruled out. However,

those processes also occur under natural resuspension conditions and are not experimental artifacts.

b) Sediment and SPM sampling and chemical analysis

Compared to the preceding study within the annular flume, a much more comprehensive sampling and chemical analysis procedure concerning PAH concentrations of sediments and suspended particulate matter samples was applied and gave important insights in contaminant dynamics during the experiments. Prior to the experiments within the annular flume, chemical analyses of all sediments regarding PAH content were performed and revealed increasing PAH concentrations in the following order (Sediment A < Sediment A/B < Sediment B) indicating excellent suitability of the chosen sediments for the following experiments as an increasing gradient of contamination was one of the main requirements of the study and a major improvement compared to the artificial, spiked sediments from the proof of concept study. Total PAH content was in accordance with other moderately and heavily contaminated sediments occurring in the field (e.g. Feiler et al. 2013, Headley et al. 2001, Witt & Gründler 2005). After exposure experiments, total PAH concentrations in sediments were by a factor of 1.6, 2 and 4.4 (Experiment with sediment A, A/B and B) lower than initial values. This behavior was attributed, on the one hand, to an altered grain size distribution after each experiment. Contaminants were most likely bound to smaller particle and were resuspended more easily than larger particles leading to a decrease of PAH concentrations in the remaining sediment. On the other hand, different degradation processes such as microbial degradation (Haritash & Kaushik 2009), adsorption and volatilization (e.g. Ravikrishna et al. 1998, Valsaraj et al. 1997) presumably occurred but were less pronounced than during the preliminary experiments (see chapter 5) due to the use of natural aged sediments. Chemical analysis of suspended particulate matter showed trends comparable to sediment analysis. However, a clear influence of exposed fish on PAHs concentrations due to resuspension attributed to swimming movement could be detected resulting in higher PAH concentrations compared to experiments without fish.

c) Biotest battery

After exposure to the three differently contaminated resuspended natural sediments (sediment A, A/B, C), significant biochemical and histological effects were observed. Concentrations of biliary metabolites of 1-hydroxypyrene, which was used to assess total PAH exposure (Ruddock et al. 2003), were increased significantly compared to reference samples and showed

an increasing gradient from the moderately contaminated sediment A towards the most contaminated sediment B. Various studies demonstrate a clear relation between PAH contamination in sediments or overlying waters and PAH metabolites in bile fluid of exposed fish (e.g. Johnson-Restrepo et al. 2008, Jung et al. 2011, Yang & Baumann 2006). These findings were strongly supported in this study by a comparable ratio of pyrene in the different matrices (a) sediment, (b) SPM samples and (c) the main metabolite 1-hydroxypyrene in bile fluid of exposed fish in the differently contaminated sediments. This ratio indicates an obvious interrelation between the level of sediment contamination and biomarker response in exposed fish. Possible uptakes paths of PAHs were not investigated in this study and other authors report that the most prominent uptake route for waterborne PAHs seems to be through the gills (e.g. Kennedy & Law 1990, Lee et al. 1972), whereas dermal uptake and uptake from food seems less important (Nichols et al. 1996, Randall et al. 1998). However, significant amounts of ingested sediment were found in guts of exposed animals although exposed fish were not fed throughout the exposure period. Supported by findings by Moermond et al. (2004) it cannot be ruled out that sediment ingestion contributed to total PAH burden during exposure experiments. Concluding, it can be stated that the bioavailable fraction is closely related to the particle bound pyrene.

In contrast to the preliminary experiments, bile metabolites of pyrene were significantly higher, even though the bioavailable fraction was significantly lower. This can be attributed to the static exposure conditions during the preliminary experiments. Respiratory volume, general metabolism and therefore also biotransformation rates are increased under physical exercise as well as under hypoxic conditions (Stevens & Randall 1967, Wood & Munger 1994). Therefore the assumption can be made, that the combination of physical exercise, SPM induced stress and reduced DO concentration in the annular flume resulted in a higher ventilation volume and led to an increased contaminant uptake and biotransformation compared to static exposure conditions. These findings furthermore strongly support the hypothesis that resuspension of contaminated sediments in case of flood or dredging events may lead to an elevated amount of readily bioavailable contaminants as well as to an increased uptake and metabolism in fish.

EROD induction in liver homogenates of exposed fish was significantly increased compared to reference samples and showed a similar but less pronounced gradient following the contamination level as found for bile metabolites. Overall, the different contamination scenarios were well reflected by the EROD activity that can be considered a reliable biomarker for the

assessment of PAH contaminated sediments. However, assets and drawbacks of EROD induction as a biomarker of exposure should be kept in mind when planning experiments. Besides species-specific differences, age and seasonal factors influence EROD activity. Furthermore, exposure time is another important factor in the determination of EROD activity as in most fish species induction occurs within the first 48 h with a maximal induction reached after less than seven days (Whyte et al. 2000).

Micronucleus formation rate was increased compared to reference samples and followed an ascending trend, from Experiment A through A/B to Experiment B. However, only after exposure to sediment B, significant differences compared to control data could be found. Overall, micronuclei induction factors were lower compared to the preceding preliminary experiments with spiked sediments. This may be attributed to a reduced bioavailability of contaminants from aged sediments as well as to the shorter exposure duration to the heavily contaminated sediment B as formation of micronuclei is depended on exposure time (Das & Nanda 1986).

Lipid peroxidation was found to be significantly increased in Experiments A and B, which indicates oxidative stress or oxidative damage of cell compounds (Carney Almroth et al. 2008) caused by those two exposure scenarios. In contrast to EROD activity and bile metabolites, no gradient linked to contaminant concentration could be derived. Presumably increased antioxidative capacities as described e.g. by Hermes-Lima and Zenteno-Savin (2002) were formed and may have compensated oxidative stress caused by readily available contaminants.

The evaluation of the biomarker battery in the preceding paragraphs demonstrates, that the current composition of biotests is a powerful tool to assess diverse effects of sediment bound contaminants on rainbow trout. It comprises tests to assess effects on different levels, e.g. EROD induction in liver tissue as a biomarker to detect the exposition and level of exposure to AhR-antagonists at an early stage of toxicity and biliary metabolites as a biomarker of exposure, and furthermore allows to assess the genotoxicity, i.e. the probability of potential long-term effects due to an irreparable genetic damage. The results clearly show (1) that the bioaccessibility of organic contaminants even after short resuspension events is influenced by increased SPM contents and (2) that associated adverse effects on aquatic organisms occur. Different contamination levels in sediments were reflected by increasing responses of assessed biomarkers of exposure (EROD, LPO, bile metabolites and micronuclei). Increased bioavailability of SPM-bound contaminants was well reflected by bile metabolites, which were

significantly elevated compared to static exposure conditions and immediate sublethal effects were well documented by elevated EROD activates. Whereas increased micronuclei frequencies in exposed rainbow trout demonstrated genotoxic effects of SPM associated contaminants and suggested potential adverse long term effects. These findings strongly support the hypothesis that resuspension of PAH contaminated sediments in case of flood or dredging events leads to an elevated amount of readily bioavailable PAHs as well as to an increased uptake and metabolism in fish.

Recently, the hypothesis that the combination of physical exercise due to higher flow velocities and additional stressors such as reduced DO concentration in addition to suspended sediment during flood events lead to an increased contaminant uptake and biotransformation compared to static exposure conditions was supported by Brinkmann et al. (2015b) who used a physiologically based toxicokinetic model (PBTK) to predict uptake of sediment-borne contaminants in fish. They were able to predict final concentrations as well as the secretion kinetics of a biliary metabolite. Furthermore, it was possible to show that exhaustive exercise during simulated flood events within the annular flume can lead to increased levels of biliary metabolites. Those findings were attributed to increased cardiac output and effective respiratory volume.

*Evaluation of the erosion process of natural sediments*

The exposure to a mixture of contaminated and moderately contaminated sediments proved to be a suitable means of controlling the desired level of contamination. Instead of using a spiking procedure to add desired contaminants to an artificial or natural sediment at a specific nominal concentration, this procedure allowed the preparation of a “semi-natural” sediment without the drawbacks of a spiked sediment, e.g. without the higher availability of contaminants due to short conditioning times. Furthermore, dynamic exposure conditions, i.e. the simulation of increasing flow and bed shear stress conditions, allowed for a much more realistic exposure scenario compared to the static exposure conditions during the preliminary experiments. Those dynamic exposure conditions are a unique feature of this hydrotoxicological approach compared to other studies with resuspended sediments.

Turbidity data and SPM samples enabled a detailed description of erosion processes during all experiments. Hence, it was possible to identify differences in erosional behavior depending on varying environmental conditions such as reduced pH-value. Further detailed information on

this sedimentological aspect can be found in the dissertation of Cofalla (2015), where further analyses regarding e.g. critical shear stress were made and consequences assessed.

*Relevance of the hydrotoxicological approach in the context of the WFD*

One of the major source of pollutants in aquatic systems are old, historically contaminated sediments. Therefore, the interaction of contaminated sediments and aquatic organisms is regarded as highly relevant for the quality of the aquatic systems in e.g. rivers, estuaries and reservoirs as well as for future operations and maintenance works as well as flood events, which might lead to the resuspension of sediments. These resuspended sediments may have negative impacts on aquatic organisms. The here gained knowledge concerning contaminated sediment transport and the associated impacts on aquatic organisms, reflected by an elevated amount of readily bioavailable PAHs as well as an increased uptake and metabolism in exposed fish, strongly supports this hypothesis.

Therefore, the necessity rises to understand the hydrotoxic interactions and the biological response during these processes to develop and operate countermeasures. This holds especially true in the context of the European Water Framework Directive, which commits European Union member states to achieve a good ecological and chemical status in European river catchments, and which recognizes the importance of sediments as a secondary long-term source for pollutants and, hence, as an important factor for water quality. The Directive on Environmental Quality Standards (EQS Directive, CEC 2008) sets environmental quality standards for different substances of concern in surface waters (see Annex 1 of the EQS). A good chemical status for e.g. a water body is reached when environmental quality standards (EQS) for certain priority substances and other pollutants are met. Furthermore, it is possible to apply those EQS for sediment and biota, instead of water. To date, SPM is not mentioned explicitly in the WFD (CEC 2000) or in the EC EQS Directive (CEC 2008) although it has a substantially influence on water quality and bioaccessibility, i.e. possible transfer of pollutants to aquatic organisms. However, environmental quality standards (EQS) for organic substances in surface waters comprise at the moment both dissolved and fractions to adsorbed SPM. This can lead to analytical inaccuracies or even problems, specifically in regard to hydrophobic compounds strongly absorbed to particles (90 % or more) when e.g. the annual average concentration is assessed (Werres et al. 2009). In a guidance document by a drafting group comprised of experts from EU member states, it was proposed to surrogate analysis of whole water sample by analysis of the SPM fraction (chapter 5.3 and 6.2, CEC 2009) and a subsequent

conversion to the concentration in the whole water to overcome this problem (Schubert et al. 2012).

Increased SPM and associated contaminants as a result of e.g. flood or dredging events are already mentioned in the Directive on Environmental Quality Standards as "losses from pollution accumulated in sediments" (EQS Directive, CEC 2008), however EQS for suspended particulate matter should be implemented within the WFD to account for the importance of SPM in risk assessment. Besides direct impacts of SPM-bound contaminants on aquatic organisms, transport and deposition of contaminated SPM may endanger the goal to achieve a good chemical and ecological status even at remote or downstream sites (Förstner et al. 2004, Hilscherova et al. 2007).

The here evaluated hydrotoxic approach may help port and dam authorities and energy companies to develop new, integrated and sustainable sediment management strategies leading to reduced costs for maintenance, expansion measures and new constructions sites.

## 7.5 **Limitations of the approach**

The preceding sections have highlighted that the interdisciplinary approach created an innovative research tool to assess the hydrotoxicological relevance of contaminated sediments after resuspension events. However, there are certain drawbacks and limitations of this innovative approach that cannot be neglected.

The annular flume, as a tool originally invented to assess the erosion and sedimentation behavior of sediments (see chapter 3.3.2), creates an endless flow within its flume. This unique feature is, on the one hand, clearly an advantage regarding the absence of pumps and sections where SPM might settle, but, on the other hand, limits the setup in certain points. Withdrawal of larger test organisms during experiments is, up to now, not possible and therefore only qualitative information on biomarker responses can be derived, whereas a kinetic monitoring of biomarkers is not possible. Furthermore, mortality of test animals always leads to a termination of a running experiment, as dead animals cannot be removed from the test stand. Furthermore, the physical structure, i.e. the dimensions of the current annular flume along with the size of the chosen test animals currently prevent the possibility to conduct joined toxicological and sedimentological studies. Results of experiments, where rainbow trout were exposed to sediments, indicate that exposed test animals have a strong influence on SPM

content as their swimming movements disturb the sediment surface and lead to a different erosion behavior compared to undisturbed erosion experiments with the same sediments. As a consequence, a simultaneous evaluation of erosion characteristics of sediments and toxicological impact of sediment remobilization on aquatic organisms could not be realized during the present study. Instead, two variants of each experimental part were conducted to be able to separately assess erosion characteristics under undisturbed conditions. Additionally, the annular flume does not allow to simulate and assess the influence of downstream transport of SPM and associated contaminants as well as the influence of dilution effects due to water exchange at a given location.

The current experimental duration of seven days as well as the associated biomarker battery does not consider potential long term effects that might follow short exposures to contaminated SPM during e.g. flood or dredging events. Those long term effects of dioxin-like compounds comprise e.g. immunotoxicity and genotoxicity and the inclusion of corresponding biomarker analyses would allow for a more comprehensive assessment of sediments and associated contaminants.

Another inherent drawback of the current design of the hydrotoxic approach is the dependence on vertebrate test animals to conduct the ecotoxicological assessments. Although the number of vertebrates necessary for obtaining mature results could be reduced significantly, each hydrotoxicological experiment still required ten animals, e.g. rainbow trout. This leads to potential high total numbers of necessary test animals for large hydrotoxicological studies, as well as to the need for (a) large animal husbandry facilities and (b) permissions of the federal authorities to conduct such experiments in accordance with the German Animal Welfare Act. Besides important ethical considerations, e.g. the general goal in ecotoxicological research that aims to replace animal testing with non-animal test methods (Pärt et al. 2010), the need for the permissions of the federal authorities complicates the hydrotoxic method to be used as a standard tool to assess contaminated sediments.

## 7.6 **Future Perspectives**

As described in the preceding chapter, the current design of the hydrotoxic approach exhibits certain drawbacks and limitations, and further method improvements, which will be detailed in this chapter, should be undertaken to optimize this innovative approach.

*The annular flume – technical modifications and alternatives*

Technical modifications of the annular flume (e.g., automatic feeding) and an increase of the dimensions to (1) allow a more realistic simulation of environmental conditions, and (2) reduce the influence of the exposed organisms on the physicochemical processes would be desirable. This would allow to minimize the influence of experimental animals on the erosion characteristics of sediments in further studies and enable combined sedimentological and toxicological experiments.

Furthermore, sediment stability assessment considering the influence of microbial communities consisting of biofilm-forming organisms such as bacteria and algae should be integrated in the current approach as suggested by Gerbersdorf et al. (2009, 2011, 2007). Those organisms play an integral role in the stabilization of riverine sediments by e.g. excreting extracellular polymeric substances (EPS) who act as a biological cement that (1) links the underlying sediment grains and thus alters their resistance to effective shear stress and (2) alters the roughness of the sediment surface resulting in local variations of the flow field and bed shear stress (Gerbersdorf et al. 2008, Lubarsky et al. 2010, Thom et al. 2013). As a consequence, it is vital to understand the influence of contaminates on those biofilm-forming organisms in the case of e.g. re-resuspension events. Lubarsky et al. (2012) demonstrated that triclosan, a widely-used antibacterial and antifungal compound, at low, environmentally relevant levels induced significant changes in bacterial community composition and diversity of impacted sediments and thus has large influence on sediment dynamics and contaminant remobilization. Therefore, the implementation of this additional parameter into the hydrotoxic approach would allow to assess the influence of contaminants on biofilm and therefore on sediment stability and would allow to account for the complex nature of sediment erosion of natural sediments and to overcome shortcomings described by Gerbersdorf and Wieprecht (2015) such as the oversimplification of ecohydraulic experiments resulting in little natural relevance.

In addition to the annular flume, straight flumes with the benefit of being simpler, both in construction and handling, could be used to gain insights into EPS-influenced erosion

characteristics of sediments, e.g. Thom and colleagues (2015) used straight flumes and natural river water to assess the erosion characteristics of biofilms under varying hydraulic and light conditions. Furthermore, Thom et al. (2013) currently investigated the possibility to implement sediment stability induced by biofilms in a morphological modelling approach that should be considered when implementing a future modelling approach into the hydrotoxic method evaluated within this thesis.

*Biomarker battery – emerging pollutants and future requirements*

The current setup of the biomarker battery clearly reflected the different contamination levels during the study and allowed to assess effects of exposure to contaminated sediments and SPM. Yet, the focus was primarily on PAHs and dioxin-like effects. Chemical analysis was also limited to PAHs and associated metabolites. To broaden the focus and assess the effects of other important contaminants commonly present in aquatic ecosystems, e.g. endocrine disrupting substances, heavy-metals, organometallic compounds and emerging contaminants such as microplastics and biocides, the set of biomarkers should by modified to be able to capture a broader range of different types of biological effects associated with those contaminants. Endpoints indicative of the exposure to metals, such as metallothioneins, endocrine disruptors, such as vitellogenin or markers of steroidogenesis, and histological investigation of ultrastructural changes should be assessed as indicators of tissue damage or alterations. An additional selection of biomarkers as proposed by Brinkmann et al. (2015a) such as hematological, nuclear changes and pathological and histopathological alterations could be integrated into the existing biomarker battery to assess potential long term effects that might follow short exposures to contaminated SPM. Furthermore, bioaccumulative substances might also be quantified in different tissues such as muscle and liver tissue of the exposed animals to experimentally confirm the hypothesis that short-term exposure to particle-bound pollutants during flood events might lead to an increased body burden. This would allow to practically implement a requirement of an annex of the EU WFD, which claims that concentrations of priority substances in sediments and tissues must not increase). Recently, Brinkmann and coworkers (Brinkmann et al. 2015a) demonstrated under static exposure conditions, i.e. without significant flow velocities, that sediment resuspension can actually lead to the accumulation of PCDD/Fs and PCBs in biota and associated adverse toxicological effects.

*Passive sampling – an innovative sampling technique*

In addition to this enhanced biomarker battery proposed in the preceding paragraph, accompanying chemical analysis should be broadened to comprise contaminants mentioned above and should comprise state-of-the-art analytic techniques such as LC-MS (Hernandez et al. 2012, Petrovic et al. 2010, Pico & Barcelo 2008). However, conventional sampling techniques, which precede any chemical analysis, are particularly problematic for contaminants present at only trace levels or with a high lipophilicity resulting in big fractions bound to either dissolved or suspended particulate matter (Greenwood et al. 2009). An implementation of passive sampling techniques, which are nowadays established as a reliable sampling technique with significant advantages for a wide range of contaminants in water (Lohmann et al. 2012, Vrana et al. 2005, Vrana et al. 2014) and sediment (Lydy et al. 2014), would allow to directly assess the time integrated bioavailable fraction of contaminants test that organisms are exposed to, and which are present near or below the limit of detection for conventional sampling techniques. Passive samplers rely on the partitioning of hydrophobic substances between the water phase and the sampler material and are therefore made of highly hydrophobic material such as silicone rubber, low density polyethylene or polyoxymethylene. Commonly, water partition coefficients ($K_{PW}$) of the samplers are used to estimate freely dissolved aqueous phase concentrations ($C_{free}$) of substances from the equilibrated concentrations of the substances in the passive sampler (Deutsch et al. 2013). Vrana et al. (2016) recently published a comprehensive interlaboratory study on the applicability of passive sampling for the monitoring of emerging pollutants such as pharmaceuticals, EDCs and biocides and concluded that although the passive sampling processes worked as expected, there is room for improvement for interlaboratory variability of results. Furthermore, passive sampling can be used in conjunction with standard bioassays: Emelogu and coworkers (2013) demonstrated, that extracts from passive samplers can be used to rapidly and economically assess the EROD induction potential. They concluded that this feasible approach can be used as a cost effective early warning signal on water quality deterioration. The importance and valuable role of passive sampling is further underlined by its mentions in various legislative regulations, such as the European WFD and the European Marine Strategy Directive (MSFD), for monitoring purposes (Mills et al. 2014) and the publication of an international standard ISO 5667-23 (2011) and is even discussed within the context of the European REACH legislation (Greenwood et al. 2009). For further details, please refer to Booij et al. (2016), who recently reviewed and evaluated passive sampling methods in the context of monitoring requirements in different countries.

In addition to the advantages discussed above, the implementation of passive sampling would furthermore eventually allow to significantly reduce the number of tests as passive sampler can be used to mimic the uptake of contaminants by biota (Greenwood et al. 2009, Mills et al. 2014). However passive sampling neglects the influence of xenobiotic metabolism in living organisms on contaminant effects and therefore also possible effects of metabolites and transformation products, and appropriate corrections factors have to be used. In addition, successful prediction of bioaccumulation based on passive sampling requires, more research for higher tropic levels as reliable species- and compound- specific bioaccumulation factor (BAF) values are often still absent (Arnot & Gobas 2006, Booij et al. 2016).

*From the annular flume to the field*

As an important addition to laboratory experiments and an extension of the interdisciplinary approach, field experiments regarding the assessment of (1) the bioavailability of SPM-bound contaminants during resuspension events and (2) physicochemical and hydraulic i.e. erosion stability parameters should be performed. Those field experiments should include *in situ* toxicity tests such as caged fish or macroinvertebrate exposure scenarios to assess, on the one hand, contaminant concentration in body tissues at the beginning and end of the experiments (Greenwood et al. 2009) and, on the other hand, ecotoxicological effects in caged animals using different biomarkers (Abrahamson et al. 2007, Barbee et al. 2008b, Hyötyläinen et al. 2002, Lindström-Seppä & Oikari 1990, Vincze et al. 2015). Chappie and Burton (2000) give a comprehensive overview and highlight the advantage that these *in situ* toxicity tests incorporate complex site-specific parameter such as SPM, dissolved oxygen, pH, salinity, and temperature, which may influence toxicity and/or bioavailability of contaminants. As recommended by Booij et al. (2016) this biota based assessment should be supplemented with passive sampling techniques to generate valuable field-based bioaccumulation factor (BAF) values that would improve the understanding of contaminant transfer to aquatic organisms. Different studies demonstrated the here proposed coupled exposure of caged aquatic organisms and passive samplers (Claessens et al. 2015, Hyötyläinen et al. 2002, Lindström-Seppä & Oikari 1990, Verweij et al. 2004) and even under conditions with elevated SPM concentrations exposure of e.g. rainbow trout did not result in mortality or in gill damage (Reid et al. 2003). Furthermore, during conditions with increased SPM load and elevated flow velocities, valuable physicochemical, hydraulic and erosions measurements with *in situ* devices should be conducted to supplement those *in situ* toxicity tests and passive sampling approaches. Noack et

al. (2015, 2014) recently demonstrated the benefits of a twofold measuring strategy combining laboratory measurements and *in situ* measurements to gain comprehensive insights into sediment stability.

Critical comparison between field experiments and findings from experiments in the annular flume would allow to further evaluate the hydrotoxicological approach and to assess the viability of an extrapolation of knowledge from laboratory experiments to field situations.

*Animal testing – a requirement for the hydrotoxicological approach?*

As an important longtime goal, the use of animal testing in hydrotoxicological studies should be reduced significantly or completely stopped. Since the introduction of the principles of humane animal experimentation by Russell & Burch (1959) and their famous 3R concept (replacement, reduction, and refinement) and the implementation of those principles in Article 7 of the European Directive 86/609/EEC (1986), *in vitro* alternatives to animal experiments to replace animal testing with non-animal test methods have strongly be forwarded in general (Hartung 2010, Pärt et al. 2010) and especially for fish in sediment toxicity assessment (Hallare et al. 2011). Next to those ethical considerations, practical considerations such as the elimination of the need for animal husbandry as well as all the simplifications of the current experimental design would be possible without experimental animals. This goal could be reached with (1) the help of modelling approaches that combine hydrodynamic models for sediment resuspension, transport and distribution (Moshenberg 2013) with PBTK models such as presented by Brinkmann et al. (Brinkmann et al. 2014, Brinkmann et al. 2015b), who developed a multi-species PBTK model to predict the bioaccumulative potential of neutral organic compounds such as hexachlorobenzene (HCB) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and (2) with passive sampling techniques and subsequent bioassays to assess bioavailability and effects of contaminants. Claessens et al. (2015) demonstrated that the combination of a passive sampling approach with a simple equilibrium partitioning model is able to predict contaminant concentrations in different environmental compartments. Booij et al. (2016) proposes that currently a combination of passive sampling techniques and bioaccumulation models seems to be the most effective approach for assessing and predicting bioaccumulation. However, those modelling as well as passive sampling approaches would initially require further experiments within the annular flume that should be supported by additional field experiments consisting of a combined exposure of passive samplers and e.g.

caged fish. This additional experimental data is required to calibrate and validate model output, which is a prerequisite for the model to be used and accepted by stakeholders and authorities.

# Scientific contributions

*Articles that contribute to the present thesis are denoted with an asterisk

## 1. Peer-reviewed articles

Brinkmann M, Eichbaum K, Kammann U, **Hudjetz S**, Cofalla C, Buchinger S, Reifferscheid G, Schüttrumpf H, Preuß TG, Hollert H (2014): Physiologically-based toxicokinetic models help identifying the key factors affecting contaminant uptake during flood events. *Aquatic toxicology* 152, 38-46

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## 2. Other journal articles

Brinkmann M, **Hudjetz S**, Herrmann H, Cofalla C, Kammann U, Hecker M, Schüttrumpf H, Schäffer A, Hollert H (2012): Floodsearch: Hochwasser im Labormaßstab ; Ökotoxikologie und Wasserbau arbeiten Hand in Hand bei der Hochwasserfolgenbewertung. *Mitteilungen der Fachgruppe Umweltchemie und Ökotoxikologie* 18, 88-91

Hollert H, Brinkmann M, **Hudjetz S**, Cofalla C, Schüttrumpf H (2014): Hochwasser - ein unterschätztes Risiko : Schadstoffe als 'Zeitbomben' im Sediment. *Biologie in unserer Zeit* 28, 44-51

## 3. Platform presentations

Stoffels, S., Brinkmann, M., **Hudjetz**, S., Freese, M., Pohlmann, J.-D., Kammann, U., Hanel, R., Hollert, H. (2012) Biomarker-basierte Untersuchung der Schadstoffbelastung des europäischen Aals in deutschen Flüssen. Wissenschaftliches Begleitprogramm zum DCF-Aal, 15.-16.10.2012, Thünen-Institut für Fischereiökologie, Hamburg.

Brinkmann, M., **Hudjetz**, S., Kuckelkorn, J., Hennig, M., Cofalla, C., Roger, S., Kammann, U., Schüttrumpf, H., Hollert, H. (2012) Uptake and effects of particle-bound polycyclic aromatic hydrocarbons (PAH) from sediment suspensions in rainbow trout

(*Oncorhynchus mykiss*, Walbaum). Proceedings, SETAC German Language Branch Annual Meeting 2012, Leipzig, Germany. Invited speaker.

Brinkmann, M. **Hudjetz**, S., Kuckelkorn, J., Hennig, M., Cofalla, C., Roger, S., Kammann, U., Hecker, M., Giesy, J., Schüttrumpf, H., Schäffer, A., Hollert, H. (2012) Can flood events affect rainbow trout? The biomarker-cascade after exposure to PAHs in sediment suspensions. Proceedings, 22nd SETAC Europe Annual Meeting 2012, Berlin, Germany.

Hollert H., Brinkmann M., **Hudjetz S**., Eichbaum K., Kuckelkorn J., Cofalla C., Roger S., Kammann U., Giesy J., Schäffer A. , Hecker M., Lennartz G. , Haag I., Gerbersdorf S., Westrich B., Wölz J. , Schüttrumpf H. (2012) Hochwasser – unterschätztes Risiko für die Erreichung des guten ökologischen Zustandes nach EU-Wasserrahmenrichtlinie, 42. Internationales Wasserbau Symposium Aachen (IWASA), January 12-13, Aachen, Germany.

Hollert, H., Brinkmann M., **Hudjetz S**., Eichbaum K., Kuckelkorn J., Cofalla C., Roger S., Kammann U., Schäffer A., Hecker M., Lennartz G., Schüttrumpf H., Wölz J. (2011) Ecotoxicological impact of re-mobilized sediments and flood events for look regulated rivers and wetlands, 2. International workshop on ecofriendly use of wetlands in the "Three Gorges Reservoir", October 21, Chongqing, China.

Brinkmann, M., **Hudjetz**, S., Kuckelkorn, J., Hennig, M., Cofalla, C., Roger, S., Kammann, U., Schüttrumpf, H., Henner, H. (2011) Aufnahme und Effekte von partikelgebundenen polyzyklischen aromatischen Kohlenwasserstoffen (PAK) von dotierten Sedimentsuspensionen in Regenbogenforellen. Proceedings, SETAC German Language Branch Annual Meeting 2011, Landau, Germany.

Cofalla, C., Roger, S., Wölz, J., **Hudjetz**, S., Brinkmann, M., Hollert, H., Schüttrumpf, H. (2010) Floodsearch - Hydrotoxic Investigations of Contaminated Sediments in an Annular Flume, in: PIANC MMX book of abstracts: 32nd PIANC Congress, 125th Anniversary, Liverpool UK, 10-14 May 2010. PIANC, London, p. 264.

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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.

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., Kammann, 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.

## 4. Poster presentations

Brinkmann, M., **Hudjetz**, S., Herrmann, H., Kamman, U., Cofalla, C., Lorke, S., Schäffer, A., Hecker, M., Schüttrumpf, H., Hollert, H. (2013) Do flood events matter? Biomarker response of rainbow trout following exposure to re-suspended sediments in an annular flume. SETAC Europe Annual Meeting 2013, Glasgow, Scotland.

Brinkmann, M., **Hudjetz**, S., Hennig, M.P., Cofalla, C., Lorke, S., Kammann, U., Hecker, M., Giesy, J.P., Schüttrumpf, H., Schäffer, A., Hollert, H. (2013) Can flood events affect rainbow trout? The biomarker-cascade after exposure to PAHs in sediment suspensions. SETAC Europe Annual Meeting 2013, Glasgow, Scotland.

Herrmann, H., **Hudjetz**, S., Brinkmann, M., Schäffer, A., Hollert, H. Cofalla, C., Lorke, S., Schüttrumpf, H., Kammann, U. (2012) Turbulent. Trübe. Toxisch? Bioverfügbarkeit und Toxizität sedimentbürtiger Schadstoffe während der Remobilisierung in einem Kreisgerinne. SETAC German Language Branch Annual Meeting 2012, Leipzig, Germany.

**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.

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, Seville, 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 remobilisation 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.

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 re- mobilisation and transport during flood events in rivers. Part B: CYP1A1 induction, micronuclei in blood cells and gene expression analysis. Proceedings, 19th SETAC Europe Annual Meeting 2009, Göteborg, Sweden.

**Hudjetz**, S., Brinkmann, M., Cofalla, C., Roger, S., Schmidt, B., Schäffer, A., Schüttrumpf, H., Hollert, H., Wölz, J. (2009) Searching for the (eco)toxicological relevance of sediment remobilisation and transport during flood events in rivers. Part A: instrumental chemical analysis and effect-directed analysis (EDA). Proceedings, SETAC German Language Branch Annual Meeting 2009, Weihenstephan, Germany.

**Hudjetz**, S., Brinkmann, M., Cofalla, C., Roger, S., Schmidt, B., Schäffer, A., Schüttrumpf, H., Hollert, H., Wölz, J. (2009) Searching for the (eco)toxicological relevance of sediment remobilisation and transport during flood events in rivers. Part A: instrumental chemical analysis and effect-directed analysis (EDA). Proceedings, 19th SETAC Europe Annual Meeting 2009, Göteborg, Sweden.

# Curriculum vitae

## 1. Personal information

| | |
|---|---|
| Name | Sebastian Hudjetz |
| Born | March 21, 1980 in Stuttgart, Germany |
| Citizenship | Germany |
| Family | married, 1 child |
| Language skills | German (native), English (proficiency), French (proficiency) |

## 2. Academic education

| | |
|---|---|
| Since February 2010 | PhD research fellow at the Department of Ecosystem Analysis, Institute for Environmental Research and the Institute of Hydraulic Engineering and Water Resources Management, both RWTH Aachen University, supervised by Prof Dr. rer. nat. Henner Hollert and Prof Dr.-Ing. Holger Schüttrumpf |
| March 2009 – February 2010 | Graduation in Biology (Diploma), thesis title: 'Effect-directed analysis for the assessment of sediment-associated environmental contaminants' supervised by Prof Dr. rer. nat. Henner Hollert and Prof Dr. rer. nat. Andreas Schäffer, Institute for Environmental Research, RWTH Aachen University, Aachen, Germany |
| 2001 – 2009 | Studies of Biology (Diploma), RWTH Aachen University, Germany<br>Major subject: Environmental Biology and Chemistry<br>Minor subject: Sanitary environmental engineering |
| 2003 | Intermediate diploma of Biology |
| 2000 – 2001 | Studies of Biology (Diploma) at Darmstadt University of Technology, Germany |

## 3. Academic work experience

| | |
|---|---|
| Since January 2013 | Research associate at the Institute of Hydraulic Engineering and Water Resources Management, RWTH Aachen University, Germany |
| April 2010 – December 2012 | Research associate at the Institute for Environmental Research, RWTH Aachen University, Germany |
| September 2008 – March 2010 | Student research assistant at the Institute for Environmental Research, RWTH Aachen University, Germany |
| November 2007 – August 2008 | Student research assistant at Development and Assessment Institute in Waste Water Technology at RWTH-Aachen University (PIA), Germany |
| January & July – September 2007 | Student research assistant at the Research Institute for Ecosystem Analysis and Assessment gaiac e.V., Aachen, Germany |
| February – March 2005<br>February – March 2006 | Student research assistant at the Institute of Plant Physiology and phytopathology, RWTH Aachen University, Germany |
| April – May 2004<br>July – August 2004 | Student research assistant at the Department of Zoology and Animal Physiology, RWTH Aachen University, Germany |
| 2003 – 2005 | Part-time work at the Office for ecology and landscape planning, Hartmut Fehr, Simmerath, Germany |
| 2001 – 2003 | Part-time work at the biological station, Düren, Germany |

## 4. Education

| | |
|---|---|
| 1999 – 2000 | Apprenticeship as paramedic, Malteser Hilfsdienst e.V. Düren, Germany |
| 1990 – 1999 | Secondary school (gymnasium), French bilingual program |
| 1986 – 1990 | Primary school, Sonnenbühl-Undingen, Germany |

## 5. Further skills and qualifications

| | |
|---|---|
| Computing | Microsoft Office, Sigmaplot, GraphPad, EndNote, SolidWorks |
| Driver's License | Class 3, sport boat license inland sailing / motor |
| Hobbies | Surfing, swimming, reading |

Aachen, 17.06.2016

(Sebastian Hudjetz)

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