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A combined hydraulic and toxicological approach to assess re-suspended sediments during simulated flood events. Part I–multiple biomarkers in rainbow trout

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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 re-suspension 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 re-suspension of pollutants and resulting toxicological impacts on aquatic organisms. A suite of different molecular, biochemical and histological markers was used to test the hypothesis that re-suspension of sediments can lead to re-mobilization of PAHs and subsequently to effects on 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 the most sensitive

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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 understanding of the re-mobilization of pollutants from sediments in support of environmental risk assessment.

Keywords Annular flume · Flood event · Genotoxicity · PAH · Re-mobilization

1 Introduction

There is general agreement that sediment pollution poses a threat to the health of aquatic ecosystems (Haag et al. 2001; Hollert et al. 2008; Westrich and Forstner 2005). Indeed, numerous concepts for risk estimation in these systems have been developed (Ahlf et al. 2002; Burton 1991, 1995; Chapman 1990, 2000; Chapman et al. 2002). 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, b; Hollert et al. 2000, 2003; Rao et al. 1990; Wölz et al. 2008). The role of sediment re-suspension, e.g., during flood events, and possible ecotoxicological effects of re-mobilized particle-bound contaminants to aquatic organisms have scarcely been investigated (Baborowski et al. 2005; Hollert et al. 2008; 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 re-mobilization of pollutants from sediments or flood plain soils (Wölz et al. 2009). In the past decade, an increasing number of extreme weather events have been observed around the globe (Schüttrumpf and Bachmann 2008), that have resulted in floods such as the 500-year flood event that occurred in 2002 in the River Elbe, Germany (Schüttrumpf and 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 understanding of the potential ecotoxicological risks associated with flood events (e.g., Huber et al. 1983).

Increased bioavailability and potential adverse effects of particle-bound compounds from these re-suspended 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 were 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 was 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 re-mobilization of dioxin-like compounds during the Elbe River flood event (Hollert et al. 2008).

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 re-mobilization 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 and Hollert 2006). One such approach is the combination of hydrodynamics and ecotoxicology, which has become an emerging field in environmental research (Hollert et al. 2008; Wölz et al. 2009). Recent studies have identified the importance of investigating the partitioning processes of particle-bound pollutants during re-suspension 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 re-suspension of Yangtze River sediments using a particle entrainment simulator (Feng et al. 2007; Yang et al. 2008).

In this proof-of-concept study, a multiple biomarker approach was used to investigate exposure to organic pollutants that were re-mobilized during a simulated flood event according to DIN 4049-3 (1994) 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 re-suspension. Thus, methodologies of ecotoxicology and hydraulic engineering were combined by means of exposing animals in an annular flume (Fig. 1), a facility that can be used to study erosion and sedimentation processes (Schweim et al. 2001). Rainbow trout were exposed to artificial sediment (OECD 218 2004) that was spiked with a mixture of PAHs during a 5-day simulated flood event. Furthermore, this study was also intended to elucidate if rainbow trout constitute a suitable test species for investigations in annular flume. For the determination of suitable endpoints for further studies, a number of bio-

Fig. 1 Annular flume at the Institute for Hydraulic Engineering and Water Resources Management, Aachen, Germany (picture: C. Cofalla)

markers at different levels of biological organization were investigated to demonstrate either exposure to or effects of sediment-bound pollutants.

2 Materials and methods

2.1 Experimental design

Rainbow trout were exposed over 5 days to simulated flood events according to the DIN 4049-3 (1994) hydrograph in an annular flume (see Fig. 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 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, catalase activity, and lipid peroxidation), gene expression analyses using quantitative real-time reverse transcription polymerase chain reaction (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.

2.2 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 as a function of bed shear stress was measured in parallel and is reported in Cofalla et al. (2010a, b). Chemical analyses of PAH concentrations in sediment and suspended matter are reported in Hudjetz et al. (2009, 2010).

2.3 Sediment preparation and spiking

The artificial sediments 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 pretreated peat (5% dw) was combined 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 days prior to erosion experiments or spiking.

Ten percent of the readily conditioned artificial sediment was 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 days, and thoroughly mixed prior to erosion experiments. The sediment mixtures were transferred to the annular flume and smoothed 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 are not expected after a consolidation time of 3 days, which was therefore also assumed in the present study.

2.4 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 $days^{-1}$ 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%, fiber 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.

2.5 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 approximately equal 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.

2.6 Preparation of homogenates and liver subcellular fractions

All steps in the preparation of tissue homogenates were carried out on ice. For measurement of 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 ε-aminocaproic acid, 0.3 M β-mercaptoethanol, and 20 µL dithiothreitol in a final volume of 100 mL distilled water) by means of an electric homogenizer (VDI 12, VWR) and subsequently centrifuged for 15 min (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.

2.7 Determination of EROD

EROD activity was measured in triplicate 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 (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 8.0) were prepared. Subsequently, 120 µl of 2 µM 7-ethoxyresorufin in HEPES-Cortland buffer were added to all wells. Plates was incubated at room temperature for 10 min in darkness prior to addition of 40 µL of 4.2 µM NADPH in HEPES-Cortland buffer. After

incubation at room temperature for 10 min in darkness, the reaction was stopped with 90 μL of 150 $\mu g L^{-1}$ 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.

2.8 Determination of glutathione-S-transferase activity

Activity of GST was determined in triplicate 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 (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 $protein^{-1}min^{-1}$.

2.9 Determination of catalase activity

CAT activity was determined in triplicate 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 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)-oxysulfate-sulphuric acid solution (Sigma). After 10 min, 250 μL of this mixture were transferred to 96-well TPP and the concentration of the remaining hydrogen peroxide was determined photometrically as the yellow peroxy titanium sulfate at 414 nm in an Infinite® 200 microplate reader (Tecan), using 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 $protein^{-1}min^{-1}$.

2.10 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 sulfate (SDS), 1.5 mL of 20% acetic acid adjusted to pH 3.5, 1.5 mL of 0.8% thiobarbituric acid 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 (approximately 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 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 as an external standard. All measurements were conducted in triplicate.

2.11 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 (Bio-Rad, Mississauga, ON, Canada). 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 (BioRad, San Leandro, CA, USA).

2.12 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 quantitative 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 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 of the target genes. Levels of gene expression were expressed relative to the average value of the respective control group.

2.13 Determination of protein concentrations

Protein concentrations for the calculation of specific enzyme activities (except for EROD) were determined in triplicate according to the Bradford method (1976), adapted to microplate measurement, using BSA as external standard. Bradford reagent was added in a ratio of 1:30-5 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.

2.14 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. For each individual fish, 4,000 erythrocytes fixed on two separate smears were examined using an epifluorescence microscope at ×1,000 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;

Table 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
EF-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 and Arukwe (2007)

Titenko-Holland et al. 1998). Results were recorded as micronucleated cells relative to the total number of cells counted.

2.15 Treatment of bile samples and HPLC analysis

PAH metabolites in bile samples were determined by a modified version of the method described by Kammann (2007), based 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^{-1} 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 high-performance liquid chromatography (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 (2007), samples were chromatographed with a flow of 0.55 mL min^{-1} on a Nucleosil 100-3 C18 (3×125 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 380 nm.

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), an 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.

2.16 Data analysis

All datasets that did not pass the Kolmogorov-Smirnov test for Gaussian distribution ($p<0.05$) or the Barlett's test for equal variances ($p<0.05$) were analyzed using nonparametric Kruskal-Wallis analysis of variance (ANOVA) on ranks ($p\leq0.001$). The datasets passing both tests were analyzed using parametric one-way ANOVA ($p\leq0.001$). The Holm-Sidak or Dunn's method was used to identify significant differences among 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 limits for all comparisons was set to at least $p\leq0.05$. 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 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 as a function of bed shear stress was measured in parallel (data not shown) and is reported in Cofalla et al. (2010a, b). Chemical analyses of PAH concentrations in sediment and suspended matter (data not shown) are reported in Hudjetz et al. (2009, 2010).

3.1 Biochemical markers

EROD activity after exposure to particle-bound PAHs during the 5-day simulated flood event was not significantly different from that of the control group, although the animals with the greatest EROD activity (approximately 45 pmol mg^{-1} min^{-1}) originated from this

Table 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

	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±0.08a	1.42±0.12a	1.43±0.14a	1.41±0.11a	1.57±0.13b	1.51±0.08a,b
Liver somatic index (LSI)	1.08±0.11a	1.06±0.19a	1.04±0.10a	1.02±0.14a	1.11±0.18b	0.85±0.12c
Liver homogenate *(S9)*						
EROD activity/pmol $mg^{-1}min^{-1}$	11.66±6.93a	n.d.	14.65±7.26a	15.49±11.88a	n.d.	n.d.
GST activity/pmol $mg^{-1}min^{-1}$	15.51±4.13a,c	14.67±2.47a,c	8.78±2.61a	9.47±2.81a	34.03±7.33b,c	43.21±11.53b
CAT activity/nmol$mg^{-1}min^{-1}$	561.6±250.90a	326.5±75.14a,c	153.8±74.73b,c	76.98±48.61b	370.4±196.5a	481.1±153.6a

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 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)

n.d. not determined

group. Similarly, no significant alteration of GST enzyme activity was detected in PAH exposed trout (see 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). 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 (Fig. 2).

3.2 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 (Fig. 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 approximately 0.4-fold ($p \leq 0.01$) in the presence of spiked 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.

3.3 Micronucleus formation

The number of micronuclei in 4,000 erythrocytes per exposed fish was determined using fluorescence microscopy (Fig. 4). Frequencies differed significantly between the control groups

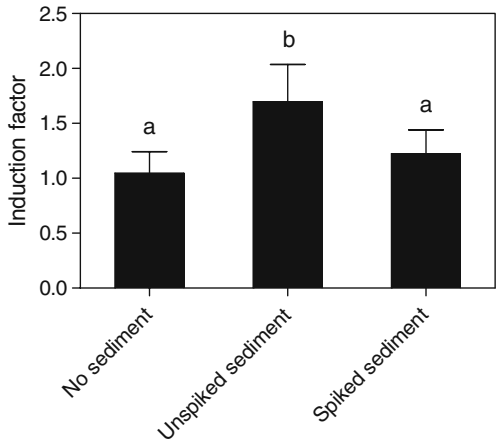

Fig. 2 Lipid peroxidation, measured as MDA equivalent concentration in the liver of rainbow trout, exposed in 5-day simulated flood events without, with unspiked and spiked sediments, respectively. Values are expressed as induction factors relative to the median of the respective control group taken from the maintenance in parallel to the treatments to establish untreated baseline values. Each test was conducted with n=15 animals. *Bars* mean value, *error bars* standard deviation. Treatment groups sharing the *same letter* do not differ significantly (Kruskal-Wallis one-way ANOVA on ranks with Dunn's method, $p \leq 0.01$)

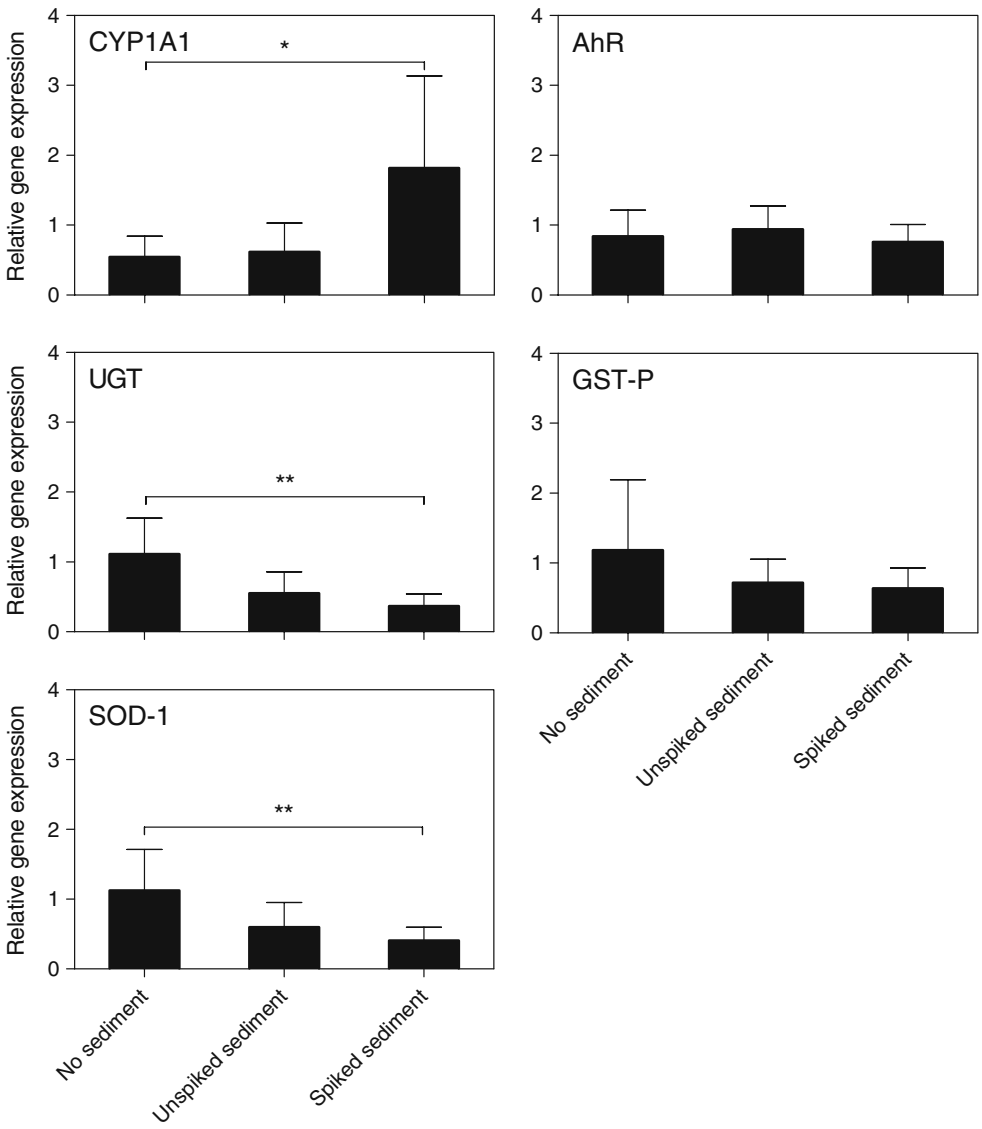

Fig. 3 Hepatic expression of the genes CYP1A1, AhRβ, GST-P, UGT and SOD-1 of rainbow trout exposed in 5-day simulated flood events without, with unspiked and spiked sediments, respectively. Each test was conducted with $n=15$ animals. *Bars* indicate the average gene expression relative to the respective control group taken from the maintenance in parallel to the experiment to establish baseline values; *error bars* the standard deviation. */**Significant alteration compared to the respective control group (Kruskal-Wallis one-way ANOVA on ranks and Dunn's method, $p \leq 0.05/0.01$)

($p \leq 0.05$, 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 ($p \leq 0.001$).

3.4 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 (Fig. 5). The concentration of 1-hydroxypyrene in bile of animals exposed to spiked sediments was significantly greater (4,596-fold) compared to animals exposed to unspiked sediments ($p \leq 0.001$), and 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 ($p \leq 0.001$).

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 (Fig. 6).

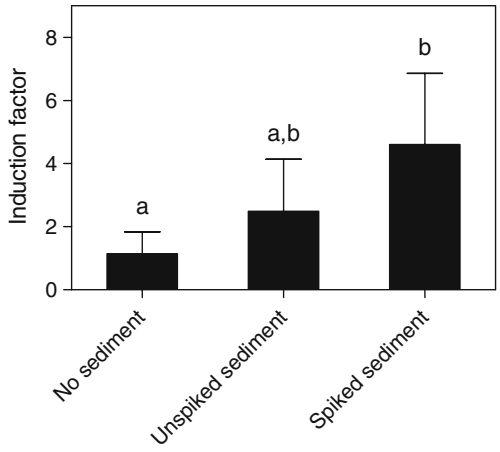

Fig. 4 Micronucleus frequency in peripheral erythrocytes of rainbow trout exposed in 5-day simulated flood events without, with unspiked and spiked sediments, respectively, expressed as induction factors relative to the median of the respective control group taken from the maintenance in parallel to the treatment to establish untreated baseline values. Each test was conducted with $n=15$ animals. *Bars* indicate the average proportion of micronucleated cells in 4,000 erythrocytes of each animal, error bars the standard deviation. Treatment groups sharing the *same letter* do not differ significantly (Kruskal-Wallis one-way ANOVA on ranks with Dunn's method, $p \leq 0.01$)

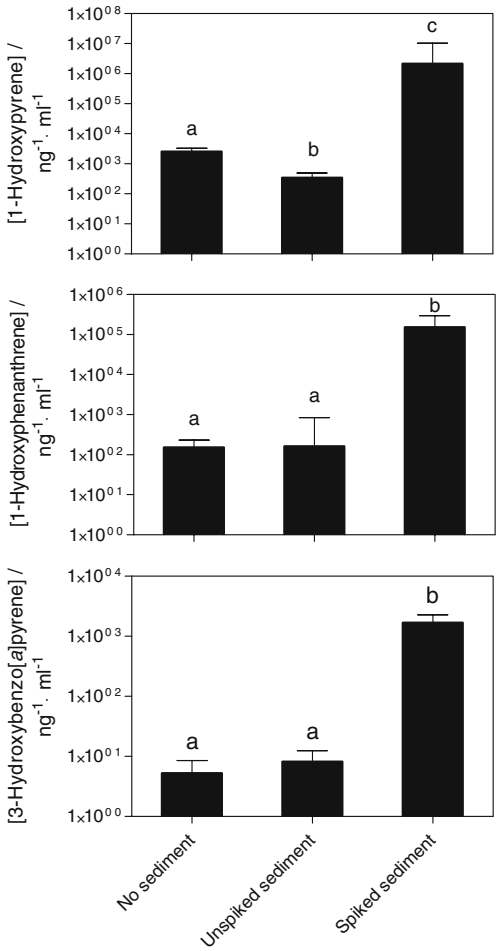

Fig. 5 Concentrations of the PAH metabolites 1-hydroxypyrene, 1-hydroxyphenanthrene and 3-hydroxybenzo[*a*]pyrene in bile of rainbow trout exposed in 5-day simulated flood events without, with unspiked and spiked sediments, respectively. Each test was conducted with $n=15$ animals. *Bars* indicate the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

4 Discussion

4.1 Identification of suitable biomarkers for use in coupled hydro-toxicological 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., EROD, GST, and 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 were 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-day 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. 1995). Thus, quantification of PAH metabolites in bile has been shown to be a very sensitive biomarker of exposure to PAH exposure (Kammann 2007). The most abundant metabolite in fish bile, 1-hydroxypyrene, can contribute up to 76% of the sum of PAH metabolites. Compared to 1-hydroxypyrene, 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-day 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 2,150.00±2,553.00,

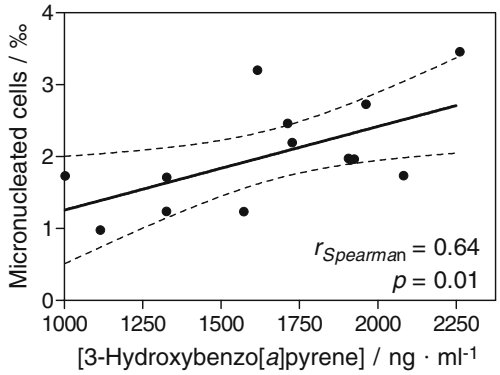

Fig. 6 Linear regression of 3-hydroxybenzo[*a*]pyrene concentrations in bile and the proportion of micronucleated cells of rainbow trout exposed in a 5-day simulated flood event with PAH spiked sediment. The tests were conducted with n=15 animals. The dashed curves represent the 95% confidence limits. 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)

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 recent years, several laboratory studies have investigated 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 days after a single intraperitoneal injection of 5.0 mg kg^{-1} body weight benzo[*a*]pyrene in *Parophrys vetulus* (Collier and 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. 2008). 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 re-suspended 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. 2004), and have been shown to cause significant induction of biotransformation enzymes in rainbow trout (Fragoso et al. 2006; Jonsson et al. 2006; Oikari et al. 2002; Ramachandran et al. 2006). Surprisingly, the activity of neither the phase I biotransformation enzyme EROD, nor the phase II enzyme GST was altered by exposure to the PAH spiked sediments during the 5-day 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. 2006) 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 days 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 and 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 might be 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 (6,990± 1,720 nmol g^{-1}) reported by Arnold et al. (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 defense 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 oxy-radicals 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. (2003; 1.80±1.57‰,) and Schultz et al. (approximately 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, the 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 (fourfold), 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. 1996a, b; Grund et al. 2010). 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, an annex to 2000/60/EC came into force, which claims that concentrations of priority substances in sediments and tissues must not increase (EC 2006). Thus, investigation of the interdependency between biota and sediments with regard to bioaccumulation during re-mobilization events is of great relevance (Förstner 2009).

4.2 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 substratum for laboratory testing. For physico-chemical sediment characteristics, as well as turbidity as a function of bed shear stress (data not shown), see Cofalla et al. (2010a, b). 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 et al. (2008) have shown that sedimentological parameters, e.g., the critical shear stress for erosion, are largely dependent on colloidal and bound extracellular polymeric substances 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 days 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 re-mobilization processes in-field, it is necessary to accurately emulate the biological and physico-chemical properties of natural sediments. Thus, additional experiments with natural sediments, either polluted or spiked, must be performed.

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

A pre-requisite for the applicability of the annular flume as a test system for coupled hydro-toxicological 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.

4.4 Potential and limitations of the current approach

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

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