In vitro bioassay tools for the toxicological evaluation of dioxins and dioxin-like compounds in sediments and biota

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der RWTH Aachen University zur Erlangung des akademischen Grades einer Doktorin der Naturwissenschaften genehmigte Dissertation

vorgelegt von

Dipl.-Biol. Kathrin Eichbaum

aus Worms

Berichter: Universitätsprofessor Dr. Henner Hollert

Universitätsprofessor Dr. Andreas Schäffer

Tag der mündlichen Prüfung: 02.02.2016

Table of contents:

Abstract

Since the middle of the 20th century, there has been increasing concern about certain historical contaminants, which due to their potential health effects (e.g. hepatotoxicity, endocrine disruption and infertility) and properties (accumulation in e.g. sediments and tissues) pose a threat to humans and wildlife. Among these contaminants, dioxin-like compounds (DLCs) can be found, including polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) as well as dioxin-like polychlorinated biphenyls (DL-PCBs). Although emissions of DLCs have been reduced considerably during the past decades, legacy pollution of sediments still constitutes a secondary pollutant source following sediment re-mobilizations (e.g. floods or dredging activities). This might cause the majority of the European surface waters not to reach the aims set by the European water framework directive (WFD). It is thus not astonishing that recently, the assessment of sediment and dredged material gained more and more attention and that new concepts have been developed such as clearly defined Environmental Quality Standards (EQS) for sediments and biota.

While sediment assessment originally simply based on classical, instrumental methods (e.g. high resolution gas chromatography - high resolution mass spectrometry (HRGC/HRMS)), these methods due to their lacking information on sediment toxicity today are often connected to ecotoxicological and ecological methods. However, those methods often exhibit relatively unspecific endpoints (e.g. growth inhibition) and thus do not allow for a comparison to concentrations measured *via* HRGC/HRMS. Because many of the toxic effects of DLCs are mediated *via* the cytosolic aryl hydrocarbon receptor (AhR), and the strengths of activation of the AhR constitutes a specific endpoint of many cell-based *in vitro* bioassays, these methods are of increasing interest to regulators and risk assessors. Biological equivalent quotients (BEQs) deduced from such assays are directly comparable to toxicity equivalents (TEQs) of HRGC/HRMS analyses.

The DioRAMA project is a joint research initiative between the Institute of Environmental Research at the RWTH Aachen University and the Department G3 of the German Federal Institute of Hydrology in Koblenz. Its main goal was to establish *in vitro* tools for the assessment of DLCs in sediment and biota to improve current risk assessment approaches. Therefore, the present thesis investigated (1) the sensitivity of various *in vitro* bioassays, the suitability of certain *in vitro* assays to be used (2) as regulatory tools, (3) to screen the uptake of DLCs by fish and (4) to be used as prioritizing tools for sediment and soil extracts. (4) Finally, the bioavailability of certain DLCs was investigated.

A literature study proved the applicability of various *in vitro* bioassays for the screening of DLCs in a multitude of complex samples, individual compounds and mixtures. To be capable of screening trace contaminants such as DLCs, *in vitro* bioassays have to be comparably sensitive like instrumental techniques. The literature data reviled that some of the *in vitro* bioassays approximated the sensitivity limits of chemical analytical methods (~0.1 pM dioxin) and hence suggests the potential suitability of these assays to be used as additionally regulatory tools.

To verify this assumption, three *in vitro* bioassays (RTL-W1 EROD, H4IIE-luc and H4IIE Micro EROD assay) were investigated with regard to their possible implementation into German guidelines for the management of dredged material. Evaluations of intra- and inter-laboratory performance and predicative power of the used bioassays based on extracts of sediments, differently contaminated with DLCs. Except the high sample throughput (RTL-W1 EROD) and the high linear range (H4IIE-luc), the H4IIE Micro EROD assay showed the overall best performance among the three assays and had a similar predictive power like HRGC/HRMS analyses. The H4IIE Micro EROD assay was highly sensitive and showed a satisfying repeatability and cross-laboratory reproducibility, independent of sample complexity. Hence, the H4IIE Micro EROD assay was proven to be highly suitable for the analysis of DLCs and to be used as ptential regulatory tool in the sediment management.

In a further study, the uptake of sediment-borne DLCs by common roach, a fish of high ecological relevance, was chemically and bio-chemically investigated using the same differently contaminated sediment samples (see above) as exposure media. Fish was either exposed to black worm - inoculated sediments (dietary eposure) or daily fed with uncontaminated worms. Both chemical and bio-chemical investigations of whole fish extracts predominantly revealed an uptake of sediment-borne DLCs by fish, independent of the sediment DLC contamination degree. BEQs indicated the uptake to be promoted by (1) the suspended matter concentration in the water column and (2) the additional ingestion of feed/sediment (only relevant for the sediment of highest DLC contamination).

While this study proved the applicability of the Micro EROD assay for challenging sample matrices such as whole fish homogenates, another study, which chemically and biochemically investigated sediment and soil samples from the river Elbe catchment area, proved the H4IIE Micro EROD assays' applicability to be used as high throughput screening tool for large sample sets. Samples of highest EROD-inducing potential, even though raw extracts (missing clean-up) were investigated, corresponded well to the *via* HRGC/HRMS detected contamination hotspots along the river. A H4IIE Micro EROD assay based limit value was deduced from the DLC concentrations of the river Elbe sediment samples and might point towards a future yes/no-decision-level in German guidelines for dredged material.

A final study investigated the bioavailabilty of polycyclic aromatic hydrocarbons (PAHs). A tenax desorption experiment was meant to close the gap between instrumental and toxicological results. It was shown that the cumulative concentrations of PAHs desorbing from the four differently contaminated sediments corresponded well to (1) the initial PAH concentrations in sediments, (2) to the effects observed in fish eggs of *D. rerio* and (3) to a certain extend to RTL-W1 BEQs. This indicated the higher contaminated sediments to pose a potentially higher threat to the aquatic environment.

The present findings might contribute to future regulatory decisions in a way that *in vitro* bioassays such as the Micro EROD assay could be implemented into German guidelines for dredged material to be used as an additional quality measure alongside classically used instrumental analysis and this way could significantly improve current sediment assessment strategies.

Zusammenfassung

Seit der Mitte des 20. Jahrhunderts wächst die Angst vor historischen Fremdstoffen, welche aufgrund ihrer potentiell schadhaften Effekte (Lebertoxizität, Hormonstörungen und Unfruchtbarkeit) und Eigenschaften (Anreicherung in Sedimenten und Geweben) Mensch und Tier gefährden können. Unter diesen Fremdstoffen findet man die Gruppe der dioxin-ähnlichen Substanzen (DLCs), bestehend aus den polychlorierten Dibenzo-*p*-dioxinen und Dibenzofuranen (PCDD/Fs) sowie den dioxin-ähnlichen polychlorierten Biphenylen (DL-PCBs). Gleich wenn die DLC Emissionen innerhalb der letzten Jahrzehnte dramatisch reduziert wurden, stellen historisch kontaminierte Sedimente nach ihrer Re-Mobilisierung (z.B. Fluten oder Baggerungen) immer noch eine Sekundärquelle für solche Substanzen dar. Dies könnte wiederum ein Nicht-Erreichen der Ziele der Wasserrahmenrichtlinie (WRRL) für Europäische Oberflächengewässer zur Folge haben. Es ist daher nicht verwunderlich, dass neuerdings die Sediment- und Baggergutbewertung als auch neue Konzepte, wie die Etablierung von klar definierten Umweltqualitätsnormen (UQN), immer mehr an Bedeutung gewinnen.

Basierte die Sedimentbewertung anfänglich lediglich auf klassischen, instrumentellen Methoden (z.B. hochauflösende Gaschromatographie - hochauflösende Massenspektrometrie (HRGC/HRMS)), so wurden diese, aufgrund ihrer fehlenden Betrachtung der Sedimenttoxizität, um umwelttoxikologische und ökologische Aspekte erweitert. Allerdings nutzen solche Methoden meist unspezifische Endpunkte (z.B. Wachstumshemmung), welche einen Vergleich mit chemisch, *via* HRGC/HRMS gemessener Konzentrationen unmöglich machen. Da jedoch viele toxische Effekte der DLCs über den zytosolischen Aryl-Hydrocarbon-Rezeptor (AhR) vermittelt werden, dessen Aktivierungsstärke wiederum der Endpunkt vieler zellbasierter *in vitro* Biotests ist, stehen solche Verfahren zunehmend im Interesse von Behörden und Risikomanagern. *In vitro* Test abgeleitete Toxizitätsäquivalente (BEQs) sind direkt vergleichbar zu vom HRGC/HRMS Analysen abgeleiteter Toxizitätsäquivalente (TEQs)

Das DioRAMA-Projekt ist eine gemeinsame Forschungsinitiative zwischen dem Institut für Umweltforschung der RWTH Aachen und dem Referat G3 der Bundesanstalt für Gewässerkunde. Sein Hauptziel ist die Etablierung von *in vitro* Tests für die Bewertung von DLCs in Sedimenten und Biota zur Verbesserung bestehender Risikobewertungskonzepte. Die vorliegende Arbeit untersuchte daher (1) die Empfindlichkeit verschiedener *in vitro* Tests, die Eignung ausgewählter *in vitro* Tests (2) als regulatorische Werkzeuge, (3) zum Nachweis der Aufnahme von DLCs in Fische und (4) zur Sediment- und Bodenextrakt Priorisierung. Final wurde die Bioverfügbarkeit ausgewählter DLCs untersucht.

Eine Literaturstudie belegte die mannigfaltigen Anwendungsmöglichkeiten von *in vitro* Tests zur DLC Analyse komplexer Proben, einzelner Substanzen und Mischungen. Um Spurenkontaminanten wie DLCs nachweisen zu können, müssen *in vitro* Tests über ähnliche Empfindlichkeit wie instrumentelle Techniken verfügen. Die Literaturrecherche konnte zeigen, dass einzelne *in vitro* Tests sich den chemisch-analytischen Empfindlichkeitsgrenzen annähern (~0,1 pM Dioxin) und deuten damit auf eine Eignung dieser Tests zur Nutzung als regulatorische Werkzeuge hin.

Um diese Annahme zu überprüfen wurden drei *in vitro* Tests (RTL-W1 EROD, H4IIE-luc und H4IIE Micro EROD Assay), hinsichtlich ihrer möglichen Implementierung in Deutsche Richtlinien zum Umgang mit Baggergut, untersucht. Die Bewertungen der Intra- und Interlabor-Performance sowie der Vorhersagekraft der angewandten Tests basierten auf Extrakten unterschiedlich stark DLC kontaminierter Sedimente. Abgesehen von höherem Probendurchsatz (RTL-W1 EROD) und einer größeren, linearen Bandbreite (H4IIE-luc), zeigte der H4IIE Micro EROD Assay die beste Performance unter allen Tests und wies eine der HRGC/HRMS Analyse vergleichbare Vorhersagekraft auf. Der Assay war hoch sensitiv und seine gute Wiederholbarkeit und labor-übergreifende Reproduzierbarkeit waren unabhängig von der Probenkomplexität. Der Assay eignet sich folglich hervorragend zur DLC Analyse und möglicherweise als zusätzliches Bewertungsinstrument im Sedimentmanagement.

Eine weitere Studie untersuchte die Aufnahme sedimentbürtiger DLCs in Rotausgen, ökologisch hochrelevanter Fische, chemisch und bio-analytisch unter Verwendung der unterschiedlich kontaminierten Sedimente (s.o.) als Expositionsmedium. Die Fische wurden entweder an Glanzwurm-inokulierten Sedimenten exponiert (Nahrungsexposition) oder täglich mit unbelasteten Würmern gefüttert. Sowohl chemische als auch bio-analytische Untersuchungen der Fischextrakte zeigten eine überwiegende, vom Sediment-Kontaminationsgrad unabhängige, DLC Aufnahme durch die Fische. BEQs zeigten, dass die Aufnahme durch (1) die Schwebstoffkonzentration im Wasser und (2) die zusätzliche Futter-/Sedimentaufnahme (nur hochbelastetes Sediment) begünstigt wurde.

Während diese Studie die Anwendbarkeit des Micro EROD Assays für anspruchsvollere Proben wie Fischhomogenatextrakte bestätigte, konnte in einer weiteren Studie mittels chemisch und bio-analytisch untersuchter Sediment- und Bodenproben des Elbe Einzugsgebiets bewiesen werden, dass der H4IIE Micro EROD Assay sich sehr gut als Hochdurchsatzverfahren für größere Probensets eignet. Trotz der Verwendung von Rohextrakten (fehlende Aufreinigung), stimmten die Proben mit den höchsten EROD Aktivität sehr gut mit instrumentell bestimmten Kontaminationshotspots längs der Elbe überein. Ein von den DLC Konzentrationen der Elbsedimentproben abgeleiteter H4IIE Micro EROD Assay Grenzwert könnte zukünftig als Ja/Nein Entscheidungswert in Deutschen Baggergutrichtlinien Einsatz finden.

Zuletzt wurde die Bioverfügbarkeit von polyzyklischen aromatischen Kohlenwasserstoffen (PAKs) untersucht. Ein Tenax Desorptionsexperiment hatte zum Ziel, die Lücke zwischen chemischen und bio-analytischen Methoden zu schließen. Die kumulative Konzentration der von den Sedimenten desorbierenden PAKs stimmte sehr gut mit (1) dem PAK Kontaminationsgrad dieser Sedimente (2) den Effekten in Fischeiern von *D. rerio* und (3) in einem gewissen Grad mit RTL-W1 BEQs überein. Dies zeigte, dass die höchstkontaminierten Sedimente den aquatischen Lebensraum stärker bedrohen.

Die Ergebnisse der vorliegenden Arbeit könnten bei zukünftigen gesetzlichen Entscheidungen dazu beitragen, dass *in vitro* Tests wie der Micro EROD Assay in deutschen Baggergutrichtlinien als zusätzliche Qualitätsmessungen neben klassischer instrumenteller Analyse Anwendung finden und so entscheidend zu einer Verbesserung bestehender Sedimentbewertungsverfahren beitragen könnten.

List of figures:

List of tables:

Chapter 1

Introduction

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

Eichbaum, K., Brinkmann, M., Buchinger, S., Hecker, M., Engwall, M., van Bavel, B., Reifferscheid, G., Hollert, H. (2013) The dioRAMA project: assessment of dioxin-like activity in sediments and fish (*Rutilus rutilus*) in support of the ecotoxicological characterization of sediments. *Journal of Soils and Sediments* 13: 770-774.

Eichbaum, K., Brinkmann, M., Buchinger, S., Reifferscheid, G., Hecker, M., Giesy, J.P., Engwall, M., van Bavel, B., Hollert, H. (2014) *In vitro* bioassays for detecting dioxin-like activity - Application potentials and limits of detection, a review. *Science of the Total Environment* 487: 37-48.

1.1 The European water framework directive (WFD)

In the year 2000, the European water framework directive (WFD) 2000/60/EG (2006) was passed by the European Parliament and the Council. The directive provides the legal framework for the future European water policy of the European member states and follows a holistic approach on a river basin scale, which is free from any administrative or spatial borders (Schulz et al. 2005). The main aim of the WFD is that all European surface waters reach a good chemical and ecological status until the year 2015, encompassing the non-deterioration, the reduction of pollution with priority substances as well as the stop of emissions and discharges (FGG-Elbe 2015).

Despite the fact that industrial and municipal emissions have been reduced considerably during the last decades (Besselink et al. 2004), monitoring programs have been shown that the majority of European surface waters will most likely not reach the aims set by the WFD (BMU 2013). Reasons for this on the ecological site are alterations of natural river morphology and lacking consistency (e.g. for migratory fish species), whereas diffuse agricultural, industrial and municipal substance discharges prevent the achievement of a good chemical status (BMU 2013). Moreover, it has been shown that until now, legacy pollution of sediments tented to be underestimated by the WFD (Hallare et al. 2011, Heise and Förstner 2006, Hollert et al. 2007), even though contaminated sediments can significantly contribute to the contamination of water so that quality standards of the WFD can probably not be achieved in catchment areas of many European rivers (Barceló and Petrovic 2007, Förstner 2008, Hollert et al. 2007). As a consequence, the WFD daughter directive (EC 2008) was introduced, which requested the concentration of 33 priority pollutants (annex, 2000/60/EC 2006) not to increase above levels set by clearly defined Environmental Quality Standards (EQS) for sediments and biota (Förstner 2009, Lepom et al. 2009).

Hence, a good ecological and chemical status of European surface water bodies according to the WFD is reached, when the concentrations of the priority substances in water, sediment and biota are below their respective EQS (Lepom et al. 2009, Wernersson et al. 2015). The time table of monitoring programs, including chemical compliance checks, follows a 6 year cycle, so that the aims of the WFD latest have to be fulfilled by the member states in management plans 2015 - 2021 and 2021 - 2027 (BMU 2013).

1.2 Sediments – an underestimated risk?

River sediments develop through erosion of parent rock and sedimentation of settling detrital, inorganic and organic particles (*Figure 1.1*). Their heterogeneous nature supports biodiversity (Barceló and Petrovic 2007), offers diverse habitat structures and nutrient sources (Hollert et al. 2000) to the aquatic environment. The high biological activity, especially at the sediment surface, makes sediments become the most effective site for transformation of organic carbon, nitrogen, phosphorus, magnesium and sulfur (Burton 1992). But their surface characteristics moreover offer a multitude of binding possibilities for organic contaminants (Berglund et al. 2001, Hollert et al. 2014) and may influence their bioavailability and accessibility (Eggleton and Thomas 2004).

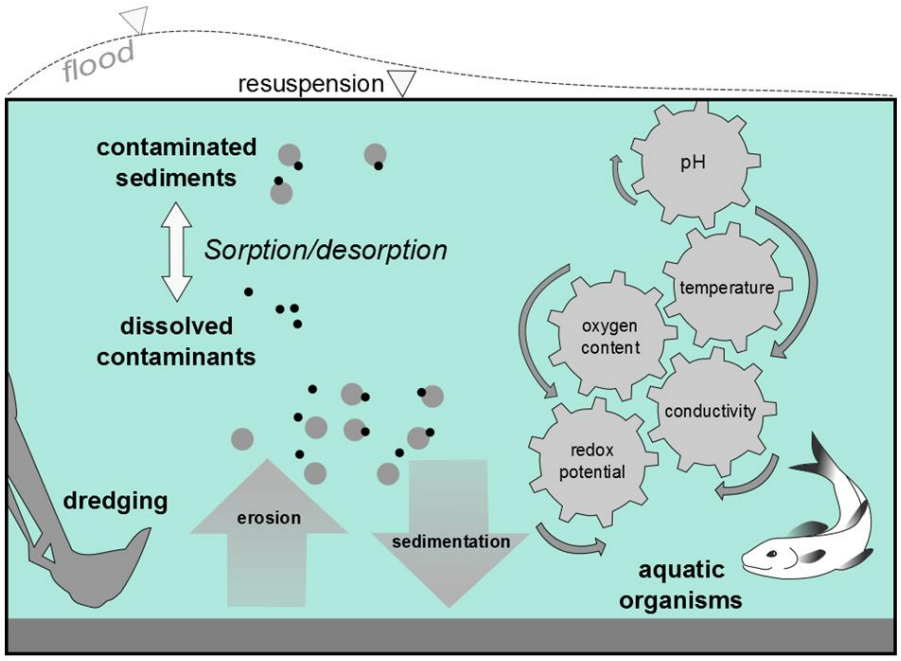


Figure 1.1 Processes of sediment development and re-suspension, sorption and desorption of organic contaminants as well as interactions with aquatic organisms and influencing limno-chemical factors. Figure recreated according to (Cofalla et al. 2012, Hollert et al. 2014).

During the 20th century, diverse anthropogenic activities of the industrial nations led to high amounts of xenobiotics that were released into the rivers, where they sorbed to sediments. Such historical contaminated sediments are immobile under normal circumstances, but following extreme hydrogeological events such as floods or dredging activities (*Figure 1.1*) can get re-mobilized and reintroduced into the water column again (Eggleton and Thomas 2004, Heise and Förstner 2006, Hollert et al. 2014), hence, can act as secondary pollutant sources (Ahlf and Förstner 2001, Hollert et al. 2014). Particular-bound or freely dissolved organic contaminants (*Figure 1.1*) are bioavailable again and this way can threaten aquatic organisms (Burton 1992),

but even pose a risk to terrestrial organisms. Frequent inundation of river associated floodplains caused a deposition of contaminants bound to particulate matter, which led to threshold exceedances in milk and meat of exposed grazing cows (Schulz et al. 2005, Stachel et al. 2005).

1.3 Stepwise and integrated sediment assessment strategies

The Evaluation of sediments becomes increasingly important in terms of (bio) remediation and decontamination of historical contaminated sites (Fent 2007) and most often is used to evaluate the risk potential of environmental samples and/or to examine the success of (bio) remediation measures (Fent 2007). While sediment evaluation initially based on chemical-analytical methods alone (Brack 2003), most of the recently developed sediment assessment approaches constitute stepwise or integrated procedures, which combine chemical, ecotoxicological and ecological perspectives.

A stepwise sediment assessment can be found in the German joint transitional arrangements for the handling of dredged material (GÜBAK 2009), where ectotoxicological test methods are performed in the case chemical concentrations of analyzed target compounds (i.e. heavy metals and selected organic contaminants) exceed action values. The ecotoxicological tools encompass acute and chronic tests with algae (*Desmodesmus subspicatus*), bacteria (*Vibrio fisheri*) and micro crustaceans (*Daphnia magna*) on lethal- and sub-lethal levels (den Besten et al. 2003, Manz et al. 2007). However, an important drawback of this stage procedure is that results of chemical analysis and ecotoxicological tests are incomparable and do not provide any information about compounds causative for a certain biologically observed effect (Manz et al. 2007).

In the group of integrated sediment assessment schemes, the most significant approach was the “sediment quality triad” (SQT), which in a weight-of-evidence approach (Chapman et al. 2002, Chapman and Hollert 2006) combines different so-called lines of evidence (LOE) (Chapman and Hollert 2006, Long and Chapman 1985). These LOE include chemical target-compound analysis to determine the chemical contamination, sediment bioassays to determine the sediment toxicity and benthic community composition to determine the impact of sediments towards the resident fauna (Chapman and Hollert 2006). A combination of different LOE has a greater informative value for the assessment of sediment than regarding single LOE. This is because the single LOE in a SQT complement each other. Concentrations of contaminants alone do not allow for toxicity estimation, while bioassays, which do allow for such estimations, do not hand information on the community structure. Information about this structure alone would not allow for estimation of any causative impacts (Chapman 1989).

Recently, many approaches for an improved integrated sediment assessment have been suggested, such as the introduction of additional LOE (Chapman and Hollert 2006).

1.4 Dioxins and dioxin-like compounds (DLCs)

Since the middle of the 20th century there has been increasing concern about exposure of humans and wildlife to certain xenobiotics that were released into the environment due to diverse anthropogenic activities. One group of environmental toxicants that is of particular interest relative to potential environmental health effects (as reviewed by White and Birnbaum 2009) and legacy contamination are dioxin-like compounds (DLCs). This group includes polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) as well as dioxin-like polychlorinated biphenyls (DL-PCBs).

These ubiquitous compounds are hydrophobic, lipophilic and resistant to biological and chemical degradation (Hilscherova et al. 2000). These properties determine their globally distribution (even polar regions) and their presence in almost every matrix (e.g. sediments, soils, wildlife, human tissue, blood and milk) and impart their propensity to bio-accumulate and bio-magnify along the food chain (Safe 1998a). The accumulation of DLCs is of special importance for top predators (fish, otters and seals), in which DLCs can accumulate to levels leading to adverse effects (Fent 2007). The range of effects is broad and may include thymic atrophy, hepatotoxicity, certain types of cancer, dermal disorders, endocrine disruption, wasting syndrome, reproductive toxicity, infertility or reduced fecundity as well as the induction of monooxygenase enzymes (Brouwer et al. 1995, Denison and Heath-Pagliuso 1998, Denison and Nagy 2003, Giesy et al. 1994a, Poland and Knutson 1982, Safe 1986, 1994, Van den Berg et al. 1998, Whyte et al. 2000). Their behavior *in vivo* furthermore depends on their organism-specific uptake, distribution and metabolism (Behnisch et al. 2001b, Safe 1986) as well as modifying factors such as species, age and reproductive status (Whyte et al. 2000).

1.4.1 Polychlorinated biphenyls (PCBs)

PCBs consist of two benzene rings, exhibiting ten possible chloral bonding sites (*Figure 1.2*). Due to the resulting various possibilities of chlorination, 209 PCB congeners are known. Within the group of PCBs, 12 congeners, the non-*ortho* PCBs 77, 81, 126 and 169 and the mono-*ortho* PCBs 105, 114, 118, 123, 156, 157, 167 and 189 build the DL-PCBs (or referred as 12-WHO-PCBs), which exhibit a molecular mode of action similar to that of dioxin (Barceló and Petrovic 2007, Safe 1998a).

The majority of PCB contamination stem from the approximately 1.3 million tons of technical PCB mixtures, used in a range of closed applications (transformers, capacitors, hydraulic fluids) and open applications (e.g. plasticizers, paints, cutting oils, flame retardants), containing approximately 1000 kg dioxin toxic equivalents (TEQs) (Breivik et al. 2002, Denison and Heath-Pagliuso 1998, Fent 2007, UNEP 1999, Wagner et al. 2014, Weber et al. 2008). Moreover, PCBs have been used as pesticides (DDT) to control the malaria carrying anopheles mosquito, but furthermore unintentionally develop during thermal processes (Anezaki and Nakano 2014, Huang et al. 2014). PCBs gained sad popularity through several, past PCB poisonings: the Japanese Yusho in the year 1968 and the Taiwanese Yu Cheng poisoning in the year 1979, which both were caused by PCB-contaminated rice oil (Masuda 2001, Yu et al. 2000). However, these incidents contributed to restrict the use of PCBs to closed systems. Despite being banned for several decades (except the use of DDT) through the Stockholm Convention (Yoder 2003), PCBs are continuously emitted into the environment through leakages from old capacitors, elastic sealants and other building materials (Fent 2007), which beside their contribution to historical contaminations illustrates their relevance to the current situation.

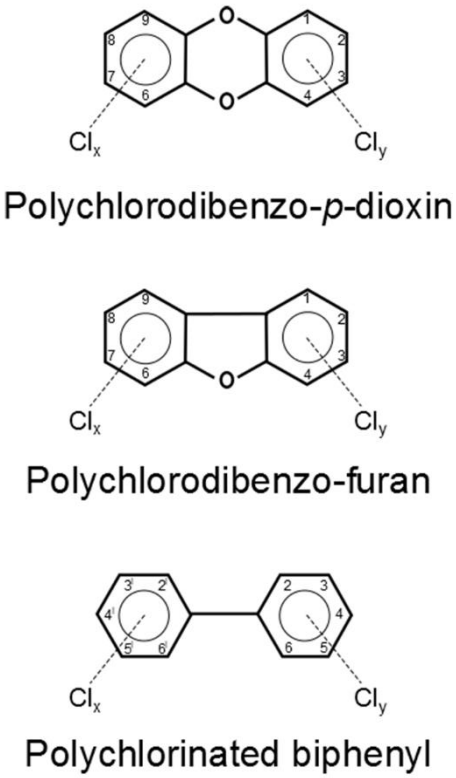


Figure 1.2 Basic molecular structures of polychlorodibenzo-*p*-dioxins (PCDD), polychlorodibenzo-furans (PCDF) and polychlorinated biphenyls (PCBs).

1.4.2 Polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs)

PCDD/Fs, collectively referred to as *dioxins*, are planar and aromatic compounds, varying in the amount and positions of chlorine atoms (*Figure 1.2*). This way, a group of 75 and 135 known PCDD and PCDF congeners can be formed (Safe 1990b). The higher the chlorination of such congeners, the higher is their persistency in the environment.

PCDD/Fs are unintentional, industrial byproducts that are mainly of anthropogenic origin. They are formed during organochlorine production, in combustion processes at low temperatures < 800 °C such as municipal, hospital and industrial waste incineration (Safe 1990b) as well as during chemical processes involving chlorine substances e.g. pulp- and paper

or magnesium production (Aarts et al. 1995, Fent 2007, Safe 1990b, 1994, UNEP 2013 , Weber et al. 2008).

The toxicity is congener-specific, which was first assessed by Poland and Glover (Poland and Glover 1973). The most toxic congener is the 2,3,7,8-Tetrachloro dibenzo-*p*-dioxin (2,3,7,8-TCDD). It is a threshold poison, meaning that the amount of receptors occupied by this congener correlates with the strength of the biological response (Fent 2007). Furthermore, 2,3,7,8-TCDD attained sad notoriety as so-called Seveso-poison following a dioxin accident in the Italian city Seveso in the year 1976. An exploded chemical reactor caused high TCDD concentrations to enter the environment, which in turn affected flora, fauna and inhabitants (Fent 2007). Due to their physical and chemical properties their half life time can add up to 10 years in humans (Denison and Heath-Pagliuso 1998), who get exposed to dioxins mainly through their daily diet (especially meat, fish, eggs and milk products), leading to average total daily intakes (TDI) of 0.3 to 5 pg TEQ/kg body weight per day (Behnisch et al. 2001a).

The status quo of PCDD/F is that inputs are declining through the banning of critical chlorine chemicals and emission control measures (Lee et al. 2007). Consequently, dioxin concentrations in environmental samples are decreasing today (Barceló and Petrovic 2007, Besselink et al. 2004), but as historical contaminants burried in sediments, dioxins still constitute critical time bombs (Hollert et al. 2014).

1.4.3 Aryl hydrocarbon receptor (AhR)-mediated toxicity

Toxic effects of DLCs are mediated *via* the aryl hydrocarbon receptor (AhR) (Bittner et al. 2006, Hankinson 1995, Olsman et al. 2007a). The AhR is a cytosolic receptor protein, which belongs to a subclass of helix-loop-helix-containing transcription factors (Giesy and Kannan 1998, Goldstein and Safe 1989). Co-planar aromatic compounds such as DLCs, but a multitude of other organic, structurally similar compounds such as polycyclic aromatic hydrocarbons (PAHs) as well as other partially known and unknown compounds (Giesy et al. 1994b, Giesy et al. 2006, Larsson et al. 2014, Poland and Knutson 1982, Song et al. 2006, Van den Berg et al. 2006, Van der Plas et al. 2001) can bind to this receptor. Following the association of ligand and receptor, the complex is translocated into the nucleus, where it forms a heterodimer with the AhR nuclear translocation protein (ARNT) and possibly additional factors (Hahn 1998). The ligand-AhR-ARNT complex binds to a specific DNA sequence, the dioxin responsive element (DRE), and with this transcriptionally activates the synthesis of AhR-responsive genes like cytochrome P_{450}1A (CYP1A) (Hilscherova et al. 2000).

Cytochromes represent a multigene family of heme-containing proteins, which are mainly present in the liver, but also in kidney, gastrointestinal tract, gills and other tissues of many organisms. They possess the ability to metabolize xenobiotics *via* Phase-I-reactions (oxidation, hydrolysis or reduction reactions), which may lead to a detoxification or to a so-called bioactivation (toxification) (Castell et al. 1997).

Despite being intensively studied, the understanding of AhR-mediated responses remains incomplete (Chen and Bunce 2004). Nevertheless, the general observation that toxic effects caused by DLCs are mainly mediated by the AhR was made by many scientists (Bittner et al. 2006, Hankinson 1995, Olsman et al. 2007b). Moreover, it has been shown that the strengths with which ligands bind to the AhR is proportional to their toxicity, the transcriptional activity as well as the AhR-mediated enzyme activities (Safe 1995). The AhR moreover has endocrine functions since it is involved in cell development, -proliferation, -differentiation and cell cycle programming (Denison and Heath-Pagliuso 1998, Fent 2007, Fernandez-Salguero et al. 1995, Schmidt et al. 1996).

1.5 CYP-based and reporter gene-based *in vitro* assays

The specific and naturally occurring mechanism of CYP1A induction by DLCs has been used in *in vitro* bioassay techniques for the characterization of dioxin-like potentials of e.g. environmental samples (Tillitt et al. 1991, Tillitt et al. 1992). As for *in vivo* effects, the responsiveness of *in vitro* systems is species or cell-line specific (Keiter et al. 2008), caused by differing binding affinities, structures, quantities and physicochemical properties of the AhR of different cell lines (Hilscherova et al. 2001, Sanderson et al. 1996).

Regarding functional AhR-based bioassays for quantification of CYP1A activity such as the 7-ethoxyresorufin-*O*-deethylase (EROD) assay, the dioxin-like potential of DLCs present in a certain sample is determined by measuring the deethylation of the artificial and exogenous substrate 7-ethoxyresorufin into fluorescent reaction product resorufin. The EROD assay can be conducted *via* different cell lines, such as fish cell lines, where the deethylation reaction is catalyzed CYP1A1 and CYP1A3 (Bols et al. 2005, Goksøyr and Förlin 1992) or rat hepatoma cell lines, where the catalyzing CYPs may be CYP1A1 and CYP1A2 (Whitlock 1999). The various application potentials of the EROD assay as well as its frequent use has led to the title "golden standard of *in vitro* bioassays" (as reviewed by Behnisch et al. 2001b). Previous studies have been shown that the EROD activating potential of some AhR agonists were well correlated to the toxicity observed *in vivo* (Safe et al. 1989). *In vitro* assays therefore are in accordance with the ethical requirements of the 3R principle (i.e. reduction, replacement, refinement),

towards a reduction of test animal numbers, a replacement of test animal-based experimentations through e.g. in vitro assays, which work on a sub-cellular level (Wernersson et al. 2015) and a general refinement of the applied methods (Russell et al. 1959).

However, the EROD assay shows certain drawbacks such as substrate inhibition e.g. in the presence of high concentrations of PCBs (Sanderson and Giesy 1998), possibly leading to false-negative results. Moreover, its linear working range compared to other assays may be limited (Behnisch et al. 2001b).

To overcome such issues, the process of AhR-mediated activation of genes has been genetically engineered by connecting the DRE of various cell lines with certain reporter genes (Lee et al. 2013). These genes may originate from firefly (*Photinus pyralis*) or from sea pen (*Renilla reniformis*) and form the enzyme luciferase, which catalyze the reaction from added luciferin to the bioluminescent oxyluciferin (Denison et al. 1988a, Denison et al. 1988b, Garrison et al. 1996, Thain et al. 2006). This reaction product is much more stable compared to EROD. Commonly used luciferase-based assays are the DR-CALUX® (Dioxin Responsive-Chemical Activated LUciferase gene eXpression) with mammalian hepatoma cell lines transfected with plasmid pGudLuc1.1, the H4IIE-luc assay with an eponymous cell line and the CALUX assay, mostly performed with mouse hepatoma cell line Hepa 1 (Villeneuve et al. 1999, Villeneuve et al. 2000). Newly developed reporter gene assays such as the CAFLUX (Chemical Activated FLUorescent protein eXpression) assay use enhanced green fluorescent protein (EGFP) from jellyfish (*Aequoria victoria*) instead of luciferase. Hence, the addition of cost intensive, exogeneous substrate is not sufficient, which results in a cheaper and faster assay methodology (Nagy et al. 2002).

1.6 Toxicity equivalents (TEQs) and relative potencies (REPs)

Commonly, results of *in vitro* bioassays are expressed in biological equivalent quotients (BEQs), which put the AhR-activating potential of e.g. an environmental sample in relation to the AhR-activating potency of the reference compound 2,3,7,8-TCDD (Ahlborg et al. 1994, Brunström et al. 1995, Engwall et al. 1996, Safe 1990b, 1998a, Van den Berg et al. 1998, Van den Berg et al. 2006). This way, bioassay-derived results become comparable with those of instrumental analysis, which can be expressed as toxicity equivalents (TEQs).

TEQs are calculated by multiplying concentrations of all single compounds that have been analyzed in an extract with their specific toxicity equivalent factor (TEF). The TEF value is a relation of the AhR-activating potential of a single compound to that of 2,3,7,8-TCDD, which exhibits a TEF of 1. WHO-TEF values since 1993 have been derived by collecting and judging

available data of different mammalian, avian and fish studies performed with DLCs (Van den Berg et al. 1998). WHO-TEF estimates are partially based on *in vivo* experiments, and thus, processes such as uptake and tissue distribution, which are negligible in cell-based assays, may not be a good representation of the relative potency (REP) specific to the cell system used (Brown et al. 2001). For this reason, REPs in general possess a better alternative compared to more unspecific TEFs (Hilscherova et al. 2001, Hilscherova et al. 2003, Kannan et al. 2008). Even though recently a number of cell line specific REP values were proposed for many single DLCs, there is still a lack of REPs for cell lines that are used less extensively. An important factor for reproducibility and applicability of cell line specific REP values is their origin. For example, it is important to state if e.g. EC_{20} or the EC_{50} values were used for REP determination, because dose-response curves of different chemicals may show a non-parallelism.

TEF or REP-based TEQ values in the case of solids are expressed as pg TEQ/ g dry mass (dm) of e.g. sediment. An exemplarily value of 1pg TEQ/g dm of sediment hence would state that 1 g of dm sediment has the same effect as if it contained 1 pg of 2,3,7,8-TCDD. By interpreting TEQs, one has to keep in mind that they neither provide any specific information regarding toxicokinetic properties of chemicals present in a mixture (shapes/slopes of their concentration-response curves), nor the tested species used to calculate the TEFs. Care must be taken when comparing TEQs of different studies, as the underlying effect levels are frequently not stated (Eichbaum et al. 2014).

Differences observed between TEQs and BEQs are often observed and can be due to several reasons: *In vitro* bioassays integrate the overall gene activating effect of all AhR agonists and antagonists present in a mixture, while instrumental analyses focuses on a selected numbers of known DLCs. Hence, non-classical and unknown AhR inducers are not taken into account by chemical analysis. Moreover, the TEQ concept assumes additivity of single DLCs, but AhR ligands can be agonistic, antagonistic or interact synergistically (Windal et al. 2005). While bioassays measure one biological endpoint, instrumental results are calculated by using TEF values, which – on the contrary - are obtained from in-depth toxicity studies (Sanctorum et al. 2007). However, bio-analytical and instrumental results are most often correlated and while bioassays are well-suited screening tools for large sample numbers, which do allow for prioritization of e.g. sediment contamination, chemical analysis allows to pinpoint the actual chemicals responsible for a biological effect.

1.7 The DioRAMA project

The DioRAMA ("**Dio**xin **R**isk **A**ssessment for sediment **M**anagement **A**pproaches") project is a joint research initiative between the Institute of Environmental Research at the RWTH Aachen University, Aachen, Germany and the Department G3 (Biochemistry/ Ecotoxicology) of the German Federal Institute of Hydrology (BfG), Koblenz, Germany and received funds from the "Title Group 05" of the German Federal Ministry of Transport, Building and Urban Development. The close interconnection between applied ecotoxicological science and regulatory needs should facilitate the establishment of *in vitro* tools for the assessment of DLCs in sediment and biota for their implementation in sediment management guidelines. While the project was coordinated by the BfG, investigations were performed at the Institute for Environmental Research.

Given the complex interactions of sediment re-suspension processes and bioavailability of sediment-associated DLCs, there is need for a better integrative understanding of the cause-effect-relationship of DLCs. Because the majority of current studies concerned with DLCs most often only focus on characterizing sediment extracts by means of *in vitro* bioassays, while disregarding bioavailability, uptake, metabolism and elimination rates of these compounds *in vivo*, the DioRAMA project aims in bridging the gap between *in vitro* and *in vivo* assessments of the effects of sediment-associated DLCs.

Bridging this gap comprises investigation of the complex cause-effect-relationships of sediment-associated DLCs in biota and to compare these with toxic potentials determined in the same samples using commonly used *in vitro* assays. The combination of *in vitro* and *in vivo* characterization of the biomarkers of exposure and biomarkers of effect will generate additional knowledge that will improve current risk assessment approaches for DLCs in biota and sediments (*Figure 1.3*), and will enable correlating laboratory with field conditions.

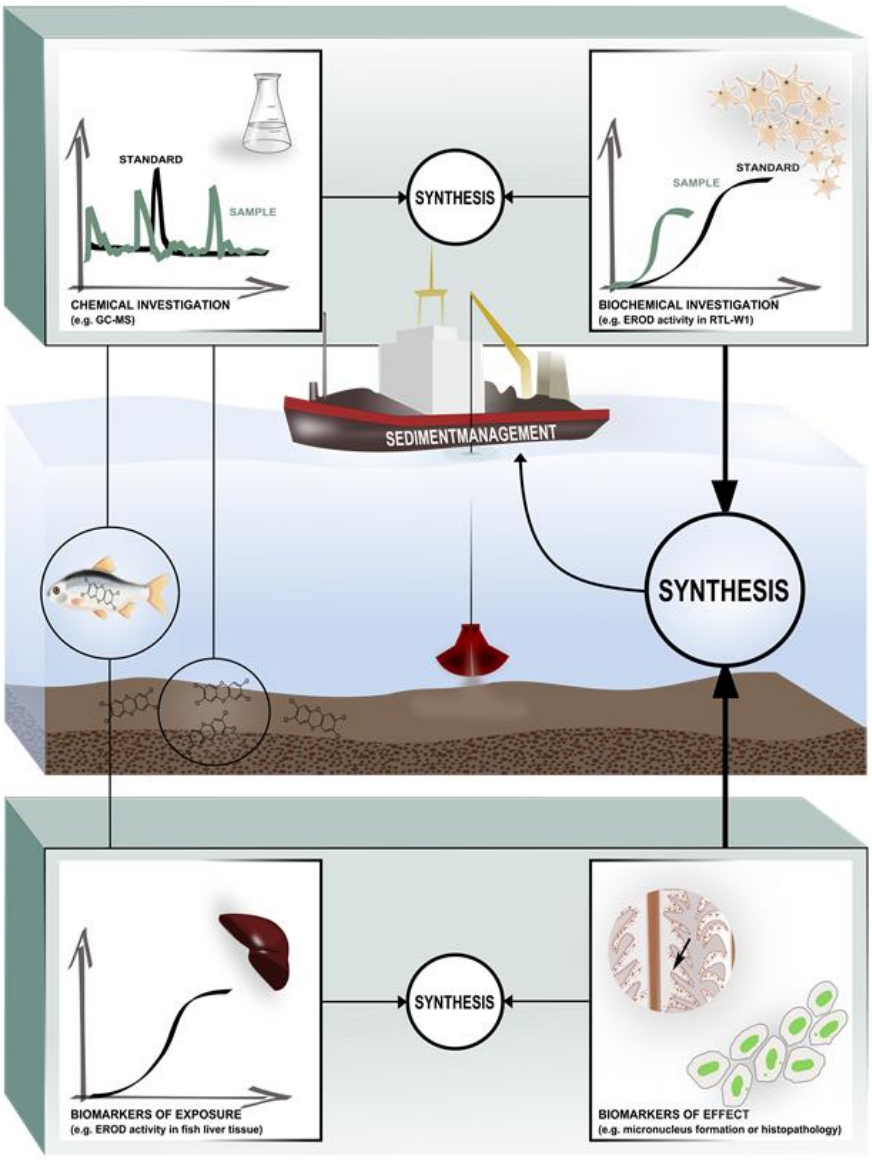


Figure 1.3 Structure of the DioRAMA project, major components of the project encompass the chemical (top left) and bio-analytical (top right) determination of dioxin-like compounds (DLCs) in fish and sediment (middle) as well as the characterization of different biomarkers of exposure (bottom left) and biomarkers of effect (bottom right) in fish exposed to sediments containing DLCs. The synthesis of all components of the project will provide new sediment risk assessment approaches to the sediment management.

1.8 Aims of the present study

The aim of the present study was to evaluate of different *in vitro* bioassays for the screening of dioxins and dioxin-like compounds (DLCs) with regard to their possible implementation into current risk assessment approaches. The investigations followed the main aims recorded in the DioRAMA project and were addressed in five chapters, encompassing a literature review of *in vitro* bioassays for detecting DLCs (Chapter 3), a validation of such assays towards their regulatory implementation (Chapter 4), a bio-analytical evidence of the uptake of DLCs by fish (Chapter 5), a chemical and exotociological analysis of the bioavailability of DLCs (Chapter 6)

and an analysis of the suitability of *in vitro* bioassays as prioritizing tools for DLCs in sediment and soil samples (Chapter 7).

1.8.1 Application potentials and limits of detection of *in vitro* bioassays for the screening of dioxin-like activity

Regarding the multitude of national and international studies on the determination of dioxin-like activity using *in vitro* bioassays, two categories of used sample types can be distinguished, including (1) complex samples and (2) individual compounds and mixtures thereof.
While the first includes environmental matrices such as sediments, soils, water, industrial emissions as well as food, feed of tissue samples, the latter is primarily used to determine relative potencies (REPs) of single compounds, which allow for attributing an integrated dioxin-like potential of e.g. a complex sample to particular compounds. The investigation of compound mixtures furthermore allows for elucidating possible interactions between chemicals and for verifying their typically assumed additive interaction (see *Section 1.6*).

Because DLCs are considered trace contaminants, one of the potential limitations of *in vitro* bioassays has been their lower sensitivity (i.e., greater detection limits (LODs)) compared to some chemical analytical approaches, which often resulted in their inability to meet analytical goals or regulatory guidelines (Simat 2007, Zhao et al. 2010). The literature review presented in chapter 3 thus, gives an overview of the multiple application potentials of in vitro bioassays, but further investigates, whether *in vitro* bioassays are equal or less sensitive compared to chemical analytical investigation techniques.

1.8.2 *In vitro* tools for the toxicological evaluation of sediments and dredged materials: cross-validation of chemical and bio-analytical methods

The management and handling of dredged materials in Germany follows guidelines that have been compiled by the Ministry of Transport and Digital Infrastructure (BMVI) under the coordination of the German Federal Institute of Hydrology (BfG) (Breitung and Keller 2010). These guidelines have been reestablished within joint transitional arrangements (GÜBAK 2009) and consider the quality of sediments from both chemical and ecotoxicological perspectives. While chemical investigations focuses on heavy metals and hydrophobic organic pollutants with high relevance for sediments and suspended particulate matter such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), ecotoxicological tests encompasses acute and chronic tests with algae

(*Desmodesmus subspicatus*), bacteria (*Vibrio fisheri*) and micro crustaceans (*Daphnia magna*) on a lethal- and sub-lethal level (den Besten et al. 2003, Manz et al. 2007).

Since these ecotoxicological methods do not provide detailed information about causative compounds responsible for the observed biological effects and are not comparable to chemical analytical results, the cross-validation study presented in chapter 4 discusses the suitability of different *in vitro* bioassays for the screening of dioxin-like activity for their implementation into German dredged material guidelines.

1.8.3 Bio-analytical and instrumental screening of the uptake of sediment-borne, dioxin-like compounds in roach (*Rutilus rutilus*)

The uptake of sediment-borne DLCs by aquatic organisms (e.g.fish) depends on the exposure pathway, include aqueous (particle-, sediment- and/or water contact *via* integument and gills) and/or dietary exposure pathways. Once taken up, rates of accumulation of DLCs on the one hand depend on the species, its developmental stage, behavior, sexual condition as well as on seasonal, environmental and climatic conditions (as reviewed by Eggleton and Thomas 2004), on the other hand are influenced by the physical chemical properties of the DLCs themselves. For example, aqueous exposure pathways exhibit a linear relationship between log K_{ow} and bioavailability of a chemical up to log K_{ow} values < 7. Log K_{ow} values > 7 result in strong binding of chemicals to e.g. sediments and thus, their bioavailability decreases (Engwall et al. 1998, Hollert et al. 2002). ´For those congeners, sediment ingestion might be primary route of uptake (Eggleton and Thomas 2004).

Chapter 5 describes a study, where a cyprinid fish, the common roach (*Rutilus rutilus*) was used to investigate the extent, to which DLC uptake in roach depends on the initial sediment DLC contamination, how sediment-specific characteristics influence this uptake and whether contaminated diet increases the uptake of DLCs compared to an uptake *via* the water phase alone. Concentrations of DLCs present in whole fish homogenates and sediments, to which fish were exposed to, were determined using two *in vitro* bioassays and verified *via* results obtained by chemical instrumental analysis.

1.8.4 Desorption and bioavailability of sediment-bound polycyclic aromatic hydrocarbons from a chemical and ecotoxicological perspective

Fractions of polycyclic aromatic hydrocarbons (PAHs), which desorb from sediments are potentially bioavailable for aquatic organisms (e.g. fish) (Mackay and Fraser 2000) and may be absorbed *via* oral or dermal exposure routes (Larsson 2009). Several congeners this way can

act mutagenic and carcinogenic (e.g. Benzo[a]pyrene) and this way threaten aquatic organisms following an uptake of desorbed PAHs. To predict environmental relevant concentrations and bioavailability of PAHs, mild extraction techniques such as extractions of the bioavailable fractions using Tenax® TA beads (Cornelissen et al. 2001, Reid et al. 2000, Schwab and Brack 2007) can be applied. Bioavailability is a complex process, including desorption, partitioning and diffusion of a compound, which along with the characteristics of the sediment, the organism, the environment and the compound itself influence, how much of the compound present in a sediment is assimilated by biota (Schwab and Brack 2007).

A classical ecotoxicological test method, which allows for bioavailability estimation and the investigation of associated embryo toxic potential of whole sediment samples, is the sediment contact assay (SCA; Hollert et al. 2003). Typical sub-lethal and lethal, teratogenic effects can be observed in fish eggs of the tropical freshwater fish *Danio rerio*, which have direct contact to the relatively unchanged sediment. The SCA represents a good alternative to fish acute toxicity testing (Lammer et al. 2009) and with this complies with the 3R principle (see *Section 1.5*). Chapter 6 represents a study, in which a combination of chemical (GC-MS) and ecotoxicological (SCA and EROD assay with RTL-W1) was used to investigate the desorption and bioavailability of certain PAHs, originating from sediments differently contaminated with PAHs.

1.8.5 Spatial variability of the pollution of sediment and soil samples with dioxin-like compounds along the river Elbe and its alluvial plain

With 148,268 km², the river Elbe catchment area is the fourth largest river catchment area in Europe (LUA 2005), serving as an important waterway, recreational area and as habitat for a diverse flora and fauna (92/43/EWG 1992).

However, sediments of the river Elbe are highly contaminated with historical persistent organic pollutants (POPs), which mainly originate from treated and untreated industrial wastewaters of industries from Bitterfeld-Wolfen, a city of the Elbe tributary Mulde catchment area (Götz and Lauer 2003, Jacobs et al. 2013, Wilken et al. 1994, Wycisk et al. 2013), but also from Czech industries such as Synthesa in Pardubice, Lovochemie in Lovosice or Spolana and Spolchemie in Neratovice (Heinisch et al. 2007, Stachel et al. 2005, Umlauf et al. 2010). Increasing lining, sealing and water level regulation of the river Elbe, led to increases in current velocities and promoted the occurrence of periodical flood events (LUA 2005) such as the Elbe flood of August 2002. Following such re-suspension events, sediments carrying POPs get remobilized and reintroduced into the water column and can contaminate Elbe associated

floodplains or downstream river regions (Burton 1992, Förstner 2009). This way, POP releases from Bitterfeld-Wolfen resulted in threshold exceedances for POPs (in particular dioxins) in milk and meat of grazing cows, exposed to sediment-loaded, frequently inundated areas (Schulz et al. 2005, Stachel et al. 2005).

Chapter 7 represents a study, in which the dioxin-like potential of river Elbe sediment samples and selected soils of the alluvial plain was compared by using the EROD assay with RTL-W1 and H4IIE cells (see *Section 1.5*). Dioxin-like potentials were discussed in the context of DLC contamination hotspots along the river as well as in the context of floodplain contaminations through sediment-borne DLCs.

Chapter 2

Material and Methods

The present chapter lists the main methods used in the present study and aims in avoiding repetition within the single chapters. Detailed information to the methods presented here as well as further methods can be taken from the single chapters.

2.1 Sediments

Sediments were collected by the German Federal Institute of Hydrology (BfG, Koblenz) during a sampling campaign in April 2012. The three sampling locations comprised one location at the river Rhine and two locations at the river Elbe (*Figure 2.1*). The sediment sampling location at the river Rhine was Ehrenbreitstein (EBR) near Koblenz, Germany, collected from stream kilometer 591.4. This sampling site has been used in former studies, as it represents a moderately contaminated sediment (Feiler et al. 2013, Heise et al. 2008, Höss et al. 2010). The two sampling locations at the river Elbe comprised Prossen/Schmilka (PR), a harbor located close to the Czech border at river kilometer 13.2 and Zollelbe (ZE), a cut-off meander (km 0.1) in the city of Magdeburg, Germany.

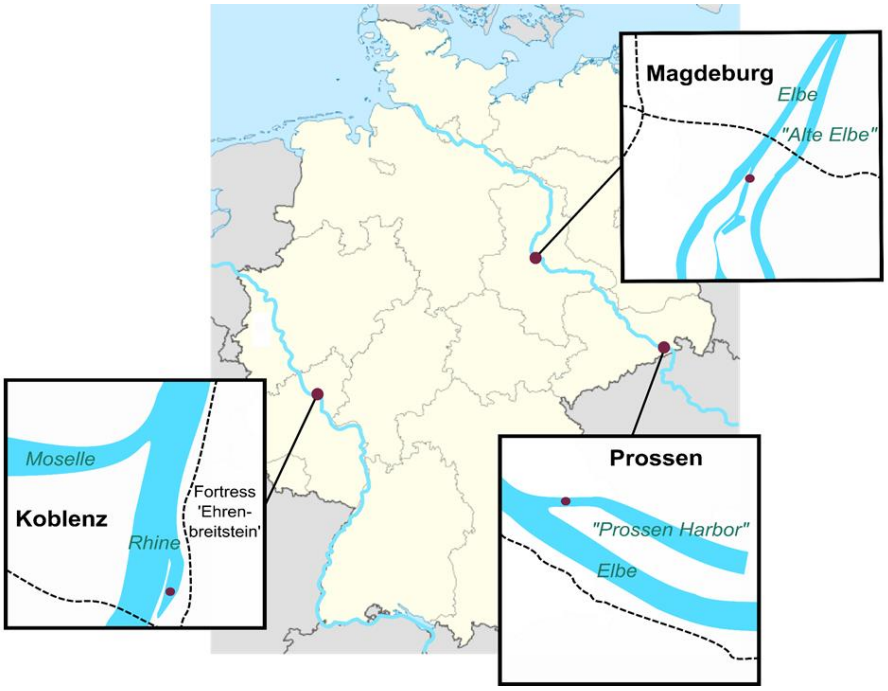


Figure 2.1 Sampling locations of sediments chosen in the DioRAMA project. Sampling locations from the river Elbe encompassed sediment samples from Zollelbe (ZE) (Magdeburg) and Prossen/Schmilka (PR), the sampling locations from the river Rhine was Ehrenbreitstein (abbreviated EBR).

Sediment PR possibly reflects the toxicological burden coming from the Czech part of the Elbe River (Stachel et al. 2011). According to our own measurements, sediment ZE contained the highest concentrations of DLCs among the three sites. Further details of the three sampling locations Ehrenbreitstein (EBR), Prossen (PR), Zollelbe (ZE) as well as the mixture (EBR/ZE) are listed in *Table 2.1*.

Table 2.1 Sampling details, locations, instrumental (b; HRGC/HRMS) and bio-analytical (RTL-W1 EROD; H4IIE Micro EROD) determined concentrations of DL-PCB and PCDD/F fractions as well as and physical chemical characteristics of sediments Ehrenbreitstein, Prossen, Zollelbe and a laboratorial manufactured sediment mixture.

	Ehrenbreitstein Harbor	10/1 mixture Ehrenbreitstein/ Zollelbe	Prossen/ Schmilka	Zollelbe/ Magdeburg
Sample acronyms	EBR	EBR/ZE	PR	ZE
River system	Rhine	-	Elbe	Elbe
Unit	main stream		main stream	meander
km	591.4		13.2	0.1
Longitude [a]	7.60792	-	14.11631	11.65087
Latitude [a]	50.35400	-	50.92776	52.13.256
Sampling date	12.04.2012	-	11.04.2012	10.04.2012
Grab	Van-Veen grab	-	Van-Veen grab	Van-Veen grab
(max. sampling depth)	(15 cm)		(15 cm)	(15 cm)
12 WHO-PCBs [ng/g dm] [b]	4.38	4.18	5.22	9.72
17 WHO-PCDD/Fs [ng/g dm] [b]	1.06	1.22	0.24	3.70
PCB $EROD_{EC25}$BEQ [pg/g dm] of PCB fractions	36.0 ± 14.7	38.4 ± 0.9	50.6 ± 9.1	192.5 ± 26.4
$EROD_{EC25}$BEQ [pg/g dm] of PCDD/F fractions	270.6 ± 77.0	180.1 ± 58.5	488.0 ± 279.8	955.8 ± 551.9
Micro $EROD_{EC25}$TEQ [pg/g dm] of PCB fractions	16.7 ± 7.1	18.6 ± 4.6	18.4 ± 6.6	76.4 ± 18.8
Micro $EROD_{EC25}$TEQ [pg/g dm] of PCDD/F fractions	63.7 ± 8.8	60.5 ± 13.9	73.9 ± 2.9	159.0 ± 32.1
TOC [g/kg]	49.6	n.a.	63.1	64.3
Percentage of sand/silt/clay [%]	4 / 79 / 17	n.a.	19 / 68 / 13	24 / 69 / 7
Loss on ignition [%]	10.6 ± 0.4	n.a.	13.1 ± 0.2	14.1 ± 0.3
Percentage of dry matter [%]	36.1 ± 0.1	n.a.	33.4 ± 0.6	34.0 ± 0.2

a = Coordinates according to the international terrestrial reference system 1998 (ITRS 98)

All sediments were sampled to a depth of 15 cm using Van-Venn grabs, filled in polyethylene buckets and immediately transferred to the Institute of Environmental Research, RWTH Aachen University, where they were thoroughly homogenized and an additional 1:10 mixture (EBR/ZE), consisting of nine parts dry weight (dw) EBR and one part dw ZE, was prepared in the laboratory to present a fine-particulate sediment contaminated with highly persistent DLCs. For simplicity, the mixture of EBR/ZE will be subsequently named sediment as well. All sediments where stored at 4 °C until further use.

2.2 Sample preparation and clean-up

All steps described in this section were conducted at the RWTH Aachen University, Aachen, Germany. Sediments were freeze-dried for 72 h (Alpha 1-4 LD plus, Martin Christ GmbH, Osterode, Germany), sieved to < 2 mm and homogenized by using a mortar and pestle. Sediments were extracted for 48 h according to the methodology described by Umlauf et al. (2004), using Soxhlet extraction (behr Labor Technik, Düsseldorf, Germany) and a solvent mixture of n-hexane/acetone (352/48; v/v). An amount of 20 g sediment (dw) was mixed with an amount of 5 g muffled sodium sulfate (99% anhydrous powder, Sigma Aldrich, Germany). Process control samples, only containing 5 g sodium sulfate, were extracted in the beginning, middle and at the end of the whole extraction cycles. Samples for chemical analysis were spiked with $^{13}C_{12}$-labeled PCDD/F standards (EPA 1613 LCS, Wellington Laboratories, Campro Scientific GmbH, Germany) and a $^{13}C_{12}$-labeled PCB standards (EPA 68C LCS, Wellington Laboratories, Germany).

Clean-up of extracts included the following steps in chronological order: desulfurization with activated copper (24 h), sulfuric acid treatment (24 h), multilayer silica column clean-up and activated carbon column clean-up (*Figure 2.2*). Each step was performed in accordance with U.S. EPA method 8290 (US-EPA 1994) with the following modifications: Multilayer silica columns were equipped in the bottom-to-top order: glass wool, 1 g of activated silica gel, 2 g of basic silica gel (30 g of sodium hydroxide dissolved in 750 ml methanol, combined together with 100 g of silica gel that was then rotary evaporated until dryness for approximately 90 min in a 55 °C water bath), 1 g of activated silica gel, 4 g of acidic silica gel, 1 g of activated silica gel and 1 g of sodium sulfate. Supelclean™ ENVI-Carb™ (Sigma Aldrich) was chosen as carbon adsorbent in the activated carbon columns. Extracts of fractions containing DL-PCBs and PCDD/Fs were aliquoted

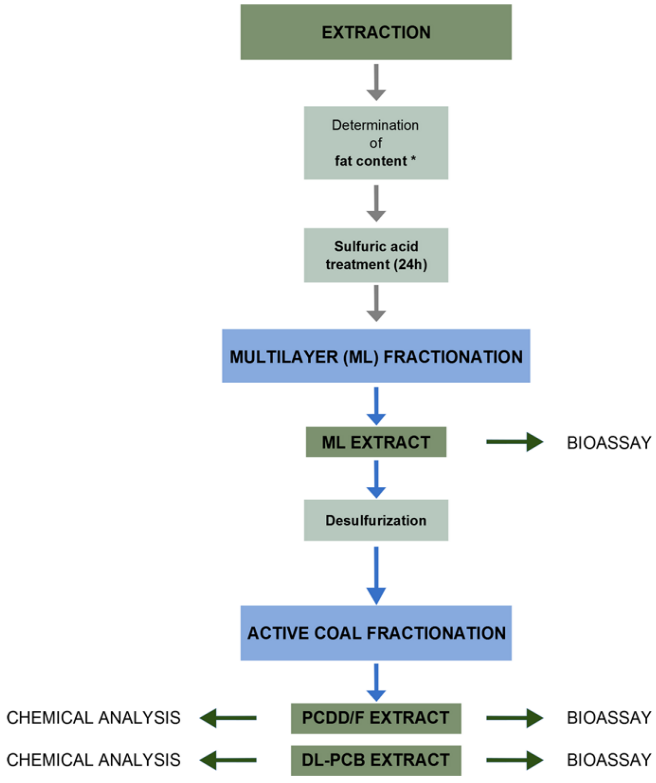


Figure 2.2 Scheme of extraction, clean-up and fractionation of sediment and biota samples as well as subsequent chemical and bio-analytical analysis.

volumetrically and stored in 4 ml vials (amber glass, 45 x 14.7 mm with Butyl/PTFE septum and screw cap, VWR International) until further use in bioassays or chemical analysis (HRGC/HRMS analysis). For bioassay purposes, extracts were reduced close to dryness under a gentle stream of nitrogen and re-dissolved in dimethyl sulfoxide (DMSO ≥ 99.5% p.a., Carl ROTH).

2.3 Bio-analytical analysis

2.3.1 The RTL-W1 EROD (7-Ethoxyresorufin-*O*-deethylase) assay

CYP1A1 induction (EROD activity) was measured using the permanent fish cell line RTL-W1 (*Oncorhynchus mykiss*, rainbow trout liver – Waterloo 1, *Figure 2.3*) (Lee et al. 1993), donated by Dr. Niels C. Bols, University of Waterloo, Canada (Bols et al. 1999).

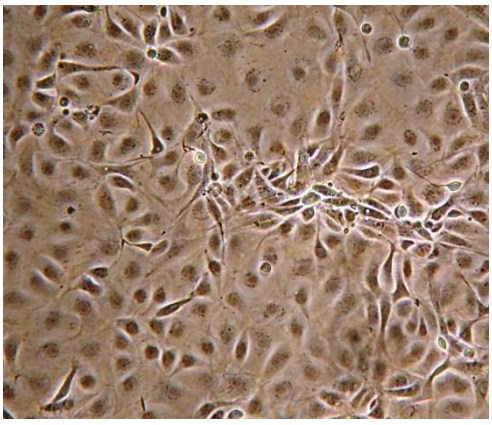


Figure 2.3 Confluent grown cell layer of permanent fish cell line RTL-W1 in a 10-fold optical magnification.

Cells were sub-cultivated weekly in Leibowitz (L15) medium, supplemented with 9% fetal bovine serum (FBS, Biochrom AG, Berlin, Germany) and a 1% penicillin-streptomycin-solution (Sigma Aldrich) and maintained at 20 °C in darkness. Passage numbers 73 to 76 were used to obtain the here presented results. Cell culture and assay were performed according to the methods described by Wölz et al. (2009) with the exception that two samples were tested in triplicate per plate and each well of a plate was adapted to a final concentration of 0.5% DMSO.

Briefly, cells were seeded in 96-well plates (TPP, Trasadingen, Switzerland) and incubated 72 h until confluence. Thereafter, medium was removed and cells were exposed to serial diluted concentrations of extracts and positive control 2,3,7,8-TCDD (3.1 pM to 100.0 pM; Promochem, Wesel, Germany), which were put on plates in triplicates and duplicates, respectively. Following a 72 h incubation time, exposure medium was removed and cells were lysed by freezing them at -80 °C for at least one hour. First, an enzyme-substrate complex consisting of EROD present in the cells and added substrate 7-ethoxyresorufin was allowed to develop within a reaction time of 10 min. Thereafter, addition of reduction equivalent NADPH caused the deethylation of the substrate, which was stopped after further 10 min through the addition of fluorescamin dissolved in acetonitrile.

After 15 min, specific EROD activity was determined by measuring the fluorescence of reaction product resorufin (extinction 544 nm, emission 590 nm) and the absorbance of fluorescamine-amine-complexes (extinction of 360 nm, emission of 460 nm, according to a method of Lorenzen and Kennedy (1997) with a multiwall-plate reader (Tecan infinite M200).

2.3.2 The H4IIE Micro EROD assay

H4IIE cells were provided by the Lower Saxony State Office for Consumer Protection and Food Safety (LaVes), were cultivated using Dulbecco's modified eagle medium with phenol red (DMEM; low glucose, Life Technologies GmbH, Schwerte, Germany) supplemented with 10% FBS (Biochrom AG) and 2% L-glutamine (2 mM, GIBCO® GlutaMAX™, Life Technologies GmbH). Passage number 26 to 50 were used for the assay, which was performed according to a protocol provided by LaVes (LAVES 2013).

Briefly, confluent cells were trypsinized and 50 µl cell suspension (200,000 cells/ml DMEM without phenol red) were seeded in a 96-well plate (96-Well, Growing surface, Sarststaedt) and incubated for 2 h in a humidified 95:5air/CO_2 atmosphere at 37 °C in darkness. Thereafter, cells were exposed to triplicates of serially (1:2) pre-diluted concentrations of extracts and positive control 2,3,7,8-TCDD (0.58 pM to 18.64 pM; Promochem, Wesel, Germany). DMSO concentration was 0.5% in all wells. Following 72 h incubation, medium was removed and 100 µl of 8 µM ethoxyresorufin (ETX) solution containing 10 µM dicumarol were added to all cell-containing wells. After 30 min, the reaction was stopped by adding 75 µl methanol (p.a.; ROTH). Plates were shaken horizontally (300 rpm) for 10 min and resorufin production was fluorometrically determined (Excitation 530 nm, Emission 590 nm) by using a multiwell plate reader (Tecan infinite M200; Tecan Germany GmbH, Crailsheim, Germany).

For the calculation of the specific EROD activity, protein was determined by using a bicinchonicacid (BCA) protein assay kit (Sigma Aldrich). A protein standard curve was prepared in the remaining ETX solution and added to the plate in a 1:2 serial dilution (3.9 – 500.0 µg/ml). Absorption was measured at 550 nm following the addition of 100 µl/well BCA solution and an incubation time of 20 min at 50 °C (Tecan infinite M200).

2.3.3 The H4IIE-luc assay

The H4IIE-luc cell line (*Figure 2.4*) was donated by Prof. Dr. John P. Giesy (University of Saskatchewan, Saskatoon, Canada) and cultivated with Dulbecco's modified eagle medium (DMEM; low glucose, Life Technologies GmbH) supplemented with 10% FBS (Biochrom AG) and 2% L-glutamine (2 mM, GIBCO® GlutaMAX™, Life Technologies GmbH,

Darmstadt, Germany). The assay was performed according to a method developed by Sanderson and Co-workers (1996). Confluent cells of passage numbers 10 to 27 were trypsinized, seeded in 96-well plates (ViewPlate™-96, Perkin Elmer, Rodgau-Jügesheim, Germany) in a density of 80,000 cells/ml DMEM and incubated for 24 h in a humidified 95:5air/CO_2 atmosphere at 37 °C in darkness.

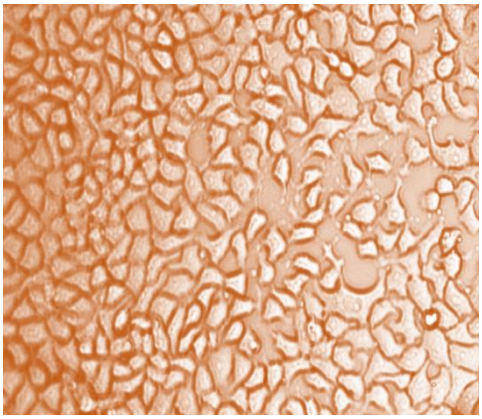


Figure 2.4 Confluent grown cell layer of cell line H4IIE-luc in a 10-fold optical magnification.

Thereafter, medium was removed (liquid handling device, IBS INTEGRA bioscience, Landquart-Davos, Switzerland) and cells were exposed to triplicates of 250 µl serially (1:3) pre-diluted concentrations of extracts and positive control (0.5% DMSO per well) for 72 h. Thereafter, plates were washed twice with phosphor buffered saline (PBS; 10 x; with 1.33 g calcium/L and1.0 g magnesium chloride/L, Sigma). The bottoms of the plates were closed with backing tape (white; for ViewPlate™-96, Unifilter™-96, PerkinElmer) and each well was equipped with 100 µl PBS and 50 µl lucilite (lucilite®, Constant Quanta™, Perkin Elmer). After 10 min, luminescence was determined using a multi-well plate reader (TECAN infinite M200).

2.3.4 Calculation of Biological Equivalent Quotients (BEQs)

Bioassay derived concentration-response curves were plotted *via* GraphPad Prism 5 (GraphPad Prism 5 Software Inc., La Jolla, CA, USA) using a non-linear regression model (dose response stimulation; log agonist vs. response). Effect concentration (EC) levels x for BEQ calculation (*Equation 2.1*) depended from the induction strength of the investigated matrix and are stated in the respective Material and Method sections of the single chapters.

$$BEQ\ [pg/g] = \frac{TCDD\ EC_x\ [pg/ml]}{extract\ EC_x\ [g/ml]} \qquad (2.1)$$

BEQs represent the mean value of three independent replicates.

2.3.5 Calculation of bio-analytical quality criteria

High throughput screening assays require adequate sensitivity, reproducibility and accuracy in order to be used as high throughput assays for the identification of samples of highest concern (2012/278/EU 2012).

Repeatability is defined as the precision under conditions, where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time (ISO/5752 2002). It is calculated as the coefficient of variation of n measurements (n = 3 in the present study).

Reproducibility can be divided into (1) *within-laboratory reproducibility* and (2) *between-laboratory reproducibility*. *Within-laboratory reproducibility* is defined as precision under conditions, where test results are obtained with the same method on identical test items in the same laboratory with different operators over a long period of time. *Between-laboratory reproducibility* is defined as precision under conditions, where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment (ISO/5752 2002). Both (1) and (2) are calculated as the coefficient of variation of two mean values, each consisting of three independent replicates.

The *z-factor* was calculated according to Zhang et al. (1999) (*Equation 2.2*):

$$z\text{-}factor = 1 - \frac{3(\sigma_s + \sigma_c)}{|\mu_s - \mu_c|} \quad (2.2)$$

With standard deviation σ and arithmetic mean μ of the sample *s* (here TCDD maximum induction) and the solvent control *c*. The factor represents the assays' dynamic range and data variation of both sample and reference compound measurement. A z-factor of 1 indicates an *ideal assay*, whereas z-factors in the ranges $1 > z \geq 0.5$ and $0.5 > z > 0$ indicate *excellent* and *double assay*, respectively. *Double assay* signifies that the separation of positive and negative control is small. The classification is based on the general assumption that the better an assay, the higher its' dynamic range and the smaller its' variability (Zhang et al. 1999).

Limit of detection (LOD) (*Equation 2.3*) and *quantification (LOQ)* (*Equation 3.4*) were determined according to MacDougall and Crummett (1980),

$$LOD = \mu_c + 3\sigma_c \quad (2.3)$$

$$LOQ = \mu_c + 10\sigma_c \quad (2.4)$$

With μ and σ being the arithmetic mean and standard deviation, respectively, of a negative control c, which in the case of this study is represented by solvent control DMSO.

2.4 Chemical analysis

2.4.1 HRGC/HRMS analysis

The HRGC/HRMS analysis of extracts, prepared by the RWTH Aachen University, was performed by mas (Münster Analytical Solutions GmbH, Münster, Germany). A capillary gas chromatograph (GC) coupled to a high resolution mass spectrometry was used (Thermo Scientific Trace Ultra GC with Thermos scientific DFS HRMS, Thermo Fisher Scientific, Bremen, Germany). The GC was equipped with a 60 m DB-5MS capillary column of 0.25 mm inner diameter and 0.25 µm film thickness (Agilent J&W, Santa Clara, CA, USA). The capillary column was used for both, PCDD/F and PCB analysis. Separate HRGC/HRMS runs at different instrumental conditions were applied for the analysis of the two compound classes.

Since the pre-cleaned extracts, provided by the RWTH Aachen, partly showed insufficient separation of the PCDD/Fs and the DL-PCBs in the matrix fish (Chapter 5) and insufficient extract purification in case of the sediments, the PCDD/F and PCB fractions of the initial clean-up were recombined and reprocessed for chemical analysis by mas. The HRGC/HRMS analyses also revealed that the hepta-, octa- and partly hexaCDD/Fs were retained within the initial clean-up. Hence, quantified congeners encompassed *2,3,7,8-tetraCDD/F*, *1,2,3,7,8-pentaCDD/F, 2,3,4,7,8-pentaCDF and most 2,3,7,8-hexaCDD/Fs*, as well as the 12 WHO-DL-PCBs, comprising the non-*ortho* PCBs *77, 81, 126* and *169* and the mono-*ortho* PCBs *105, 114, 118, 123, 156, 157, 167* and *189*. However, we could show that the comparably low TEF values of these retained congeners make them negligible for TEQ calculation.

Quantification of PCDD/Fs and PCBs was performed *via* isotope dilution and method of the internal standard, based on the labeled PCDD/F and PCB standards added by the RWTH Aachen prior to the initial clean-up. Overall recoveries of the internal standards through both clean-up procedures were determined by means of labeled recovery standards added prior to the instrumental analysis. Based on blanks and sample dry masses, LOQs for PCDD/Fs and PCBs were below 2 and 1 pg WHO_{2005}TEQ/g for fish (Chapter 4) and sediment samples, respectively.

Recoveries of the $^{13}C_{12}$-labelled tetra- through hexaCDD/F quantification standards were in the range of 3 – 128% and 15 – 106% for fish (Chapter 5) and sediment samples, respectively. Recoveries of the DL-PCBs ranged from 27 – 135% and 4 – 120% for fish (Chapter 5) and sediment samples, respectively. Recoveries of PCDD/Fs and DL-PCBs in the three process controls ranged from 11 - 102% and from 52 - 108%, respectively.

2.4.2 Calculation of Toxicity Equivalent Quotients (TEQs)

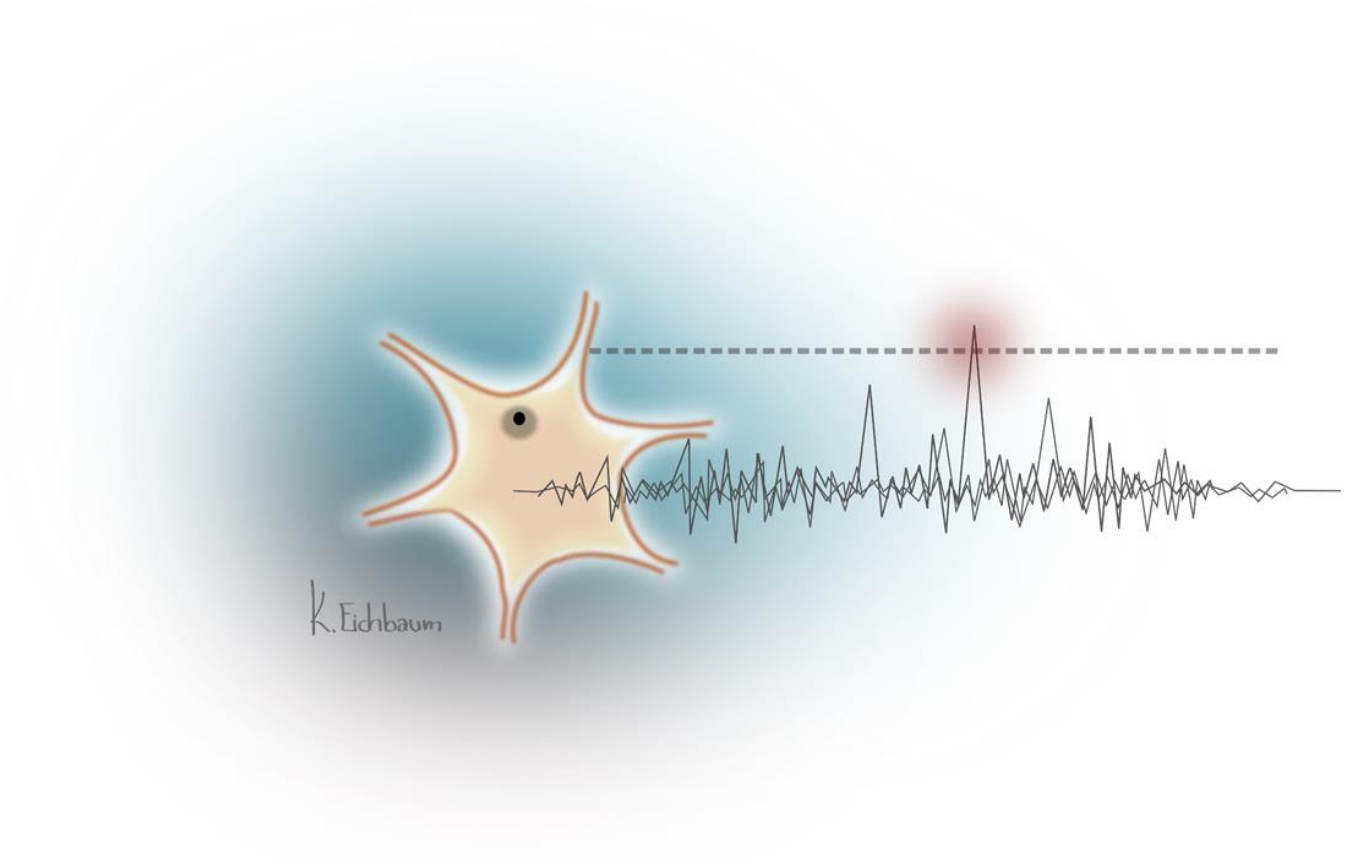
When the results of various *in vitro* assays were compared with instrumental derived TEQs, these TEQs were calculated on a toxicity equivalent factor (TEF) basis using the mammalian WHO-TEFs from 2005 (Van den Berg et al. 2006). The cconcentration of congener *i*, measured *via* HRCG/HRMS, was multiplied with its respective TEF (*Equation. 2.5*). This was repeated with all chemically investigated congeners and finally, the sum of all congeners and their TEFs was built. TEQ calculation did not include congeners below the analytical detection limit.

$$TEQ\ [pg/g] = \sum conc_i * TEF_i\ (REP_{i(x)}) \quad (2.5)$$

When the results of single *in vitro* assays were compared with instrumental derived TEQs, these TEQs were calculated on a relative potency (REP) basis. The calculation of REP-based TEQs is according to that of TEF-based TEQs, with the exception that all congeners' concentrations *i* are multiplied with a respective, assay- and cell line-specific literature REP value, which ideally has been determined under the same assay conditions *x* (*Equation 2.5*) as used in the present study. For EROD- and RTL-W1-specific REP-based TEQs, REPs were taken from Clemons et al. (1997) and represented 72 h-EC_{50} values, whereas REPs deduced from 72 h-EC_{20} values were taken for H4IIE (Behnisch et al. 2002) and H4IIE-luc (Lee et al. 2013) -specific REP-based TEQs, respectively.

Chapter 3

Application potentials and limits of detection of *in vitro* bioassays for the screening of dioxin-like activity



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

Eichbaum, K., Brinkmann, M., Buchinger, S., Reifferscheid, G., Hecker, M., Giesy, J.P., Engwall, M., van Bavel, B., Hollert, H. (2014) *In vitro* bioassays for detecting dioxin-like activity - Application potentials and limits of detection, a review. *Science of the Total Environment* 487: 37-48.

3.1 Abstract

Use of *in vitro* assays as screening tool to characterize contamination of a variety of environmental matrices has become an increasingly popular and powerful toolbox in the field of environmental toxicology.

While bioassays cannot entirely substitute analytical methods such as gas chromatography-mass spectrometry (GC-MS), the increasing improvement of cell lines and standardization of bioassay procedures enhances their utility as bio-analytical pre-screening tests prior to more targeted chemical analytical investigations. Dioxin receptor-based assays provide a holistic characterization of exposure to dioxin-like compounds (DLCs) by integrating their overall toxic potential, including potentials of unknown DLCs not detectable via e.g. GC-MS. Hence, they provide important additional information with respect to environmental risk assessment of DLCs.

This review summarizes different *in vitro* bioassay applications for detection of DLCs and considers the comparability of bioassay and chemical analytically derived toxicity equivalents (TEQs) of different approaches and various matrices. These range from complex samples such as sediments through single reference to compound mixtures. A summary of bioassay derived detection limits (LOD) showed a number of current bioassays to be equally sensitive as chemical methodologies, but moreover revealed that most of the bio-analytical studies conducted to date did not report their LODs, which represents a limitation with regard to low potency samples.

Keywords: TEQ-approach • LOD • dioxin • effect directed analysis • exposure characterization

3.2 Fields of application of *in vitro* bioassays

A multitude of national and international studies have been conducted that focused on the determination of dioxin-like activities using *in vitro* bioassays with a wide variety of sample types including two major categories: (1) Complex samples and (2) individual compounds (pure reference compounds) and mixtures thereof. The following sections introduce sample types of these two categories, starting with complex environmental samples.

3.2.1 Measuring dioxin-like activity in complex samples

3.2.1.1 Limnic sediment samples

Since sediments maybe an important sink and source of DLCs, the evaluation of polluted sediments is an integral part of sediment risk assessment and a popular field of environmental science. A host of studies was conducted that investigated the dioxin-like activity of sediments of various rivers, tributaries or small streams (Behnisch et al. 2010, Chen et al. 2010, David et al. 2010, Heimann et al. 2011, Hilscherova et al. 2001, Hilscherova et al. 2003, Hollert et al. 2002, Huuskonen et al. 2000, Kannan et al. 2008, Keiter et al. 2008, Kinani et al. 2010, Koh et al. 2004, Murk et al. 1996, Song et al. 2006, Suares Rocha et al. 2010, Windal et al. 2005, Wölz et al. 2008, Wölz et al. 2010a, Wölz et al. 2010b). Others focused on sediments from lakes (Engwall et al. 1998, Hofmaier et al. 1999, Khim et al. 1999b, Koh et al. 2005) and coastal areas (Anderson et al. 1999b, Anderson et al. 1999a, Chen et al. 2010, David et al. 2010, Gale et al. 2000, Hurst et al. 2004, Kannan et al. 2008, Khim et al. 1999a, Koh et al. 2002, Koh et al. 2004, Sanctorum et al. 2007, Song et al. 2006, Thain et al. 2006, Wölz et al. 2009) or screened the potential of DLCs in SPM (Engwall et al. 1996, Engwall et al. 1997, Koh et al. 2004, Veilens et al. 1992). Soil and sediment organic matter constituents have also been investigated (Bittner et al. 2006, Larsson et al. 2013).

Most of these studies related their biological toxicity equivalents (BEQs) to chemical analytically determined toxicity equivalents (TEQs) concentrations of DLCs in the same matrix. BEQs and TEQs of acid-treated extract fractions thereby most often were in good accordance. Different approaches have been developed to progressively enhance the comparability of biologically measured potentials and instrumentally determined quantities of DLCs. In order to distinguish between rapidly metabolized PAHs and more persistent compounds (e.g. dioxins and PCBs) that remain highly active after elongated exposure times, two studies conducted the EROD assay with PLHC-1 cells using two different exposure times (4 and 24 h, respectively) (David et al. 2010, Kinani et al. 2010). Both used benzo[a]pyrene

(BaP) as standard in the 4-hours-exposure and 2,3,7,8-TCDD as standard in the 24-hours-exposure experiments. TEQs as well as BaP equivalents (BEQs) (both based on EC_{20} values, which were proven to show smaller variability compared to EC_{50} values) were in good accordance with chemical findings in both studies (TEQs $r^2 = 0.84$ and 0.97, BEQs $r^2 = 0.98$ and 0.99, respectively). Other scientists, who used a P_{450} reporter gene system (RGS assay) with the cell line 101L first correlated their bioassay results with total PAHs ($r^2 = 0.47$), but finally found a much better correlation with BEQs ($r^2 = 0.63$) (Andersson et al. 1999). Other studies documented BEQs (between 3.62 and 7.92 ng TEQ/g dm sediment), which were not correlated with their respective TEQs, which only accounted for approximately 5% of bioassay-derived potentials (Heimann et al. 2011). Here the authors concluded that BEQs were dominated by PAHs and unidentified pollutants. These findings were supported by many others (Gale et al. 2000, Hilscherova et al. 2001, Hurst et al. 2004, Keiter et al. 2008, Sanctorum et al. 2007, Song et al. 2006, Suares Rocha et al. 2010), which supports the approach of BEQs with BaP as positive control as applied by David et al. (2010) and Kinani et al. (2010). But care has to be taken regarding the use of BaP as a control because unlike 2,3,7,8-TCDD, the potencies of BaP and other PAHs are sensitive to culture conditions, which indicates that the BEQ approach appears to be more variable compared to the TEQ approach (Bols et al. 1999).

The fact that acid labile compounds may affect the comparability between bioassay and chemical analytical results indicates additional clean-up to be necessary when investigating complex environmental samples. A need for such clean-up procedures was proven by a study that correlated TEQs (EC_{20}) with H4IIE-luc BEQs of both crude and cleaned-up extracts (Hilscherova et al. 2003). While the correlation between TEQs and BEQs of crude extracts was moderate ($r^2 = 0.72$), a good correlation was observed among TEQs of the cleaned-up extract ($r^2 = 0.94$). Nevertheless, care has to be taken during the clean-up of extracts. A study by Villeneuve et al. (2002) indicated that following a 1 h treatment with concentrated H_2SO_4 acid-breakdown products of PAHs and other compounds were formed, which produced dioxin-like responses *in vitro*. This indicates that a longer acid treatment (the authors suggested a duration of 10 h or longer) followed by a water rinse should serve as an effective method to completely eliminate dioxin-like responses caused by the acid labile fraction (Villeneuve et al. 2002).

When focusing on sediments as DLC containing matrixes, many of the above mentioned studies have shown the ability of various *in vitro* bioassays to detect contamination sources. For instance, Hilscherova and co-workers (2003) could identify a 10-100 fold greater concentration of H4IIE-luc derived BEQs downstream the Tittabawassee River than those determined upstream. The same trend (5 to 10-fold) was observed for soils of the respective associated river

banks. Both results were confirmed by instrumental analysis, which revealed that PCDD/Fs were the critical contaminants causing the dioxin-like activity observed *via* the H4IIE-luc. The same bioassay indicated contamination sources of sediments and floodplain soils of the Saginaw River, Michigan, USA, which exceeded the screening concentration of 50 pg TEQ/g dm soil that was suggested by the Agency for Toxic Substances and Disease Registry ATSDR (Kannan et al. 2008). Other studies using mass balance analysis also reported successful identification of causative substances using bio analytical techniques (H4IIE-luc assay) by testing different extract fractions and comparing those with chemical analytical determined results (Koh et al. 2004, Otte et al. 2013). Thereby, PCDD/Fs were found to be responsible for the majority of the dioxin-like activity measured in sediment extracts of the Hyeongsan River, Korea, while PCBs and PAHs contributed a relatively small proportion to the overall activity (Koh et al. 2004). In the contrast, the 16 EPA PAHs explained between 47 and 118% of the H4IIE-luc assay derived BEQs in sediment extracts of the Elbe River, Germany (Otte et al. 2013). By comparing the H4IIE-luc assay with 3 other assays (EROD with H4IIE wild type (wt) and PHLC-1 wt and CALUX with RLT2.0), was the least variable and most sensitive biotest and lead to similar conclusions as those that would have been made based on extensive instrumental analyses (Hilscherova et al. 2001).

3.2.1.2 Coastal sediment samples

Extracts of coastal sediments, including samples from German (Wölz et al. 2009), Scottish (Thain et al. 2006), Belgian (Sanctorum et al. 2007), French (David et al. 2010) and UK coastal areas (Hurst et al. 2004), as well as samples from various bays alongside the USA (Anderson et al. 1999a, Gale et al. 2000, Kannan et al. 2008), Korea (Khim et al. 1999a, Koh et al. 2004, Koh et al. 2005) and Japan (Kannan et al. 2008), revealed significant dioxin-like activity. For sediment extracts from the North Sea general low contamination levels were observed (around 0.1 pg CALUX-TEQ/g sediment) while at the mouth of two rivers (the Yser and the Scheldt) 100-fold greater concentrations were measured (10 - 42 pg CALUX-TEQ/g dw sediment) (Sanctorum et al. 2007). In a study of Baltic Sea sediment cores a combinatory approach applying bioassay (EROD with RTL-W1) and chemical analytical methods indicated a significant hazard potential at site. The authors hypothesized that benthic organisms or animals living in close contact these sediments might be at risk (Wölz et al. 2009). A different study that compared results obtained by screening both cleaned-up and whole extracts of sediments from the East Shetland basin using the DR-CALUX® determined dioxin-like potentials in some areas that were potentially harmful to organisms (Thain et al. 2006). Those areas, according to the authors, require targeted chemical analyses of a range of known potential candidate

compounds to identify the causative agents. According to results obtained by the DR-CALUX® in combination with a clean-up procedure the vast majority of the dioxin-like activity in the East Shetland sediments was attributable to labile compounds such as PAHs (Thain et al. 2006). Equal results were obtained by Hurst et al. (2004) for sediments sampled along the coastal line of the United Kingdom. BEQs ranged between 1.0 and 106.0 pg CALUX-TEQ/g dm sediment and the majority of sediments contained levels of DLCs above concentrations that are considered to possess a low risk to aquatic organisms. Like for the previously mentioned studies, stream and inland sampling locations from Korean coastal areas were found to contain greater concentrations of DLCs than offshore sites, as was identified by using the H4IIE-luc assay (Koh et al. 2005). When BEQs were compared to chemical instrumental findings it was found that BEQs were consistently greater than TEQs. On average, the known concentrations of DLCs present in the extracts accounted for only 30% of the total bioassay responses observed.

Some studies investigated extracts of typical sediment constituents to evaluate the potential interaction of these with a number of bioassays. For example, Bittner et al. (2006) used the EROD and CALUX assays with the wild type and genetically engineered H4IIE cell line to investigate the dioxin-like potential of humic acids (HA). They reported that different treatments of HA (organic extraction, alkali solution) resulted in different dioxin-like potentials in both assays, which was unexpected due to the missing dioxin-like structure of HA. The calculated REP_{HA} was $6x10^{-8}$ and, thus, equates an environmental relevant concentration. These findings again illustrate the presence of numerous unknown AhR ligands in environmental samples.

In summary, the above mentioned examples show that *in vitro* bioassay methodologies constitute an important tool in support of environmental risk assessments. Moreover, most of these results suggests that instrumental chemical analysis alone (based on the concentrations of identified target analytes) cannot completely estimate the total dioxin-like potency of DLCs. However, care needs to be taken when using bioassays to assess dioxin-like activities of sediment extracts due to potential interactions of non-dioxin-like components with these tests.

3.2.1.3 Soil samples

One particular topic of interest in context with the assessment of DLCs in soils is the deposition of such contaminants on floodplains during flood events. Such studies are often closely related to those focusing on river sediments. For instance, floodplain soils from the river Rhine, Germany (Wölz et al. 2011), Saginaw and Shiawassee Rivers and Saginaw Bay, Michigan (Kannan et al. 2008) and those collected along the Tittabawassee River, Michigan,

USA (Hilscherova et al. 2003) were investigated as part of environmental risk assessments of DLCs in these watersheds. All these studies revealed SPM deposited during flood events to cause contamination of inundated sites. Related studies are those focusing on agricultural soils that are in proximity to electronic waste recycling sites, such as the Taizhou area, China (Shen et al. 2007, Shen et al. 2009). In case of the study of Shen and coworkers (2007), TEQs and BEQs correlated well (r^2 = 0.96) and PCBs were proven to cause 98% of the dioxin-like potential in the Taizhou area. Anderson on the other hand, who investigated clayey soils contaminated with PAHs that were collected from an old gasworks plant in Sweden with respect to a large-scale bioremediation (Andersson et al. 2009). By using the CALUX assay, they could prove an increasing dioxin-like potential in bioavailable fractions after 274 days of soil remediation (compared to day 0), which according to the authors was most likely was caused by a chemically detected release of previously sorbed PAHs.

3.2.1.4 Sewage sludge samples

Since tons of sewage sludge are produced worldwide every year and the capacity for incineration does not fill the demands, sludge has been used for landfilling or as fertilizer on farmland. In doing so, its release may cause a threat to the environment because a multitude of environmental contaminants can remain in the sludge after their removal from waste water (Engwall et al. 1999). Therefore, the ecotoxicological investigation of sewage sludge is of great relevance in studies concerned with dioxin-like activities. For instance, Hofmaier et al. (1999) analyzed sewage sludge extracts originating from two waste water treatment plants (WTP) in Selbitztal (Germany) *via* the Micro EROD, whereas Engwall and co-workers (1999) used the chicken embryo hepatocyte (CEH) bioassay to determine the dioxin-like potential of sewage sludge from different WTPs in Sweden. Both studies concluded the combination of bioassays and chemical analysis to be a well suited tool for the screening of organic residual materials.

The DR-CALUX and the EROD were used to investigate pharmaceutical-containing sewage sludge from Sweden. The authors could prove that an anaerobic treatment caused an increase in the levels of acid resistant AhR agonists, while an aerobic treatment did not affect the levels of these agonists (Gustavsson et al. 2004, Gustavsson et al. 2007). Additionally, the uptake of DLCs in carrots, oilseed rape seeds, zucchinis and cucumbers grown in soil amended with sewage sludge from those Swedish WTPs was estimated. A sewage sludge-amendment in moderate application rates (below 10 tons dm/ha) did not yield notably high carrot DLC concentrations, but the authors pointed out that a risk estimation is complicated due to a missing correlation between application rates and sludge-borne DLCs and their resulting concentrations in carrots (Engwall and Hjelm 2000).

3.2.1.5 Water samples

Previous studies have investigated dioxin-like potentials in different water sample types, including ground- (Schirmer et al. 2004a), waste- (Kobayashi et al. 2003, Ma et al. 2005, Shen et al. 2001, Zacharewski et al. 1995), pore- (Koh et al. 2002, Koh et al. 2004, Murk et al. 1996), stream- (Shen et al. 2001, Villeneuve et al. 1997) and surface water (Rastall et al. 2004). Ground water can be used to analyze the mobility of pollutants present in soils of e.g. industrial areas and their possible transition into the ground water body. Ground water samples in the area of Zeitz, a large contaminated site in Germany where oil and lignite were refined to produce fuel, lubricants and benzene all caused EROD induction in RTL-W1 cells, which demonstrated that *in vitro* bioassays can be used as an early warning tool to initiate a more detailed cause-analysis and to guide subsequent chemical identification in water samples (Schirmer et al. 2004b).

A study that investigated industrial and municipal wastewater-containing lake water samples (Taihu Lake, China) reported CALUX-TEQ values between 134 and 232 pg/l, which exceeded the US EPA national primary drinking water standards maximum contaminant level of dioxin (30 pg/l) by a factor of 4.5 – 7.7 (Shen et al. 2001). Some studies determined the AhR-activating potential of bioavailable DLCs sampled using semipermeable membrane devices (SPMDs) serving as passive samplers for lipophilic chemicals such as DLCs in river water (Villeneuve et al. 1997). It could be demonstrated that this approach was well suited to estimate the risk posed by DLCs to fish. Sediments and pore water from several locations in the Netherlands were screened for their ability to induce AhR-mediated gene expression in H4IIE cells using the EROD and CALUX assays. The luciferase inducing potential (CALUX) of organic extracts from 450 mg sediment aliquots or 250 µl pore water aliquots corresponded well with the instrumentally determined degree of pollution of the sediment with DLCs.

The authors furthermore pointed out that the usage of pore water as a matrix in DLC studies has the advantage to be more rapid due to the need for fewer clean-up steps (Murk et al. 1996).

3.2.1.6 Samples of Human blood, food and feed

Due to their lipophilic nature and low degradability, many DLCs accumulate in animal and human tissues up to concentrations, which can cause adverse effects. Blood samples therefore widely have been evaluated using bioassays. Specifically, blood serum levels of AhR ligands in different human populations (Long et al. 2006, Olsman et al. 2007b, Pauwels et al. 2000, Schecter et al. 1999, Van Wouwe et al. 2004). Because the main exposure route to dioxins and related compounds for humans is through the diet (Fent 2007), the characterization of DLCs in food and feed represents an important tool for human health risks assessment of these chemicals. Many of the studies conducted to date were concerned with bioassay investigation

of fish, e.g. retail fish from local markets in China and supermarkets in Japan, as well as fish oil from North Sea herring and fish oil used for feed ingredients from several manufacturers in Japan (Hasegawa et al. 2007, Nording et al. 2005, Tsutsumi et al. 2003, Wei et al. 2010).

3.2.1.7 Samples of air emissions and combustion products

Air emission samples originate from various sources and recently belong to the popular fields of research of DLCs (Arrieta et al. 2003, Clark et al. 1999, Franzén et al. 1988, Gierthy and Crane 1985, Hamers et al. 2000, Hofmaier et al. 1999, Kasai et al. 2006, Klein et al. 2006, Kobayashi et al. 2003, Li et al. 1999, Mason 1994, Till et al. 1997). Klein and Coworkers (2006) investigated gas- and particulate phases from ambient air, sampled in an urban and rural location in the Greater Toronto Area, Canada *via* the AhR assay with H1L6.1c1 cells. They found a distinct correlation between the AhR-binding potency and the concentration of PAHs, as ascertained by other studies of APM such as traffic exhaust (Hamers et al. 2000), vehicle exhaust and urban air (Arrieta et al. 2003, Franzén et al. 1988, Hamers et al. 2000, Klein et al. 2006, Mason 1994). Moreover, according to the authors, it was the first study in which APM was sampled between seasons over two years (Klein et al. 2006).

A further interesting attempt of this topic was the investigation of AhR ligands in cigarette smoke. The results indicated that there were more AhR ligands in the smoke of one cigarette (10 mg tar) than expected. Levels of one cigarette exceeded the tolerable daily intake (TDI) of dioxin (1 - 4 pg/kg/d) suggested by the WHO up to 656 times (Kasai et al. 2006).

3.2.2 Measuring dioxin-like activity of individual compounds and mixtures

The use of assay-specific REP values can enhance the comparability between chemical and bio-analytical results when assessing DLCs in environmental or human samples. A series of studies that investigated the correlation among different bioassays moreover demonstrated that they can be in good accordance when screening single reference compounds. Hence, the continuing determination of dioxin-like potencies of single compounds with various different cell lines is essential.

One compound class that already has been analyzed in this context are PAHs (Behnisch et al. 2003, Bols et al. 1999, Kennedy et al. 1996, Machala et al. 2001, Villeneuve et al. 2002). For example, Machala et al. (2001) investigated 30 individual PAHs using the CALUX assay with two different exposure times (6 and 24 h) in order to characterize their metabolism *in vitro*. The authors measured the largest DLC potential after 6 hours of exposure time.

The substance class of PCBs has also been explored regarding their dioxin-like potential using *in vitro* bioassays (Aarts et al. 1995, Behnisch et al. 2003, Brown et al. 2001, Kennedy et al. 1996, Sanderson et al. 1996, Schneider et al. 1995, Tillitt et al. 1991, Zeiger et al. 2001). In the process, both *DL-PCBs* (mouse hepatoma cell line H1L1 (Brown et al. 2001), human hepatoblastoma cell line HepG2 (Zeiger et al. 2001), rat hepatoma cell line H4IIE (Tillitt et al. 1991), primary cell cultures of CEH (Kennedy et al. 1996)) and *non-dioxin like PCBs* (NDL-PCBs) (CEH (Kennedy et al. 1996)) were investigated, as well as several NDL-PCBs in combination with DL-PCBs in order to discover possible interactions among the two categories. In doing so, some studies could prove antagonistic effects of certain NDL-PCBs on their dioxin-like counterparts (Aarts et al. 1995, Sanderson et al. 1996).

Various congeners of PCDD/Fs (Brown et al. 2001, Garrison et al. 1996, Murk et al. 1996, Tillitt et al. 1991, Villeneuve et al. 2000) as well as brominated and fluorinated analogs (Behnisch et al. 2003, Brown et al. 2001, Olsman et al. 2007a, Samara et al. 2009) or nitro- (Schneider et al. 1995), methyl- and alkyl-substituted (Behnisch et al. 2003) analogs were investigated. Polychlorinated naphthalenes (PCNs) were frequently analyzed (Behnisch et al. 2003, Blankenship et al. 2000, Hanberg et al. 1991, Schneider et al. 1995, Villeneuve et al. 2000) and found to be equally active as enzyme inducers as certain DL-PCBs (Hanberg et al. 1991). Furthermore, commonly used flame retardants, the polybrominated diphenyl ethers (PBDEs) were proven to act dioxin-like (Behnisch et al. 2003, Chen et al. 2001, Hanberg et al. 1991, Schneider et al. 1995). In one study, BEQs of the chicken embryo hepatocyte (CEH) EROD of single tested PBDEs correlated well with those obtained by using the Micro EROD assay ($r^2 = 0.89$) (Chen et al. 2010).

Tetrachlorostilbenes, polychlorinated azobenzenes, azoxybenzenes, trans-stilbenes (Schneider et al. 1995), ß-naphthoflavone (Lee et al. 1993), NSO-heterocyclic PAHs (Hinger et al. 2011), DDT metabolites (Wetterauer et al. 2012) as well as pentabromophenols (PBPs) (Behnisch et al. 2003, Schneider et al. 1995) were investigated sporadically.

3.3 Limit of detection (LOD)

3.3.1 LOD and limit of quantification (LOQ) in chemical analysis

In terms of instrumental chemical analysis, the LOD is defined as the lowest concentration of an analyte in a sample that the analytical process can reliably detect (MacDougall and Crummett 1980), meaning that the signal of the analyte is statistically different from a blank (Bradlaw et al. 1980, Keith et al. 1983). Various methods have been described to calculate the LOD (Currie 1968, Mandel and Stiehler 1957). According to the IUPAC gold book, the LOD of an instrumental analysis is calculated by the mean of the blank measures plus the standard deviation of the blank measures multiplied by a numerical factor chosen according to the confidence level desired (IUPAC 2006) (*Figure 3.1*). The majority of studies set this numerical factor to a value of three standard deviations, but in general this value depends on the definition used. In the case that a single sample is analyzed for which there is no blank data, the LOD of chemical methods is based on the peak to peak noise measured on the base line close to the actual or expected analyte peak (MacDougall and Crummett 1980).

The limit of quantification (LOQ) is frequently calculated by the mean of the blank plus ten times the standard deviation (Keith et al. 1983) or, in rare cases, only six times the standard deviation (Bradlaw et al. 1980). It is defined as the lowest concentration of an analyte that can be determined with acceptable precision and accuracy under the stated operational conditions of the used method (e.g. bioassay or high resolution (HRGC/HRMS)) (Whyte et al. 2004). Only signals above the LOQ can be quantified (*Figure 3.1*). Signals > LOD but < LOQ are significantly detectable but not quantifiable. Signals less than the LOD hence should be reported as not detected (ND) with the limit of detection given in parentheses. Signals in the region of detection should be measured and reported as numbers with the limit of detection in parentheses (MacDougall and Crummett 1980).

3.3.2 LOD and LOQ in bio-analytical analysis

Since most *in vitro* bioassay studies compared their results with those obtained by instrumental chemical analysis, the bioassays LOD should also be stated.

In terms of *in vitro* bioassays, the definition of the LOD is very similar to that of chemical analytical methods with two exceptions: (1) the signal of an analyte equates the bioassay specific endpoint, which may be measured as fluorescence (e.g. in the EROD or CAFLUX assays) or luminescence (e.g. in the DR-CALUX or CALUX assays); and (2) the blank to which the LOD definition refers to equates the negative control or the solvent control (e.g. dimethyl

sulfoxide (DMSO), isooctane and/or isopropanol) of the bioassay (*Figure 3.1*). For instance, Murk and coworkers (1996) who investigated sediments and pore water from the Netherlands *via* the CALUX assay set their blank value at the DMSO response. As a consequence, the LOD of most studies is expressed as the effect of the lowest concentration of the standard (the standard is typically 2,3,7,8-TCDD) that can be statistically separated from the effect of the control.

The LOQ of a bioassay can be defined according to chemical analysis, with two exceptions: (1) the blank again equates a negative or a solvent control; and (2) the signal level of a sample can only be related to the same signal level caused by the positive control e.g. 2,3,7,8-TCDD (*Figure 3.1*). The respective concentration of 2,3,7,8-TCDD, which is necessary to cause this certain level is used to describe the potency of the sample. Thus, bioassay results can just be expressed as equivalents instead of actual concentrations.

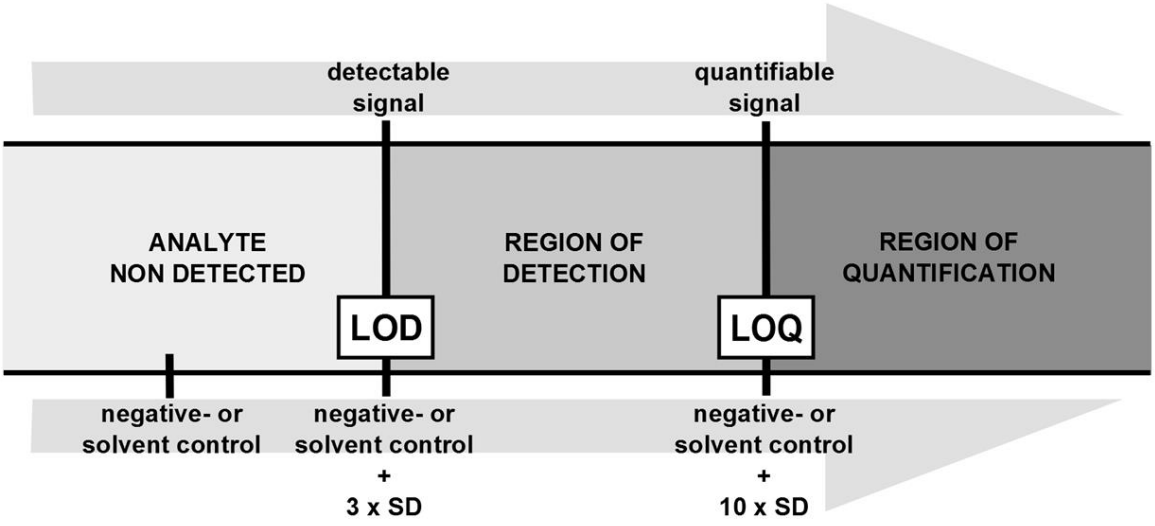


Figure 3.1 Schematic diagram of limit of detection (LOD) and limit of quantification (LOQ) as well as their determination, regions of detectable, quantifiable and non-detectable analyte signals, SD = standard deviation, diagram modified according to (MacDougall and Crummett 1980).

According to our literature review, most of the *in vitro* bioassay studies used to quantify DLCs did not report their LOD or LOQ. However, this additional information is of critical importance as it enables scientists to decide whether the presented assay and the respective cell line reach the sensitivity goals required for e.g. the screening of samples with very low levels of DLCs (Whyte et al. 2000, Whyte et al. 2004).

3.3.3 Chemically and bioassay derived LODs

Conventional GC-MS analysis has been able to achieve detection limits for 2,3,7,8-TCDD in the range of 1 pg/g (Rappe 1984), or more recently, LODs in the parts per quadrillion range (Fernandes et al. 2004, Focant et al. 2002, Patterson et al. 1987, US-EPA 1994). This might in parts be similar to LODs achieved by bio detection methods including the Micro EROD, the DR-CALUX and the CEH assay (Behnisch et al. 2001b). For instance, the limits of quantification derived from CALUX and HRGC/HRMS in a study focusing on animal feed were approximately 0.50 pg CALUX-TEQ/g lipid and 0.25 pg TEQ/g lipid, respectively (as reviewed by Behnisch et al. 2001b). This is supported by Schoeters and colleagues (2004), who performed the CALUX assay and reported LOD and LOQ values similar to those of chemical analysis

The actual LOD of a compound (as measured *via* chemical analysis) differs from the so-called method LOD, which in the case of bio-analytical methods is, among others, influenced by matrix effects in a complex mixture (Bhavsar et al. 2007). These matrix effects can be caused by chemicals, which influence each other or chemicals such as heavy metals, which are capable of e.g. inhibit the EROD enzyme (Oliveira et al. 2004, Viarengo et al. 1997) and thereby lessening signal strength. For this reason, bioassay detected LOQs for the positive control 2,3,7,8-TCDD and the extract, which technically should be in agreement, most commonly differ from one another (Whyte et al. 2004). While signals that are near or less than the LOD constitute a "yes/no-decision" when using bioassays (Armbruster and Pry 2008), the contribution of non-detected compounds in chemical analysis is often estimated by commonly presenting half their LOD (Windal et al. 1998).

3.3.4 LOD influencing factors and LOD enhancement

The LOD depends on several factors, including the type of cell-line, the positive control, the solvent carrier, the extract preparation and measured endpoint, the exposure time as well as multiple laboratory test conditions such as temperature.

An enhancement of the LOD of bioassays can be achieved by altering these factors in a certain manner and can significantly increase their utility as prioritization tools prior to more detailed instrumental analysis (Zhao et al. 2010).

As mentioned above, the LOD of a bioassay is species- and cell-line specific (Giesy et al. 2002, Hilscherova et al. 2000, Keiter et al. 2008). Recombinant cell lines stably transfected with vectors containing easily measurable reporter genes (i.e., luciferase or EGFP) are

frequently reported to be more sensitive than bioassays performed with the respective wt cell line (Brouwer et al. 1995, Garrison et al. 1996, Sanderson et al. 1996). Additionally, the type of endpoint used determines the sensitivity of a test, and thus, the LOD. Some bioassays are based on measurement of light emission to visualize the response. For those signals the most sensitive detectors exist. For this reason and due to the additional fact that visualization systems such as the luciferase enzyme have a high turnover rate (meaning that a few enzyme molecules are sufficient to produce a detectable signal) those assays are characterized by very low detection limits (Sanderson et al. 1996, Willett et al. 1997). For instance, Sanderson et al. (1996) described a three-fold improvement in sensitivity (i.e. the minimal detection limit) of H4IIE-luc cells relative to the wt cells with detection limits of 0.2 and 0.6 fmol 2,3,7,8-TCDD/ well, respectively (*Table 3.1*). This was confirmed by results of Murk and Co-workers (1996), who reported the CALUX assay with H4IIE-luc cells to be slightly more sensitive than the Micro EROD assay with H4IIE wt cells. According to the authors this was mainly due to the missing substrate inhibition within the CALUX assay. Furthermore, the LOD of the Micro EROD described by Sanderson (1996) demonstrated a 50-fold enhancement of the LOD compared to those reported by other studies (Kennedy et al. 1993, Tillitt et al. 1991). As reviewed by Brouwer and co-workers (1995) the sensitivity of such genetically engineered cell bioassays can be enhanced by increasing the number of dioxin response elements regulating the expression of the desired reporter gene and by increasing the number of copies of the expression plasmid in the cell by amplification. Additionally, the concentration of AhR in those cells can be elevated by introducing constitutively active AhR cDNA expression vectors (Brouwer et al. 1995). Concerning the solvent carrier, it could be proven that the use of isooctane instead of DMSO, which may be cytotoxic above concentrations of 1%, enhanced the sensitivity of the bioassay conducted with both, crude and cleaned-up extracts (Bradlaw and Casterline 1979).

LODs are mainly linked to the available sample size (e.g. chemical analysis typically requires a sample amount of 5 - 10 g for solids and liquids (Harrison and Eduljee 1999)) with greater volumes being able to be concentrated more during extraction process, thus resulting in a decreased LOD. Applied sample extract clean-up procedures increased the sensitivity in both chemical and biological analysis by eliminating interfering substances like acid labile PAHs (Harrison & Eduljee 1999) (see *Sections 3.2.1.1* and *3.2.1.2*).

Multiple laboratory test conditions may have various effects on a bioassays' LOD. For example, recent studies have shown that even a different temperature during the performance can decrease the LOD by increasing the level of reporter gene activity. For instance, Zhao and colleagues (2010) who performed the CAFLUX assay (*Table 3.1*) observed a 2- to 3-fold

Table 3.1 List of several *in vitro* bioassay studies. Stated are the bioassay detection limits with 2,3,7,8-TCDD as reference substance (LOD), the EC_{50} values for 2,3,7,8-TCDD, the cell lines, benchmarks, endpoints, solvent carriers and their final amounts in the assay, replicates (n) and coefficients of variation (CV).

Reference	Cell line (origin)	Benchmark (volume/assay)	Endpoint	Bioassay	LOD [pM]	EC_{50}TCDD [pM]	Solvent carrier (final amount)	n	CV [%]
(Schwirzer et al. 1998)	H4IIE	96 well plate (100 µl)	EROD activity	**Macro EROD**	**1.9**	-	DMSO/Isopropanol (4/ 1; 0.5%)	2	25
(Behnisch et al. 2002)		96 well plate (-)			**0.3**	5	DMSO (0.4%)	3	26
(Tillitt et al. 1991)		Petri dish,15x100 mm (10000 µl)			**3.1**	17	Isooctane (1.0%)	5 4	3.7
(Hanberg et al. 1991)	H4IIE	Culture plate, 20 cm^2 (3000 µl)	EROD activity	Micro EROD	**10**	47	DMSO (0.5%)	3 0	-
(Sanderson et al. 1996)		96 well plate (-)			**2.4**	20	Isooctane (-)	7	10-25
Lab code 7*		96 well plate (-)			**3.1**	6.2	DMSO/Isopropanol (4 : 1; 50% (v/v))	-	10 - 15
(Behnisch et al. 2002)		96 well plate (-)			**0.3**	14	DMSO (0.5%)	3	20
(Hurst et al. 2004)		96 well plate (-)			**0.4**	-	DMSO (0.4%)	3	< 30
Lab code 2*	H4IIE-luc (H4IIE)	96 well plate (-)	luminiscence	DR-CALUX	**0.3**	10	DMSO (0.8%)	3	15
Lab code 4*		96 well plate (-)			**0.3**	10	DMSO (0.4%)	3	-
Lab code 5*		96 well plate (-)			**0.6**	7.6	DMSO (0.4%)	3	12

* laboratories, which participated in an intra-laboratory comparison of dioxin-like compounds in food (Engwall & Van Bavel 2004)

Table 3.1 (continued) List of several *in vitro* bioassay studies. Stated are the bioassay detection limits with 2,3,7,8-TCDD as reference substance (LOD), the EC_{50} values for 2,3,7,8-TCDD, the cell lines, benchmarks, endpoints, solvent carriers and their final amounts in the assay, replicates (n) and coefficients of variation (CV).

Reference	Cell line (origin)	Benchmark (volume/assay)	Endpoint	Bioassay	LOD [pM]	EC_{50}TCDD [pM]	Solvent carrier (final amount)	n	CV[%]
Lab code 9*	H4IIE-luc (H4IIE)	96 well plate (-)	Luminescence	DR CALUX	**0.3**	10	DMSO (0.4%)	3	-
Lab code 23*		96 well plate (-)			**0.3**	10	DMSO (0.4%)	-	15
(Schoeters et al. 2004)	H4IIE-luc (H4IIE)	96 well plate (100 µl)	Luminescence	CALUX	**0.4**	-	DMSO (1.0%)	-	10-26
(Jeong et al. 2005)	Hepa 1c1c7 (H1L1.1c2)	24 well plate (500 µl)			**0.1**	10	DMSO (1.0%)	6	5.2
(Murk et al. 1996)	H4IIE-luc (H4IIE)	24 well plate (500 µl)			**1**	10	DMSO (0.5%)	3	-
Lab code 21*	Hepa1.12cR	96 well plate (-)			**2**	60	DMSO (0.1%)	-	-
Lab code 28*	Hepa1c1c7	96 well plate (-)			**1**	25	DMSO (1.0%)	3	-
(Zhao et al. 2010)	H1G1.1c3 (Hepa1c1c7)	96 well plate (-)	GFP fluorescence	CAFLUX	**1**	-	DMSO (1.0%)	3	-
(Nagy et al. 2002)	H1G1.1c3 (Hepa1c1c7)	96 well plate (100 µl)			**1**	18	DMSO (1.0%)	3	-
Lab code 20*	H1G1.1c3	96 well plate (-)			**1**	7	DMSO (1.0%)	-	-

* laboratories, which participated in an intra-laboratory comparison of dioxin-like compounds in food (Engwall & Van Bavel 2004)

Table 3.1 (continued) List of several *in vitro* bioassay studies. Stated are the bioassay detection limits with 2,3,7,8-TCDD as reference substance (LOD), the EC_{50} values for 2,3,7,8-TCDD, the cell lines, benchmarks, endpoints, solvent carriers and their final amounts in the assay, replicates (n) and coefficients of variation (CV).

Reference	Cell line (origin)	Benchmark (volume/assay)	Endpoint	Bioassay	LOD [pM]	EC_{50}TCDD [pM]	Solvent carrier (final amount)	n	CV[%]
(Song et al. 2006)	H4IIE-luc (H4IIE)	96 well plate (250 µl)	Luminescence	H4IIE-luc assay	**1.9**	31	-	3	-
(Sanderson et al. 1996)		96 well plate (-)			**0.8**	5.6	Isooctane (-)	7	10-30
(Aarts et al. 1995)	Hepa 1c1c7 (H1L1.1c7)	6 well plate (3000 µl)	Luminescence	Luciferase induction assay	**0.6**	-	DMSO (0.1%)	-	-
(Garrison et al. 1996)	Hepa 1c1c7 (H1L1.1c2)	6 well plate (-)			**0.1**	20	DMSO (0.1%)	3	-
Lab code 19*	101L (HepG2)	96 well plate (-)	Luminescence	P_{450}reporter gene system	**7.8**	-	Isooctane (1.0%)	3	-
Lab code 22*	RTL-W1	96 well plate (-)	EROD activity	EROD assay	**1**	5	DMSO (1.0%)	-	-
(Richter et al. 1997)	RLT 2.0 (RTH-149)	96 well plate (-)	Luminescence	RLT2.0 assay	**4**	64	DMSO/Isooctane (0.1 %)	3	-
(Niwa et al. 1975)	H4IIE	Petri dish, Ø 60mm (2500 µl)	AHH activity	AHH-enzyme assay	**4**	230	DMSO (0.2%)	2	-
(Bradlaw et al. 1980)		Petri dish, 60x15 mm (4000 µl)			**20**	385	DMSO (0.3%)	6	-

* laboratories, which participated in an intra-laboratory comparison of dioxin-like compounds in food (Engwall & Van Bavel 2004)

greater fluorescence of EGFP at a cell incubation temperature of 33 °C compared to that measured at 37 °C. Finally, the sensitivity of a bioassay is not only dependent on the LOD but also on the reliable production of reproducible and full concentration-response-curves and EC_x values (Sanderson et al. 1996). From an environmental risk assessment perspective this additional information can tell us much more about the potency and efficacy of a single compound or a mixture than it would be the case for instrumental analysis alone

3.3.5 Comparison of cell line and bioassay-specific LODs

The aim of this section is to summarize LODs obtained by using different assays and types of cell lines (*Table 3.1*). Moreover, results from an inter-laboratory comparison study that was conducted and organized by Magnus Engwall and Bert van Bavel from the Örebro University in Sweden were discussed (Engwall and Van Bavel 2004).

The aim of the inter-laboratory validation of bioassays was to determine: (1) the accordance of BEQ and TEQ values determined for 3 different types of samples; (2) the differences of TEQs among different bioassays; (3) the inter-laboratory variance and (4) the occurrence of additive effects in different bioassays. In brief, between December 2003 and April 2004 22 laboratories from Sweden, Norway, Denmark, Germany, the Netherlands, Belgium, Italy, the United Kingdom, USA, Canada, Japan, Taiwan and New Zealand participated in the above described validation study. Three types of samples were sent to all participating laboratories: sample 1 was a freeze-dried and homogenized sample of salmon muscle tissue, samples 2 and 3 were capsules containing a standard PCB and PCDD/F mixture, respectively. Participating laboratories were asked to extract the samples *via* their own methodologies and investigate them *via* their preferential *in vitro* bioassays (*Table 3.1*).

Comparing all results listed in *Table 3.1*, the data of inter-laboratory comparison collected by Engwall and Van Bavel (2004) corresponded well with the data previously reported in the literature (Behnisch et al. 2002, Hanberg et al. 1991, Hurst et al. 2004, Jeong et al. 2005, Murk et al. 1996, Nagy et al. 2002, Sanderson et al. 1996, Schoeters et al. 2004, Schwirzer et al. 1998, Zhao et al. 2010). The EROD assay with RTL-W1 as well as the related Micro EROD with H4IIE were the most TCDD-sensitive tests among the various assays with EC_{50} values of 5 pM 2,3,7,8-TCDD (*Table 3.1*). Detection limits of all applied assays ranged between 0.1 and 20 pM 2,3,7,8-TCDD, with the CALUX and DR-CALUX assays possessing the highest overall sensitivity (lowest LODs) of all listed test systems. The highest overall LOD (least sensitivity) was received by a rather obsolete method using high volume (4 ml) petri dishes. Nevertheless, surprisingly low LODs of up to 3.1 pM TCDD could be achieved by using those larger volume

approaches. In general, all luminescence-based bioassays showed the lowest LODs (*Table 3.1*). Coefficients of variance seem to be lower within these luminescence assays compared to e.g. EROD-based assays (*Table 3.1*). Some of those assays e.g. the P_{450} reporter gene system appear to be less favorable since their LODs are about 10 to 20-fold greater compared to the related assays.

The intra-bioassay and intra-laboratory comparability was excellent for the DR-CALUX, the CALUX and CAFLUX assays with mean LODs of 0.4, 0.9 and 1 pM, respectively. The relatively small data base for each of the assays should be considered and again depicts the already mentioned difficulty of non-stated LODs of *in vitro* bioassay studies.

3.4 Conclusion

In recent years the use of *in vitro* bioassays for the characterization of dioxin-like activities in environmental samples and other matrices as well as for individual chemicals and mixtures has become an increasingly popular field of research. There exist a multitude of possible applications for these *in vitro* bioassays, ranging from support of environmental risk assessments to food safety. In this context, the improvement of the sensitivity of *in vitro* assay technologies is a growing field, especially in consideration of genetically engineered cell lines in so far as those are typically more sensitive compared to the respective wt cells.

Regarding the sensitivity of *in vitro* bioassays they are increasingly competing with chemical analytical quantification technologies such as GC-MS with LODs of up to 0.1 pM 2,3,7,8-TCDD. While chemical investigations can give a more detailed view regarding specific quantities of different compounds present in e.g. complex environmental mixtures, bioassays are better suited to pre-screen those mixtures and hence, to identify the most dioxin-like active samples. In this context, bioassays have the distinct advantage that they can detect the overall dioxin-like potential of a sample including chemicals that cannot be analyzed by chemical analytical techniques.

We strongly recommend a standardized presentation of *in vitro* bioassay results enabling an estimate of its sensitivity. These results should include effect levels (especially in case BEQs are stated), the linear working range as well as the calculated LOD.

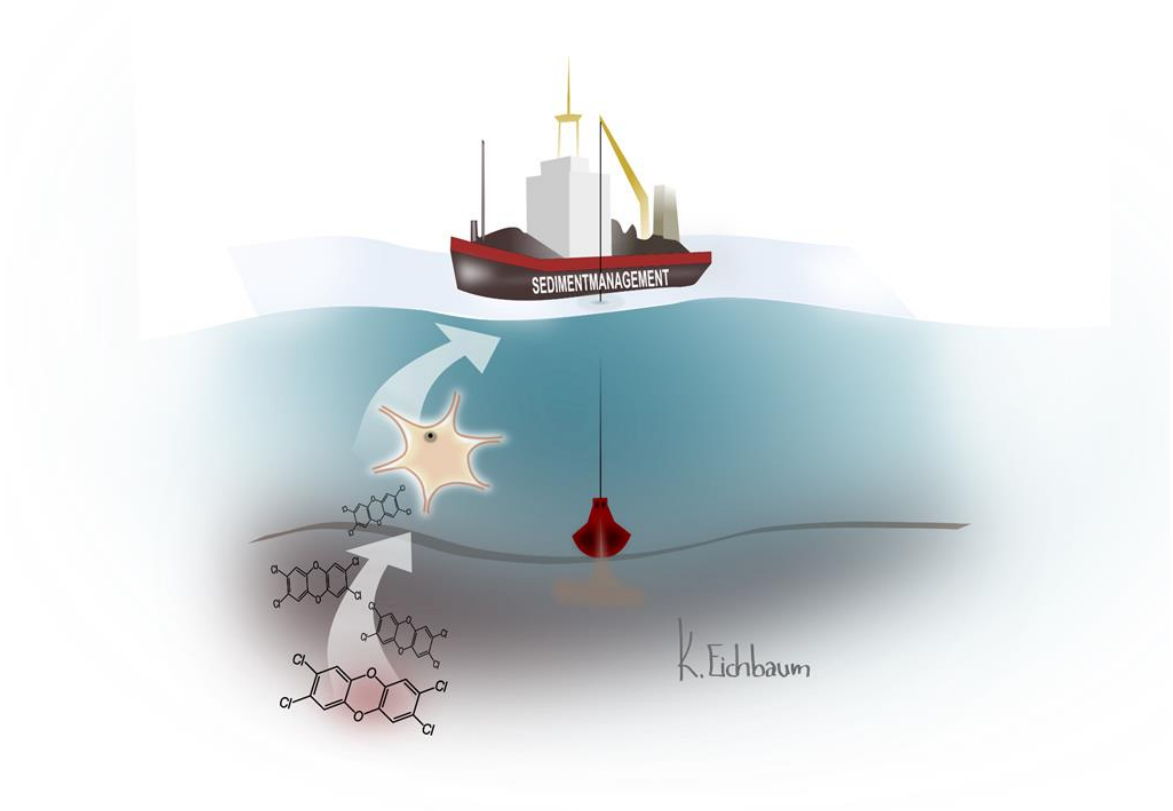
3.5 Acknowledgements

The present review forms a part of the DioRAMA project ("**dio**xin **R**isk **A**ssessment for sediment **M**anagement **A**pproaches"), which received funds from the "Title Group 05" of the German Federal Government. M.B. received a personal stipend from the German National

Academic Foundation (“Studienstiftung des deutschen Volkes”). Prof. Giesy was supported by the Canada Research Chair program, a Visiting Distinguished Professorship in the Department of Biology and Chemistry and State Key Laboratory in Marine Pollution, City University of Hong Kong, the 2012 "High Level Foreign Experts" (#GDW20123200120) program, funded by the State Administration of Foreign Experts Affairs, the P.R. China to Nanjing University and the Einstein Professor Program of the Chinese Academy of Sciences. Prof. Hollert was supported by the Chinese 111 Program (College of Environmental Science and Engineering and Key Laboratory of Yangze Water environment, Ministry of Education, Tongji University).

Chapter 4

In vitro tools for the toxicological evaluation of sediments and dredged materials: cross-validation of chemical and bio-analytical methods



Parts of this chapter have been submitted to the Journal of Soils and Sediments:

Eichbaum, K., Brinkmann, M., Nuesser, L., Gembé, C., Ohlig, M., Buchinger, S., Reifferscheid, G., Hecker, M., Giesy, J.P., Hollert, H. (submitted) *In vitro* tools for the toxicological evaluation of sediments and dredged materials: cross-validation of chemical and bio-analytical methods. *Journal of Soils and Sediments*.

4.1 Abstract

The implementation of *in vitro* bioassays for the screening of dioxin-like compounds (DLCs) into management guidelines of dredged material is of increasing interest to regulators and risk assessors. This study reports on a cross-validation between four independent laboratories. A bioassay battery consisting of RTL-W1 (7-Ethoxy-resorufin-*O*-deethylase; EROD), H4IIE (Micro EROD) and H4IIE-luc cells was used to assess aryl hydrocarbon receptor mediated effects of sediments from two major European rivers, differently contaminated with DLCs. Each assay was validated by characterization of its limit of detection (LOD) and quantification (LOQ), z-factor, reproducibility and repeatability. DLC concentrations were measured using high-resolution gas chromatography-high-resolution mass spectrometry (HRGC/HRMS) and compared to bioassay-specific responses *via* toxicity equivalents (TEQs) on intra- and inter-laboratory levels.

The Micro EROD assay exhibited the best overall performance among the bioassays. It was ranked excellent (z-factor = 0.54), reached a repeatability < 25%, was highly comparable ($r^2 = 0.87$) and reproducible (17%) between two laboratories and was well correlated ($r^2 = 0.803$) with TEQs. Its LOD and LOQ of 0.5 and 0.7 pM 2,3,7,8-TCDD, respectively, approached LOQs of HRGC/HRMS measurements. In contrast, cell lines RTL-W1 and H4IIE-luc produced LODs > 0.7 pM 2,3,7,8-TCDD, LOQs > 1.7 pM 2,3,7,8-TCDD and repeatability > 30%.

Based on the data obtained, the Micro EROD assay is the most favourable bio-analytical tool and *via* a Micro EROD-based limit value would allow for the assessment of sediment DLC concentrations, thus it could be considered for the implementation into testing and management guidelines for dredged materials.

Keywords: EROD • Micro EROD • BEQ • sediment management • EQS • ERA

4.2 Introduction

Industrial and municipal emissions to rivers have been reduced considerably during the last decades due to regulations such as the German water legislation and the European water framework directive (WFD) (Besselink et al. 2004). However, as sediments serve as sinks for persistent and bio-accumulative contaminants, they remain to constitute important sources by re-introducing particulate-bound organic pollutants back into the water phase through events such as dredging or flood events (Burton 1992). Among sediment-bound pollutants, the group of dioxin-like compounds (DLCs) represents one of the most relevant groups of legacy contaminants. This group includes polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) as well as dioxin-like polychlorinated biphenyls (DL-PCBs), which have the potential to cause adverse effects to wildlife and humans (as reviewed by White and Birnbaum 2009). Through frequently conducted sediment dredging activities, which are required and unavoidable for the maintenance of navigable waterways, fisheries, hot spot controlling and flood defense (Breitung and Keller 2010), sediment-bound pollutants can be re-mobilized and transferred into surface waters where they become bioavailable again (Burton 1992). Thus, dredging may be contradictive to the aim of the WFD (management plan; 2000/60/EG 2009) and most likely prevent achieving the frameworks' goal of a "good ecological status" (Barceló and Petrovic 2007, Förstner 2008, Hallare et al. 2011).

Recently, several attempts have been undertaken to progressively improve environmental risk assessment (ERA) approaches to enhance the quality of rivers, especially the quality of water. For instance, the WFD daughter directive requested the concentrations of 33 priority pollutants (annex, 2000/60/EC 2006) not to increase in water (Hollert et al. 2009) and with this, the establishment of environmental quality standards (EQSs) for sediment and biota proceeded (Förstner et al. 2008). EQSs, also referred to as action or trigger values, are important tools in sediment assessment frameworks (Apitz and Power 2002) for identifying effects or no effects of sediment-borne contaminants (Wenning and Ingersoll 2002). They define measures, such as disposal or habitat construction, to be undertaken with dredged materials (Manz et al. 2007).

The management and handling of dredged materials in Germany follows guidelines that have been compiled by the Ministry of Transport and Digital Infrastructure (BMVI) under the coordination of the German Federal Institute of Hydrology (BfG) (Breitung and Keller 2010). Waterways located outside the jurisdiction of the Water and Shipping administration (WSV) are subjected to the regulations of the respective German Federal states (den Besten et al. 2003). Two directives of the management of dredged material on both, federal inland (HABAB 2000) and coastal (HABAK 1999) waterways have been established and reestablished within joint

transitional arrangements (GÜBAK 2009). For disposal of dredged materials, characteristics of dredging and relocation sites have to be comparable and evaluated according to economic and ecological aspects (Breitung and Keller 2010).

The assessment of sediment quality is based on chemical analysis and ectotoxicological test methods. Chemical analysis in this context focuses on pollutants such as heavy metals and hydrophobic organic pollutants such as polycyclic aromatic hydrocarbons (PAHs) and PCBs, which all are known to be of high relevance for sediments and suspended particulate matter. In case of hydrophobic organic pollutants, seven so-called indicator PCBs (i-PCBs; IUPAC No. 28, 52, 101, 118, 138, 153 und 180) are required to be analyzed, and of which mono-*ortho* PCB 118 belongs to the 12 DL-PCBs.

Ecotoxicological tools encompass acute and chronic tests with algae (*Desmodesmus subspicatus*), bacteria (*Vibrio fisheri*) and micro crustaceans (*Daphnia magna*) on a lethal- and sub-lethal level (den Besten et al. 2003, Manz et al. 2007). However, these tests do not provide information about causative compounds responsible for the observed biological effects and are not comparable to chemical analytical results. Bioassays, which belong to the most important lines of evidence in support of integrated sediment assessment schemes such as the sediment quality triad (SQT) (Chapman and Hollert 2006, Hollert et al. 2002) overcome such issues by being complementary to chemical results and by taking synergetic/antagonistic factors into account (Ahlf et al. 2002).

In this respect, scientists increasingly discuss the role of *in vitro* bioassays for a biological effect-based assessment in decision making frameworks (Ahlf et al. 2002, Besselink et al. 2004, den Besten et al. 2003, Förstner et al. 2008). In environmental matrices, PCDD/Fs are found in much lower concentrations than PCBs, which in turn complicates their instrumental analysis (Van Bavel 1995). However, bioassays measuring dioxin-like activity provide the advantage that PCDD/Fs in contrast to PCBs produce higher signals, which relatively to chemical analysis enhances the signal.

Since the year 2004, successful implementations of *in vitro* assays for the screening of DLCs in form of the DR-CALUX assay can be found in the Dutch dredging guideline for coastal sediments, which formerly only included chemical analysis. Here, a biological equivalent quotient (BEQ) signal value of 50 ng BEQ/g dry weight (dw) sediment has been set, which - if exceeded – involves further, detailed investigations (Manz et al. 2007). In German legislation, *in vitro* assays as semi-quantitative methods prior to quantitative instrumental analysis have only been established in the field of food analysis, where BEQs allow for a simple yes/no-decision (2012/252/EU 2012).

The present study addresses the question if specific *in vitro* assays for the detection of dioxin-like effects could be of added value for the assessment of sediment quality in the context of dredging activities in German waterways. Based on the results of the most reliable bioassay and possibilities of a threshold definition are presented and discussed.

4.3 Materials and methods

4.3.1 Design of the cross-validation study

The cross-validation study followed the scheme of *Figure 4.1*. Participating laboratories encompassed (1) the Institute for Environmental Research, RWTH University, Aachen, Germany, (2) the Federal Institute for Hydrology (BfG), Koblenz, Germany, and (3) the BfG contract laboratory GBA (Gesellschaft für Bioanalytik mbH) and Münster Analytical Solutions (mas), Münster, Germany, which in the following are abbreviated as (1) lab 1, (2) lab 2, (3) lab 2* and (4) lab 3. The Institute for Environmental Research, RWTH University, Aachen, Germany was the main laboratory, were most of the present study's work has been conducted.

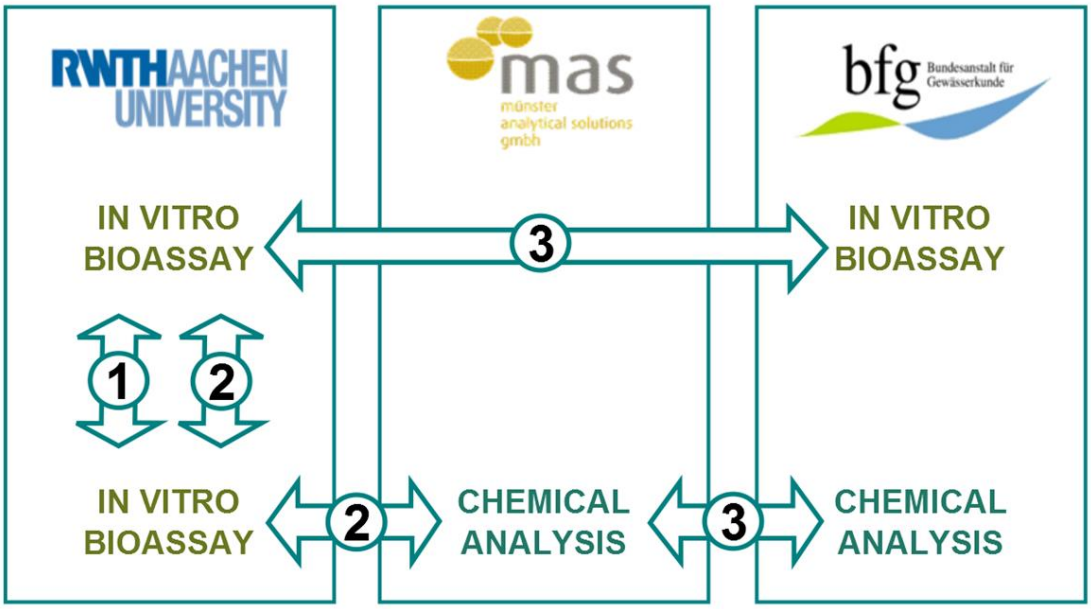


Figure 4.1 Conceptual drawing of the DioRAMA cross validation study. The three boxes constitute the participating partners: Institute for environmental research of the RWTH Aachen University, Münster Analytical Solutions (mas) and the Federal Institute of Hydrology (BfG). Arrows indicate intra- (1) and inter-laboratory (3) as well as method (2) comparisons.

4.3.1.1 Method comparisons

Firstly, a method comparison (number 2; *Figure 4.1*) of three bioassays, including the RTL-W1 EROD assay, the H4IIE Micro EROD assay and the H4IIE-luc assays, was intra-

laboratorial conducted by lab 1 (main laboratory). Bioassays were validated by means of a set of criteria (*Table 4.1*) such as repeatability, limits of detection (LOD) and quantification (LOQ), different levels of effect concentrations (EC) of the 2,3,7,8-TCDD standard, sample induction strengths relative to positive control as well as z-factors. Raw extracts, multilayer fractions (containing the sum of dioxin-like PCBs and PCDD/Fs) as well as DL-PCB and PCDD/F fractions of differently contaminated sediments served as a basis for bioassay validation.

In a further method comparison, results of bioassay and HRGC/HRMS measurements were compared (number 1; *Figure 4.1*) via toxicity equivalents (TEQs) and biological equivalents (BEQs), respectively.

4.3.1.2 Intra-laboratory comparison

Repeatability of results for sediment extracts, which increased in the order RTL-W1 < H4IIE-luc < H4IIE (*Table 4.1*), disqualified the RTL-W1 EROD assay as reliable screening tool and indicated the need for further investigations using the H4IIE-luc assay. These included intra-laboratory comparisons of test results achieved by two operators in lab 1 (number 1; *Figure 4.1*) and aimed at uncovering possible operator-related variations. Because, H4IIE-luc repeatability obtained through operator 2 did not meet the requirements of a repeatability < 25% (2012/278/EU 2012), the H4IIE-luc assay was disqualified for a further investigations.

4.3.1.3 Inter-laboratory comparisons

Finally, an inter-laboratory comparison (number 3; *Figure 4.1*) was conducted by two different operators of lab 1 and 2 using the H4IIE Micro EROD assay, which previously turned out to exhibit the best overall performance (*Table 4.1*).

On the chemical analytical side, a further inter-laboratory comparison of high resolution gas chromatography – high resolution mass spectrometry (HRGC/HRMS) results was performed between lab 2* and 3.

4.3.2 Sediment samples

Sediment sampling was conducted by lab 2 in April 2012. Sample locations and details can be found in *section 2.1*.

4.3.3 Sample preparation, extraction and clean-up

The preparation of sediment samples as well as their extraction and clean-up were conducted in lab 1. Details can be found in *section 2.2*. For cross-validation purposes, 20 g of each freeze-dried sediment were sent to lab 2*, where an appropriate extraction and clean-up was performed.

4.3.4 Bio-analytical analysis

Details on the RTL-W1 EROD (7-Ethoxyresorufin-*O*-deethylase), the H4IIE Micro EROD and the H4IIE-luc assays are given in *sections 2.3.1* and *2.3.3*, respectively.
For inter-laboratory comparison purposes, personnel of lab 2 were trained in Micro EROD assay performance in lab 1. Frozen H4IIE cells (passage number 22), aliquots of extract fractions, 96-well plates and stock solutions required for test performance were sent to lab 2. Plates were measured using the same protocol (Tecan Infinite M200 Pro) and data was then sent to and evaluated by lab 1 personnel.

4.3.4.1 Calculation of Biological Equivalent Quotients (BEQs)

BEQs were calculated according to *equation 2.1* with *x* being the 25% effect concentration level (see *section 2.3.4*). All bio-analytical data from intra- and inter-laboratory comparisons were evaluated by the same operator.

4.3.4.2 Calculation of bio-analytical quality criteria

Quality criteria, including repeatability, reproducibility, z-factor, limit of detection (LOD) and quantification (LOQ), were calculated as presented in *section 2.3.5*.

4.3.5 Chemical analysis

4.3.5.1 HRGC/HRMS analyses

HRGC/HRMS analyses of extracts prepared by lab 1 was performed by lab 3. Details on the analysis of PCDD/Fs and DL-PCBs can be found in *section 2.4.1*. Inter-laboratorial conducted HRGC/HRMS results were evaluated by different operators of laboratories 2* and 3.

4.3.5.2 Calculation of Toxicity Equivalent Quotients (TEQs)

REP-based TEQ were calculated by use of *equation 2.5* given in *section 2.4.2*. This section moreover gives additional information to the literature-specific *x*-values used in *equation 2.5*.

4.3.6 Bio-analytical threshold value derivation from chemical data

To connect the present study's results with German dredged material directives, we conducted an attempt to derive a bio-analytical threshold value (H4IIE Micro EROD assay) from chemical analytical data. TEQ values (sum of WHO_{2005} DL-PCB and PCDD/F TEQs) from an Elbe length profile study, conducted by Stachel and Co-workers (2011) were collected and a 75% percentile of all data points, including TEQs of the present studies' Elbe sampling locations Prossen and Magdeburg (ZE), was determined and served as limit value for stronger contaminated locations.

4.3.7 Data analysis and presentation

All graphs and correlation analyses (Pearson correlation; $p < 0.05$) were calculated using GraphPad Prism 5. Illustrations were created using the vector graphic program Inkscape 0.91. A one-tailed Student's t-test ($p < 0.05$) was performed using Sigma Stat 12.0 to statistically analyze differences between intra- and inter-laboratorial derived bioassay results. In case of uneven variances, Welch's correction ($p < 0.005$) was applied for Student's t-test adjustment.

4.4 Results

4.4.1 Physical chemical characterization of sediments

Generally, Elbe sediments PR and ZE, which on average exhibited higher percentages of sand (21%) and smaller percentages of silt (69%) and clay (10%) clearly differed from Rhine sediment EBR (4, 79 and 17% of sand, silt and clay, respectively). In contrast to EBR (49.6 g/kg TOC), they had higher amounts of TOC (63.7 g/kg), confirmed by their slightly higher losses on ignition (13.6 and 10.6%, respectively).

WHO-PCDD/F concentrations (data not shown) increased in the order EBR < PR < EBR/ZE < ZE (0.03, 0.24, 1.22 and 3.70 ng/g dw, respectively), whereas WHO-DL-PCB concentrations increased in the order EBR < EBR/ZE < PR < ZE (3.50, 4.18, 5.22 and 9.72 ng/g dw, respectively). When expressed as toxicity equivalents (TEQ, Van den Berg et al. 2006) both PCDD/F and DL-PCB TEQs increased in the order EBR < PR < EBR/ZE < ZE. PCDD/F TEQs were 5.13, 5.49, 17.53 and 84.22 ng/g dw and DL-PCB TEQs were 2.66, 2.95, 4.04 and 5.85 ng/g dw for EBR, PR, EBR/ZE and ZE, respectively.

4.4.2 Cross-validation study

4.4.2.1 Method comparisons

This section focuses on the general handling and performance of the EROD, Micro EROD and H4IIE-luc assay (refer to assays marked with "a", *Table 4.1)* as well as the method comparison between bioassay and HRGC/HRMS-derived BEQs and TEQs, respectively.

The H4IIE Micro EROD assay showed an average z-factor of 0.54, an average repeatability (coefficient of variation (CV) of three independent measurements) of < 25% for both sediment extracts and TCDD (*Table 4.1*) with an LOD and LOQ of 0.5 and 0.7 pM 2,3,7,8-TCDD, respectively.

The average LOD and LOQ of the H4IIE-luc assay was 0.7 and 2.1 pM 2,3,7,8-TCDD, respectively. Repeatability for sediment extracts, which was independent of the different fractions (raw, multilayer, DL-PCB and PCDD/F) and 2,3,7,8-TCDD was high and averaged at 31 and 39%, respectively. The quotient of the average EC_{25}TCDD and the average EC_{10}TCDD levels was highest (2.6) for the H4IIE-luc assay (*Table 4.1*).

RTL-W1 EROD assay average LODs and LOQs of 0.94 and 1.72 pM 2,3,7,8-TCDD (*Table 4.1*), respectively were high compared to the remaining assays. The assay's repeatability was highest (36%), but independent of the different fractions tested (raw, multilayer, DL-PCB and PCDD/F). The CV among replicate experiments with the single substance 2,3,7,8-TCDD was lesser with a value of 30% (*Table 4.1*). The assays' overall z-factor was 0.36 and was accompanied by the overall highest standard deviation among the three assays.

The comparability between TEQs and BEQs increased from the H4IIE-luc ($r^2 = 0.642$) to the EROD ($r^2 = 0.779$) to the Micro EROD assay ($r^2 = 0.803$). The percentage of relative potency (REP)-based TEQs in Micro EROD BEQs amounted up to 49% (*Figure 4.2*). Thereby, it was proven that REP-based TEQs explained a greater part (26.5%) of BEQs than it was the case for routinely used WHO_{2005}TEQs (16.0%, data not shown).

Table 4.1 Bio-analytical quality criteria achieved for three different *in vitro* bioassays (RTL-W1 EROD, H4IIE Micro EROD and H4IIE-luc assay) including effect concentrations (EC) levels of positive control 2,3,7,8-TCDD, limit of detection (LOD) and quantification (LOQ), z-factor, repeatability and reproducibility, n.a. = not analyzed.

	In vitro bioassay				
	$EROD_a$	**Micro $EROD_a$**	**Micro $EROD_b$**	**H4IIE-luc_a**	**H4IIE-luc_c**
cell line	*RTL-W1*	*H4IIE*	*H4IIE*	*H4IIE-luc*	*H4IIE-luc*
passages used	*60-77*	*26-50*	*26-30*	*10-26*	*19-27*
number of tests	*273*	*75*	*16*	*71*	*48*
EC_{10}TCDD [pM]					
Mean ± SD	2.53 ± 0.82	2.39 ± 0.76	1.80 ± 0.20	1.21 ± 0.87	1.03 ± 0.60
Min/Max	0.72 / 6.25	1.09 / 5.99	1.49 / 2.20	0.26 / 6.00	0.11 / 2.73
EC_{25}TCDD [pM]					
Mean ± SD	4.72 ± 1.32	3.59 ± 1.28	3.08 ± 0.35	2.52 ± 0.85	2.71 ± 1.40
Min/Max	1.81 / 12.08	1.61 / 10.35	2.58 / 3.69	0.72 / 4.22	0.77 / 7.67
LOD [pM]					
Mean ± SD	0.94 ± 0.61	0.45 ± 0.32	0.43 ± 0.16	0.78 ± 0.63	0.73 ± 0.91
Min/Max	0.01 / 5,41	0.07 / 2.05	0.06 / 0.61	0.06 / 2.96	0.01 / 4.72
LOQ [pM]					
Mean ± SD	1.72 ± 1.28	0.69 ± 0.36	0.72 ± 0.18	2.32 ± 2.05	2.12 ± 2.27
Min/Max	0.02 / 13.05	0.17 / 2.47	0.20 / 1.01	0.16 / 9.93	0.06 / 10.86
z-factor					
Mean ± SD	0.36 ± 0.46	0.54 ± 0.22	0.72 ± 0.10	0.67 ± 0.14	0.64 ± 0.25
Min/Max	-5.13 / 0.99	-0.06 / 1.00	0.58 / 0.90	0.27 / 0.90	-0.88 / 0.91
Repeatability [%]					
Extract/TCDD	36 / 30	24 / 23	12 / 10	31 / 39	57 / 38
Reproducibility [%]					
Extract/TCDD	n.a.	$17_{(bl)}$ / $2_{(bl)}$	$17_{(bl)}$ / $2_{(bl)}$	$17_{(wl)}$ / $22_{(wl)}$	$17_{(wl)}$ / $22_{(wl)}$

a = operator 1, lab 1, Method comparison
b = operator 2, lab 2, Inter-laboratory comparison
c = operator 2, lab 1, Intra-laboratory comparison
(bl) = between-laboratory reproducibility; (wl) = within-laboratory reproducibility

4.4.2.2 Intra-laboratory comparison

Results obtained by the different operators with the H4IIE-luc assay were highly comparable. The intra-laboratory validation study achieved LODs that did not significantly differ ($p = 0.339$) from each other following Welch's correction ($p < 0.0001$). However, repeatability differed considerably between operator 1 (31%) and 2 (57%), respectively. The within-laboratory reproducibility was independent of the different fractions and amounted to 22%**.** There was a highly significant ($r^2 = 0.942$) correlation between H4IIE-luc BEQs obtained by two different operators (*Figure 4.3a)*. Among the different sediments, both operators characterized sediment ZE and its fractions to possess the highest overall AhR-activating potential (*Figure 4.3a*).

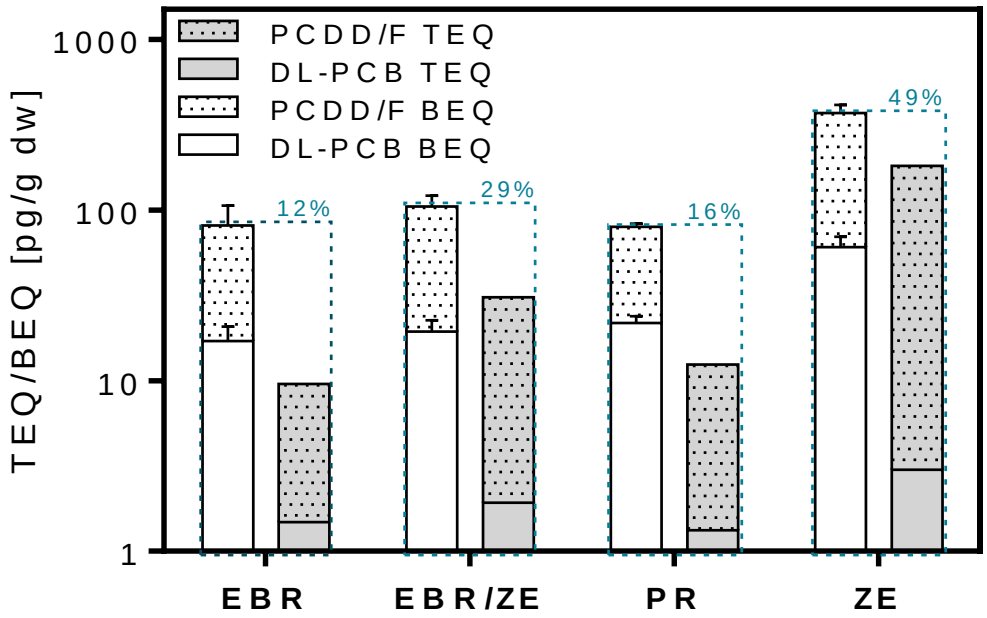


Figure 4.2 Comparison of bio-analytical (BEQs) and instrumentally derived toxicity equivalents (TEQs) of DL-PCB and PCDD/F extract fractions of four sediments. EBR = Ehrenbreitstein (Rhine), PR = Prossen (Elbe), ZE = Zollelbe (Elbe) and a 1:10 mixture (EBR/ZE) consisting of one dry weight part EBR and 9 dry weight parts ZE. BEQs were determined on an EC_{25} level *via* the H4IIE Micro EROD assay, while TEQs were calculated using H4IIE Micro EROD assay-specific relative potencies (REP). The overall share of TEQs in respective BEQs is given in percentages.

4.4.2.3 Inter-laboratory comparisons

Results obtained for the H4IIE Micro EROD assays were highly comparable between both operators and laboratories. A one-tailed student's t-test, which due to unequal variances was adapted by Welch's correction ($p < 0.005$), indicated inter-laboratorial achieved LODs to not significantly differ from each other ($p = 0.752$).

Repeatability achieved by both operators was lower for DL-PCB than for PCDD/F fractions and averaged to 24 and 12% for lab 1 and 2, respectively. The between-laboratory reproducibility for sediment extracts and single substance 2,3,7,8-TCDD averaged to 17 and 2%, respectively. Average LOD and LOQ values of the Micro EROD assay were 0.4 and 0.7 pM 2,3,7,8-TCDD, respectively (*Table 4.1*). Furthermore, the correlation of Micro EROD assay results obtained by different laboratories was significant ($r^2 = 0.87$, *Figure 4.3b*). Both operators and laboratories found highest AhR-activating potential for fractions of sediment ZE.

Concerning the inter-laboratory comparison of HRGC/HRMS measurements, a highly significant correlation was found between DL-PCB and PCDD/F congener concentrations determined in sediments PR and EBR ($r^2 = 0.933$; *Figure 4.3c*), even though extracts were differently extracted and cleaned.

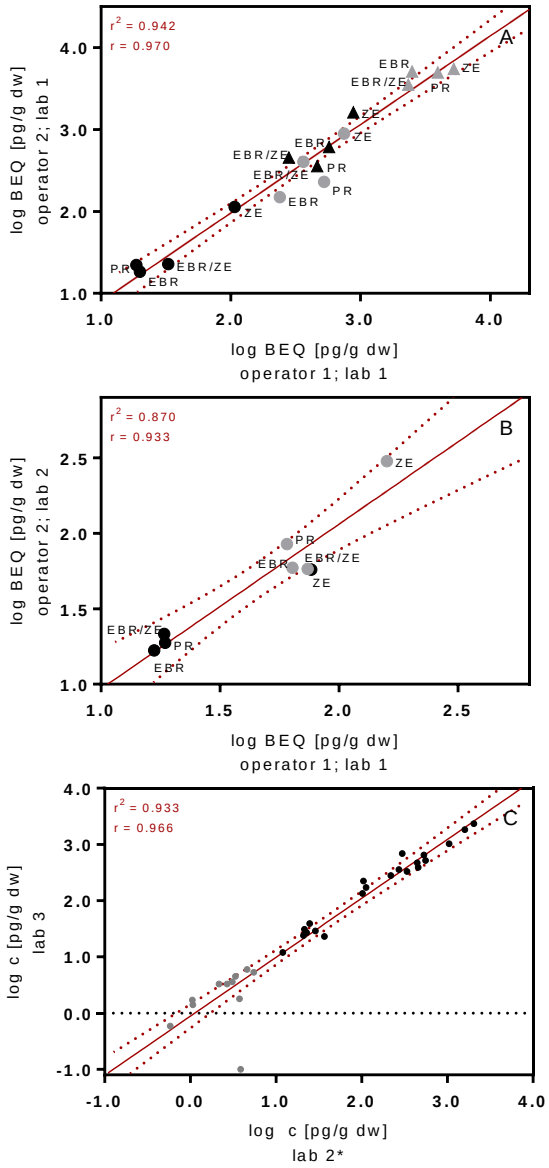


Figure 4.3a-c Pearson correlations of an intra-laboratory comparison *via* H4IIE-luc EC_{25}BEQs **(a)** as well as inter-laboratory comparisons *via* H4IIE Micro EROD EC_{25}BEQ **(b)** and instrumental determined concentrations **(c)** in four sediments extracts and fractions thereof. EBR = Ehrenbreitstein (Rhine), PR = Prossen (Elbe), ZE = Zollelbe (Elbe) and a 1:10 mixture (EBR/ZE) consisting of one dry weight part EBR and 9 dry weight parts ZE. Black and grey circles show BEQs **(a, b)** and single congener concentrations **(c)** determined for sediment DL-PCB and PCDD/F fractions, respectively. Black and grey triangles show BEQs determined for multilayer fractions and raw extracts, respectively. Linear regression line is depicted with its 95% confidence interval (dashed red line). Lab 1 = RWTH Aachen University, lab 2 = German Federal Institute for Hydrology (BfG); lab 2* = BfG contract laboratory; lab 3 = Münster Analytical Solutions (mas).

4.4.2.4 Summarized consideration of bio-analytical results

Multilayer and raw fractions were only analysed using the EROD and H4IIE-luc assays (data not shown). In both assays, the sum of activities of DL-PCB and PCDD/F fractions was lower than total activity measured in multilayer extracts (by 73 and 82% for DL-PCB and PCDD/F fractions, respectively). Regarding intra- and inter-laboratorial achieved BEQs of the three

different assays, they all indicated sediment ZE and fractions thereof to possess the overall highest toxicity. The fish cell line RTL-W1 produced the highest and the mammalian wild type cell line H4IIE the lowest BEQs (*Figure 4.4a and b*). Logarithmic BEQs of the EROD assay correlated well with those determined *via* the H4IIE-luc ($r^2 = 0.930$) and Micro EROD assay ($r^2 = 0.910$). Logarithmic BEQs of the H4IIE-luc and Micro EROD assays correlated well, too ($r^2 = 0.900$).

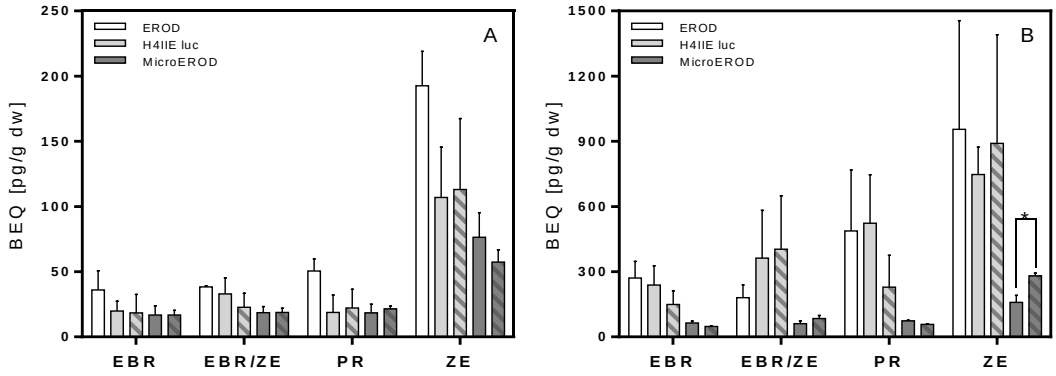


Figure 4.4a, b Biological toxicity equivalents (BEQs) obtained for DL-PCB **(a)** and PCDD/F **(b)** fractions of three sediments from Ehrenbreitstein (EBR), Prossen (PR) and Zollelbe (ZE) as well as a mixture (EBR/ZE) consisting of 9 dry weight parts EBR and one dry weight part ZE. BEQs were obtained using the RTL-W1 EROD, H4IIE-luc and H4IIE Micro EROD assays. Dashed bars show results of intra- (H4IIE-luc) and inter-laboratory (H4IIE Micro EROD) comparisons. Bars show mean values of three independent replicates with standard deviations. Asterisks show significant differences between results obtained by different operators, which was analyzed using a Students t-test ($p < 0.05$).

Intra- (H4IIE-luc) and inter-laboratory (Micro EROD) BEQs were comparable ($p = 0.008$). Only the Micro EROD BEQs obtained for ZE PCDD/F fraction significantly differed between the two operators and laboratories (*Figure 4.4b*).

4.4.3 Bio-analytical threshold value derivation from chemical data

When combining the results obtained for Elbe sediments analyzed by this study with TEQ values from a more extensive data set that includes multiple locations analyzed along the Elbe River (Stachel et al. 2011), the top 25% most contaminated sediments were clearly separated from the remaining samples by a TEQ limit value (LV) of 35 pg/g dw (*Figure 4.5a*). Linear regression analysis of Micro EROD BEQs and TEQs determined for Rhine and Elbe sediment DL-PCB and PCDD/F fractions obtained during the present study (*Figure 4.5b*) resulted in a respective BEQ_{LV} of 145 pg BEQ/g dw sediment.

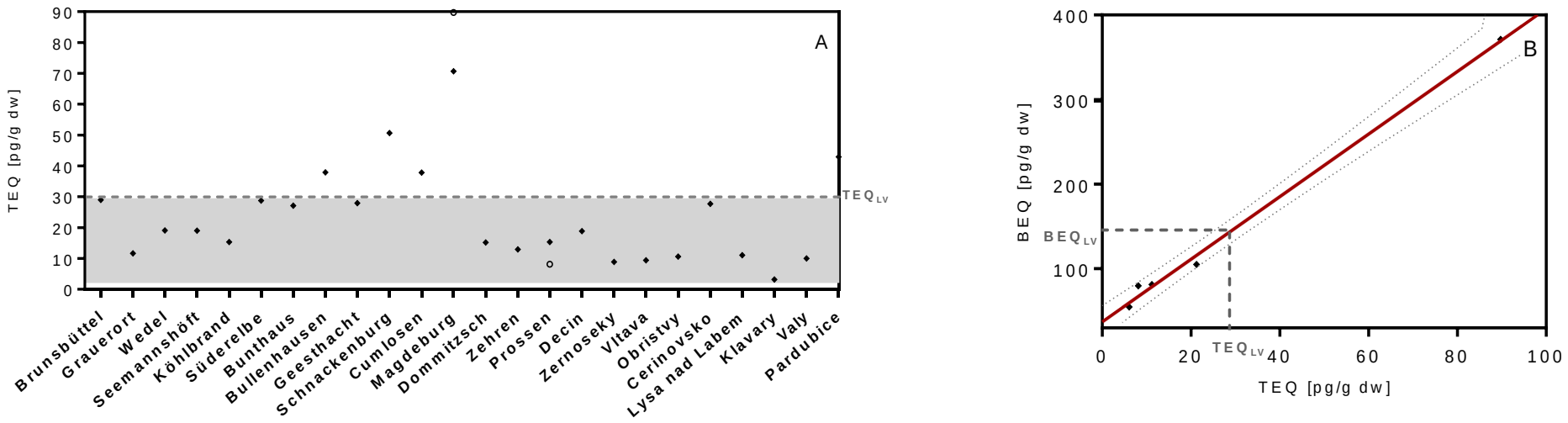


Figure 4.5a, b (**A**) Sum of DL-PCB and PCDD/F WHO_{2005} toxicity equivalents (TEQs) measured *via* HRGC/HRMS in sediment extracts of an Elbe length profile sampling campaign conducted by Stachel and Coworkers (2011), sampling locations follow the rivers' course, dashed line represents a TEQ limit value (LV) derived from the 75% percentile (grey area) of all data points, blank dots at locations Magdeburg and Prossen represent TEQs measured in the present study. (**B**) Linear Correlation of TEQs [pg/g dw] and biological equivalents (BEQs); TEQs represent the sum of DL-PCB and PCDD/F measured in sediments of rivers Elbe and Rhine of the present study, whereas BEQs show the AhR-activating potential of the respective DL-PCB and PCDD/F fractions analyzed using the Micro EROD assay with H4IIE cells. Through the TEQ_{LV} derived in (**B**), a limit value for BEQ calculation was determined (dashed line, BEQ_{LV}).

4.5 Discussion

4.5.1 Cross-validation study

4.5.1.1 Method comparisons

Each of the performed assays was able to detect dioxin-like activity in complex samples. Altogether, according to our findings, the Micro EROD assay with the cell line H4IIE constitutes the preferable bio-analytical screening tool among the examined assays. The Micro EROD assay according to its average z-factor of 0.54 could be classified as *excellent*. Its average repeatability < 25% corresponds to aforementioned regulatory recommendations (2012/278/EU 2012) and the here applied two sample-plate layout allowed for the simultaneous testing of 16 samples per cycle. The assays' most promising criteria were its remarkably low LOD and LOQ of 0.5 and 0.7 pM 2,3,7,8-TCDD (*Table 4.1*), respectively, which approach the limits achieved by instrumental analysis such as HRGC/HRMS.

In contrast to cell line H4IIE, a two sample-plate layout turned out to be inappropriate using H4IIE-luc cells due to cross-talk of adjacent wells during the luminescence measurements (Puga et al. 2009). This limited the number of samples/cycle to six. Although luminescence is known to be one of the most sensitive endpoints (Sanderson et al. 1996, Willett et al. 1997), the average LOD of 0.8 pM 2,3,7,8-TCDD was higher than expected and the average LOQ of 2.3 pM 2,3,7,8-TCDD could not compete with those of the remaining assays. While the assay's average repeatability was satisfactory for complex samples (31%), it was unexpectedly high (39%) for standard 2,3,7,8-TCDD. The distance between the average EC_{25}TCDD and the average EC_{10}TCDD showed that the H4IIE-luc assay covered the widest concentration range among the analysed assays *(Table 4.1*), hence might compensate for time-consuming range finding tests prior to the actual assay.

The RTL-W1 EROD assay allows for the testing of up to 36 samples per cycle, which constitutes the assays' most promising feature and equates 3 to 5-times the testing capacity of mammalian cells such as H4IIE and H4IIE-luc. High sample numbers require long test periods, which due to differing culture conditions (e.g. lower temperature, no need for culture in CO_2 atmosphere) are much better tolerated by the fish cell RTL-W1 compared to their mammalian relatives. RTL-W1 cells are very slow growing (one doubling after 72 h) and have stable cytochrome concentrations even at high passage numbers. Hence, they allow for the testing of large numbers of samples using a single subculture (Lee et al. 2013), which decreases subculture-related variability. Nevertheless, repeatability of the RTL-W1 EROD assay was

higher than formerly determined (2012/278/EU 2012, Besselink et al. 2004, Engwall and Van Bavel 2004) and showed values of 36 and 30% for extracts and single substance 2,3,7,8-TCDD respectively. Hence, repeatability increased with increasing sample complexity, which corresponds to previous observations of Besselink et al. (2004).

Moreover, care has to be taken when samples with low EROD-inducing potential have to be evaluated because of the assay's relatively high average LOD and LOQ of 0.9 and 1.7 pM 2,3,7,8-TCDD (*Table 4.1*), respectively. The fact that the z-factor of 0.36 was accompanied by the overall highest standard deviation most likely indicates high intra-assay fluctuations of positive and negative control (Zhang et al. 1999) due to the z-factors' high sensibility towards variability. In conclusion, the EROD assay (RTL-W1) constitutes an assay particularly suitable for the pre-screening of large sampling sets and, despite its comparably high detection limits, promptly can be used to detect samples of highest concern.

4.5.1.2 Intra-laboratory comparison

Intra-laboratory results obtained for the H4IIE-luc assay were highly comparable ($r^2 = 0.942$) between two operators of the same laboratory (*Figure 4.3a*), but repeatability for sediment extracts of 31 and 57% achieved by operator 1 and 2, respectively, was different and indicated the assays' high variability. The within-laboratory reproducibility for sediment extracts of 17% (in contrast to the percentage of repeatability) corresponded to recommendations set by European guidelines (2012/278/EU 2012) and most-likely was due to the fact that test results of both operators and laboratories was evaluated by the same operator (*Table 4.1*). Results generated by both operators indicated that sediment ZE and its fractions possessed the overall highest AhR-activating potential (*Figure 4.3a*), which demonstrated the suitability of the H4IIE-luc assay to be used as prioritizing tool in sediment evaluations.

4.5.1.3 Inter-laboratory comparisons

The inter-laboratory comparison of the H4IIE Micro EROD assay showed comparable results ($r^2 = 0.87$) between both operators and laboratories, but showed slightly higher deviations than the intra-laboratory comparison (*Figure 4.3a and b*). The between laboratory reproducibility averaged to 17%, indicating the H4IIE Micro EROD as a useful cross-laboratory method. A standard (here 2,3,7,8-TCDD), which delivers highly comparable repeated measures, is one basic requirement for implementing an assay as a regulatory tool. For the standard, between-laboratory reproducibility and repeatability on an EC_{25}TCDD level were 2 and 16% (calculated from respective standard deviations depicted in *Table 4.1*), respectively,

and thus are smaller than the recommendations of regulatory guidelines (2012/278/EU 2012) and performance of reported by other studies (Engwall and Van Bavel 2004).

In general, the variability observed for this assay was below the inter-laboratory variability that was achieved results using the CALUX assay. The reproducibility for 2,3,7,8-TCDD and sediment extracts reached values of 14 and 20%, respectively in case of the CALUX assay (Besselink et al. 2004). However, the fact that results of both laboratories were calculated by the same operator most likely lowered the intra-assay variation. For instance, Engwall and Van Bavel (2004) concluded for their inter-laboratory bioassay comparison study that different evaluation methods conducted by the participating laboratories influenced inter-laboratory variance. To lower this influencing factor, the authors strongly recommended standardized evaluation methods (Engwall and Van Bavel 2004).

Average LOD and LOQ values of 0.4 and 0.7 pM 2,3,7,8-TCDD were comparable to former studies (as reviewed by Eichbaum et al. 2014), and indicate that the H4IIE Micro EROD is a highly suitable screening tool for complex samples with low dioxin-like activity. The inter-assay variability of EC_{10}TCDD values was very high compared to EC_{25}TCDD levels, which possibly indicates EC_{25} values to be the more reliable effect level for sample evaluation (*Table 4.1*). Finally, both operators found the highest overall induction potential for fractions of sediment ZE, again showing the suitability as prioritization tool and supporting the results of the remaining assays (*Figure 4.3b*).

Concerning HRGC/HRMS measurements conducted by different operators and laboratories, a highly significant correlation ($r^2 = 0.933$, *Figure 4.3c*) revealed that extraction, clean-up and analytical methods applied by labs 1 and 2* were comparable and robust. However, for future cross-method comparisons, it is strongly recommended that extracts have the same origin. This is because findings of an intra- and inter-laboratory comparison study conducted by Besselink et al. (2004) revealed that different extraction and clean-up methodologies distinctly influenced TEQs and BEQs and most likely increased reproducibility.

Although DL-PCBs and PCDD/Fs show high and low concentrations, respectively, (*Figure 4.3 a-c*) bio-analytical results show low and high induction levels for DL-PCBs and PCDD/Fs, respectively, which reveals their high sensitivity towards dioxins. PCDDF/s so far are not present among the target compounds of guidelines for dredged material (HABAB 2000, HABAK 1999). However, this compound class essentially influences the overall induction potential in the group of DLCs and thus the future implementation of *in vitro* bioassays for the

screening of environmental trace contaminations with the highly relevant PCDD/Fs should be considered.

4.5.1.4 3.2.4 Summarized consideration of bio-analytical results

Only the H4IIE Micro EROD assay showed the same order of contamination levels (*Figure 4.4a and b*) for DL-PCBs (EBR < EBR/ZE < PR < ZE) and PCDD/Fs (EBR < PR < EBR/ZE < ZE) as the results from the chemical analysis (see *Table 2.1*). Furthermore, this assay exhibited the smallest amount of unexplained percentages of 26.5% when compared to chemical analytical results (*Figure 4.2)*. Discrepancies between TEQs and BEQs were considerably higher than Guideline recommendations of ± 20% (2012/278/EU 2012), which may be explained through antagonistic or synergistic effects, contradicting the additive character of the TEQ approach (Safe 1998a, b). Moreover, complex environmental mixtures are known to contain a certain fraction of dioxin-like inducers non-targeted by chemical analysis (Engwall and Van Bavel 2004).

EROD and H4IIE-luc assays, by which multilayer fractions containing both DL-PCBs and PCDD/Fs were investigated, showed that the sum of the activity of the two fractions (DL-PCB, PCDD/F) was lower than the total activity measured in multilayer extracts (73 and 82%, respectively; data not shown). This observation, although opposite to former findings (Manz et al. 2007), may indicate the presence of antagonistic substances in the multilayer fractions or possible compound losses during the fractionation process.

In general, BEQs of the different assays were comparable when DL-PCB fractions were investigated, while they differed for PCDD/Fs (*Figure 4.4a and b*), indicating the bioassays differing sensitivities towards PCDD/Fs.

4.5.2 Bio-analytical threshold value derivation from chemical data

The present study's sampling location ZE (Magdeburg) was found to be among the top 25% of the most contaminated sediments of the river Elbe. Regarding the TEQ_{LV} of 35 pg/g dw, sediment ZE with TEQs of 70 and 90 pg/g dw as determined in the previous and in the present study, respectively, clearly separated from other sampling locations along the river such as sediment PR (Prossen/Schmilka). The TEQ_{LV} was chosen in an arbitrary manner, and thus, could also be based on any other percentile of choice (*Figure 4.5a*). The BEQ_{LV} of 145 pg BEQ/g dw sediment, deduced from a linear correlation of BEQs and TEQs determined in the present study (*Figure 4.5b*), has to be considered as preliminary due to the limited data available for the bioassay to date (*Figure 4.5b)*.

Hence, further bio-chemical and instrumental sediment evaluations with DL-PCB and PCDD/F fractions would have to be conducted to strengthen the basis for such *in vitro* assay sediment evaluations. On this basis, sediments could be evaluated and ranked using simple, rapid and low-cost intensive *in vitro* bioassays such as the H4IIE Micro EROD assay.

4.6 Conclusion

The H4IIE Micro EROD assay showed the best performance within the investigated bioassays. It was ranked *excellent* (z-factor = 0.54), possessed a satisfactory samples/cycle-number, its repeatability < 25% was independent of sample complexity and its remarkably low LOD and LOQ of 0.5 and 0.7 pM 2,3,7,8-TCDD, respectively approached the limits achieved by instrumental analysis, with which the assay was highly comparable ($r^2 = 0.803$). Bioassay results were highly reproducible (17%) and comparable ($r^2 = 0.87$) between operators and laboratories. While all bioassays reliably indicated sediment ZE and its fractions to possess the overall highest dioxin-like potential among the four chosen sediments, only cell line H4IIE showed the same sequence in sediment contamination as it was determined by HRGC/HRMS.

In contrast, the RTL-W1 EROD assay due to its high sample/cycle-number was more suitable for the pre-screening and prioritization of large sampling sets and the H4IIE-luc assay due to its widest concentration range may compensate for time-consuming range finding tests.

With an exemplarily set limit value, derived from former determined river Elbe sediment TEQs, we could moreover deduce a H4IIE Micro EROD-based limit value that might be used as an additional quality measure for the assessment of sediments.

To improve future cross-method comparisons, we strongly recommend extracts for instrumental and *in vitro* analyses to have the same origin. Furthermore, bio-analytical methods should be standardized and strictly be conducted and evaluated by well-trained personnel.

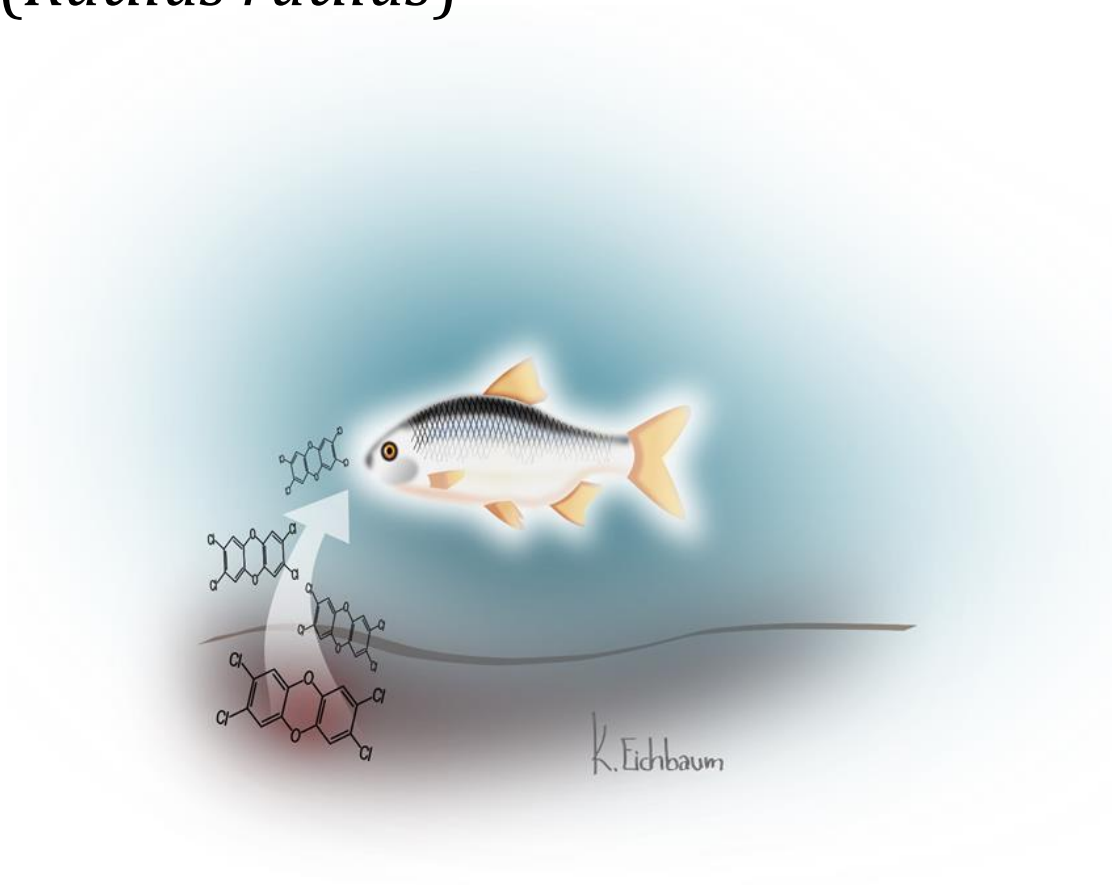
4.7 Acknowledgements

The present work forms a part of the dioRAMA project (“dioxin Risk Assessment for sediment Management Approaches”), which received funds from the “Title Group 05” of the German Federal Government. M.B. received a personal stipend from the German National Academic Foundation (“Studienstiftung des Deutschen Volkes”). Profs. Dr. Giesy and Hecker were supported by the Canada Research Chair program. Furthermore, Dr. Giesy was supported by a Visiting Distinguished Professorship in the Department of Biology and Chemistry and State Key Laboratory in Marine Pollution, City University of Hong Kong, the 2012 "High Level

Foreign Experts" (#GDW20123200120) program, funded by the State Administration of Foreign Experts Affairs, the P.R. China to Nanjing University and the Einstein Professor Program of the Chinese Academy of Sciences. Prof. Dr. Hollert was supported by the Chinese 111 Program (College of Environmental Science and Engineering and Key Laboratory of Yangtze Water environment, Ministry of Education, Tongji University). Special thanks go to Dr. Stephan Hamm and Dr. Armin Maulshagen from mas (Münster Analytical Solutions GmbH), Münster, Germany for the HRGC/HRMS measurements and their kind contribution to this work.

Chapter 5

Bio-analytical and instrumental screening of the uptake of sediment-borne, dioxin-like compounds in roach (*Rutilus rutilus*)



Parts of this chapter have been submitted to Environmental Science and Pollution Research:

Eichbaum, K., Brinkmann, M., Nuesser, L., Buchinger, S., Reifferscheid, G., Hecker, M., Codling, G., jones, P., Giesy, J.P., Hollert, H. (submitted) Bio-analytical and instrumental screening of the uptake of sediment-borne, dioxin-like compounds in roach (*Rutilus rutilus*). *Environmental Science and Pollution Research*

5.1 Abstract

To examine the uptake of dioxin-like compounds (DLCs), common roach (*Rutilus rutilus*) were exposed for 28 days to differently contaminated sediments from two major European rivers in a purpose-built facility. Dietary transfer of DLCs was investigated by exposing fish to sediments inoculated or non-inoculated with black worms (*Lumbriculus variegatus*).

Dioxin-like polychlorinated biphenyles (DL-PCBs) and polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) measured *via* high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) in sediments and whole fish were used to calculate toxicity equivalent quotients (TEQs). TEQs were compared with biological toxicity equivalents quotients (BEQs) determined *via* the 7-Ethoxyresorufin-*O*-deethylase (EROD) assay, performed with mammalian (H4IIE) and fish (RTL-W1) liver cell lines.

TEQs and BEQs indicated an uptake of sediment-borne DLCs by roach, which was independent of sediment contamination levels. For most sediment treatments, DLC uptake did not increase with time. Highest congener-specific uptake (DL-PCB 123) was 10-fold compared to control. Exposure to worm-inoculated sediment of highest overall DLC contamination caused a 2-fold (TEQ and H4IIE BEQ) greater uptake of DLCs by fish compared to the respective non-inoculated treatment. H4IIE cells showed the greatest sensitivity (0.37 ± 0.25 pM TCDD) and the strongest correlation with TEQs ($r^2 = 0.79$), hence, seem to be best suited for DLC screening of sediments and biota, amended by compound specific instrumental analysis if required.

Keywords: Dioxin • EROD • Micro EROD • BEQ • Common roach

5.2 Introduction

Sediments are well known but poorly understood sources of pollutants for aquatic environments. Their interfaces constitute areas of intense recycling of organic carbon and persistent organic pollutants (POPs) (Berglund et al. 2001) and their characteristics can influence bioavailability and accessibility of POPs (Eggleton and Thomas 2004).

Among the POPs, the so-called dioxin like compounds (DLCs) are of particular concern because they are persistent, toxic and bioaccumulative (Hilscherova et al. 2000). DLCs share similarities in structure and bind to the Aryl hydrocarbon receptor (AhR). Although this group comprises a large variety of contaminants, many of which are still unknown, the term "DLCs" in the present work exclusively refers to 12 dioxin-like polychlorinated biphenyls (DL-PCBs), the 7 polychlorinated dibenzo-*p*-dioxins (PCDDs) and the 10 dibenzofurans (PCDFs) with 2,3,7,8-chlorosubstitution, when considering results of instrumental analyses.

While PCBs were produced for various applications such as pesticide additives, fluids in capacitors and transformers as well as lubricants in cutting oils, PCDD/Fs are undesired industrial byproducts, which, among others, can be formed during incineration, chemical processes involving chlorine and paper bleaching processes (Aarts et al. 1995, Safe 1994, Weber et al. 2008). Despite being banned for several decades (Stockholm Convention; Yoder 2003) PCBs are continuously emitted into the environment through leakages from old capacitors, elastic sealants and other building materials, whereas the emission of PCDD/Fs decreased in recent years (Besselink et al. 2004) through banning of critical chlorine chemicals and emission control measures (Lee et al. 2007). DL-PCBs and PCDD/Fs are persistent and toxic organic compounds, differing in number and position of chlorine atoms bound to their basic aromatic structures. Because so much research has been done on PCBs they are a useful reference chemical for use in studies. Due to their physical chemical properties, these contaminants are globally distributed and can be found in almost every matrix including sediments, soils, wildlife, human tissue, blood and milk. Their potential to bioaccumulate and biomagnify along the food chain endangers wildlife, the environment, but also human beings (Fent 2007). Exposure to DLCs, which exceeds a hazardous level, can cause wasting syndrome, reduced fecundity, hepatic damage, dermal disorders, thymic atrophy, immunotoxicity, endocrine disruption and reproductive toxicity (Safe 1986, Whyte et al. 2000).

All DLCs, including 2,3,7,8-tetrachloro dibenzo-*p*-dioxin, which is considered the most toxic congener (Safe 1990, 1994), bind to the AhR. The AhR is a ligand-activated transcription factor in the Per-Amt-Sim (PAS) family of proteins that mediates the pleiotropic expression of a suite of genes and is believed to regulate most, if not all, adverse effects associated with

exposure to DLCs. Among the downstream effects of the AhR induction is expression of enzymes involved with xenobiotic metabolism (Okey 2007). One prototypic biomarker for the activation of the AhR by DLCs in vertebrates is induction of the phase I biotransformation enzyme cytochrome P_{450}1A (CYP1A). Within this group, member CYP1A1 7-ethoxy-resorufin-*O*-deethylase (EROD) can be found. EROD develops in vertebrate cells that have been exposed to environmental sample extracts and can be quantified by determining both, the amount of EROD-catalyzed fluorescent reaction product resorufin (built following addition of the artificial substrate 7-Ethoxyresorufin) and the amount of protein present at the moment of reaction. EROD constitutes the endpoint of both, the RTL-W1 EROD and the H4IIE Micro EROD assay used in this study.

These assays possess a certain predictive ability and might therefore serve as screening tools for the detection of DLCs in various environmental matrices (2012/252/EU 2012, Eichbaum et al. 2014). They represent supporting bio-analytical methods for classical, instrumental analysis of individual DLC congeners. One advantage of bio-analytical characterization of DLCs in complex mixtures such as sediment or tissue samples is that they provide a more realistic, ecotoxicological relevant exposure assessment and allow for both, the integration of all interactions among DLC congeners and detection of inducers not monitored in compound specific instrumental analyses (Giesy et al. 1997, Wernersson et al. 2015). Bio-analytical and instrumental results can be compared by using the approach of toxicity equivalent quotients (TEQs) and biological equivalent quotients (BEQs) (Safe 1998a).

When considering exposure of aquatic organisms to sediment-borne DLCs, different exposure pathways include aqueous (particle-, sediment- and/or water contact *via* integument and gills) and dietary exposure. Rates of accumulation are dependent on the species, its developmental stage, behavior and sexual condition as well as season, environment and climatic conditions (as reviewed by Eggleton and Thomas 2004) but are also influenced by physical chemical properties of compounds. For example, the octanol/water partitioning coefficient ($\log K_{ow}$) can be used to determine the affinity of a compound to biotic tissue or fat. For aqueous exposure pathways, a linear relationship between $\log K_{ow}$ and bioavailability of a chemical up to $\log K_{ow}$ values < 7 has been found. $\log K_{ow}$ values > 7 result in strong binding of chemicals to e.g. sediments and thus, their bioavailability decreases (Engwall et al. 1998, Hollert et al. 2002). Concerning the accumulation of DLCs, foodstuff of animal origin is one of the main sources of POPs (van Leeuwen et al. 2000) and that feeding, movement and burrow formation can increase contaminant release from sediments. Moreover, sediment ingestion might be primary routes for congeners exhibiting high $\log K_{ow}$ values (Eggleton and Thomas 2004).

In the present study a cyprinid fish, the common roach (*Rutilus rutilus*) was studied. This species is abundant in Eurasian lakes and rivers (Jamet and Desmolles 1994) and thus a species of high ecotoxicological relevance. To determine the extent to which DLC uptake in roach depends on the initial sediment contamination and/or sediment-specific characteristics, individuals were exposed to sediments, differently contaminated with DLCs (Feiler et al. 2013, Höss et al. 2010). Exposure of roach to sediments to which oligochaetes had been added, aimed in determining whether contaminated diet increases uptake of DLCs compared to uptake *via* the water phase alone. All exposure scenarios were applied to create more realistic exposure pathways of DLCs in aquatic systems. Whether an uptake of DLCs by roach is detectable by means of bio-analytical methods was investigated by analyzing homogenates of fish and sediments by means of two *in vitro* bioassays and by verifying those results *via* results obtained by chemical instrumental analysis.

5.3 Materials and methods

5.3.1 Study design

In a 28-days exposure experiment, juvenile common roach were exposed to three sediments and a 1:10 mixture of two of these sediments, which differed in characteristics and level of DLC pollution. The dietary uptake of DLCs in fish was investigated by comparing the uptake following the exposure of fish to either worm-inoculated or non-inoculated sediment treatments. Finally, sediment and biota samples were extracted, subjected to clean-up and bio-analytically investigated using the 7-Ethoxyresorufin-*O*-deethylase (EROD) assay with RTL-W1 cells and the Micro EROD assay with H4IIE cells. Concentrations of 29 DLCs were determined using capillary gas chromatography/high resolution mass spectrometry (HRGC/HRMS).

5.3.2 Sediments

Details on sediment samples can be taken from *table 2.1* and s*ection 2.1*. Exposure experiments to sediments ZE and EBR/ZE were conducted in summer 2012 (07.06.2012 - 14.07.2012), those of sediments PR and EBR in autumn 2012 (24.10.2012 - 30.11.2012).

5.3.3 Fish

Juvenile common roach were obtained from a pond aquaculture of a local supplier (Inquadro, Aachen, Germany), transported to RWTH Aachen University and transferred to aerated 1,000 L tanks. Fish were maintained under flow-through conditions in tap water (approx. 15 °C; pH 7.8 ± 0.2; NH_3 < 0.1 mg/L) with a water exchange rate of 0.5 – 1/d. Light and dark phases were 12 h each. Fish were fed ad libitum with frozen chironomids (Aquahobby, Peine, Germany) and allowed to acclimatize for at least one month. A total of 156 fish were used and distributed among the exposure units in similar dimensions, on average 118 ± 5 mm length and 36 ± 6 g wet body mass (wm). Fish were used in accordance to the Animal Welfare Act and with permission of the federal and local authorities, registration no. 84-02.04.2011.A368.

5.3.4 Experimental conditions

Exposures of fish were conducted in accordance with OECD test guideline 305 with the most important deviations including the lack of true flow-through conditions and depuration time as well as the exposure to sediment (Ahlf et al. 2002). Experiments were conducted in a purpose-built exposure facility in an air-conditioned room at the Institute for Environmental Research, RWTH Aachen University. The exposure facility, which enabled simultaneous testing of three sediments, consisted of six independent exposure systems, each consisting of five 100 L aquariums. Four aquaria were used as exposure tanks and one as a sump (*Figure 5.1*).

The latter enabled continuously pumping and recirculation of water to the individual aquariums at a rate of about 10 L/min. Water was aerated (approx. 20 L/min) and temperature was maintained at 17.0 ± 0.6 °C by use of stainless steel heat exchangers connected to a re-circulating chiller *via* the sumps. Light/dark phases were 12 h each.

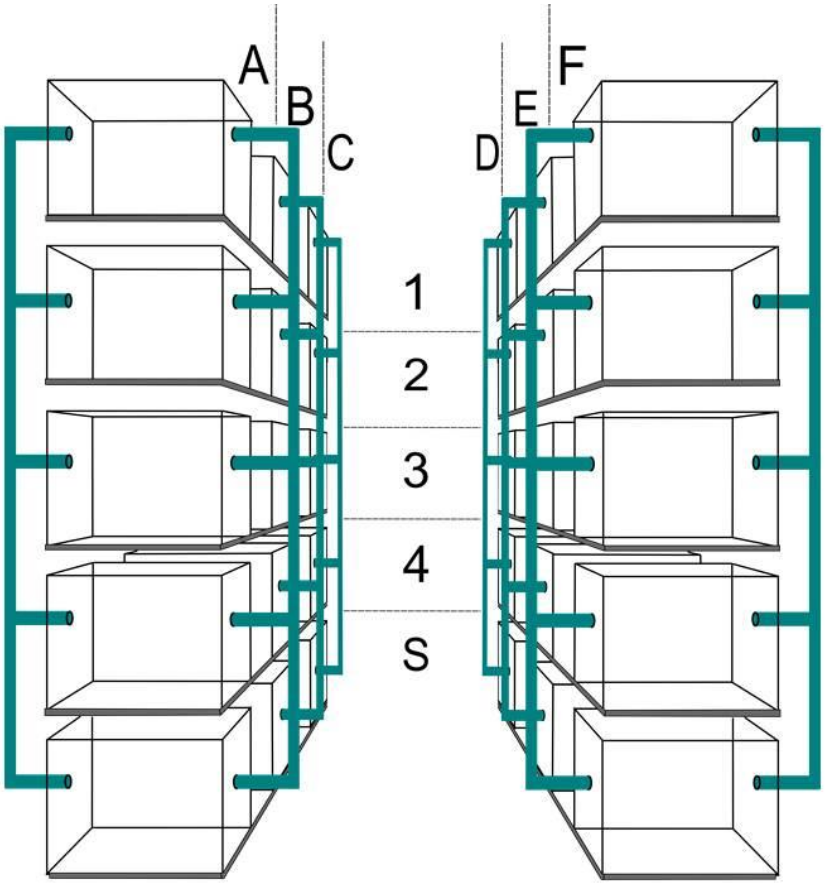


Figure 5.1 Technical drawing of the exposure facility composed of six independent systems (A - F) and respective aquariums (1 - 4); each system is equipped with a pump sump (S), in which regulation of oxygen and temperature, as well as the permanent measurement of temperature and turbidity took place.

5.3.5 Experimental set up

5.3.5.1 Exposure scenarios

Individual roach were exposed to sediments under two different exposure scenarios: (1) Sediments EBR, EBR/ZE, PR and ZE that were inoculated with 100 g wm black worms (*Lumbriculus variegatus*, Fauna topics GmbH, Marbach, Germany) and which are referred as (+) approaches, and (2) non-inoculated sediments ZE and PR, which are referred to as (-) approaches. Here, fish were daily fed with uncontaminated black worms at a rate of 1% collective body mass (body mass per aquarium). Feeding was only performed on populated aquariums.

5.3.5.2 Conduct of exposure experiments

Each homogenized sediment was tested in four pseudo-replicate tanks connected *via* a sump. Sediment (8 kg wet mass (wm)/replicate) was covered with tap water (approx. 75 L) avoiding re-suspension. Sediments were allowed to consolidate over ten days. In case of (+) scenarios,

sediments were inoculated with 10 g wm of living black worms (*L. variegatus*) per replicate before consolidation. Following this period, six individuals of (*R. rutilus*) from the maintenance tank were transferred to each replicate tank (except the sump), and in the case of (-) scenarios were fed daily with 1 g wm of living black worms. Following an exposure period of 10 days, fish were transferred to new tanks that had been subjected to the same 10 days consolidation period as described above, to maintain stable sediment contaminant concentrations. This procedure was repeated until an exposure period of 28 days was reached.

5.3.5.3 Daily measurement of limno-chemical parameters

During both, consolidation and exposure periods limno-chemical parameters were daily measured in four pseudo-replicates and the sump of each test system, respectively. Parameters that were consistent among replicate tanks of a system until the first fish transfer were thereafter only monitored in the sump. Daily measured limno-chemical parameters included: *temperature* and *conductivity* (conductivity electrode LF91 KLE 1/T, WTW, Weilheim, Germany), *dissolved oxygen* (DO meter HI 9146, Hanna Instruments, Kehl, Germany), *pH* (pH meter, Mettler Toledo AG, Schwerzenbach, Switzerland), *redox potential* (ORP 15, VWR international, Darmstadt, Germany) and *turbidity* (turbidimeter, Ratio/XR, HACH company, Loveland, Colorado, U.S.A.). The *concentration of alkaline earth metals* (i.e. total hardness) was determined in 100 ml water filtrates (0.7 µm glass-fiber filters, Macherey und Nagel GmbH & Co.KG, Düren, Germany) containing both an indicator buffer tablet (Merck, Darmstadt Germany) and 1 ml of ammonia (32%, Carl Roth GmbH + Co.KG, Karlsruhe, Germany). Samples were titrated (25 ± 0.075 ml, 20 °C, Brand, Germany) with Titriplex solution B (Merck) until colour change.

5.3.5.4 Sediment and biota sampling

Aliquots of the three sediments and the mixture were taken prior to the exposure experiments. Fish samples were taken on day 4, 7, 14 and 28. Specifically, all fish from one tank (in case no mortality occurred: six individuals) were removed (starting with the first tank on day 4 and ending up with the fourth tank on day 28), anaesthetized using benzocaine and killed by exsanguination. Standard lengths and mass of fish were determined. Fish were wrapped in aluminium foil and stored at -80 °C until further use. Six randomly chosen animals from the maintenance tank were treated the same way and used as “summer” and “autumn” controls.

Condition factor (K) of each fish was determined according to equation 5.1 (Fulton 1902, Iannuzzi et al. 1995). Where N represents the numerical factor 5, L is standard length measured in millimetres and W is mass measured in grams.

$$K = 10^N * W * L^{-3} \quad (5.1)$$

5.3.6 sample preparation, extraction and clean-up

Preparation, extraction and clean-up of sediment and biota samples were performed according to *section 2.2*, but the following additional steps and/or modifications were applied for the biota samples:

Homogenates of whole fish (consisting of six frozen animals from one treatment (refer to *section 5.3.5.2*) were prepared by mixing in a Philips blender (2096/00, Aschaffenburg, Germany) for 10 min under addition of deionized water. After freeze-drying of the biota samples, these were sieved < 2 mm and masses of fractions > 2 mm of fish homogenates (mostly containing bones and scales) were determined gravimetrically. Under the assumption that wet mass (wm) equates dry mass (dm) in the fraction > 2 mm, re-calculation of the initial homogenate fresh masses was conducted. For extraction of biota samples, 3 g dm of sample were mixed with the same amount of sodium sulfate as was used for sediment samples (5 g).

The clean-up of biota samples included the steps of the gravimetrical determination of the fat content, which was not applied for sediments (refer to *Figure 2.2*).

5.3.7 Bio-analytical and HRGC/HRMS analysis

5.3.7.1 The RTL-W1 EROD and H4IIE Micro EROD assay

The EROD assay with cell lines RTL-W1 and H4IIE were performed according to *sections 2.3.1* and *2.3.2*, respectively.

5.3.7.2 Chemical analysis by means of HRGC/HRMS

The HRGC/HRMS analyses of sediment and biota extracts was performed as described in *section 2.4.1*.

5.3.7.3 Calculation of TEQs and BEQs

The calculation of BEQs was made according to equation 2.1 in presented in *section 2.3.4*, whereby due to the partially very low efficacy of the samples, *x* in *equation 2.1* was the

concentration at 10% effect level. TEF-based TEQs were calculated according to *equation 2.5* with WHO_{2005}TEFs.

5.3.8 Data analysis and presentation

All plots and linear correlation analyses (Pearson correlation; $p < 0.05$) were conducted in GraphPad Prism 5 (La Jolla, CA, USA). Bio-analytical data was processed *via* Excel (Microsoft Office Excel 2003) and concentration-response curves were plotted using GraphPad Prism using a non-linear regression and a dose-response stimulation (log agonist vs. response). Statistical analyses were conducted by use of the software Sigma Stat 3. Normally distributed (Kolmogorov-Smirnov test, $p < 0.05$) data sets with equal variances (Levene's test, $p < 0.05$) were analyzed by use of parametric one-way ANOVA (Dunnett's test; $p < 0.01$). Data sets that were not normally distributed were analyzed with a Kruskal-Wallis ANOVA on ranks ($p < 0.01$) was performed with Dunn's test as post-hoc test ($p < 0.01$). A Students t-test ($p < 0.05$) was used to statistically analyze the impact of contaminated feed. All graphical drawings were produced using the vector graphic program Inkscape 0.48.

5.4 Results

The following sections will give the results of general sediment characteristics, experimental conditions during the exposure experiments, fish condition/ mortality as well as instrumentally and bio-analytically derived results.

5.4.1 Characterization of sediments

Despite the missing natural origin of the lab-prepared 1:10 mixture EBR/ZE, this mixture will also be termed "*sediment"* to simplify matters.

The Elbe sediments PR and ZE clearly differed from Rhine sediment EBR in terms of composition. Generally, Elbe sediments were characterized by a greater amount of sand and thus, lesser proportions of silt and clay. Despite this smaller fraction of fine particulate matter, these sediments had greater concentrations of TOC and higher losses on ignition (refer to *Table 2.1*).

Generally, HRGC/HRMS measurements showed that sediments PR, EBR and the mixture EBR/ZE were equally contaminated with 12 DL-PCBs and 17 PCDD/Fs, whereas sediment ZE showed an approximately 2 and 4-fold higher contamination, respectively (refer to *Table 2.1*). RTL-W1 EROD and H4IIE Micro EROD BEQs determined for DL-PCB and PCDD/F fractions

were magnitudes higher than the concentrations determined *via* HRGC/HRMS with a comparable higher signal strengths for PCDD/Fs than for DL-PCB fractions.

5.4.2 Limno-chemical parameters

Suspended matter concentrations of sediments EBR and EBR/ZE (consisting of 9 parts EBR) were approximately twice of those observed for the remaining scenarios (*Figure 5.2*). The non-inoculated (-) approaches of sediments PR and ZE showed lower particulate matter concentrations as the respective (+) approaches (*Figure 5.2*). In general, particulate matter concentrations were highest for all EBR containing scenarios and all (+) scenarios.

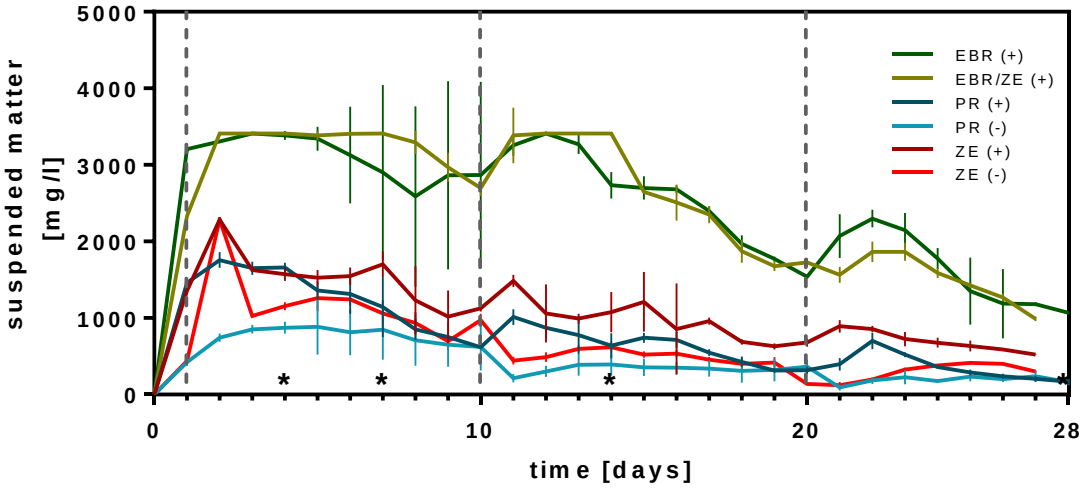


Figure 5.2 Course of the suspended particulate matter concentration during a 28-days experiment with common roach (R. rutilus) exposed to six sediment approaches: EBR = Ehrenbreitstein, PR = Prossen, ZE = Zollelbe; EBR/ZE = mixture consisting of 9 dry mass (dm) parts EBR and one dm parts ZE. Approaches marked with (-) in contrast to the remaining ones, were not inoculated with black worm. Graphs represent the concentrations measured in four aquariums of one system (one approach) and error bars show standard deviations thereof. Asterisks mark dates of fish sampling, each performed on one of the four aquariums of the respective sediment approaches. Dashed lines mark dates of a fish transfer to freshly consulidated sediment.

Average temperature and dissolved oxygen concentrations during exposure to the 6 treatments were 17 ± 0.6 °C and 8.3 ± 1.4 mg/L, respectively. Redox potential, conductivity, pH and water hardness along the six treatments averaged to 175.5 ± 17.0 mV, 342.2 ± 17.8 µS*cm^{-3}, 7.83 ± 0.42 and 78.5 ± 6.2, respectively.

5.4.3 Mortality and condition of test animals

Mortality was observed in all treatment groups, particularly at the end of the 28-day exposure period, reaching values between 4% (EBR, PR, PR (-)) and 25% (ZE). Highest mortalities of 21% (EBR/ZE, ZE (-)) and 25% were observed for fish exposed to sediments containing ZE sediment. Health effects during the exposure experiments have not been observed, partly

because high turbidity complicated such observations. Control fish originating from the tap water filled maintenance tank did not show mortality or any effects.

The index of condition K of fish in the summer and autumn run was 1.8 ± 0.1 and 1.9 ± 0.2, respectively. A student's t-test between condition indices of fish prior to and at the end of each experimental period as well as between fish of different feeding strategies showed no significant changes in condition.

5.4.4 Bio-analytical and HRGC/HRMS analysis

5.4.4.1 Bio-analytical quality criteria

Overall, 78 measurements were performed with extracts of fish in both assays. Thereby, limits of detection (LOD) and quantification (LOQ) exhibited values of 0.37 ± 0.25 and 0.57 ± 0.27 pM TCDD in the H4IIE Micro EROD, respectively and 1.20 ± 0.71 and 2.03 ± 1.24 pM TCDD in the RTL-W1 EROD assay, respectively. In contrast to the H4IIE Micro EROD assay, an overlap between LOQ and EC_{10}TCDD became obvious for RTL-W1 cells. While the maximum EROD induction strengths of sediment fractions averaged to 70 ± 18% and 80 ± 9% in the H4IIE Micro EROD and RTL-W1 EROD assay, respectively, induction strengths of fish homogenate extracts only averaged to 54 ± 18% and 59 ± 13% in the H4IIE Micro EROD and RTL-W1 EROD assay, respectively.

5.4.4.2 BEQs and TEQs of whole fish extracts with respect to the sediment type

Concentrations of BEQs and TEQs, reported on a lipid mass basis, in fish exposed to the four sediments to which *L. variegatus* had been added (referred as (+)) are given in *Figure 5.3*. Thereby, BEQs in fish exposed to EBR (+) exhibited a temporal dependency, which was significant compared to the control in both the RTL-W1 EROD ($r^2 = 0.808$) and H4IIE Micro EROD ($r^2 = 0.864$) assay. BEQs measured in fish exposed to the remaining sediments exhibited a similar trend: A marked uptake (day 4) was followed by a slight decrease in concentration of BEQ (day 7) followed by an increase in concentration of BEQ (day 14), which in turn was followed by another decrease in concentrations of BEQ (day 28).

Concentrations of BEQs in fish on sediment EBR (+) were significant higher compared to the control from day 7 (H4IIE) and 14 on (RTL-W1). However, there was no clear tendency observed for fish exposed to EBR/ZE (+). Concentrations of BEQs in fish at day 14 were the only ones significantly different from the control in both assays. In contrast to concentrations of BEQs determined *via* the RTL-W1 EROD assay, H4IIE BEQs in fish exposed to PR (+) on all sampling dates were significantly different from the control (ANOVA, Dunnett's test;

$p < 0.01$). The patterns of BEQs determined for fish exposed to ZE (+) and that of EBR/ZE (+) were similar for both assays. More detailed, BEQs were significantly different from the control on days 4 and 7 for H4IIE cells and day 14 for RTL-W1 cells (*Figure 5.3*).

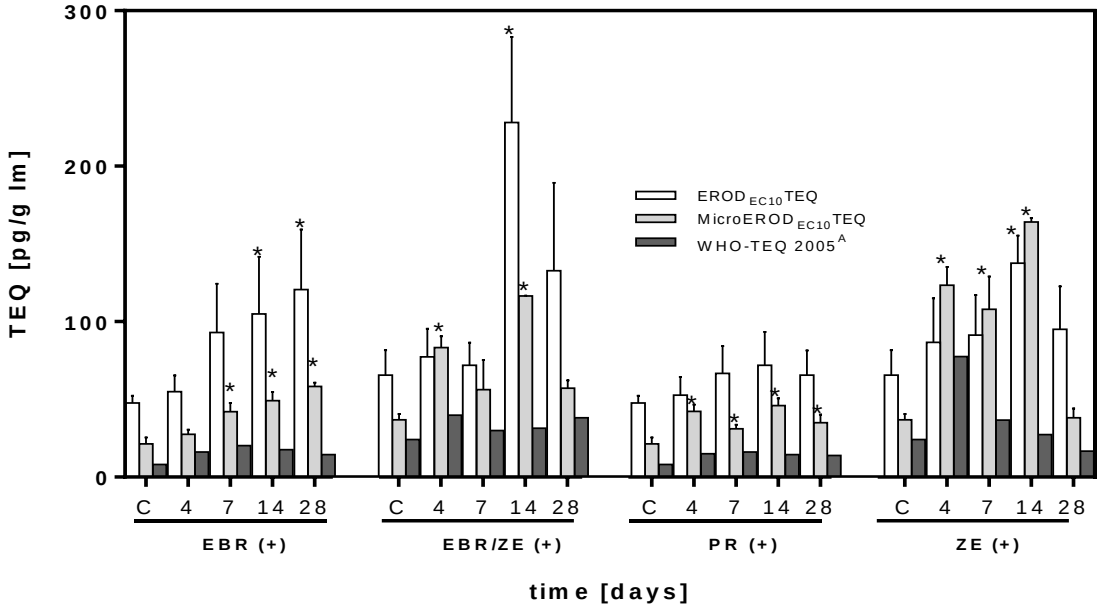


Figure 5.3 Lipid mass (lm) normalized, instrumentally (HRGC/HRMS; WHO2005TEQs; A = congeners below the detection limit were excluded) and bio-analytically (RTL-W1 EROD; H4IIE Micro EROD) determined toxicity equivalents (TEQs) of whole fish homogenates of common roach (R. rutilus), exposed to four different worm-inoculated sediments for 28 days. EBR = Ehrenbreitstein, PR = Prossen, ZE = Zollelbe; EBR/ZE = mixture consisting of 9 dry mass (dm) parts EBR and one dm parts ZE. White (RTL-W1 EROD assay) and light grey (H4IIE Micro EROD assay) bars represent TEQs of three independent biological replicates, calculated on EC10 basis. Error bars show standard deviations thereof. Asterisks mark results significantly different to C = control (parametric one-way analysis of variance, ANOVA, Dunnett's test; $p < 0.01$ with Kolmogorov-Smirnov and Levene's test as pre-tests).

Concentrations of BEQs in fish on sediment EBR (+) were significant higher compared to the control from day 7 (H4IIE) and 14 on (RTL-W1). However, there was no clear tendency observed for fish exposed to EBR/ZE (+). Concentrations of BEQs in fish at day 14 were the only ones significantly different from the control in both assays. In contrast to concentrations of BEQs determined *via* the RTL-W1 EROD assay, H4IIE BEQs in fish exposed to PR (+) on all sampling dates were significantly different from the control (ANOVA, Dunnett's test; $p < 0.01$). The patterns of BEQs determined for fish exposed to ZE (+) and that of EBR/ZE (+) were similar for both assays. More detailed, BEQs were significantly different from the control on days 4 and 7 for H4IIE cells and day 14 for RTL-W1 cells (*Figure 5.3*).

The comparability of BEQs determined by use of both assays was inversely proportional to sediment DLC concentrations. While linear correlation between the two assays was not significant ($r^2 = 0.11$) when fish exposed to ZE (+) were included, correlation was significant ($r^2 = 0.32$) when fish exposed to ZE (+) were excluded. BEQs determined *via* both assays and

respective WHO_{2005}TEQs showed comparable trends. (*Figure 5.3)*. Looking at the results of all three methods, concentrations of TEQs and BEQs in the roaches increased from sediments EBR (+) and PR (+) over EBR/ZE (+) to ZE (+).

In total, 29 ± 18% of the RTL-W1 and 44 ± 15% of the H4IIE BEQs could be explained through the WHO_{2005}TEQs. Thereby, the correlation between H4IIE BEQs and TEQs ($r^2 = 0.62$) was distinctly higher than that of RTL-W1 BEQs and TEQs ($r^2 = 0.25$).

5.4.4.3 Classification of DLC uptake on basis of environmental quality standards

In 2013 regulation 2013/39/EU entered into force (2013/39/EU 2013), which established an environmental quality standard (EQS) of 6.5 pg TEQ/g fm for DLCs in biota. To compare the present data with this EQS lipid mass (lm) data underlying *Figure 5.3* was normalized to fresh mass (fm) (data not shown). BEQs ranged from 1.8 to 4.9 pg BEQ/g fm and 1.0 to 5.5 pg BEQ/g fm in the RTL-W1 EROD and H4IIE Micro EROD assays, respectively. Chemical TEQs ranged from 0.7 to 3.6 pg TEQ/g fm for the sum of WHO-PCDD/Fs and DL-PCBs.

5.4.4.4 DLC uptake as a function of ingestion

The worms were not chemically investigated, but an uptake of sediment by *L. variegatus* could be observed through the skin a few hours following their transfer on the sediments (*Figure 5.4*).

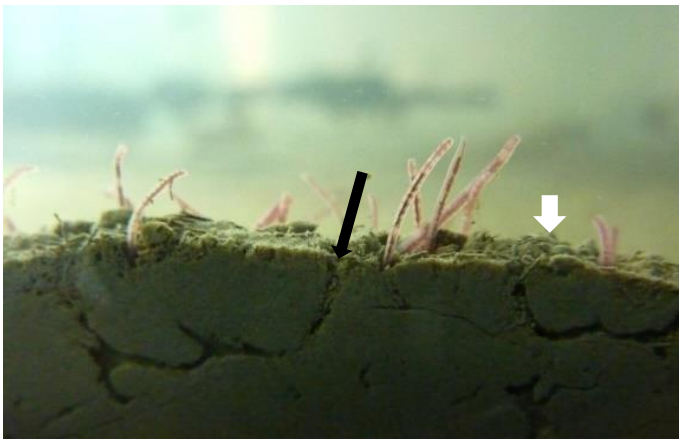


Figure 5.4 Tunnels (black arrow) and feces (white arrow) of black worms (Lumbriculus variegatus) one day after the sediment has been inoculation.

On a relative basis, uptake factors aligned both seasonal (summer; autumn) and methodological (BEQs; TEQs) differences among fish exposed to the different scenarios. Uptake factors of fish exposed to sediment PR predominantly showed uptake factors > 1.

The general pattern of uptake factors determined for fish on PR (*Figure 5.5a*) and ZE (*Figure 5.5b*) differed in that way that uptake factors determined for fish exposed to PR (*Figure 5.5a*) did not show an temporal trends and were relatively consistent among the different methods (EROD, Micro EROD and chemical analysis). However, the altitude of uptake factors of fish exposed to sediment ZE ((-) 1.8; (+) 2.0) on average did not significantly differ from those calculated for fish on PR.

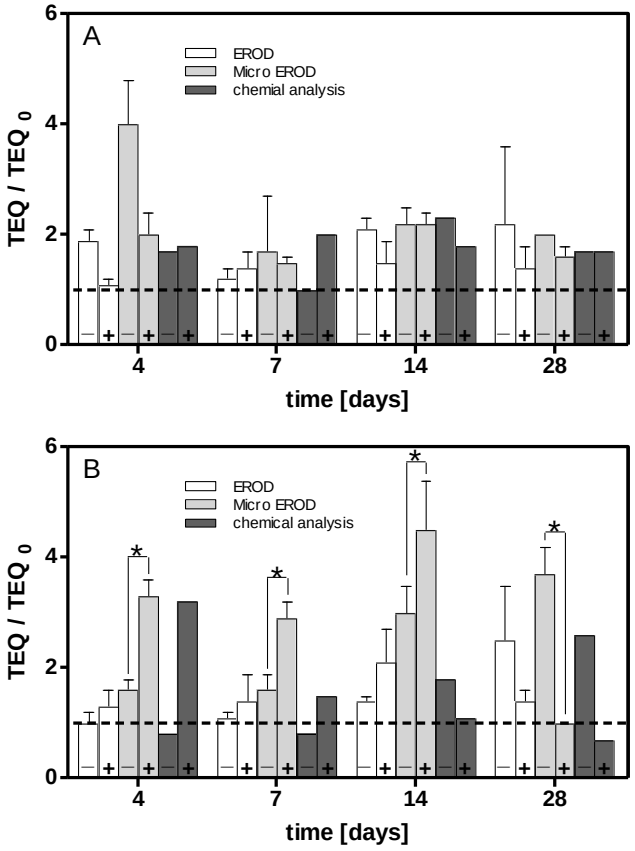


Figure 5.5 Comparison between instrumentally (HRGC/HRMS, WHO_{2005}TEQs) and bio-chemically (RTL-W1 EROD, H4IIE Micro EROD) determined factors (TEQ/TEQ control) of fish exposed to sediment Prossen (a) and Zollelbe (b) under consideration of presence (+) and absence (-) of contaminated feed. Dashed line marks the level above which uptake took place. *White* (RTL-W1 EROD assay) and *light grey* (H4IIE Micro EROD assay) bars represent TEQs of three independent biological replicates, calculated on an EC_{10} basis. Error bars show standard deviations thereof. Asterisks mark significant differences between (-) and (+) approaches analyzed by using a student's t-test ($p < 0.05$).

In contrast to uptake factors determined for fish on sediment PR (+) and PR (-), uptake factors in fish exposed to sediment ZE in the presence of worms in the sediment were significant (student's t-test, $p < 0.05$) higher than in the absence of worms. In total, the uptake factors in fish on ZE (+) were 2.1-fold (TEQs) and 1.8-fold (BEQs, at least for the H4IIE Micro EROD BEQs) higher than uptake factors in fish on ZE (-).

The instrumental derived uptake factors determined for ZE (-) showed an opposite temporal trend compared to uptake factors in fish exposed to ZE (+). More detailed, BEQs determined

by both EROD assays showed a temporal increase for the ZE (-) approach, while in fish on the ZE (+) approach, except day 14) a high initial uptake of DLCs was followed by a decrease. In contrast to the ZE (+) approach, H4IIE Micro EROD BEQs determined in fish exposed to ZE (-) correlated well with instrumental data ($r^2 = 0.99$; n = 4).

5.4.4.5 Congener-specific uptake following 28 days of exposure

This section focuses on concentrations of individual DLCs and their uptake by roach following 28 days of exposure to four (+) sediments. DLC congeners, their octanol-water portioning coefficient (log K_{ow}), water solubility and initial sediment concentrations are given in *Table 5.1*, accompanied by their respective uptake factors in roach.

DL-PCB uptake factors ranged between 0.5 (ZE (+)) and 4.8 (EBR/ZE (+)) (*Table 5.1).* In total, only four of the factors were < 1. On the chemical site, PCB 81 and 169 occurred at small concentrations or were not detected in sediments, thus were not detected in roach. The remaining non-*ortho* congeners were present in sediments at relatively small (PCB 126) or high (PCB 77) concentrations, but exhibited equivalent uptake factors in roach. For mono-*ortho* PCBs, most of the uptake factors were independent of initial concentrations in the sediments. For instance, the concentration of PCB 118 among the four sediments was, on average, 75 times the concentrations of PCB 114. Nevertheless, uptake factors calculated for fish were equivalent.

The largest uptake factors among all chosen congeners were observed for PCB 123. Despite small initial concentrations of this congener in all sediments, uptake factors of as much as 10-fold higher compared to control were observed (*ZE (+) day 4*, *Figure 5.6*).

5.4.4.6 Temporal congener-specific uptake depending on sediment characteristics

The temporal patterns of uptake of DL-PCB by roach exposed to sediment mixture EBR/ZE (+) were compared with those obtained for each sediment EBR (+) or ZE (+) alone (*Figure 5.6*). Predicted concentrations of the 12 DL-PCBs in sediment EBR/ZE, deduced from the 1:10 mixing ratio, on average only differed by 12% from the actual concentrations.

While in fish exposed to EBR (+) most of the congeners exhibited a slight increase in uptake factors over time, fish exposed to ZE (+) showed a high initial uptake, followed by a decrease.

Fish exposed to the mixture exhibited the same high initial uptake as observed for fish exposed to ZE sediment. These uptake factors moreover showed comparable values ranging from 1.7 to 9.0 and 2.7 to 10.0 for EBR/ZE (+) and ZE (+), respectively.

Table 5.1 Log K_{ow} values, water solubilities as well as uptake factors (in fish after 28 days of exposure to four sediments and normalized to concentration of fish control) and initial concentrations (in sediments) of non-*ortho* (77, 81,126, 169) and mono-*ortho* (105, 114, 118, 123, 156, 157, 167, 189) PCBs and several PCDD/Fs. EBR = Ehrenbreitstein, PR = Prossen, ZE = Zollelbe; EBR/ZE = mixture consisting of 9 dry mass (dm) parts EBR and one dm parts ZE. Uptake factors were calculated on a dry mass (dm) basis.

			Uptake factors (c/c_0) in fish exposed to sediment				**Sediment congener concentration [pg/g dm]**			
	log K_{ow} [a]	**Water solubility at 25 °C** [a,c]	**EBR**	**EBR/ZE**	**PR**	**ZE**	**EBR**	**EBR/ZE**	**PR**	**ZE**
PCB 77	6.63 [b]	0.0298	2.5	1.6	2.1	1.7	297	422	357	948
PCB 81	6.34	0.0532	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	7	12	28
PCB 126	6.98	0.0094	1.5	2.2	1.0	1.0	25	35	24	50
PCB 169	7.41 [b]	0.0025	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
PCB 105	6.79 [b]	0.0136 [b]	1.6	1.5	1.3	1.4	446	453	391	939
PCB 114	6.98	0.0094	1.5	1.4	1.2	1.4	23	32	29	62
PCB 118	7.12 [b]	0.0071	1.5	1.4	1.2	1.4	1600	1970	2330	5300
PCB 123	6.98	0.0094	3.8	4.8	3.1	2.9	21	34	23	81
PCB 156	7.60 [b]	0.0017	1.6	1.9	1.4	0.9	533	607	1030	1170
PCB 157	7.62	0.0016	1.5	2.0	1.1	0.9	102	121	223	162
PCB 167	7.50 [b]	0.0021	1.4	1.9	1.2	0.7	339	369	518	706
PCB 198	8.27	0.0003	1.4	1.9	1.3	0.5	112	132	279	270
12378-PentaCDD	6.64 [b]	0.0009	1.4	*n.a.*	1.2	*n.a.*	1.1	2.8	0.7	4.4
2378-TetraCDF	6.63	0.0019	2.3	2.6	1.7	1.8	0.6	5.8	0.5	3.0
12378-PentaCDF	7.27	0.0003	*n.a.*	4.3	*n.a.*	4.1	2.2	6.6	3.7	67.7
23478-PentaCDF	7.27	0.0003	2.6	3.8	1.8	2.8	3.1	7.4	4.5	81.1

a = estimated values (unless highlighted with [b]) from the US EPA data base "EPISuite"; b = experimentally determined value from the EPI database ;

c = estimated from the respective log K_{ow}; n.d. = not detectable; n.a. = not analyzable (in case congener was not detectable in the control group)

The high initial uptake by fish exposed to the mixture was followed by a constant baseline, which stayed unaffected by the decrease of uptake factors as it was observed for fish on ZE (+).

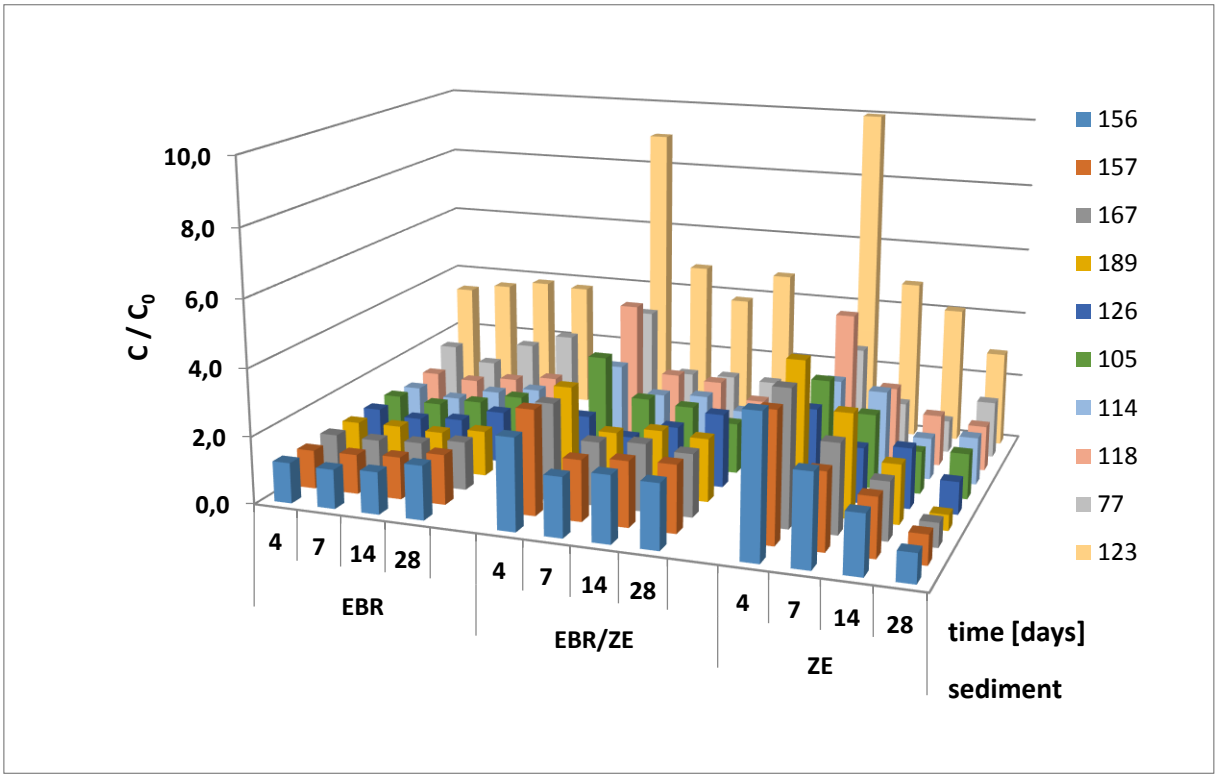


Figure 5.6 Temporal course of the control normalized uptake of 12 WHO-DL-PCB congeners (except PCB 169) in common roach (R. rutilus) exposed to sediments EBR = Ehrenbreitstein, ZE = Zollelbe and a 9:1 dry mass mixture thereof (EBR/ZE) for 28 days. Kongeners (legend) were determined instrumentally (HRGC/HRMS).

5.5 Discussion

The following section will characterize the investigated sediments, discuss the condition and mortality of fish based on the experimental conditions. In particular, instrumentally and bio-analytically derived results will be discussed with respect to method comparability as well as with regard to congener-specific and dietary uptake behaviour of DLCs in fish.

5.5.1 Characterization of sediments

The Elbe sediments PR and ZE clearly differ from Rhine sediment EBR since they exhibited relatively lesser proportions of silt and clay, greater concentrations of TOC and higher losses on ignition (refer to *Table 2.1*), indicating a higher number of possible DLC binding sites of Elbe sediments, which in principle allow for higher concentrations of contaminants.

Instrumentally (HRGC/HRMS) and bio-analytically (RTL-W1 EROD and H4IIE Micro EROD) derived results both should the same trend with sediment ZE to be distinctly higher

contaminated with DLCs compared to the remaining sediments. Thereby, BEQs were magnitudes higher than TEQs, indicating effects of additional dioxin-like compounds, not targeted by chemical analysis.

5.5.2 Limno-chemical parameters

Limno-chemical parameters provide important information on the environmental conditions during animal experiments, and moreover form the basis for the availability of xenobiotics and reflect sediment dynamics.

Sediments differed greatly with respect to their percentage of fine particulate matter (refer to *Table 2.1*). This characteristic influenced turbidity during the exposure experiments and, hence, the amount of particulate matter present in the water phase and most likely the amount of particle-bound DLCs. EBR as a fine particulate sediment influenced all EBR containing treatments in that way that highest particulate matter concentrations were found in these treatments (*Figure 5.2*). Particulate matter concentrations moreover were dependent on fish behaviour: In contrast to the non-inoculated (-) approaches, fish in the respective (+) approaches began to feed on the worms after their transfer, and in turn caused higher re-suspension of sediment (*Figure 5.5a, b*). Because suspended matter concentrations are known to influence DLC uptake kinetics from water (Ahlf et al. 2002), comparably higher concentrations in EBR containing scenarios and all (+) scenarios most likely have led to a higher DLC uptake in fish through the water phase.

Average temperature and dissolved oxygen concentrations during exposure met criteria required by OECD 305 with temperature changes < 2 °C and dissolved oxygen concentrations $> 60\%$ saturation (OECD 2011). Redox potential and conductivity were inconspicuous, the pH stayed neutral to slightly alkaline and the average value of the total water hardness equated 1.4 mmol Calcium oxide/mL and thus was classified as soft (Breitung and Keller 2010). Hence, all limno-chemical parameters were in the ranges of tolerance of common roach.

5.5.3 Mortality and condition of test animals

Whether *mortality* was caused by xenobiotics released from the different sediments is unclear, but it can be excluded that they were due to limno-chemical conditions, which were comparable among treatment groups and in the range of tolerance (refer to *section 5.5.2*). Health effects neither have been observed during the exposure experiments, nor in control fish in the maintenance tank.

The index of condition K gives information on the fish's fitness and nutritional status. It assumes that the heavier the fish in relation to its standard length, the better its condition (Kortet et al. 2003). K values of fish of summer and autumn run corresponded to former K values measured for common roach (Jamet and Desmolles 1994, Kortet et al. 2003) and did not show any experimental-related changes.

These findings and the fact that K constantly was in the range of *excellent, good* and *fair* (K = 1.6; 1.4 and 1.2, respectively) (Iannuzzi et al. 1995), shows that test animals did not suffer from any stress caused by experimental or environmental conditions.

5.5.4 Bio-analytical and HRGC/HRMS analysis

5.5.4.1 Bio-analytical quality criteria

LODs and LOQs determined for overall 78 measurements indicated the H4IIE Micro EROD to be the most sensitive assay. The use of EC_{10} values for BEQ calculation is appropriate as long as these values are well above assay-specific LODs and LOQs, which was the case using the H4IIE Micro EROD assay, but an overlap between LOQ and EC_{10}TCDD became obvious for RTL-W1 cells. Hence, RTL-W1 BEQs have to be evaluated with care. Maximum H4IIE and RTL-W1 EROD induction strengths observed for sediment and biota showed that equal efficacy of sample and standard (Villeneuve et al. 2000) was not reached for most fish extracts, reflecting difficult initial test conditions of this matrix.

5.5.4.2 BEQs and TEQs of whole fish extracts depending on the sediment type

The following section aims of exploring the question whether different DLC contaminations of sediments influenced uptake kinetics of those compounds into biota (*Figure 5.3*).

When interpreting BEQs derived in this study, it has to be considered that the insufficient initial clean-up possibly has led to impurity-related false-positive effects in extracts of fish and sediment. Due to the missing PCB and dioxin separation in fish extracts, these substance classes cannot be interpreted separately (see *section 2.4.1*). However, acording to Hasegawa (Hasegawa et al. 2007) and our own experience, concentrations of BEQs accounting for the sum of DL-PCBs and PCDD/Fs correlate better with respective concentrations of TEQs as compared to concentrations of BEQs and TEQs of the single fractions. An explanation for that is that dioxins are much more potent in activating the AhR as compared to DL-PCBs. Hence, fish homogenate extracts solely allowed for evaluating the overall dioxin-like potential of the sum of DL-PCBs and PCDD/Fs present in these samples.

Previous studies revealed that fine-grained bottom sediments such as EBR can act as reservoirs by reducing toxicity potential to aquatic organisms. Due to their sorptive nature they accumulate contaminants more effectively (as reviewed by Eggleton and Thomas 2004). But once re-suspended (*Figure 5.2*) this reservoir can become a source, which in the case of fish exposed to EBR (+), could have led to temporal increasing concentrations of BEQs as measured in both assays. Trends observed for the remaining sediments could be explained by feeding behaviour. As aforementioned, fish began to feed on the worms as soon as they were transferred to sediments containing worms. This transfer took place on days 0, 10 and 20. Sampling days 4 and 14, which are located closest in time to these transfer activities, exhibited the overall highest concentrations of BEQs (*Figure 5.3*).

By comparing BEQs measured in exposed and in control fish (*Figure 5.3*), a performed ANOVA partly could prove significant uptakes of DLCs in exposed fish. This uptake appeared from day 4 on, especially using the H4IIE Micro EROD assay. However, the most significant uptakes could be determined in fish exposed to EBR (+) using both EROD assays. This supports the aforementioned hypothesis that due to its fine particulate characteristic, sediment EBR generates relatively higher concentration of DLC loaded particulate matter to the exposed fish. The fact that fish exposed to all ZE containing sediment treatments (i.e. ZE (+) and EBR/ZE (+)) could indicate that the high DLC concentrations present in sediment ZE caused higher uptakes in fish exposed to the mixture due to the uptake supporting characteristics of sediment EBR, present in the mixture. But in this circumstance, it should be mentioned that DL-PCB and PCDD/F concentrations on a lipid mass (lm) basis average were 4.6-fold higher in autumn than in summer control fish, although the absolute lipid mass for both groups of fish were similar. Hence, similarities of DLC uptake in fish exposed to ZE (+) and mixture EBR/ZE (+) could also reflect seasonal differences.

The fact that the correlation between the degree of sediment contamination with DLCs and the altitude of BEQs was only significant when fish exposed to ZE (+) was excluded, reflects the mismatch of both EROD assays in fish of this treatment. H4IIE Micro EROD BEQs, which generally were smaller compared to RTL-W1 EROD BEQs in this treatment were clearly higher. Hence, it might be assumed that highly contaminated sediment ZE led to high DLC uptakes in fish, which in case of the EROD assay with RTL-W1 could have caused inhibitory effects (Behnisch et al. 2001b, Lorenzen et al. 1997). However, WHO_{2005}TEQs measured in fish of this treatment, from day 7 on, speak against this hypothesis. Regarding the general trend of TEQs and BEQs to increase from fish exposed to sediments EBR (+) and PR (+) over

EBR/ZE (+) to ZE (+), this trend more or less reflected the initial trend of sediment contamination with DLCs.

TEQ and BEQ comparisons moreover could prove the higher suitability of the Micro EROD assay to be compared with chemically derived results. Differences between BEQs and TEQs could be due to synergism and/or the presence of compounds in the extracts not targeted by chemical analysis (Zacharewski et al. 1989). TEQs and H4IIE Micro EROD BEQs were well correlated and moreover, their correlation was higher than previously determined for TEQs and BEQs measured in whole fish samples from Saginaw Bay, Michigan, USA ($r^2 = 0.44$), where the unexplained percentage of TEQs in BEQs amounted for 75%. (Giesy et al. 1997).

Taking all these results into account and the fact that for the cell line RTL-W1 LOQs partly overlapped with EC_{10} values, the H4IIE Micro EROD is the appropriate assay to be compared to TEQs calculated from instrumental quantification of individual congeners.

5.5.4.3 Classification of DLC uptake on an environmental quality standards basis

Fresh mass normalized RTL-W1 EROD and H4IIE Micro EROD BEQs as well as TEQs for the sum of WHO-PCDD/Fs and DL-PCBs, deduced from *Figure 5.3* (data not shown), indicated internal fish DLC concentrations to be less than the threshold EQS set by regulation 2013/39/EU (2013/39/EU 2013), although the uptake of DLCs by roach was most likely promoted by the contaminated feed present in all sediments.

5.5.4.4 DLC uptake as a function of ingestion

In order to examine the hypothesis that ingestion of contaminated feed is a relevant route of exposure for fish, different feeding scenarios (see *section 5.3.5.1*) were applied. So far, only fish exposed to sediments containing worms (assigned by (+)) has been discussed. Those results in the following were compared with fish exposed to sediments that did not contain worms (assigned by (-)). An uptake of sediment by *L. variegatus* apparent through the skin, suggests that DLCs could have been passed to fish through the worms' guts (*Figure 5.4*). But due to the missing chemical analysis of this matrix, it is not proven that DLCs were accumulated in the worms.

The general assumptions for the uptake of DLCs included that DLC uptake in fish: (1) exposed to (+) treatments was higher than for fish on (-) treatments, (2) has a temporal dependency and (3) increases with the DLC contamination of the sediment. For a better comparison between bio-analytically and instrumentally derived results, BEQs and TEQs in fish of each sampling date were normalized to the respective BEQs and TEQs determined in the control fish, resulting in unit less *uptake factors*.

The alignment of previously observed (*Figure 5.3*) seasonal and methodological differences created a better comparability among the treatments. Fish exposed to sediment PR predominantly showed an uptake of DLCs (uptake factors > 1). The fact that patterns of uptake factors determined for fish on PR (*Figure 5.5a*) and ZE (*Figure 5.5b*) distinctly differed from one another, points towards but does not verify assumption (3). Although sediment ZE showed the highest initial concentration of DLCs, average altitudes of uptake factors of fish exposed to sediments ZE and PR did not significantly differ from one another. But, it is likely that species such as rainbow trout, which exhibit a higher body fat content, would have exhibited higher uptakes of DLCs compared to roach.

In contrast to fish exposed to sediment PR, the presence of worms in sediment ZE caused significantly higher uptake factors than without worms. This was the case for both H4IIE Micro EROD BEQs and TEQs. On the one hand this observation equates assumption (1) and shows that the initial sediment contamination with DLCs determines a comparably higher uptake of DLCs by the fish, on the other hand these observations correspond to previous findings of Rubinstein et al. (1984). In this study, the dietary uptake of sediment-borne PCBs by spot croaker was chemically investigated and it has been demonstrated that fish exposed to PCB-contaminated sediments and daily fed with polychaetes from the same sediment accumulated more than twice as much whole-body concentrations of PCBs after 20 days than fish exposed to similar conditions but fed with uncontaminated polychaetes (Rubinstein et al. 1984).

Instrumental derived uptake factors for ZE (-) and (+) showed opposite temporal trends, demonstrating that fish on sediment, which did not include worms, constantly accumulated DLCs over time, while fish that fed on worms had a higher initial uptake of DLCs followed by a distinct decrease. The high initial uptake of DLCs most likely caused a higher degradation of DLCs through xenobiotic enzymes, which in turn could have led to the aforementioned decrease of uptake factors.

5.5.4.5 Internal DLC concentrations in context to former studies

BEQs in fish exposed to four (+) sediments averaged to 1.8 to 4.9 pg/g fm and 1.0 to 5.5 pg/g fm based on the RTL-W1 EROD and H4IIE Micro EROD assay, respectively and with this were highly comparable to BEQs determined in whole fish and seafood samples (0.1 to 4.5 pg/g fm) using DR-eco screen cells (Kojima et al. 2011). Moreover, minimum concentrations of congeners 105 and 118 found in filets of roach from the Baltic Sea (Burreau et al. 2004) corresponded to control fish concentrations determined in this study. Both examples imply that concentrations of DLCs in roach during this study might correspond to background contamination. The percentage of individual DL-PCBs and PCDD/Fs found in the present study

was highly comparable to those observed in previous studies, where PCB 77 was the most abundant congener among the non-*ortho* substituted PCBs and PCB 118, 105 and 156 being the most abundant congeners among the mono-*ortho* substituted PCBs (Hasegawa et al. 2007), whereas 2,3,7,8-TCDF was the most abundant congener among the dioxins (Hasegawa et al. 2007, Zacharewski et al. 1989). The relative abundance of the 12 DL-PCBs in this study furthermore highly corresponded to previous analyses of PCB patterns in filets of three cyprinid species from the river Po (Viganò et al. 2000).

5.5.4.6 Congener-specific uptake following 28 days of exposure

This section focuses on concentrations of individual DLCs and their uptake by roach following 28 days of exposure to four (+) sediments. DLC congeners, their octanol-water portioning coefficient (log K_{ow}), water solubility and initial sediment concentrations are given in *Table 5.1*, accompanied by their respective uptake factors in roach.

DL-PCB uptake factors predominantly showed an uptake (uptake factors < 1) after 28 days. This corresponds to previous findings of Blanco et al. (2007), who found that all DL-PCB except PCB 169 accumulated in fish. The non-*ortho* substituted PCBs 77, 81, 126 and 169 are more potent DLCs than are their mono-*ortho* analogues and thus, of greater concern. However, some studies have found that planar PCBs 77, 126 and 169 accumulate to a lesser extent than might be expected based on their structure (Engwall 1995, Van Bavel et al. 1996). PCB 81 and 169, although present in sediments and relatively potent, according to their uptake factors were not of toxicological concern for common roach. Concerning the equal uptake factors for PCB 126 and 77 in roach it should be mentioned that on a cellular level, relative potencies (REPs) for PCB 77 and 126 in RTL-W1 and H4IIE cells correspond to 60 and 1170 times the average REP of all mono-*ortho* DL-PCBs, respectively (Behnisch et al. 2002, Clemons et al. 1997). Assuming that these *in vitro* REPs are comparable to potentials which occur *in vivo*, PCB 126 and 77 would be expected to pose the greatest potential risk to fish from all 12 WHO-DL-PCBs. Many examples have shown that an uptake of DLCs from sediment into biota cannot reliable be deduced from the sediments' initial contamination level of DLC, which in turn complicates the determination of environmental quality standards.

The observation that PCB 123 caused the highest overall uptake factors in roach is supported by a study which revealed considerable concentrations of PCB 123 in liver and muscle tissue of eels (*Anguilla anguilla*) from the Camargue Nature Reserve, France, compared to the remaining mono-*ortho* DL-PCBs (Oliveira Ribeiro et al. 2008). Uptake factors observed might be due to the small log K_{ow} value and relatively high water solubility of PCB 123, compared to the other mono-*ortho* PCB congeners. Moreover, higher chlorinated PCBs like PCB 123 are

known to possess a higher potential to biomagnify (Porte and Albaigés 1994). Hence, beside the non-*ortho* relates 77 and 126, PCB 123 is predicted to have the highest potential to adversely affect the aquatic fauna.

Although scientists have revealed DL-PCBs to bioaccumulate more efficiently than PCDD/Fs (Blanco et al. 2007, Isosaari et al. 2002), uptake factors of PCDD/Fs in roach were comparable to those determined for DL-PCBs and exhibited values between 1.2 and 4.3, which slightly correlated ($r^2 = 0.44$) with their initial concentrations in the sediments. This might demonstrate that the uptake kinetics of PCDD/Fs and DL-PCBs follow different principles and that the uptake of dioxin is a function of how much sediment s ingested. Thus, it can be concluded that in addition to log K_{ow}, water solubility and initial concentration of DLCs in the sediments, other factors influence the uptake of DLCs (*Table 5.1*).

5.5.4.7 Temporal congener-specific uptake depending on sediment characteristics

Both redox potential and pH can accelerate desorption, partitioning and bacterial degradation, thereby increasing bioavailability of organic chemicals (Eggleton and Thomas 2004). It is therefore likely that sediments of different origins, such as sediments EBR and ZE can differentially transform contaminants into more bioavailable or toxic forms. Predicted concentrations of the 12 DL-PCBs in sediment EBR/ZE nearly equated the excepted concentrations, demonstrating that uptake factors measured in fish on differing sediments, are the result of sediment-specific characteristics and not a result of varying distributions of congeners.

The high initial uptake (*Figure 5.6*) which has been observed in fish exposed to sediment ZE (+) and the mixture (EBR/ZE (+) was previously shown in fish exposed to sediments containing PCBs (Rubinstein et al. 1984). And although uptake factors in fish exposed to the mixture were expected to be similar to those determined for fish exposed to EBR (+), since there was a higher proportion of EBR (+) present in the mixture, uptake factors more or less were comparable to those of fish exposed to ZE (+). One exception was that the baseline behind the high initial uptake was higher in fish exposed to the mixture, which could reflect the nine dm parts EBR present in the mixture, which due to their fine-particulate character promote high DLC concentrations in the water column.

Results of this study indicate that: (1) the initial uptake of PCBs by fish is controlled by sediment ZE (+), the sediment with higher concentrations of DL-PCBs and (2) characteristics of sediment EBR promote the uptake of DL-PCBs originating from sediment ZE (+). Hence, the uptake of DLCs by roach after 28 days of exposure is dependent on sediment-specific characteristics rather than the initial congener concentration.

5.6 Conclusion

HRGC/HRMS and bioassay derived uptake factors predominantly indicated an uptake (factors > 1) of sediment-borne DLCs by common roach. Calculation of factors eliminated seasonal (summer; autumn) and methodological (HRGC/HRMS; bioassay) differences observed for TEQs and BEQs. BEQs in fish exposed to sediment EBR (+) increased with time, whereas BEQs of fish exposed to the remaining sediments reflected suspended matter concentrations caused by fish transfer and feeding activities. In contrast to fish exposed to sediment PR, contaminated feed (+) on average caused a 2.1 (TEQs) and 1.8-fold (H4IIE BEQs) higher DLC uptake in fish exposed to sediment ZE compared to feeding with uncontaminated worms (-). This indicates that contaminated feed only promotes the uptake of DLCs by roach exposed to a highly contaminated sediment.

Results based on both TEQs and BEQs revealed that the uptake of DLCs was largely independent of the initial concentrations of DLCs in sediments. This was further confirmed by a comparison of the pattern of uptake of DL-PCBs by fish exposed to lesser contaminated sediment from EBR (+), the more contaminated sediment from ZE (+) and a 1:10 mixture (+) thereof. This demonstrated that uptake of DL-PCBs by roach after 28 days of exposure depends rather on sediment-specific characteristics than on initial concentrations of DLCs in the sediment. Congener-specific conciderations of the uptake of DL-PCBs by roach indicated PCB congeners 123, 77 and 126 among all of the DL-PCBs to pose the highest risk to roach.

All bioassay and HRGC/HRMS derived equivalents were less than the EQS of 6.5 pg TEQ/g fm for DLCs in biota. Organic extracts from fish turned out to be a complicated matrix for BEQ calculations due to low induction strengths. However, especially H4IIE BEQs showed a high sensitivity (LOD = 0.37 ± 0.25 pM TCDD) and a high correlation with TEQs ($r^2 = 0.62$). The authors therefore suggest, using the H4IIE Micro EROD as bio-analytical alternative or amendment for congener specific instrumental sediment and biota screening analysis.

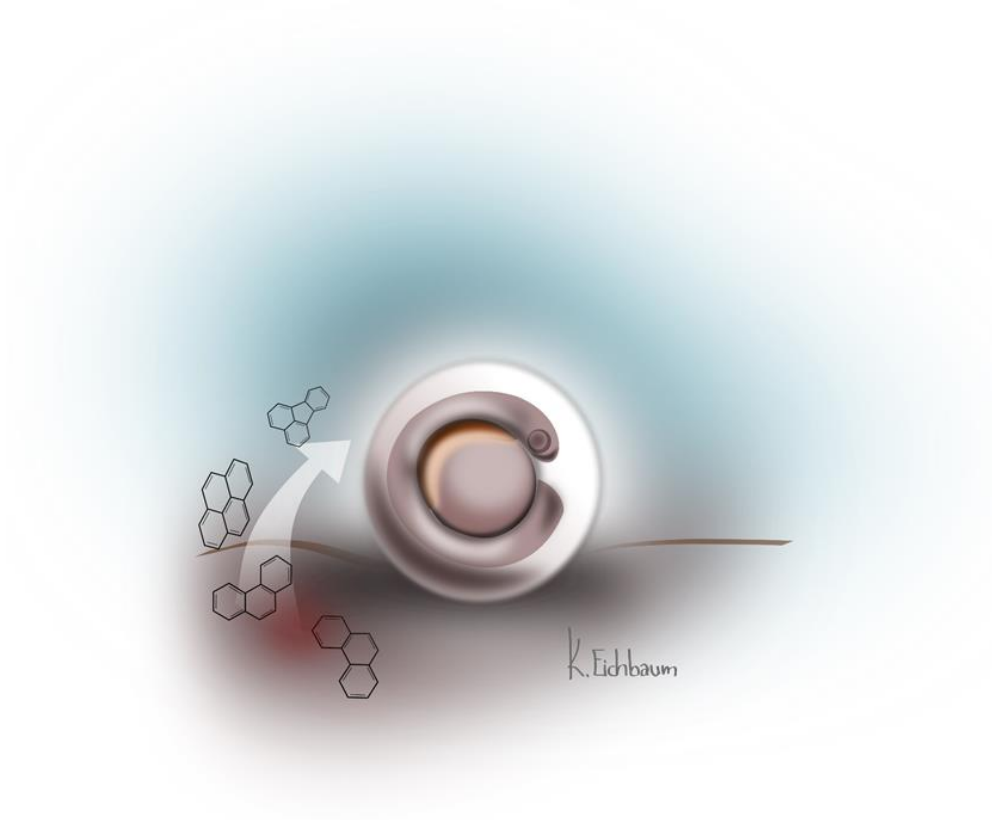
5.7 Acknowledgements

The present work forms a part of the DioRAMA project (“dioxin Risk Assessment for sediment Management Approaches”) that received funds from the German Federal Ministry of Transport and Digital Infrastructure. MB received a personal stipend from the German National Academic Foundation (“Studienstiftung des deutschenVolkes”). HH was supported by the Chinese 111 Program (College of Environmental Science and Engineering and Key Laboratory of Yangtze Water environment, Ministry of Education, Tongji University). JPG was supported

by the Canada Research Chair program, the 2012 "High Level Foreign Experts" (#GDT20143200016) program, funded by the State Administration of Foreign Experts Affairs, the P.R. China to Nanjing University and the Einstein Professor Program of the Chinese Academy of Sciences. The research was supported by a Discovery Grant from the Natural Science and Engineering Research Council of Canada (Project # 326415-07) and a grant from the Western Economic Diversification Canada (Project # 6578, 6807 and 000012711). The authors wish to acknowledge the support of an instrumentation grant from the Canada Foundation for Infrastructure and would like to specially thank MSc Miriam Zimmer and MSc Mathias Reininghaus for their contribution to the exposure experiments. The authors in particular would like to thank Dr. Stephan Hamm and Dr. Armin Maulshagen from mas (münster analytical solutions GmbH), Münster, Germany for the HRGC/HRMS measurements and their kind contribution to this work.

Chapter 6

Desorption and bioavailability of sediment-bound polycyclic aromatic hydrocarbons from a chemical and ecotoxicological perspective



Desorption and bioavailability of sediment-bound polycyclic aromatic hydrocarbons from a chemical and ecotoxicological perspective

Miriam Zimmer[1] • Kathrin Eichbaum[1] • Markus Brinkmann[1] • Henner Hollert[1,2,3,4]

[1] Department of Ecosystem Analysis, Institute for Environmental Research, ABBt – Aachen Biology and Biotechnology, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany

[2] Key Laboratory of Yangtze River Environment of Education Ministry of China, College of Environmental Science and Engineering, Tongji University, Shanghai, China

[3] College of Resources and Environmental Science, Chongqing University, China.

[4] School of Environment, Nanjing University, China

6.1 Abstract

Bioavailability is a complex processes, depending of sediment-, compound- and species-specific properties. For bioavailability investigations, a combination of a mild extraction technique with tenax and adjacent cell- and organism-based ecotoxicological tests, including the 7-Ethoxyresorufin-*O*-deethylase (EROD) assay with RTL-W1 cells and the sediment contact assay (SCA) with eggs of *Danio rerio*, was used. The desorption of polycyclic aromatic hydrocarbons (PAHs) from four sediments, differently contaminated with PAHs, was analysed for 53 days in tenax containing sediment/water batch systems.

Predominantly desorbing congeners phenanthrene, fluoranthen and pyrene desorbed from the sediments in a bi-phasic manner, consisting of rapidly and slowly desorbing fraction, of which the first corresponded well to the initial sediment contamination levels. Rates of desorption were correlated with sediment organic matter contents ($r^2 = 0.97$, $p = 0.05$) and compound characteristics ($r^2 = 0.85$, $p = 0.05$). All tenax extracts showed cumulative, temporal decreasing dioxin-like activity in the EROD assay, corresponding to the cumulative desorption of phenanthrene, flouranthene and pyrene from the four sediments. Since these congeners are non-inducers for RTL-W1 cells, other dioxin-like compounds most likely caused the observed effects. In the SCA, lethal and sub-lethal effects were highest in embryos exposed to the highly PAH contaminated sediments, whereby the latter predominantly included disturbed or lacking pigmentations of skin and eyes. Besides PAHs, other sediment-borne compounds such as dioxins might have caused these effects.

A combination of mild extraction and cellular and organismic ecotoxicological tests served to investigate bioavailability of sediment-bound compounds and allowed for an improved sediment risk assessment.

Keywords: Desorption • bioavailability • polycyclic aromatic hydrocarbons • tenax

6.2 Introduction

Sediments may act as long-term reservoirs of sediment-bound organic pollutants such as polycyclic aromatic hydrocarbons (PAHs) (Förstner et al. 2008, Hollert et al. 2014). In aquatic systems, PAHs are relatively persistent and ubiquitously distributed (Burns et al. 1997, Laflamme and Hites 1978). While low-molecular PAHs have a higher water solubility and are thus often dissolved in the water-phase (Gocht and Grathwohl 2004), high-molecular PAHs show higher sorption to particles (Larsson 2009). PAH fractions desorbing from sediment are potentially bioavailable for aquatic organisms (e.g. fish) (Mackay and Fraser 2000) and may be absorbed *via* oral or dermal exposure routes (Larsson 2009). Although PAH in general show low acute toxicity, several congeners can be mutagenic and carcinogenic (e.g. Benzo[a]pyrene).

In order to determine the overall concentrations of contaminants present in a sediment, exhaustive extraction procedures are often applied (Brown et al. 2007, Giesy et al. 2002, Gómez-Ariza et al. 2002, Hilscherova et al. 2001, Li et al. 1999). However, due to the physical chemical properties of organic pollutants, their aqueous concentrations are much lower than when compared to concentrations determined using exhaustive extraction procedures (Alexander 2000, Dossier 2011). Hence, exhaustive extraction procedures do not have a great value for predicting environmental relevant concentrations and bioavailability.

Towards a greater informative value of natural occurring contaminant concentrations, scientists progressively used less exhaustive techniques such as extractions of bioavailable fractions using e.g. Tenax® TA beads (Cornelissen et al. 2001, Reid et al. 2000, Schwab and Brack 2007). Tenax® TA is an organic polymer with an average pore size of 200 nm, which due to its hydrophobic properties and porous adsorbent surface binds volatile and dissolved hydrophobic organic pollutants (Zhao and Pignatello 2004).

Desorption of organic compounds from sediment in general shows at least a bi-phasic kinetic, consisting of a rapidly desorbing phase (also referred as labile fraction), followed by slowly desorbing phase (also referred as non-labile fraction) (Cornelissen et al. 1997, Gocht and Grathwohl 2004, van Noort et al. 2003). The rapidly desorbing fractions, which desorb during the first days (desorption rate constants > 0.1 h^{-1}), are assumed to be decisive in terms of bioavailability, whereas the slowly desorbing fractions have a smaller influence (Brack et al. 2009, Cornelissen et al. 1997, Cornelissen et al. 2001, Lamoureux and Brownawell 1999). Bioavailability is a complex process, including desorption, partitioning and diffusion of a compound, which along with the characteristics of the sediment, the organism, the environment and the compound itself influence, how much of the compound present in a sediment is assimilated by biota (Schwab and Brack 2007).

Bioavailability can be investigated using both chemical and biological methods. One ecotoxicological test method is the 7-ethoxyresorufin-*O*-deethylase (EROD) assay with permanent fish cell line RTL-W1 (Rainbow trout liver - Waterloo1), which is the most commonly applied method to quantify the expression of cytochrome P_{450} 1A ($CYP_{450}1A$) *in vitro* (Behnisch et al. 2001b). The test principle is to measure the induction strength of CYP1A through EROD activity. Following deethylation of the exogenous substrate 7-ethoxyresorufin through EROD, the resulting reaction product resorufin can be fluorometrically measured. After normalization to protein concentrations, the specific EROD activity can be calculated by the amount of resorufin formed by the proteins within a certain reaction time (Lorenzen and Kennedy 1993, Lorenzen et al. 1997). Because the test presumes a solvent exchange with dimethyl sulfoxide (DMSO), results cannot be discussed with respect to bioavailability. Nevertheless, the test uncovers the dioxin-like potential of the desorbed compounds during a desorption experiment on a cellular level.

Ecotoxicological tests such as the sediment contact assay (SCA) allow for the determination of the embryotoxic potential of whole sediment samples towards fish eggs of the tropical freshwater fish *Danio rerio*. Eggs of *D. rerio*, in which the larval development can be easily observed (Scholz et al. 2008) represent a good alternative to fish acute toxicity testing (Lammer et al. 2009). Typical teratogenic effects in the test may include sub-lethal and lethal effects. Sub-lethal effects *inter alia* include reduced or lacking heartbeat, blood circulation and/or pigmentation, edema as well as malformations of the eyes, fins, and/or the vertebral column of the embryo, while lethal effects encompass coagulation, missing heartbeat, missing somites and the non-detachment from the yolk sack (Braunbeck et al. 2005, DIN 2001, Hollert et al. 2003, ISO 1996, Nagel 2002). Because the embryos have direct contact to the sediment, they reflect bioavailability of compounds in relatively unchanged sediments (Feiler et al. 2005, Hollert et al. 2003, Seiler et al. 2008). Hence, the SCA is a promising tool in search for more realistic exposure scenarios, which represent bioaccessibility (Zielke et al. 2011), the amount of compounds potentially available following desorption (Semple et al. 2004).

The present study investigated kinetics of PAHs desorbing from differently contaminated sediments in sediment/ water batch systems under standardized conditions using tenax as a sorbent. Towards an extrapolation of the instrumentally determined PAH concentrations to natural scenarios, sediments were additionally tested on their embryotoxic potential using the sediment contact assay (SCA) and tenax extracts were bio-analytically investigated using the EROD assay after a solvent exchange (DMSO, < 0.5%). The study focused on the following questions: (1) Do contaminant concentrations measured in sediments reflect bioavailable

concentrations? (2) Do sediment and compound properties influence the desorption behavior of PAHs? (3) Do ecotoxikological test results reflect chemical detected available PAH fractions?

6.3 Materials and methods

6.3.1 Study design

Desorption kinetics of polycyclic aromatic hydrocarbons (PAHs) from three freeze-dried sediments from Ehrenbreitstein (EBR, river Rhine), Prossen (PR, river Elbe) and Zollelbe (ZE, river Elbe) as well as from a 1:10 dry weight (dw) mixture of sediments from ZE and EBR (refer to *Table 2.1*) were analyzed using Tenax® TA in small-scaled sediment/water systems. Desorbed PAHs were extracted from the Tenax® TA and quantified using gas chromatography – mass spectrometry (GC-MS). The dioxin-like potential of these extracts was further investigated using the 7-Ethoxyresorufin-*O*-deethylase (EROD) assay with the permanent fish cell line RTL-W1. To investigate the bioavailability of xenobiotics present the three freeze-dried sediments and the 1:10 mixture, a sediment contact assay (SCA) with fish eggy from *Danio rerio* was conducted. Results of the desorption kinetics, the EROD and SCA assay were compared and discussed.

6.3.2 PAH desorption experiments

For desorption experiments, simple sediment/water systems, including sediments EBR, PR, ZE and the mixture EBR/ZE, were prepared in 100 ml brown glass bottles with PTFE-septum containing screw caps according to a previously described method (van Noort et al. 2003). Each bottle was equipped with 1 g dry weight (dw) of sediment, 70 ml ultrapure water (LiChrosolv ® Water for Chromatography, Merck, Darmstadt, Germany), 1 mg the of the biocide mercury chloride (HgCl2; Mercury(II)chlorid, 5 g, 215465, ACS reagent, ≥ 99.5%, Sigma Aldrich, Crailsheim, Germany) and 0.6 g pre-cleaned Tenax® TA beads (Ø 60 - 80 mesh, 177 – 250 µm, Porous Polymer Adsorbent matrix Tenax® TA, 11982 SUPELCO, Sigma Aldrich) were, put into PTFE gauze bags. For pre-cleaning, Tenax® TA was rinsed three times in the order: water (LiChrosolv ® Water; Merck), acetone (p.a.; Roth, Karlsruhe, Germany) and n-hexane (p.a.; Roth) and dried at 75 °C overnight (van Noort et al. 2003). Two process control bottles only contained 70 ml ultrapure water and Tenax® TA beads in PTFE gauze bags. All bottles were horizontally shaken (GFL 3017, Gesellschaft für Labortechnik GmbH, Burgwedel, Germany) with 126 rpm and incubated at room temperature for 53 days in darkness.

Tenax® TA beads were sampled on days 2, 4, 7, 14, 28 and 53. Tenax® TA beads were removed and transferred into 250 ml beakers and adjacently replaced by new beads. For extraction, the sampled Tenax® TA beads were three times shaken with 30 ml n-hexane (p.a.; Roth). Water was removed by leading the extracts through sodium sulfate (anhydrous, Sigma) containing funnels. Extracts were collected in 100 ml round bottom flasks and rotary evaporated close to dryness. Exact volumes were adjusted in n-hexane (Roth) using 1 ml volumetric flasks (1 ml; VWR, Darmstadt, Germany) and extracts were stored in 1.5 ml brown glass vials with PTFE-caps (4 ml; VWR) at 4 °C until further use.

6.3.3 Gas chromatography – mass spectrometry (GC-MS)

PAH concentrations in extracts of Tenax® TA beads were measured according to DIN ISO 18287 under slight modifications (DIN 2006) on an Agilent Technologies GC system (7890 A GC system and 5975 C inert XL MSD with Triple-Axis-Detector, Agilent Technologies Deutschland GmbH, Böblingen, Germany). A HP-5ms capillary column (19091S-433, 30 m x 0.25 m, 0.25 µm film thickness, Agilent Technologies) was used to separate the compounds. Carrier gas helium had a flow rate 1 ml/min and the temperature program followed the order: 60 °C (2 min isothermal), 60 – 120 °C increasing with a rate of 30 °C/min, 120 – 300 °C increasing with a rate of 5 °C/min and 300 °C (15 min isothermal). The interface between the GC and the MS had a temperature of 295 °C. The injection temperature was 300 °C and the injection volume 1 µl.

The quantitative analysis of PAHs was conducted in SIM (selected ion monitoring) mode. Mass spectrometric detector parameters including time intervals and selected ions with qualifying fragments given in parenthesis for the 16 EPA-PAHs were the following: *naphthalene*, 6 – 9 min, 128 (102); *acenaphtylene*, 9 - 12.5 min, 152 (76), *acenaphthene*, 9 - 12.5 min, 154 (80); *fluorene*, 12.5 - 16 min, 166 (140); *phenanthrene, anthracene*, 16 - 20 min, 178 (152, 89); *fluoranthene, pyrene*, 20 - 25 min, 202 (101); *benzo[a]anthracene, chrysene*, 25 - 32 min, 228 (114); *benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene*, 32 - 37 min, 252 (126*); indeno(1,2,3-cd)pyrene, benzo[g,h,i]perylen*, 37 - 55 min, 276 (138); d*ibenzo[a,h]anthracene*, 37 - 55 min, 278 (139).

6.3.4 Calculation of desorption

For determination of PAH desorption rates, Tenax-desorbed PAH concentrations were cumulatively subtracted from total sediment concentrations at the beginning of the experiment

and transformed into relative values. Desorption was calculated according to *equation 6.1* by using the following two-compartment model equation (Cornelissen et al. 1998):

$$\frac{S_{(t)}}{S_0} = F_{rap} \cdot e^{-k_{rap} \cdot t} + F_{slow} \cdot e^{-k_{slow} \cdot t} \quad (6.1)$$

With S_0 and $S_{(t)}$ being the sediment-sorbed amounts at the experimental beginning and at time point t(h), respectively. F_{rap} and F_{slow} are the rapidly and slowly desorbing fractions, respectively, accompanied by their rapid and slow desorption constants k_{rap} and k_{slow}, respectively. The time point, at which the rapidly desorbing fraction was exhausted, and thus the slowly desorbing fraction exceeded the rapidly desorbing fraction, was calculated using the Newtonian approximation technique depicted in *equations 6.2* and *6.3*.

$$F_{rap} - F_{rap} \cdot e^{-k_{rap} \cdot t} = F_{slow} - F_{slow} \cdot e^{-k_{slow} \cdot t} \quad (6.2)$$

$$0 \approx F_{rap} \cdot e^{-k_{rap} \cdot t} \quad (6.3)$$

6.3.5 Ecotoxicological analyses

6.3.5.1 The RTL-W1 EROD assay

The RTL-W1 EROD assay was performed as described in *section 2.3.1*. BEQs were calculated according to *section 2.3.4*, whereby *x* in *equation 2.1* was the concentration at the 25% effect level.

6.3.5.2 Sediment contact assay (SCA)

The sediment contact assay (SCA) was performed according to the German DIN 15088 (DIN 2009) for the fish embryo test (FET) with *Danio rerio*, adapted to sediment testing introduced by Hollert (2003) and described in detail in Zielke et al. (2011). Fish maintenance was equal to maintenance and egg production conditions described in Schiwy et al. (2015).

Sediment (EBR, PR, EBR/ZE and ZE) and controls were prepared one day before the SCA. For this, freeze-dried sediments were serially diluted with quartz sand (W4, Quarzwerke, Germany) in 20 ml crystallization glasses so that percentages between 6.3 and 100% sediment and concentrations between 26.8 and 428.6 mg dw sediment/ml were reached, respectively (*Table 6.1*). Due to the high volume of sediment EBR, 7 ml of artificial water were given to all treatments, including the controls, instead of the prescribed 5 ml. Artificial water (294.0 mg/l $CaCl_2$ 2 H_2O, 123.3 mg/l $MgSO_4$ 7 H_2O, 63.0 mg/l $NaHCO_3$ and 5.5 mg/l KCl) was prepared according to ISO 7346/3 (ISO 1996). As negative controls eight aqueous controls, simply consisting of 7 ml artificial water, and four quartz sand controls, consisting of 3 g sand covered

with 7 ml artificial water were prepared. As positive controls four aqueous controls (7 ml artificial water with 3.7 µg/ml 3,4-dichloroanilin) and two quartz sand controls (3 g sand covered with 7 ml 3.7 µg/ml 3,4-dichloroaniline solution) were prepared. On the day of their preparation, all control approaches singly contained pure artificial water, whereas the 3,4-dichloroanilin solution was added on the actual test day. All approaches were covered with self-adhesive foil (Nunc, Roskilde, Denmark) to prevent water evaporation and horizontally shaken with 50 rpm at 26 °C for 24 hours.

Table 6.1 Amount of quartz sand, sediment and artificial water and resulting dry weight (dw) sediment concentrations (dw) applied in the sediment contact assay (SCA), which was performed with fish eggs of *D. rerio*.

Sediment [%]	Sediment [g]	Sand [g]	Artificial water [ml]	Concentration [mg dw/ml]
100.0	3.00	0.00	7.0	428.57
50.0	1.50	1.50	7.0	214.29
25.0	0.75	2.25	7.0	107.14
12.5	0.38	2.62	7.0	53.57
6.3	0.19	2.81	7.0	26.79

Thereafter, normally developed fish eggs in an eight-cell-stadium were transferred to artificial water (ISO 1996) and thereafter transferred to each treatment (5 eggs/replicate; 15 eggs/treatment). Overall, 60 and 30 eggs were transferred to the negative and positive controls, respectively. Approaches again were covered with self-adhesive foil and incubated on a horizontal shaker at 50 rpm and 26 °C for 48 hours.

Prior to the test evaluation, eggs were collected from the treatments using a 5 ml pipette and transferred to 24-well plates (TPP, Trasadingen, Switzerland) and optically analyzed using an inverse microscope at 40 and 100-fold magnification. Investigated effects included sub-lethal (i.e. weak heartbeat/blood circulation, edema, deformed embryo/ fins/vertebral column, missing/malformed pigments of skin/eyes) and lethal effects (i.e. coagulation, missing heartbeat, missing somites, non-detachment from the yolk sack) (Braunbeck et al. 2005, DIN 2001, Hollert et al. 2003, ISO 1996, Nagel 2002). The SCA is regarded valid, if mortality of fish exposed to negative aqueous and quartz sand controls is $\leq$ 10%, the mortality of fish exposed to positive aqueous control ranges between 20 and 90% and the egg fertilization rate was $\geq$ 70% (DIN 2009). In total, three replicates were conducted for each sediment.

6.3.6 Data analysis and presentation

Desorption data were fitted to a two-compartment model. EROD assay data was processed *via* Excel (Microsoft Office Excel 2003) and concentration-response curves were plotted using GraphPad Prism 5 software (La Jolla, CA, USA) using a non-linear regression and a dose-response stimulation (log agonist vs. response). All graphs, curve fittings and correlation analyses (Pearson correlation; $p = 0.05$) were conducted using GraphPad Prism 5.

6.4 Results

6.4.1 Sediment PAH contamination

Sediments EBR, PR and ZE differed in their concentrations of 16 EPA-PAHs (*Figure 6.1*).

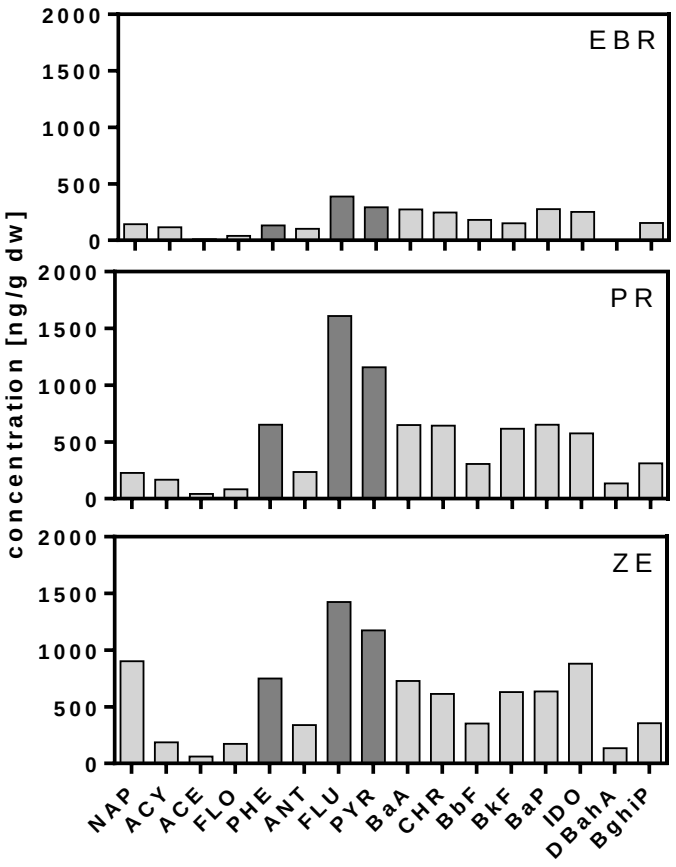


Figure 6.1 Concentrations of 16-EPA PAHs in three sediments from Ehrenbreitstein (EBR, top), Prossen (PR, middle) and Zollelbe (ZE, bottom) measured using gas chromatography – mass spectrometry (GC-MS). Bars represent results of a single measurement. Darker grey bars mark congeners, which predominantly desorbed from the sediments during a desorption experiment; dw = dry weight; naphthalene (NAP), acenaphtylene (ACY), acenaphthene (ACE), fluorine (FLO), phenanthrene (PHE), anthracene (ANT), fluoranthene (FLU), pyrene (PYR), benzo[a]anthracene (BaA), chrysene (CHR), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), indeno(1,2,3-cd)pyrene (IDO), dibenzo[a,h]anthracene (DBahA), benzo[g,h,i]perylen (BghiP).

The PAH contamination of Elbe sediments PR and ZE were comparable with total concentrations of 8.1 and 9.3 µg/g dry weight (dw), respectively, whereas slightly contaminated sediment EBR showed a 3-fold lower total contamination of 2.8 µg/g dw. However, congener distribution in the sediments was nearly independent of their origin with most dominant congeners including fluoranthene, pyrene and phenathrene averaging to 35.7 ± 6.5%. Total concentrations of all 16-EPA-PAHs in the mixture EBR/ZE (data not shown) were 3.7 µg/g dw, and on basis of the detected concentration of PAHs in sediments EBR and ZE, on average only deviated 15.6% from the expected concentrations. Total contamination of the sediments with PAHs thus increased in the order EBR < EBR/ZE < PR < ZE.

6.4.2 Desorption of polycyclic aromatic hydrocarbons (PAHs)

6.4.2.1 Desorption rates of PAHs

Concerning the overall desorption of the 16 EPA-PAHs, only phenanthrene, fluoranthen, pyrene and fluorene were detected in the water. In all three sediments and the 1:10 mixture, the predominantly desorbed congeners, including fluranthene, pyrene and phenanthrene, showed similar bi-phasic desorption kinetics (*Figure 6.2a - d*).

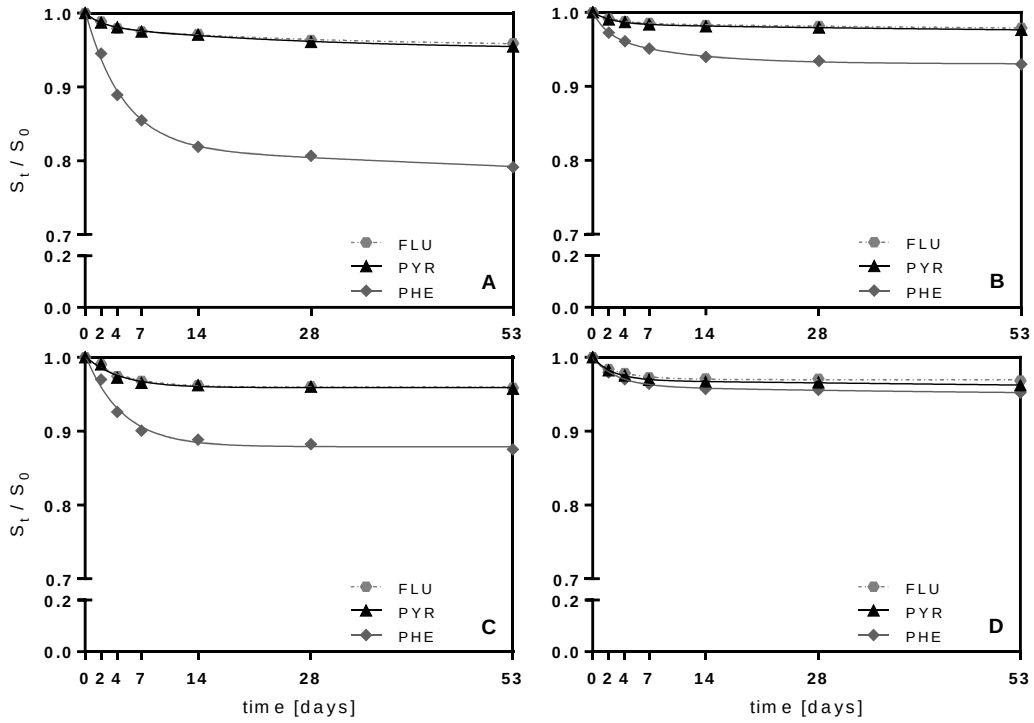


Figure 6.2 a-d Kinetics of the relative desorption of phenanthrene (PHE), fluoranthene (FLU) and pyrene (PYR) from three freeze-dried sediments from Ehrenbreitstein (EBR; A), Prossen (PR; B) and Zollelbe (ZE; D) as well as from a 1:10 sediment mixture (EBR/ZE; C) during 53 days; sediment-sorbed amounts at the sampling date (St) at the end of the experiment (S0).

These kinetics consisted of a high initial desorption (rapidly desorbing fraction), which after approximately 7 days was followed by a low desorption (slowly desorbing fraction). The desorption kinetics of fluoranthene and pyrene were similar for each sediment. More precise, approximately 5% of the initial sediment concentration were desorbed from the sediments during 53 days (*Figure 6.2a - d*). The respective rapidly desorbing fraction proportionally exhibited percentages < 4% for all sediments (*Table 6.2*) and was exhausted approximately after 50 days for all sediments, except for ZE, for which the time point of exhaustion was approximately 75 days (*Table 6.2*).

Table 6.2 Percentages, concentrations (C_{Frap}) and dates of exhaustion (Frap exh.) of the rapidly desorbing fractions (F_{rap}) as well as initial concentrations (C_0) of phenanthrene (PHE), fluoranthene (FLU) and pyrene (PYR) desorbed from three freeze-dried sediments from Ehrenbreitstein (EBR), Prossen (PR) and Zollelbe (ZE) as well as from a 1:10 sediment mixture (EBR/ZE) during 53 days.

Sediment	Compound	F_{rap} [%]	C_0 [ng/g dw]	C_{Frap} [ng/g dw]	Time $_{Frap\ exh.}$ [days]
BR	PHE	18.59	133.11	24.74	93
	FLU	2.63	387.87	10.21	60
	PYR	2.58	292.05	7.54	64
	sum	**23.81**	**813.03**	**42.50**	
PR	PHE	5.67	651.50	36.93	68
	FLU	1.52	1610.36	24.54	50
	PYR	1.67	1158.32	19.30	48
	sum	**8.86**	**3420.18**	**80.77**	
ZE	PHE	4.01	748.36	29.99	57
	FLU	2.75	1424.94	39.20	45
	PYR	3.11	1174.97	36.55	46
	sum	**9.87**	**3348.28**	**105.75**	
EBR/ZE	PHE	12.04	223.95	26.96	83
	FLU	4.05	597.66	24.22	81
	PYR	4.08	439.64	17.95	72
	sum	**20.18**	**1261.26**	**69.14**	

For phenanthrene, the total desorption during 53 days was 20, 12 and 6% for sediments EBR, EBR/ZE and PR, respectively, whereas it was similar to that of fluoranthene and pyrene for sediment ZE (*Figure 6.2a - d*). The rapidly desorbing fractions of phenanthrene in detail increased in the order 4.0, 5.7, 12.0 and 18.6% for sediments ZE, PR, EBR/ZE and EBR,

respectively (data nor shown). However, with regard to initial phenanthrene sediment concentrations (refer to *Section 6.4.1*), the rapidly desorbing fractions showed concentrations increasing in the order 24.7, 27.0, 30.0 and 36.9 ng/g dw for sediments EBR, EBR/ZE, ZE and PR, respectively (*Table 6.2*). The calculated time point, at which the rapidly desorbing fraction of phenanthrene is exhausted, was approximately 60 days for sediments PR and ZE, while it exhibited values of 83 and 93 days for sediments EBR/ZE and EBR, respectively (*Table 6.2*).

The concentrations of rapidly desorbed fractions of phenanthrene, fluoranthen and pyrene increased in the order 42.5, 69.1, 80.8 and 105.8 ng/g dw for sediments EBR, EBR/ZE, PR and ZE (*Table 6.2*), respectively.

The cumulative concentration of the desorbed sum of phenanthrene, fluoranthene and pyrene during 53 days increased in the order 56.9, 71.3, 106.9 and 125.4 ng/g dw for sediments EBR, EBR/ZE, PR and ZE, respectively (*Figure 6.3b*) and with this corresponded to the sequence found for the initial, total contamination of the four sediments with 16-EPA PAHs (refer to *Section 6.4.1*).

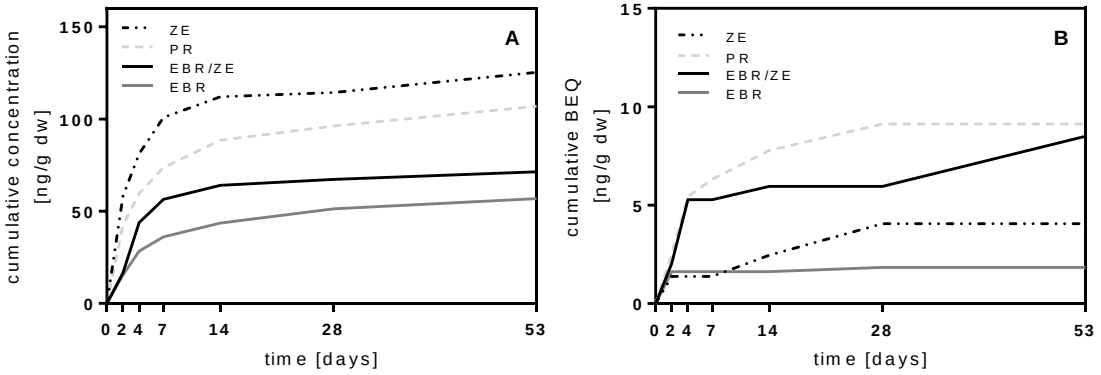


Figure 6.3 a, b Cumulative concentration of the sum of phenanthrene, fluoranthene and pyrene desorbed from three freeze-dried sediments from Ehrenbreitstein (EBR), Prossen (PR) and Zollelbe (ZE) as well as from a 1:10 sediment mixture (EBR/ZE) during 53 days (A). Cumulative biological equivalents (BEQ) measured in four sediment extracts from EBR, PR, ZE and EBR/ZE during a 53-days desorption experiment. BEQs were determined using the 7-Ethoxyresorufin-O-deethylase (EROD) assay with permanent fish cell line RTL-W1 and were the result of three independent replicates.

The final sum of phenanthrene, fluoranthene and pyrene desorbed from sediment ZE after 53 days equated more than two-times the amount desorbed from sediment EBR. Equivalent to the relative desorption kinetics (*Figure 6.2a - d*), the amount of desorbed PAHs was higher at the beginning than at the end of the experiment (*Figure 6.3b*).

6.4.2.2 Desorption rates versus characteristics of sediments

Slowly and rapidly desorbing constants, calculated for phenanthrene, fluoranthene and pyrene, all should a linear dependency to the organic matter amounts of sediments EBR, PR and ZE, respectively (*Figure 6.4*).

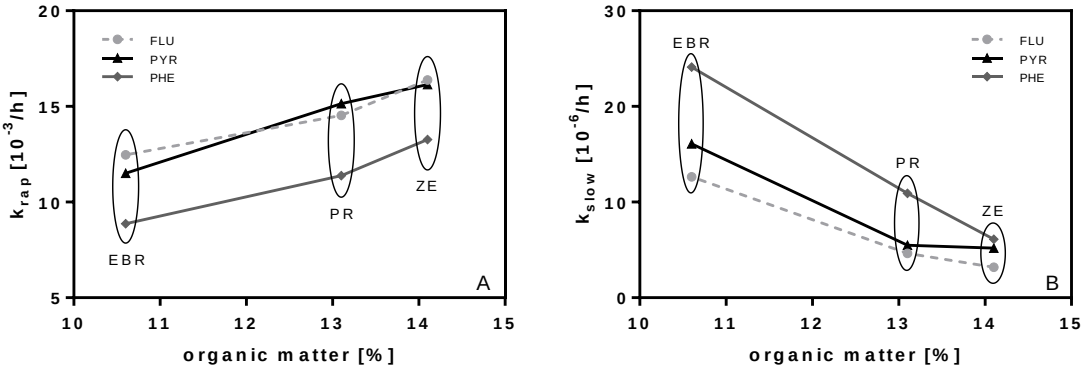


Figure 6.4 Correlation of the desorption rates of rapidly (k_{rap}; **A**) and slowly (k_{slow}; **B**) desorbing fractions with the percentage of organic matter of three sediments (represented as circles) from Ehrenbreitstein (EBR), Prossen (PR) and Zollelbe (ZE), desorbing compounds included phenanthrene (PHE), fluoranthene (FLU) and pyrene (PYR).

While the desorption rate of rapidly desorbing fractions (k_{rap}) of the three compounds increased with the percentage of sediment organic matter, the desorption rate of slowly desorbing fractions (k_{slow}) decreased with percentage of sediment organic matter. Despite the correlation ($p = 0.05$) of k_{rap} and the sediment organic matter was good for phenanthrene ($r^2 = 0.974$) and fluoranthene ($r^2 = 0.956$), it was only found to be significant for pyrene ($r^2 = 0.994$). In contrast, correlation ($p = 0.05$) of k_{slow} and the sediment organic matter was only significant for phenanthrene ($r^2 = 0.999$), while a good correlation was found for fluranthene ($r^2 = 0.981$) and pyrene ($r^2 = 0.936$).

6.4.2.3 Desorption rates versus characteristics of compounds

The three investigated compounds phenanthrere, fluoranthene and pyrene exhibit (estimated, EpiSuit) water solubilities of 0.67, 0.13 and 0.22 mg/l, respectively, while their experimental determined log K_{ow} values are 4.45, 5.16 and 4.88, respectively (US-EPA 2015). These two compound characteristics were well correlated (average $r^2 = 0.85 \pm 0.19$) with their desorption rates determined for all three sediments.

6.4.3 Ecotoxicological analyses

6.4.3.1 Results obtained using the 7-Ethoxyresorufin-O-deethylase (EROD) assay

Regarding the results obtained by using the EROD assay with cell line RT-W1, cumulative biological equivalents (BEQs) could be detected for all investigated sediment tenax extracts. BEQs in tenax extracts increased in the following order EBR < ZE < EBR/ZE < PR with final values of 1.84, 4.06, 8.50 and 9.13 ng/g dw after 53 days, respectively (*Figure 6.3b*). Hence, the sediment mixture EBR/ZE unexpectedly showed a higher EROD induction potential compared to the single sediments EBR and ZE, respectively. Nevertheless, overall lowest and highest EROD inductions were found for extracts of sediments EBR and PR, respectively, which corresponds to the trends of the cumulative desorbed concentrations measured for the sum of phenanthrene, fluoranthene and pyrene (*Figure 6.3a*).

6.4.3.2 Results obtained using the sediment contact assay (SCA)

The SCA was ranked valid due to mortalities < 10% for the negative controls and mortalities > 10% for the positive controls, respectively (DIN 2009). Eggs in negative controls were normally developed throughout the three replicates, meaning that after 48 hours, the embryos spontaneously moved, had a distinctly structured spinal cord with clearly visible somites. Furthermore, their eyes and skin were pigmented and both heart beat and blood stream were visible. Some eggs hatched, which can occur after a 48-hours incubation.

The four examined sediments caused a broad range of both sub-lethal and lethal effects in embryos of *Danio rerio*. All listed effects (refer to *Section 6.3.5.2*) could be observed with the exception of any kind of spinal deformations. Both mortality and teratogenic effects, however, in most of the test runs showed no distinct dose-response-relationships. Thus, effect concentration levels were not determined. Therefore, effects will be expressed in minimum and maximum percentages in the following.

Minimum and maximum effect percentages ranged between 9.4 and 37.8% for sediment EBR, 0.0 and 33.0% for the mixture EBR/ZE, 8.9 and 66.0%for sediment PR and 6.7 and 68.1% for sediment ZE. Moreover, for each of the four different sediments the maximum effect level was reached at a concentration of 214.29 mg dw/ml medium. Except EBR/ZE, each sediment caused effects in the lowest concentration of 26.79 mg dw/ml medium. In this context, a noteworthy observation was that for all sediments the effect level distinctly decreased when the highest concentration of 428.57 mg dw/ml medium (equates 100% freeze-dried sediment) was reached.

With respect to the observed sub-lethal effects observed for embryos exposed to PR and ZE, the predominant effect in embryos were anomalies in pigmentation of eyes and skin. More precise, 67 ± 17% and 56 ± 22% of all effects amounted for missing pigmentation of skin and/or eyes, respectively, and in 30% of these cases, both eyes and skin were affected at the same time. In contrast, sediments EBR and EBR/ZE did not cause any specific, predominant effects. With respect to lethality, all of the freeze-dried sediments showed an embryotoxic potential with sediment PR exhibiting the overall highest potential. Maximum mortality summed up to 11.3% for EBR, 13.7% for mixture EBR/ZE, 18.9% for ZE and 35.6% for PR.

6.5 Discussion

6.5.1 Sediment PAH contamination

Total PAH concentrations measured in the three sediments and the mixture, indicated sediments ZE and EBR to possess the overall highest and lowest contamination level, respectively (*Figure 6.1*). Thereby, congeners fluoranthene, pyrene and phenathrene, which in a consecutive desorption experiment were proven to predominantly desorb from the sediments, showed the highest proportion of approximately 36%. This might indicate that high abundance of compounds present in a sediment leads to comparably higher availability, but there are further sediment- and compound-specific characteristics, influencing bioavailability (refer to *Sections 6.4.2.2* and *6.4.2.3*). The fact that PAH concentrations in sediment mixture EBR/ZE only deviated 15.6% from the, on basis of the PAH concentrations present in sediments EBR and ZE, expected concentrations, proved the 1.10 mixing ratio of sediments to be precise. A failure in mixing, thus can be excluded by considering the present study's results for the mixture EBR/ZE.

6.5.2 Desorption of polycyclic aromatic hydrocarbons (PAHs)

6.5.2.1 Desorption rates of PAHs

Only a few EPA-PAHs desorbed from the sediments in significant concentrations. Consequently, the non-desorbing substances were strongly bound to the sediment-particles and could not be considered as potentially bioavailable contaminants. The desorbing compounds, in contrast to the high-molecular and hardly soluble PAHs, had log K_{ow} values < 4.9 and mostly were three-ring-systems, which are the most water soluble congeners among the 16-EPA PAHs (Fent 2007, Gocht and Grathwohl 2004). Hence, in all of the investigated sediments, fluoranthene, pyrene and phenanthrene can be considered as potentially bioavailable

contaminants. This corresponds to the general observation that low-molecular PAHs such as phenanthere and anthracene possess a greater mobility in the environment than larger, more hydrophobic PAHs (Mackay et al. 1992, Shor et al. 2003, Sutherland et al. 1995).

Moreover, phenanthrene, fluoranthene, and pyrene showed the overall highest degradation rates during a bioremediation experiment, indicating their high bioavailability in sediment/water systems (Ghosh et al. 2003). An extensive literature review (n = 100) performed by Oen and al (2006) revealed that for native sediments the average percentage of the rapidly desorbing fractions of phenanthrene and pyrene were 22 and 29%, respectively. In the present desorption experiment, these percentages accounted for 10 ± 7 and 3 ± 1% for phenanthrene and pyrene, respectively. Hence, the rapidly desorbing fractions of these congeners were much lower compared to the average values of a multitude of studies. Only the rapidly desorbing fraction of phenanthrene desorbed from sediment EBR (19%) corresponds to those findings.

In a desorption study, using the present desorption approach, the percentages of high-molecular PAHs, including benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene and benzo[a]pyrene, desorbed from *inter alia* sediments of the river Rhine, and desorption percentage were comparable to those determined for phenanthrene (122%) (van Noo$_r$t et al. $_2$003). Further studies also found a significant desorption of those high-molecular PAHs from native sediments (Shor et al. 2003). It is therefore assumable that the extraction technique (a sequence of water, hexane and acetone) of Van Noort et al. (2003), in contrast to that of the present study, could have caused a better recovery of high-molecular PAHs. It is also conceivable that the applied freeze-drying process of sediments could have caused and alteration of desorption kinetics (Zielke et al. 2011). These examples thus could explain the rapidly desorbing fractions of phenanthrene and pyrene in the present study to be lower compared to the literature data collected by Oen et al. (Oen et al. 2006).

Desorbing compound concentrations could be divided into rapidly and slowly desorbing fractions by using a two-compartment-model. The 53-days desorption experiment showed that the rapidly desorbing fraction of desorbed compounds, which in contrast to the slowly desorbing fraction is of high importance in terms of bioavailability (Cornelissen et al. 1997, Pignatello and Xing 1995), mostly constituted a minor part compared to their respective initial sediment concentrations (*Figure 6.2 a - d, Table 6.1*), which corresponds to previous findings, where approximately 40% of PAHs were released quickly and 60% slowly (Talley et al. 2002).

In river systems rapidly desorbing fractions can occur during short-term remobilizations of contaminants caused by e.g. dumping of dredged material (Förstner et al. 2008). Those fractions for fluoranthene and pyrene as well as their time point of exhaustion was comparable in all

sediments. In contrast, distinct differences were observed for phenanthrene. Here, desorption from the lowest contaminated sediment was the highest and *vice versa*. Former studies have proven the desorption of phenanthrene from native sediments to be highly variable for sediments with different properties (Shor et al. 2003).

However, by taking the initial sediment concentrations of phenanthrene into account, all sediments the risk through bioavailable phenathrene increased in the order EBR, EBR/ZE, ZE and PR. This sequence nearly equated the sequence of sediment contamination (refer to *Section 6.4.1*), and also the cumulative concentration of rapidly desorbing phenanthrene, fluoranthene and pyrene showed this sequence, showing that the initial sediment concentration is an important factor when assessing compound bioavailability.

Nevertheless, while the rapidly desorbing phenanthrene from highly contaminated sediments PR and ZE was exhausted after two months, this progress took about three months for slightly contaminated sediments EBR/ZE and EBR (*Table 6.1*), indicating that despite their lower contamination levels, sediments EBR and EBR/ZE pose a risk, which is comparably long-lasting. A previous study has shown that miniaturized desorption experiments are transferrable to large-scale experimental setups (Schwab and Brack 2007), suggesting that desorption experiments may also be transferable to field scenarios. However, natural occurring sediment re-mobilization processes, such as hydrogeological events (e.g. floods) only last for a few days and compounds, which during such events enter the water phase, are subjected to dilution, decreasing their risk potential. In general, the present experiment showed that with respect to sediment risk assessment, the initial sediment concentration is of high importance when evaluating bioavailability, but further compound- and sediment specific characteristics have to be considered as well.

6.5.2.2 Desorption versus characteristics of sediments

A comparison of rapidly and slowly desorbing fractions of phenanthrene, fluoranthene and pyrene with the sediments' organic matter percentages showed that desorption strongly depends on sediment-specific characteristics. The higher the organic content of the sediment, the higher was the resulting rate of the rapidly desorbing fractions of phenanthrene, fluoranthene and pyrene, respectively (*Figure 6.4a*). This observation is opposite to previous findings, that the higher the organic portion in a sediment, the stronger the binding of organic compounds (Latimer et al. 1999, Reid et al. 2000). However, desorption of PAHs depends on compound origin and characteristics (Thorsen et al. 2004). Nevertheless, parameters for the estimation of the desorption such as the equilibrium portioning coefficient, in models have been shown to

overestimate (partly by a factor of 100) the aqueous concentration of planar PAHs (McGroddy et al. 1996) indicating that these compounds may be unexpectedly strong sorbed to sediments.

Large molecules due to their steric hindrance and interaction on multiple points can for examples be very difficult to desorb (Pignatello and Xing 1995). Moreover, desorption depends on the composition of the sediment organic matter (Kukkonen et al. 2004). PAHs for example desorb much faster when associated with pitch particles than with coal or coke particles (Ghosh et al. 2003) and the presence of detrital plant debris, have been shown to dramatically influence their desorption from natural sediments (Rockne et al. 2002). Previous studies furthermore emphasized the dependency of desorption from „aging processes“ of sediments and assumed that „aging" complicates the determination to which extent the desorption of PAHs from sediment is influenced by compound and sediment-specific properties (Shor et al. 2003). Hence, other sediment-characteristic influencing components beside the percentage of organic matter cannot be completely excluded. By taking into account that the rapidly desorbing fraction is of much higher importance for bioavailability compared to the slowly desorbing fraction (Cornelissen et al. 1997, Xing et al. 1996), sediment ZE with the highest organic percentage during e.g. a flood would pose the highest risk to the environment through desorbed, bioavailable compounds. The assumption that the organic matter content of a sediment allows for a rough estimation of the extent of desorption especially would be applicable for the rate of rapidly desorbing pyrene, which according to our statistical findings showed a significant correlation ($r^2 = 0.994$) to the sediment organic percentage and corresponds to findings of Oen et al. (2006) with a significant correlation of $r^2 = 0.82$ for pyrene.

Slow desorption is thought to be caused by hindered diffusion of organic compounds through sediment organic matter and/or micro pores (Cornelissen et al. 1998, Farrell et al. 1999, Pignatello and Xing 1995). Here, the rates of slowly desorbing fractions of phenanthrene, fluoranthene and pyrene decreased with increasing organic matter content in the sediment. Good correlations were found for the slow desorbing rates of the three PAH congeners and the organic matter content, which corresponds to previous findings (Oen et al. 2006). In general, the slightly contaminated sediment EBR showed the potentially highest risk of contamination of the surrounding water through desorbed, bioavailable compounds compared to highly contaminated sediments ZE and PR. Nevertheless, this observation from an ecotoxicological perspective is of minor importance, because the concentration of the slowly desorbing fractions are comparably low, thus it takes a long time until these fractions are available for organisms (Kukkonen et al. 2004, Ten Hulscher et al. 2003). Moreover, with regard to river systems, dilution would make the slowly desorbing fraction negligible for sediment risk assessment.

6.5.2.3 Desorption rates versus characteristics of compounds

As the previous chapter could show, the desorbtion of phenanthrene, fluoranthene and pyrene depended on the sediment-specific characteristics such as the organic matter percentage. When the desorption rates of the three compounds determined for four different sediments were compared with the compounds' water solubility and log K_{ow} value, respectively, good correlations were found for each combination, indicating the rate of desorption to increase with an increasing water solubility and a decreasing log K_{ow}, respectively. It is thus not surprising that the compound with the lowest log K_{ow} and the highest water solubility (US-EPA 2015), phenanthrene, showed the overall highest desorption from the four sediments.

With respect to sediment evaluation, these good correlations proved that the desorption and in turn the bioavailability of compounds depends on both, the sediment-specific as well as the compound-specific characteristics (Reid et al. 2000). However, with regard to risk assessment on basis of the three compounds' log K_{ow} values, it is important to know that the higher the log K_{ow}, the higher is the compounds' potential to accumulate in organisms as well as in the food chain (Mackay and Fraser 2000, Reid et al. 2000). It is thus questionable if the three compounds, even though they were the most available ones among all 16-EPA PAHs, would tend to accumulate in biota, which would increase their environmental risk potential. Nevertheless, studies criticizing commonly conducted tenax desorption experiments to singly analyze predominantly unpolar target-compounds, questioned the risk caused by polar compounds possessing a higher significance in terms of bioavailability (Brack et al. 2009).

6.5.3 Ecotoxicological analyses

6.5.3.1 Results obtained using the 7-Ethoxyresorufin-*O*-deethylase (EROD) assay

Chemically based desorption experiments alone are not able to predict bioavailability, because the potential harm of adsorbed contaminants following their bioavailability depends on complex processes such as resorption, transport und metabolism in the cells of an organism (Brack et al. 2009). Therefore, the EROD assay with cell line RTL-W1 was applied to show the effects of the potentially bioavailable fraction of a cellular basis. Tenax extracts of each of the four investigated sediments showed dioxin-like activity, while process controls did not show any of such effects (data not shown). The EROD activity thereby showed a kinetic, which was comparable to the previously discussed PAH desorption kinetics. More precise, higher EROD activities were detected in the first days compared to the activities at the end of the experiment, so that RTL-W1 BEQs formed a saturation curve. In detail, highest and lowest EROD induction was found for sediments PR and EBR, respectively, which primary gives the impression that

EROD activity increased with the increase of the cumulative desorbed concentrations (*Figure 6.3a*).

But, unexpectedly low inductions of tenax extracts originating from sediment ZE were found, diminishing the hypothesis that increased cumulative concentrations of desorbed PAHs led to increased EROD activities. Moreover, tenax extracts of the mixture EBR/ZE, consisting of nine parts dry weight (dw) EBR and one part dw ZE, showed inductions greater as inductions of tenax extracts from the single sediments EBR and ZE, respectively. This result may indicate the presence of synergistic compounds present in tenax extracts of the mixture, which led to an increase in EROD activity compared to extracts of the initial sediments.

By interpreting the present bioassay-derived results, it is moreover important to mention that for permanent fish cell line RTL-W1, relative potency (REP) measurements of single compounds could prove, that only high-molecular PAHs, including benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno(1,2,3-cd)pyrene, benzo[g,h,i]perylen and benzo[a,h]anthracene, possessed a demonstrable EROD-inducing potential (Bols et al. 1999). Further studies proved phenanthrene to be a non-inducer for cell line RTL-W1 (Billiard et al. 2004) and phenanthrene, fluoranthene and pyrene to be non-inducer in fish liver cell line PLHC-1 (Villeneuve et al. 2002). Hence, the four PAH congeners, desorbed from the sediments (i.e. fluorene, phenanthrene, fluoranthene and pyrene), most likely did not cause the EROD activity measured in the extracts. For this, it was not possible to compare the present BEQs with instrumental-derived toxicity equivalents (TEQs) in a mass-balance approach.

The fact that via GC-MS measures only detected four PAHs, which according to the abovementioned REP studies are not responsible for the observed effects in the EROD assay and previous studies indeed could prove high-molecular PAHs to desorb from sediments (Shor et al. 2003, van Noort et al. 2003), lead to the assumption that: (1) other dioxin-like non-targeted compounds were present in the tenax extracts, which led to EROD-induction and/or (2) the assay was able to even detect small concentrations of desorbed, high-molecular EPA-PAHs not detected by using GC-MS.

Summarizing these results, tenax extracts partly reflected the levels of cumulative desorbed PAHs measured by means of GC-MS, but possibly indicated the presence of dioxin-like compounds non-targeted by chemical analysis. Because the EROD assay used DMSO as a solvent carrier, the assays' results do not reflect any bioavailability (Reid et al. 2000), thus the EROD assay rather reflects the possible overall receptor-mediated effects worst-case scenario

of all desorbing compounds over a 53-days' time range. The use of DMSO hence most likely overestimates the cell-based fate of the bioavailable fraction (Brack et al. 2009).

6.5.3.2 Sediment contact assay (SCA)

Although tenax extraction techniques are considered to reflect bioavailability, the complex process of bioavailability encompasses further organism- and species-specific properties (Reid et al. 2000). Meaning that contaminant concentration alone most likely is insufficient to predict the environmental risk of sediments.

Because the SCA was ranked valid, results obtained for eggs exposed to sediments are reliable. The effect of egg coagulation is relatively unspecific compared to other effects. Negative controls, on which eggs were normally developed distinctly differed from eggs exposed to the four replicates. These differences were found throughout the three conducted replicates. Sub-lethal effects encompassed all listed effects (see *Section 6.3.5.2*), except malformations of the spinal column. Some of the eggs, independent of the sediment to which they were exposed to, were coagulated after 48 hours. Coagulation can be the result of lacking oxygen (Strecker et al. 2011) and cannot completely excluded due to sediment-related oxygen attrition and a missing oxygen control during the exposure time. An increase in effect percentage with increasing sediment concentration could not be found, which indicates, that the dilution of sediment through the quartz sand did not diminish the effects. However, highest effect levels were reached in the second highest sediment concentration, except for the mixture EBR/ZE. The unexpected result that pure sediment (highest concentration) showed less effects compared to the remaining quartz sand diluted approaches, possibly indicates that the quartz sand increased the bioavailability of desorbing compounds from the four sediments.

The maximum sub-lethal effect levels of approximately 35% for sediment EBR and the mixture EBR/ZE, 66% for sediment PR and 68% for sediment ZE, hence more or less reflected the sequence of the initial PAH contamination degree of these sediments (refer to *Section 6.4.1*) and more or less reflected the cumulative desorbing fractions of the sum of compounds desorbing from these sediments (*Figure 6.3a*). It is therefore assumable that the higher the initial PAH sediment contamination and the respective amount of the bioavailable PAH fraction, the higher the effect levels on a fish embryo level. Although the desorbed fraction decreases with time, the bioavailability increases with time (Reid et al. 2000), which means that the SCA covers the most harmful time range in terms of desorption. Nevertheless, results obtained by using the SCA are rather complicated to transfer to other aquatic species such as benthal oranisms, because accumulation is promoted by additional uptake pathways such as ingestion (Landrum 1989). Ingestion of sediment-associated PAHs especially is of high

importance for stronger bond, high-molecular PAH congeners (Kukkonen et al. 2004, Landrum 1989, Ten Hulscher et al. 2003). For this, low-molecular PAHs, which have been shown to rapidly desorb from the present sediments during the 53-days desorption experiment, most likely were the more accessible congeners in the SCA.

Nevertheless, it is likely that beside the PAH congeners instrumentally investigated here, other sediment-borne bioavailable compounds caused the observed effects. Compounds such as polychlorinated dibenzo-*p*-dioxins and dibenzo furans (PCDD/Fs), polychlorinated biphenyls (PCBs) as well as a multitude of dioxin-like compounds could for instance account for those effects (Cantrell et al. 1998, Sundberg et al. 2005). While embryos of fish eggs exposed EBR and the mixture EBR/ZE did not show any predominant effects, 67% and 56% of all effects in embryos exposed to highly PAH contaminated sediments PR and ZE, respectively, amounted for missing pigmentation of skin and/or eyes and in 30% of these cases, both eyes and skin were affected at the same time. A number of PAHs are known to cause such teratogen effects in early-life stages of *D. rerio* (Barron et al. 2004). The lack of protective pigmentation possibly leads to an increased sensitivity of the fish embryos towards UV light (Kosmehl et al. 2006). Beside the sub-lethal effects, all of the investigated sediments showed an embryotoxic potential with maximum mortality of 36% for embryos exposed to sediment PR and approximately 15% for embryos exposed to the remaining sediments. Besides the PAH congeners investigated here, PCBs could possibly have led to this increased mortality (Hollert et al. 2003, Westerlund et al. 2000). Again, the relatively higher PAH contaminated sediments PR and ZE led to higher mortality, indicating a correlation between general sediment contamination degree and lethal effects.

6.6 Conclusion

The PAH congeners phenathrene, fluoranthene and pyrene predominantly desorbed from the four sediments during 53 days, thus, were considered as most potentially bioavailable contaminants. Desorption, which occurred in a bi-phasic manner (rapidly and slowly desorbing fraction) in its intensity corresponded to the initial sediment contamination. Although rapid desorption lasted longer for slightly than for the highly contaminated sediments, the latter ones with much higher short-time concentrations of desorbing PAHs, most likely possess a greater environmental risk potential e.g. during floods. Rates of desorption were dependent from sediment-specific (here: percentage of sediment organic matter) and compound-specific (here: water solubility, log K_{ow}) characteristics, with higher organic sediments PR and ZE causing the

highest rates of desorption and thus, indicating a higher potential risk to the aquatic environments during e.g. floods.

Extracts from the desorption experiment all showed dioxin-like activity and the temporal course of cumulative BEQs corresponded to that of the cumulative PAH desorption of phenanthrene, pyrene and fluoranthene. These congeners, however, are non-inducers for cell line RTL-W1, raising the question if high-molecular PAHs, which probably stayed un-detected in the extracts, caused those effects *in vitro* or if other dioxin-like and non-targeted compounds could have caused these effects. Increased cumulative concentrations of desorbed PAHs did not lead to increased EROD activities. Due to the use of DMSO as a solvent carrier, the EROD assay did not reflect bioavailability but rather a worst-case scenario of all desorbing compounds over 53 days. In the SCA assay sub-lethal and lethal effect levels were highest in embryos exposed to sediments PR and ZE, highly contaminated with PAHs, and more or less corresponded to the intensity of desorption from all sediments, leading to the assumption that initial sediment contamination and intensity of desorption determine the extent of embryotoxic effects. It is likely that beside the analyzed PAH congeners, other sediment-borne bioavailable compounds such as dioxins or led to the observed effects.

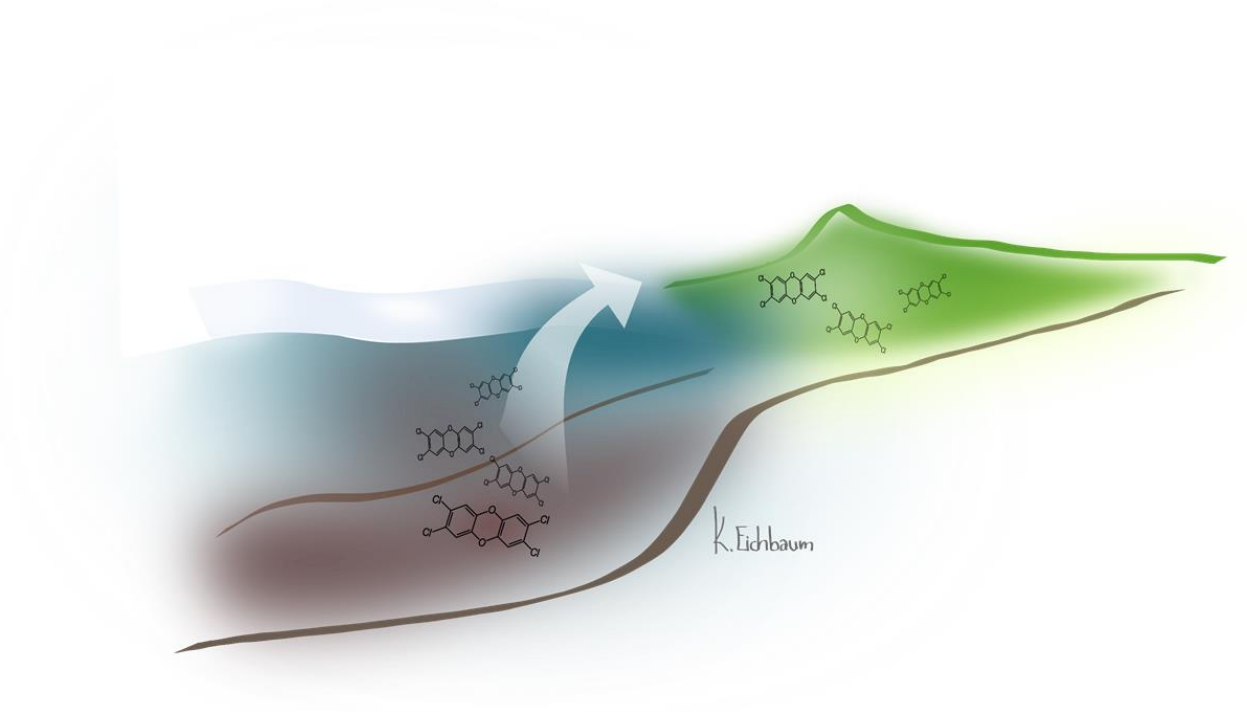
Consequently, the applied ecotoxicological test systems partly supported the instrumental findings, partly uncovered deficits of those quantifying analyses and with this pointed towards an improved sediment risk assessment. In terms of bioavailability and toxicity, tenax extraction combined with subsequent ecotoxicological testing allows for prioritization of contaminated sediments. Since the rapidly desorbing fraction is considered to reflect bioavailability, future experiments could be shortened to a couple of days to reduce the experimental expense.

6.7 Acknowledgements

The present work forms a part of the DioRAMA project (“dioxin Risk Assessment for sediment Management Approaches”) that received funds from the German Federal Ministry of Transport and Digital Infrastructure. MB received a personal stipend from the German National Academic Foundation (“Studienstiftung des deutschenVolkes”). HH was supported by the Chinese 111 Program (College of Environmental Science and Engineering and Key Laboratory of Yangtze Water environment, Ministry of Education, Tongji University). The authors wish to especially thank MSc Miriam Zimmer for conducting the desorption experiment and the adjacent GC-MS measurement.

Chapter 7

Spatial variability of the pollution of sediment and soil samples with dioxin-like compounds along the river Elbe and its alluvial plain



Parts of this chapter have been submitted to Environmental Science Europe

Eichbaum, K., Brinkmann, M., Winkens, K., Umlauf, G., Stachel, B., Buchinger, S., Reifferscheid, G., Möhlenkamp, C., Weber, R., Hollert, H. (submitted) Spatial variability of the pollution of sediment and soil samples with dioxin-like compounds along the river Elbe and its alluvial plain *Environmental Science Europe*

7.1 Abstract

The river Elbe catchment area is the fourth largest catchment area in Europe. Frequently occurring flood events can cause sediments contaminated with dioxin-like compounds (DLCs) to be re-transferred into the water phase, where they can threaten the aquatic environment. The present work applied two 7-ethoxyresorufin-*O*-deethylase (EROD) bioassays with RTL-W1 and H4IIE cells for screening DLCs in raw extracts of sediment samples from a longitudinal sampling program in 2008 and soil samples of inundated floodplains taken in 2003. Bioassay data were compared with chemical analysis data and discussed in the context of contamination hotspots and the Elbe flood in August 2002.

Dioxin-like activities of sediment and soil samples were comparable and showed similar trends in both assays (reproducibility < 23%; r^2= 0.586). Samples KS 11 (a former dumping site of sewage sludge in the North Sea), Schnackenburg, Prossen, Lysa nad Labem and soils of the Mulde tributary floodplain showed highest EROD-inductions, which corresponded to contamination hotspots along the Elbe. Near-river soil samples of floodplains consistently showed higher dioxin-like activities than distant samples, indicating that more frequent inundations led to higher contaminations. The trend of highest EROD-induction of samples Schnackenburg, Prossen and Lysa nad Labem was further confirmed following normalization of bioassay results to total organic carbon (TOC) contents. Correlation between bioassay and chemical data was weak, most likely due to the missing clean-up of extracts analyzed via bioassays.

Since prioritized samples were in good accordance between both cell lines, we conclude that bioassays can provide important information for the assessment of contaminated sediments and soils in addition to chemical investigations.

Keywords: Micro EROD assay • EROD assay • Toxicity Equivalent Quotient • REP

7.2 Introduction

The river Elbe extends from the Krkonoše Mountains, Czech Republic, to the North Sea at Cuxhaven, Germany (FGG-Elbe 2015). The river's most important tributaries include Vltava, Saale, Havel, Mulde, Schwarze Elster and Eger. With 148,268 km², the river Elbe catchment area is the fourth largest river catchment area in Europe (LUA 2005). It serves as an important waterway, recreational area and as habitat for a diverse flora and fauna (92/43/EWG 1992), including migratory fish species such as European eel (*Anguilla anguilla*) and Atlantic salmon (*Salmo salar*) (IKSE 1992-1995, 1996-2010). However, increasing lining, sealing and water level regulation in the tidal part of the river and along its course, especially in the Czech upper reaches of the river Elbe, led to increases in current velocities and promoted the occurrence of periodical flood events (LUA 2005) like the Elbe flood in August 2002, which until today belongs to the most significant inundations of Central Europe (UFZ 2003).

Apart from such flood events, the Elbe ecosystem is threatened by contamination through e.g. persistent organic pollutants (POPs), which – due to their physical and chemical properties – adsorbed to sediments. Following floods or other hydrogeological events such as dredging and/or bioturbation, sediments get remobilized and reintroduced into the water column and can contaminate Elbe associated floodplains or downstream river regions (Burton 1992, Förstner 2009). Certain POP contamination in the river Elbe significantly exceed the pollution levels of other major European rivers (IKSE 1992-1995) and mainly originate from treated and untreated industrial wastewaters of industries in the Bitterfeld area producing magnesium and organochlorine compounds (including HCH, DDT) (Götz and Lauer 2003, Jacobs et al. 2013, Wilken et al. 1994, Wycisk et al. 2013)or Czech industries such as Synthesa in Pardubice, Lovochemie in Lovosice or Spolana and Spolchemie in Neratovice (Heinisch et al. 2007, Stachel et al. 2004, Umlauf et al. 2010) . The POPs releases from industries in Bitterfeld-Wolfen, a city of the Mulde catchment area, are the major source for contamination of land and floodplains of the Elbe river in Germany (Brack et al. 1999, Götz et al. 2007, Stachel et al. 2004, Umlauf et al. 2005, Umlauf et al. 2010, Wölz et al. 2008), which resulted in threshold exceedances for POPs (in particular dioxins) in milk and meat of exposed grazing cows (Schulz et al. 2005, Stachel et al. 2005).

Within the group of POPs, one can find the so-called dioxin-like compounds (DLCs), which include polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), as well as dioxin-like polychlorinated biphenyls (DL-PCBs). While PCDD/Fs (collectively referred to as *dioxins*) are unintentional, industrial byproducts that are mainly formed from anthropogenic origin, formed during organochlorine production, in combustion processes as

well as during e.g. pulp- and paper or magnesium production when chlorine is applied (Fent 2007, Safe 1990a, UNEP 2013 , Weber et al. 2008). The majority of PCB contamination stem from the approximately 1.3 million tons of technical PCB mixtures used in a range of closed applications (transformers, capacitors, hydraulic fluids) and open applications (e.g. plasticizers, paints, cutting oils, flame retardants) containing approximately 1000 kg dioxin toxic equivalents (TEQs) (Breivik et al. 2002, Denison and Heath-Pagliuso 1998, Fent 2007, UNEP 1999, Wagner et al. 2014, Weber et al. 2008). Unintentional PCB are formed in thermal processes and organochlorine chemicals such as certain pigments or pesticides (Anezaki and Nakano 2014, Huang et al. 2014, UNEP 2013). Besides their partitioning to solids, DLCs accumulate in the food chain (biomagnification) and in this way also threaten humans and wildlife (Safe 1998a) by causing cancer, immune toxicity, neurotoxicity, teratogenicity, developmental toxicity, disruption of the endocrine system, reproduction and fertility (Denison and Nagy 2003, Fent 2007, Hilscherova et al. 2000, Safe 1990a, 1994).

On the cellular level, DLCs bind with high affinity to the Aryl hydrocarbon receptor (AhR). This cytosolic receptor belongs to a multimeric protein complex and binds DLCs and similar compounds with coplanar structures with high affinity (Fent 2007, Hilscherova et al. 2000). It has been shown that many of the toxic effects caused by DLCs are mediated by the AhR (Safe 1998b) and that the binding affinity of a ligand is proportional to its toxicity, transcriptional activity and AhR-mediated enzyme activities (Safe 1995). A ligand binding to the AhR causes AhR-associated heat shock proteins to dissociate from the complex and its adjacent translocation into the nucleus, where it forms a dimer with the AhR nuclear translocation protein (ARNT). The ligand-AhR-ARNT complex binds to dioxin responsive elements (DRE) and leads to transcriptional activation and the synthesis of e.g. cytochrome P_{450}-dependent monooxygenases (CYPs) (Hilscherova et al. 2000). CYPs are enzymes of phase I xenobiotic metabolism-, which catalyze the oxidation, reduction and hydroxylation of xenobiotics (Fent 2007). The subfamily CYP1A and especially its individual enzyme CYP1A1 is one of the most important enzymes in ecotoxicology and has been successfully used as biomarker for analyzing the pollutant exposure of e.g. fish (Whyte et al. 2000).

The most commonly applied method to quantify the expression of CYP1A *in vitro* is the measurement of 7-ethoxyresorufin-*O*-deethylase (EROD) activity (Behnisch et al. 2001b). It can be performed with different cell lines including the permanent fish liver cell line RTL-W1 (Rainbow trout liver - Waterloo1) and the wild-type rat hepatoma cell line H4IIE. The test principle is to measure the induction strength of CYP1A through EROD activity. Following deethylation of the exogenous substrate 7-ethoxyresorufin through EROD, the resulting

reaction product resorufin can be fluorometrically measured. After normalization to protein concentrations, the specific EROD activity can be calculated by the amount of resorufin formed by the proteins within a certain reaction time (Kennedy et al. 1993). Bioassays such as the EROD assay are cost-efficient screening tools, allowing for the analysis and prioritization of a multitude of samples and with this overcome some of the limitations of classical instrumental analysis (Wernersson et al. 2015).

Extracts of environmental samples can be evaluated by comparing their response in the EROD assay to the responses of one of the strongest inducers, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Such results are commonly expressed as so-called biological equivalents (BEQs), enabling a direct comparison to results of chemical analysis, expressed as toxicity equivalents (TEQs) (Safe 1998a, b, Van den Berg et al. 1998, Van den Berg et al. 2006). TEQs are the sum of all single DLC concentrations present in an environmental extract multiplied with their corresponding relative potencies (REPs), representing the congener's induction potential related to TCDD, which by definition has a REP of 1. This approach in the present study enabled the comparison between bioassay and chemical analysis derived concentrations of DLCs in soil and sediment samples along the river Elbe and selected alluvial plains.

7.3 Material and Methods

7.3.1 Study design

Freeze-dried sediment samples of an Elbe longitudinal sampling program of the year 2008 and soil samples from Elbe associated flood areas Glinde, Wörlitz and Mulde, which were sampled in the year 2003 following the Elbe flood of August 2002, were extracted by means of pressurized liquid extraction (PLE) and bio-analytically investigated by means of the EROD assay with the fish liver cell line RTL-W1 and the Micro-EROD assay with the rat hepatoma cell line H4IIE. *In vitro* results were discussed in the context of contamination hotspots along the river's course and the flood event of 2002. Finally, the obtained BEQs were compared with chemically/instrumentally determined concentrations, which had been analyzed in former studies (Stachel et al. 2006, Stachel et al. 2011, Umlauf et al. 2010).

7.3.2 Sampling and composition of sediment and soil samples

All freeze-dried sediment and soil samples along with the corresponding measured total organic carbon (TOC) contents, grain size distributions and concentrations of PCDD/Fs and DL-PCBs were provided by the Joint Research Center in Ispra, Italy. Details of both, sampling

and analysis of sediment and soil samples have been published elsewhere (Stachel et al. 2006, Stachel et al. 2011, Umlauf et al. 2010). *Figure 7.1* provides an overview of the Elbe longitudinal profile samples of 2008 (red dots; *Figure 7.1*), as well as the inundated flood areas (boxes 1, 2 and 3 for areas Wörlitz, Glinde and Mulde, respectively; *Figure 7.1*).

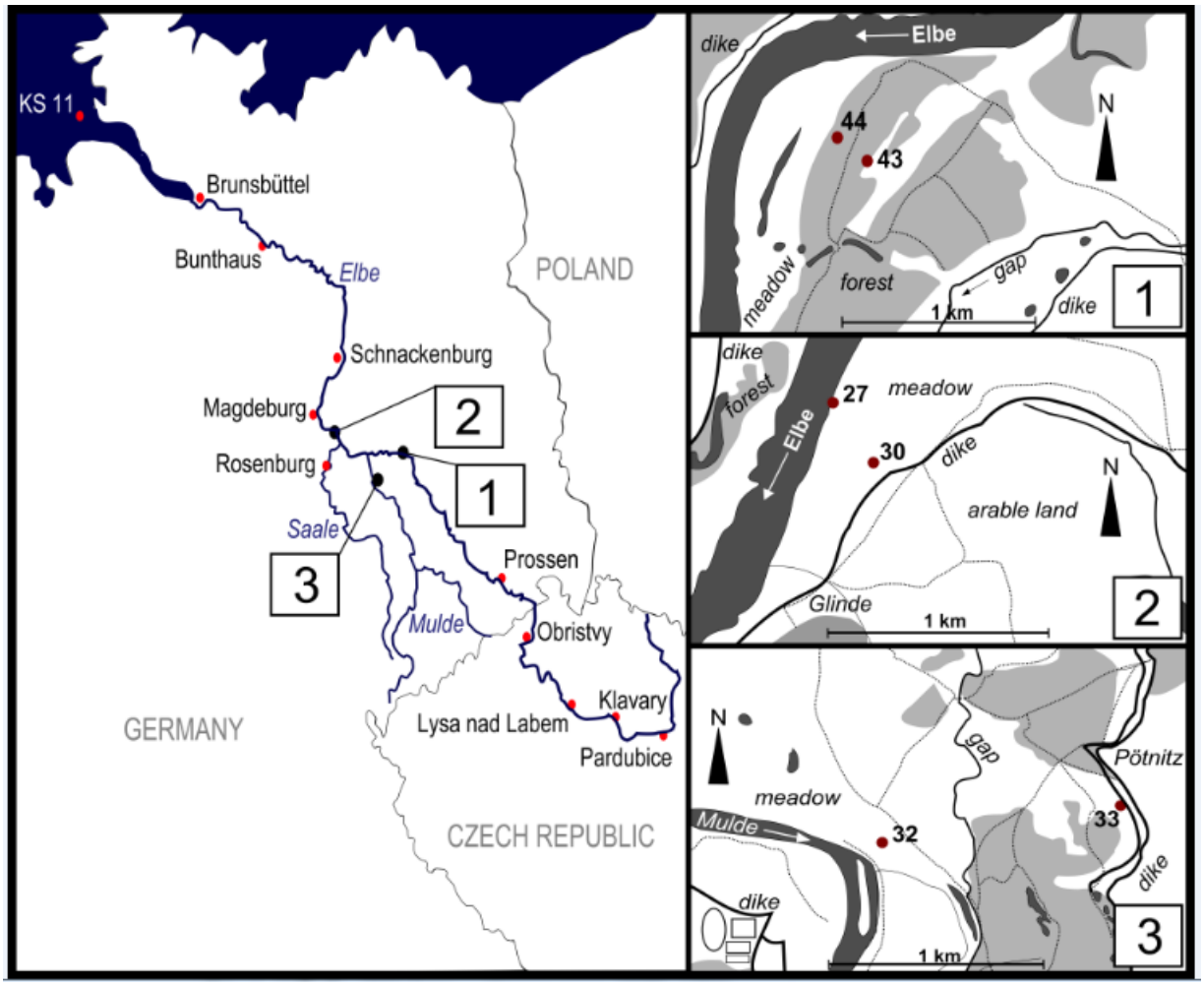


Figure 7.1 Map of sediment and soil sampling locations. Red dots (left side) show sediment sampling locations of an Elbe longitudinal profile sampling program in the year 2008; Numbered boxes (left side) show locations of the three inundated floodplains Wörlitz (1), Glinde (2) and the tributary Mulde (3), which are shown in detail on the right side; the map was designed according to (Stachel et al. 2006).

7.3.3 Extraction

Freeze-dried samples were stored at 4 °C until analysis. Sediments and soils were sieved < 2 mm and homogenized before 10 g dry weight (dw) of each sample were mixed with fire-dried quartz sand and extracted by means of PLE (E-916, BÜCHI Labortechnik GmbH, Flawil, Switzerland). Extraction was conducted according to short notes of BÜCHI Labortechnik GmbH, Flawil, Switzerland (BÜCHI 2009), using an acetone/*n*-hexane mixture (v/v; 50/50; p.a.; Roth, Karlsruhe, Germany) at 100 °C and a pressure of 120 bar. Each extraction consisted

of two cycles, including a heat-up (1 min), a hold (10 min) and a discharge phase (2 min). In total, three process controls, containing quartz sand only, were extracted at the beginning, in the middle and at the end of the whole sample extraction procedure.

Extracts were rotary evaporated down to a volume of approximately 1 ml and gravimetrically divided into two equal parts. These aliquots were transferred into amber glass vials with PTFE septum-containing screw caps (VWR, Darmstadt, Germany) and were later on used for chemical and bio-analytical purposes. For the first purpose, extracts were stored in *n*-hexane, whereas the latter one was blown down to dryness completely under a gentle nitrogen stream and re-dissolved in dimethyl sulfoxide (DMSO, p.a.; Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

7.3.4 Chemical extract analysis

7.3.4.1 PCDD/Fs and DL-PCBs

Details on the extraction and subsequent determination of PCDD/Fs and DL-PCBs measured *via* high resolution gas chromatography – high resolution mass spectrometry (HRGC/HRMS) can be found elsewhere (Stachel et al. 2006, Stachel et al. 2011, Umlauf et al. 2010).

7.3.4.2 16 US EPA PAHs

Concentrations of 16 US EPA PAH in sediment and soil extracts were analyzed by the Federal Institute of Hydrology (BfG) in Koblenz, Germany. Quantitative analysis was performed *via* gas chromatography - mass spectrometry (GC/MS; GC 6890- MS 5977N Agilent Technologies Deutschland GmbH, Böblingen, Germany) over a HP5-MS capillary column (30m x 0.25mm; 0.25 µm film thickness, Agilent). The mass selective detector (MSD) was operated in single ion monitoring (SIM) mode. The temperature program of the GC was as follows: 60 °C (2.5 min isothermal) with 20 °C/min to 130 °C, 4 °C/min to 320 °C (1 min isothermal). The quadrupole temperature was 150 °C, whilst the injection temperature was 230 °C. Samples were 1:10 fold diluted with *n*-heptane and PAH concentrations were interpolated from a dilution series of external standards (2.5, 5, 10, 25, 50, 100, 200, 500 and 1000 pg/µl) using MassHunter software (Agilent).

7.3.5 Bio-chemical extract analysis

7.3.5.1 EROD assay with RTL-W1 cells

The RTL-W1 assay was performed according to *section 2.3.1* with the exceptions that only one sample was tested per plate and that DMSO concentrations in each well were < 1%.

7.3.5.2 The Micro EROD assay with H4IIE cells

The H4IIE Micro EROD assay was performed according to *section 2.3.2*.

7.3.6 Data analysis and representation

All graphical drawings were produced using the vector graphic program Inkscape 0.48. Bio-analytical data was processed *via* Excel (Microsoft Office Excel 2003) and concentration-response curves were plotted using GraphPad Prism 5 software (La Jolla, CA, USA) using a non-linear regression and a dose-response stimulation (log agonist vs. response). BEQs were calculated according to *equation 2.1* with *x* being the concentration at a 25% effect level. TEQs were calculated according to *equation 2.5*. Details on *x* used in *equation 2.5* are given in *section 2.4.2* Limits of detection (LODs) and quantification (LOQs) were calculated according to *equations 2.3* and *2.4*, respectively. Z-factors were calculated as given in *equation 2.2* and reproducibility as described in *section 2.3.5*.

Statistical analysis was conducted using Sigma Plot 12.0 software. Normality was analyzed by means of a Shapiro-Wilks test ($p < 0.5$). Differences between BEQs of Elbe length profile samples were analyzed using a repeated measures one-way ANOVA ($p < 0.005$) with Tukey's multiple comparison test ($p = 0.05$) as post-hoc test, Elbe inundated flood area samples with respect to their distance to the river were compared via a two-tailed student's t-test ($p < 0.05$; $p = 0.05$). All reported linear correlation coefficients were calculated as Pearson's correlation coefficients ($p = 0.05$).

7.4 Results

7.4.1 General consideration of bioassay results

Altogether, 51 measurements performed with the RTL-W1 EROD and H4IIE Micro EROD assay resulted in average limits of detection (LOD) of 1.86 ± 0.84 and 0.24 ± 0.06 pM TCDD, whereas limits of quantification (LOQ) reached values of 4.59 ± 2.00 and 0.39 ± 0.09 pM TCDD, respectively. The average z-factor of 51 measurements summed up to 0.77 ± 0.16 and

0.66 ± 0.26 for the RTL-W1 EROD and H4IIE Micro EROD, respectively. The average coefficients of variation (CV), taken from the LOD, LOQ and z-factor calculations, accounted for 23.2 ± 1.5% and 42.6 ± 3.2% for the RTL-W1 EROD and H4IIE Micro EROD assay, respectively. The reproducibility of three independent replicates on average was 28.6 ± 16.2 and 21.7 ± 9.1 for the RTL-W1 EROD and H4IIE Micro EROD assay, respectively.

7.4.2 EROD-activity of Elbe length profile sediment samples from 2008

Figure 7.2 shows that all tested raw extracts, including Elbe longitudinal profile samples, and samples of inundated flood areas possessed dioxin-like activity. Process controls, which were tested in concentrations similar to the highest extract concentrations, caused no such effects (data not shown).

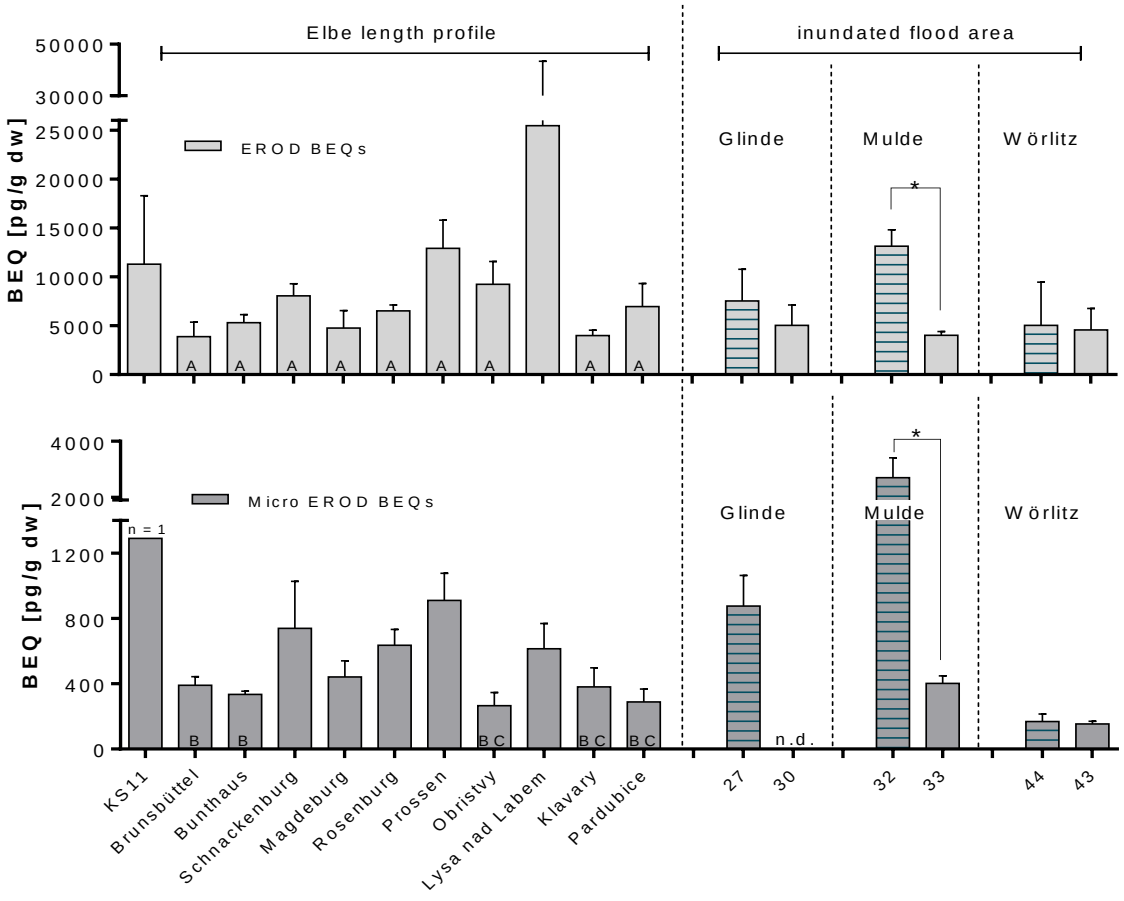


Figure 7.2 Biological equivalents (BEQs) of sediment and soil raw extracts, produced via the RTL-W1 EROD (upper part, lighter grey bars) and H4IIE Micro EROD bioassays (lower part). Elbe longitudinal profile sediment samples (left side, darker grey bars) were taken in the year 2008, while soil samples of the inundated flood area were taken in the year 2003. All sampling sites are depicted in *Figure 7.1*. Bars show average of three independent replicates with corresponding standard deviations. Dashed bars mark near-river samples. Capital letters show the results of one-way ANOVA ($p < 0.005$) with Tukey's multiple comparison test ($p = 0.05$): Significant differences

compared to Lysa nad Labem (A), Prossen (B) and Schnackenburg (C). Asterisks mark samples which, according to student's t-test (p = 0.05), were significantly different (p = 0.05); n.d. = not determined.

RTL-W1 EROD BEQs ranged from 3990 to 25470 pg/g dry weight (dw) sediment and with this, were constantly higher compared to H4IIE Micro EROD BEQs, which ranged from 150 to 2700 pg/g dw sediment (*Figure 7.2*). Nevertheless, equal trends in sample activities could be observed between the two assays. For instance, both methods identified the samples KS 11, Schnackenburg, Prossen and Lysa nad Labem as those with the highest dioxin-like potential. In the RTL-W1 EROD assay, the sample Lysa nad Labem showed the overall highest EROD-inducing potential and was proven (ANOVA with Tukey's comparison test; $p < 0.05$) to be significantly different from all remaining Elbe longitudinal profile samples, except sample KS11.

Regarding H4IIE Micro EROD assay results, five samples (highlighted with B; *Figure 7.2*) were significantly different (ANOVA; $p < 0.05$ with Tukey's method; $p = 0.05$) from sampling site Prossen, whereas three samples (marked with C; *Figure 7.2*) turned out to be significantly different from those of the location Schnackenburg.

7.4.3 EROD-activty of inundated flood area samples from the year 2003

For each of the inundated floodplain soils from the areas Glinde, Mulde and Wörlitz, two sampling sites existed, of which one was located close to (striped bars, *Figure 7.2*) and the other one with greater distance to the respective river system (*Figure 7.1*). For each of the three sampling areas, both bioassays indicated higher dioxin-like activities of samples with smaller distance to the river compared to their more distant counterparts. For the Mulde area, this difference was proven to be significant (two-tailed student's t-test; $p = 0.05$) using both, the RTL-W1 EROD ($p = 0.007$) and H4IIE Micro EROD ($p = 0.034$) assay. Furthermore, sample 32 of the Mulde area was the sample with the highest overall induction potential in the H4IIE Micro EROD assay, and in the RTL-W1 EROD assay it was among the highly inducing samples. Rear dike sample Mulde 33, which got inundated once after a dike break during the flood in August 2002 (Stachel et al. 2006), showed lower REP-based TEQs for both cell lines compared to Mulde sample 32 (*Table 7.1*), which was located next to the Mulde River. BEQs determined in composite samples of the transects increased in the order Wörlitz < Glinde < Mulde using both assays, but generally were in in the same order of magnitude like the BEQs determined for Elbe sediment samples (*Figure 7.2*).

7.4.4 Comparison of BEQs and REP-based TEQs

While the correlation (Pearson, $p = 0.05$) of RTL-W1 EROD and H4IIE Micro EROD BEQs was significant ($r^2 = 0.343$), neither the RTL-W1 EROD ($r^2 = 0.003$), nor the H4IIE Micro EROD assay ($r^2 = 0.237$) correlated with their REP-based TEQ values presented in *Table 7.1*.

Table 7.1 Sampling types, total organic carbon (TOC) content, grain size distributions as well as RTL-W1 and H4IIE relative potency (REP)-based toxicity equivalents (TEQs) of sediment samples of the river Elbe and soil samples of Elbe associated flood areas; all sampling sites are depicted in *Figure 7.1*; n.a. = not analyzed; PAH = polycyclic aromatic hydrocarbons; PCDD/F = polychlorinated dibenzo-p-dioxins and dibenzofurans; DL-PCB = dioxin-like polychlorinated biphenyls; sampling types, TOC, Fraction and concentrations of DL-PCBs and PCDD/Fs according to (Stachel et al. 2006, Stachel et al. 2011, Umlauf et al. 2010).

				RTL-W1 REP-based TEQs [pg/g dw]		**H4IIE REP-based TEQs [pg/g dw]**	
Sample	Type	TOC [% dw]	Fraction < 20µm [% dw]	PAH TEQ[a]	DL-PCB PCDD/F TEQ[b]	PAH TEQ[c]	DL-PCB PCDD/F TEQ[c]
KS 11	EP	2.2	n.a.	n.a.	69.5	n.a.	34.4
Brunsbüttel	EP	1.7	26	n.a.	111.0	n.a.	49.9
Bunthaus	FDS	4.8	45	478.3	110.3	624.5	49.1
Schnackenburg	FDS	5.8	46	550.1	200.7	698.9	90.2
Magdeburg	FDS	5.7	69	253.0	292.0	315.4	128.6
Rosenburg	FDS	4.8	68	605.9	154.4	754.9	67.3
Prossen	FDS	7.1	78	1058.0	45.1	1318.2	21.6
Obristvy	FDS	5.6	44	555.8	34.1	699.6	15.4
Lysa n. Labem	FDS	6.9	33	795.5	36.1	1019.3	15.7
Klavary	EP	3.7	n.a.	n.a.	11.3	n.a.	4.7
Pardubice	EP	3.2	n.a.	595.5	141.5	746.0	51.6
Glinde 27	TS	8.1	n.a.	826.4	1201.9	1044.0	522.7
Glinde 30	TS	9.5	n.a.	639.0	2071.6	845.3	919.6
Mulde 32	TS	7.9	n.a.	n.a.	2168.5	n.a.	980.3
Mulde 33	TS	13	n.a.	n.a.	372.8	n.a.	164.0
Wörlitz 44	TS	6.2	n.a.	558.1	120.9	742.6	51.3
Wörlitz 43	TS	9.0	n.a.	623.8	127.1	827.2	54.2

a = according to Bols et al. (1999) deduced from EC_{50} following 24 h of incubation; b = according to Clemons et al. (1997) deduced from EC_{50} following 72 h of incubation; c according to Behnisch et al. (2003) deduced from EC_{20} following 72 h of incubation
EP = individual samples; FDS = composite samples of freshly deposited sediments (FDS);TS = transect sample (16 individual samples)

TEQs were much lower compared to RTL-W1 EROD BEQs, so that only 13 ± 5% of BEQs could on average be explained through the respective TEQs. In contrast, H4IIE Micro EROD assay specific TEQs explained 219 ± 102% of the respective BEQs. Total RTL-W1 EROD TEQs comprised 58% PAHs and 42% PCDD/Fs, total H4IIE Micro EROD TEQs 92% PAHs and 8% PCDD/Fs, respectively. For the inundated flood areas, the PCDD/F percentage in total TEQs increased.

7.4.5 TOC-normalized EROD-activity of Elbe length profile samples

Following a TOC normalization (*Figure 7.3*) H4IIE BEQs in more than half of the longitudinal samples were proven to be significantly smaller (repeated measures one-way ANOVA, $p < 0.05$ with Tukey's multiple comparison test, $p = 0.05$) than BEQs in extracts from the sampling sites Schnackenburg, Prossen and Lysa nad Labem.

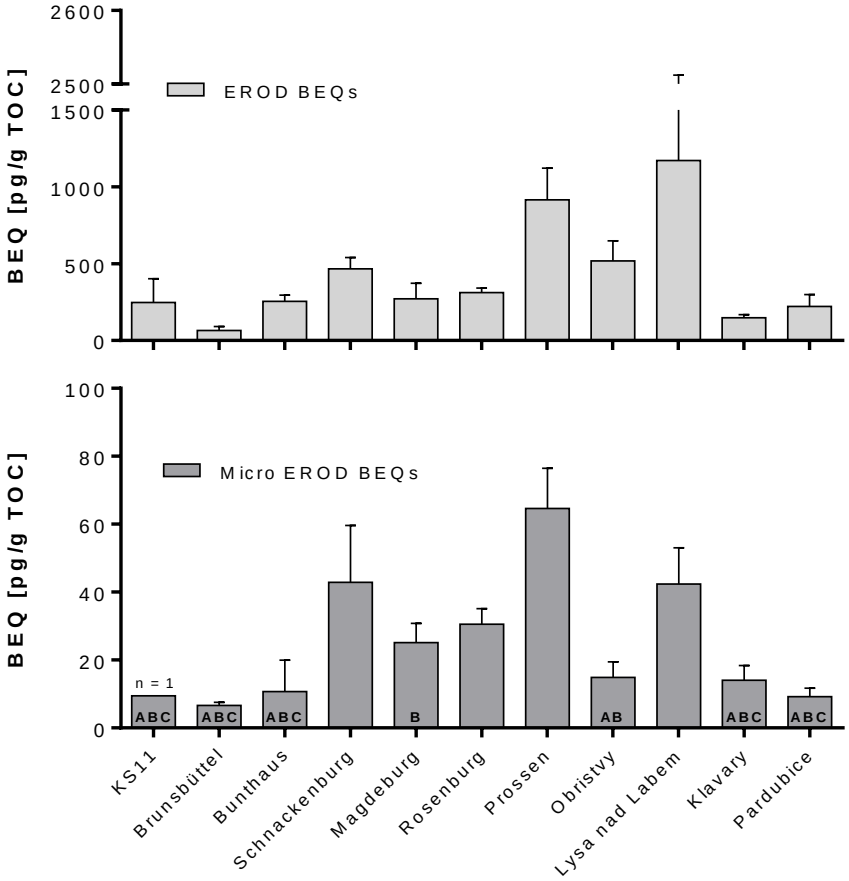


Figure 7.3 Total organic carbon (TOC)-normalized biological equivalents (BEQs) of sediment raw extracts from an Elbe longitudinal profile (sampled in 2008 and depicted in *Figure 7.1*), produced via the RTL-W1 EROD (upper part, lighter grey bars) and H4IIE Micro EROD assay (lower part, darker grey bars). Bars show average values from three independent replicates with corresponding standard deviations. Capital letters show the results of a one-way ANOVA ($p < 0.05$) with Tukey's multiple comparison test ($p = 0.05$): Significant different compared to samples Schnackenburg (A), Prossen (B) and/or Lysa nad Labem (C).

Although a repeated measures one-way ANOVA ($p < 0.05$) with Tukey's multiple comparison test ($p = 0.05$) did not show any significances when conducted with the RTL-W1 data, similar trends for higher TOC-normalized BEQs in the samples Schnackenburg, Prossen and Lysa nad Labem could be observed.

7.5 Discussion

7.5.1 General consideration of bioassay results

Average LOD and LOQ calculations from 51 bioassay measurements proved the H4IIE Micro EROD assays to allow for the detection of much lower concentrations compared to the RTL-W1 EROD assay. This indicates that the assay to be more suitable for the investigation of weak AhR inducers. According to the average z-factor determined for 51 measurements, both assays could be classified as excellent, with a good separation of the highest standard concentration and the negative control (Zhang et al. 1999). Whereas the average CV calculated from the LOD, LOQ and z-factors showed that the Micro EROD assay is less subjected to variability than the RTL-W1 EROD assay. According to the results of reproducibility calculations, both assays were located within an acceptable range of reproducibility (2012/252/EU 2012).

7.5.2 EROD-activity of Elbe length profile sediment samples from 2008

Although RTL-W1 EROD BEQs were constantly higher compared to H4IIE Micro EROD BEQs, equal trends in sample activities using both assays could prove their predictive potential. Samples KS 11, Schnackenburg, Prossen and Lysa nad Labem were the samples of highest dioxin-like potential. Lysa nad Labem showed the overall highest EROD-inducing potential from cell line RTL-W1, which correspond to findings of Behnisch et al. (2010), who investigated desulfurized and cleaned sediment extracts of sediment from Brunsbüttel, Magdeburg, Rosenburg, Lysa nad Labem and Klavary (sample aliquots were used in the present study) *via* the DR-CALUX® with the cell line H4IIE-luc. Behnisch and co-workers (2010) received BEQs between 1300 and 8240 pg/g dw sediment and identified location Lysa nad Labem as overall highest AhR-activating extract. This increased potential most likely originated from historical pollution of organochlorine industries in the Czech Republic as shown by (Heinisch et al. 2007) including the plant "Spolana" in Neratovice located upstream, which until 1986 produced the organochlorine herbicide 2,4,5-trichlorophenoxyacetic acid known as source for dioxin from Agent Orange assessments (Stachel et al. 2004, Stellman et

al. 2003, Van Thuong et al. 2014) and from contamination at 2,4,5-T production sites (Weber and Varbelow 2013).

Considering the "dioxin transport hypothesis", DLC-loaded particles may have been transported along the river and in turn caused the contamination of downstream areas (Lick 2009, Stachel et al. 2011). The assumption that an emitter around the sampling site Neratovice served as a source of DLC contamination for downstream river region Lysa nad Labem is supported by the lower RTL-W1 EROD and H4IIE Micro EROD BEQs from sampling sites Klavary and Pardubice located upstream (*Figure 7.2*). On the contrary, only the REP-based TEQs in sediment samples from the location Klavary support this assumption with regard to chemical analyses, while those of sample Pardubice are distinctly higher compared to TEQs for Lysa nad Labem in both bioassays (*Table 7.1*). For a more robust assignment of DLC pollution and sources, PCDD/F and DL-PCBs and other DLCs congener patterns in sediments and patterns in (former) chemical processes will need to be compared.

North Sea sampling site KS11 was located in a place of former ocean dumping for sewage sludge from the city of Hamburg (Umlauf et al. 2010). Sewage sludge is known to carry high loads of DLCs, which due to anaerobic conditions in sewage sludge digestion tanks are less affected by degradation (Schramm et al. 1995). Umlauf et al. (2010) reported that North Sea reference samples, un-affected by former dumping activities (not investigated here), in contrast to sample KS 11 did not show elevated PCDD/F and PCB contaminations. Previous investigations of sediment cores of the Baltic Sea could prove that dumping site extracts exhibited and in contrast to a reference site 5-fold higher EROD-inducing potential in cell line RTL-W1 (Wölz et al. 2009). It is therefore assumable that the high EROD-inducing potential of sample KS 11 in the present study was a result of former dumping activities. It is further known that river sediments continuously lead to a contamination of the North Sea (Stachel et al. 2003, Stachel et al. 2011) with special regard to contaminants originating from the Bitterfeld industrial region (Götz and Lauer 2003, Stachel et al. 2005, Umlauf et al. 2010). Hence, sample KS 11 most likely also reflects contamination through the river Elbe.

Regarding H4IIE Micro EROD assay results, sampling sites Prossen and Schnackenburg significantly differed from the remaining sampling sites. The German sampling site Prossen, located next to the Czech-German border, most likely represents particle-bound pollutant loads from the Czech Republic that had been transported downstream (UFZ 2003). For instance, the Czech city Ustí nad Labem located upstream of the sampling site hosts the chemical plant "Spolchemie", which until the year 2000 was a strong PCB emittent (Heinisch et al. 2007). The increased BEQ of 740 pg/g dw measured in sample Schnackenburg corresponds well to the

sample's increased WHO_{2005}TEQ of 51 pg/g dw, which formerly had been analyzed by Umlauf et al. (2010). According to the authors, this comparably higher TEQ results from DLC loads transported downstream from the city of Magdeburg, which in that study turned out to possess the overall highest WHO_{2005}TEQ of 68 pg/g dw (Umlauf et al. 2010).

7.5.3 EROD-activity of inundated flood area samples from the year 2003

The fact that both bioassays indicated higher dioxin-like activities of near-river bank samples of transects Glinde, Wörlitz and Mulde compared to their more distant counterparts, could indicate that these areas were more frequently inundated and thus exhibited higher concentrations of DLCs. This hypothesis is supported for transects Glinde and Mulde by comparing their REP-based TEQs of the two sampling sites (*Table 7.1*). However, it has formerly been proven that, e.g., the distribution of PCB congeners on an equal sampling area may vary between 1 and 84% (Barceló and Petrovic 2007). Hence, the difference between one transect's samples could also be due to landscape-related variabilities (Stachel et al. 2005). Nevertheless, for the Mulde area, the difference between BEQs of samples close to and far from the Mulde water level was proven to be significant in both assays.

The comparably high dioxin-like activity of sample 32 of the Mulde in both assays was expected due to the well-known pollution background of the Mulde River. Its high dioxin loads are mainly caused by its tributary Spittelwasser. This tributary contains high concentrations of xenobiotics, which most likely originate from discharges of industrial sites such as the historic release from magnesium production (Götz and Lauer 2003, Jacobs et al. 2013) or those of the ion exchanger wofatit in Bittefeld-Wolfen (Brack et al. 1999). In a study of Stachel et al. (2007) sediment samples of tributary Spittelwasser produced WHO_{2005}TEQs of 1260 pg/g dw sediment, whilst 180000 pg/g dw WHO_{2005}TEQ were found for its associated floodplain. POPs in this area repeatedly and demonstrably contaminated inundated floodplains (Brack et al. 1999, Brack et al. 2002, Götz et al. 2007, Krüger and Gröngröft 2003, Stachel et al. 2004, Umlauf et al. 2005, Umlauf et al. 2010) as well as distant downstream river regions of the river Elbe until the North Sea (Götz and Lauer 2003). Heise et al. (2008) showed that 70 - 80% of dioxin contamination in sediments from Hamburg harbor can be ascribed to the dioxin contamination of the Mulde catchment area. REP-based TEQs of Mulde samples 32 and 33 (*Table 7.1*) on the one hand correspond to the bio-analytical findings (*Figure 7.2*), on the other hand both methods proved that the frequently flooded sampling site Mulde 32 was more contaminated compared to its once flooded counterpart.

According to the initial expectations and the REP-based TEQs for these areas (*Table 7.1*), inundated flood area samples of the transect Glinde in both bioassays showed higher BEQs compared to the transect Wörlitz (*Figure 7.2*). Wörlitz in contrast to the remaining transects is located upstream the Elbe tributaries Mulde and Saale (*Figure 7.1*) and, thus stayed unaffected by high contamination loads, which developed during the flood in August 2002 (Götz and Lauer 2003). In contrast, an in August 2002 inundated Mulde storage reservoir in Bitterfeld, a sink for particulate matter-bound DLCs (Klemm et al. 2005), caused high SPM loads of 10000 t/d (Stachel et al. 2011) and in turn contaminated downstream river regions such as the transect Glinde. Although the Saale tributary Bode showed relatively high PCDD/F WHO_{2005}TEQs of 102 pg/g dw (Stachel et al. 2004, Stachel et al. 2011, Umlauf et al. 2010), the contamination potential of river Saale is rather low compared to that of the Mulde River (Götz and Lauer 2003, Stachel et al. 2011, Umlauf et al. 2005). Hence, the comparably high BEQs for the transect Glinde most likely reflect contamination of the Mulde catchment area. Since the dioxin-like activities of sediment and floodplain samples were in the same order of magnitude (*Figure 7.2*), it might be assumed that the floodplain samples reflect Elbe sediment contaminations caused by frequent inundations and re-mobilized sediments. Although no data was available, representing the condition of floodplains prior to the flood event of August 2002 (Stachel et al. 2011), various studies support the assumption that frequent inundations led to contaminations of floodplains. For instance, recently it has been shown that frequently inundated floodplain soils of the river Rhine showed higher EROD-inducing potentials in cell line RTL-W1 compared to soil samples of infrequently inundated floodplains (Schulze et al. 2014). The authors view suspended particulate matter (SPM) and re-mobilized sediments from the river Rhine as the reason for the observed floodplain contaminations (Schulze et al. 2014). This re-mobilization of sediments with historical contaminations was further proven in a study, which investigated the dioxin-like potential of SPM extracts from rivers Rhine and Neckar by using the DR-CALUX assay with the rat hepatoma cell line H4L1.1c4 and the EROD assay with the cell line RTL-W1. The dioxin-like activity of SPM sampled during flood event peaks was on average 8-fold higher compared to that of SPM sampled during the high water runoff (Wölz et al. 2008). Increased PCDD/F values, which have been found in eels (*Anguilla anguilla*) directly after the Elbe flood in August 2002 furthermore prove the hazard potential of re-mobilized contaminated sediments (Stachel et al. 2007).

7.5.4 Comparison of BEQs and REP-based TEQs

The discrepancy between BEQs of both assays and REP-based TEQs may have different reasons: For instance, AhR inducers that do not by definition belong to the group of DLCs such as PAHs, heterocyclic PAHs (Brack et al. 2005, Hinger et al. 2011, Otte et al. 2013, Wölz 2005) or chemical compounds that were not targeted by chemical analysis may be present in the extracts, leading to higher BEQs compared to TEQs. Furthermore, incubation times used for REP calculation (Behnisch et al. 2003, Bols et al. 1999) did not always correspond to the incubation time (72 h) applied in the present study, hence the REP-based TEQ calculation always hides failures. Moreover, the dioxin-like activity of e.g. some PAHs may vary with differing culture conditions (Bols et al. 1999), meaning that only a bioassay strictly adapted to the method used for REP calculation would give BEQs comparable to REP-based TEQs.

Matrix components may develop during exhaustive extraction techniques such as pressurized liquid extraction (PLE), which may influence the remaining compounds' effect potentials (Barceló and Petrovic 2007, Brack et al. 2000, Larsson 2009). Since bioassays reflect the integrated effect potential of all compounds present in an extract (Behnisch et al. 2001a), synergism or antagonism may cause the TEQ-concept, which assumes additivity, to fail (Safe 1998b, Van den Berg et al. 2006). Polyhalogenated (PX)DD/Fs, polychlorinated naphtalenes and many more compounds, which have not been part of the present TEQ calculation, exhibit dioxin-like properties (Behnisch et al. 2001b, 2003, Hasegawa et al. 2007, Safe 1998b, Till et al. 1997, Van den Berg et al. 2006) and thus explain the case that TEQs are much lower compared to BEQs as it was the case for RTL-W1 EROD BEQ to TEQ comparisons. The considerably high variability of the percentage of TEQs explaining H4IIE Micro EROD BEQs makes an explanation difficult.

The share of different contaminant groups (i.e. PAHs and PCDD/Fs) in EROD and Micro EROD BEQs confirmed the frequent observation in raw extracts that PAHs cause the overall largest dioxin-like activity compared to PCDD/Fs (Barceló and Petrovic 2007, Behnisch et al. 2001a, Keiter 2007, Villeneuve et al. 2002). Acid labile compounds such as PAHs, which were present in high concentrations in the here investigated raw extracts, most likely impaired the comparability between BEQs and REP-based TEQs. Hence, a clean-up and a H_2SO_4 treatment, which according to Villeneuve and co-authors (Villeneuve et al. 2002) at least should last 10 h, would have removed the PAHs but not destroyed PCDD/F, PCB and other higher chlorinated DLCs. The fact that for inundated flood areas, the PCDD/F percentage in total TEQs increased, indicated a greater importance of this contaminant group for the terrestrial sampling areas.

7.5.5 TOC-normalized EROD-activity of Elbe length profile samples

The TOC fraction of a sediment sample is composed of dissolved organic carbon (DOC) and particulate organic carbon (POC) (Meybeck 1982). The presence or absence of TOC distinctly influences how chemicals associate in the sediment. From an ecotoxicological perspective, one of the most important characteristics of TOC is its ability to sorb and desorb organic compounds like PCDD/Fs, PCBs and PAHs (Schumacher 2002). The extraction method applied in the present study is considered to be exhaustive (Seiler et al. 2008). Hence, bio-analytical results obtained for the sediment extracts reflect a worst-case-scenario, which through normalization with the sample's TOC contents (*Figure 7.3*) can partly be transformed into more realistic, naturally occurring scenarios and provides a basis for predicting toxicity of POPs to aquatic organisms (Ni et al. 2008).

Since some studies reported positive correlations between the concentrations of certain POPs and the TOC content (Chen et al. 2006, Hinga 2003), but concentrations of POPs do not well correlate with the overall dry weight of a sediment (Di Toro et al. 1991), TOC-normalized data might be more relevant as dry weight normalized data. Following a TOC normalization (*Figure 7.3*), both assays showed samples Schnackenburg, Prossen and Lysa nad Labem to exhibit highest dioxin-like activities among all Elbe lengths profile samples. These samples moreover exhibited the overall highest TOC amounts among the longitudinal samples (*Table 7.1*). Such increased TOC percentages are typical for depositional areas where organic matter accumulates (Michelsen 1992) and most likely indicate an organic contamination (Ni et al. 2008). These findings support the aforementioned assumptions of samples Schnackenburg, Prossen and Lysa nad Labem being contamination hotspots among the longitudinal samples.

7.6 Conclusions

All Elbe longitudinal profile samples caused dioxin-like activity using both, the RTL-W1 EROD and H4IIE Micro EROD assay. In spite of higher RTL-W1 EROD BEQs compared to H4IIE Micro EROD BEQs, both assays showed equal trends in sample dioxin-like potentials. More precisely, samples KS 11, Schnackenburg, Prossen and Lysa nad Labem belonged to the samples with highest dioxin-like potentials, which partly was supported by statistical findings (ANOVA with Tukey's comparison test; $p < 0.005$) and further confirmed following normalization of bioassay results to the sample's TOC contents. In this context, different contamination sources in the course of the Czech Republic as well as industrial site Bitterfeld have been discussed.

Soil samples of the inundated flood areas Wörlitz, Glinde and Mulde, of which only the latter two ones demonstrably have been affected by contaminant loads of the Saale and Mulde catchment areas during the Elbe flood of August 2002, all caused dioxin-like activity. For each of the three areas, both bioassays indicated the respective near-river samples to possess higher dioxin-like activities than their more distant counterparts, which most likely was caused by more frequent inundations at the riverbank region. For the Mulde inundated flood area samples, this difference was found to be significant in both, the RTL-W1 EROD and the H4IIE Micro EROD assay. Because the dioxin-like activities of sediment and floodplain samples were in the same order of magnitude, it is assumed that Elbe sediments contaminated the floodplains through frequent inundations.

Altogether, the RTL-W1 EROD and H4IIE Micro EROD showed LODs of 1.7 and 0.2 pM TCDD, and LOQs of 4.6 and 0.4, respectively. This proved the H4IIE Micro EROD assay to be much more suitable for the screening of weak AhR inducers. Average z-factors < 1 classified both assays as excellent with a very good separation of positive and negative control. Furthermore, with a reproducibility of 19 and 22% both, the RTL-W1 EROD and H4IIE Micro EROD assay were in an acceptable range of reproducibility. While the correlation (Pearson, $p = 0.05$) between both assay results was significant ($r^2 = 0.343$), neither the RTL-W1 EROD ($r^2 = 0.003$), nor the H4IIE Micro EROD assay ($r^2 = 0.237$) correlated with their respective REP-based TEQs, which most likely was caused by the presence of a multitude of non-classical AhR inducers in the raw extracts. Our results let us assume that chemical data alone may be insufficient for an overall assessment of dioxin-like activity of sediment and soil samples and that bio-analytical methods such as the EROD assays applied here can hand important additional information in particular for biota which might also be affected by PAH-triggered DLC effects.

7.7 Acknowledgements

The present work forms a part of the DioRAMA project (“dioxin Risk Assessment for sediment Management Approaches”) and received funds from the German Federal Ministry of Transport and Digital Infrastructure. MB received a personal stipend from the German National Academic Foundation (“Studienstiftung des deutschen Volkes”). HH was supported by the Chinese 111 Program (College of Environmental Science and Engineering and Key Laboratory of Yangtze Water environment, Ministry of Education, Tongji University).

Chapter 8

Discussion

8.1 General discussion

The chapters of the present thesis have emphasized the utility of different *in vitro* bioassays for the detection of dioxin-like compounds (DLCs) in various matrices to possess a particularly predicative power, comparable to classical applied chemical analytical methods.

Altogether, the studies could give an overview of the state of the art of different *in vitro* bioassays for the detection of DLCs (Chapter 3), could prove the suitability of *in vitro* bioassays to (a) be used as predicative, prioritizing bio-analytical tool within frameworks for the assessment of sediments and dredged material (Chapter 4), (b) to be suitable to predict the uptake of DLCs from sediments by fish (Chapter 5) and (c) to be used as high-throughput sediment and soil sample screening tools (Chapter 7). A combination of sediment desorption experiments and adjacent ecotoxicological tests (Chapter 6) could provide further information for a final consideration of the toxicity of the four differently DLC contaminated sediments, which built the basis of the present study's *in vitro* bioassay validations. All these results will be interconnected and critically discussed in the present chapter.

8.2 Bioassays for the detection of DLCs

The use of *in vitro* bioassays for the characterization of dioxin-like activities in various environmental matrices such as sediments is of increasing interest to researchers, regulators and risk assessors. To be reliable, bio-analytical screening tools have to keep up with classical analytical methods such as HRGC/HRMS by exhibiting high sensitivities (low LOD and LOQ) and by providing highly repeatable and reproducible results (2012/252/EU 2012, Eichbaum et al. 2014).

A summary of bioassay-derived quality criteria (Chapter 3) revealed that LODs, which unfortunately turned out to be rarely stated, ranged between 0.1 and 20 pM 2,3,7,8-TCDD for various bioassays. Thereby, the luminescence-based bioassays (CALUX and DR-CALUX) possessed the lowest LODs and thus the highest overall sensitivity (*Table 3.1*). In contrast, EROD-based assays (RTL-W1, H4IIE) belonged to the most TCDD-sensitive tests with EC_{50}TCDD values of approximately 5 pM (*Table 3.1*), but their repeatability seemed to be lower compared to the luminescence assays (*Table 3.1*) (Chapter 3).

By considering the performance of the three bioassays used in the present study as a whole (results obtained in Chapters 4, 5 and 7), the luminescence-based H4IIE-luc assay in contrast to the above stated result (Chapter 3) did not show a higher sensitivity than the EROD-based assays. Overall LOD and LOQ values were 0.7 and 2.1 pM 2,3,7,8-TCDD, respectively.

Furthermore, the assays' repeatability (~35%) and comparability to instrumental-derived TEQs ($r^2 = 0.6$) were relatively poor, but at least the repeatability was independent of sample complexity (raw, multilayer, DL-PCB and PCDD/F, single substance 2,3,7,8-TCDD). Because the H4IIE-luc assays' samples number/test cycles was satisfactory and the H4IIE-luc assay among the three investigated assays covered the widest linear concentration range, there is no need for time-consuming pre-screening test, which especially is beneficial for the prioritization of big sampling sets.

The RTL-W1 EROD assay allows for the highest sample number/test cycle among the investigated assays, which underlines its high suitability for the high-throughput screening of environmental samples. Nonetheless, the assay exhibited the overall highest LOD and LOQ of 1.3 and 2.8 pM 2,3,7,8, respectively (Chapters 4, 5 and 7), of which the latter one partly overlapped with the determined effect levels of sample and standard. This gives concern about the assays' suitability for the analysis of samples possessing weak AhR-activating potential. The assays' overall repeatability of 31% was comparable to that achieved by the H4IIE-luc assay and was independent of sample complexity (see above) and sample matrix (sediment and fish homogenate sample). The assay showed a relatively poor correlation with instrumental-derived TEQs ($r^2 = 0.5$), which disqualifies it to be used in regulatory frameworks, which in a first step are based on results of chemical analysis.

Except the high sample throughput (RTL-W1) and the high linear range (H4IIE-luc), the H4IIE Micro EROD assay showed the overall best performance among the three investigated assays. According to its average z-factor of approximately 0.6 (Chapters 4 and 7) it was ranked excellent, exhibited the overall lowest LOD and LOQ values of 0.4 and 0.6 pM 2,3,7,8-TCDD, respectively and showed the best overall repeatability of < 25%, which was independent of sample complexity and matrix (see above) (Chapters 4 and 7) and in accordance to regulatory recommendations (2012/252/EU 2012). Its comparability to instrumental-derived TEQs was good ($r^2 = 0.7$) and pointed towards its high suitability to be implemented in regulatory guidelines.

8.3 Is the H4IIE Micro EROD assay an applicable screening tool?

8.3.1 General performance

A cross-validation (Chapter 4) revealed the H4IIE Micro EROD assay to constitute the most preferable bio-analytical screening tool among all examined assays. The assay was classified excellent, was characterized by a repeatability < 25%, which corresponds to regulatory

requirements (2012/278/EU 2012). The Micro EROD assay in comparison to the remaining assays in all studies (Chapters 4, 5 and 7) was less subjected to variability. Moreover, its sample number per test cycle was satisfying and allows for high throughput screenings. Its high sensitivity (Chapters 4, 5 and 7) approached that achieved by instrumental analysis and makes the assay highly suitable for the analysis of samples possessing a low dioxin-like activity.

Inter-laboratory results of the H4IIE Micro EROD assay (Chapter 4) were highly correlated ($r^2 = 0.87$) and highly reproducible for complex mixtures (17%) and single compounds (2%) between different operators and laboratories, suggesting the H4IIE Micro EROD to be a reliable cross-laboratory method and full filling the basic requirement for implementing the assay as a regulatory tool. To maintain the Micro EROD assays' validity over long periods of time, assay performance and evaluation have to strictly follow cross-laboratory standardized methods (Engwall and Van Bavel 2004).

8.3.2 Matrix-related variations

The basic requirement for the declaration of a certain bio-analytically observed effect is that the chosen effect concentration level is well above the assay-specific detection limits, especially well above the LOQ. In one study (Chapter 5), the EC_{10} level was used for BEQ calculation, due to very low EROD-induction strengths of pooled whole fish homogenate samples of *R. rutilus.* Here, the Micro EROD assay, in contrast to the remaining assays, showed no overlapping neither between the LOQ and the samples' EC_{10} value nor between the LOQ and the EC_{10}TCDD. This example revealed the high sensitivity of the Micro EROD assay and its potential to evaluate samples with a weak AhR-activating potential. Nevertheless, low altitudes of the dose-response-curves of the fish homogenate samples (~54%) most likely complicated or distorted the calculation of H4IIE BEQs, which assumes equal efficacy (i.e. equal altitude) of sample and standard. (Villeneuve et al. 2000). This could points towards matrix-dependent weaknesses in the H4IIE Micro EROD assay, but the fact that a remarkably good repeatability of 16% was reached for the complex, DL-PCBs and PCDD/Fs containing fish homogenate extracts (Chapter 5), contradicts this assumption.

For the sediment fractions (Chapter 4), the maximum sample induction strengths averaged to (~70%) and with this, in contrast to the fish homogenate samples, complied with the required equal efficacy of sample and standard (Villeneuve et al. 2000). Repeatability of the complex sediment DL-PCB (32%) and PCDD/F (15%) fractions did not differ from those determined for single substance 2,3,7,8-TCDD (23%) so that the regulatory requirement of a repeatability

< 25% (2012/278/EU 2012) was met. Summarizing this, matrix-related variations of the H4IIE Micro EROD assay derived results in general should be considered as low.

8.3.3 Predictive power with regard to sediment evaluation

8.3.3.1 Sediments chosen for the DioRAMA project bioassay cross-validation

By cross-laboratory analyses by means of the H4IIE Micro EROD assay, highly contaminated sediment ZE could clearly be identified as sample of highest concern among the four observed sediments (Chapter 4). Moreover, calculated H4IIE BEQs reflected the initially, chemically determined sequence of the concentration levels of DL-PCBs (EBR < EBR/ZE < PR < ZE) and PCDD/Fs (EBR < PR < EBR/ZE < ZE) found in the four chosen sediments. The H4IIE Micro EROD assay in general was highly comparable to instrumental derived TEQs and produced the lowest range of unexplained percentages from TEQs in BEQs (Chapter 4). This evidences the assays' high potential to be implemented in sediment management guidelines. Compared to environmentally occurring concentrations of other DLCs, those of PCDD/Fs are very low. However, *in vitro* bioassays possess a high sensitivity towards dioxins, what especially with respect to German guidelines for dredged material (GÜBAK 2009), which so far do not provide a screening of dioxins, could constitute an interesting additional evaluation tool.

8.3.3.2 Sediments and soils of the Elbe catchment area

By screening raw extracts of sediment samples from a Elbe lengths profile and soil from the Elbe inundated flood area (Chapter 7), the H4IIE Micro EROD assay identified sediment samples KS 11 (a place of former ocean dumping for sewage sludge from the city of Hamburg), Schnackenburg, Prossen (same location used in Chapters 4 and 5) and Lysa nad Labem as potentially highest DLC contaminated samples. This partly corresponded to previous bio-analytical investigations (Behnisch et al. 2010) and previously demonstrated contamination hotspots along the river Elbe (Götz and Lauer 2003, Heinisch et al. 2007, Stachel et al. 2004, Stachel et al. 2005, Stellman et al. 2003, Umlauf et al. 2010, Van Thuong et al. 2014, Weber and Varbelow 2013).

Furthermore, the assays indicated higher dioxin-like activities of near-river bank samples compared to more distant samples of the investigated floodplains, which corresponded to analytical findings and most likely indicated a higher contamination of these near-river bank samples with particle-bound DLCs, most likely caused by demonstrably more frequent inundations (Stachel et al. 2005) of these samples.

Although, the H4IIE Micro EROD assay demonstrated some contamination hotspots among the sediment and soil samples of the Elbe catchment area, the correlation with instrumental results was poor and proved a clean-up and H_2SO_4 treatment (which was not applied for the bio-analytically investigated extracts) to be essential (Villeneuve et al. 2002) for a functioning and reliable mass-balance approach.

8.3.4 Possible regulatory application of the H4IIE Micro EROD assay

An arbitrary chosen TEQ_{LV} of 35 pg/g dw sediment, determined on basis of chemical data for various Elbe sediment sampling sites (Chapter 4) could prove sampling location ZE to be among the top 25% of the most contaminated sediments of the river Elbe, clearly separated from other sampling locations such as sediment sample PR. By means of a linear correlation of available TEQs and respective H4IIE BEQs, a BEQ_{LV} of 145 pg BEQ/g dw sediment was deduced, which could allow for a simple, rapid and low-cost evaluation of sediments and dredged materials based on bioassays.

8.4 Summary assessment of the four "DioRAMA" sediment samples

The present section aims in final assessment of the toxicity of the four chosen sediments by means of all chemical and toxicological insights gained in the different chapters (*Table 8.1*).

8.4.1 Sediment assessment *via* chemical methods

Chemical assessment of the four chosen sediments took place on basis of both exhaustive (*Section 8.4.1.1*) and mild extraction techniques (*Section 8.4.1.2*). While the exhaustive extraction techniques (Soxhlet and pressurized liquid extraction) constituted a worst-case scenario (Seiler et al. 2008), the mild extraction techniques rather reflected the bioavailable fraction of compounds present in the four sediments (Brack et al. 2000, Cornelissen et al. 2001, Reid et al. 2000).

8.4.1.1 Exhaustive extraction and HRGC/HRMS measurements

From a chemical perspective, sediment ZE showed the overall strongest contamination with both DL-PCBs and PCDD/Fs (Chapter 4, *Table 8.1*) and together with sediment PR was among the sediments, which were strongest contaminated with 16-EPA PAHs (Chapter 6, *Table 8.1*). German dredged material guidelines (GÜBAK 2009), which like other staged regulatory

instructions use chemical data as basic information for subsequent decisions, would classify sediment ZE as potentially more hazardous relative to the remaining sediments.

8.4.1.2 Mild extractions and GC-MS measurements

The mild extraction techniques (tenax, Chapter 6) were meant to close the gap between instrumental and ecotoxicological results towards a sediment assessment with respect to bioavailability.

Among the analyzed 16-EPA PAHs, the high water soluble congeners fluoranthene, pyrene and phenathrene (Fent 2007, Gocht and Grathwohl 2004) predominantly desorbed from the sediments and thus were considered as potentially most bioavailable contaminants within this group. Results of their cumulative desorbing concentrations showed that higher amounts desorbed from sediments PR and ZE compared to the remaining sediments (Chapter 6, *Table 8.1*). Hence, the cumulative concentrations of desorbed PAHs corresponded well to the initial sediment PAH contamination degree and verified the high value of the initial contamination for assessing bioavailability.

The time point of exhaustion of the rapidly desorbing fraction showed the opposite trend. Here, the rapidly desorbing fraction, which in contrast to the slowly desorbing fraction is of high value for the bioavailability (Cornelissen et al. 1997, Pignatello and Xing 1995), was more long-lasting for slightly contaminated sediments EBR and EBR/ZE compared to highly contaminated sediments PR and ZE. (Chapter 6, *Table 8.1*). Even though this is suggestive of a relatively higher risk potential caused by re-mobilization of the slightly contaminated sediments (e.g. during a flood), it has to be mentioned that sediment re-mobilization processes such as floods only last for a few days and desorbed compounds, which anyhow decrease with time, are subjected to dilution, lowering their environmental risk potential. According to this, the highly contaminated sediments PR and ZE during a flood (during the first days of re-mobilization) would possess the highest contamination potential of the surrounding environment due to their relatively higher total concentrations of desorbed compounds.

8.4.2 Sediment assessment *via* ecotoxicological methods

With the following ecotoxicological results (*Table 8.1*), the initial instrumental derived classifications should be investigated and critically discussed.

8.4.2.1 Sediment assessment *via* cell-based *in vitro* bioassays

Regarding the *in vitro* bioassay derived results from the RTL-W1 EROD, the H4IIE Micro EROD and the H4IIE-luc assay, they all showed DL-PCB and PCDD/F fractions of sediment sample ZE to possess the overall highest activity among the four sediment samples (Chapter 4, *Table 8.1*). Apart of the fact that bioassay-specific BEQs showed different altitudes (highest and lowest BEQs were obtained by using the RTL-W1 EROD and H4IIE Micro EROD assay, respectively), they corresponded well with the chemical instrumental derived classification of sediment ZE to exhibit the overall highest DLC concentrations (*Section 8.4.1.1*).

8.4.2.2 Sediment assessment on an organism level

Exposure experiments conducted with *Rutilus rutilus* (Chapter 5**)** demonstrated a statistically significant, bio-analytically determined uptake of sediment-borne DLCs by roach on all sediments independent of their initial contamination degree with DLCs, however, season-related effects cannot completely be excluded in this connection. BEQs and TEQs measured in fish were assumed to reflect DLC background contamination levels, because they were well below the EQS for biota (2013/39/EU 2013) and corresponded to previously measured BEQs and TEQs in whole fish homogenates of various origins (Burreau et al. 2004, Hasegawa et al. 2007, Kojima et al. 2011, Viganò et al. 2000).

Unexpectedly, the most significant and temporal dependent bio-analytically determined uptake of DLCs by common roach was observed for slightly contaminated sediment EBR. Suspended particulate matter, which showed the overall highest concentration for EBR containing treatments and which is known to carry organic contaminants such as DLCs (Eggleton and Thomas 2004), most likely supported this higher and temporally increasing uptake of DLCs *via* the water phase.

Regarding the different feeding scenarios, BEQs determined in fish exposed to worm-containing sediments more or less reflected the feeding behaviour of the test animals (see Chapter 5). BEQs mostly increased following a fish transfer, which possibly reflects intense initial feeding activities of fish and thus high initial uptakes of DLCs. Such higher uptakes could have led to induction of defense mechanisms against DLCs through xenobiotic enzymes, which could explain the relatively lower DLC concentrations measured on the subsequent sampling dates. While the presence or absence of worms in sediment PR did not alter the uptake of sediment-borne DLCs by common roach, the presence of worms in highest DLC contaminated sediment ZE caused a 2-fold (TEQ and H4IIE BEQ) greater uptake of DLCs by fish compared to the respective non-inoculated treatment. This indicates that the uptake of DLCs is promoted through ingestion of food (or sediment) as it was previously observed (Rubinstein et al. 1984),

but that this is only valid for very strong contaminated sediments such as sediment ZE. Fish exposure experiments thus gave no clear picture of the risk potential of the differently contaminated sediments.

Mortality, which occurred during the above exposure experiments of *Rutilus rutilus*, was highest for sediments EBR/ZE and ZE (Chapter 5). Because both EQS for biota (2013/39/EU 2013) and results of former studies (Kojima et al. 2011, Hasegawa et al. 2007, Burreau et al. 2004, Viganò et al. 2000) showed that DLC concentrations determined in *R. rutilus* reflected a background DLC contamination level and the acute toxicity of DLCs in general has to be considered low (Fent 2007), lethality most likely was caused by other compound classes such as heavy metals (Di Giulio and Hinton 2008). Copper for example is known to cause lethality in fish, probably by disrupting the osmoregulatory function of the gills (Erickson et al. 1996).

In this connection, it is assumable that the limno-chemical parameters measured during the experiments did not directly affect the fish, but possibly indirectly influenced the toxicity of compounds such as heavy metals, which could have caused the observed mortality effects.

Desulfurized raw extracts of the four sediments were analysed in the fish embryo toxicity (FET) test with eggs of *D. rerio* (unpublished data). Experiments were conducted by M.Sc. Yvonne Müller (Institute for Environmental research, RWTH University, Aachen) according to DIN EN ISO 15088 (DIN 2009). According to the EC_{50} values for mortality, which were determined after 48 and 96 hours of incubation, the extract of sediment EBR exhibited the highest embryotoxic potential compared to the other sediment extracts (*Table 8.1*). However, regarding the respective dose-response-curves (*Figure 8.1*), the curve obtained for extract of sediment ZE showed a noticeably steeper course compared to the remaining ones, which in contrast to the calculated EC_{50} values shows the extracts' fast effectiveness in a very small range of concentration.

The sediment contact assay (SCA) with eggs from *Danio rerio* was conducted with extracts gained in desorption experiments (*Section 3.4.1.2*, Chapter 6). Maximum sub-lethal effect levels observed in embryos of *Danio rerio* were lowest (35%) for sediments EBR and EBR/ZE and highest for sediments PR (66%) and ZE (68%) (Chapter 6, *Table 8.1*). Hence, they more or less reflected the sequence of the initial PAH contamination degree as well as the sequence of the concentrations of the cumulative desorbed PAHs.

Table 8.1 Concluding presentation of the most important experimental and analytical results obtained for the four sediments from Ehrenbreitstein (EBR) at the river Rhine and sediments from Prossen/Schmilka (PR) and Zollelbe/Magdeburg at the river Elbe as well as a sediment mixture (EBR/ZE) consisting of nine parts dry weight EBR and one part dry weight ZE. PCDD/F = polychlorinated dibenzo-p-dioxin and dibenzofuran, DL-PCB = dioxin-like polychlorinated biphenyl, PAHs = polycyclic aromatic hydrocarbons, * = cumulative concentration of desorbed PAHs after 53 days, Frap = rapidly desorbing fraction, BEQ = biological equivalent quotient, EC = effect concentration, SCA = sediment contact assay, FET = fish embryo toxicity test, A = desulfurized, B = native, C = freeze-dried; A simple classification of sediment toxicity was made by setting the maximum value of each category to 100% and by converting the remaining ones relative to that value, (red = 80 – 100% of the maximum value, green = 20 - 80% of the maximum value, yellow = 0 – 20% of the maximum value).

Category	Parameter	Organism /Cell line	Assay	Matrix	EBR	EBR/ZE	PR	ZE
Chemistry	Conc. of 17 WHO-PCDD/F [ng/g dw]	-	-	PCDD/F extract	1.1	1.2	0.2	3.7
Chemistry	Conc. of 12 WHO-PCB [ng/g dw]	-	-	DL-PCB extract	4.4	4.2	5.2	9.7
Chemistry	Conc. of 16-EPA PAHs [ng/g dw]	-	-	raw extract [A]	813.0	1261.3	3420.2	3348.3
Desorption	Cumulative conc. of PAHs* [ng/g dw]	-	-	tenax extract	56.9	71.3	106.9	125.4
Desorption	Frap exhausted [days]	-	-	tenax extract	72	79	55	49
Cell test	BEQ [pg/g dw]	H4IIE	Micro EROD	DL-PCB extract	16.7	18.6	18.4	76.4
Cell test	BEQ [pg/g dw]	H4IIE	Micro EROD	PCDD/F extract	63.7	60.5	73.9	159.0
Cell test	BEQ [pg/g dw]	H4IIE-luc	H4IIE-luc	DL-PCB extract	19.9	33.0	18.7	107.0
Cell test	BEQ [pg/g dw]	H4IIE-luc	H4IIE-luc	PCDD/F extract	238.9	362.8	523.4	747.3
Cell test	BEQ [pg/g dw]	RTL-W1	EROD	DL-PCB extract	36.0	38.4	50.6	192.5
Cell test	BEQ [pg/g dw]	RTL-W1	EROD	PCDD/F extract	270.5	180.1	488.0	955.8
Organism	max. mortality [%]	*R. rutilus*	(exposition)	sediment [B]	16.7	83.3	16.7	83.4
Organism	max. sub-lethal effect [%]	*D. rerio*	SCA	sediment [C]	37.8	33.0	66.0	68.1
Organism	max. mortality [%]	*D. rerio*	SCA	sediment [C]	11.3	13.7	35.6	18.9
Organism	EC50 (48h) mortality [mg/ml]	*D. rerio*	FET	raw extract [A]	10.4	13.4	12.3	14.1
Organism	EC50 (96h) mortality [mg/ml]	*D. rerio*	FET	raw extract [A]	10.0	13.3	12.1	10.8

While embryos exposed to slightly PAH contaminated sediments EBR and EBR/ZE did not show any predominant effects, more than half of the observed effects in the embryos exposed to highly PAH contaminated sediments PR and ZE accounted for missing pigmentation of skin and/or eyes. Many PAHs can alter and/or disturb the embryonal pigmentation (Barron et al. 2004), which during the later development can lead to an increased sensitivity of the fish towards UV light (Kosmehl et al. 2006).

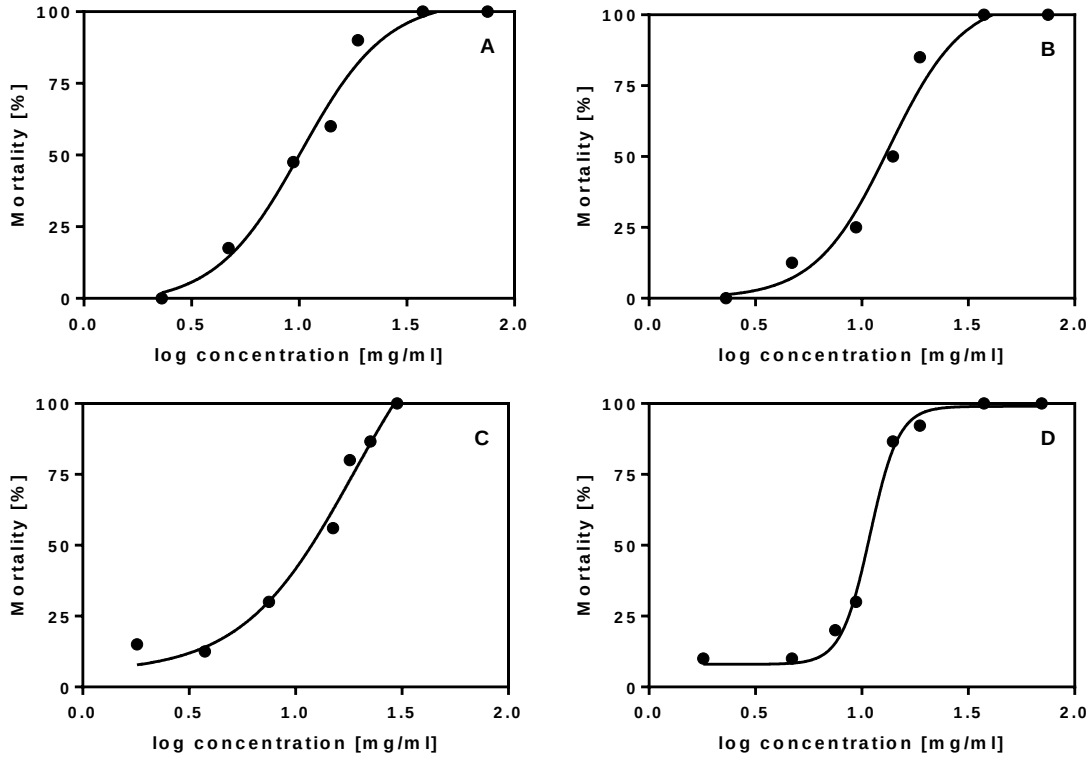


Figure 8.1 Mortality of fish embryos (*D. rerio*) exposed to different concentrations of desulfurized raw extracts of four sediments for 96 hours (dots represent the mean values of three independent replicates). Sediments included location Ehrenbreitstein (A) at the river Rhine, and locations Prossen (C) and Zollelbe (D) at the river Elbe as well as a sediment mixture (B) consisting of nine parts dry weight Ehrenbreitstein and one part dry weight Zollelbe, tested concentrations never exceeded 0.5% DMSO.

Embryos exposed to sediments PR and ZE moreover showed a higher maximum mortality (Chapter 6, *Table 8.1*), compared to that of embryos exposed to the remaining sediments. Some PAHs, PCBs and PCDD/Fs, which generally were higher concentrated in sediments PR and ZE, but also heavy metals are known to possess an embryotoxic potential (Cantrell et al. 1998, Hollert et al. 2003, Sundberg et al. 2005, Westerlund et al. 2000) and thus may have led to the increased mortality of fish embryos exposed to those sediments.

Rapidly desorbing fractions in general are considered to be more important in terms of bioavailability (Cornelissen et al. 1997, Pignatello and Xing 1995), while slowly desorbing fractions need a long time until they are available for organisms (Kukkonen et al. 2004, Ten

Hulscher et al. 2003). The SCA with its incubation time of 48 h therefore covers the most harmful time range in terms of bioavailability (Zielke et al. 2011).

Summarizing all results of the present sediment assessment, both chemical and ecotoxicological results predominantly showed sediment sample ZE to possess the highest contamination/toxic potential. But sediment sample PR, especially in the conducted desorption experiments and tests on an organism level showed an elevated bioavailable/toxic potential. The conducted *in vitro* bioassays, owing to their specificity of DLCs, showed a good accordance with the exhaustive extracted and instrumentally analyzed DL-PCBs and PCDD/Fs. All those results show the importance of an integrated sediment assessment (Wernersson et al. 2015).

8.5 Conclusion and Outlook

The present thesis could prove the Micro EROD assay with rat hepatoma cell line H4IIE to possess the overall best performance and the best accordance with results based on HRGC/HRMS among all investigated *in vitro* bioassays. The H4IIE Micro EROD assay was shown to be highly sensitive, since its LOD and LOQ corresponded well to the lowest overall limits found in a comprehensive literature review and approximate the limits achieved by instrumental analysis. A cross-laboratory *in vitro* bioassay comparison moreover verified the assays' excellent reproducibility, comparability as well as its predicative power in terms of evaluating extract fractions of sediments, which are differently contaminated with DLCs.

HRGC/HRMS and H4IIE derived results of whole fish homogenates predominantly indicated an uptake of sediment-borne DLCs by common roach, which was largely independent of the initial concentrations of DLCs in sediments and did not exceed the biota EQS of 6.5 pg TEQ/g fm. The fact that the uptake of DLCs by fish (1) only temporally increasing when fish was exposed to the sediment of slightest contamination but highest concentration of dissolved organic matter and (2) BEQs in fish corresponded to fish transfer and feeding activities, suggests that the uptake of DLCs by common roach is promoted by the particle concentration in the water column. Fish exposed to the sediment of highest DLC contamination containing black worms on average showed a 2-fold higher uptake compared to fish exposed to the same sediment but daily fed with uncontaminated worms. This suggests that the uptake of DLCs by common roach, additionally to the suspended matter concentration, is promoted by ingestion of feed/sediment. Although, fish extracts caused lower EROD-induction strengths than sediment extracts, H4IIE BEQs in fish extracts showed a good repeatability and

comparability to HRGC/HRMS derived results and thus proved the suitability of the H4IIE Micro EROD assay to investigate comparably challenging sample matrices.

By analyzing large sampling sets from the Elbe catchment area the H4IIE Micro EROD assay, even though complex raw extracts of sediments and soils were investigated, possessed a good predicative and prioritizing power for samples, which according to their DLC concentrations constituted contamination hotspots. A H4IIE Micro EROD assay limit value, which was deduced from DLC concentrations of those contamination hotspots, could be used as simple yes/no level and this way could be used as a simple, quick and low-cost prioritization tool for the assessment of sediments and dredged material. The results of the present thesis might contribute to future regulatory decisions in that way that *in vitro* bioassays could be implemented into German guidelines for dredged material to be used as an additional quality measure beside traditionally used instrumental analysis.

References

92/43/EWG (1992): Richtlinie des Rates der Europäischen Gemeinschaften zur Erhaltung der natürlichen Lebensräume sowie der wildlebenden Tiere und Pflanzen.

2000/60/EC (2006): Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 - establishing a framework for Community action in the field of water policy. Off. J. Eur. Union. 2000; L327:1–73.

2000/60/EG (2009): Bewirtschaftungsplan nach Artikel 13 der Richtlinie 2000/60/EG für den deutschen Teil der Flussgebietseinheit Elbe (FGG Elbe)

2012/252/EU (2012): Commission Regulation (EU) No 252/2012 of 21 March 2012 laying down methods of sampling and analysis for the official control of levels of dioxins, dioxin-like PCBs and non-dioxin-like PCBs in certain foodstuffs and repealing Regulation EC1883/2006, Off. J. Eur. Union.

2012/278/EU (2012): Verordnung (EU) Nr. 278/2012 der Kommission vom 28. März 2012 zur Änderung der Verordnung EG152/2009 hinsichtlich der Bestimmung der Gehalte an Dioxinen und polychlorierten Biphenylen.

2013/39/EU (2013): Directive 2013/39/EU of the European Parliament and the European Council from August 12th, amending Directives 2000/60/EG and 2008/105/EG with respect to priority pollutants in the field of water policy.

Aarts JMMJG, Denison MS, Cox MA, Schalk MAC, Garrison PM, Tullis K, de Haan LHJ, Brouwer A (1995): Species-specific antagonism of Ah receptor action by 2,2′,5,5′-tetrachloro- and 2,2′,3,3′,4,4′-hexachlorobiphenyl. Environ. Toxicol. Pharm. 293, 463-474

Ahlborg UG, Becking GC, Birnbaum LS, Brouwer A, Derks H, Feeley M, Golor G, Hanberg A, Larsen JC, Liem AKD, Safe SH, Schlatter C, Waern F, Younes M, Yrjänheikki E (1994): Toxic equivalency factors for dioxin-like PCBs: Report on WHO-ECEH and IPCS consultation, December 1993. Chemosphere 28, 1049-1067

Ahlf W, Förstner U (2001): Managing Contaminated Sediments. J. Soils Sediments 1, 30-36

Ahlf W, Hollert H, Neumann-Hensel H, Ricking M (2002): A guidance for the assessment and evaluation of sediment quality - a German Approach based on ecotoxicological and chemical measurements. J. Soils Sediments 2, 37-42

Alexander M (2000): Aging, Bioavailability, and Overestimation of Risk from Environmental Pollutants. Environ. Sci. Technol. 34, 4259-4265

Anderson JW, Jones JM, Hameedi J, Long E, Tukey RH (1999a): Comparative analysis of sediment extracts from NOAA's bioeffects studies by the biomarker, P450 Reporter Gene System. Mar. Environ. Res. 48, 407-425

Anderson JW, Zeng EY, Jones JM (1999b): Correlation between response of human cell line and distribution of sediment polycyclic aromatic hydrocarbons and polychlorinated biphenyls on Palos Verdes Shelf, California, USA. Environ. Toxicol. Chem. 18, 1506-1510

Andersson E, Rotander A, Kronhelm T, Berggren A, Ivarsson P, Hollert H, Engwall M (2009): AhR agonist and genotoxicant bioavailability in a PAH-contaminated soil undergoing biological treatment. Environ. Sci. Pollut. Res. Int. 16, 521-530.

Andersson PL, Blom A, Johannisson A, Pesonen M, Tysklind M, Berg AH, Olsson PE, Norrgren L (1999): Assessment of PCBs and Hydroxylated PCBs as Potential Xenoestrogens: In Vitro Studies Based on MCF-7 Cell Proliferation and Induction of Vitellogenin in Primary Culture of Rainbow Trout Hepatocytes. Arch. Environ. Contam. Toxicol. 37, 145-150

Anezaki K, Nakano T (2014): Concentration levels and congener profiles of polychlorinated biphenyls, pentachlorobenzene, and hexachlorobenzene in commercial pigments. Environ. Sci. Pollut. Res. Int. 21, 998-1009.

Apitz S, Power E (2002): From risk assessment to sediment management an international perspective. J. Soils Sediments 2, 61-66

Armbruster DA, Pry T (2008): Limit of blank, limit of detection and limit of quantitation. The Clinical biochemist. Reviews / Australian Association of Clinical Biochemists 29 Suppl 1, S49-52

Arrieta DE, Ontiveros CC, Li W-W, Garcia JH, Denison MS, McDonald JD, Burchiel SW, Washburn BS (2003): Aryl hydrocarbon receptor-mediated activity of particulate organic matter from the Paso del Norte airshed along the U.S.-Mexico border. Environ. Health Perspect. 111(10), 1299–1305

Barceló D, Petrovic M (Editors), 2007: Sustainable Management of Sediment Resources. Sediment Quality and Impact Assessment of Pollutants, **Vol 1:**, Amsterdam

Barron MG, Heintz R, Rice SD (2004): Relative potency of PAHs and heterocycles as aryl hydrocarbon receptor agonists in fish. Mar. Environ. Res. 58, 95-100

Behnisch P, Umlauf G, Stachel B, Felzel E, Brouwer B (2010): Bio/chemical analysis of sediments from the Elbe River, the North Sea and from several tributaries. Organohal. Comp. 72, 2

Behnisch PA, Hosoe K, Sakai S (2001a): Combinatorial bio/chemical analysis of dioxin and dioxin-like compounds in waste recycling, feed/food, humans/wildlife and the environment. Environ. Int. 27, 495-519

Behnisch PA, Hosoe K, Sakai S (2001b): Bioanalytical screening methods for dioxins and dioxin-like compounds -- a review of bioassay/biomarker technology. Environ. Int. 27, 413-439

Behnisch PA, Hosoe K, Brouwer A, Sakai S (2002): Screening of Dioxin-Like Toxicity Equivalents for Various Matrices with Wildtype and Recombinant Rat Hepatoma H4IIE Cells. Toxicol. Sci. 69, 125-130

Behnisch PA, Hosoe K, Sakai S (2003): Brominated dioxin-like compounds: in vitro assessment in comparison to classical dioxin-like compounds and other polyaromatic compounds. Environ. Int. 29, 861-877

Berglund O, Larsson P, Ewald G, Okla L (2001): Influence of trophic status on PCB distribution in lake sediments and biota. Environ. Pollut. 113, 199-210

Besselink HT, Schipper C, Klamer H, Leonards P, Verhaar H, Felzel E, Murk AJ, Thain J, Hosoe K, Schoeters G, Legler J, Brouwer B (2004): Intra- and interlaboratory calibration of the DR CALUX® bioassay for the analysis of dioxins and dioxin-like chemicals in sediments. Environ. Toxicol. Chem. 23, 2781-2789

Bhavsar SP, Fletcher R, Hayton A, Reiner EJ, Jackson DA (2007): Composition of Dioxin-like PCBs in Fish: An Application for Risk Assessment. Environ. Sci. Technol. 41, 3096-3102

Billiard SM, Bols NC, Hodson PV (2004): In vitro and in vivo comparisons of fish-specific CYP1A induction relative potency factors for selected polycyclic aromatic hydrocarbons. Ecotox. Environ. Safe. 59, 292-299

Bittner M, Janošek J, Hilscherová K, Giesy JP, Holoubek I, Bláha L (2006): Activation of Ah receptor by pure humic acids. Environ. Toxicol. 21, 338-342

Blanco SL, Sobrado C, Quintela C, Cabaleiro S, González JC, Vieites J (2007): Dietary uptake of dioxins (PCDD/PCDFs) and dioxin-like PCBs in Spanish aquacultured turbot (*Psetta maxima*). Food Addit. Contam. 24, 421-428

Blankenship AL, Kannan K, Villalobos SA, Villeneuve DL, Falandysz J, Imagawa T, Jakobsson E, Giesy JP (2000): Relative Potencies of Individual Polychlorinated Naphthalenes and Halowax Mixtures To Induce Ah Receptor-Mediated Responses. Environ. Sci. Technol. 34, 3153-3158

BMU (2013): Die Wasserrahmenrichtlinie - Eine Zwischenbilanz zur Umsetzung der Maßnahmenprogramme 2012, Referat Öffentlichkeitsarbeit, Bundesministerium für Umwelt, Naturschutz und Reaktorsicherheit (BMU; Hrsg.), Berlin, Germany

Bols NC, Schirmer K, Joyce EM, Dixon DG, Greenberg BM, Whyte JJ (1999): Ability of Polycyclic Aromatic Hydrocarbons to Induce 7-Ethoxyresorufin-o-deethylase Activity in a Trout Liver Cell Line. Ecotox. Environ. Safe. 44, 118-128

Bols NC, Dayeh VR, Lee LEJ, Schirmer K (2005): Use of fish cell lines in the toxicology and ecotoxicology of fish. Piscine cell lines in environmental toxicology. Biochem. Mol. Biol. Fish. 6, 43-84

Brack W, Altenburger R, Ensenbach U, Möder M, Segner H, Schüürmann G (1999): Bioassay-Directed Identification of Organic Toxicants in River Sediment in the Industrial Region of Bitterfeld (Germany)—A Contribution to Hazard Assessment. Arch. Environ. Contam. Toxicol. 37, 164-174

Brack W, Segner H, Möder M, Schüürmann G (2000): Fixed-effect-level toxicity equivalents—a suitable parameter for assessing ethoxyresorufin-O-deethylase induction potency in complex environmental samples. Environ. Toxicol. Chem. 19, 2493-2501

Brack W, Schirmer K, Kind T, Schrader S, Schüürmann G (2002): Effect-directed fractionation and identification of cytochrome P4501A-inducing halogenated aromatic hydrocarbons in a contaminated sediment. Environ. Toxicol. Chem. 21, 2654-2662

Brack W (2003): Effect-directed analysis: a promising tool for the identification of organic toxicants in complex mixtures? Anal. Bioanal. Chem. 377, 397-407

Brack W, Schirmer K, Erdinger L, Hollert H (2005): Effect-directed analysis of mutagens and ethoxyresorufin-O-deethylase inducers in aquatic sediments. Environ. Toxicol. Chem. 24, 2445-2458

Brack W, Bandow N, Schwab K, Schulze T, Streck G (2009): Identifizierung toxischer Verbindungen in Sedimenten: Ansätze zur Integration von Wirkung und Bioverfügbarkeit. Umweltwiss. Schadst.-Forsch. 21, 240-244

Bradlaw JA, Casterline JL (1979): Induction of enzyme activity in cell culture: a rapid screen for detection of planar polychlorinated organic compounds. J. Assoc. Off. Anal. Chem. 62, 904-916

Bradlaw JA, Garthoff LH, Hurley NE, Firestone D (1980): Comparative induction of aryl hydrocarbon hydroxylase activity in vitro by analogues of dibenzo-p-dioxin. Food Cosmet. Toxicol. 18, 627-635

Braunbeck T, Boettcher M, Hollert H, Kosmehl T, Lammer E, Leist E, Rudolf M, Seitz N (2005): Towards an alternative for the acute fish LC50 test in chemical assessment: The fish embryo toxicity test goes multi-species - An update. Altex 22, 87-102

Breitung V, Keller M (2010): Management von schadstoffbelasteten Feinsedimenten in Bundeswasserstraßen. Umweltwiss. Schadst.-Forsch. 22, 645-650

Breivik K, Sweetman A, Pacynaa JM, Jones K (2002): Towards a global historical emission inventory for selected PCB congeners - a mass balance approach 1. Global production and consumption. Sci. Tot. Environ. 290, 181–198

Brouwer A et al. (1995): Functional aspects of developmental toxicity of polyhalogenated aromatic hydrocarbons in experimental animals and human infants. Eur. J. Pharm. Environ. Toxicol. Pharm. 293, 1-40

Brown DJ, Chu M, Van Overmeire I, Chu A, Clark GC (2001): Determination of REP Values for the CALUX® Bioassay and Comprison to the WHO TEF Values. Organohal. Comp. 53, 3

Brown DJ, Orelien J, Gordon JD, Chu AC, Chu MD, Nakamura M, Handa H, Kayama F, Denison MS, Clark GC (2007): Mathematical Model Developed for Environmental Samples: Prediction of GC/MS Dioxin TEQ from XDS-CALUX Bioassay Data. Environ. Sci. Technol. 41, 4354-4360

Brunström B, Engwall M, Hjelm K, Lindqvist L, Zebühr Y (1995): EROD induction in cultured chick embryo liver: A sensitive bioassay for dioxin-like environmental pollutants. Environ. Toxicol. Chem. 14, 837-842

BÜCHI (2009): Extraction of Sediment using the SpeedExtractor E-916 for the determiantion of Polycyclic Aromatic Hydrocarbons (PAH), Short Note. BÜCHI Switzerland

Burns WA, Mankiewicz PJ, Bence AE, Page DS, Parker KR (1997): A principal-component and least-squares method for allocating polycyclic aromatic hydrocarbons in sediment to multiple sources. Environ. Toxicol. Chem. 16, 1119-1131

Burreau S, Zebühr Y, Broman D, Ishaq R (2004): Biomagnification of polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) studied in pike (*Esox lucius*), perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) from the Baltic Sea. Chemosphere 55, 1043-1052

Burton A (Editor), 1992: Sediment Toxicity Assessment. Lewis Publishers, Inc., Chelsea, Chapter 1-3, 15 pp

Cantrell SM, Joy-Schlezinger J, Stegeman JJ, Tillitt DE, Hannink M (1998): Correlation of 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced apoptotic cell death in the embryonic vasculature with embryotoxicity. Toxicol. Appl. Pharm. 148, 24-34

Castell JV, Gómez-Lechón MJ, Ponsoda X, Bort R (1997): In vitro Investigation of the Molecular Mechanisms of Hepatotoxicity. In: Seiler JP , Vilanova E (Editors), Applied Toxicology: Approaches Through Basic Science. Archives of Toxicology. Springer Berlin Heidelberg, pp. 313-321

Chapman PM (1989): Current approaches to developing sediment quality criteria. Environ. Toxicol. Chem. 8, 589-599

Chapman PM, McDonald BG, Lawrence GS (2002): Weight-of-Evidence Issues and Frameworks for Sediment Quality (And Other) Assessments. Hum. Ecol. Risk Assess. 8, 1489-1515

Chapman PM, Hollert H (2006): Should the Sediment Quality Triad Become a Tetrad, a Pentad, or Possibly even a Hexad? J. Soils Sediments 6, 4-8

Chen G, Konstantinov AD, Chittim BG, Joyce EM, Bols NC, Bunce NJ (2001): Synthesis of Polybrominated Diphenyl Ethers and Their Capacity to Induce CYP1A by the Ah Receptor Mediated Pathway. Environ. Sci. Technol. 35, 3749-3756

Chen G, Bunce NJ (2004): Interaction between halogenated aromatic compounds in the Ah receptor signal transduction pathway. Environ. Toxicol. 19, 480-489

Chen L, Yu C, Shen C, Zhang C, Liu L, Shen K, Tang X, Chen Y-Y (2010): Study on adverse impact of e-waste disassembly on surface sediment in East China by chemical analysis and bioassays. J. Soils Sediments 10, 359-367

Chen S-J, Luo X-J, Mai B-X, Sheng G-Y, Fu J-M, Zeng EY (2006): Distribution and Mass Inventories of Polycyclic Aromatic Hydrocarbons and Organochlorine Pesticides in Sediments of the Pearl River Estuary and the Northern South China Sea. Environ. Sci. Technol. 40

Clark GC, Chu M, Touati D, Rayfield B, Stone J, Cooke M (1999): A Novel Low-Cost Air Sampling Device (AmbStack Sampler) and Detection System (CALUX Bioassay) for Measuring Air Emissions of Dioxin, Furan, and PCB on a TEQ Basis Tested With a Model Industrial Boiler. Organohal. Comp. 40, 79-83

Clemons JH, Dixon DG, Bols NC (1997): Derivation of 2,3,7,8-TCDD toxic equivalence factors (TEFs) for selected dioxins, furans and PCBs with rainbow trout and rat liver cell lines and the influence of exposure time. Chemosphere 34, 1105-1119

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. J. Soils Sediments 12, 429-442

Cornelissen G, Rigterink H, Vrind BA, ten Hulscher DTEM, Ferdinandy MMA, van Noort PCM (1997): Two-stage desorption kinetics and in situ partitioning of hexachlorobenzene and dichlorobenzenes in a contaminated sediment. Chemosphere 35, 2405-2416

Cornelissen G, van Noort PCM, Govers HAJ (1998): Mechanism of Slow Desorption of Organic Compounds from Sediments: A Study Using Model Sorbents. Environ. Sci. Technol. 32, 3124-3131

Cornelissen G, Rigterink H, ten Hulscher DEM, Vrind BA, van Noort PCM (2001): A simple Tenax® extraction method to determine the availability of sediment-sorbed organic compounds. Environ. Toxicol. Chem. 20, 706-711

Currie LA (1968): Limits for qualitative detection and quantitative determination. Application to radiochemistry. Anal. Chem. 40, 586-593

David A, Gomez E, Aït-Aïssa S, Rosain D, Casellas C, Fenet H (2010): Impact of Urban Wastewater Discharges on the Sediments of a Small Mediterranean River and Associated Coastal Environment: Assessment of Estrogenic and Dioxin-like Activities. Arch. Environ. Contam. Toxicol. 58, 562-575

den Besten P, de Deckere E, Babut M, Power B, DelValls TA, Zago C, Oen AP, Heise S (2003): Biological effects-based sediment quality in ecological risk assessment for European waters. J. Soils Sediments 3, 144-162

Denison MS, Fisher JM, Whitlock JP (1988a): Inducible, receptor-dependent protein-DNA interactions at a dioxin-responsive transcriptional enhancer. Proc. Natl. Acad. Sci. U.S.A. 85, 2528-2532

Denison MS, Fisher JM, Whitlock JP (1988b): The DNA recognition site for the dioxin-Ah receptor complex. Nucleotide sequence and functional analysis. J. Biol. Chem. 263, 17221-17224

Denison MS, Heath-Pagliuso S (1998): The Ah Receptor: A Regulator of the Biochemical and Toxicological Actions of Structurally Diverse Chemicals. Bull. Environ. Contam.Toxicol. 61, 557-568

Denison MS, Nagy SR (2003): Activation of the Aryl Hydrocarbon Receptor by structurally diverse Exogenous and Endogenous Chemicals. Annu. Rev. Pharm. Toxicol. 43, 309-334

Di Giulio RT, Hinton DE (2008): The Toxicology of Fishes. Taylor and Francis Group, Boca Raton, USA

Di Toro DM, Zarba CS, Hansen DJ, Berry WJ, Swartz RC, Cowan CE, Paquin PR (1991): Technical basis for establishing sediment quality criteria for nonionic organic chemicals using equilibrium partitioning. Environ. Toxicol. Chem. 10(12), 1541-1583.

DIN (2001): DIN 38415-6: Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung: Suborganismische Testverfahren (Gruppe T) Teil 6: Giftigkeit gegenüber Fischen: Bestimmung der nicht akut giftigen Wirkung von Abwasser auf die Entwicklung von Fischeiern über Verdünnungsstufen (T 6) Deutsches Institut für Normung e. V., Berlin

DIN (2006): DIN ISO 18287; Bodenbeschaffenheit - Bestimmung der polycyclischen aromatischen Kohlenwasserstoffe (PAK) - Gaschromatographisches Verfahren mit Nachweis durch Massenspektrometrie (GC-MS) DIN Deutsches Institut für Normung e.V.

DIN (2009): DIN EN ISO 15088. Wasserbeschaffenheit - Bestimmung der akuten Toxizität von Abwasser auf Zebrafisch-Eier (Danio rerio); Deutsche Fassung EN ISO 15088:2008, Deutsches Institut für Normung e.V.; Beuth Verlag GmbH; Berlin, 20 S.

Dossier E (2011): Dioxin and Dioxin-Like PCBs EQS dossier - Polychlorinated Dibenzo-p-dioxins (PCDDs), polychlorinated Dibenzofurans (PCDFs), and dioxin-like polychlorinated Biphenyls (DL-PCBs)

EC (2008): Priority substances and certain other pollutants (according to Annex II of the Directive 2008/105/EC) http://ec.europa.eu/environment/water/water-framework/priority_ substances.htm.

Eggleton J, Thomas KV (2004): A review of factors affecting the release and bioavailability of contaminants during sediment disturbance events. Environ. Int. 30, 973-980

Eichbaum K, Brinkmann M, Buchinger S, Reifferscheid G, Hecker M, Giesy JP, Engwall M, van Bavel B, Hollert H (2014): In vitro bioassays for detecting dioxin-like activity — Application potentials and limits of detection, a review. Sci. Tot. Environ. 487, 37-48

Engwall E 1995: Toxicity Assessment of Lipophilic extracts from Environemntal samples. Ph.D. Thesis, Uppsala University, Uppsala, Sweden

Engwall M, Broman D, Brunström B, Ishaq R, Näf C, Zebühr Y (1996): Toxic potencies of lipophilic extracts from sediments and settling particulate matter (SPM) collected in a PCB-contaminated river system. Environ. Toxicol. Chem. 15, 213-222

Engwall M, Broman D, Näf C, Zebühr Y, Brunström B (1997): Dioxin-like compounds in HPLC-fractionated extracts of marine samples from the east and west coast of Sweden: Bioassay- and instrumentally-derived TCDD equivalents. Mar. Pollut. Bull. 34, 1032-1040

Engwall M, Naf C, Broman D, Brunstrom B (1998): Biological and chemical determination of contaminant levels in settling particulate matter and sediments - A Swedish river system before, during, and after dredging of PCB-contaminated lake sediments. Ambio 27, 7

Engwall M, Brunström B, Näf C, Hjelm K (1999): Levels of dioxin-like compounds in sewage sludge determined with a bioassay based on erod induction in chicken embryo liver cultures. Chemosphere 38, 2327-2343

Engwall M, Hjelm K (2000): Uptake of dioxin-like compounds from sewage sludge into various plant species – assessment of levels using a sensitive bioassay. Chemosphere 40, 1189-1195

Engwall M, Van Bavel B (2004): The second round of Interlaboratory comparison of Dioxin-like Compounds in Food using Bioassays. Man Technology Environment Research Centre, Department of Natural Sciences, Örebro University, Sweden, pp. 314

Erickson RJ, Benoit DA, Mattson VR, Leonard EN, Nelson HP (1996): The effects of water chemistry on the toxicity of copper to fathead minnows. Environ. Toxicol. Chem. 15, 181-193

Farrell J, Grassian D, Jones M (1999): Investigation of Mechanisms Contributing to Slow Desorption of Hydrophobic Organic Compounds from Mineral Solids. Environ. Sci. Technol. 33, 1237-1243

Feiler U, Ahlf W, Hoess S, Hollert H, Neumann-Hensel H, Meller M, Weber J, Heininger P (2005): The SeKT Joint Research Project: Definition of reference conditions, control sediments and toxicity thresholds for limnic sediment contact tests. Environ. Sci. Pollut. Res. 12, 257–258

Feiler U, Höss S, Ahlf W, Gilberg D, Hammers-Wirtz M, Hollert H, Meller M, Neumann-Hensel H, Ottermanns R, Seiler T-B, Spira D, Heininger P (2013): Sediment contact tests as a tool for the assessment of sediment quality in German waters. Environ. Toxicol. Chem. 32, 144-155

Fent K (Editor), 2007: Ökotoxikologie. Georg Thieme Verlag, Stuttgart, New York

Fernandes A, White S, D'Silva K, Rose M (2004): Simultaneous determination of PCDDs, PCDFs, PCBs and PBDEs in food. Talanta 63, 1147-1155

Fernandez-Salguero P, Pineau T, Hilbert DM, McPhail T, Lee SS, Kimura S, Nebert DW, Rudikoff S, Ward JM, Gonzalez FJ (1995): Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor. Science 268, 722-726

FGG-Elbe (2015): Backgroundinformations to the management plans, retrieved September 24 2015, from http://fgg-elbe.de/hintergrundinformationen.html, Flussgebietsgemeinschaft (FGG) Elbe, Magdeburg, Germany

Focant JF, Eppe G, Pirard C, Massart AC, André JE, De Pauw E (2002): Levels and congener distributions of PCDDs, PCDFs and non-ortho PCBs in Belgian foodstuffs: Assessment of dietary intake. Chemosphere 48, 167-179

Förstner U (2008): Differences in policy response to similar scientific findings—examples from sediment contamination issues in River Basin Management Plans. J. Soils Sediments 8, 214-216

Förstner U, Ahlf W, Calmano W (2008): Entwicklung von Qualitätskriterien für Gewässersedimente. Universitätsbibliothek der Technischen Universität Hamburg-Harburg.

Förstner U (2009): Sediments and priority substances in river basins. J. Soils Sediments 9, 89-93

Franzén B, Haaparanta T, Gustafsson J-Å, Toftgård R (1988): TCDD receptor ligands present in extracts of urban air particulate matter induce aryl hydrocarbon hydroxylase activity and cytochrome P-450c gene expression in rat hepatoma cells. Oxford University Press

Fulton T (1902): Rate of growth of seas fishes. Sci. Invest. Fish. Div. Scot. Rept. 20

Gale RW, Long ER, Schwartz TR, Tillitt DE (2000): Evaluation of planar halogenated and polycyclic aromatic hydrocarbons in estuarine sediments using ethoxyresorufin-O-deethylase induction of H4IIE cells. Environ. Toxicol. Chem. 19, 1348-1359

Garrison PM, Tullis K, Aarts JMMJG, Brouwer A, Giesy JP, Denison MS (1996): Species-Specific Recombinant Cell Lines as Bioassay Systems for the Detection of 2,3,7,8-Tetrachlorodibenzo-p-dioxin-like Chemicals. Toxicol. Sci. 30, 194-203

Ghosh U, Zimmerman JR, Luthy RG (2003): PCB and PAH Speciation among Particle Types in Contaminated Harbor Sediments and Effects on PAH Bioavailability. Environ. Sci. Technol. 37, 2209-2217

Gierthy JF, Crane D (1985): In vitro bioassay for dioxinlike activity based on alterations in epithelial cell proliferation and morphology. Fundam. Appl. Toxicol. 5, 754-759

Giesy, Ludmig JP, Tillitt DE (1994a): Embryolethality and deformities in colonial, fish-eating, water birds of the Great Lakes region - Assessing Causality. Environ. Sci. Technol. 28, 128A-135A

Giesy JP, Ludmig JP, Tillitt DE (1994b): Dioxins, Dibenzofurans, PCBs and Colonial, Fish-eating Water Birds, pp. 254-307. In: Schecter A (Hrsg.), Dioxin and Health. Plenum Press, New York

Giesy JP, Jude DJ, Tillitt DE, Gale RW, Meadows JC, Zajieck JL, Peterman PH, Verbrugge DA, Sanderson JT, Schwartz TR, Tuchman ML (1997): Polychlorinated dibenzo-p-dioxins, dibenzofurans, biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents in fishes from Saginaw Bay, Michigan. Environ. Toxicol. Chem. 16, 713-724

Giesy JP, Kannan K (1998): Dioxin-Like and Non-Dioxin-Like Toxic Effects of Polychlorinated Biphenyls (PCBs): Implications For Risk Assessment. Crit. Rev. Toxicol. 28, 511-569

Giesy JP, Hilscherova K, Jones PD, Kannan K, Machala M (2002): Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. Mar. Pollut. Bull. 45, 3-16

Giesy JP, Kannan K, Jones PD, Blankenship AL (2006): PCBs and Related Compounds, Chapter 11, pp. 245-331. In: Carr J (Hrsg.), Endocrine Disruptors: Biological Basis for Health Effects in Wildlife and Humans. Oxford University Press, New York, pp. 477

Gocht T, Grathwohl P (2004): Polyzyklische aromatische Kohlenwasserstoffe aus diffusen Quellen. Umweltwiss. Schadst.-Forsch. 16, 245-254

Goksøyr A, Förlin L (1992): The cytochrome P-450 system in fish, aquatic toxicology and environmental monitoring. Aquat. Toxicol. 22, 287-311

Goldstein JA, Safe S (1989): Mechanism of action and structure-activity relationships for the chlorinated dibenzo-p-dioxins and related compounds. Halogenated biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins and Related Products. In: Kimbrough RD , Jensen AA (Hrsg.). Elsevier, Amsterdam, The Netherlands, pp. 239-293

Gómez-Ariza JL, Bujalance M, Giráldez I, Velasco A, Morales E (2002): Determination of polychlorinated biphenyls in biota samples using simultaneous pressurized liquid extraction and purification. J. Chromatogr. A 946, 209-219

Götz R, Lauer R (2003): Analysis of Sources of Dioxin Contamination in Sediments and Soils Using Multivariate Statistical Methods and Neural Networks. Environ. Sci. Technol. 37, 5559-5565

Götz R, Bauer O-H, Friesel P, Herrmann T, Jantzen E, Kutzke M, Lauer R, Paepke O, Roch K, Rohweder U, Schwartz R, Sievers S, Stachel B (2007): Vertical profile of PCDD/Fs, dioxin-like PCBs, other PCBs, PAHs, chlorobenzenes, DDX, HCHs, organotin compounds and chlorinated ethers in dated sediment/soil cores from flood-plains of the river Elbe, Germany. Chemosphere 67, 592-603

GÜBAK (2009): Gemeinsame Übergangsbestimmungen zum Umgang mit Baggergut in Küstengewässern GÜBAK-WSV.

Gustavsson LK, Klee N, Olsman H, Hollert H, Engwall M (2004): Fate of ah receptor agonists during biological treatment of an industrial sludge containing explosives and pharmaceutical residues. Environ. Sci. Pollut. Res. Int. 11, 379-387.

Gustavsson LK, Hollert H, Jönsson S, Van Bavel B, Engwall M (2007): 14Reed beds receiving industrial sludge containing nitroaromatic compounds. Env. Sci. Poll. Res. Int. 14, 202-211.

HABAB (2000): Handlungsanweisung für den Umgang mit Baggergut im Binnenland (HABAB-WSV), BfG-1251.

HABAK (1999): Handlungsanweisung für den Umgang mit Baggergut im Küstenbereich (HABAK-WSV), BfG-1100.

Hahn ME (1998): The aryl hydrocarbon receptor: A comparative perspective. Comp. Biochem. Physiol. Part C: Pharmacology, Toxicology and Endocrinology 121, 23-53

Hallare A, Seiler T-B, Hollert H (2011): The versatile, changing, and advancing roles of fish in sediment toxicity assessment—a review. J. Soils Sediments 11, 141-173

Hamers T, van Schaardenburg MD, Felzel EC, Murk AJ, Koeman JH (2000): The application of reporter gene assays for the determination of the toxic potency of diffuse air pollution. Sci. Tot. Environ. 262, 159-174

Hanberg A, Stahlberg M, Georgellis A, de Wit CA, Ahlborg UG (1991): Swedish Dioxin Survey: Evaluation of the H-4-II E Bioassay for Screening Environmental Samples for Dioxin-Like Enzyme Induction. Pharm. Toxicol. 69, 442-449

Hankinson O (1995): The Aryl Hydrocarbon Receptor Complex. Annu. Rev. Pharm. Toxicol. 35, 307-340

Harrison RO, Eduljee GH (1999): Immunochemical analysis for dioxins — progress and prospects. Sci. Tot. Environ. 239, 1-18

Hasegawa J, Guruge KS, Seike N, Shirai Y, Yamata T, Nakamura M, Handa H, Yamanaka N, Miyazaki S (2007): Determination of PCDD/Fs and dioxin-like PCBs in fish oils for feed ingredients by congener-specific chemical analysis and CALUX bioassay. Chemosphere 69, 1188-1194

Heimann W, Sylvester M, Seiler T-B, Hollert H, Schulz R (2011): Sediment toxicity in a connected oxbow lake of the Upper Rhine (Germany): EROD induction in fish cells. J. Soils Sediments 11, 1279-1291

Heinisch E, Kettrup A, Bergheim W, Wenzel S (2007): Persistent chlorinated hydrocarbons, source-oriented monitoring in aquatic media. 6. Strikingly high contaminated sites. Fresen. Environ. Bull. 16, 1248–1273

Heise S, Förstner U (2006): Risks from Historical Contaminated Sediments in the Rhine Basin. In: Kronvang B, Faganeli J , Ogrinc N (Editors), The Interactions Between Sediments and Water. Springer Netherlands, pp. 261-272

Heise S, Krüger F, Baborowski M, Stachel B, Götz R, Förstner U 2008: Bewertung von Risiken durch feststoffgebundene Schadstoffe im Elbeinzugsgebiet, Hamburg

Hilscherova K, Machala M, Kannan K, Blankenship A, Giesy JP (2000): Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. Environ. Sci. Pollut. Res. Int. 7, 159-171.

Hilscherova K, Kannan K, Kang Y-S, Holoubek I, Machala M, Masunaga S, Nakanishi J, Giesy JP (2001): Characterization of dioxin-like activity of sediments from a Czech River Basin. Environ. Toxicol. Chem. 20, 2768-2777

Hilscherova K, Kannan K, Nakata H, Hanari N, Yamashita N, Bradley PW, McCabe JM, Taylor AB, Giesy JP (2003): Polychlorinated Dibenzo-p-dioxin and Dibenzofuran Concentration Profiles in Sediments and Flood-Plain Soils of the Tittabawassee River, Michigan. Environ. Sci. Technol. 37, 468-474

Hinga KR (2003): Degradation rates of low molecular weight PAH correlate with sediment TOC in marine subtidal sediments. Mar. Pollut. Bull. 46, 466-474

Hinger G, Brinkmann M, Bluhm K, Sagner A, Takner H, Eisenträger A, Braunbeck T, Engwall M, Tiehm A, Hollert H (2011): Some heterocyclic aromatic compounds are Ah receptor agonists in the DR-CALUX assay and the EROD assay with RTL-W1 cells. Environ. Sci. Pollut. Res. Int. 18, 1297-1304.

Hofmaier A, Schwirzer S, Wiebel F, Schramm K-W, Wegenke M, Kettrup A (1999): Bioassay zur Bestimmung von TCDD-Toxizitätsäquivalenten (TEQ) von Umweltproben und Reststoffen. Umweltwiss. Schadst.-Forsch. 11, 2-8

Hollert H, Dürr M, Erdinger L, Braunbeck T (2000): Cytotoxicity of settling particulate matter and sediments of the Neckar River (Germany) during a winter flood. Environ. Toxicol. Chem. 19, 528-534

Hollert H, Dürr M, Olsman H, Halldin K, van Bavel B, Brack W, Tysklind M, Engwall M, Braunbeck T (2002): Biological and Chemical Determination of Dioxin-like Compounds in Sediments by Means of a Sediment Triad Approach in the Catchment Area of the River Neckar. Ecotox. 11, 323-336

Hollert H, Keiter S, König N, Rudolf M, Ulrich M, Braunbeck T (2003): A new sediment contact assay to assess particle-bound pollutants using zebrafish (*Danio rerio*) embryos. J Soil Sediment 3, 197-207

Hollert H, Heise S, Keiter S, Heininger P, Förstner U (2007): Wasserrahmenrichtlinie — Fortschritte und Defizite. Umweltwiss. Schadst.-Forsch. 19, 58-70

Hollert H, Ernst M, Seiler TB, Wölz J, Braunbeck T, Kosmehl T, Keiter S, Grund S, Ahlf W, Erdinger L, Dürr M (2009): Strategien zur Sedimentbewertung – ein Überblick. Umweltwiss. Schadst.-Forsch. 21, 160-176

Hollert H, Brinkmann M, Hudjetz S, Cofalla C, Schüttrumpf H (2014): Hochwasser – ein unterschätztes Risiko. BIUZ 44, 44-51

Höss S, Ahlf W, Fahnenstich C, Gilberg D, Hollert H, Melbye K, Meller M, Hammers-Wirtz M, Heininger P, Neumann-Hensel H, Ottermanns R, Ratte HT, Seiler TB, Spira D, Weber J, Feiler U (2010): Variability of sediment-contact tests in freshwater sediments with low-level anthropogenic contamination – Determination of toxicity thresholds. Environ. Pollut. 158, 2999-3010

Huang J, Gao J, Yu G, Yamazaki N, Deng S, Wang B, Weber R (2014): Unintentional formed PCDDs, PCDFs, and DL-PCBs as impurities in Chinese pentachloronitrobenzene products. Environ. Sci. Pollut. Res. Int.

Hurst MR, Balaam J, Chan-Man YL, Thain JE, Thomas KV (2004): Determination of dioxin and dioxin-like compounds in sediments from UK estuaries using a bio-analytical approach: chemical-activated luciferase expression (CALUX) assay. Mar. Pollut. Bull. 49, 648-658

Huuskonen SE, Tuvikene A, Trapido M, Fent K, Hahn ME (2000): Cytochrome P4501A Induction and Porphyrin Accumulation in PLHC-1 Fish Cells Exposed to Sediment and Oil Shale Extracts. Arch. Environ. Contam. Toxicol. 38, 59-69

Iannuzzi TJ, Bonnevie NL, Wenning RJ (1995): An evaluation of current methods for developing sediment quality guidelines for 2,3,7,8-tetrachlorodibenzo-p-dioxin. Arch. Environ. Contam. Toxicol. 28, 366-377

IKSE (1992-1995): Das erste Aktionsprogramm (Sofortprogramm) zur Reduzierung der Schadstofffrachten in der Elbe und ihrem Eizugsgebiet für den Zeitraum 1992 bis 1995. http://www.ikse-mkol.org/index.php?id=406

IKSE 1996-2010: Die Elbe ist wieder ein lebendiger Fluss, Magdeburg

ISO (1996): ISO 7346/3: Water quality -- Determination of the acute lethal toxicity of substances to a freshwater fish [Brachydanio rerio, Hamilton-Buchanan (Teleostei, Cyprinidae)] - Part 1: Static method; Part 2 - Semi-Static Method. Iso Guideline.

ISO/5752 (2002): Guide to the use of repeatability, reproducibility and trueness estimates in measurement uncertainty estimation.

Isosaari P, Vartiainen T, Hallikainen A, Ruohonen K (2002): Feeding trial on rainbow trout: comparison of dry fish feed and Baltic herring as a source of PCDD/Fs and PCBs. Chemosphere 48, 795-804

IUPAC (2006). In: Nic M, Jirat J, Kosata B , Jenkins A (Hrsg.), Last update: 2012-08-19; version: 2.3.2., http://goldbook.iupac.org

Jacobs P, Krautter N, Diesner K (2013): Frachtreduzierung Spittelwasser – Abschlussbericht. Im Auftrag der Landesanstalt für Altlastenfreistellung des Landes Sachsen-Anhalt.

Jamet J-L, Desmolles F (1994): Growth, Reproduction and Condition of Roach (*Rutilus rutilus*), Perch (*Perca fluviatilis*) and Ruffe (*Gymnocephalus cernuus*) in Eutrophic Lake Aydat (France). Int. Rev. Gesamt. Hydrobiol. Hydrogr. 79, 305-322

Jeong S-H, Cho J-H, Park J-M, Denison MS (2005): Rapid Bioassay for the Determination of Dioxins and Dioxin-like PCDFs and PCBs in Meat and Animal Feeds. J. Anal. Toxicol. 29, 156-162

Kannan K, Yun SH, Ostaszewski A, McCabe JM, Mackenzie-Taylor D, Taylor AB (2008): Dioxin-Like Toxicity in the Saginaw River Watershed: Polychlorinated Dibenzo-p-Dioxins, Dibenzofurans, and Biphenyls in Sediments and Floodplain Soils from the Saginaw and Shiawassee Rivers and Saginaw Bay, Michigan, USA. Arch. Environ. Contam. Toxicol. 54, 9-19

Kasai A, Hiramatsu N, Hayakawa K, Yao J, Maeda S, Kitamura M (2006): High levels of dioxin-like potential in cigarette smoke evidenced by in vitro and in vivo biosensing. Cancer Res. 66, 7143-7150

Keiter S 2007: Der Fischrückgang in der Donau - Ein Modell zur Bewertung der Belastung von Sedimenten, Ruprecht-Karls-Universität Heidelberg, Heidelberg, 208 pp

Keiter S, Grund S, Van Bavel B, Hagberg J, Engwall M, Kammann U, Klempt M, Manz W, Olsman H, Braunbeck T, Hollert H (2008): Activities and identification of aryl hydrocarbon receptor agonists in sediments from the Danube river. Anal. Biochem. 390, 2009-2019

Keith LH, Crummett WB, Deegan J, Libby RA, Taylor JK, Wentler G (1983): Principles of environmental analysis. Anal. Chem. 55, 2210-2218

Kennedy SW, Lorenzen A, James CA, Collins BT (1993): Ethoxyresorufin-O-deethylase and Porphyrin Analysis in Chicken Embryo Hepatocyte Cultures with a Fluorescence Multiwell Plate Reader. Anal. Biochem. 211, 102-112

Kennedy SW, Lorenzen A, Jones SP, Hahn ME, Stegeman JJ (1996): Cytochrome P4501A Induction in Avian Hepatocyte Cultures: A Promising Approach for Predicting the Sensitivity of Avian Species to Toxic Effects of Halogenated Aromatic Hydrocarbons. Toxicol. Appl. Pharm. 141, 214-230

Khim JS, Kannan K, Villeneuve DL, Koh CH, Giesy JP (1999a): Characterization and Distribution of Trace Organic Contaminants in Sediment from Masan Bay, Korea. 1. Instrumental Analysis. Environ. Sci. Technol. 33, 4199-4205

Khim JS, Villeneuve DL, Kannan K, Lee KT, Snyder SA, Koh C-H, Giesy JP (1999b): Alkylphenols, polycyclic aromatic hydrocarbons, and organochlorines in sediment from Lake Shihwa, Korea: Instrumental and bioanalytical characterization. Environ. Toxicol. Chem. 18, 2424-2432

Kinani S, Bouchonnet S, Creusot N, Bourcier S, Balaguer P, Porcher J-M, Aït-Aïssa S (2010): Bioanalytical characterisation of multiple endocrine- and dioxin-like activities in sediments from reference and impacted small rivers. Environ. Pollut. 158, 74-83

Klein GP, Hodge EM, Diamond ML, Yip A, Dann T, Stern G, Denison MS, Harper PA (2006): Gas-phase ambient air contaminants exhibit significant dioxin-like and estrogen-like activity in vitro. Environ. Health Perspect. 114, 697-703

Klemm W, Greif A, Broekaert JAC, Siemens V, Junge FW, van der Veen A, Schultze M, Duffek A (2005): A Study on Arsenic and the Heavy Metals in the Mulde River System. Acta Hydroch. Hydrob. 33, 475-491

Kobayashi Y, Hall A, Hiraoka M, Ashieda K, Nakanishi T, Yamada T, Ogiwara K, Uechi T, Hughes B, Inoue N (2003): Evaluation of AH-immunoassay as a screening method for dioxins and Co-PCBs in environmental samples, 60. Federal Environmental Agency, Vienna, Austria, 4 pp

Koh CH, Khim JS, Villeneuve DL, Kannan K, Giesy JP (2002): Analysis of trace organic contaminants in sediment, pore water, and water samples from Onsan Bay, Korea: instrumental analysis and in vitro gene expression assay. Environ. Toxicol. Chem. 21, 1796-1803

Koh CH, Khim JS, Kannan K, Villeneuve DL, Senthilkumar K, Giesy JP (2004): Polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs) and 2,3,7,8-TCDD equivalents (TEQs) in sediment from the Hyeongsan River, Korea. Environ. Pollut. 132, 489-501

Koh CH, Khim JS, Villeneuve DL, Kannan K, Johnson BG, Giesy JP (2005): Instrumental and bioanalytical measures of dioxin-like and estrogenic compounds and activities associated with sediment from the Korean coast. Ecotox. Environ. Safe. 61, 366-379

Kojima H, Takeuchi S, Tsutsumi T, Yamaguchi K, Anezaki K, Kubo K, Iida M, Takahashi T, Kobayashi S, Jin K, Nagai T (2011): Determination of dioxin concentrations in fish and seafood samples using a highly sensitive reporter cell line, DR-EcoScreen cells. Chemosphere 83, 753-759

Kortet R, Taskinen J, Sinisalo T, Jokinen I (2003): Breeding-related seasonal changes in immunocompetence, health state and condition of the cyprinid fish, *Rutilus rutilus*, L. Biol. J. Linnean Soc. 78, 117-127

Kosmehl T, Hallare AV, Reifferscheid G, Manz W, Braunbeck T, Hollert H (2006): A novel contact assay for testing genotoxicity of chemicals and whole sediments in zebrafish embryos. Environ. Toxicol. Chem. 25, 2097-2106

Krüger F, Gröngröft A (2003): The Difficult Assessment of Heavy Metal Contamination of Soils and Plants in Elbe River Floodplains. Acta Hydroch. Hydrob. 31, 436-443

Kukkonen JVK, Landrum PF, Mitra S, Gossiaux DC, Gunnarsson J, Weston D (2004): The role of desorption for describing the bioavailability of select polycyclic aromatic hydrocarbon and polychlorinated biphenyl congeners for seven laboratory-spiked sediments. Environ. Toxicol. Chem. 23, 1842-1851

Laflamme RE, Hites RA (1978): The global distribution of polycyclic aromatic hydrocarbons in recent sediments. Geochim. Cosmochim. Acta 42, 289-303

Lammer E, Carr GJ, Wendler K, Rawlings JM, Belanger SE, Braunbeck T (2009): Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? . Comp. Biochem. Physiol. C-Toxicol. Pharm. 149

Lamoureux EM, Brownawell BJ (1999): Chemical and biological availability of sediment-sorbed hydrophobic organic contaminants. Journal Name: Environ. Toxicol. Chem. 18, 1733-1741; Conference: 218th Meeting of the American Chemical Society, Las Vegas, NV (US), 09/07/1997--09/11/1997; Other Information: PBD: Aug 1999

Landrum PF (1989): Bioavailability and toxicokinetics of polycyclic aromatic hydrocarbons sorbed to sediments for the amphipod Pontoporeia hoyi. Environ. Sci. Technol. 23, 588-595

Larsson M 2009: Integrated Bioassay- and Chemical Analysis in Risk Assessment of Remediated PAH-Contaminated Soils. Licentiate Thesis Thesis, Örebro University, Örebro, 1-25 pp

Larsson M, Hagberg J, Rotander A, Bavel B, Engwall M (2013): Chemical and bioanalytical characterisation of PAHs in risk assessment of remediated PAH-contaminated soils. Environ. Sci. Pollut. Res. Int., 1-10.

Larsson M, Hagberg J, Giesy JP, Engwall M (2014): Time-dependent relative potency factors (REPs) for polycyclic aromatic hydrocarbons and their derivatives in the H4IIE-luc bioassay. Environ. Toxicol. Chem.

Latimer JS, Davis WR, Keith DJ (1999): Mobilization of PAHs and PCBs from In-Place Contaminated Marine Sediments During Simulated Resuspension Events. Estuar. Coast. Shelf Sci. 49, 577-595

LAVES (2013): Mikro-EROD-Bioassay zur Bestimmung von Dioxinen und dioxinähnlichen Substanzen (gekürzte Fassung), 03-110-MAA-M-11EROD V4, LAVES (Lower Saxony State Office for Consumer Protection and Food Safety), pp. 5

Lee KT, Hong S, Lee JS, Chung KH, Hilscherová K, Giesy JP, Khim JS (2013): Revised relative potency values for PCDDs, PCDFs, and non-ortho-substituted PCBs for the optimized H4IIE-luc in vitro bioassay. Environ. Sci. Pollut. Res. Int., 1-10.

Lee LEJ, Clemons JH, Bechtel DG, Caldwell SJ, Han K-B, Pasitschniak-Arts M, Mosser DD, Bols NC (1993): Development and characterization of a rainbow trout liver cell line expressing cytochrome P450-dependent monooxygenase activity. Cell Biol. Toxicol. 9, 279-294

Lee S-J, Choi S-D, Jin G-Z, Oh J-E, Chang Y-S, Shin SK (2007): Assessment of PCDD/F risk after implementation of emission reduction at a MSWI. Chemosphere 68, 856-863

Lepom P, Brown B, Hanke G, Loos R, Quevauviller P, Wollgast J (2009): Needs for reliable analytical methods for monitoring chemical pollutants in surface water under the European Water Framework Directive. J. Chromatogr. A 1216, 302-315

Li W, Wu WZ, Barbara RB, Schramm KW, Kettrup A (1999): A new enzyme immunoassay for PCDD/F TEQ screening in environmental samples: Comparison to micro-EROD assay and to chemical analysis. Chemosphere 38, 3313-3318

Lick W (Editor), 2009: Sediment and Contaminant Transport in Surface Waters, London, New York

Long ER, Chapman PM (1985): A Sediment Quality Triad: Measures of sediment contamination, toxicity and infaunal community composition in Puget Sound. Mar. Pollut. Bull. 16, 405-415

Long M, Andersen BS, Lindh CH, Hagmar L, Giwercman A, Manicardi G-C, Bizzaro D, Spanò M, Toft G, Pedersen HS, Zvyezday V, Bonde J, Bonefeld-Jorgensen EC (2006): Dioxin-like activities in serum across European and Inuit populations. BioMed Central Ltd.

Lorenzen A, Kennedy SW (1993): A fluorescence-based protein assay for use with a microplate reader, 214. Elsevier, Kidlington, ROYAUME-UNI

Lorenzen A, Kennedy SW, Bastien LJ, Hahn ME (1997): Halogenated aromatic hydrocarbon-mediated porphyrin accumulation and induction of cytochrome P4501A in chicken embryo hepatocytes, 53. Elsevier, Amsterdam, PAYS-BAS

LUA (2005): Umsetzung der Wasserrahmenrichtline, Bericht zur Bestandsaufnahme für das Land Brandenburg, Landesumweltamt Brandenburg, Potsdam

Ma M, Li J, Wang Z (2005): Assessing the Detoxication Efficiencies of Wastewater Treatment Processes Using a Battery of Bioassays/Biomarkers. Arch. Environ. Contam. Toxicol. 49, 480-487

MacDougall D, Crummett WB (1980): Guidelines for data acquisition and data quality evaluation in environmental chemistry. Anal. Chem. 52, 2242-2249

Machala M, Vondráček J, Bláha L, Ciganek M, Neča J (2001): Aryl hydrocarbon receptor-mediated activity of mutagenic polycyclic aromatic hydrocarbons determined using in vitro reporter gene assay. Mutat .Res-Gen. Toxicol. Environ. Mut. 497, 49-62

Mackay D, Shiu WY, Ma KC (1992): Illustrated Handbook of Physical-Chemical Properties and Environmental Fate for Organic Chemicals: Vol. II, Polynuclear Aromatic Hydrocarbons, Polychlorinated Dioxins, and Dibenzofurans. Lewis Publishers, Chelsea, MI

Mackay D, Fraser A (2000): Bioaccumulation of persistent organic chemicals: mechanisms and models. Env. Poll. 110, 375-391

Mandel J, Stiehler RD (1957): Evaluation of analytical methods by the sensitivity criteria. J. Res. Nat. Bur. Stand. 53, 4

Manz W, Krebs F, Schipper CA, Den Besten PJ 2007: Status of ecotoxicological assessment of sedient and dredged material in Germany and the Netherlands - Dutch-German Exchange (DGE) on dredged material

Mason GG (1994): Dioxin-receptor ligands in urban air and vehicle exhaust. Environ. Health Perspect. 102 111-116

Masuda Y (2001): Fate of PCDF/PCB congeners and change of clinical symptoms in patients with Yusho PCB poisoning for 30 years. Chemosphere 43, 925-930

McGroddy SE, Farrington JW, Gschwend PM (1996): Comparison of the in situ desorption sediment-water partitioning of polycyclic aromatic hydrocarbons and polychlorinated biphenyls. Environ. Sci. Technol. 30, 172–7

Meybeck M (1982): Carbon, nitrogen, and phosphorus transport by world rivers. Journal Name: Am. J. Sci.; (United States); Journal Volume: 282:4, Medium: X; Size: Pages: 401-450

Michelsen TC (1992): Technical Information Memorandum - Organic Carbon Normalization of Sediment Data. Washington Department of Ecology, Sediment Management Unit,Publication No. 05-09-050, p. 13.

Murk AJ, Legler J, Denison MS, Giesy JP, van de Guchte C, Brouwer A (1996): Chemical-Activated Luciferase Gene Expression (CALUX): A Novel in Vitro Bioassay for Ah Receptor Active Compounds in Sediments and Pore Water. Toxicol. Sci. 33, 149-160

Nagel R (2002): DarT: The embryo test with the zebrafish Danio rerio - a general model in ecotoxicology and toxicology. Altex 19, 38-48

Nagy SR, Sanborn JR, Hammock BD, Denison MS (2002): Development of a Green Fluorescent Protein-Based Cell Bioassay for the Rapid and Inexpensive Detection and Characterization of Ah Receptor Agonists. Toxicol. Sci. 65, 200-210

Ni H-G, Lu F-H, Luo X-L, Tian H-Y, Zeng EY (2008): Riverine inputs of total organic carbon and suspended particulate matter from the Pearl River Delta to the coastal ocean off South China. Mar. Pollut. Bull. 56, 1150-1157

Nording M, Sporring S, Wiberg K, Björklund E, Haglund P (2005): Monitoring dioxins in food and feedstuffs using accelerated solvent extraction with a novel integrated carbon fractionation cell in combination with a CAFLUX bioassay. Anal. Bioanal. Chem. 381, 1472-1475

OECD (2011): OECD 305, Guidlines for Testing of Chemicals - Bioaccumulation in Fish: Aqueous and Dietary Exposure. 68

Oen AMP, Breedveld GD, Kalaitzidis S, Christanis K, Cornelissen G (2006): How quality and quantity of organic matter affect polycyclic aromatic hydrocarbon desorption from Norwegian harbor sediments. Environ. Toxicol. Chem. 25, 1258-1267

Okey AB (2007): An Aryl Hydrocarbon Receptor Odyssey to the Shores of Toxicology: The Deichmann Lecture, International Congress of Toxicology-XI. Toxicol. Sci. 98, 5-38

Oliveira M, Santos MA, Pacheco M (2004): Glutathione protects heavy metal-induced inhibition of hepatic microsomal ethoxyresorufin O-deethylase activity in *Dicentrarchus labrax* L. Ecotox. Environ. Safe. 58, 379-385

Oliveira Ribeiro CA, Vollaire Y, Coulet E, Roche H (2008): Bioaccumulation of polychlorinated biphenyls in the eel (Anguilla anguilla) at the Camargue Nature Reserve – France. Environ. Pollut. 153, 424-431

Olsman H, Engwall M, Kammann U, Klempt M, Otte J, van Bavel B, Hollert H (2007a): Relative differences in aryl hydrocarbon receptor-mediated response for 18 polybrominated and mixed halogenated dibenzo-P-dioxins and -furans in cell lines from four different species. Environ. Toxicol. Chem. 26, 2448-2454

Olsman H, Van Bavel B, Björnfoth H, Hardell L, Lindström G, Engwall M (2007b): CALUX-TEQs, PCDD/F and PCB in SFE-extracts of human adipose tissue from breast cancer patients, Örebro University, Department of Natural Sciences

Otte JC, Keiter S, Faßbender C, Higley EB, Suares Rocha P, Brinkmann M, Wahrendorf D-S, Manz W, Wetzel MA, Braunbeck T, Giesy JP, Hecker M, Hollert H (2013): Contribution of Priority PAHs and POPs to Ah Receptor-Mediated Activities in Sediment Samples from the River Elbe Estuary, Germany. PLoS ONE 8(10), 11

Patterson DG, Hampton L, Lapeza CR, Belser WT, Green V, Alexander L, Needham LL (1987): High-resolution gas chromatographic/high-resolution mass spectrometric analysis of human serum on a whole-weight and lipid basis for 2,3,7,8-tetrachlorodibenzo-p-dioxin. Anal. Chem. 59, 2000-2005

Pauwels A, Cenijn PH, Schepens PJ, Brouwer A (2000): Comparison of chemical-activated luciferase gene expression bioassay and gas chromatography for PCB determination in human serum and follicular fluid. Environ. Health Perspect., pp. 553-557

Pignatello JJ, Xing B (1995): Mechanisms of slow sorption of organic chemicals to natural particles. Env. Sci. Technol. 30, 1-11

Poland A, Glover E (1973): Chlorinated Dibenzo-p-dioxins: Potent Inducers of δ-Aminolevulinic Acid Synthetase and Aryl Hydrocarbon Hydroxylase. Mol. Pharm. 9, 736-747

Poland A, Knutson JC (1982): 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. Annu. Rev. Pharm. Toxicol. 22, 517-554

Porte C, Albaigés J (1994): Bioaccumulation patterns of hydrocarbons and polychlorinated biphenyls in bivalves, crustaceans, and fishes. Arch. Environ. Contam. Toxicol. 26, 273-281

Puga A, Ma C, Marlowe JL (2009): The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways. Biochem. Pharm. 77, 713-722

Rappe C (1984): Analysis of polychlorinated dioxins and furans. Environ. Sci. Technol. 18, 78A-90A

Rastall AC, Neziri A, Vukovic Z, Jung C, Mijovic S, Hollert H, Nikcevic S, Erdinger L (2004): The identification of readily bioavailable pollutants in lake shkodra/skadar using semipermeable membrane devices (SPMDs), bioassays and chemical analysis. Environ. Sci. Pollut. Res. Int. 11, 240-253.

Reid BJ, Jones KC, Semple KT (2000): Bioavailability of persistent organic pollutants in soils and sediments—a perspective on mechanisms, consequences and assessment. Environ. Pollut. 108, 103-112

Rockne KJ, Shor LM, Young LY, Taghon GL, Kosson DS (2002): Distributed Sequestration and Release of PAHs in Weathered Sediment: The Role of Sediment Structure and Organic Carbon Properties. Environ. Sci. Technol. 36, 2636-2644

Rubinstein NI, Gilliam WT, Gregory NR (1984): Dietary accumulation of PCBs from a contaminated sediment source by a demersal fish (*Leiostomus xanthurus*). Aquat. Toxicol. 5, 331-342

Russell WMS, Burch RL, Hume CW (1959): The principles of humane experimental technique, 238 pp

Safe S, Zacharewski T, Safe L, Harris M, Yao C, Holcomb M (1989): Chlorinated Dioxins and Related Compounds Validation of the AHH indoction bioassay for the determination of 2,3,7,8-TCDD toxic equivalents. Chemosphere 18, 941-946

Safe SH (1986): Comparative toxicology and mechanism of action of polychlorinated dibenzo-p-dioxins and dibenzofurans. Annu. Rev. Pharm. Toxicol. 26, 371-399

Safe SH (1990a): Polychlorinated Biphenyls (PCBs), Dibenzo-p-Dioxins (PCDDs), Dibenzofurans (PCDFs), and Related Compounds: Environmental and Mechanistic Considerations Which Support the Development of Toxic Equivalency Factors (TEFs). Annu. Rev. Toxicol. 21, 51-88

Safe SH (1990b): Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: Environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). Journal Name: Critical Reviews in Toxicology; (USA); Journal Volume: 21:1, Medium: X; Size: Pages: 51-88

Safe SH (1994): Polychlorinated Biphenyls (PCBs): Environmental Impact, Biochemical and Toxic Responses, and Implications for Risk Assessment. Annu. Rev. Toxicol. 24, 87-149

Safe SH (1995): Modulation of gene expression and endocrine response pathways by 2,3,7,8-tetrachlorodibenzo-p-dioxin and related compounds. Pharm. Ther. 67, 247-281

Safe SH (1998a): Limitations of the toxic equivalency factor approach for risk assessment of TCDD and related compounds. Teratog. Carcinog. Mutagen. 17, 285-304

Safe SH (1998b): Development validation and problems with the toxic equivalency factor approach for risk assessment of dioxins and related compounds. J. Anim. Sci. 76, 134-41

Samara F, Gullett BK, Harrison RO, Chu AC, Clark GC (2009): Determination of relative assay response factors for toxic chlorinated and brominated dioxins/furans using an enzyme immunoassay (EIA) and a chemically-activated luciferase gene expression cell bioassay (CALUX). Environ. Int. 35, 588-593

Sanctorum H, Windal I, Hanot V, Goeyens L, Baeyens W (2007): Dioxin and Dioxin-Like Activity in Sediments of the Belgian Coastal Area (Southern North Sea). Arch. Environ. Contam. Toxicol. 52, 317-325

Sanderson JT, Aarts JMMJG, Brouwer A, Froese KL, Denison MS, Giesy JP (1996): Comparison of Ah Receptor-Mediated Luciferase and Ethoxyresorufin-O-deethylase Induction in H4IIE Cells: Implications for Their Use as Bioanalytical Tools for the Detection of Polyhalogenated Aromatic Hydrocarbons. Toxicol. Appl. Pharm. 137, 316-325

Sanderson JT, Giesy JP (1998): Functional Response Assays in Wildlife Toxicology, pp. 5272-5297. In: Meyers RA (Hrsg.), Encyclopedia of Environmental Analysis and Remediation. Inc. John Wiley and Sons, New York

Schecter AJ, Sheu SU, Birnbaum LS, DeVito MJ, Denison MS, Päpke O (1999): A Comparison and Discussion of two differing Methods of measuring dioxin-like Compounds: Gas Chromatography-Mass Spectrometry and the CALUX bioassay - Implications for Health Studies. Organohal. Comp. 40, 4

Schirmer K, Bopp S, Russold S, Popp P (2004a): Dioxin-ähnliche Wirkungen durch Grundwasser am Industriestandort Zeitz. Grundwasser 9, 33-42

Schirmer K, Dayeh VR, Bopp S, Russold S, Bols NC (2004b): Applying whole water samples to cell bioassays for detecting dioxin-like compounds at contaminated sites. Toxicol. 205, 211-221

Schiwy S 2015: Development and application of sediment contact bioassays for the investigation of acute and mechanism-specific toxicity with Danio rerio embryos, RWTH Aachen University, Aachen, 194 pp

Schmidt JV, Su GH, Reddy JK, Simon MC, Bradfield CA (1996): Characterization of a murine AhR null allele: involvement of the Ah receptor in hepatic growth and development. Proc. Natl. Acad. Sci. U.S.A. 93, 6731-6736

Schneider UA, Brown MM, Logan RA, Millar LC, Bunce NJ (1995): Screening assay for dioxin-like compounds based on competitive binding to the murine hepatic Ah receptor. 1. Assay development. Environ. Sci. Technol. 29, 2595-2602

Schoeters G, Goyvaerts MP, Ooms D, Van Cleuvenbergen R (2004): The evaluation of dioxin and dioxin-like contaminants in selected food samples obtained from the Belgian market: comparison of TEQ measurements obtained through the CALUX bioassay with congener specific chemical analyses. Chemosphere 54, 1289-1297

Scholz S, Fischer S, Gündel U, Küster E, Luckenbach T, Voelker D (2008): The zebrafish embryo model in environmeltal risk assessment-applications beyond acute toxicity testing. Environ Sci & Pollut Res 15, 394-404

Schramm KW, Henkelmann B, Kettrup A (1995): PCDD/F sources and levels in River Elbe sediments. Water Res. 29, 2160-2166

Schulz A-J, Wiesmüller T, Appuhn H, Stehr D, Severin K, Landmann D, Kamphues J (2005): Dioxin concentration in milk and tissues of cows and sheep related to feed and soil contamination. J. Anim. Physiol. Anim. Nutr. 89, 72-78

Schulze T, Ulrich M, Maier D, Maier M, Terytze K, Braunbeck T, Hollert H (2014): Evaluation of the hazard potentials of river suspended particulate matter and floodplain soils in the Rhine basin using chemical analysis and in vitro bioassays. Environ. Sci. Pollut. Res., 1-15

Schumacher BA (2002): Methods for the determination of total organic carbon (TOC) in soils and sediments. United States Environmental Protection Agency, Environmental Sciences Division, National Exposure Research Laboratory, 25

Schwab K, Brack W (2007): Large volume TENAX® extraction of the bioaccessible fraction of sediment-associated organic compounds for a subsequent effect-directed analysis. J. Soils Sediments 7, 178-186

Schwirzer SMG, Hofmaier AM, Kettrup A, Nerdinger PE, Schramm K-W, Thoma H, Wegenke M, Wiebel FJ (1998): Establishment of a Simple Cleanup Procedure and Bioassay for Determining 2,3,7,8-Tetrachlorodibenzo-p-dioxin Toxicity Equivalents of Environmental Samples. Ecotox. Environ. Safe. 41, 77-82

Seiler T-B, Schulze T, Hollert H (2008): The risk of altering soil and sediment samples upon extract preparation for analytical and bio-analytical investigations – a review. Anal. Bioanal. Chem 390, 1975–1985

Semple KT, Doick KJ, Jones KC, Burauel P, Craven A, Harms H (2004): Defining bioavailability and bioaccessibility of contaminated soil and sediment is complicated. Environ. Sci. Technol. 38, 228A-231A

Shen C, Huang S, Wang Z, Qiao M, Tang X, Yu C, Shi D, Zhu Y, Shi J, Chen X, Setty K, Chen Y-Y (2007): Identification of Ah Receptor Agonists in Soil of E-waste Recycling Sites from Taizhou Area in China. Environ. Sci. Technol. 42, 49-55

Shen C, Chen Y-Y, Huang S, Wang Z, Yu C, Qiao M, Xu Y, Setty K, Zhang J, Zhu Y, Lin Q (2009): Dioxin-like compounds in agricultural soils near e-waste recycling sites from Taizhou area, China: Chemical and bioanalytical characterization. Environ. Int. 35, 5

Shen JH, Gutendorf B, Vahl HH, Shen L, Westendorf J (2001): Toxicological profile of pollutants in surface water from an area in Taihu Lake, Yangtze Delta. Toxicol. 166, 71-78

Shor LM, Rockne KJ, Taghon GL, Young LY, Kosson DS (2003): Desorption Kinetics for Field-Aged Polycyclic Aromatic Hydrocarbons from Sediments. Environ. Sci. Technol. 37, 1535-1544

Simat TJ (2007): Vortrag: Kombinierte Anwendung von chemischer Analytik und Biotests auf Verpackungsmaterialien. In: BfR-Symposium – 50 Jahre Kunststoffkommission B (Hrsg.)

Song M, Jiang Q, Xu Y, Liu H, Lam PKS, O'Toole DK, Zhang Q, Giesy JP, Jiang G (2006): AhR-active compounds in sediments of the Haihe and Dagu Rivers, China. Chemosphere 63, 1222-1230

Stachel B, Ehrhorn U, Heemken OP, Lepom P, Reincke H, Sawal G, Theobald N (2003): Xenoestrogens in the River Elbe and its tributaries. Environ. Pollut. 124, 497-507

Stachel B, Götz R, Herrmann T, Krüger F, Knoth W, Papke O, Rauhut U, Reincke H, Schwartz R, Steeg E, Uhlig S (2004): The Elbe flood in August 2002 - Occurrence of polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans (PCDD/F) and dioxin-like PCB in suspended particulate matter (SPM), sediment and fish. Water Sci. Technol. 50, 309-316

Stachel B, Jantzen E, Knoth W, Kruger F, Lepom P, Oetken M, Reincke H, Sawal G, Schwartz R, Uhlig S (2005): The Elbe flood in August 2002 - Organic contaminants in sediment samples taken after the flood event. J. Environ. Sci. Health Part A-Toxic/Hazard. Subst. Environ. Eng. 40, 265-287

Stachel B, Christoph EH, Götz R, Herrmann T, Krüger F, Kühn T, Lay J, Löffler J, Päpke O, Reincke H, Schröter-Kermani C, Schwartz R, Steeg E, Stehr D, Uhlig S, Umlauf G (2006): Contamination of the alluvial plain, feeding-stuffs and foodstuffs with polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans (PCDD/Fs), dioxin-like polychlorinated biphenyls (DL-PCBs) and mercury from the River Elbe in the light of the flood event in August 2002. Sci. Tot. Environ. 364, 96-112

Stachel B, Christoph EH, Götz R, Herrmann T, Krüger F, Kühn T, Lay J, Löffler J, Päpke O, Reincke H, Schröter-Kermani C, Schwartz R, Steeg E, Stehr D, Uhlig S, Umlauf G (2007): Dioxins and dioxin-like PCBs in different fish from the river Elbe and its tributaries, Germany. J. Hazard. Mater. 148, 199-209

Stachel B, Mariani G, Umlauf G, Götz R 2011: Dioxine und PCBs in Feststoffen aus der Elbe, ihren Nebenflüssen und der Nordsee (Längsprofilaufnahme 2008)

Stellman JM, Stellman SD, Christian R, Weber T, Tomasallo C (2003): The extent and patterns of usage of Agent Orange and other herbicides in Vietnam. Nature 422, 681-687

Strecker R, Seiler TB, Hollert H, Braunbeck T (2011): Oxygen requirements of zebrafish (*Danio rerio*) embryos in embryo toxicity tests with environmental samples. Comp. Biochem. Physiol. Part C: Toxicol. Pharm. 153, 318-327

Suares Rocha P, Azab E, Schmidt B, Storch V, Hollert H, Braunbeck T (2010): Changes in toxicity and dioxin-like activity of sediments from the Tietê River (São Paulo, Brazil). Ecotox. Environ. Safe. 73, 550-558

Sundberg H, Ishaq R, Åkerman G, Tjärnlund U, Zebühr Y, Linderoth M, Broman D, Balk L (2005): A bio-effect directed fractionation study for toxicological and chemical characterization of organic compounds in bottom sediment. Toxicol. Sci. 84, 63-72

Sutherland JB, Rafii F, Khan AA, Cerniglia CE (1995): In Microbial Transformation and Degradation of Toxic Organic Chemicals. Wiley-Liss, New York

Talley JW, Ghosh U, Tucker SG, Furey JS, Luthy RG (2002): Particle-Scale Understanding of the Bioavailability of PAHs in Sediment. Environ. Sci. Technol. 36, 477-483

Ten Hulscher TEM, Postma J, den Besten PJ, Stroomberg GJ, Belfroid A, Wegener JW, van Noort PCM (2003): Application of Tenax extraction to measure bioavailability of sorbed organic contaminants to soil and sediment inhabiting organisms. Environ. Toxicol. Chem. 22, 2258-2265

Thain JE, Hurst MR, Thomas KV (2006): Determination of Dioxin-like Activity in Sediments from the East Shetland Basin. Organohal. Comp. 68 185-188

Thorsen WA, Cope WG, Shea D (2004): Bioavailability of PAHs: Effects of Soot Carbon and PAH Source. Environ. Sci. Technol. 38, 2029-2037

Till M, Behnisch P, Hagenmaier H, Bock KW, Schrenk D (1997): Dioxinlike components in incinerator fly ash: A comparison between chemical analysis data and results from a cell culture bioassay. Environ. Health Perspect. 105, 1326-1332

Tillitt DE, Giesy JP, Ankley GT (1991): Characterization of the H4IIE rat hepatoma cell bioassay as a tool for assessing toxic potency of planar halogenated hydrocarbons in environmental samples. Environ. Sci. Technol. 25, 87-92

Tillitt DE, Ankley GT, Giesy JP, Ludwig JP, Kurita-Matsuba H, Weseloh DV, Ross PS, Bishop CA, Sileo L, Stromborg KL, Larson J, Kubiak TJ (1992): Polychlorinated biphenyl residues and egg mortality in double-crested cormorants from the great lakes. Environ. Toxicol. Chem. 11, 1281-1288

Tsutsumi T, Amakura Y, Nakamura M, Brown DJ, Clark GC, Sasaki K, Toyoda M, Maitani T (2003): Validation of the CALUX bioassay for the screening of PCDD/Fs and dioxin-like PCBs in retail fish. The Analyst 128, 486-492

UFZ 2003: Schadstoffbelastung nach dem Elbe-Hochwasser 2002- Ermittlung der Gefährdungspotentiale an Elbe und Mulde, UFZ Umweltforschungszentrum Leipzig-Halle GmbH, Magdeburg

Umlauf G, Christoph EH, Bidoglio G (2004): Schadstoffuntersuchungen nach dem Hochwasser vom August 2002 – Ermittlung der Gefährdungspotenziale an Elbe und Mulde (Endbericht BMBF-FKZ PJT 0330492). In: Geller W, Ockenfeld K, Boehme M , Knoechel A (Hrsg.), ISBN: 3-00-013615-0

Umlauf G, Bidoglio G, Christoph EH, Kampheus J, Krüger F, Landmann D, Schulz AJ, Schwartz R, Severin K, Stachel B, Stehr D (2005): The Situation of PCDD/Fs and Dioxin-like PCBs after the Flooding of River Elbe and Mulde in 2002. Acta Hydroch. Hydrob. 33, 543-554

Umlauf G, Mariani G, Skejo H, Mueller A, Amalfitano L, Stachel B, Goetz R (2010): Dioxins and dioxin-like PCBs in solid material from the river Elbe, its tributaries and from the North Sea. Organohal. Comp. 72, 95-99

UNEP (1999): Guidelines for the Identification of PCBs and Materials Containing PCBs.

UNEP (2013): Toolkit for Identification and Quantification of Releases of Dioxins, Furans and Other Unintentional POPs under Article 5 of the Stockholm Convention on Persistent Organic Pollutants.

US-EPA (1994): Method 8290 - Analysis of Polychlorinated dibenzodioxins and poly-chlorinated dibenzofurans by high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS).

US-EPA (2015): http://www.epa.gov/oppt/exposure/pubs/episuitedl.htm

Van Bavel B 1995: Analytical and Environmental Chemistry of Polychlorinated Dibenzodioxins, Dibenzofurans and Biphenyls. Ph.D. Thesis, Umeå University, S-90187 Umeå, Sweden

Van Bavel B, Andersson P, Wingfors H, Rappe C, Tysklind M, Bergqvist P-A, Ahgren J, Norrgren L (1996): Multivariate modeling of pcb bioaccumulation in three-spined stickleback (*Gasterosteus aculeatus*). Environ. Toxicol. Chem. 15, 947-954

Van den Berg M et al. (1998): Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environ. Health Perspect. 106, 775-792

Van den Berg M, Birnbaum LS, Denison MS, De Vito M, Farland W, Feeley M, Fiedler H, Hakansson H, Hanberg A, Haws L, Rose M, Safe S, Schrenk D, Tohyama C, Tritscher A, Tuomisto J, Tysklind M, Walker N, Peterson RE (2006): The 2005 World Health Organization Reevaluation of Human and Mammalian Toxic Equivalency Factors for Dioxins and Dioxin-Like Compounds. Toxicol. Sci. 93, 223-241

Van der Plas SA, Lutkeschipholt I, Spenkelink B, Brouwer A (2001): Effects of subchronic exposure to complex mixtures of dioxin-like and non-dioxin-like polyhalogenated aromatic compounds on thyroid hormone and vitamin A levels in female Sprague-Dawley rats, 59. Oxford University Press, Oxford, ROYAUME-UNI

van Leeuwen FXR, Feeley M, Schrenk D, Larsen JC, Farland W, Younes M (2000): Dioxins: WHO's tolerable daily intake (TDI) revisited. Chemosphere 40, 1095-1101

van Noort PC, Cornelissen G, ten Hulscher TE, Vrind BA, Rigterink H, Belfroid A (2003): Slow and very slow desorption of organic compounds from sediment: influence of sorbate planarity. Water Res. 37, 2317-2322

Van Thuong N, Hung NX, Mo NT, Thang NM, Huy PQ, Van Binh H, Nam VD, Van Thuy N, Son LK, Minh NH (2014): Transport and bioaccumulation of polychlorinated dibenzo-p-dioxins and dibenzofurans at the Bien Hoa Agent Orange hotspot in Vietnam. Environ. Sci. Pollut. Res. Int., 1-11.

Van Wouwe N, Windal I, Vanderperren H, Eppe G, Xhrouet C, Massart AC, Debacker N, Sasse A, Baeyens W, De Pauw E, Sartor F, Van Oyen H, Goeyens L (2004): Validation of the CALUX bioassay for PCDD/F analyses in human blood plasma and comparison with GC-HRMS. Talanta 63, 1157-1167

Veilens E, Brunström B, Broman D, Näf C, Zeböhr Y, Dencker L (1992): Extracts from settling particulate matter collected in the Stockholm archipelago waters: Embryolethality, immunotoxicity and erod-inducing potency of fractions containing aliphatics/monoaromatics, diaromatics or polyaromatics. Environ. Toxicol. Chem. 11, 1441-1449

Viarengo A, Bettella E, Fabbri R, Burlando B, Lafaurie M (1997): Heavy metal inhibition of EROD activity in liver microsomes from the bass Dicentrarchus labrax exposed to organic xenobiotics: Role of GSH in the reduction of heavy metal effects. Mar. Environ. Res. 44, 1-11

Viganò L, Arillo A, Aurigi S, Corsi I, Focardi S (2000): Concentrations of PCBs, DDTs, and TCDD Equivalents in Cyprinids of the Middle Po River, Italy. Arch. Environ. Cont. Toxicol. 38, 209-216

Villeneuve DL, Crunkilton RL, DeVita WM (1997): Aryl hydrocarbon receptor-mediated toxic potency of dissolved lipophilic organic contaminants collected from Lincoln Creek, Milwaukee, Wisconsin, USA, to PLHC-1 (Poeciliopsis lucida) fish hepatoma cells. Environ. Toxicol. Chem. 16, 977-984

Villeneuve DL, Richter CA, Blankenship AL, Giesy JP (1999): Rainbow trout cell bioassay-derived relative potencies for halogenated aromatic hydrocarbons: Comparison and sensitivity analysis. Environ. Toxicol. Chem. 18, 879-888

Villeneuve DL, Kannan K, Khim JS, Falandysz J, Nikiforov VA, Blankenship AL, Giesy JP (2000): Relative Potencies of Individual Polychlorinated Naphthalenes to Induce Dioxin-Like Responses in Fish and Mammalian <i>In Vitro Bioassays. Arch. Environ. Contam. Toxicol. 39, 273-281

Villeneuve DL, Khim JS, Kannan K, Giesy JP (2002): Relative potencies of individual polycyclic aromatic hydrocarbons to induce dioxinlike and estrogenic responses in three cell lines. Environ. Toxicol. 17, 128-137

Wagner U, Schneider E, Watson A, Weber R (2014): Management of PCBs from Open and Closed Applications – Case Study Switzerland, http://www.global-chemicals-waste-platform.net/fileadmin/files/doc/Management_of_PCBs_Case_Study_Switzerland.pdf

Weber R et al. (2008): Dioxin- and POP-contaminated sites—contemporary and future relevance and challenges. Environ. Sci. Pollut. Res. Int. 15, 363-393.

Weber R, Varbelow G (2013): The Dioxin/POPs legacy of pesticide production in Hamburg: Part 1 Securing of the production area. Env. Sci. Pollut. Res. 20, 1918-1924

Wei X, Ching LY, Cheng SH, Wong MH, Wong CKC (2010): The detection of dioxin- and estrogen-like pollutants in marine and freshwater fishes cultivated in Pearl River Delta, China. Environ. Pollut. 158, 2302-2309

Wenning RJ, Ingersoll CG (2002): Summary of the SETAC Pellston Workshop on Use of Sediment Quality Guidelines and Related Tools for the Assessment of Contaminated Sediments. 17-22 August 2002, Fairmont, Montana, USA. Society of Environmental Toxicology and Chemistry (SETAC), Pensacola FL, USA

Wernersson A-S et al. (2015): The European technical report on aquatic effect-based monitoring tools under the water framework directive. Environ. Sci. Eu. 27, 1-11

Westerlund L, Billsson K, Andersson PL, Tysklind M, Olsson P-E (2000): Early life-stage mortality in zebrafish (*Danio rerio*) following maternal exposure to polychlorinated biphenyls and estrogen. Env. Toxicol. Chem. 19, 1582-1588

Wetterauer B, Ricking M, Otte JC, Hallare AV, Rastall AC, Erdinger L, Schwarzbauer J, Braunbeck T, Hollert H (2012): Toxicity, dioxin-like activities, and endocrine effects of DDT metabolites—DDA, DDMU, DDMS, and DDCN. Environ. Sci. Pollut. Res. Int. 19, 403-415.

White SS, Birnbaum LS (2009): An Overview of the Effects of Dioxins and Dioxin-Like Compounds on Vertebrates, as Documented in Human and Ecological Epidemiology. J. Environ. Sci. Health, Part C: Environ. Carcinog. Ecotox. Rev. 27, 197-211

Whitlock JPJ (1999): Induction of cytochrome P4501A1. Annu. Rev. Pharm. Toxicol. 39, 103-125

Whyte JJ, Jung RE, Schmitt CJ, Tillitt DE (2000): Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure, 30. Informa Healthcare, London, ROYAUME-UNI

Whyte JJ, Schmitt CJ, Tillitt DE (2004): The H4IIE Cell Bioassay as an Indicator of Dioxin-like Chemicals in Wildlife and the Environment. Crit. Rev. Toxicol. 34, 1-83

Wilken M, Walkow F, Jager E, Zeschmar-Lahl B (1994): Flooding area and sediment contamination of the river Mulde, an Elbe-influx, with PCDD/F and other organic pollutants. Chemosphere 29, 2237-2252.

Willett KL, Gardinali PR, Sericano JL, Wade TL, Safe SH (1997): Characterization of the H4IIE Rat Hepatoma Cell Bioassay for Evaluation of Environmental Samples Containing Polynuclear Aromatic Hydrocarbons (PAHs). Arch. Environ. Contam. Toxicol. 32, 442-448

Windal I, Eppe G, Catherine Gridelet A, De Pauw E (1998): Supercritical fluid extraction of polychlorinated dibenzo-p-dioxins from fly ash: the importance of fly ash origin and composition on extraction efficiency. J. Chromatogr. A 819, 187-195

Windal I, Denison MS, Birnbaum LS, Van Wouwe N, Baeyens W, Goeyens L (2005): Chemically Activated Luciferase Gene Expression (CALUX) Cell Bioassay Analysis for the Estimation of Dioxin-Like Activity: Critical Parameters of the CALUX Procedure that Impact Assay Results. Environ. Sci. Technol. 39, 7357-7364

Wölz J 2005: Entwicklung von Fraktionierungsmethoden zur Untersuchung des Dioxin-ähnlichen Potenzials von Hochwasserschwebstoffen, Ruprecht-Karls-Universität, Heidelberg, 93 pp

Wölz J, Engwall M, Maletz S, Olsman Takner H, van Bavel B, Kammann U, Klempt M, Weber R, Braunbeck T, Hollert H (2008): Changes in toxicity and Ah receptor agonist activity of suspended particulate matter during flood events at the rivers Neckar and Rhine — a mass balance approach using in vitro methods and chemical analysis. Environ. Sci. Pollut. Res. Int. 15, 536-553.

Wölz J, Borck D, Witt G, Hollert H (2009): Ecotoxicological characterization of sediment cores from the western Baltic Sea (Mecklenburg Bight) using GC–MS and biotests. J. Soils Sediments 9, 400-410

Wölz J, Brack W, Moehlenkamp C, Claus E, Braunbeck T, Hollert H (2010a): Effect-directed analysis of Ah receptor-mediated activities caused by PAHs in suspended particulate matter sampled in flood events. Sci. Tot. Environ. 408, 3327-3333

Wölz J, Fleig M, Schulze T, Maletz S, Lübcke-von Varel U, Reifferscheid G, Kühlers D, Braunbeck T, Brack W, Hollert H (2010b): Impact of contaminants bound to suspended particulate matter in the context of flood events. J. Soils Sediments 10, 1174-1185

Wölz J, Schulze T, Lübcke-von Varel U, Fleig M, Reifferscheid G, Brack W, Kühlers D, Braunbeck T, Hollert H (2011): Investigation on soil contamination at recently inundated and non-inundated sites. J. Soils Sediments 11, 82-92

Wycisk P, Stollberg R, Neumann C, Gossel W, Weiss H, Weber R (2013): Integrated Methodology for Assessing the HCH Groundwater Pollution at the Multi-Source Contaminated Mega-Site Bitterfeld/Wolfen. Env. Sci. Pollut. Res. 20, 1907-1917

Xing B, Pignatello JJ, Gigliotti B (1996): Competitive Sorption between Atrazine and Other Organic Compounds in Soils and Model Sorbents. Environ. Sci. Technol. 30, 2432-2440

Yoder AJ (2003): Lessons from Stockholm: Evaluating the Global Convention on Persistent Organic Pollutants. Ind. J. Global Legal Stud. 10, 113-156

Yu M-L, Guo YL, Hsu C-C, Rogan WJ (2000): Menstruation and reproduction in women with polychlorinated biphenyl (PCB) poisoning: long-term follow-up interviews of the women from the Taiwan Yucheng cohort. Int. J. Epidemiol. 29, 672-677

Zacharewski T, Safe L, Safe S, Chittim B, DeVault D, Wiberg K, Bergqvist PA, Rappe C (1989): Comparative analysis of polychlorinated dibenzo-p-dioxin and dibenzofuran congeners in Great Lakes fish extracts by gas chromatography-mass spectrometry and in vitro enzyme induction activities. Environ. Sci. Technol. 23, 730-735

Zacharewski TR, Berhane K, Gillesby BE, Burnison BK (1995): Detection of Estrogen- and Dioxin-like Activity in Pulp and Paper Mill Black Liquor and Effluent Using in vitro Recombinant Receptor/Reporter Gene Assays. Environ. Sci. Technol. 29, 2140-2146

Zeiger M, Haag R, Höckel J, Schrenk D, Schmitz H-J (2001): Inducing Effects of Dioxin-like Polychlorinated Biphenyls on CYP1A in the Human Hepatoblastoma Cell Line HepG2, the Rat Hepatoma Cell Line H4IIE, and Rat Primary Hepatocytes: Comparison of Relative Potencies. Toxicol. Sci. 63, 65-73

Zhang J-H, Chung TDY, Oldenburg KR (1999): A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J. Biomol. Screen. 4, 67-73

Zhao B, Baston DS, Khan E, Sorrentino C, Denison MS (2010): Enhancing the response of CALUX and CAFLUX cell bioassays for quantitative detection of dioxin-like compounds. Sci. China Chem. 53, 1010-1016

Zhao D, Pignatello JJ (2004): Model-aided characterization of Tenax®-ta for aromatic compound uptake from water Env. Tox. and Chem. 23, 1592-1599

Zielke H, Seiler T-B, Niebergall S, Leist E, Brinkmann M, Spira D, Streck G, Brack W, Feiler U, Braunbeck T, Hollert H (2011): The impact of extraction methodologies on the toxicity of sediments in the zebrafish (*Danio rerio*) embryo test. J. Soils Sediments 11, 352-363

Acknowledgements

I would like to express my thanks to everyone who actively or passively contributed to this thesis, with specials thanks to:

Prof. Dr. Henner Hollert for the possibility to conduct the present work in his working group Ecosystem Analysis at the Institute for Environmental Research of the RWTH Aachen. Thank you for being my first referee and supervisor, for all your good suggestions and the good and fruitful discussion we had. Thank you for offering me the opportunity to participate in national and international conferences as well as to gain experience in other scientific institutions. It was an honor to plan, accompany and celebrate your performance as a DJ at the "Night of the Profs" ;-)

Prof. Dr. Andreas Schäffer for agreeing to act as my second referee.
Thank you for your interest in my project.

Dr. Thomas-Benjamin Seiler, for numerous useful tips and tricks especially for Endnote ;).

Dr. Georg Reifferscheid, Dr. Sebastian Buchinger, Dipl. -Ing. Denise Spira, Dipl. -Ing. Christel Möhlenkamp and Marina Ohlig from the German Federal Institute of Hydrology (BfG) for the great team work during the last years. Without you the present work wouldn't have been possible. Thank you so much for all the fruitful discussions we had on our meetings, for planning and physically supporting our sediment sampling tours, for the measurement of PAHs in our sediment and soil sample extracts and for the excellent and fast performance in our cross-laboratory experiments.

Dr. Stephan Hamm and Dr. Armin Maulshagen from Münster Analytical Solutions GmbH (mas) for their eager assistance and contribution to this work. Thank you for the chemical analysis of PCDD/Fs and DL-PCBs in whole fish homogenates and sediments, for telephone conversations and for giving me the opportunity to visit your company.

Dr. Gunther Umlauf from the European Commission und Dr. Burkhard Stachel from the Ministry of Urban Development and Environment of the City of Hamburg for supporting me since the beginning of my Diploma thesis. Thank you for providing big sampling sets of sediment and soil from the Elbe catchment area and for last-minute corrections through postal channels.

Dr. Burkhard Schmidt and Dr. Björn Scholz Starke for patiently answering all my chemical analytical as well as statistical questions. Thank you for your precious time, for sharing your expertise with me and for giving new perspectives.

Prof. Dr. Markus Hecker, Prof. Dr. John P. Giesy, Garry Codling, PhD and Ass. Prof. Dr. Paul Jones from the University of Saskatchewan, Canada for their constant willingness to contribute to the discussions and the corrections of my manuscripts. Thank you for providing the H4IIE-luc cell line. I appreciated your constructive critics and countless good advices. You really helped me a lot improving my English texts. Thank you for supervising my master candidate Nora Niehus during her stay in Saskatoon.

Dr. Niels C. Bols from the University of Waterloo and Dr. Ines Thiem from the Lower Saxony State Office for Consumer Protection and Food Safety (LaVes) for providing the RTL-W1 and H4IIE cell lines, respectively.

Dipl.-Biol. Andrzey Schiwy and Kerstin Winkens, M.Sc. for showing me the handling of the H4IIE cells and the Micro EROD assay. Thank you Kerstin for being such a good friend in spite of the distance.

Yvonne Müller, M.Sc. for testing the DioRAMA sediment samples in the fish egg assay with *D. rerio*.

Dipl. –Biol. Nora Hoeltzenbein for her eager proof-reading service and especially for being such a good friend. You're my greatest idol of a self-confident and smart woman.

The German Academic Exchange Service (DAAD) and the Ministry of Education and Research (BMBF) for funding my exchange via the Group of eight (Go8) Australia-Germany Joint Research Cooperation Scheme.
In this connection I want to thank:
Dr. Janet Yat-Man Tang from the National Research Centre for Environmental Toxicology (Entox) for being my supervisor during my stay. Thank you for the pleasant working and travelling environment.
Jennifer Bräunig, M.Sc. for giving me a wonderful and cozy home and for introducing me to the entire team of Entox. You grew so close to my heart during that days. Thank you so much again!

The German Federal Ministry of Transport and Digital Infrastructure (BMVI), for funding the DioRAMA project *via* the German Federal Institute of Hydrology (BfG).

My colleague Markus Brinkmann, who worked with me in the DioRAMA project. Thank you for being honest and reliable. Your passion in the field of environmental toxicology is adorable. Thank you for our Bavarian and Fake-Spanish discussions we held over our monitors and which frequently drove our colleagues crazy. Thank you for all the catchy tunes ("sag mir quando", "Komm zurüüüück, ich bin allein…", "Feliz Navidad") and for my funniest memory of you and two-component glue "Du bist ja doch ein Mensch!"

Mathias Reininghaus, M.Sc., my colleague and good friend, for the endless mudrakeing hours you spend in our facility (wet, cold and dirty). Thank you for being the most empathetic guy I ever met, warm-hearted, diligent and funny. Thank you for all the little breaks "die Steine sind in mir…"!
I recently started calling myself "Karin Knecht" just to get over your departure. I wish you all the best for your future and the enviable life style you chose. See you at the wine festival ;)

Leonie Nüßer, M.Sc. for being such a treasure, good colleague and friend. Thank you for all the assays you performed for my project. Thank you for being my good adviser during countless cigarettes in front of the office. Laughter with you is such a big win "Traube Nüßchen". Maybe one day "Juliane" will turn into something, which so far, sadly, only exists in the animal kingdom ;).

Mirkko Flecken, M.Sc. for fitting so good in all the little gaps and corners of our exposure facility ;)
All jokes aside, thank you very much for all the hard work!

Carolin Gembé, M.Sc. for choosing me as a supervisor for both her bachelor and master theses. Thank you for your trust in me as a supervisor and for conducting the intra-laboratory studies side-by-side with me. It was nice seeing you develop. Thank you for funny talks and cigarettes.

Miriam Zimmer, M.Sc. for conducting desorption experiments and the measurement of PAHs.
Thank you for your stamina to stay with me in the laboratory during endless hours of sediment and biota extract fractionations and clean-ups and
for giving me an understanding how tasty sushi actually can be.

Nora Niehus, M.Sc. for her stay in Saskatoon and the performed HRGC/HRMS measurements. Thank you very much for your resilience and willingness to conduct kinetic overnight measurements.

The 156 roaches for giving me the opportunity to investigate the uptake of sediment-borne dioxin-like compounds.

The whole Institute for Environmental Research for the great working atmosphere, the support and reliability. Many thanks to the people who work in the secretariat, in the IT-team and as technical assistants. You make the Institute work!
I especially want to thank my recent and former office dudes Dipl.-Biol. Tilman Floehr, Dr. Sabrina Schiwy, Hongxia Xiao, M.Sc., Dipl.-Biol. Jochen Kuckelkorn, Dr. Beat Thalmann, Dipl.-Biol. Henriette Meyer-Alert for their company. Working, laughing and celebrating with you was such an honor.
I would also like to take this opportunity to thank Dr. Martina Roß-Nickoll, Prof. Dr. Andreas Schäffer and Dr. Burkhard Schmidt for sharing their knowledge with me and for giving me countless good advices.

Telse Bauer for being such a warm-hearted and modest character. Thank you for all the answered questions, good advices and your precious time. You make us PhD candidates feel like home in the Institute!

My painting group members Pia, Peter, Carmen, Jessica, Paul, Nico and John as well as my voluntary colleagues Gregor, Dorit and Yvonne. Although I often was tired after work, you recharged me with your loveliness, creativity and uniqueness. The jump from the academic pretentious world into a relaxed, sometimes unexpectedly self-mocking atmosphere was great and facilitated the everyday laboratory work tremendously.

Jesus Elibar for being a special friend in my life. ¡Gracias por acercarme la selva amazónica a través de tus correos!

Daniela, Christoph, Scheitel, Houdini, Pief, Kathi, Anna, Laura for being my beloved friends. Thank you for being honest, loving and sympathetic. Thank you for all the little words and situations that made me feel like I'm a little bit in love. Promise never to care about distances!

Hippo, Annika and Manu, my hometown friends for your support, honesty, love and especially for swaggering with me („Hast du denn schon ne eigene Praxis?" [...] „Naja, isch versteh' net völ davon, aba et is ima gut nen Arzt zu kennen").

Peter and Karin Ohrndorf for still being by my side, for your warm-hearted character, your support, your belief in me and for countless postcards from all over the world.

My entire family (Mama, Papa, Christine, Isa, Heiner and Zelda). Without you, your love, your support and belief in me, I would not be where I am now. *Ich hab euch lieb!*

Björn Deutschmann, M.Sc., my "Master of hearts", for being my readily visited base camp, for all the adorable meals you served while we had good talks. Thank you for being a good colleague especially during exhausting sediment samplings. Thank you for being my opaque but sensitive friend with an ambient noise, which surrounds you and helps me calming my mind in strenuous times.

KATHRIN EICHBAUM • WORRINGERWEG 1 • D-52064 AACHEN, GERMANY
PHONE +49 (0) 241/ 80-26686 • E-MAIL KATHRIN.EICHBAUM@RWTH-AACHEN.DE

Curriculum vitae

Name	Kathrin Eichbaum
Date of Birth	05th of November 1985
Place of Birth	Worms, Germany

EDUCATION

01/2012-2016	Doctoral thesis in Biology at the Department of Ecosystem Analysis, Institute for Environmental Research, RWTH Aachen University, Germany (Prof. Dr. Henner Hollert) Title: "In vitro bioassay based evaluation of sediments differently contaminated with dioxins and dioxin-like compounds"
10/2010-09/2011	Diploma thesis, RWTH University, Department for Ecosystem Analysis, Institute for Environmental Research, RWTH Aachen University, Germany (Prof. Dr. Henner Hollert) Title: "Dioxin-like activity of sediment samples from the Elbe River and soil samples from the Elbe associated flood area"
10/2005-10/2010	Studies of Biology at the RWTH Aachen University; Germany Main subjects: ecotoxicology/ecology, environmental medicine/hygiene, plant physiology and soil ecology

PROFESSIONAL EXPERIENCE

05/2015-06/2015	Research visit at the National Research Centre for Environmental Toxicology (ENTOX), Brisbane, QLD, Australia
12/2011-07/2015	Graduate assistant, Department for Ecosystem Analysis, Institute for Environmental Research, RWTH Aachen University, (Prof. Dr. Hollert)
06/2011-10/2011	Student assistant, Department for Ecosystem Analysis, Institute for Environmental Research, RWTH Aachen University, (Prof. Dr. Hollert)
10/2006-08/2010	Student assistant, Research Institute for Ecosystem Analysis and Assessment (gaiac), RWTH Aachen University

ADDITIONAL EXPERIENCE

Since 03/2012	Voluntary service as a painting course leader for the association of physically and multiple handicapped people (VKM), Aachen, Germany
03/2011	Local Organizing Committee of the SETAC Europe Young Environmental Scientists (YES) Meeting, Aachen, Germany
07/2010-08/2010	Ecological field trip to Åndalsnes, Norway
07/2009-09/2009	Voluntary service at the ecological station "Jatun Sacha" in Tena, Ecuador
10/2003-11/2003	Voluntary service at the veterinary clinic for animals, Betzdorf, Germany

Scientific contribution

**Publications contributing to this thesis are highlighted with asterisks.*

Research articles in international peer-reviewed journals

Brinkmann, M., Koglin, S., Eisner, B., Wiseman, S., Hecker, M., Eichbaum, K., Thalmann, B., Buchinger, S., Reifferscheid, G., Hollert, H. (2016) Characterization of transcriptional responses to dioxins and dioxin-like contaminants in roach (*Rutilus rutilus*) using whole transcriptome analysis. *Aquatic Toxicology* 541: 412-423.

Schiwy, A., Brinkmann, M., Thiem, I., Guder, G., Winkens, K., Eichbaum, K., Nüßer, L., Thalmann, B., Buchinger, S., Reifferscheid, G., Seiler, T.-B., Hollert, H. (2015) Determination of the dioxin-like potential of single substances, complex mixtures and environmental samples in the Micro-EROD assay with H4IIE cells. *Nature Protocols* 10 (11): 1728-1741.

*Eichbaum, K., Brinkmann, M., Buchinger, S., Reifferscheid, G., Hecker, M., Giesy, J.P., Engwall, M., van Bavel, B., Hollert, H. (2014) *In vitro* bioassays for detecting dioxin-like activity — Application potentials and limits of detection, a review. *Science of the Total Environment* 487:37-48.

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

Brinkmann, M., Eichbaum, K., Buchinger, S., Reifferscheid, G., Bui, T., Schaeffer, A., Hollert, H., Preuss, T.G. (2014) Understanding receptor-mediated effects in rainbow trout: *in vitro-in vivo* extrapolation using physiologically based toxicokinetic models. *Environmental Science and Technology* 48:3303-3309.

Liu, L., Chen, L., Shao, Y., Zhang, L., Floehr, T., Xiao, H., Yan, Y., Eichbaum, K., Hollert, H., Wu, L. (2014) Evaluation of the Ecotoxicity of Sediments from Yangtze River Estuary and Contribution of Priority PAHs to Ah Receptor-Mediated Activities. *PLoS ONE* 9(8): e104748. doi:10.1371/journal.pone.0104748.

*Eichbaum, K., Brinkmann, M., Buchinger, S., Hecker, M., Engwall, M., van Bavel, B., Reifferscheid, G., Hollert, H. (2013) The dioRAMA project: assessment of dioxin-like activity in sediments and fish (*Rutilus rutilus*) in support of the ecotoxicological characterization of sediments. *Journal of Soils and Sediments* 13:770-774.

Research articles submitted or accepted for publication

*Eichbaum, K., Brinkmann, M., Winkens, K., Umlauf, G., Stachel, B., Buchinger, S., Reifferscheid, G., Möhlenkamp, C., Weber, R., Hollert, H. (submitted) Spatial variability of the pollution of sediment and soil samples with dioxin-like compounds along the Elbe River and its alluvial plain Submitted to *Environmental Science Europe.*

*Eichbaum, K., Brinkmann, M., Nüßer, L., Buchinger, S., Reifferscheid, G., Codling, G., jones, P., Giesy, J.P., Hecker, M., Hollert, H. (submitted) Bio-analytical and instrumental screening of the uptake of sediment-borne, dioxin-like compounds in roach (*Rutilus rutilus*). *Submitted to Environmental Science and Pollution Research.*

*Eichbaum, K., Brinkmann, M., Nüßer, L., Gembé, C., Ohlig, M., Buchinger, S., Reifferscheid, G., Giesy, J.P., Hecker, M., Hollert, H. (submitted) *In vitro* tools for the toxicological evaluation of sediments and dredged materials: cross-validation of chemical and bio-analytical methods. *Submitted to Journal of Soils and Sediments*.

Brinkmann, M., Eichbaum, K., Reininghaus, M., Koglin, S., Kammann, U., Baumann, L., Segner, H., Zennegg, M., Buchinger, S., Reifferscheid, G., Hollert, H. (submitted) Towards science-based sediment quality standards for dioxin-like compounds – effects of field-collected sediments in rainbow trout (*Oncorhynchus mykiss*). *Submitted to Aquatic Toxicology*.

Brinkmann, M., Schlechtriem, C., Reininghaus, M., Eichbaum, K., Buchinger, S., Reifferscheid, G., Hollert, H., Preuss, T.G. (submitted) Cross-species extrapolation of uptake and disposition of neutral organic chemicals using a multi-species physiologically based toxicokinetic model. *Submitted to Environmental Science and Technology.*

Platform Presentations

Brinkmann, M., Eichbaum, K., Buchinger, S., Reifferscheid, G., Bui, T., Schäffer, A., Hollert, H., Preuss, T.G. (2014) Physiologically based toxicokinetic models for *in vitro-in vivo* extrapolation of receptor-mediated effects in rainbow trout. Proceedings, SETAC North America Annual Meeting 2014, Vancouver, Canada.

Eichbaum, K., Brinkmann, M., Buchinger, S., Reifferscheid, G., Nüßer, L., Hollert, H. (2014) Bioanalytische Untersuchung der Aufnahme sedimentbürtiger, dioxin-ähnlicher Substanzen in Rotaugen (*Rutilus rutilus*). Proceedings, SETAC GLB Annual Meeting 2014, Gießen.

Brinkmann, M., Eichbaum, K., Buchinger, S., Reifferscheid, G., Bui, T., Schäffer, A., Hollert, H., Preuss, T.G. (2014) Physiologisch-basierte toxikokinetische Modelle zur *In vitro-in vivo*-Extrapolation Rezeptor-vermittelter Effekte in Fischen. Proceedings, SETAC GLB Annual Meeting 2014, Gießen.

Brinkmann, M., Eichbaum, K., Buchinger, S., Reifferscheid, G., Bui, T., Schäffer, A., Hollert, H., Preuss, T.G. (2014) Physiologically based toxicokinetic models for *in vitro-in vivo* extrapolation of receptor-mediated effects in rainbow trout. Proceedings, 24th SETAC Europe Annual Meeting 2014, Basel, Switzerland.

Eichbaum, K., Seiler, T.-B., Keiter, S., Winkens, K., Brinkmann, M., Umlauf, G., Stachel, B., Hollert, H. (2012) Dioxin-like activity of sediment samples from the Elbe River and soil samples from the Elbe associated flood area. Proceedings, 22th 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.P., 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), 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", Chongqing, China.

Eichbaum K., Seiler T.-B., Keiter S., Umlauf G., Stachel B., Hollert H. (2011) Dioxin-ähnliche Wirksamkeit von Sedimentproben der Elbe und Feststoffproben angrenzender Auenflächen. SETAC GLB 16thAnnual Meeting, Landau, Germany

Posters

Zimmer, M., Eichbaum, K., Brinkmann, M., Buchinger, S., Reifferscheid, G., Hollert, H. (2014)Desorption und Bioverfügbarkeit von polyzyklischen aromatischen Kohlenwasserstoffen aus natürlichen Sedimenten. Proceedings, SETAC GLB Annual Meeting 2014, Gießen.

Zimmer M., Eichbaum K., Brinkmann M., Buchinger S., Reifferscheid G. and Hollert H. (2014) Desorption and bioavailability of polychlorinatedbiphenyls, polycyclic aromatic hydrocarbons and heterocyclic compounds present in sediments, Proceedings, SETAC Europe 24thAnnual Meeting, Basel, Switzerland

Eichbaum, K., Brinkmann, M., Buchinger, S., Hecker, M., Engwall, M., van Bavel, B., Reifferschied, G., Hollert, H. (2013) The dioRAMA joint project – Methods for the detection of dioxin-like chemicals in risk assessment and management of contaminated sediments. Proceedings, SETAC Europe 23th Annual Meeting, Glasgow, Scotland.

Eichbaum K., Kerstin Winkens, Seiler T.-B., Brinkmann M., Umlauf G., Stachel B., Reifferscheid G., Buchinger S., Hollert H. (2012) Assessing the dioxin-like activity of sediment and soil samples from the Elbe and its associated flood area - Bioassays as an alternative for chemical analysis?, Magdeburger Gewässerschutzseminar (MGSS) 2012, Hamburg, Germany

Eichbaum, K., Seiler, T.-B., Keiter, S., Umlauf, G., Stachel, B. and Hollert, H. (2011) Dioxin-like activity of Sediments from the Elbe River and associated flood areas, Proceedings, SETAC Europe 21th Annual Meeting, Milan, Italy