Time-related alterations and other confounding factors in direct sediment contact tests

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

vorgelegt von

Diplom-Biologe

Hanno Zielke

aus Solingen

Berichter: Universitätsprofessor Dr. Henner Hollert
apl. Professor Dr. Hans-Toni Ratte

Tag der mündlichen Prüfung: 12.07.2011

Diese Dissertation ist auf den Internetseiten der Hochschulbibliothek online verfügbar.

Abstract

Sediments are an important sink for and source of environmental pollutants. Because of these two qualities, sediments have been acknowledged in environmental risk assessment and corresponding regulations. In case of the European Water Framework Directive (EWFD), for instance, sediments were addressed by means of an amendment published in 2008. In order to adequately response to sediment contamination within the context of this legal obligation, however, there is still a need for research in several key fields. One open question is the correlation between the effects of pollutants in sediments and factors which can potentially confound such effects. The present thesis aimed to investigate potential confounding impacts in order to identify the importance of the given influences on biotests results and eventually the assessment of sediment contamination. Impact of contact-time on results obtained in biotests appeared to be of particular priority. Hence, time-dependence of data acquired in direct sediment contact tests (SCTs) was the major focus of the given studies. Further investigated were the impacts of test vessel material, extraction methodology, and difference between single- and multi-metal exposures.

A literature survey and two experimental studies were carried out in order to address the time-dependence of results obtained in SCTs. The main experimental investigation compared the impact of time on the effects of 3,4-dichloroaniline (DCA), fluoranthene (FA) and pentachlorophenol (PCP) in the zebrafish (*Danio rerio*) embryo SCT, the nematode SCT with *Caenorhabditis elegans,* and the bacterial SCT with *Arthrobacter globiformis*. The second experimental study assessed the impact of short-term (two week) contact-time between sediments and pollutants on bioaccumulation of FA and PCP in the earthworm species *Lumbriculus variegatus.*

All three studies concurrently revealed that time can severely impact results of SCTs. Literature indicated a complete loss of initially observable effects within weeks and statistically significant changes in effects in different SCT which mostly utilised invertebrates. These changes in effects included both increases and decreases, depending on the respective combination of species, substances, sediments, and additional accompanying parameters. In the two experimental studies, the impact of time was sufficiently severe that distinct changes of results were observed. In the case of the fish embryo SCT, a complete loss of originally observed toxicity of all three substances was recorded within two to sixteen weeks. In the nematode contact test, low number of juveniles in comparison to controls indicated toxicity in all four tested combinations of sediments and substances. Reproduction of worms exposed to FA-spiked natural sediment increased in the test conducted after 4 weeks of contact between FA and the sediment, and reached reproduction in the controls after six weeks. In the other three combinations of sediment and substance that were tested in the nematode SCT, no changes were observed until the end of experiments after six weeks. Toxicity of PCP remained unchanged in the bacterial SCT over the course of 44 weeks in both sediments. Accumulation of PCP in earthworms significantly decreased in FA-spiked natural sediment within 13 days of ageing and exhibited no change in PCP-spiked artificial sediment and both FA-spiked sediments.

In an additional study, four different sediment extraction methodologies and one corresponding SCT were intercompared with regard to biological effectiveness on zebrafish embryos: Soxhlet extraction (SOX), membrane dialysis extraction (MDE), hydroxypropyl-β-cyclodextrin extraction (HPCD) and Tenax TA® extraction (TNX). TNX was identified as a putatively suitable method for the prediction of toxicological bioavailability in the SCT with zebrafish embryos. SOX, MDE and HPCD overestimated bioaccessibility, as was expected for SOX and MDE. HPCD is a methodology that was reported to be predictive of bioaccessibility, but induced effects at least as great as effects induced by the two vigorous

extraction methodologies Soxhlet and MDE. This result may be explained by short sediment-pollutant contact-time, and indicated that results obtained from different extraction methodologies can be severely altered by time.

In an investigation comparing the impact of glass or plastic test vessel material on results in the aqueous zebrafish embryo test, glass vessels were shown to be better suited for testing of DCA. In the case of plastic, 16 hours of DCA-vessel contact prior to embryo exposure resulted in an almost complete loss of effects in the tested concentrations, whereas in glass vessels, an LC_{50} could still be obtained. These results confirm that currently discussed measures, such as semi-static tests, passive dosing and flow-through renewal, are necessary to minimise the influence of vessel material. Furthermore, experimental timing, i.e. the exact time test compounds are introduced into test vessels, can be crucial for the outcomes of experiments.

The final study assessed uptake, accumulation, and corresponding changes in transcript abundance of selected genes in zebrafish embryos exposed to Cd-, Cu-, Ni- and Zn-spiked sediment. In terms of potentially confounding impacts, multi- vs. single-metal exposures were compared. Results emphasized the great relevance of Cu as toxicant on fish. Furthermore, it was shown that multi-metal exposure can impact uptake and bioaccumulation, most likely by means of inhibition and competition. This correlation was established for the first time concerning the zebrafish embryo sediment contact test. Literature indicated that this impact can be further influenced by additional confounding factors like pH or time. Transcript abundances were not sensitive to these changes. This finding could indicate that molecular biomarkers are more robust in their response to confounding factors than other endpoints, but needs to be further investigated.

In most studies in the present thesis, a recurring comparison was conducted on the impact of sediment type, either artificial or natural sediment, on results in spiking-based investigations of sediment contamination. Results indicated no preference for either type, and depended, again, on the specific combination of species and substance. It is concluded that both artificial and natural sediments are very useful tools with advantages as well as inherent limitations. A parallel application should be considered in spiking-based assessments.

The observed significant alterations of results induced by time concurrently indicated a need to developed research strategies to sustainably address time-dependence as potential confounding impact. Difference between exposures to single substances or mixtures was also identified as a relevant impact. Both identified influences might result in severe over- or underestimations of risks associated with contamination of sediments.

However, acknowledgement of factors impacting results in environmental assessment creates a novel challenge. Assessing confounding impacts apparently calls for more tests, whereas economical and animal welfare considerations prioritise a minimisation of test demands. Hence, a three step, long-term strategy is proposed, which can eventually reduce necessity for extensive testing and address confounding impacts at the same time: a) Pilot-studies on the targeted confounding impacts (such as given in the present thesis), b) Integrative studies to assess the interlinking of the addressed impacts between different organisational levels and c) Inclusion of the confounding impacts into holistic, predictive models. In addition, the novel idea of adverse outcome pathways was identified as promising conceptual strategy to characterise and understand the mechanisms of confounding impacts.

To realise such long-term research strategies on the organisational level, concentrated cooperational efforts in research frameworks are necessary. Current EU-founded projects such as MODELKEY provide examples how frameworks can be conceived that are developed to address the challenge posed by confounding impacts. The impact of time, in particular, was identified to be sufficiently severe to justify its inclusion into such frameworks.

Zusammenfassung

Sedimente können sowohl Senken als auch Quellen für Schadstoffe in der Umwelt sein. Ihre große Bedeutung wird in der Chemikalienrisikobewertung sowie den dazugehörigen gesetzlichen Regularien berücksichtigt, beispielsweise in der 2008 veröffentlichten Tochterrichtlinie zur Europäischen Wasserrahmenrichtlinie. Hieraus ergibt sich die rechtlich verbindliche Notwendigkeit, Sedimentbelastung angemessen zu erfassen, zu bewerten und gegebenenfalls notwendige Maßnahmen zu ergreifen. Um dieser Herausforderung gerecht werden zu können, besteht dringender Forschungsbedarf in Bezug auf Schlüsselfaktoren, die im Zusammenhang mit Sedimentbelastung und der dazugehörigen Bewertung stehen. Ein solcher wichtiger, noch unzureichend erforschter Aspekt sind die Faktoren, welche die Wirkung oder die Bestimmung der Wirkung von Umweltschadstoffen beeinflussen können (confounding factors).

Um die Relevanz ergebnisverändernder Faktoren für die Sedimentbewertung einzuschätzen, wurden in der vorliegenden Dissertation mehrere potentielle Einflussfaktoren auf die Ergebnisse in Biotests untersucht. Der Hauptfokus lag dabei auf der Zeitabhängigkeit der Ergebnisse direkter Sedimentkontakttestsysteme (SKTs). Weitere untersuchte Einflüsse waren Testgefäßmaterialien, Extraktionsmethoden sowie Unterschiede zwischen der Exposition mit einzelnen Schwermetallen und einem Schwermetallgemisch.

Eine Literaturstudie und zwei experimentelle Studien beschäftigten sich mit der Zeitabhängigkeit der Effekte in SKTs. In der experimentellen Hauptstudie wurden die Effekte von 3,4-Dichloranilin (DCA), Fluoranthen (FA) und Pentachlorphenol (PCP) im SKT mit Zebrabärblingsembryonen (*Danio rerio*), im Nematoden-SKT mit *Caenorhabditis elegans* und im Bakterien-SKT mit *Arthrobacter globiformis* untersucht. Die zweite experimentelle Studie ermittelte den Einfluss, den eine Erhöhung der Kontaktzeit zwischen Schadstoffen und Sedimenten um zwei Wochen auf die Bioakkumulation von FA und PCP in der Oligochaetenart *Lumbriculus variegatus* hatte.

Alle drei Studien belegten übereinstimmend, dass der Einflussfaktor Zeit die Ergebnisse direkter Sedimentkontakttests erheblich verändern kann. In der Literatur wurden sowohl ein vollständiger Verlust ursprünglich festgestellter Mortalität innerhalb weniger Wochen als auch statistisch signifikante, zeitbedingte Ergebnisveränderungen belegt. Als Veränderungen wurden abhängig von der jeweils untersuchten Kombination aus Spezies, Substanz, Sediment und zusätzlicher Begleitparameter sowohl zu- als auch abnehmende Effekte beschrieben. Ebenso konnten in beiden experimentellen Studien zeitabhängige Veränderungen der Ergebnisse nachgewiesen werden. Im SKT mit Zebrafischembryonen wurde ein vollständiger Verlust der ursprünglich beobachteten Mortalität innerhalb von zwei bis sechzehn Wochen festgestellt. Im SKT mit Nematoden stieg die Anzahl juveniler Würmer nach Belastung mit FA-dotiertem natürlichem Sediment innerhalb von lediglich sechs Wochen auf Kontrollniveau an. In den anderen drei getesteten Kombinationen aus Sediment und Substanz wurde über sechs Wochen gleichbleibend Toxizität festgestellt. Im Bakterien-SKT blieb die durch PCP bedingte Toxizität über 44 Wochen unverändert. Die Akkumulation von PCP in Regenwürmern aus natürlichem Sediment sank innerhalb von 13 Tagen signifikant und blieb im Fall des mit PCP dotierten künstlichen und der beiden mit FA dotierten Sedimente unverändert.

In einer weiteren Studie wurden vier verschiedene Methoden zur Sedimentextraktion miteinander und mit dem entsprechenden SKT hinsichtlich ihrer biologischen Wirksamkeit auf Zebrabärblingsembryonen verglichen: Soxhletextraktion (SOX), Membrandialyseextraktion (MDE), Hydroxypropyl-β-cyclodextrinextraktion (HPCD) und Tenax TA®-Extraktion (TNX). TNX wurde als mögliche Methode zur Vorhersage der toxikologischen Bioverfügbarkeit für Zebrafischembryonen identifiziert, während SOX, MDE und HPCD diese überschätzten. Für SOX und MDE entsprach dieses Ergebnis den Erwartungen. Im Fall von HPCD könnte die Ursache eine kurze Kontaktzeit zwischen Sedimenten und Schadstoffen vor Beginn der Versuche sein. HPCD wird in der Literatur als Extraktionsmethode zur Abbildung der Bioverfügbarkeit beschrieben, führte jedoch in der vorliegenden Studie zu Effekten, die mindestens so hoch waren wie die der beiden untersuchten erschöpfenden Extraktionsmethoden Soxhlet und MDE. Dieses Ergebnis zeigt, dass sowohl die mit einzelnen Extraktionsmethoden erzielten Resultate als auch Unterschiede zwischen Extraktionsmethoden durch den Faktor Zeit erheblich und signifikant verändert werden können.

Der Vergleich zwischen den Testgefäßmaterialien Glas und Plastik anhand der Wirkung von DCA im Fischembryotest mit dem Zebrabärbling ergab, dass Gefäße aus Glas für diese Substanz besser geeignet sind. Nach einer sechzehnstündigen Kontaktzeit zwischen DCA und Plastik vor Beginn der eigentlichen Exposition konnten im getesteten Konzentrationsbereich lediglich noch minimale Effekte festgestellt und kein LC_{50} bestimmt werden. In Glasgefäßen war nach 16 h Vorexposition die Mortalität nur geringfügig reduziert, und ein LC_{50} konnte bestimmt werden. Die direkte Exposition der Embryonen nach der Einbringung des DCA in die Testgefäße ergab sowohl für Glas als auch für Plastik einen LC_{50} im Erwartungsbereich. Diese Ergebnisse bestätigen, dass die aktuell in der Literatur diskutierten Maßnahmen zur Reduktion derartiger Einflüsse des Testmaterials notwendig sind. Beispiele für derartige Maßnahmen sind semistatische Ansätze, passive Dosierung oder Durchflusssysteme. Weiterhin konnte gezeigt werden, dass der Zeitpunkt der Einbringung von Chemikalien in das Testsystem Ergebnisse entscheidend beeinflussen kann. Dies sollte bei experimentellen Planungen berücksichtigt werden.

Die letzte Studie untersuchte Aufnahme, Akkumulation und dazugehörige Veränderungen der Expressionsmuster ausgewählter Gene in Zebrafischembryonen nach Belastung mit metalldotierten (Cd, Cu, Ni und Zn) Sedimenten. Im Hinblick auf potentiell ergebnisverändernde Faktoren wurden die Unterschiede zwischen der Belastung mit einzelnen Metallen und einem Metallgemisch betrachtet. Die Ergebnisse belegen die hohe Relevanz, die Kupfer als Schadstoff für Fische hat. Weiterhin wurde gezeigt, dass die Exposition gegenüber einem Metallgemisch die Aufnahme und die Bioverfügbarkeit im Vergleich zur Einzelexposition senken kann. In der Literatur konnten Hinweise gefunden werden, dass dieser Einfluss zusätzlich von pH und Zeit abhängig sein kann. Die untersuchten Expressionsmuster reagierten auf beide Expositionsarten nicht unterschiedlich. Dieser Befund könnte darauf hindeuten, dass diese molekularen Biomarker weniger sensitiv gegenüber ergebnisverändernden Einflüssen sind, erfordert jedoch weiterführende Untersuchungen.

Der Vergleich von natürlichen und künstlichen Sedimenten war ein wiederkehrender Aspekt in den meisten der durchgeführten Studien. Der Einfluss ergebnisverändernder Faktoren war jeweils abhängig von der getesteten Kombination aus Spezies und Substanz. Die Ergebnisse zeigten, dass beide Sedimenttypen für die Sedimentbewertung vergleichbar gut geeignet sind. Daher sollte in zukünftigen Studien zur dotierungsbasierten Sedimentbewertung die Möglichkeit einer parallelen Verwendung beider Sedimenttypen in Betracht gezogen werden.

Die Untersuchungen, die im Rahmen der vorliegenden Arbeit durchgeführt wurden, verdeutlichen die Notwendigkeit, die Zeitabhängigkeit als potentiellen Einflussfaktor auf Ergebnisse in der Sedimentbewertung zu berücksichtigen und entsprechende Forschungsstrategien zu entwickeln. Mögliche Unterschiede zwischen Testergebnissen nach einer Belastung mit Einzelsubstanzen oder mit Gemischen wurden als weiterer relevanter Faktor identifiziert. Beide Einflüsse können in erheblichen Unter- oder Überschätzungen des mit sedimentassoziierten Schadstoffen verbundenen Risikopotentials resultieren.

Aus der Relevanz der in der vorliegenden Studie identifizierten potentiell ergebnisverändernden Einflüsse ergibt sich die neue Herausforderung, dass eine Berücksichtigung dieser Faktoren in der Sedimentbewertung eine erhebliche Erhöhung der Anzahl notwendiger Untersuchungen nach sich ziehen würde. Dies steht im Widerspruch zu ökonomischen Überlegungen und Tierschutzbemühungen. Daher wird eine dreistufige Langzeitstrategie vorgeschlagen: a) Grundlagenstudien zu den jeweiligen Einflussfaktoren (analog zu den Studien in der vorliegenden Arbeit), b) Integrative Studien, um Abhängigkeiten und Wechselwirkungen in Bezug auf die Einflussfaktoren auf verschiedenen Organisationsebenen zu charakterisieren und c) Integration der untersuchten Einflussfaktoren in mathematische Modelle. Zusätzlich wurden das neue Konzept der adversen Schadwirkungspfade (adverse outcome pathways) als vielversprechende Strategie identifiziert, um ergebnisbeeinflussende Faktoren zu charakterisieren und zu verstehen.

Derartige langzeitorientierte Forschungsprojekte können nur in konzentrierten Forschungsverbundprojekten realisiert werden. EU-finanzierte Großprojekte, wie etwa das MODELKEY-Verbundprojekt, sind Beispiele für die Konzeption solcher Forschungsverbünde. Der ergebnisverändernde Einfluss insbesondere des Faktors Zeit wurde als hinreichend relevant identifiziert, um seine zukünftige Berücksichtigung in derartigen Verbundprojekten zu rechtfertigen.

**4   Impact of storage time on bioaccumulation of
sediment-bound pollutants in the sediment
contact test with *Lumbriculus variegatus***   **75**

Figures

Tables

Chapter 1

Introduction

1.1   Relevance of contaminated sediments

Pollution of aquatic environments represents one of today's prime challenges for the protection of ecosystems, biodiversity, human health and water as essential resource (Schwarzenbach et al. 2006). According to Fent (2007) and Schwarzenbach et al. (2006), approximately 100,000 chemicals are in use world-wide, 30,000-70,000 on a daily basis. Some 500-1,000 new compounds are added to this number each year (Fent 2007). The ubiquitous presence of anthropogenic chemicals inevitably results in releases into the environment, both constantly and due to accidental spills (Schwarzenbach et al. 2006).

A number of severe accidents and environmental impacts have sharpened public perception and sparked interest in and funding of environmental research in the past 50 years. Infamous examples are the fire in Schweizerhalle with the subsequent release of pesticides into the River Rhine in 1986, the chlorine production plant accident in Seveso in 1976, and the "silent spring", i.e. the impact of DDT on bird egg shell hardness and subsequent reductions in singing bird populations (Giger 2009, Khan & Cutkomp 1982, Lincer 1975, Meharg 1994, Rattner 2009).

Once released, chemicals partition between water, soil, air, and water-borne sediments, and can also become available to biological receptors (Jaffe 1991). As environmental compartment, sediments can be both a sink and a source for pollutants (Ahlf et al. 2002, Eggleton & Thomas 2004, Hilscherova et al. 2010, Wölz et al. 2009). In consequence, contaminations in sediments can considerably impact water quality and the ecological status of water bodies (Burton 1991). The great environmental relevance of contaminated sediments resulting from this dual quality has, however, only recently fully been acknowledged on the regulatory level in Europe (Förstner 2009, Hollert et al. 2009). The European Water Framework Directive (EWFD) demands a good ecological and chemical status of water bodies in terms of anthropogenic contamination (EC 2000). Until implementation of an amendment in 2008, the directive addressed only water bodies, not sediments (EC 2008). In particular, the Member States of the European Union are now obliged to monitor sediment contamination and to ensure that the existing levels of 33 priority pollutants in sediments and biota will not increase above levels given by environmental quality standards defined in the regulation (EC 2008). This integration on the regulatory level results in a legal obligation to assess the impact of sediment-bound chemicals.

1.2   Binding and availability of sediment associated contaminants

After initial contamination, hydrophobic organic pollutants do not stay freely available in sediments and soils (Alexander 2000). With increasing contact time, chemicals tend to bind to the sediment matrix and form bound residues (e.g. Cornelissen et al. 1998a, Menchai et al. 2008, Puglisi et al. 2009, Reid et al. 2000a, Xu et al. 2008). This process is known as sequestration whereas the observable phenomenon is termed ageing (Alexander 2000). Key determinants of sequestration are the organic matrix together with structural micro- and nanopores (Alexander 2000, Cornelissen et al. 1998b, Hatzinger & Alexander 1995, Kukkonen et al. 2003, Nam & Alexander 1998, Nam et al. 1998, Northcott &Jones 2000). Other potentially relevant determinants may be particulate carbon and non-aqueous phase liquids (Luthy et al. 1997). The individual impact of each determinant is controversially discussed and also depends on the particular sediment composition (Huang et al. 2003). Other processes like degradation (Brennan et al. 2009, Lei et al. 2005, You et al. 2009) or re-mobilisation (Van Hoof et al. 2001, Wölz et al. 2008) also determine the time-related fate of substances in sediments.

This time-dependent decrease in chemical availability results in a corresponding decrease in bioavailability (Alexander 2000, Ehlers & Luthy 2003). In brief, bioavailability is the availability of a given substance for absorption or metabolisation by ecological receptors (Brack et al. 2009, ISO 2005; also refer to chapter 2). A recent definition given by Brack et al. (2009) distinguishes between three key processes of bioavailability:

a) Environmental activity or bioaccessibility, which is independent of organisms and refers to the aforementioned desorbing fractions.

b) Environmental bioavailability, which means the uptake of desorbable and dissolved molecules into the organisms, driven by chemical activity.

c) Toxicological bioavailability, which is the internal availability in terms of toxicokinetics within an organism and also includes observable effects.

1.3 Assessment of contaminated sediments

As a result of the difference between chemical availability and bioavailability, chemical concentration alone is not indicative of the adverse impacts of a given pollutant (Chapman 1990). Substances present in sediments often have lesser impacts on organisms than would be expected with regard to their toxic potential (Ehlers & Loibner 2006). This issue is addressed in sediment assessment by means of biotests and chemical methods that are predictive for bioaccessibility (Ahlf et al. 2002, Brack et al. 2009, Feiler et al. 2005).

Biotests give responses in relation to bioavailability on multiple organisational levels such as chemical uptake, molecular reactions, cellular reactions, whole organisms, populations and ecosystems (Ankley et al. 2010). Different exposure scenarios are applied in order to assess sediments in biotests: Pore water, aqueous eluates, organic extracts and whole sediment tests (Ahlf et al. 2002, Burton 1991). Organic extraction and, in particular, direct sediment contact tests, were applied in the present thesis.

1.3.1 Direct sediment contact tests

Tests in which organisms are directly exposed to sediments (Sediment Contact Tests; SCTs) are the primarily addressed exposure scenario in the present thesis. Whole-sediment exposure in SCTs represents the most realistic scenario to simulate *in situ* exposure conditions in the laboratory (Feiler et al. 2005). In SCTs, factors such as species dependence, compound characteristics, sediment properties and environmental impacts are integrated and expressed as biological effects (e.g. Blaha et al. 2010, Conder et al. 2004, Dillon et al. 1994, Duft et al. 2003, Eklund et al. 2010, Feiler et al. 2009, Hallare et al. 2011, Höss et al. 2010, Ingersoll et al. 1995, Kosmehl et al. 2006, Lee et al. 2004, Schmitt et al. 2010, Traunspurger et al. 1997, Turesson et al. 2007, Weber et al. 2006). In addition, SCTs ensure lower alteration of the sample compared to commonly applied strategies for sediment assessment based on extraction, as reviewed by Seiler et al. (2008).

With regard to the three processes of bioavailability discussed by Brack et al (2009), SCTs can either represent environmental bioavailability by means of uptake and bioaccumulation, or toxicological bioavailability by observing or measuring specific effects.

1.3.2   Sediment extraction

Extraction is a second exposure scenario regularly applied in the assessment of sediment contamination (e.g. Arditsoglou & Voutsa 2008, De la Cal et al. 2008b, Hallare et al. 2005, Karlsson et al. 2008, Kosmehl et al. 2007, Qiao et al. 2008, Wölz et al. 2008). Procedures range from vigorous methods for exhaustive extraction to procedures that have been specifically developed to predict bioaccessibility and yield only a limited fraction of pollutants (e.g. Brack et al. 2009, Cornelissen et al. 2001b, Hallare et al. 2011, Luque de Castro & Garcia-Ayuso 1998, Reid et al. 2000b, Seiler et al. 2006, 2008).

Extracts are often further processed by chemical analyses in order to obtain the total extractable concentrations of chemicals by vigorous methodologies or to directly predict bioaccessibility in the case of biomimetic methodologies (Brack 2003, Cornelissen et al. 2001b, Cuypers et al. 2002, Seiler et al. 2008). When applied in biotests as exposure scenario, extracts represent either a worst-case scenario in the case of vigorous extraction methodologies or, in the case of biomimetic extractions, express bioaccessibility on the level of biological effect (Brack et al. 2009, Hallare et al. 2005, Oikari et al. 2002, Schwab & Brack 2007, Wölz et al. 2008).

1.4   Confounding impacts

Effects in biotests with sediments depend on many interdependent factors. Table 1.1 gives an overview on relevant factors without claiming to be exhaustive. Firstly, effects of chemicals in sediments are determined by chemical properties of the substance and the affected organisms. Environmental factors, such as temperature, pH, and dissolved organic matter, further influence effects. Hence, investigations of sediment contamination account for these factors on a regular basis (e.g. Du Laing et al. 2009, Lair et al. 2009, Namiesnik & Rabajczyk 2010), because the co-occurrence of natural stressors can mask the adverse effects of anthropogenic stressors (Liess & von der Ohe 2005, Schäfer et al. 2007).

In addition, factors remain that can confound results observed in biotest, but are not addressed on a regular basis. However, in consequence of the legal obligation to assess the contamination of sediments, all factors that can potentially determine results obtained in tests to assess sediment contamination need to be investigated.

Tab. 1.1 Selection of factors that can determine the effects obtained in biotests with sediments

Sediment parameters / characteristics	Lab-based factors	Substance	Organism	Environmental factors
Geochemical composition[1]	Condition of test sediment (freeze-dried vs. native)[5]	Log KoW[11]	Mode of life (burrowing, sediment-feeding, epibenthic etc.)[14]	Temperature[10]
Organic matter[1]		Solubility[11]		pH[18]
Distribution of particle sizes[1]	Type of test sediment (natural vs. artificial)[2,6]	Volatility[11]	Pathways available for uptake[14]	Oxygen saturation[3]
Porosity (structural micro- and nanopores)[1]	Exposure scenario (whole sediment, eluate, pore water, extract)[7]	Equilibrium/partitioning coefficients (in relation to sediments or organisms)[12]	Behaviour[15]	Hardness[19]
Structure of bacterial community[2]	Material of test vessels[8]	Persistence[11]	Habitat/Biotope[14]	Dissolved organic carbon[20]
Bacterial abundance[2]	Storage time[9]	Concentration[11]	Life cycle[16]	Suspended particulate matter[21]
Oxygen saturation[3]	Storage temperature[10]	Toxicity/Efficacy[11]	Metabolic activity/Potential for detoxification / biotransformation / biodegradation[17]	Environmental events (e.g. flood events)[22]
Content/amount of particulate carbon[4]		Potential to form toxic metabolites[13]		Presence of other compounds (interactions)[23]
Non-aqueous phase liquids[4]			Genetic structure/pool of animal community	Ecosystem[24]
				Sediment-pollutant contact time[9]

[1] Alexander 2000, Northcott &Jones 2000
[2] Goedkoop et al. 2005, Verrhiest et al. 2002
[3] Airas et al. 2008, Madsen et al. 1996
[4] Luthy et al. 1997
[5] refer to chapter 6
[6] also refer to chapters 3, 4, 5, and 7
[7] Ahlf et al. 2002, Burton 1991, Hollert et al. 2009, also refer to chapter 6
[8] Lammer et al. 2009b, refer to chapter 5

[9] e.g. Beiras et al. 1998, Conder et al. 2004, Jones et al. 2008, Lee et al. 2004, Leweke 1999, Sormunen et al. 2009a, also refer to chapter 2-4
[10] Dillon et al. 1994, Leweke 1999, Sormunen et al. 2009a
[11] General substance characteristics
[12] Reichenberg &Mayer 2006, Smith et al. 2010
[13] Puglisi et al. 2009

[14] Moermond et al. 2007a, Puglisi et al. 2009, also refer to chapter 3
[15] Ryder et al. 2004
[16] DeLorenzo &De Leon 2010
[17] Brennan et al. 2009, Puglisi et al. 2009, Semple et al. 2007, Whyte et al. 2000
[18] Di Toro et al. 2005, Simpson & Batley 2006
[19] Brinkman & Hansen 2007, Komjarova & Blust 2009a

[20] Burnison et al. 2006, Haitzer et al. 1998
[21] Keiter et al. 2006, Wölz et al. 2008
[22] Hilscherova et al. 2010, Wölz et al. 2009
[23] Borgmann et al. 2008, Komjarova & Blust 2009b, Rodrigues et al. 2010, also refer to chapter 7
[24] Moermond et al. 2007a

The present thesis combines studies which addressed the impact of such non-target factors in the investigation of sediment-bound pollutants, and how these impacts can change the outcome of tests used to assess the risks of sediment-associated pollutants. In particular, the following aspects are addressed with respect to all investigated impacts:

a) Identify if there is an impact on biotest results by the addressed factor.

b) Evaluate if the respective confounding impact is sufficiently great to be relevant for the assessment of sediment contamination.

c) Identify common patterns in the induced alterations of results that are independent of test systems, for instance, if increasing contact time between a sediment and a given chemical always results in a decrease of effects.

d) Develop recommendations on future strategies to address the given impact.

In detail, the present thesis aims to assess the potential of five impacts to relevantly change the results of biotests (Fig. 1.1):

a) Contact time between sediments and pollutants (Chapters 2-4)

b) Material of test vessels (Chapter 5)

c) Methodology of extraction (Chapter 6)

d) Exposure with mixtures compared to single substances (Chapter 7)

e) Type of sediment (Chapters 3, 4, 6 and 7)
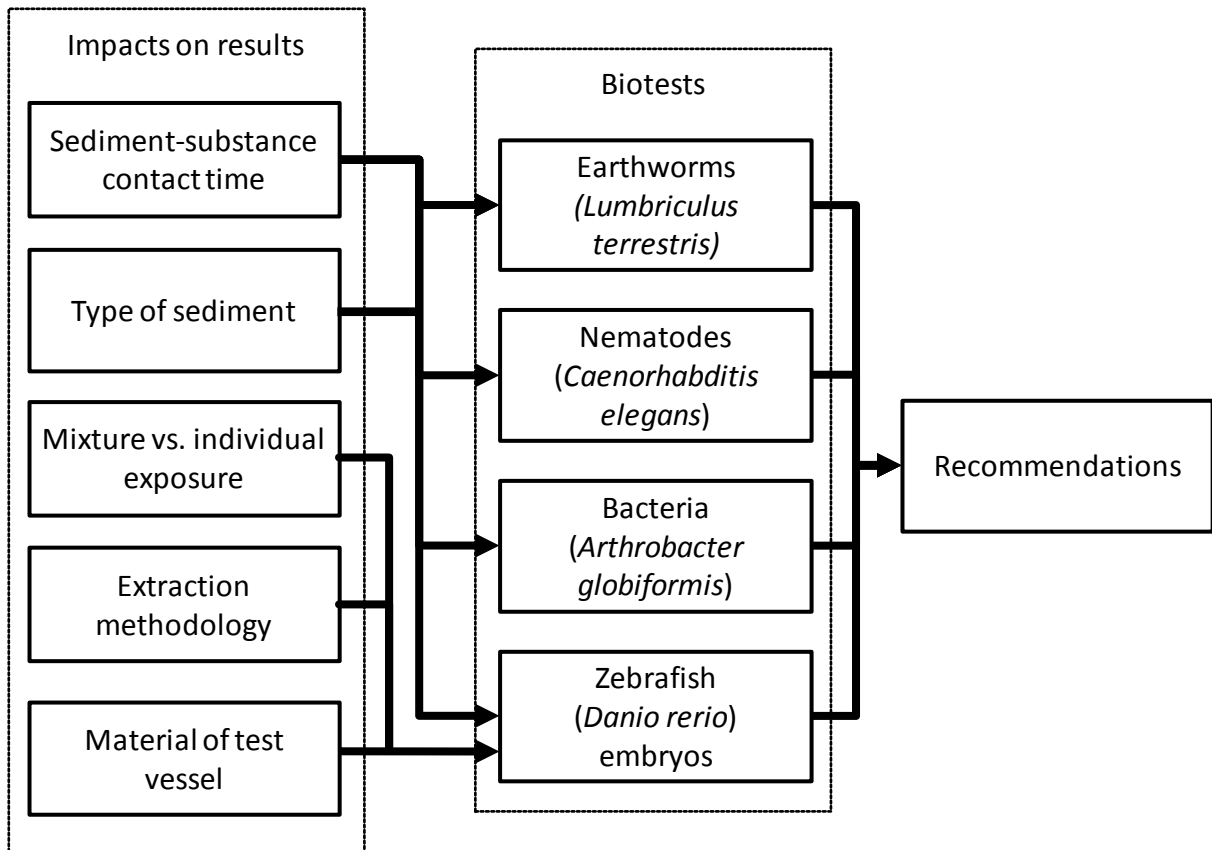
Fig. 1.1 Potential impacts on results in biotest with sediments addressed within the present thesis.

1.4.1 Impact of sediment-pollutant contact time

Temporal impacts were the main focus of the present thesis. Chemicals can time-dependently bind to the sediment matrix, and in consequence, chemical and biological availability often decreases with time (Alexander 2000, Cornelissen et al. 1998b, Hatzinger & Alexander 1995, Kukkonen et al. 2003, Nam & Alexander 1998, Nam et al. 1998, Northcott &Jones 2000). Similarly, abiotic factors vary greatly in time (Solimini et al. 2009). Literature addresses the time-dependence of uptake and bioaccumulation in SCTs to some extent (e.g. Chai et al. 2008, Conrad et al. 2002, Leppänen & Kukkonen 2000, Morrison et al. 2000, Saghir et al. 2007, Sormunen et al. 2009a, Zhong & Wang 2006). However, time-dependence of observable effects in SCTs has received insufficient coverage, and it is unknown how exactly the impact of time translates into biological effects obtained in biotests.

To highlight the importance of temporal effects, Chapter 2 provides an overview of the time-dependence of results obtained in direct sediment contact tests by conducting a comprehensive survey of the international literature.

Chapter 3 gives the results of an effect-focused pilot study which assessed the time-dependence of biological effects in the three direct sediment contact tests with zebrafish (*Danio rerio*) embryos, the soil bacterium *Arthrobacter globiformis* and the nematode *Caenorhabditis elegans*. These Test systems were chosen to represent different modes of life and access to sediment-bound chemicals.

The zebrafish embryo sediment contact test was introduced by Hollert et al. (2003) as an addition to the original aqueous fish embryo test protocol described by Nagel et al. (2002) and updated by Braunbeck et al. (2005) and Lammer et al. (2009a). The aqueous version of the test is mandatory in waste water effluent testing in Germany (DIN 2001) and is internationally standardised (ISO 2007). The zebrafish is a well-established model organism and its development has been described in detail (Kimmel et al. 1995). This makes the test system an excellent tool to assess the impact of pollutants on the embryogenesis of an aquatic vertebrate. The BCT accounted for the impact on sediment-associated bacteria. *Arthrobacter* sp. dominates the group of aerobic, chemoheterotrophic soil- and sediment-living bacteria. The assay allows assessment of toxicity *in situ*, without need for separation of the bacteria from the solid material (Ahlf 2007, DIN 2002, Neumann-Hensel & Melbye 2006, Rönnpagel et al. 1995, Standardisation 2009).

The sediment-dwelling bacterivorous nematode *Caenorhabditis elegans* represented organisms that are in external contact with the tested sediment and also ingest, potentially extract and bioaccumulate particle-bound pollutants (Höss et al. 2009). The test has been successfully used for investigating freshwater sediments (Comber et al. 2006, Comber et al. 2008, Höss et al. 2009, Traunspurger et al. 1997). A standardised test guideline is available (ISO 2010).

All three test systems also are components of a comprehensive SCT battery established by the recent German joint research framework project SeKT (Sediment Kontakt Test; Feiler et al. 2009, Feiler et al. 2005, Höss et al. 2010).

Three chemicals representing different classes of substances were tested for their response to temporal impact in the three applied tests: Fluoranthene (FA) is a polyaromatic hydrocarbon and used as reference substance in ageing and sediment sorption studies (e.g. Hawthorne et al. 2002, Moermond et al. 2007a, Tang & Alexander 1999, Van Noort et al. 2003). Pentachlorophenol (PCP) is a bioaccumulative halogenated aromatic hydrocarbon (Hanna et

al. 2004, Maenpaa et al. 2008, Puglisi et al. 2009). The halogenated aniline 3,4-dichloroanilin (DCA) is applied as the positive control in the fish embryo test (DIN 2001, Nagel 2002). FA and PCP are listed as priority pollutants in Annex II of the amended European Water Framework Directive (EC 2008).

The study presented in chapter 4 also addressed the impact of sediment-pollutant contact time, but the focus was shifted from directly observable effects to bioaccumulation in the earthworm species *Lumbriculus variegatus*, which is a common test species utilised in investigations on bioaccumulation (Conrad et al. 2002, Leppänen & Kukkonen 2000, Maenpaa et al. 2008, Sijm et al. 2000, Sormunen et al. 2009a, Van der Heijden & Jonker 2009, Van Hoof et al. 2001, You et al. 2009, You et al. 2006). In this study, the influence of time on bioaccumulation was again investigated using FA- and PCP-spiked sediments.

1.4.2 Impact of test vessel material

Range-finding tests conducted prior to the experiments on time-dependence indicated a need to also investigate the potential impact of the plastic material of the applied test vessels. Previous studies indicated that organic chemicals can potentially adsorb into plastic materials (Dahlstrom et al. 2004, Koutsopoulos et al. 2007, Palmgren et al. 2006). Chapter 5 assessed the applicability of plastic vessels in the assessment of organic substances on the basis of the effects of 3,4-dichloroanilin in the fish embryo test with zebrafish (*Danio* rerio). In order to focus on the potential impact of vessel material, rather than sediment-vessel interactions, this was the only study within the present thesis that investigated aqueous solutions rather than sediments or sediment extracts.

1.4.3 Impact of methodology

Chapter 6 gives a study that was carried out to assess the impact resulting from differences between the exposure scenarios of extraction and direct sediment contact. Results from a sediment contact test protocol and four extraction methodologies were intercompared on the level of observable biological effects. Most extraction methods have so far been characterised and compared in relation to chemically determined uptake and accumulation of pollutants (e.g. Cornelissen et al. 2001a, De la Cal et al. 2008a, Moermond et al. 2007b, Sormunen et al.

2009b, Swindell & Reid 2006, Ten Hulscher et al. 2003a), but not with regard to biological effects.

Membrane dialysis extraction (MDE), a Soxhlet extraction procedure (SOX), hydroxypropyl-β-cyclodextrin extraction (HPCD) and Tenax®-TA extraction (TNX) were assessed using mortality in the fish embryo test (FET) with zebrafish (*Danio rerio*). SOX is a widely known and commonly applied technique (Bjorklund et al. 2002, Luo et al. 2009, Luque de Castro & Garcia-Ayuso 1998, Wölz et al. 2008). Several protocols for SOX have been shown to yield extracts containing all leachable contaminant fractions at high recovery rates (Luque de Castro & Garcia-Ayuso 1998, Seiler et al. 2008). MDE is a passive leaching procedure that provides exhaustive extracts regarding non-covalently bound contaminants without using any auxiliary energy sources and, thus, effectively reduces the risk of loss of volatile or thermally labile substances (Huckins et al. 1990, Macrae &Hall 1998, Seiler et al. 2006). Hydroxyl-propyl-β-cyclodextrin extraction (HPCD) and Tenax®-TA extraction (TNX) are both techniques reported to provide extracts which represent the bioaccessible fractions of pollutants (Cornelissen et al. 2001b, De la Cal et al. 2008b, Reid et al. 2000b, Schwab & Brack 2007, Ten Hulscher et al. 2003b, Van der Heijden & Jonker 2009).

Sediments spiked with a mixture of six organic substances were investigated. The mixture contained 2,4 dinitrophenol, diuron, fluoranthene, nonylphenol, parathion and pentachlorophenol. Samples originated from the framework project SeKT (Feiler et al. 2009, Feiler et al. 2005, Höss et al. 2010), allowing for a comparison to direct sediment contact test data.

1.4.4 Impact of exposure to mixtures or single substances

Effects of pollutants can greatly differ depending on whether the test organisms are exposed to one single substance or multiple stressors (Hecky et al. 2010, Mothersill & Seymour 2010, Pelletier et al. 2006, Sundback et al. 2010). Because metals are an ubiquitous and persistent class of pollutants (Boyd 2010, Dell'Anno et al. 2003), they can often occur in mixtures in the environment (Borgmann et al. 2008, Komjarova & Blust 2009b, Rodrigues et al. 2010). In order to address the potential impact of exposure to single substances or mixtures on results obtained in SCTs, a study focusing on metals was conducted with Cd, Cu, Ni and Zn-spiked sediments (Chapter 7). This study compared reactions of zebrafish embryos to single- or

multiple-metal exposure in terms of uptake, bioaccumulation, and abundance of metal-related transcripts.

Abundances of metal-related transcripts were investigated in order to identify impacts of the different exposures on the mechanistic level (Menzel et al. 2009). Abundances of mRNA for metallothioneins (MT1 and MT2) were used as indication of the magnitude of exposure to metals (Chen et al. 2007, Chen et al. 2004, Craig et al. 2009a, Craig et al. 2009b, Gonzalez et al. 2006, Roesijadi 1992). Expression levels of superoxide dismutase (sod1) and heat shock proteins (hsp70 and hsp90α1) were used as indicators of cellular stress (Blechinger et al. 2002, Gonzalez et al. 2006, Krone et al. 2003, Pierron et al. 2009). CYP1A was used as an indicator of exposure to dioxin-like chemicals that could modulate responses through the aromatic hydrocarbon receptor (AhR) and expression of GST was used as an indicator of biotransformation activity (Brack & Schirmer 2003, Hollert et al. 2002, Oikari et al. 2002, Whyte et al. 2000).

1.4.5   Choice of sediment type

The application of natural (field-sampled) and artificial (formulated) sediment was compared in chapters 3, 4, 6 and 7. Both sediment types can be used to assess spiked sediments, as long as the necessary controls are carried out, and carry a number of advantages and disadvantages linked to inherently different properties, such as the composition of organic matter, bacterial abundance and bacterial diversity (Fleming et al. 1998, Goedkoop et al. 2005). In order to contribute to the controversial discussion of which sediment type should be preferred, both types were applied in all studies in the present thesis that investigated sediment.

1.5   Animal usage

The aspect of animal usage is addressed because animals were utilised in several of the given studies. The European regulation on the Registration, Evaluation and Authorisation of Chemicals (REACh) explicitly calls for implementation and promotion of alternative methods to minimise animal tests (EC 2006). However, usage of test animals will drastically increase, to an estimate of about 141 million test animals, as result of the regulation (Hartung & Rovida

2009). This number is based on the 140.000 substances that have been pre-registered within the first phase of REACh, contradicting the original EU-estimates of 30.000 substances. Economically, costs of animal tests would add up to a total of approximately 9.5 billion € (Hartung & Rovida 2009). In contrast, recent annual animal usage in the EU is approximately 90,000 animals and 60 million €. These numbers clearly underline the need to develop alternative methods in accordance with the so-called "three R's" (Refinement, Replacement, Reduction; Russell & Burch 1959), in addition to economic considerations.

The studies given in chapters 3, 5, 6 and 7 all applied the fish embryo test (FET) with zebrafish (*Danio rerio*) or the respective FET protocol for whole-sediment exposure (Hollert et al. 2003). The FET has been explicitly developed as an alternative to acute toxicity tests with adult fish (Braunbeck et al. 2005, Nagel 2002). It is an alternative method because adult fish are replaced by early life stages that lack fully developed nervous systems. The 48 h standard protocol of the FET is not subjected to animal welfare legislation because legal status is given only to either hatched or self-feeding fish. In Germany, the FET has mandatorily replaced the acute fish test with the golden ide (*Leuciscus idus melanotus*) in wastewater testing and the corresponding regulations (DIN 2001, German Federal Ministry of Justice 2005). The FET is also internationally standardised and, thus, applicable in the assessment of chemicals (ISO 2007).

The given studies utilising the FET were conducted to optimise interpretation of results obtained in the test. A better understanding of external influences that can potentially alter results in the FET, such as time-dependence or materials of the utilised test vessels, can help increase acceptance and promote application of the FET and, thus, the replacement of tests with adult fish. In addition to optimisation of the FET, long-term assessment strategies need to be evaluated in context of animal welfare. Hence, the proposed strategies to address confounding impacts given in the final discussion are also evaluated regarding a long-term reduction of test animals.

1.6   References

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 Soil Sediment 2, 37-42

Ahlf W (2007): 'Optimisation of the solid-contact test with Arthrobacter globiformis. J Soil Sediment 7, 67-67

Airas S, Leppanen M, Kukkonen JVK (2008): Effects of temperature and oxygen concentration in sediment toxicity testing. Ecotoxicology and Environmental Safety 70, 475-482

Alexander M (2000): Aging, bioavailability, and overestimation of risk from environmental pollutants. Environ Sci Technol 34, 4259-4265

Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung MW, Johnson RD, Mount DR, Nichols JW, Russom CL, Schmieder PK, Serrrano JA, Tietge JE, Villeneuve DL (2010): Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environ Toxicol Chem 29, 730-741

Arditsoglou A, Voutsa D (2008): Determination of phenolic and steroid endocrine disrupting compounds in environmental matrices. Environ Sci Pollut R 15, 228-236

Beiras R, His E, Seaman MNL (1998): Effects of storage temperature and duration on toxicity of sediments assessed by Crassostrea gigas oyster embryo bioassay. Environ Toxicol Chem 17, 2100-2105

Bjorklund E, Holst C, Anklam E (2002): Fast extraction, clean-up and detection methods for the rapid analysis and screening of seven indicator PCBs in food matrices. TRAC-Trend Anal Chem 21, 39-52

Blaha L, Hilscherova K, Cap T, Klanova J, Machat J, Zeman J, Holoubek I (2010): Kinetic bacterial bioluminescence assay for contact sediment toxicity testing: relationships with the matrix composition and contamination. Environ Toxicol Chem 29, 507-514

Blechinger SR, Warren JT, Kuwada JY, Krone PH (2002): Developmental toxicology of cadmium in living embryos of a stable transgenic zebrafish line. Environ Health Persp 110, 1041-1046

Borgmann U, Norwood WP, Dixon DG (2008): Modelling bioaccumulation and toxicity of metal mixtures. Hum Ecol Risk Assess 14, 266-289

Boyd RS (2010): Heavy Metal Pollutants and Chemical Ecology: Exploring New Frontiers. J Chem Ecol 36, 46-58

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 (2003): Effect-directed identification of oxygen and sulfur heterocycles as major polycyclic aromatic cytochrome P4501A-Inducers in a contaminated sediment. Environ Sci Technol 37, 3062-3070

Brack W, Bandow N, Schwab K, Schulze T, Streck G (2009): Bioavailability in effect-directed analysis of organic toxicants in sediments. TRAC-Trend Anal Chem 28, 543-549

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

Brennan AA, Harwood AD, You J, Landrum PF, Lydy MJ (2009): Degradation of fipronil in anaerobic sediments and the effect on porewater concentrations. Chemosphere 77, 22-28

Brinkman SF, Hansen DL (2007): Toxicity of cadmium to early life stages of brown trout (Salmo trutta) at multiple water hardnesses. Environ Toxicol Chem 26, 1666-1671

Burnison BK, Meinelt T, Playle R, Pietrock M, Wienke A, Steinberg CEW (2006): Cadmium accumulation in zebrafish (Danio rerio) eggs is modulated by dissolved organic matter (DOM). Aquat Toxicol 79, 185-191

Burton GA (1991): Assessing the toxicity of fresh-water sediments. Environ Toxicol Chem 10, 1585-1627

Chai YZ, Davis JW, Saghir SA, Qiu XJ, Budinsky RA, Bartels MJ (2008): Effects of aging and sediment composition on hexachlorobenzene desorption resistance compared to oral bioavailability in rats. Chemosphere 72, 432-441

Chapman PM (1990): The sediment quality triad approach to determining pollution-induced degradation. Sci Total Environ 97-8, 815-825

Chen WY, John JAC, Lin CH, Lin HF, Wu SC, Chang CY (2004): Expression of metallothionein gene during embryonic and early larval development in zebrafish. Aquat Toxicol 69, 215-227

Chen WY, John JAC, Lin CH, Chang CY (2007): Expression pattern of metallothionein, MTF-1 nuclear translocation, and its DNA-binding activity in zebrafish (Danio rerio) induced by zinc and cadmium. Environ Toxicol Chem 26, 110-117

Comber SDW, Conrad AU, Hoss S, Webb S, Marshall S (2006): Chronic toxicity of sediment-associated linear alkylbenzene sulphonates (LAS) to freshwater benthic organisms. Environ. Pollut. 144, 661-668

Comber SDW, Rule KL, Conrad AU, Hoss S, Webb SF, Marshall S (2008): Bioaccumulation and toxicity of a cationic surfactant (DODMAC) in sediment dwelling freshwater invertebrates. Environ. Pollut. 153, 184-191

Conder JM, La Point TW, Steevens JA, Lotufo GR (2004): Recommendations for the assessment of TNT toxicity in sediment. Environ Toxicol Chem 23, 141-149

Conrad AU, Comber SD, Simkiss K (2002): Pyrene bioavailability; effect of sediment-chemical contact time on routes of uptake in an oligochaete worm. Chemosphere 49, 447-454

Cornelissen G, Rigterink H, Ferdinandy MMA, Van Noort PCM (1998a): Rapidly desorbing fractions of PAHs in contaminated sediments as a predictor of the extent of bioremediation. Environ Sci Technol 32, 966-970

Cornelissen G, Van Noort PCM, Covers HAJ (1998b): 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 (2001a): A simple tenax® extraction method to determine the availability of sediment-sorbed organic compounds. Environmental Toxicology and Chemistry 20, 706-711

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

Craig PM, Galus M, Wood CM, McClelland GB (2009a): Dietary iron alters waterborne copper-induced gene expression in soft water acclimated zebrafish (Danio rerio). Am J Physiol-Reg I 296, R362-R373

Craig PM, Hogstrand C, Wood CM, McClelland GB (2009b): Gene expression endpoints following chronic waterborne copper exposure in a genomic model organism, the zebrafish, Danio rerio. Physiol Genomics 40, 23-33

Cuypers C, Pancras T, Grotenhuis T, Rulkens W (2002): The estimation of PAH bioavailability in contaminated sediments using hydroxypropyl-ß-cyclodextrin and Triton X-100 extraction techniques. Chemosphere 46, 1235-1245

Dahlstrom M, Jonsson PR, Lausmaa J, Arnebrant T, Sjogren M, Holmberg K, Martensson LGE, Elwing H (2004): Impact of polymer surface affinity of novel antifouling agents. Biotechnol Bioeng 86, 1-8

De la Cal A, Eljarrat E, Grotenhuis T, Barcelo D (2008a): Tenax (R) extraction as a tool to evaluate the availability of polybrominated diphenyl ethers, DDT, and DDT metabolites in sediments. Environmental Toxicology and Chemistry 27, 1250-1256

De la Cal A, Eljarrat E, Grotenhuis T, Barcelo D (2008b): Tenax (R) extraction as a tool to evaluate the availability of polybrominated diphenyl ethers, DDT, and DDT metabolites in sediments. Environ Toxicol Chem 27, 1250-1256

Dell'Anno A, Mei ML, Ianni C, Danovaro R (2003): Impact of bioavailable heavy metals on bacterial activities in coastal marine sediments. World J Microb Biot 19, 93-100

DeLorenzo ME, De Leon RG (2010): Toxicity of the Insecticide Etofenprox to Three Life Stages of the Grass Shrimp, Palaemonetes pugio. Archives of Environmental Contamination and Toxicology 58, 985-990

Di Toro D, McGrath J, Hansen D, Berry W, Paquin P, Mathew R, Wu KB, Sanatore RC (2005): Predicting sediment toxicity using a sediment Biotic Ligand Model: Methodology and initial application. Environ Toxicol Chem 24, 2410-2427

Dillon TM, Moore DW, Jarvis AS (1994): The effects of storage-temperature and time on sediment toxicity. Arch Environ Con Tox 27, 51-53

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 (2002): DIN 38412-48: Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlamm-untersuchung: Testverfahren mit Wasserorganismen (Gruppe L) Teil 48: *Arthrobacter globiformis* Kontakttest für kontaminierte Feststoffe. Deutsches Institut für Normung e. V., Berlin

Du Laing G, Rinklebe J, Vandecasteele B, Meers E, Tack FMG (2009): Trace metal behaviour in estuarine and riverine floodplain soils and sediments: A review. Sci Total Environ 407, 3972-3985

Duft M, Schulte-Oehlmann U, Tillmann M, Markert B, Oehlmann J (2003): Toxicity of triphenyltin and tributyltin to the freshwater mudsnail Potamopyrgus antipodarum in a new sediment biotest. Environ Toxicol Chem 22, 145-152

EC (2000) Directive 2000/60/EC of the european parliament and of the councilof 23 October 2000establishing a framework for Community action in the field of water policy. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2000:327:0001:0072:EN:PDF, last accessed 21.03.2011

EC (2006) Regulation No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32006R1907:EN:PDF, last accessed 09.03.2011

EC (2008) Directive 2008/105/EC of the european parliament and of the council of 16 December 2008 on environmental quality standards in the field of water policy,

amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:348:0084:0097:EN:PDF, last accessed 09.03.2011

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

Ehlers GAC, Loibner AP (2006): Linking organic pollutant (bio)availability with geosorbent properties and biomimetic methodology: A review of geosorbent characterisation. and (bio)availability prediction. Environ. Pollut. 141, 494-512

Ehlers LJ, Luthy RG (2003): Contaminant bioavailability in soil and sediment. Environ Sci Technol 37, 295A-302A

Eklund B, Elfstrom M, Gallego I, Bengtsson B-E, Breitholtz M (2010): Biological and chemical characterization of harbour sediments from the Stockholm area. J Soil Sediment 10, 127-141

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 R 12, 257-258

Feiler U, Ahlf W, Fahnenstich C, Gilberg D, Hammers-Wirtz M, Höss S, Hollert H, Melbye K, Meller M, Neumann-Hensel H, Ratte HT, Seiler TB, Spira D, Weber J, Heininger P 2009: Final report SeKT framework project - Definition von Referenzbedingungen, Kontrollsedimenten und Toxizitätsschwellenwerten für limnische Sedimentkontakttests - SeKT

Fent K (2007): Ökotoxikologie, 3. Georg Thieme Verlag, Stuttgart

Fleming RJ, Holmes D, Nixon SJ (1998): Toxicity of permethrin to Chironomus riparius in artificial and natural sediments. Environ Toxicol Chem 17, 1332-1337

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

German Federal Ministry of Justice (2005): Gesetz über Abgaben für das Einleiten von Abwasser in Gewässer (Abwasserabgabengesetz - AbwAG)

Giger W (2009): The Rhine red, the fish dead-the 1986 Schweizerhalle disaster, a retrospect and long-term impact assessment. Environ Sci Pollut R 16, 98-111

Goedkoop W, Widenfalk A, Haglund AL, Steger K, Bertilsson S (2005): Microbial characterization of artificial sediment and comparisons with natural sediments - Implications for toxicity testing. Environ Toxicol Chem 24, 2725-2733

Gonzalez P, Baudrimont M, Boudou A, Bourdineaud JP (2006): Comparative effects of direct cadmium contamination on gene expression in gills, liver, skeletal muscles and brain of the zebrafish (Danio rerio). Biometals 19, 225-235

Haitzer M, Hoss S, Traunspurger W, Steinberg C (1998): Effects of dissolved organic matter (DOM) on the bioconcentration of organic chemicals in aquatic organisms - A review. Chemosphere 37, 1335-1362

Hallare AV, Kosmehl T, Schulze T, Hollert H, Kohler HR, Triebskorn R (2005): Assessing contamination levels of Laguna Lake sediments (Philippines) using a contact assay with zebrafish (Danio rerio) embryos. Sci Total Environ 347, 254-271

Hallare AV, Seiler TB, Hollert H (2011): The versatile, changing, and advancing roles of fish in sediment toxicity assessment-a review. J Soil Sediment 11, 141-173

Hanna K, de Brauer C, Germain P (2004): Cyclodextrin-enhanced solubilization of pentachlorophenol in water. Journal of Environmental Management 71, 1-8

Hartung T, Rovida C (2009): Chemical regulators have overreached. Nature 460, 1080-1081

Hatzinger PB, Alexander M (1995): Effect of aging of chemicals in soil on their biodegradability and extractability. Environ Sci Technol 29, 537-545

Hawthorne SB, Poppendieck DG, Grabanski CB, Loehr RC (2002): Comparing PAH availability from manufactured gas plant soils and sediments with chemical and biological tests. 1. PAH release during water desorption and supercritical carbon dioxide extraction. Environ Sci Technol 36, 4795-4803

Hecky RE, Mugidde R, Ramlal PS, Talbot MR, Kling GW (2010): Multiple stressors cause rapid ecosystem change in Lake Victoria. Freshwater Biol 55, 19-42

Hilscherova K, Dusek L, Sidlova T, Jalova V, Cupr P, Giesy JP, Nehyba S, Jarkovsky J, Klanova J, Holoubek I (2010): Seasonally and regionally determined indication potential of bioassays in contaminated river sediments. Environ Toxicol Chem 29, 522-534

Hollert H, Duerr 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. Ecotoxicology 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, Ernst M, Ahlf W, Duerr M, Erdinger L, Grund S, Keiter S, Kosmehl T, Seiler T-B, Woelz J, Braunbeck T (2009): Strategies for assessing sediment toxicity - a review. Umweltwiss Schads Forsch 21, 160-176

Höss S, Jansch S, Moser T, Junker T, Rombke J (2009): Assessing the toxicity of contaminated soils using the nematode Caenorhabditis elegans as test organism. Ecotox Environ Safe 72, 1811-1818

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 WL, Ping PA, Yu ZQ, Fu HM (2003): Effects of organic matter heterogeneity on sorption and desorption of organic contaminants by soils and sediments. Applied Geochemistry 18, 955-972

Huckins JN, Tubergen MW, Lebo JA, Gale RW, Schwartz TR (1990): Polymeric film dialysis in organic-solvent media for cleanup of organic contaminants. Journal of the Association of Official Analytical Chemists 73, 290-293

Ingersoll CG, Ankley GT, Benoit DA, Brunson EL, Burton GA, Dwyer FJ, Hoke RA, Landrum PF, Norbergking TJ, Winger PV (1995): Toxicity and bioaccumulation of sediment-associated contaminants using fresh-water invertebrates - a review of methods and applications. Environ Toxicol Chem 14, 1885-1894

ISO (2005): ISO 11074: Soil quality -- Vocabulary. Iso Guideline

ISO (2007): ISO 15088: Water quality — Determination of the acute toxicity of waste water to zebrafish eggs (*Danio rerio*). Iso Guideline

ISO (2010): ISO 10872: Water quality - Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of *Caenorhabditis elegans* (Nematoda). Iso Guideline

Jaffe R (1991): Fate of hydrophobic organic pollutants in the aquatic environment - a review. Environmental Pollution 69, 237-257

Jones RP, Hassan SM, Rodgers JH (2008): Influence of contact duration on sediment-associated copper fractionation and bioavailability. Ecotox Environ Safe 71, 104-116

Karlsson J, Sundberg H, Akerman G, Grunder K, Eklund B, Breitholtz M (2008): Hazard identification of contaminated sites - ranking potential toxicity of organic sediment extracts in crustacean and fish. J Soil Sediment 8, 263-274

Keiter S, Rastall A, Kosmehl T, Wurm K, Erdinger L, Braunbeck T, Hollert H (2006): Ecotoxicological assessment of sediment, suspended matter and water samples in the upper Danube River - A pilot study in search for the causes for the decline of fish catches. Environ Sci Pollut R 13, 308-319

Khan HM, Cutkomp LK (1982): In vitro studies of DDT, DDE and ATPase as related to avian eggshell thinning. Arch Environ Con Tox 11, 627-633

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995): Stages of embryonic development of the zebrafish. Developmental Dynamics 203, 253-310

Komjarova I, Blust R (2009a): Effects of Na, Ca, and pH on the Simultaneous Uptake of Cd, Cu, Ni, Pb, and Zn in the Zebrafish Danio rerio: A Stable Isotope Experiment. Environ Sci Technol 43, 7958-7963

Komjarova I, Blust R (2009b): Multimetal Interactions between Cd, Cull, Ni, Pb, and Zn Uptake from Water in the Zebrafish Danio rerio. Environ Sci Technol 43, 7225-7229

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

Kosmehl T, Krebs F, Manz W, Braunbeck T, Hollert H (2007): Differentiation between bioavailable and total hazard potential of sediment-induced DNA fragmentation as measured by the comet assay with zebrafish embryos. J Soil Sediment 7, 377-387

Koutsopoulos S, Patzsch K, Bosker WTE, Norde W (2007): Adsorption of trypsin on hydrophilic and hydrophobic surfaces. Langmuir 23, 2000-2006

Krone PH, Evans TG, Blechinger SR (2003): Heat shock gene expression and function during zebrafish embryogenesis. Semin Cell Dev Biol 14, 267-274

Kukkonen JVK, Landrum PF, Mitra S, Gossiaux DC, Gunnarsson J, Weston D (2003): Sediment characteristics affecting desorption kinetics of select PAH and PCB congeners for seven laboratory spiked sediments. Environ Sci Technol 37, 4656-4663

Lair GJ, Zehetner F, Fiebig M, Gerzabek MH, van Gestel CAM, Hein T, Hohensinner S, Hsu P, Jones KC, Jordan G, Koelmans AA, Poot A, Slijkerman DME, Totsche KU, Bondar-Kunze E, Barth JAC (2009): How do long-term development and periodical changes of river-floodplain systems affect the fate of contaminants? Results from European rivers. Environ. Pollut. 157, 3336-3346

Lammer E, Carr GJ, Wendler K, Rawlings JM, Belanger SE, Braunbeck T (2009a): Is the fish embryo toxicity test (FET) with the zebrafish (Danio rerio) a potential alternative for the fish acute toxicity test? Comp Biochem Phys C 149, 196-209

Lammer E, Kamp HG, Hisgen V, Koch M, Reinhard D, Salinas ER, Wendler K, Zok S, Braunbeck T (2009b): Development of a flow-through system for the fish embryo toxicity test (FET) with the zebrafish (Danio rerio). Toxicol in Vitro 23, 1436-1442

Lee JS, Lee BG, Luoma SN, Yoo H (2004): Importance of equilibration time in the partitioning and toxicity of zinc in spiked sediment bioassays. Environ Toxicol Chem 23, 65-71

Lei L, Khodadoust AP, Suidan MT, Tabak HH (2005): Biodegradation of sediment-bound PAHs in field contaminated sediment. Water Res 39, 349-361

Leppänen MT, Kukkonen JVK (2000): Effect of sediment-chemical contact time on availability of sediment-associated pyrene and benzo[a]pyrene to oligochaete worms and semi-permeable membrane devices. Aquat Toxicol 49, 227-241

Leweke EC (1999): Einfluss verschiedener Lagerungsbedingungen auf die ökotoxikologische Wirkung von Kunst- und Süßwassersedimenten - Untersuchungen mit *Daphnia*

magna, *Enchytraeus albidus*, *Chironomus riparius* und *Tubifex tubifex*. Cuvillier Verlag Göttingen, 194 pp

Liess M, von der Ohe PC (2005): Analyzing effects of pesticides on invertebrate communities in streams. Environ Toxicol Chem 24, 954-965

Lincer JL (1975): DDE-induced eggshell-thinning in american kestrel - comparison of field situation and laboratory results. Journal of Applied Ecology 12, 781-793

Luo JP, Ma M, Liu C, Zha JM, Wang ZJ (2009): Impacts of particulate organic carbon and dissolved organic carbon on removal of polycyclic aromatic hydrocarbons, organochlorine pesticides, and nonylphenols in a wetland. J Soil Sediment 9, 180-187

Luque de Castro MD, Garcia-Ayuso LE (1998): Soxhlet extraction of solid materials: an outdated technique with a promising innovative future. Analytica Chimica Acta 369, 1-10

Luthy RG, Aiken GR, Brusseau ML, Cunningham SD, Gschwend PM, Pignatello JJ, Reinhard M, Traina SJ, Weber WJ, Westall JC (1997): Sequestration of hydrophobic organic contaminants by geosorbents. Environ Sci Technol 31, 3341-3347

Macrae JD, Hall KJ (1998): Comparison of methods used to determine the availability of polycyclic aromatic hydrocarbons in marine sediment. Environ Sci Technol 32, 3809-3815

Madsen EL, Mann CL, Bilotta SE (1996): Oxygen limitations and aging as explanations for the field persistence of naphthalene in coal tar-contaminated surface sediments. Environ Toxicol Chem 15, 1876-1882

Maenpaa K, Sorsa K, Lyytikainen M, Leppanen MT, Kukkonen J (2008): Bioaccumulation, sublethal toxicity, and biotransformation of sediment-associated pentachlorophenol in Lumbriculus variegatus (Oligochaeta). Ecotox Environ Safe 69, 121-129

Meharg AA (1994): Ecological impact of major industrial-chemical accidents, Reviews of Environmental Contamination and Toxicology, Vol 138. Reviews of Environmental Contamination and Toxicology, pp. 21-48

Menchai P, Van Zwieten L, Kimber S, Ahmad N, Rao PSC, Hose G (2008): Bioavailable DDT residues in sediments: Laboratory assessment of ageing effects using semi-permeable membrane devices. Environ. Pollut. 153, 110-118

Menzel R, Swain SC, Hoess S, Claus E, Menzel S, Steinberg CEW, Reifferscheid G, Sturzenbaum SR (2009): Gene expression profiling to characterize sediment toxicity - a pilot study using Caenorhabditis elegans whole genome microarrays. Bmc Genomics 10

Moermond CTA, Roessink I, Jonker MTO, Meijer T, Koelmans AA (2007a): Impact of polychlorinated biphenyl and polycyclic aromatic hydrocarbon sequestration in sediment on bioaccumulation in aquatic food webs. Environ Toxicol Chem 26, 607-615

Moermond CTA, Roessink I, Jonker MTO, Meijer T, Koelmans AA (2007b): Impact of polychlorinated biphenyl and polycyclic aromatic hydrocarbon sequestration in sediment on bioaccumulation in aquatic food webs. Environmental Toxicology and Chemistry 26, 607-615

Morrison DE, Robertson BK, Alexander M (2000): Bioavailability to earthworms of aged DDT, DDE, DDD, and dieldrin in soil. Environ Sci Technol 34, 709-713

Mothersill C, Seymour C (2010): Eco-systems biology-From the gene to the stream. Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis 687, 63-66

Nagel R (2002): DarT: The embryo test with the zebrafish Danio rerio - a general model in ecotoxicology and toxicology. Altex 19, 38-48

Nam K, Alexander M (1998): Role of nanoporosity and hydrophobicity in sequestration and bioavailability: Tests with model solids. Environ Sci Technol 32, 71-74

Nam K, Chung N, Alexander M (1998): Relationship between organic matter content of soil and the sequestration of phenanthrene. Environ Sci Technol 32, 3785-3788

Namiesnik J, Rabajczyk A (2010): The speciation and physico-chemical forms of metals in surface waters and sediments. Chemical Speciation and Bioavailability 22, 1-24

Neumann-Hensel H, Melbye K (2006): Optimisation of the solid-contact test with Artbrobacter globiformis. J Soil Sediment 6, 201-207

Northcott GL, Jones KC (2000): Experimental approaches and analytical techniques for determining organic compound bound residues in soil and sediment. Environ. Pollut. 108, 19-43

Oikari A, Fragoso N, Leppanen H, Chan T, Hodson PV (2002): Bioavailability to juvenile rainbow trout (Oncorynchus mykiss) of retene and other mixed-function oxygenase-active compounds from sediments. Environ Toxicol Chem 21, 121-128

Palmgren JJ, Monkkonen J, Korjamo T, Hassinen A, Auriola S (2006): Drug adsorption to plastic containers and retention of drugs in cultured cells under in vitro conditions. Eur J Pharm Biopharm 64, 369-378

Pelletier E, Sargian P, Payet J, Demers S (2006): Ecotoxicological effects of combined UVB and organic contaminants in coastal waters: A review. Photochemistry and Photobiology 82, 981-993

Pierron F, Bourret V, St-Cyr J, Campbell PGC, Bernatchez L, Couture P (2009): Transcriptional responses to environmental metal exposure in wild yellow perch (Perca flavescens) collected in lakes with differing environmental metal concentrations (Cd, Cu, Ni). Ecotoxicology 18, 620-631

Puglisi E, Vernile P, Bari G, Spagnuolo M, Trevisan M, de Lillo E, Ruggiero P (2009): Bioaccessibility, bioavailability and ecotoxicity of pentachlorophenol in compost amended soils. Chemosphere 77, 80-86

Qiao M, Huang S, Wang Z (2008): Partitioning characteristics of PAHs between sediment and water in a shallow lake. J Soil Sediment 8, 69-73

Rattner BA (2009): History of wildlife toxicology. Ecotoxicology 18, 773-783

Reichenberg F, Mayer P (2006): Two complementary sides of bioavailability: Accessibility and chemical activity of organic contaminants in sediments and soils. Environ Toxicol Chem 25, 1239-1245

Reid BJ, Jones KC, Semple KT (2000a): Bioavailability of persistent organic pollutants in soils and sediments - a perspective on mechanisms, consequences and assessment. Environ. Pollut. 108, 103-112

Reid BJ, Stokes JD, Jones KC, Semple KT (2000b): Nonexhaustive cyclodextrin-based extraction technique for the evaluation of PAH bioavailability. Environ Sci Technol 34, 3174-3179

Rodrigues SM, Henriques B, Coimbra J, da Silva EF, Pereira ME, Duarte AC (2010): Water-soluble fraction of mercury, arsenic and other potentially toxic elements in highly contaminated sediments and soils. Chemosphere 78, 1301-1312

Roesijadi G (1992): Metallothioneins in metal regulation and toxicity in aquatic animals. Aquat Toxicol 22, 81-114

Rönnpagel K, Liss W, Ahlf W (1995): Microbial bioassays to assess the toxicity of solid-associated contaminants. Ecotox Environ Safe 31, 99-103

Russell WMS, Burch RL (1959): The principles of humane experimental technique. The principles of humane experimental technique, xiv+238p pp

Ryder K, Temara A, Holdway DA (2004): Avoidance of crude-oil contaminated sediment by the Australian seastar, Patiriella exigua (Echinodermata : Asteroidea). Mar. Pollut. Bull. 49, 900-909

Saghir SA, Bartels MJ, Budinsky RA, Harris EE, Clark AJ, Staley JL, Chai Y, Davis JW (2007): Effect of organic carbon content, clay type, and aging on the oral bioavailability of hexachlorobenzene in rats. Environ Toxicol Chem 26, 2420-2429

Schäfer RB, Caquet T, Siimes K, Mueller R, Lagadic L, Liess M (2007): Effects of pesticides on community structure and ecosystem functions in agricultural streams of three biogeographical regions in Europe. Sci Total Environ 382, 272-285

Schmitt C, Balaam J, Leonards P, Brix R, Streck G, Tuikka A, Bervoets L, Brack W, van Hattum B, Meire P, de Deckere E (2010): Characterizing field sediments from three European river basins with special emphasis on endocrine effects - A recommendation for Potamopyrgus antipodarum as test organism. Chemosphere 80, 13-19

Schwab K, Brack W (2007): Large volume tenax (R) extraction of the bioaccessible fraction of sediment-associated organic compounds for a subsequent effect-directed analysis. J Soil Sediment 7, 178-186

Schwarzenbach RP, Escher BI, Fenner K, Hofstetter TB, Johnson CA, von Gunten U, Wehrli B (2006): The challenge of micropollutants in aquatic systems. Science 313, 1072-1077

Seiler TB, Rastall AC, Leist E, Erdinger L, Braunbeck T, Hollert H (2006): Membrane dialysis extraction (MDE): A novel approach for extracting toxicologically relevant hydrophobic organic compounds from soils and sediments for assessment in biotests. J Soil Sediment 6, 20-29

Seiler TB, 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, Wick LY, Harms H (2007): Microbial interactions with organic contaminants in soil: Definitions, processes and measurement. Environ. Pollut. 150, 166-176

Sijm D, Kraaij R, Belfroid A (2000): Bioavailability in soil or sediment: exposure of different organisms and approaches to study it. Environ. Pollut. 108, 113-119

Simpson SL, Batley GE (2006): Predicting Metal Toxicity in Sediments: A Critique of Current Approaches. Integrated Environmental Assessment and Management 3, 18-31

Smith KEC, Dom N, Blust R, Mayer P (2010): Controlling and maintaining exposure of hydrophobic organic compounds in aquatic toxicity tests by passive dosing. Aquatic Toxicology 98, 15-24

Solimini AG, Ptacnik R, Cardoso AC (2009): Towards holistic assessment of the functioning of ecosystems under the Water Framework Directive. TRAC-Trend Anal Chem 28, 143-149

Sormunen AJ, Leppanen MT, Kukkonen JVK (2009a): Examining the role of temperature and sediment-chemical contact time on desorption and bioavailability of sediment-associated tetrabromo diphenyl ether and benzo(a)pyrene. Ecotox Environ Safe 72, 1234-1241

Sormunen AJ, Leppanen MT, Kukkonen JVK (2009b): Examining the role of temperature and sediment-chemical contact time on desorption and bioavailability of sediment-associated tetrabromo diphenyl ether and benzo(a)pyrene. Ecotoxicology and Environmental Safety 72, 1234-1241

Standardisation IOf (2009): ISO 10871: Water quality - Determination of the inhibition of dehydrogenase activity of *Arthrobacter globiformis* - Solid contact test using the redox dye resazurine. Iso Guideline

Sundback K, Alsterberg C, Larson F (2010): Effects of multiple stressors on marine shallow-water sediments: Response of microalgae and meiofauna to nutrient-toxicant exposure. Journal of Experimental Marine Biology and Ecology 388, 39-50

Swindell AL, Reid BJ (2006): Comparison of selected non-exhaustive extraction techniques to assess PAH availability in dissimilar soils. Chemosphere 62, 1126-1134

Tang JX, Alexander M (1999): Mild extractability and bioavailability of polycyclic aromatic hydrocarbons in soil. Environ Toxicol Chem 18, 2711-2714

Ten Hulscher TEM, Postma J, Den Besten PJ, Stroomberg GJ, Belfroid A, Wegener JW, Faber JH, Van Der Pol JJC, Jan Hendriks A, Van Noort PCM (2003a): Tenax extraction mimics benthic and terrestrial bioavailability of organic compounds. Environmental Toxicology and Chemistry 22, 2258-2265

Ten Hulscher TEM, Postma J, Den Besten PJ, Stroomberg GJ, Belfroid A, Wegener JW, Faber JH, Van Der Pol JJC, Jan Hendriks A, Van Noort PCM (2003b): Tenax extraction mimics benthic and terrestrial bioavailability of organic compounds. Environ Toxicol Chem 22, 2258-2265

Traunspurger W, Haitzer M, Hoss S, Beier S, Ahlf W, Steinberg C (1997): Ecotoxicological assessment of aquatic sediments with Caenorhabditis elegans (nematoda) - A method for testing liquid medium and whole-sediment samples. Environ Toxicol Chem 16, 245-250

Turesson EU, Stiernstrom S, Minten J, Adolfsson-Erici M, Bengtsson BE, Breitholtz M (2007): Development and reproduction of the freshwater harpacticoid copepod Attheyella crassa for assessing sediment-associated toxicity. Aquat Toxicol 83, 180-189

Van der Heijden SA, Jonker MTO (2009): PAH bioavailability in field sediments: Comparing different methods for predicting in situ bioaccumulation. Environ Sci Technol 43, 3757-3763

Van Hoof PL, Kukkonen JVK, Landrum PF (2001): Impact of sediment manipulation on the bioaccumulation of polycyclic aromatic hydrocarbons from field-contaminated and laboratory-dosed sediments by an oligochaete. Environ Toxicol Chem 20, 1752-1761

Van Noort PCM, Cornelissen G, ten Hulscher TEM, 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

Verrhiest GJ, Cortes S, Clement B, Montuelle B (2002): Chemical and bacterial changes during laboratory conditioning of formulated and natural sediments. Chemosphere 46, 961-974

Weber J, Kreutzmann J, Plantikow A, Pfitzner S, Claus E, Manz W, Heininger P (2006): A novel particle contact assay with the yeast Saccharomyces cerevisiae for ecotoxicological assessment of freshwater sediments. J Soil Sediment 6, 84-91

Whyte JJ, Jung RE, Schmitt CJ, Tillitt DE (2000): Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. Crit Rev Toxicol 30, 347-570

Wölz J, Engwall M, Maletz S, Takner HO, 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 R 15, 536-553

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

Xu YP, Gan J, Wang ZJ, Spurlock F (2008): Effect of aging on desorption kinetics of sediment-associated pyrethroids. Environ Toxicol Chem 27, 1293-1301

You J, Landrum PF, Lydy MJ (2006): Comparison of chemical approaches for assessing bioavailability of sediment-associated contaminants. Environ Sci Technol 40, 6348-6353

You J, Brennan A, Lydy MJ (2009): Bioavailability and biotransformation of sediment-associated pyrethroid insecticides in Lumbriculus variegatus. Chemosphere 75, 1477-1482

Zhong H, Wang WX (2006): Influences of aging on the bioavailability of sediment-bound Cd and Zn to deposit-feeding sipunculans and soldier crabs. Environ Toxicol Chem 25, 2775-2780

Chapter 2

Time-dependence of results in direct sediment contact tests

Hanno Zielke[1], Thomas-Benjamin Seiler[1] and Henner Hollert[1]

[1]Department of Ecosystem Analysis, Institute for Environmental Research, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany

2.1 Relevance of direct sediment contact test in sediment assessment

Direct sediment contact tests (SCTs), also referred to as whole-sediment toxicity tests, directly integrate all factors that are relevant for the impact of a given pollutant, such as dependence on test species, compound characteristics, sediment properties and environmental factors, and translate them into biological effects (e.g. Blaha et al. 2010, Conder et al. 2004, Dillon et al. 1994, Duft et al. 2003, Feiler et al. 2009, Höss et al. 2010a, Ingersoll et al. 1995, Jones et al. 2008, Lee et al. 2004, Re et al. 2009, Ryder et al. 2004, Sae-Ma et al. 1998, Seiler et al. 2010, Turesson et al. 2007). Thus, SCTs directly represent the bioavailability of a contaminant to the test species on the level of effect. In addition, as reviewed by Seiler et al. (2008), these test systems reduce alterations of the sample compared to most other current strategies for sediment assessment, in particular extraction with organic solvents. The lab-based evaluation of chemicals and field monitoring can therefore greatly benefit from the application of SCTs. In the context of REACh (EC 2006) and the amended water framework directive (EC 2008), effect-focused assessment tools can provide data on potential biological impact if first-line screening indicates a need for further, more detailed assessment measures. In terms of field monitoring, SCTs are invaluable markers of biological impact (e.g.Eklund et al. 2010, Hilscherova et al. 2010, Krcmova et al. 2009, Re et al. 2009, Schmitt et al. 2010). However, in order to fully benefit from these advantages and properly interpret the resulting biological data, potential influences on the effects recorded using SCTs need to be investigated. An important step towards this end has been made by the German SEKT project framework, which defined reference conditions, control sediments and toxicity thresholds for a battery of six test systems (Feiler et al. 2009, Feiler et al. 2005, Höss et al. 2010b). The present comment aims to show that the time between an initial contamination event (or spiking in case of lab-based assessment of chemicals) and the actual exposure in an SCT is a factor with potentially great impact on SCT results.

2.2 Impact of time and concepts of availability

After initial contamination, hydrophobic organic pollutants do not remain freely available in sediments and soils (Alexander 2000). With increasing contact time, chemicals bind to the sediment matrix and eventually form bound residues, often accompanied by decreasing

chemical extractability and availability for uptake into organisms (e.g.Cornelissen et al. 1998a, Menchai et al. 2008, Puglisi et al. 2009, Reid et al. 2000a, Xu et al. 2008). This process is known as sequestration whereas the observable phenomenon is termed ageing (Alexander 2000). Key determinants of sequestration are the organic matrix together with structural micro- and nanopores (Alexander 2000, Cornelissen et al. 1998b, Hatzinger & Alexander 1995, Kukkonen et al. 2003, Nam & Alexander 1998, Nam et al. 1998, Northcott & Jones 2000b). Other potentially relevant determinants may be particulate carbon and non-aqueous phase liquids (Luthy et al. 1997). The individual impact of each determinant is, however, controversially discussed and also depends on the particular sediment composition (Huang et al. 2003). Other processes like degradation (Brennan et al. 2009, Lei et al. 2005, You et al. 2009) or re-mobilisation (Van Hoof et al. 2001, Wölz et al. 2008) also play important roles for the time-related fate of substances, in addition to sequestration.

Due to on-going sequestration, ageing compounds are partly still freely available, partly reversibly and partly irreversibly bound (Northcott & Jones 2000b, Pignatello & Xing 1996). Studies that utilise kinetic desorption to investigate ageing differentiate between fast, slowly and very slowly desorbing fractions (e.g. Chen et al. 2010, Cornelissen et al. 2000, Cui et al. 2010, Semple et al. 2003, Sormunen et al. 2009, Sormunen et al. 2010). The freely available or fast desorbing fractions have sometimes been referred to as "bioavailable" (Cornelissen et al. 2001, Reid et al. 2000b, Ten Hulscher et al. 2003). In case of studies relating and comparing chemical extractability to uptake by organisms, the term was often used to imply that a substance is not only chemically available but can also be accessed by organisms.

However, the term "bioavailability" and all of its implications and aspects are still being discussed and in the process of final definition (Ehlers & Loibner 2006, Semple et al. 2004). Semple et al. (2004) were the first to establish the distinction between bioavailability and bioaccessibility. According to this definition, compounds readily and directly available for uptake by an organism are termed "bioavailable", whereas the term "bioaccessible" additionally includes substances that are available as soon as an organism has access to them (Semple et al. 2004). A further important step was made by Reichenberg and Mayer (2006), who defined bioavailability as a dual concept that integrates accessibility and chemical activity, with accessibility being the mass of a contaminant that is available for uptake into an organism and chemical activity representing the substance's potential to undergo physico-chemical processes. However, as Reichenberg and Mayer (2006) also noted, this approach focuses on physic-chemical aspects, not the equally important biological level.

A recent definition was given by Brack et al. (2009) on the basis of ISO 11074, which defined bioavailability as "the degree to which chemicals present in the soil (or sediment) may be absorbed or metabolized by human or ecological receptors or are available for interaction with biological systems'' (ISO 2005). Within this definition of bioavailability, Brack et al. (2009) distinguished between three key processes, effectively including the previous concepts of Semple et al. (2004) and Reichenberg and Meyer (2006): a) environmental activity or bioaccessibility, which is independent of organisms and refers to the aforementioned desorbing fractions, b) environmental bioavailability, which means the uptake of desorbable and dissolved molecules into the organisms, driven by chemical activity and c) toxicological bioavailability, which is the internal availability in terms of toxicokinetics within an organism and also includes observable effects. With regard to these definitions, most biomimetic extraction methods like e.g. hydroxylpropyl-β-cyclodextrin or Tenax TA$^®$ extraction provide data on bioaccessibility rather than bioavailability (Zielke et al. 2011).

Within this context, it is of central importance that chemical behaviour cannot be directly translated to biological impact. Bioavailability on each of the three levels discussed by Brack et al (2009) may vary largely for a given compound, depending on sediment parameters, species and route of exposure (e.g. via water phase, sediment contact, gut-fluid extraction, etc.; Alexander 2000, Ehlers & Loibner 2006, Reid et al. 2000a). Therefore, no test system alone is sufficient to assess "bioavailability" as a whole, but can only provide information on one aspect of this complex relationship.

Biomimetic extractions are promising tools to assess bioaccessibility, whereas SCTs can either represent environmental bioavailability by means of uptake and bioaccumulation, or toxicological bioavailability by observing or measuring specific effects. Most studies that utilise SCTs accounted only for either uptake and bioaccumulation or direct effects, rather than both at the same time. Both aspects are clearly interlinked and most SCTs could be easily adapted to account for both. Due to this differentiation, although scientific literature addresses the time-dependence of uptake and bioaccumulation in SCTs as well as of biomimetic methodologies assessing bioaccessibility to some extent (e.g. Chai et al. 2008, Conrad et al. 2002, Jones et al. 2008, Leppänen & Kukkonen 2000, Morrison et al. 2000, Saghir et al. 2007, Sormunen et al. 2009, Xu et al. 2008, Zhong & Wang 2006), time-dependence of observable effects in SCTs has received insufficient coverage.

2.3 Time-dependency of results in effect-focused sediment contact tests

2.3.1 Existing Literature

As sediment-pollutant contact time has been demonstrated to impact chemical availability, it is apparent that time also influences the effects obtained in SCTs. In addition to ageing, the integrative capacity of SCTs causes time-dependence in these systems to also all other processes that have a potential to vary with time, such as degradation (Brennan et al. 2009, Lei et al. 2005, You et al. 2009), re-mobilisation (Van Hoof et al. 2001, Wölz et al. 2008) or the formation of toxic metabolites (Puglisi et al. 2009). The crucial points are, however, how the well-characterised time-dependence at the chemical level translates into SCT results, i.e. directly observable or measurable biological endpoints, whether distinct exposure routes impact time-related effect patterns for a given species, and whether the effects of a given pollutant change simultaneously for different taxa. It can be reasonably assumed that feeding and living habits strongly influence time-dependence, just as they influence uptake and accumulation in general (Ehlers & Loibner 2006).

In order to build a basis for a targeted approach this comment now provides a brief overview of existing literature. However, publications dealing with time-dependence of biological effects are comparatively rare, and even more so for organisms of higher complexity (Fig. 2.1). Furthermore, some of the identified publications have a different focus and address time-dependent changes of observable effects only secondarily.

In general, most studies point towards decreasing effects within a time frame of few weeks to several months.

With respect to metals, studies concentrated on invertebrates. Lee and co-workers (2004) tested a zinc-spiked natural sediment aged between 5 and 20 days at 20 ± 1 °C and described a significant twofold decrease of amphipod (*Leptocheirus plumulosus*) mortality. Accounting for partitioning and the role of metal speciation, the authors attribute this reduced effects to the decreasing concentration of dissolved zinc and conclude that, among other factors, short equilibration time accentuates metal uptake and resulting effects via the dissolved phase.

Jones et al. (2008) spiked sediments with different concentrations of copper, stored them at 25 ± 1 °C and tested them after 2, 14, 28, 42 and 60 d, respectively, in 10 d amphipod (*Hyalella azteca*) assays. Significant declines in the mortality of amphipods became evident up to 42 d in all copper concentrations in two of the three tested sediments and for one of three concentrations in the third sediment. Mortality decreased by as much as 87 % within 60 days.
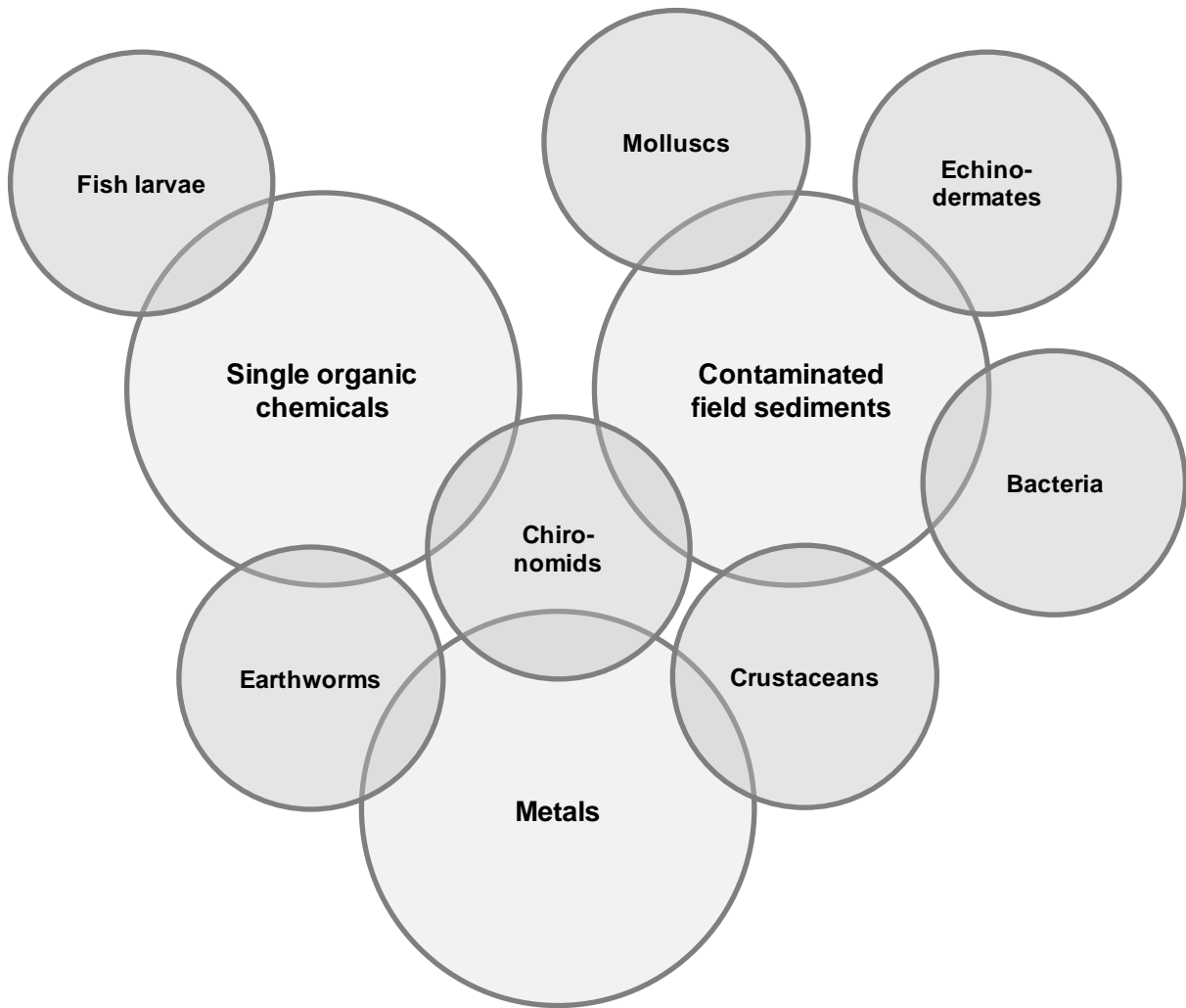
Fig. 2.1 Schematic overview of zoological taxa and types of samples investigated in literature for potential impacts of time on direct sediment contact test results.

In accordance with the two aforementioned studies, survival of midge larvae (*Chironomus tentans*) exposed to sediment-associated cadmium stored at 4 °C in the dark increased significantly when tested after 1, 31, 79 and 120 d of storage, although total Cd concentrations in the bulk sediment remained unchanged (Sae-Ma et al. 1998). For instance, survival in the highest concentration changed from 16 % to 40 % within 119 days.

Several other publications described approaches that focus on one specific organic pollutant or type of chemical. One of the few studies with an explicit focus on the effect of storage duration on toxicity utilised oyster (*Crassostrea gigas*) embryos (Beiras et al. 1998). Unfortunately, eluates were applied instead of a SCT procedure, limiting transferability of the results to SCTs. Still, a loss of toxicity was evident between 1 to 15 and to 30 days of storage at 4 °C. This decrease was more pronounced in less-polluted sediments. Additionally, deep-freeze-drying (-196 °C) lead to high toxicities of all eluates in comparison to the applied

controls regardless of origin and contamination level. The authors advise sediment storage at -20 °C if samples have to be kept for longer than a week before being applied in a biotest.

In the special case of trinitrotoluol (TNT), an ageing-related decrease of toxicity of as much as 100 % on the earthworm *Tubifex tubifex* was shown between 1, 8, and 29 days (Conder et al. 2004). The authors argue that this decrease can primarily be attributed to the high degradability of TNT. Sediments in this study were first dried, then re-wetted and subsequently stored at $23 \pm 1°C$ with a day/night rhythm of 16/8 hours. The authors concluded that for many sublethal toxicity tests, such as an earthworm contact assay (28 d), concentrations at the end of the experiment may deviate from the initially applied concentrations by orders of magnitude. However, sediment quality deteriorated with time independently of the spiked TNT. Therefore, they suggest the use of intermediate ageing periods after initial spiking (8-14 d).

Although the study of Xu et al. (2007) focused primarily on comparing different partition coefficients, it also includes LC_{50} values for *Chironomus tetans* exposed to a pyrethroid-spiked natural bulk sediment, which was spiked at 4 °C and aged for 30 or 90 days. The results indicated a tendency towards increasing LC_{50} values with increasing contact time, up to approximately a two-fold increase.

Ryder et al. (2004) reported a behavioural experiment, showing that seastars (*Patiriella exigua*) avoided oil-contaminated sediments. Seastar tolerance of oiled sediments significantly increased by a maximum factor 6-7 over 32 days as oil content decreased due to ageing effects.

One interesting approach addressed ageing-dependent CYP1A induction using EROD activity in fingerling rainbow trouts (Oncorhynchus mykiss; Oikari et al. 2002). Test sediments were spiked with PAHs and aged for 24 h and one month, respectively, at 4 °C in the dark. No significant effect of ageing was apparent.

The study of Guthrie-Nichols et al. (2003) linked ageing-related changes in SOM structure to toxicity of sediment humin fractions after ageing for 60 and 120 days at 25 °C. Toxicity to bacteria (*Vibrio* sp.) in a luminescence assay was completely lost over the course of the experiment. The LC_{50} of sediment pre-aged for additional 120 d changed from 0.65 % dilution to non-toxic, and the LC_{50} of sediments freshly spiked directly prior to the definite 120 d experimental period decreased from 0.12 % (0 d) to 1.43 % (60 d) and finally became non-toxic after 120 d.

Although a clear tendency is apparent, not all results published on ageing in sediments concur that the time-dependent change is necessarily a decrease. Dillon et al. (1994) stored an

estuarine sediment containing elevated levels of heavy metals, pesticides, petroleum and chlorinated hydrocarbons at -22 °C, 4 °C, and 25 °C. They found a significant increase in toxicity to mysid shrimps (*Mysidopsis bahia*) within 2-12 weeks of ageing. Although toxicity decreased significantly at week 20, this effect was plausibly attributed to a lower sensitivity of the shrimps in general as shown by an accompanying decrease of reference mortality. Organisms in this study were exposed using a suspended particulate phase assay. This might explain the increasing mortality, as it represented an inherent step of remobilisation in the experimental setup, potentially raising compound bioavailability. Still, this case clearly shows that the time-dependence of bioassay effects is too complex to be described completely by the assumption that decreasing effects are the result. As Alexander (2000) noted for soils, ageing (and in consequence time-dependence in general) undeniably impacts toxicity tests, but the direction of this change cannot be easily predicted.

This realisation was underlined by a systematic approach covering Cu-spiked sediments as well as field sediments with a complex contamination pattern applied in the PhD study of Leweke (1999). After storage for 0, 1, 3 and 9 weeks at both 4°C and -18°C, SCTs as well as pore water tests with *Daphnia magna*, *Enchytraeus albidus*, *Chironomus riparius* and *Tubifex tubifex* were carried out. For the Cu-spiked artificial sediment, 10 out of 12 tests gave significantly different results between unstored sediments and sediments stored for 9 weeks. Generally, adverse impacts showed a tendency to decrease with time in the applied SCTs. However, effects in the pore-water tests mostly increased with time. Because significant changes but no definite relationship between time and effect was observed for one particular Cu-spiked natural sediment with respect to test species and exposure scenario (i.e. direct contact or pore water), Leweke (1999) concluded that no final recommendation on optimal storage duration of Cu-spiked sediment can be made. To further complicate this relationship, a dependence on temperature was observed in parallel. Overall toxicity of spiked sediments increased when samples were stored at -18°C compared to 4 °C. In contrast, field sediments stored at -18 °C turned out to be less toxic.

2.3.2   Conclusion from literature

Increasing sediment-substance contact time resulted in decreasing biological effects in more studies (Beiras et al. 1998, Conder et al. 2004, Jones et al. 2008, Lee et al. 2004, Leweke 1999, Ryder et al. 2004, Sae-Ma et al. 1998) than in increasing effects (Dillon et al. 1994,

Leweke 1999). At the same time, a need to further address and investigate this influence is apparent. In addition, several issues were identified that appear to be of special importance within the context of time-dependence: (a) experimental timing, (b) the rate and speed of time-dependent changes, (c) type of assessment (i.e. investigation of field samples or lab-based spiking), and (d) correlation with test and storage temperature.

The most important conclusion appears to be that time-dependence of SCT results is directly linked to the question of experimental timing. In case of spiking-based assessment, current concepts recommend starting exposure at one defined point of time after initial spiking (Beiras et al. 1998, Conder et al. 2004, Northcott & Jones 2000a). For instance, OECD guideline 218 for the spiking of sediments suggests one week of equilibration (OECD 1984). Whereas such an approach is often inevitable due to experimental necessities, the literature reviewed above clearly indicates that the influence of time has at least to be considered and thoroughly discussed. If possible, several timepoints should be tested in all spiking-based experiments, whenever allowed by temporal and economical circumstances. However, in context of regulatory guidelines like REACh and the European Water Framework Directive, strategies are required that reduce demands for additional testing rather than to increase it.

In addition, the exact rate and speed of time-dependent changes are greatly relevant factors. Knowing these rates for a given substance or class of pollutants after an initial contamination event or spiking procedure would not greatly improve our understanding of bioassay results, and allow for a more precise assessment of acute contamination events, e.g. by industrial accidents. However, because literature indicates that exact time factors individually depend on the test species, test parameters, assessed substance and sediment characteristics, it is apparent that such tests cannot be carried out for every possible combination of parameters. Hence, the key long-term goal has to be to gather sufficient data in order to develop mathematical models encompassing time-dependence and other potentially confounding factors.

The difference between assessment of field samples and lab-based assessment which utilises spiking is one more point to consider. From a comparison of spiked and field-contaminated sediments at different storage temperatures, Leweke (1999) concluded that data on the impact of storage temperature of spiked sediments cannot be directly transferred to storage of contaminated field sediments. The same is likely true for time-dependence, and poses a great challenge for sediment risk assessment. In this context, it is interesting to note that many studies linked time-dependence and either test or storage temperature. Both impacts are interrelated to great degree, which result in increased complexity, clearly calling for new tools to integrate and account for this interdependency.

2.4 Inclusion of time-dependence into sediment assessment

In order to address the challenges that arise from the recognition of time-dependence and accompanying factors as important influence on SCTs, we recommend a three step strategy:

Firstly, as literature clearly shows, knowledge on the time-dependence of effects in SCTs is scarce. Chironomids and earthworms have been best characterised in literature, whereas data on vertebrates are scarce (Fig. 1). There is also a lack of data on time-dependence of the results obtained in assessment of single organic substances in SCTs (Fig. 1). Further research is necessary by means of studies which systematically assess this interrelationship, such as the study by Leweke (1999). In a first step towards this end, a recent effect-focused pilot study compared effects of the organic chemicals 3,4-dichloroaniline, fluoranthene and pentachlorophenol in three SCTs from the SeKT project framework in regard to the influence of sediment-pollutant contact time on biological results.

Secondly, effect-focused data have to be directly linked to chemical data, in regard to the initial questions how chemical patterns of time-dependence translate into time-dependence of effects. The best solution can be studies which integrate time-dependence on the level of effect with chemical fate and accessibility. Interesting studies that could provide the basis for such integrative approaches have been described by Puglisi (2009) and Moermond (2007). The study of Puglisi et al. (2009) on the impact of PCP in compost-amended soils provides an excellent example of an integrative approach that accounted for bioaccessibility (via mild extraction) as well as two different endpoints of bioavailability, bioaccumulation and biological effect in both earthworms and bacteria with the inclusion of time-dependence. Moermond et al. (2007) investigated the impact of ageing on PCBs in a model ecosystem and accounted for the Soxhlet-extractable fraction, bioaccessibility by means of 6 h Tenax TA$^{®}$ extraction, and bioaccumulation. The concept could be easily adapted to include toxicological bioavailability for one or several species. In general, successful integrative strategies like the triad concept (Chapman 1990, 1996, 2000) simply need to be expanded in order to encompass time-dependence. In their opinion paper on the optimisation and future application of the triad approach, Chapman and Hollert (2006) suggested an expansion of the original triad to account for additional stressors. However, these studies are very time-intensive and cannot be carried out for every combination of substance, sediment, organism and environmental parameters.

Hence, thirdly an inclusion of time-dependence not only into integrative assessment strategies, but eventually into predictive tools like modelling is needed. Chapman and Hollert (2006) addressed modelling as a strategy to include into future triad approaches. Recent

ecotoxicological models were developed to include multiple stressors and confounding factors (Loos et al. 2010, Park et al. 2008). The AQUATOX model, for instance, was explicitly developed to account for time-dependent variations of exposure (Park et al. 2008). Such developments show that once reliable base-data have been gathered, modelling can be the most promising tool to solve the challenges presented by time-dependence as well as other confounding factors.

2.5   Conclusion

The present literature survey revealed that sediment-pollutant contact time can severely impact results derived from direct sediment contact tests. In order to adequately address this influence, we propose a three step long-term strategy. This strategy comprises of a) pilot-studies on time-dependence of biological effects, b) integrative studies on basis of the triad concept combining bioaccessibility, environmental bioavailability, and toxicological bioavailability to assess the interlinkage of time-dependence and c) inclusion of time-dependence and other potentially confounding impacts into holistic ecological and ecotoxicological modelling. Such predictive models can in turn minimise test demands and support regulatory decisions.

2.6 References

Alexander M (2000): Aging, bioavailability, and overestimation of risk from environmental pollutants. Environ Sci Technol 34, 4259-4265

Beiras R, His E, Seaman MNL (1998): Effects of storage temperature and duration on toxicity of sediments assessed by Crassostrea gigas oyster embryo bioassay. Environ Toxicol Chem 17, 2100-2105

Blaha L, Hilscherova K, Cap T, Klanova J, Machat J, Zeman J, Holoubek I (2010): Kinetic bacterial bioluminescence assay for contact sediment toxicity testing: relationships with the matrix composition and contamination. Environ Toxicol Chem 29, 507-514

Brack W, Bandow N, Schwab K, Schulze T, Streck G (2009): Bioavailability in effect-directed analysis of organic toxicants in sediments. TRAC-Trend Anal Chem 28, 543-549

Brennan AA, Harwood AD, You J, Landrum PF, Lydy MJ (2009): Degradation of fipronil in anaerobic sediments and the effect on porewater concentrations. Chemosphere 77, 22-28

Chai YZ, Davis JW, Saghir SA, Qiu XJ, Budinsky RA, Bartels MJ (2008): Effects of aging and sediment composition on hexachlorobenzene desorption resistance compared to oral bioavailability in rats. Chemosphere 72, 432-441

Chapman PM (1990): The sediment quality triad approach to determining pollution-induced degradation. Sci Total Environ 97-8, 815-825

Chapman PM (1996): Presentation and interpretation of sediment quality triad data. Ecotoxicology 5, 327-339

Chapman PM (2000): The Sediment Quality Triad: then, now and tomorrow. Int J Environ Pollut 13, 351-356

Chapman PM, Hollert H (2006): Should the sediment quality triad become a tetrad, a pentad, or possibly even a hexad? J Soil Sediment 6, 4-8

Chen S, Xu Y, Wang Z (2010): Assessing desorption resistance of PAHs in dissolved humic substances by membrane-based passive samplers. J Colloid Interface Sci 350, 348-54

Conder JM, La Point TW, Steevens JA, Lotufo GR (2004): Recommendations for the assessment of TNT toxicity in sediment. Environ Toxicol Chem 23, 141-149

Conrad AU, Comber SD, Simkiss K (2002): Pyrene bioavailability; effect of sediment-chemical contact time on routes of uptake in an oligochaete worm. Chemosphere 49, 447-454

Cornelissen G, Rigterink H, Ferdinandy MMA, Van Noort PCM (1998a): Rapidly desorbing fractions of PAHs in contaminated sediments as a predictor of the extent of bioremediation. Environ Sci Technol 32, 966-970

Cornelissen G, Van Noort PCM, Covers HAJ (1998b): Mechanism of slow desorption of organic compounds from sediments: A study using model sorbents. Environ Sci Technol 32, 3124-3131

Cornelissen G, Rigterink H, Van Noort PCM, Govers HAJ (2000): Slowly and very slowly desorbing organic compounds in sediments exhibit Langmuir-type sorption. Environ Toxicol Chem 19, 1532-1539

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

Cui XY, Hunter W, Yang Y, Chen YX, Gan J (2010): Bioavailability of sorbed phenanthrene and permethrin in sediments to Chironomus tentans. Aquat Toxicol 98, 83-90

Dillon TM, Moore DW, Jarvis AS (1994): The effects of storage-temperature and time on sediment toxicity. Arch Environ Con Tox 27, 51-53

Duft M, Schulte-Oehlmann U, Tillmann M, Markert B, Oehlmann J (2003): Toxicity of triphenyltin and tributyltin to the freshwater mudsnail Potamopyrgus antipodarum in a new sediment biotest. Environ Toxicol Chem 22, 145-152

EC (2006) Regulation No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32006R1907:EN:PDF, last accessed 25.01.2011

EC (2008) Directive 2008/105/EC of the european parliament and of the council of 16 December 2008 on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:348:0084:0097:EN:PDF, last accessed 25.01.2011

Ehlers GAC, Loibner AP (2006): Linking organic pollutant (bio)availability with geosorbent properties and biomimetic methodology: A review of geosorbent characterisation. and (bio)availability prediction. Environ. Pollut. 141, 494-512

Eklund B, Elfstrom M, Gallego I, Bengtsson B-E, Breitholtz M (2010): Biological and chemical characterization of harbour sediments from the Stockholm area. J Soil Sediment 10, 127-141

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 R 12, 257-258

Feiler U, Ahlf W, Fahnenstich C, Gilberg D, Hammers-Wirtz M, Höss S, Hollert H, Melbye K, Meller M, Neumann-Hensel H, Ratte HT, Seiler TB, Spira D, Weber J, Heininger P 2009: Final report SeKT framework project - Definition von Referenzbedingungen, Kontrollsedimenten und Toxizitätsschwellenwerten für limnische Sedimentkontakttests - SeKT

Guthrie-Nichols E, Grasham A, Kazunga C, Sangaiah R, Gold A, Bortiatynski J, Salloum M, Hatcher P (2003): The effect of aging on pyrene transformation in sediments. Environ Toxicol Chem 22, 40-49

Hatzinger PB, Alexander M (1995): Effect of aging of chemicals in soil on their biodegradability and extractability. Environ Sci Technol 29, 537-545

Hilscherova K, Dusek L, Sidlova T, Jalova V, Cupr P, Giesy JP, Nehyba S, Jarkovsky J, Klanova J, Holoubek I (2010): Seasonally and regionally determined indication potential of bioassays in contaminated river sediments. Environ Toxicol Chem 29, 522-534

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 (2010a): Variability of sediment-contact tests in freshwater sediments with low-level anthropogenic contamination - Determination of toxicity thresholds. Environ. Pollut. 158, 2999-3010

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 (2010b): Variability of freshwater sediment contact tests in sediments with low anthropogenic contamination - Determination of toxicity thresholds. Environmental Pollution, doi:10.1016/j.envpol.2010.05.013

Huang WL, Ping PA, Yu ZQ, Fu HM (2003): Effects of organic matter heterogeneity on sorption and desorption of organic contaminants by soils and sediments. Applied Geochemistry 18, 955-972

Ingersoll CG, Ankley GT, Benoit DA, Brunson EL, Burton GA, Dwyer FJ, Hoke RA, Landrum PF, Norbergking TJ, Winger PV (1995): Toxicity and bioaccumulation of sediment-associated contaminants using fresh-water invertebrates - a review of methods and applications. Environ Toxicol Chem 14, 1885-1894

ISO (2005): ISO 11074: Soil quality -- Vocabulary. Iso Guideline

Jones RP, Hassan SM, Rodgers JH (2008): Influence of contact duration on sediment-associated copper fractionation and bioavailability. Ecotox Environ Safe 71, 104-116

Krcmova K, Robertson D, Cveckova V, Rapant S (2009): Road-deposited sediment, soil and precipitation (RDS) in Bratislava, Slovakia: compositional and spatial assessment of contamination. J Soil Sediment 9, 304-316

Kukkonen JVK, Landrum PF, Mitra S, Gossiaux DC, Gunnarsson J, Weston D (2003): Sediment characteristics affecting desorption kinetics of select PAH and PCB congeners for seven laboratory spiked sediments. Environ Sci Technol 37, 4656-4663

Lee JS, Lee BG, Luoma SN, Yoo H (2004): Importance of equilibration time in the partitioning and toxicity of zinc in spiked sediment bioassays. Environ Toxicol Chem 23, 65-71

Lei L, Khodadoust AP, Suidan MT, Tabak HH (2005): Biodegradation of sediment-bound PAHs in field contaminated sediment. Water Res 39, 349-361

Leppänen MT, Kukkonen JVK (2000): Effect of sediment-chemical contact time on availability of sediment-associated pyrene and benzo[a]pyrene to oligochaete worms and semi-permeable membrane devices. Aquat Toxicol 49, 227-241

Leweke EC (1999): Einfluss verschiedener Lagerungsbedingungen auf die ökotoxikologische Wirkung von Kunst- und Süßwassersedimenten - Untersuchungen mit *Daphnia magna*, *Enchytraeus albidus*, *Chironomus riparius* und *Tubifex tubifex*. Cuvillier Verlag Göttingen, 194 pp

Loos M, Ragas AMJ, Plasmeijer R, Schipper AM, Hendriks AJ (2010): Eco-SpaCE: An object-oriented, spatially explicit model to assess the risk of multiple environmental stressors on terrestrial vertebrate populations. Sci Total Environ 408, 3908-3917

Luthy RG, Aiken GR, Brusseau ML, Cunningham SD, Gschwend PM, Pignatello JJ, Reinhard M, Traina SJ, Weber WJ, Westall JC (1997): Sequestration of hydrophobic organic contaminants by geosorbents. Environ Sci Technol 31, 3341-3347

Menchai P, Van Zwieten L, Kimber S, Ahmad N, Rao PSC, Hose G (2008): Bioavailable DDT residues in sediments: Laboratory assessment of ageing effects using semi-permeable membrane devices. Environ. Pollut. 153, 110-118

Moermond CTA, Roessink I, Jonker MTO, Meijer T, Koelmans AA (2007): Impact of polychlorinated biphenyl and polycyclic aromatic hydrocarbon sequestration in sediment on bioaccumulation in aquatic food webs. Environ Toxicol Chem 26, 607-615

Morrison DE, Robertson BK, Alexander M (2000): Bioavailability to earthworms of aged DDT, DDE, DDD, and dieldrin in soil. Environ Sci Technol 34, 709-713

Nam K, Alexander M (1998): Role of nanoporosity and hydrophobicity in sequestration and bioavailability: Tests with model solids. Environ Sci Technol 32, 71-74

Nam K, Chung N, Alexander M (1998): Relationship between organic matter content of soil and the sequestration of phenanthrene. Environ Sci Technol 32, 3785-3788

Northcott GL, Jones KC (2000a): Developing a standard spiking procedure for the introduction of hydrophobic organic compounds into field-wet soil. Environ Toxicol Chem 19, 2409-2417

Northcott GL, Jones KC (2000b): Experimental approaches and analytical techniques for determining organic compound bound residues in soil and sediment. Environ. Pollut. 108, 19-43

OECD (1984): Guideline for Testing of Chemicals 207: Earthworm, Acute Toxicity Tests. Organisation for Economic Cooperation and Developement

Oikari A, Fragoso N, Leppanen H, Chan T, Hodson PV (2002): Bioavailability to juvenile rainbow trout (Oncorynchus mykiss) of retene and other mixed-function oxygenase-active compounds from sediments. Environ Toxicol Chem 21, 121-128

Park RA, Clough JS, Wellman MC (2008): AQUATOX: Modeling environmental fate and ecological effects in aquatic ecosystems. Ecol Model 213, 1-15

Pignatello JJ, Xing BS (1996): Mechanisms of slow sorption of organic chemicals to natural particles. Environ Sci Technol 30, 1-11

Puglisi E, Vernile P, Bari G, Spagnuolo M, Trevisan M, de Lillo E, Ruggiero P (2009): Bioaccessibility, bioavailability and ecotoxicity of pentachlorophenol in compost amended soils. Chemosphere 77, 80-86

Re A, Freitas R, Sampaio L, Rodrigues AM, Quintino V (2009): Estuarine sediment acute toxicity testing with the European amphipod Corophium multisetosum Stock, 1952. Chemosphere 76, 1323-1333

Reichenberg F, Mayer P (2006): Two complementary sides of bioavailability: Accessibility and chemical activity of organic contaminants in sediments and soils. Environ Toxicol Chem 25, 1239-1245

Reid BJ, Jones KC, Semple KT (2000a): Bioavailability of persistent organic pollutants in soils and sediments - a perspective on mechanisms, consequences and assessment. Environ. Pollut. 108, 103-112

Reid BJ, Stokes JD, Jones KC, Semple KT (2000b): Nonexhaustive cyclodextrin-based extraction technique for the evaluation of PAH bioavailability. Environ Sci Technol 34, 3174-3179

Ryder K, Temara A, Holdway DA (2004): Avoidance of crude-oil contaminated sediment by the Australian seastar, Patiriella exigua (Echinodermata : Asteroidea). Mar. Pollut. Bull. 49, 900-909

Sae-Ma B, Meier PG, Landrum PF (1998): Effect of extended storage time on the toxicity of sediment-associated cadmium on midge larvae (Chironomus tentans). Ecotoxicology 7, 133-139

Saghir SA, Bartels MJ, Budinsky RA, Harris EE, Clark AJ, Staley JL, Chai Y, Davis JW (2007): Effect of organic carbon content, clay type, and aging on the oral bioavailability of hexachlorobenzene in rats. Environ Toxicol Chem 26, 2420-2429

Schmitt C, Balaam J, Leonards P, Brix R, Streck G, Tuikka A, Bervoets L, Brack W, van Hattum B, Meire P, de Deckere E (2010): Characterizing field sediments from three European river basins with special emphasis on endocrine effects - A recommendation for Potamopyrgus antipodarum as test organism. Chemosphere 80, 13-19

Seiler TB, 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

Seiler TB, Ahlf W, Fahnenstich C, Gilberg D, Melbye K, Meller M, Hammers-Wirtz M, Heininger P, Hollert H, Höss S, Neumann-Hensel H, Ottermanns R, Ratte HT, Spira D, Weber J, Feiler U (2010): SeKT joint research: Investigation of formulated and natural spiked sediment samples in limnic sediment contact assays. in prep.

Semple KT, Morriss AWJ, Paton GI (2003): Bioavailability of hydrophobic organic contaminants in soils: Fundamental concepts and techniques for analysis. European Journal of Soil Science 54, 809-818

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

Sormunen AJ, Leppanen MT, Kukkonen JVK (2009): Examining the role of temperature and sediment-chemical contact time on desorption and bioavailability of sediment-associated tetrabromo diphenyl ether and benzo(a)pyrene. Ecotox Environ Safe 72, 1234-1241

Sormunen AJ, Tuikka AI, Akkanen J, Leppanen MT, Kukkonen JVK (2010): Predicting the Bioavailability of Sediment-Associated Spiked Compounds by Using the Polyoxymethylene Passive Sampling and Tenax(A (R)) Extraction Methods in Sediments from Three River Basins in Europe. Arch Environ Con Tox 59, 80-90

Ten Hulscher TEM, Postma J, Den Besten PJ, Stroomberg GJ, Belfroid A, Wegener JW, Faber JH, Van Der Pol JJC, Jan Hendriks A, Van Noort PCM (2003): Tenax extraction mimics benthic and terrestrial bioavailability of organic compounds. Environ Toxicol Chem 22, 2258-2265

Turesson EU, Stiernstrom S, Minten J, Adolfsson-Erici M, Bengtsson BE, Breitholtz M (2007): Development and reproduction of the freshwater harpacticoid copepod Attheyella crassa for assessing sediment-associated toxicity. Aquat Toxicol 83, 180-189

Van Hoof PL, Kukkonen JVK, Landrum PF (2001): Impact of sediment manipulation on the bioaccumulation of polycyclic aromatic hydrocarbons from field-contaminated and laboratory-dosed sediments by an oligochaete. Environ Toxicol Chem 20, 1752-1761

Wölz J, Engwall M, Maletz S, Takner HO, 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 R 15, 536-553

Xu YP, Spurlock F, Wang ZJ, Gan J (2007): Comparison of five methods for measuring sediment toxicity of hydrophobic contaminants. Environ Sci Technol 41, 8394-8399

Xu YP, Gan J, Wang ZJ, Spurlock F (2008): Effect of aging on desorption kinetics of sediment-associated pyrethroids. Environ Toxicol Chem 27, 1293-1301

You J, Brennan A, Lydy MJ (2009): Bioavailability and biotransformation of sediment-associated pyrethroid insecticides in Lumbriculus variegatus. Chemosphere 75, 1477-1482

Zhong H, Wang WX (2006): Influences of aging on the bioavailability of sediment-bound Cd and Zn to deposit-feeding sipunculans and soldier crabs. Environ Toxicol Chem 25, 2775-2780

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 Soil Sediment 11, 352-363

Chapter 3

A question of timing - Time-dependence of results in three sediment contact test systems using fish embryos, bacteria and nematodes

Hanno Zielke[1], Carina Adamzyk[1], Matthias Berens[1], Rachel Conrad[1], Mackenzie Gerringer[2], Johanna Oellers[1], Richard Ottermanns[3], Nina Rehage[1], Anne J. Schneider[1], Anne Simon[1], Denise Spira[4], Emily Weigel[5], Wolfgang Ahlf[6], Ute Feiler[4], Thomas-Benjamin Seiler[1] and Henner Hollert[1]

[1]Department of Ecosystem Analysis, Institute for Environmental Research (Biology V), RWTH Aachen University, Aachen, Germany

[2]Whitman College, Walla Walla, Washington, USA

[3]Department of Environmental Biology and Chemodynamics, Institute for Environmental Research (Biology V), RWTH Aachen University, Aachen, Germany

[4]Department of Biochemistry and Ecotoxicology, German Federal Institute of Hydrology, Koblenz, Germany

[5]Georgia Institute of Technology, Atlanta, Georgia, USA

[6]Institute of Environmental Technology and Energy Economics, Technical University Hamburg-Harburg, Germany

3.1 Abstract

3.1.1 Purpose

Direct sediment contact tests (SCTs) are excellent tools for effect-focused sediment assessment. The availability of sediment-bound pollutants is often time-dependent. Results obtained in SCTs can thus be expected to depend on the time that has passed since the initial contamination, or spiking. Hence, the present pilot study investigated the time-dependency of effects in SCTs.

3.1.2 Materials and methods

Three organic pollutants (3,4-dichloroaniline, fluoranthene and pentachlorophenol) were spiked into one natural and one artificial sediment and stored at 15 °C. The samples were tested directly after spiking and at regular intervals in three SCTs, the fish embryo sediment contact test (FCT) with the zebrafish *Danio rerio*, the bacterial contact test (BCT) with the soil bacterium *Arthrobacter globiformis*, and the nematode contact test (NCT) with *Caenorhabditis elegans.*

3.1.3 Results and discussion

Results confirmed that effects in SCTs can be time-dependent. Exposure directly after spiking gave mortality in all spiked samples in the FCT. These effects were completely lost within 2-16 weeks of storage after spiking at 15°C. A similar loss became evident for FA-spiked natural sediment in the NCT. PCP was the only substance inducing measurable effects in the BCT. These effects did not change over the course of 44 weeks. However, variations in the bacterial reactions between days of treatments became evident by directly plotting the increase of fluorescence instead of normalised data. These variations have not been reported for this test system before and need to be taken into account for the proper interpretation of BCT results.

3.1.4 Conclusions

The present effect-focused pilot study showed that the time-dependence of effects in SCTs can influence results. Hence, time-dependence needs to be considered in study design and interpretation of results. The exact time-frame and direction of this dependency, however, have to be individually determined for each set of chemicals, model organisms, sediments, and is further influenced by other environmental factors.

3.2   Introduction

Direct sediment contact tests (SCTs), also referred to as whole-sediment toxicity tests, directly integrate numerous factors relevant for the impact of sediment-bound pollutants. Factors such as species dependence, compound characteristics, sediment properties and environmental impacts are expressed as biological effects (e.g. Blaha et al. 2010, Conder et al. 2004, Dillon et al. 1994, Duft et al. 2003, Eklund et al. 2010, Feiler et al. 2009, Höss et al. 2010, Ingersoll et al. 1995, Kosmehl et al. 2006, Lee et al. 2004, Traunspurger et al. 1997, Weber et al. 2006). SCTs are of great ecological relevance because they do not overestimate risk-potential, but rather directly express bioavailability on the level of effect (Feiler et al. 2005, Hollert et al. 2009). In addition, SCTs ensure lower alteration of the sample compared to commonly applied strategies for sediment assessment based on extraction, as reviewed by Seiler et al. (2008). Hence, SCTs are excellent tools for effect-focused sediment assessment which in turn allow for subsequent decisions if further assessment measures are necessary. Especially in the context of REACh and the amended European water framework directive (EWFD, EC 2000, 2006, 2008), such tools are called upon in laboratory screening of single chemicals with regard to their effects in sediments and to monitor field samples for biological impact.

Binding of pollutants to sediments is time-dependent. With increasing time, sequestration occurs and pollutants become less available, a phenomenon termed ageing (Alexander 2000, Hatzinger und Alexander 1995, Kelsey et al. 1997). Scientific literature thoroughly covers ageing of substances in sediments as well as the time-dependent uptake at the chemical level (e.g. Chai et al. 2008, Conrad et al. 2002, Jones et al. 2008, Leppänen & Kukkonen 2000, Morrison et al. 2000, Saghir et al. 2007, Sormunen et al. 2009, Xu et al. 2008, Zhong & Wang

2006). Sediment-pollutant contact time also influences effects in SCTs. The integrative capacity of SCTs causes this time-dependency to account for more than ageing, though, because processes like degradation (Brennan et al. 2009, Lei et al. 2005, Puglisi et al. 2009, You et al. 2009) or re-mobilisation (Hilscherova et al. 2010, Van Hoof et al. 2001, Wölz et al. 2008) are also covered and translated into biological endpoints. A small number of studies have elucidated the time-dependencies of SCT results (Conder et al. 2004, Dillon et al. 1994, Jones et al. 2008, Lee et al. 2004, Oikari et al. 2002, Ryder et al. 2004, Sae-Ma et al. 1998, Xu et al. 2007). However, these investigations have often focused on particular relationships like avoidance of oil-containing sediments by seastars (Ryder et al. 2004) or time-dependency of pollutant-induced CYP1A induction in juvenile rainbow trouts (Oikari et al. 2002). In order to fully benefit from the advantages of SCTs, there is an apparent need to collect more data to better understand how effects measured in these tests depend on time.

The present pilot study was carried out in order to assess the time-dependence of the effects of the three organic pollutants 3,4-dichloroaniline (DCA), fluoranthene (FA) and pentachlorophenol (PCP) in three different SCTs. The three biotest systems were the fish embryo sediment contact test (FCT) with zebrafish (*Danio rerio*) embryos (Hollert et al. 2003), the bacterial contact test (BCT) with *Arthrobacter globiformis* (Ahlf 2007, DIN 2002, ISO 2009, Neumann-Hensel & Melbye 2006, Rönnpagel et al. 1995) and the nematode contact test (NCT) with *Caenorhabditis elegans* (Höss et al. 2009, Traunspurger et al. 1997).

Test systems were chosen to represent different modes of life and access to sediment-bound chemicals. The FCT was introduced by Hollert et al. (2003) as an addition to the original aqueous fish embryo test protocol described by Nagel et al. (2002) and updated by Braunbeck et al. (2005) and Lammer et al. (2009). The aqueous version of the test is mandatory in waste water effluent testing in Germany (DIN 2001) and is internationally standardised (ISO 2007). The zebrafish is a well-established model organism and its development has been described in detail (Kimmel et al. 1995). This makes the test system an excellent tool to assess the impact of pollutants on the embryogenesis of an aquatic vertebrate.

The BCT was included into the test battery in order to account for the impact on sediment-associated bacteria. *Arthrobacter* sp. dominates the group of aerobic, chemoheterotrophic soil- and sediment-living bacteria. The assay allows assessment of toxicity *in situ*, without need for separation of the bacteria from the solid material (Ahlf 2007, DIN 2002, Neumann-Hensel & Melbye 2006, Rönnpagel et al. 1995, Standardisation 2009).

The sediment-dwelling bacterivorous nematode *Caenorhabditis elegans* has been successfully used as a test organism for investigating freshwater sediments (Comber et al. 2006, Comber et

al. 2008, Höss et al. 2009, Traunspurger et al. 1997). A standardised test guideline is available (ISO 2010). In the present study, the NCT represented organisms that are in external contact with the tested sediment and also ingest and potentially extract particle-bound pollutants. Because nematodes are primary consumers, bioaccumulation is a third potential pathway for uptake of chemicals (Höss et al. 2009).

All three spiked chemicals represented different classes of substances: Fluoranthene (FA) is an example of polyaromatic hydrocarbons, pentachlorophenol (PCP) of halogenated aromatic hydrocarbons and 3,4-dichloroanilin (DCA) of halogenated anilines. DCA is applied as the positive control in the FCT (DIN 2001, Nagel 2002). Its chemical fate has been analysed in detail in sediment-water systems (Heim et al. 1995). FA is used as reference substance in ageing and sediment sorption studies (e.g. Hawthorne et al. 2002, Moermond et al. 2007, Tang & Alexander 1999, Van Noort et al. 2003). PCP is known to bioaccumulate and to be very toxic (Hanna et al. 2004, Maenpaa et al. 2008, Puglisi et al. 2009). FA and PCP are listed as priority pollutants in Annex II of the amended EWFD (EC 2008).

The present study compared one natural and one artificial sediment in all three test systems in order to investigate the impact of sediment type on the time-dependency of SCT results. The two sediments chosen were suggested as references for their respective sediment type by the German SeKT (Sediment KontaktTest) framework project. SeKT compared six different SCTs and assessed applicable reference sediments and toxicity thresholds for these test systems (Feiler et al. 2009, Feiler et al. 2005, Höss et al. 2010). The three SCTs in the present study have all been part of the SeKT test battery.

3.3   Material and methods

3.3.1   Sediments, substances and spiking

Natural sediment was sampled with a Van Veen grab from a depth of 5 m at Altrip, a back water of the river Rhine near Worms (river km 416.9, Rhineland-Palatinate, Germany) in August 2008 and May 2009. Total organic carbon was 34 g/kg (Feiler et al. 2009). Sediment composition was 0.8 % gravel, 1.3 % sand, 74.1 % silt and 23.4 % clay. Chemical analyses revealed no relevant background contamination. Details on chemical concentrations are given in Höss et al. (2010) and the final report of the SeKT framework project (Feiler et al. 2009).

Artificial sediment was prepared according to OECD guideline 218 (OECD 2004) except for kaolin clay content. Clay content was reduced from 20 % to 5 % and replaced with quartz sand (F36; Quarzwerke Frechen, Frechen, Germany) because greater clay content has been shown to negatively impact the NCT (Feiler et al. 2009). Sediment composition was 89 % quartz sand, 5 % kaolin clay (Sigma-Aldrich, Steinheim, Germany), 5 % peat (Klasmann-Deilmann GmbH, Geeste, Germany) and 1 % $CaCO_3$ (Sigma).

Sediment dry weight was 32-34 % (natural) and 58-63 % (artificial), determined by drying a defined amount of sediment for 14 h at 105 °C and measuring weight loss. PH values were 7.5 (natural) and 6.7 (artificial). Prior to spiking, sediments were stored in a darkroom at 4 °C. In order to exclude background effects by residual contamination (natural sediment) or formulation (artificial sediment), unspiked samples were tested as sediment controls in all experiments.

Substance purities were ≥ 98.0 % (DCA), ≥ 99.0 % (FA) and ≥ 98.9 % (PCP; all obtained from Sigma). Log K_{ow} values of the chemicals are 2.7 (DCA), 3.0 (PCP) and 5.2 (FA Sediments were spiked according to the OECD Guidelines 207 (OECD 1984) and 218 (OECD 2004) in three spiking replicates per sample. 10 % of sediment total wet weight were dried for 14 h at 105 °C. Acetone (p.a., ≥ 99.5 %, Sigma) was used as solubiliser. Per gramme sediment dry weight, 200 - 250 µl acetone were applied and completely evaporated at room temperature for three days before remixing the spiked portion with the remaining 90% sediment. To exclude background effects caused by either residual acetone or the drying procedure, solubiliser controls containing no spike and a process control consisting of 90 % wet and 10 % dry sediment but no acetone were prepared in parallel. In order to cover time-dependency directly on from the initial "contamination event", the first biotests were always carried out immediately after spiking. Samples were stored as native (wet) sediment at 15 °C $\pm$ 1 °C in darkness.

3.3.2   Sediment contact test with *Danio rerio* embryos
Fish culture

Zebrafish were maintained according to Braunbeck et al. (2005) in 30 L aquaria at 26 $\pm$ 1°C in hatching groups of 12 males and 8 females. Photoperiod was adjusted to 10 h of darkness and 14 h of light. Aquaria were filled with charcoal filtered tap water. Water temperature was 26 $\pm$ 1 °C with an oxygen saturation of ≥ 80 % and pH-value was 7.8 $\pm$ 0.5. Water hardness

was 11 °dH. Tank water was exchanged once a week. The fish were daily fed dry flakes (TetraMin™, Tetra GmbH, Melle, Germany) and *Artemia* sp. nauplii (Great Salt Lake Artemia Cysts, Sanders, Ogden, USA).

Embryo collection

For spawning, glass dishes were transferred into the tanks on evenings before experiments. In order to prevent egg predation, dishes were covered with a stainless steel grid with a mesh opening size of 1 mm through which the eggs could drop. Plastic plant imitations were attached to the mesh in order to stimulate mating. Spawning occurred within 0.5 - 1 h after the onset of illumination.

Test

The sediment contact test with zebrafish embryos was based on the aqueous fish embryo test according to DIN 38415-6 (DIN 2001) and the methods given in Nagel (2002) and Lammer et al (2009). Modifications for use in sediment assessment are given in detail in Hollert et al. (2003). Artificial water prepared according to ISO 7346/3 (ISO 1996) was used as the test medium (294.0 mg/l $CaCl_2 \cdot 2 H_2O$, 123.3 mg/l $MgSO4 \cdot 7 H_2O$, 63.0 mg/l $NaHCO_3$ and 5.5 mg/l KCl). The test was carried out in 6-well plates with 3 g wet weight test sediment and 5 ml artificial water per 5 fish embryos and well, and 20 embryos (4 wells) per sample or control. In addition, the following controls were carried out in each test:

- Quartz sand negative controls (20 eggs, 3 g quartz sand F36 + artificial water)
- Quartz sand positive controls (10 eggs, 3.7 mg/l DCA freshly applied to water phase + 3 g quartz sand F36 + artificial water),
- Aqueous negative controls (40 eggs, artificial water only)
- Aqueous positive controls (20 eggs, artificial water + 3.7 mg/l freshly applied DCA)

Sediment samples were weighed into plates one day before exposure, covered with self-adhesive foil (Nunc, Roskilde, Denmark), and placed on a horizontal shaker over night at 50 rpm and 26 ± 1 °C. Only fertilized and normally developed eggs which were at least in the 8-cell stage were selected for testing using a binocular microscope (SMZ 1500, Nikon, Düsseldorf, Germany). Selected eggs were then transferred into the wells containing the

prepared sediment. Wells were re-covered with self-adhesive foil and incubated for 48 h on a horizontal shaker at 50 rpm and $26 \pm 1\,°C$. After exposure, eggs were collected from the sediment by means of a 5 ml-pipette. In order to aid re-collection of eggs from sediment, wells were illuminated with a cold light source (KL 1500 LCD, Schott, Mainz, Germany). Eggs were then briefly rinsed in artificial water to remove remaining sediment particles and evaluated for effects (i.e. mortality) by means of an inverse microscope (Eclipse TS100, Nikon, Düsseldorf, Germany). Mortality criteria were (a) coagulation, (b) lack of heartbeat, (c) missing somite development and (d) failure of tail detachment from the yolk sack (Braunbeck et al. 2005, DIN 2001, Hollert et al. 2003, ISO 1996, Nagel 2002).

Validity criteria in the FCT

In accordance with test guidelines (DIN 2001, ISO 2007, Nagel 2002), results of positive controls and quartz positive controls (3.7 mg/l DCA) had to be $\geq 20\,\%$ as a control for the validity of a given test. Literature reports a mortality of $68 \pm 24\,\%$ for this concentration in a 16 lab-study (DIN 2001) and $LC_{50,DCA}$ of 3.3 ± 0.5 mg/l (DIN 2001) as well as 1.6 to 2.4 mg/l (Lammer et al. 2009). Negative, quartz negative, sediment, process and solubiliser controls were regarded valid if the mortality did not exceed 10 % (DIN 2001, ISO 2007, Nagel 2002). As further quality criterion, egg fertilisation rate was required to be $\geq 70\,\%$ in order for a test to be carried out (Lammer et al. 2009).

3.3.3   Bacterial contact test

The bacterial contact test with *Arthrobacter globiformis* was carried out according to DIN 38412 L 48 (DIN 2002) and ISO 10871 (ISO 2009), adapted to include the optimisations proposed by Neumann-Hensel and Ahlf (Ahlf 2007, Neumann-Hensel & Melbye 2006). The test strain was obtained from the Institute of Environmental Technology and Energy Economics (Technical University Hamburg-Harburg, Germany) and is also available at the German collection of microorganisms (DSM, Braunschweig; strain No. 20124). Frozen stock cultures were incubated in 50 ml sterile (autoclaved for 20 min at 121 °C) growth medium containing 10.0 g/l casein peptone, 5.0 g/l yeast extract (both Sigma), 5.0 g/l D(+)-glucose and 5.0 g NaCl (both Merck KGaA, Darmstadt, Germany) on a horizontal shaker for 8 h at

30 °C and 150 rpm in the dark. 1 ml of this pre-culture was transferred into 50 ml sterile growth medium and again incubated at 30 °C for 16 h at 100 rpm in the dark. Afterwards, optical density at 600 nm (OD_{600}) was measured and adjusted to 0.4 by dilution. This corresponds to approximately 10^8 cells/ml. Subsequently, 4 % v/v DMSO were added to 1 ml of bacterial solution as anti-freezing agent and stored at -80° C until usage.

For tests, pre-cultures were prepared by transferring 100 µl of stock culture into 50 ml of test medium and incubating overnight for 14 - 16 h on a horizontal shaker at 150 rpm and 30 °C. Growth medium diluted at a ratio of 1:3 was used as test medium. Both media were autoclaved for 20 min at 121 °C. In order to prepare test inocula, OD_{600} was measured and the pre-culture was diluted to an OD_{600} of 0.2. The diluted pre-culture was incubated again under the same conditions until OD_{600} was 0.4 ± 0.04, i.e. bacteria were in logarithmic phase of growth.

Tests were carried out in 24-well plates. Each well received 0.6 g of sediment sample. Subsequently, 0.6 ml of distilled water were added into each well, plates were covered with oxygen-permeable self-adhesive foil, and shaken for 24 h at 240 rpm. Directly prior to the addition of bacteria, native microorganisms were deactivated by heating the plates two times to 85°C for 20 min in a water bath. Then, 0.4 ml of inoculum with an $OD_{600} = 0.4 \pm 0.04$ were added into each well (Fig. 3.1). Afterwards, the plates were incubated on a horizontal shaker for 2 h at 30 °C and 150 rpm in the dark. Into each well, 0.8 ml of the redox-active dye resazurine (45 mg/l, Sigma) in 1 M MOPS buffer (3-(N-morpholino)-propansulfonic acid, $C_7H_{15}NO_4S$, ≥ 99 %, Roth, Karlsruhe, Germany; pH adjusted to 8.2 with 10 M NaOH) were added in no/low light conditions. The plates were then shaken for an additional 60 min at 30°C. During this time, dehydrogenase activity was monitored by measuring fluorescence of resorufine (em. 535 nm, exc. 590 nm; manual amplification factor 75; Tecan Infinite M 200 Fluorescence Reader, Tecan Austria GmbH Grödig/Salzburg, Austria) formed by resazurine reduction every 15 min.
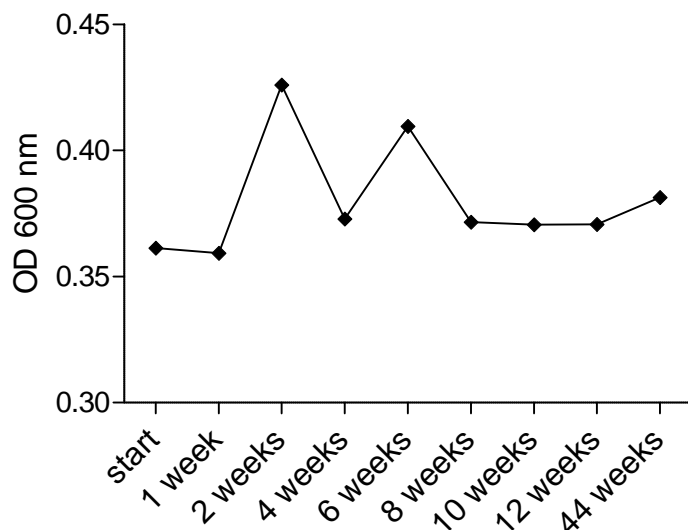
Fig. 3.1 OD_{600nm} of the bacterial solution applied in the bacterial contact test for different times of treatment

Calibration, controls and validity

Five additional controls were carried out in all tests in addition to sediment-related controls (sediment, process and solubiliser controls, see above):

(1) Blanks (whole wet sediment + medium, no bacteria) both with sediments containing spiked organic compounds to account for fluorescence effects of test substances, and without spike to test for residual bacteria after pasteurisation.

(2) Growth controls (water + bacteria) to show uninhibited resorufine production.

(3) Quartz negative control (quartz sand + bacteria) to provide data on bacterial growth in a system containing a solid phase comparable in between tests with different sediments

(4) Quartz positive control (quartz sand + bacteria + 600 mg benzalkonium chloride/kg sediment dry weight). Benzalkonium chloride (BAC) is recommended as positive control (DIN 2002, ISO 2009). It was applied at concentration of 600 mg/kg dry weight (EC_{50} according to DIN 38412-48).

In modification to the protocol provided by guidelines (DIN 2002, ISO 2009), quartz sand (F36, Milisil, Quarzwerke Frechen, Germany) washed with distilled water was substituted for quartz powder (W4, Quarzwerke) in quartz controls, because F36 quartz sand was also a primary component of the applied OECD 218 artificial sediment, thus allowing for better comparison. Sand was adjusted to 33 % humidity with bi-distilled water.

In order for a test to be valid and included in the results, fluorescence had to increase in the test period from 0 min to 60 min by a factor of at least 5 (DIN 2002).

Measuring fluorescence directly in the wells containing sediment carries the risk that matrix-related effects could quench fluorescence (Ahlf 2007). In order to account for such quenching effects, calibrations were run for both tested sediments. Equimolar resazurine (45 mg/l) and resorufine (42 mg/l, both 0.196 mM, Sigma) solutions were prepared in MOPS buffer and applied to wet natural and artificial sediment, quartz sand and distilled water. Fluorescence was measured and the slope of each calibration line was determined.

Evaluation

Dehydrogenase inhibition was calculated in relation to sediment controls and the quartz sand negative control for each sample by first subtracting blank slopes from measured sample slopes using equation 1:

$$S_{M-B} = S_M - S_B \qquad (1)$$

S_{MB} = Slope of sample after subtracting blanks

S_M = Measured slope between 0 and 60 min

S_B = Slope of blanks between 0 and 60 min

Next, calibration was accounted for by using equation 2:

$$S_N = \frac{S_{MB}}{S_{Cal}} \qquad (2)$$

S_N = normalised fluorescence slope between 0 and 60 min

S_{Cal} = Slope of calibration line

Finally, inhibitions were related to controls with equation 3:

$$I = 100 - \left[\frac{Sn}{Sc} \times 100 \right] \qquad (3)$$

I = Inhibition of bacterial dehydrogenase activity in percent

S_c = Slope of control between 0 and 60 min

3.3.4 Nematode contact test

The NCT was carried out according to ISO 10872 (ISO 2010) and the methods given in Höss et al. (2009) and Traunspurger et al. (1997). In order to create a food-culture for *C. elegans*, 200 µl of *Escherichia coli* stock solution were incubated in 20 ml LB-medium over-night at 37 °C on a horizontal shaker. In the next morning, a sterile agar plate was inoculated with the E. coli over-night suspension by means of a spatula and incubated for 8 h at 37 °C. 8 h later, an agar piece of approximately 1 cm² from an agar culture containing *C. elegans* dauer larvae was transferred onto the food-culture plate and incubated for 3 d at 21 °C (worm culture plate).

For each sample and control, 0.5 g sediment were transferred into glass vials in triplicate. In addition, triplicates of aqueous positive control (benzalkonium chloride (160 mg/l) and aqueous negative controls (M9-medium) were prepared. One day before exposure, a second food culture of *E. coli* was prepared by incubating 20 µl *E. coli* stock solution in 50 ml LB-medium for 17-22 h at 37 °C on a horizontal shaker. For exposure, 200 µl of this food culture were transferred into 6 ml M9-medium and adjusted to 12000 ± 600 formazine adsorption units (FAU) by measurement of OD_{600nm} and subsequent dilution. After adjustment, 0.5 ml food culture were added to each sample vial. In case of the aqueous controls, a food solution with a concentration of 1000 ± 50 FAU was used.

Juvenile worms were collected from worm culture plate by filtration through two filters with mesh openings of 10 µm and 5 µm. Ten worms were transferred into each sample vial. All samples were then incubated at 21 °C.

After 96 h of exposure, worms were stained by addition of 1 ml bengal rose (300 mg/l; Sigma) to each sample and subsequent heating at 80 °C for 15-20 min. For evaluation, worms were extracted by mixing the sediment with 2 ml Ludox TM50 (Sigma) and centrifugation at 800 rpm. This step was repeated three times. The total 6 ml of ludox were transferred into a petri-dish and juveniles were counted by means of a binocular microscope (SMZ 1500, Nikon).

3.4   Results

3.4.1   Fish embryo sediment contact test

FCT controls were valid with regard to the definitions given above. Mean mortalities ± standard deviations were 3.6 ± 4.9 % (aqueous negative control, n = 21), 73.7 ± 21.0 % (aqueous positive control, n = 21), 5.8 ± 12.7 % (quartz sand negative control, n = 21), 72.1 ± 28.0 % (quartz sand positive control, n = 20), 6.0 ± 5.4 % (natural sediment control, n = 21), 3.4 ± 5.9 % (artificial sediment control, n = 19), 3.6 ± 5.5 % (solubiliser control, natural sediment, n = 21), 1.2 ± 3.0 % (process control, natural sediment, n = 13), 4.5 ± 5.1 % (solubiliser control, artificial sediment, n = 19) and 3.9 ± 3.0 % (process control, artificial sediment, n = 13).

In all FCTs, effects decreased from the start of experiments until mortality was below the validity threshold 10 % (Fig. 3.2a-c). Although concentrations were based on pre-tests and had been chosen to induce 100 % effect, initial mortality was below 100 % in all tests except for the exposure to PCP in artificial sediment.

In natural sediment, mortality of DCA decreased from 94.4 ± 9.6 % to 7.1 ± 7.5 % within 9 weeks (Fig. 3.2a). Mortality of PCP decreased from 72.2 ± 6.3 % to 3.3 ± 2.9 % within 5 weeks (Fig. 3.2b). Effects of FA started at 24.6 ± 12.6 % and decreased to 0.0 ± 0.0 % within 16 weeks (Fig. 3.2c).

In artificial sediment, the decrease was 15.1 ± 21.7 % to 5.6 ± 3.5 % within 2 weeks (DCA), 100.0 ± 0.0 % to 0.0 ± 0.0 % within 9 weeks (PCP) and 51.7 ± 12.6 % to 2.7 ± 4.6 % within 16 weeks (FA), respectively (Fig. 3.2a-c). In case of PCP in artificial sediment, the decrease was less linear than in the other experiments: Mortality decreased to 11.7 ± 12.6 % after two weeks, but increased again to 28.3 ± 24.7 % in week three and 40.8 ± 33.0 % in week four. However, this increase was not statistically significant (one-way ANOVA, $p \leq 0.05$).

In case of all three chemicals, initial mortality was distinctly different between natural and artificial sediment (Fig. 3.2a-c). The greatest difference of initial mortality between both sediment types was observed for DCA (94.4 ± 9.6 % in natural sediment vs. 15.1 ± 21.7 % in artificial sediment).

a 3,4-Dichloroaniline

b Fluoranthene

c Pentachlorophenol

● Natural sediment
○ Artificial sediment
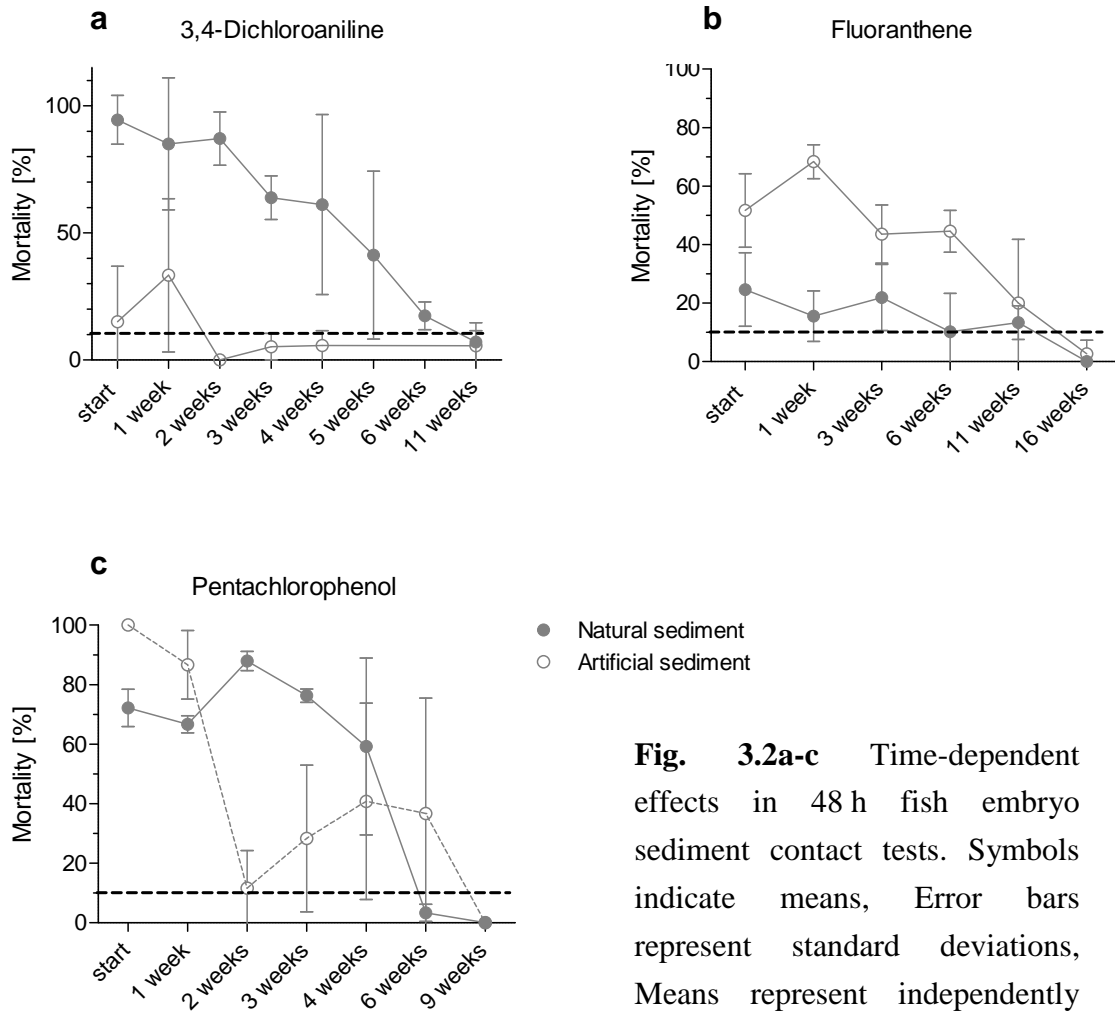
Fig. **3.2a-c** Time-dependent effects in 48 h fish embryo sediment contact tests. Symbols indicate means, Error bars represent standard deviations, Means represent independently spiked and tested treatments (n = 3).

3.4.2 Bacterial sediment contact test

Inhibition of PCP in comparison to the sediment controls varied between 59.4 % and 100.7 % in both sediments at all timepoints from 0 to 12 weeks, and decreased to between 34.2 % and 66.3 % in the test after 44 weeks (Fig. 3.3). In relation to the quartz negative control, inhibition was consistently 80.5 % - 100.3 % (Fig. 3.4). Figure 3.5 compares the non-normalised data, i.e. the slopes of fluorescence increase. These data show that the fluorescence increase of the spiked sediments did not strongly vary between time points including the test after 44 weeks, whereas fluorescence slopes in the growth controls and the quartz sand negative controls were greatly variable. Slopes varied by as much as a factor of 10 in the quartz sand negative control.
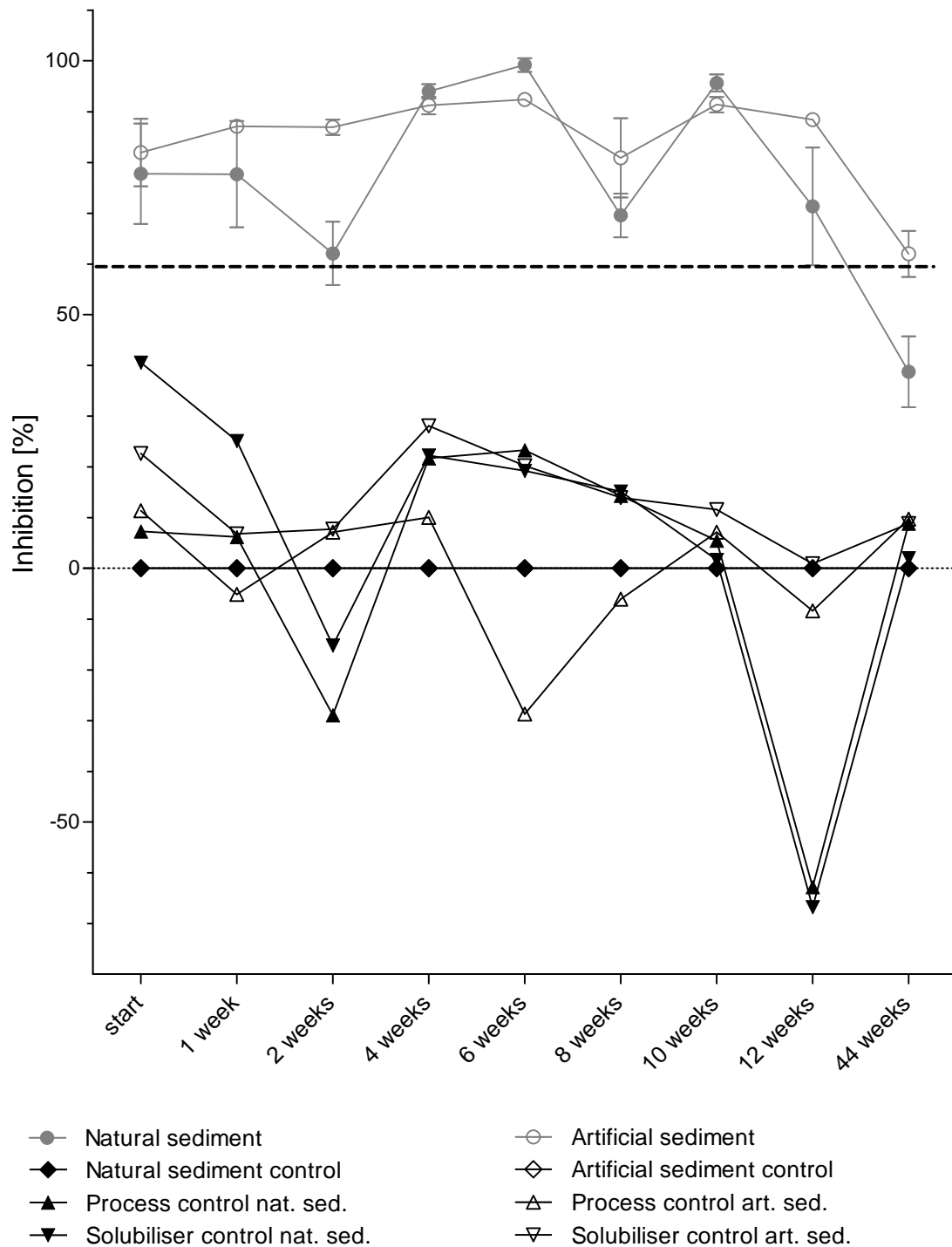
Fig. 3.3 Time-dependent effects of PCP in bacterial contact test with *Arthrobacter globiformis* normalised to sediment controls. Broken line shows effect threshold of 60 %; Symbols indicate means; Error bars represent standard deviations; Means represent independently spiked and tested treatments (n = 3) and controls (n°=°1), each further tested in four on-plate replicates (n = 4). Please refer to text for details on controls and experimental design. Note that mortality apparently dropped to/below 60 % in week 44.
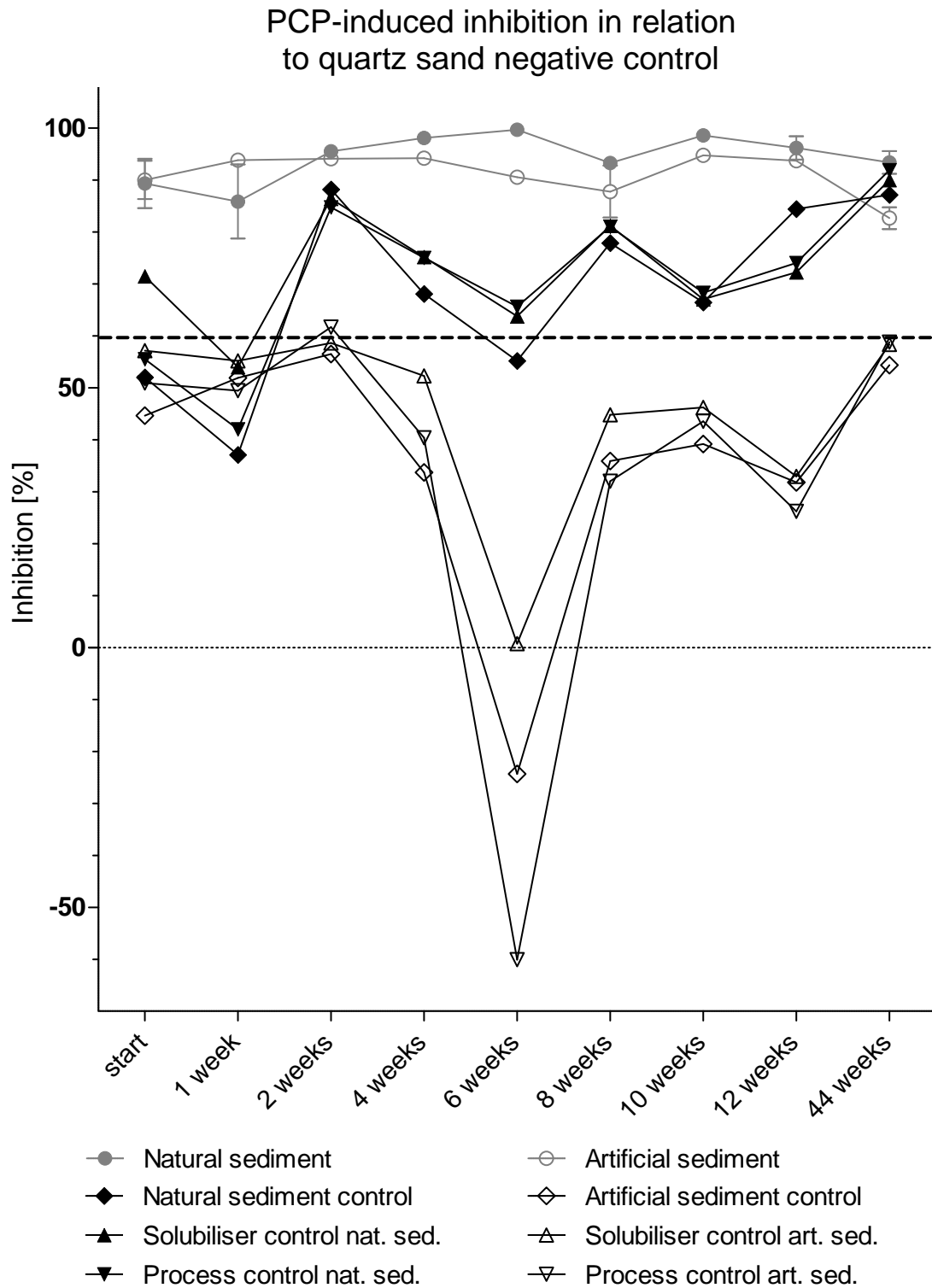
Fig. 3.4 Time-dependent effects of PCP in bacterial contact test with *Arthrobacter globiformis* normalised to quartz sand negative control; Broken line shows effect threshold of 60 %; Symbols indicate means; Error bars represent standard deviations; Means represent independently spiked and tested treatments (n = 3) and controls (n°=°1), each further tested in four on-plate replicates (n = 4); Please refer to text for details on controls and experimental design.

Variations of fluorescence between days of treatment in the BCT

Legend:
- ● Nat. sediment, spiking replicate 1
- ◕ Nat. sediment, spiking replicate 2
- ◔ Nat. sediment, spiking replicate 3
- ◆ Natural sediment control
- ▲ Process control nat. sed.
- ▼ Solubiliser control nat. sed.
- + Quartz sand positive control
- × Quartz sand negative control
- ◇ Artificial sediment control
- △ Process control art. sed.
- ▽ Solubiliser control art. sed.
- ○ Art. sediment, spiking replicate 1
- ◑ Art. sediment, spiking replicate 2
- ◐ Art. sediment, spiking replicate 3
- ∗ Growth control

Fig. 3.5 Non-normalised slopes of fluorescence increase from 0 to 60 min; Symbols indicate means; Error bars represent standard deviations; Means represent independently spiked treatments and controls each tested in four on-plate replicates (n = 4). Please refer to text for details on controls and experimental design. Note that fluorescence increase of PCP did not change in week 44. Further note correlation between the variations of fluorescence increase.

In comparison to the sediment controls, dehydrogenase inhibition induced by DCA in the BCT varied between experiments, but remained below the effect threshold of 60 % at all times (Fig. 3.6). No ageing experiment was carried out with FA because pre-tests revealed no effects on dehydrogenase activity.
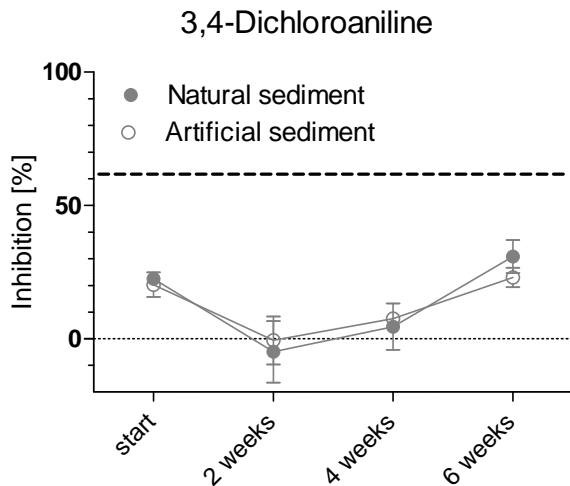
Fig. 3.6 Time-dependent effects of DCA in the bacterial contact test with *Arthrobacter globiformis*. Broken line shows effect threshold of 60 %; Symbols indicate means; Error bars represent standard deviations; Means represent independently spiked and tested treatments (n = 3), each further tested in four on-plate replicates (n = 4).

3.4.3 Nematode sediment contact test

Mean number ± standard deviation of juvenile worms in the controls of the artificial sediment in the NCT was 343 ± 166 (sediment control, n = 12), 356 ± 222 (solubiliser control, n = 12) and 241 ± 174 (process control, n = 12; Fig. 3.7a). Both PCP and FA impacted reproduction, resulting in a total of 32 ± 45 (FA, n = 12) and 6 ± 3 (PCP, n = 12) juvenile worms for all four timepoints. Both reductions were significantly different in comparison to all three controls (Kruskal-Wallis one-way ANOVA on ranks, Student-Newman-Keuls post-hoc test, $p \leq 0.05$; parametric tests: Kolmogorov-Smirnov (normality of distribution), Levene (variance homogeneity)).

In case of natural sediment, the number of worms increased steadily in all controls (Fig. 3.7b). Mean number of juveniles in all three controls was 72 ± 130 (n = 3) at the start of the experiment and increased to 502 ± 166 (n = 3) after two weeks, 1007 ± 349 (n = 3) after four weeks and 1295 ± 226 (n = 3) after six weeks.
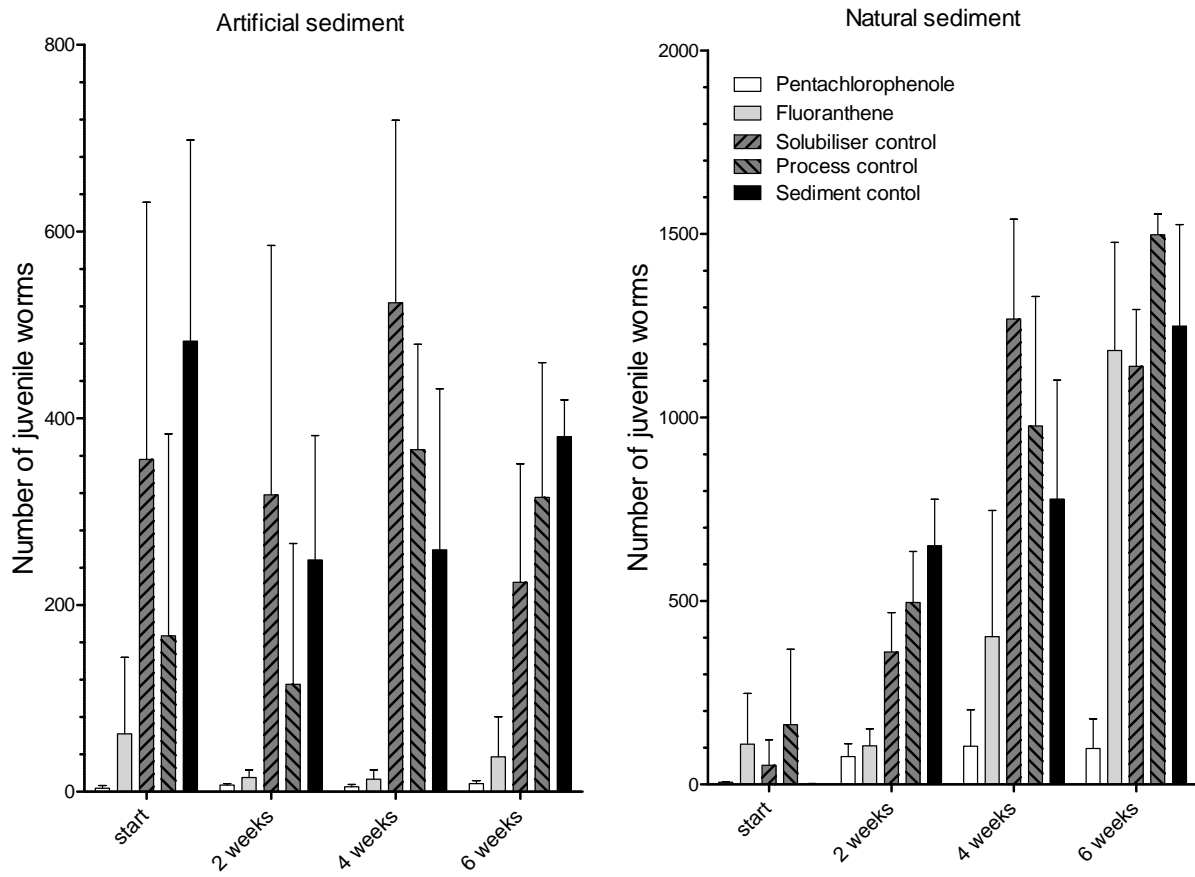
Fig. 3.7 a,b Time-dependent effects in 96 h nematode contact test with *Caenorhabditis elegans*; Columns indicate means; Error bars represent standard deviations; means represent independently spiked and tested treatments (n = 3) and controls (n°=°1), each further tested in three in-test replicates (n = 3).

Sediment spiked with PCP inhibited this increase and lead to a mean number of 71 ± 70 (n = 12) worms over the course of all tested time points. Exposure to FA resulted in 110 ± 138 (n = 3) juveniles at the start of the study and 105 ± 47 (n = 3) after two weeks. After week 2, reproduction increased to 403 ± 344 (n = 3) juvenile worms in week 4 and reached the same level as in the controls in week 6 (1182 ± 294, n = 3). The difference between the number of juveniles in each control and in FA-spiked natural sediment in week 2 was statistically significant (one-way ANOVA, Holm-Sidak post-hoc test, $p \leq 0.05$; parametric tests: Kolmogorov-Smirnov (normality of distribution), Levene (variance homogeneity)).

3.5 Discussion

The aim of the present investigation was to assess the impact of sediment-pollutant contact time on data obtained in SCTs. The results confirmed that biological effects can indeed be affected by time-related changes. These changes depended on the test system as well as substance and sediment type. A complete loss of the initially measured effects of all three test substances occurred in the FCT after 2 to 16 weeks. A loss of effect also occurred in the NCT, but only for FA in natural sediment. Comparable changes have been described in the literature for selected organic pollutants. A time-dependent decrease of TNT toxicity on the earthworm *Tubifex tubifex* of as much as 100 % was shown by Conder et al. (2004) after 4 weeks of sediment-pollutant contact time. Xu et al. (2007) found approximately a two-fold increased LC_{50} values for *Chironomus tetans* exposed to a pyrethroid after 13 weeks. However, time-dependent change does not always result in decreased effects. Dillon et al. (1994) found a significant 30 %-increase in mortality of mysid shrimps (*Mysidopsis bahia*) after exposure to a contaminated field sediment within 2-12 weeks of storage. All these results underline that time-dependence needs to be individually determined individually for each combination of substance and test system.

In the FCT, effects of FA displayed the slowest decrease, even though the four-ring polycyclic aromatic hydrocarbon FA has a log K_{OW} of 5.2, highest of the three applied substances. In contrast, no effects were observed after only two weeks in embryos exposed to DCA-spiked artificial sediment (log $K_{ow,DCA}$ = 2.67). However, the exact number of weeks between the first test and the eventual loss of effects likely depended on a number of factors. As outlined in the introduction, SCTs integrate several complex and diverse factors and translate them into easily obtainable biological results. However, this advantage simultaneously prevents predictions on how exactly a complex interrelation, like the influence of sediment-pollutant contact time, impacts results. The cause of the observed differences in time-dependence might be either species mode of life, i.e. potential exposure routes, or the mode of action of a given chemical. The exact time-frames observed in the present study likely further depended on the initially applied concentrations, which were different for each respective substance and test, on initial mortalities, and on the selected test intervals. A definite cause for the time-dependence or exact values cannot be named here, because the experiments represented an effect-focused pilot-study and no chemical analyses were carried out. The general tendencies show, however, that time-dependents can greatly affect the reactions of SCT systems.

FA and DCA did not induce dehydrogenase inhibitions in the BCT, but PCP persistently caused effects for 44 weeks. Further experiments are needed in order to attribute this to probable causes such as higher bioaccessibility of PCP due to the different spatial limits of bacteria (Semple et al. 2007) or production of toxic metabolites (Puglisi et al. 2009), which could have masked a decrease of dehydrogenase inhibition induced by PCP itself.

In order to obtain an inhibition, the current evaluation procedure according to BCT guidelines is to normalise the slope of fluorescence increase to an appropriate control (DIN 2002, ISO 2009, Neumann-Hensel & Melbye 2006). However, application of this procedure caused effects of PCP to seemingly decrease in week 44, and results varied to a high degree (Fig. 3.3 and 3.4). Direct plotting of the non-normalised slopes revealed that the variations were caused by different bacterial reactions between treatments. Fluorescence varied by as much as 10-fold between treatment days in the quartz sand negative and the growth controls (Fig 3.5). This mode of plotting confirmed that PCP disturbed bacterial reaction and constantly caused effects independent of time. However, it also raised questions concerning the interpretation of results obtained in the BCT. The applied evaluation procedure accounted for quenching effects of the chemical (by means of blanks without bacteria) and sediment matrix (by calibration; Ahlf 2007). Potential impacts of the solubiliser, spiking process, unspiked sediment and indigenous bacteria were also addressed by the respective controls. Therefore, the variations must have been caused by a yet unknown factor that influenced bacterial reactions on the individual day of exposure. Variations of the bacterial concentration at the beginning of exposure were considered as the potential cause of these different reactions. The applied protocol was based on the guideline DIN 38412-48 (DIN 2002) and accordingly used bacteria from an overnight culture for the tests. Neumann-Hensel (2006) proposed an optimisation of this protocol by application of freeze-dried bacteria. However, even though this protocol can shorten test time, it is unlikely to solve the problem of the described variations. This is because in either protocol, bacteria are applied at an OD_{600nm} of 0.40 ± 0.04. This is the determining step for the initial bacterial concentration. As confirmed by correlation analyses ($r^2 = 0.267$ (OD600/growth control) and $r^2 = 0.350$ (OD600/quartz sand negative control); two-tailed Spearman correlation, 95 % confidence interval), no relationship existed between initial OD (Fig 3.1) and the fluorescence slopes of the quartz negative and the growth control (Fig 3.5). The application of quartz sand instead of quartz powder also represents an unlikely source of the variability, because variations also occurred in the aqueous growth controls. Fluorescence increase was greater in the quartz sand control,

but this can be explained by the greater surface area available for bacterial growth provided by the sand.

To our knowledge, similar variations have previously not been reported. Because the potential to influence normalised results is very high, we strongly recommend controlling raw data, i.e. fluorescence slopes, for potential variations when applying and evaluating the BCT. Still, the test provides a valuable first-line procedure in terms of SCTs. As shown by PCP, disturbances in the bacteria-sediment system can lead to a reduction of fluorescence increase which is independent of time, despite great variations in the controls (Fig. 3.5).

The time-dependent development of effects in the natural and the artificial sediment did not show a common tendency in the three SCTs. Effects on fish embryos exposed to spiked artificial sediment either decreased more rapidly (DCA, FA) or more slowly (PCP) than in natural sediment. In the NCT, a decrease and eventual complete loss of effects in comparison to the controls only became apparent for FA-spiked natural sediment within six weeks after initial spiking. These findings might partially be explained by the different exposure routes relevant for the two model organisms. Pollutants that bind more rapidly to binding sites in artificial sediment than in natural sediment also become less available at a faster rate for passive uptake, e.g. by diffusion into zebrafish eggs. However, these substances could be more readily available by more active processes, e.g. gut-fluid extraction in a nematode worm. Again, this represents just one potential explanation, calling for future, chemical-analytical investigations.

With regard to the loss of effects in the NCT, it was striking that all controls in natural sediment displayed effects at the start of the experiment (Fig 3.7b). In weeks two, four and six of storage, the number of juveniles increased steadily. Nematodes might have been sensitive to residual contamination in the controls of natural sediment with an unknown compound whose effect also decreased time-dependently, but this warrants further investigation. Again, this effect in the controls of the natural sediment stresses the need to account for time-dependence in order to properly interpret test results.

Natural sediments have advantages that cannot be easily mimicked by artificial sediment, i.e. composition of the organic matter as well as bacterial abundance and diversity, resulting in a better simulation of "natural" conditions in general (Fleming et al. 1998, Goedkoop et al. 2005). However, reference sites can always be contaminated and thus rendered unsuitable for further collection of samples (Dillon et al. 1994). The application of natural sediments also bears the risk of alteration during storage and pre-treatment processes (Seiler et al. 2008). Artificial sediments, on the other hand, can be applied as a stable reference to relate to,

because they consistently have the same composition and provide reproducible test conditions. However, artificial sediments cannot fully mimic all properties of and conditions in natural sediments (Fleming et al. 1998, Goedkoop et al. 2005, Verrhiest et al. 2002).

The present effect-focused pilot study proved that the relationship between SCT results obtained by the use of different sediment types also depends on the test systems. This relationship needs to be individually determined in order to achieve transferability or extrapolation of data from artificial to natural sediment and vice versa.

3.6   Conclusion and Outlook

The observed time-dependencies clearly indicate that the influence of sediment-pollutant contact time has to be considered in the interpretation of SCT results. This holds true for field samples as well as spiked sediments applied in regulatory assessment. This finding is inherently linked to the question of experimental timing. For instance, OECD guideline 218 for the spiking of sediments suggests one week of equilibration (OECD 2004).

Accordingly, contamination history of field samples should be related to effects in the context of the time-dependence of SCT results. Of course, field samples are subjected to more potentially-influencing environmental factors than laboratory-spiked samples that could mask time-dependent changes (Liess & von der Ohe 2005, Seiler et al. 2008, Solimini et al. 2009, Van Hoof et al. 2001)

With regard to the present results, it seems questionable if it is sufficient to test at only one point of time. If possible, we suggest that several timepoints should be tested in all spiking-based experiments, whenever allowed by the temporal and economic circumstances.

3.7 References

Ahlf W (2007): 'Optimisation of the solid-contact test with Arthrobacter globiformis. J Soil Sediment 7, 67-67

Blaha L, Hilscherova K, Cap T, Klanova J, Machat J, Zeman J, Holoubek I (2010): Kinetic bacterial bioluminescence assay for contact sediment toxicity testing: relationships with the matrix composition and contamination. Environ Toxicol Chem 29, 507-514

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

Brennan AA, Harwood AD, You J, Landrum PF, Lydy MJ (2009): Degradation of fipronil in anaerobic sediments and the effect on porewater concentrations. Chemosphere 77, 22-28

Chai YZ, Davis JW, Saghir SA, Qiu XJ, Budinsky RA, Bartels MJ (2008): Effects of aging and sediment composition on hexachlorobenzene desorption resistance compared to oral bioavailability in rats. Chemosphere 72, 432-441

Comber SDW, Conrad AU, Hoss S, Webb S, Marshall S (2006): Chronic toxicity of sediment-associated linear alkylbenzene sulphonates (LAS) to freshwater benthic organisms. Environ. Pollut. 144, 661-668

Comber SDW, Rule KL, Conrad AU, Hoss S, Webb SF, Marshall S (2008): Bioaccumulation and toxicity of a cationic surfactant (DODMAC) in sediment dwelling freshwater invertebrates. Environ. Pollut. 153, 184-191

Conder JM, La Point TW, Steevens JA, Lotufo GR (2004): Recommendations for the assessment of TNT toxicity in sediment. Environ Toxicol Chem 23, 141-149

Conrad AU, Comber SD, Simkiss K (2002): Pyrene bioavailability; effect of sediment-chemical contact time on routes of uptake in an oligochaete worm. Chemosphere 49, 447-454

Dillon TM, Moore DW, Jarvis AS (1994): The effects of storage-temperature and time on sediment toxicity. Arch Environ Con Tox 27, 51-53

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 (2002): DIN 38412-48: Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlamm-untersuchung: Testverfahren mit Wasserorganismen (Gruppe L) Teil 48: *Arthrobacter globiformis* Kontakttest für kontaminierte Feststoffe. Deutsches Institut für Normung e. V., Berlin

Duft M, Schulte-Oehlmann U, Tillmann M, Markert B, Oehlmann J (2003): Toxicity of triphenyltin and tributyltin to the freshwater mudsnail Potamopyrgus antipodarum in a new sediment biotest. Environ Toxicol Chem 22, 145-152

EC (2000) Directive 2000/60/EC of the european parliament and of the council of 23 October 2000establishing a framework for Community action in the field of water policy. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2000:327:0001:0072:EN:PDF, last accessed 21.03.2011

EC (2006) Regulation No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93

and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32006R1907:EN:PDF, last accessed 09.03.2011

EC (2008) Directive 2008/105/EC of the european parliament and of the council of 16 December 2008 on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:348:0084:0097:EN:PDF, last accessed 09.03.2011

Eklund B, Elfstrom M, Gallego I, Bengtsson B-E, Breitholtz M (2010): Biological and chemical characterization of harbour sediments from the Stockholm area. J Soil Sediment 10, 127-141

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 R 12, 257-258

Feiler U, Ahlf W, Fahnenstich C, Gilberg D, Hammers-Wirtz M, Höss S, Hollert H, Melbye K, Meller M, Neumann-Hensel H, Ratte HT, Seiler TB, Spira D, Weber J, Heininger P 2009: Final report SeKT framework project - Definition von Referenzbedingungen, Kontrollsedimenten und Toxizitätsschwellenwerten für limnische Sedimentkontakttests - SeKT

Fleming RJ, Holmes D, Nixon SJ (1998): Toxicity of permethrin to Chironomus riparius in artificial and natural sediments. Environ Toxicol Chem 17, 1332-1337

Goedkoop W, Widenfalk A, Haglund AL, Steger K, Bertilsson S (2005): Microbial characterization of artificial sediment and comparisons with natural sediments - Implications for toxicity testing. Environ Toxicol Chem 24, 2725-2733

Hanna K, de Brauer C, Germain P (2004): Cyclodextrin-enhanced solubilization of pentachlorophenol in water. Journal of Environmental Management 71, 1-8

Hawthorne SB, Poppendieck DG, Grabanski CB, Loehr RC (2002): Comparing PAH availability from manufactured gas plant soils and sediments with chemical and biological tests. 1. PAH release during water desorption and supercritical carbon dioxide extraction. Environ Sci Technol 36, 4795-4803

Heim K, Schuphan I, Schmidt B (1995): Behaviour of C-14 4-nitrophenol and C-14 3,4-dichloroaniline in laboratory sediment-water systems: 2. Desorption experiments and identification of sorptive fraction. Environ Toxicol Chem 14, 755-761

Hilscherova K, Dusek L, Sidlova T, Jalova V, Cupr P, Giesy JP, Nehyba S, Jarkovsky J, Klanova J, Holoubek I (2010): Seasonally and regionally determined indication potential of bioassays in contaminated river sediments. Environ Toxicol Chem 29, 522-534

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, Ernst M, Ahlf W, Duerr M, Erdinger L, Grund S, Keiter S, Kosmehl T, Seiler T-B, Woelz J, Braunbeck T (2009): Strategies for assessing sediment toxicity - a review. Umweltwiss Schads Forsch 21, 160-176

Höss S, Jansch S, Moser T, Junker T, Rombke J (2009): Assessing the toxicity of contaminated soils using the nematode Caenorhabditis elegans as test organism. Ecotox Environ Safe 72, 1811-1818

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

Ingersoll CG, Ankley GT, Benoit DA, Brunson EL, Burton GA, Dwyer FJ, Hoke RA, Landrum PF, Norbergking TJ, Winger PV (1995): Toxicity and bioaccumulation of sediment-associated contaminants using fresh-water invertebrates - a review of methods and applications. Environ Toxicol Chem 14, 1885-1894

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 (2007): ISO 15088: Water quality — Determination of the acute toxicity of waste water to zebrafish eggs (*Danio rerio*). Iso Guideline

ISO (2009): ISO 10871: Water quality - Determination of the inhibition of dehydrogenase activity of *Arthrobacter globiformis* - Solid contact test using the redox dye resazurine. Iso Guideline

ISO (2010): ISO 10872: Water quality - Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of *Caenorhabditis elegans* (Nematoda). Iso Guideline

Jones RP, Hassan SM, Rodgers JH (2008): Influence of contact duration on sediment-associated copper fractionation and bioavailability. Ecotox Environ Safe 71, 104-116

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995): Stages of embryonic development of the zebrafish. Developmental Dynamics 203, 253-310

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

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 Phys C 149, 196-209

Lee JS, Lee BG, Luoma SN, Yoo H (2004): Importance of equilibration time in the partitioning and toxicity of zinc in spiked sediment bioassays. Environ Toxicol Chem 23, 65-71

Lei L, Khodadoust AP, Suidan MT, Tabak HH (2005): Biodegradation of sediment-bound PAHs in field contaminated sediment. Water Res 39, 349-361

Leppänen MT, Kukkonen JVK (2000): Effect of sediment-chemical contact time on availability of sediment-associated pyrene and benzo[a]pyrene to oligochaete worms and semi-permeable membrane devices. Aquat Toxicol 49, 227-241

Liess M, von der Ohe PC (2005): Analyzing effects of pesticides on invertebrate communities in streams. Environ Toxicol Chem 24, 954-965

Maenpaa K, Sorsa K, Lyytikainen M, Leppanen MT, Kukkonen J (2008): Bioaccumulation, sublethal toxicity, and biotransformation of sediment-associated pentachlorophenol in Lumbriculus variegatus (Oligochaeta). Ecotox Environ Safe 69, 121-129

Moermond CTA, Roessink I, Jonker MTO, Meijer T, Koelmans AA (2007): Impact of polychlorinated biphenyl and polycyclic aromatic hydrocarbon sequestration in sediment on bioaccumulation in aquatic food webs. Environ Toxicol Chem 26, 607-615

Morrison DE, Robertson BK, Alexander M (2000): Bioavailability to earthworms of aged DDT, DDE, DDD, and dieldrin in soil. Environ Sci Technol 34, 709-713

Nagel R (2002): DarT: The embryo test with the zebrafish Danio rerio - a general model in ecotoxicology and toxicology. Altex 19, 38-48

Neumann-Hensel H, Melbye K (2006): Optimisation of the solid-contact test with Artbrobacter globiformis. J Soil Sediment 6, 201-207

OECD (1984): Guideline for Testing of Chemicals 207: Earthworm, Acute Toxicity Tests. Organisation for Economic Cooperation and Developement

OECD (2004): OECD guideline 218: Sediment-water chironomid toxicity using spiked sediment. Organisation for Economic Cooperation and Development

Oikari A, Fragoso N, Leppanen H, Chan T, Hodson PV (2002): Bioavailability to juvenile rainbow trout (Oncorynchus mykiss) of retene and other mixed-function oxygenase-active compounds from sediments. Environ Toxicol Chem 21, 121-128

Puglisi E, Vernile P, Bari G, Spagnuolo M, Trevisan M, de Lillo E, Ruggiero P (2009): Bioaccessibility, bioavailability and ecotoxity of pentachlorophenol in compost amended soils. Chemosphere 77, 80-86

Rönnpagel K, Liss W, Ahlf W (1995): Microbial bioassays to assess the toxicity of solid-associated contaminants. Ecotox Environ Safe 31, 99-103

Ryder K, Temara A, Holdway DA (2004): Avoidance of crude-oil contaminated sediment by the Australian seastar, Patiriella exigua (Echinodermata : Asteroidea). Mar. Pollut. Bull. 49, 900-909

Sae-Ma B, Meier PG, Landrum PF (1998): Effect of extended storage time on the toxicity of sediment-associated cadmium on midge larvae (Chironomus tentans). Ecotoxicology 7, 133-139

Saghir SA, Bartels MJ, Budinsky RA, Harris EE, Clark AJ, Staley JL, Chai Y, Davis JW (2007): Effect of organic carbon content, clay type, and aging on the oral bioavailability of hexachlorobenzene in rats. Environ Toxicol Chem 26, 2420-2429

Seiler TB, 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, Wick LY, Harms H (2007): Microbial interactions with organic contaminants in soil: Definitions, processes and measurement. Environ. Pollut. 150, 166-176

Solimini AG, Ptacnik R, Cardoso AC (2009): Towards holistic assessment of the functioning of ecosystems under the Water Framework Directive. TRAC-Trend Anal Chem 28, 143-149

Sormunen AJ, Leppanen MT, Kukkonen JVK (2009): Examining the role of temperature and sediment-chemical contact time on desorption and bioavailability of sediment-associated tetrabromo diphenyl ether and benzo(a)pyrene. Ecotox Environ Safe 72, 1234-1241

Standardisation IOf (2009): ISO 10871: Water quality - Determination of the inhibition of dehydrogenase activity of *Arthrobacter globiformis* - Solid contact test using the redox dye resazurine. Iso Guideline

Tang JX, Alexander M (1999): Mild extractability and bioavailability of polycyclic aromatic hydrocarbons in soil. Environ Toxicol Chem 18, 2711-2714

Traunspurger W, Haitzer M, Hoss S, Beier S, Ahlf W, Steinberg C (1997): Ecotoxicological assessment of aquatic sediments with Caenorhabditis elegans (nematoda) - A method for testing liquid medium and whole-sediment samples. Environ Toxicol Chem 16, 245-250

Van Hoof PL, Kukkonen JVK, Landrum PF (2001): Impact of sediment manipulation on the bioaccumulation of polycyclic aromatic hydrocarbons from field-contaminated and laboratory-dosed sediments by an oligochaete. Environ Toxicol Chem 20, 1752-1761

Van Noort PCM, Cornelissen G, ten Hulscher TEM, 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

Verrhiest GJ, Cortes S, Clement B, Montuelle B (2002): Chemical and bacterial changes during laboratory conditioning of formulated and natural sediments. Chemosphere 46, 961-974

Weber J, Kreutzmann J, Plantikow A, Pfitzner S, Claus E, Manz W, Heininger P (2006): A novel particle contact assay with the yeast Saccharomyces cerevisiae for ecotoxicological assessment of freshwater sediments. J Soil Sediment 6, 84-91

Wölz J, Engwall M, Maletz S, Takner HO, 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 R 15, 536-553

Xu YP, Spurlock F, Wang ZJ, Gan J (2007): Comparison of five methods for measuring sediment toxicity of hydrophobic contaminants. Environ Sci Technol 41, 8394-8399

Xu YP, Gan J, Wang ZJ, Spurlock F (2008): Effect of aging on desorption kinetics of sediment-associated pyrethroids. Environ Toxicol Chem 27, 1293-1301

You J, Brennan A, Lydy MJ (2009): Bioavailability and biotransformation of sediment-associated pyrethroid insecticides in Lumbriculus variegatus. Chemosphere 75, 1477-1482

Zhong H, Wang WX (2006): Influences of aging on the bioavailability of sediment-bound Cd and Zn to deposit-feeding sipunculans and soldier crabs. Environ Toxicol Chem 25, 2775-2780

Chapter 4

Impact of storage time on bioaccumulation of sediment-bound pollutants in the sediment contact test with *Lumbriculus variegatus*

Anne Simon[1*], Hanno Zielke[1], Burkhardt Schmidt[2], Andreas Schaeffer[2], Thomas-Benjamin Seiler[1], Jussi V.K. Kukkonen[3] and Henner Hollert[1]

[1]Department of Ecosystem Analysis, Institute for Environmental Research (Biology V), RWTH Aachen University, Aachen, Germany

[2]Department of Environmental Biology and Chemodynamics, Institute for Environmental Research (Biology V), RWTH Aachen University, Aachen, Germany

[3]University of Eastern Finland, Department of Biology, Joensuu, Finland

4.1 Abstract

The purpose of the present study was to investigate the influence of short-term contact time between sediments and pollutants on the uptake and bioaccumulation of pentachlorophenol (PCP) and fluoranthene (FA) from sediment. The partitioning of FA and PCP was compared in a system composed of sediment, water and organisms. Two different sediments, a natural (Höytiäinen Lake, Finland) and an artificial sediment (OECD 218) were applied. Bioaccumulation of PCP was observed in both sediments. No bioaccumulation of FA was recorded independent of storage period of 5 or 18-21 days. Bioaccumulation of PCP in natural sediment was significantly less than in artificial sediment after a storage period of 13 days. No change in bioaccumulation was observed in artificial sediments between 5 and 21 days of storage.

As shown by bioaccumulation of PCP, short-term contact between sediment and pollutant can significantly change bioaccumulation. Further tests assessing bioaccumulation should account for the potential impact of time.

4.2 Introduction

As result of present and past use of anthropogenic chemicals, pollutants have been introduced into the environment, accumulated in soil and sediments and pose a potential hazard (Coulston & Kolbye , do Nascimento et al. 2004, Hong et al. 2005, Kobayashi et al. 1979, Muir & Eduljee 1999, Sandau et al. 2002).

The freely-dissolved amount of environmental chemicals is best available for benthic organisms. However, hydrophobic organic substances tend to bind to sediments or soils (Alexander 2000, Hatzinger & Alexander 1995, Kelsey et al. 1997). This time-dependent decrease in availability results in a corresponding decrease in bioavailability (Alexander 2000, Ehler and Luthy 2003).

The present study assessed potential time-dependent changes in the bioaccumulation of the organic pollutants pentachlorophenol (PCP) and fluoranthene (FA) after a contact time between sediment and substance of 5 or 18-21 days.

Bioaccumulation, defined as the increase of a chemical in an organism relative to the concentration in the ambient medium or the food, consists of several processes and integrates all possible exposure routes (Egeler et al. 1999). Bioaccumulation of any pollutant depends on environmental conditions, the chemical nature of the pollutant, and the biological characteristics of the target organisms (Escher & Hermens 2004, Fisher et al. 1999, Mäenpää et al. 2008, Nikkila et al. 2003, Streit 1993). Therefore, clear predictions of bioaccumulation cannot be made simply by extrapolating known environmental concentrations of the chemical (Chapman et al. 1990). For example, pH and sediment organic matter affect the bioavailability and, consequently, the toxicity of pollutants in an aquatic environment (Mäenpää et al. 2008). Furthermore, biotransformation of the bioaccumulated chemical may affect the toxic outcome in the organism (Livingstone 1998). Consequently, it has been proposed that the toxic effects should be linked to the internal chemical concentration of the chemical in the organism (McCarty & Mackay 1993). Hence, measuring the bioaccumulation in benthic organisms can provide detailed information on the hazard of chemicals for aquatic ecosystems (Egeler et al. 1999).

To date, many studies have been published on bioaccumulation in *Lumbriculus variegatus* (Kraaij et al. 2002, Kukkonen & Landrum 1994, Kukkonen 2002, Landrum et al. 2002, Leppänen & Kukkonen 1998, 2004, Lyytikäinen et al. 2007, Mäenpää et al. 2008, Mount et al. 1999, Nikkila et al. 2003, Sormunen et al. 2008, Van Hoof et al. 2001). However, the potential of contact time between sediments and chemicals has not been previously addressed. The present study also investigated the impact of time in relation to two types of sediment: A natural sediment from Lake Höytiäinen in Eastern Finland and an artificial sediment according OECD Guideline 218 (OECD 2004).

4.3   Materials and methods

4.3.1   Sediments

Artificial sediment was prepared according to OECD guideline 218 (OECD 2004). Sediment composition was 75 - 76 % quartz sand (NFQ, Nilsiä quartz (100-600 μm), Sibelco Nordic Oy Ab., Espoo, Finland), 20 ± 1 % kaolin clay (Sigma-Aldrich, Steinheim, Germany), 5 ± 1 % peat (Klasmann-Deilmann GmbH, Geeste, Germany), and 0,48 % $CaCO_3$ (Sigma).

Natural sediment was sampled from unpolluted Lake Höytiäinen (Eastern Finland) (Leppänen & Kukkonen 2000, Nikkila et al. 2003). No major industry is in the catchment area, and contaminant profiles reflect only atmospheric precipitation (Leppänen & Kukkonen 2004, Ristola et al. 1996). The Lake Höytiäinen sediment was collected from a depositional area (depth, 18 m) using a pump connected to a benthic dredge. The total organic carbon (TOC) content was 3.66 % ($\pm$0.03 % SD, n = 3; Carlo Erba elemental analyzer, model 1106; Milan, Italy), and fine particles (particle diameter, < 63 μm) constituted 90 % ($\pm$0.01 %, n = 3) of the dry weight. The wet density was 1.12 g ml^{-1} ($\pm$0.001 g ml^{-1}, n = 3). Previous chemical analyses revealed no relevant background contamination. Details on chemical concentrations are given in Cornelissen et al. (2004). Before usage, the Lake Höytiäinen sediment was sieved through a 1 mm mesh and stored in a polypropylene container at 4 °C.

Sediment dry weight was 13.3 % (natural) and 64.5 % (artificial), determined by drying a defined amount of sediment 14 h at 105 °C and measuring weight loss. PH values were 7.5 $\pm$0.1 (natural) and 6.95 $\pm$ 0.5 (artificial). Prior to spiking, sediments were stored in a darkroom at 4 °C.

4.3.2 Test organisms

Worms were maintained at 20 °C $\pm$ 1 °C in aerated 5 l plastic aquaria containing artificial freshwater (ISO 7346/3; International Standardisation 1996). The photoperiod was adjusted to 16 h (using a yellow-light source > 500 nm), and shredded, pre-soaked, paper towels were placed along the bottom of the aquaria. Water was weekly renewed and constant aeration was provided. Worms were fed at least three times a week with TetraMin$^{®}$ (Tetra GmbH, Melle, Germany) flakes ad libidum. Worms used in the experiments were collected from the culture aquaria and were placed in 1 l beakers filled with artificial freshwater adjusted to pH 6.99 with a phosphate buffer (1.0 mmol $Na_2HPO_4 \cdot 2H_2O$ and 1.0 mmol $Na_2HPO_4 \cdot 4H_2O$ per litre). Mean wet weight of worms was 6.13 mg.

4.3.3 Chemicals and spiking of sediments

Both [^{14}C]-labelled chemicals were spiked into the sediments at a concentration of 10,000 dpm/g sediment dry weight, confirmed through liquid scintillation counting (Win

Spectral liquid scintillation counter (LSC), Wallac, Turku, Finland). Radioactive [^{14}C]-PCP (specific activity 10.4 mCi/mmol, chemical purity >98 %) and[^{14}C]-FA (specific activity 45 mCi/mmol, chemical purity >99 %) were obtained from Sigma Aldrich Chemical Company (Munich, Germany).Stock solutions were prepared in acetone (PCP) and methanol (FA) with a total activity of 30.81 µCi µl^{-1} (PCP) and 175.38 µCi µl^{-1} (FA), respectively.

Höytiäinen sediment

Chemicals were added drop-wise to the sediments while mixing with a rotating metal blade connected to a drill (Black & Decker, KD 163 E) for 2 h at room temperature(20 °C ± 1 °C).

Artificial sediment

8.7 g of quartz sand (1 % of the total weight of artificial sediment) were spiked with the stock solutions. The solubiliser was allowed to completely evaporate. After 3 days, the dried quartz sand was added to the sediments. Sediments were then mixed and slowly stirred for 2 h at room temperature. After spiking, sediments were stored in a darkroom at 4 °C until usage.

4.3.4   Test procedure

Spiked sediment samples were transferred into glass jars either 5 days(NS I, natural sediment and AS I, artificial sediment), 18 days (NS II), or 21 days (AS II) after spiking and allowed to settle for 24 h before the addition of worms. Six days of total contact time were chosen in order to investigate bioavailability from freshly contaminated sediments. The experimental unit consisted of a 50 ml glass jar containing 25.0 ± 0.5 g of sediment wet weight. Artificial freshwater was decanted onto the sediments with minimum sediment disturbance, and the sediment surface was cautiously cut with a Pasteur pipette to ensure easy burrowing for the worms. Six worms were introduced per beaker and exposed to the same ^{14}C-PCP and ^{14}C-FA concentration in both sediments for 14 days. Bioaccumulation of the sediment-associated chemicals was measured after 24, 48, 96, 168 and 336 h of exposure. It was assumed that worms were exposed to the pollutants in the sediments through all relevant routes(Nikkila et al. 2003), i.e. dissolved fractions in the interstitial and overlying water, and sediment- and

suspended particle-bound fractions. It was further assumed that the total body burden directly reflected the total bioavailable portion of the compounds (Nikkila et al. 2003).

Evaporated water was replaced every other day by adding aerated artificial freshwater. At each sampling time, each test unit was processed in triplicate to determine substance concentrations in the overlying water, worms, and sediment. Per sample, 5 ml of overlying water were mixed with 5 ml of LSC cocktail (InstaGel^{TM}Plus, Packard BioSience B.V., Groningen, The Netherlands). The remaining water was removed and a100-400 mg sediment sample was transferred into 20 ml LSC-vials, mixed with 1 ml of tissue dissolvent (Soluene®-350, Packard BioSience B.V., Groningen, The Netherlands), and stored overnight in an oven (Memmert) at 50 °C. On the next day, 12 ml of LSC cocktail (UltimaGold^{TM}, Packard BioSience B.V., Groningen, The Netherlands) were added and the contents of vials were homogenized (Vortex Genie^{R}2, Scientific Industries^{TM}, New York, USA). Sediment dry weight was determined by weighing 2 g wet weight (ww) of sediment and drying it at 50 °C for 14 h. Finally, the content of each jar was sieved through 200 µm mesh (Retsch, Haan, Germany) to collect the worms. Worms were placed in petri-dishes with 6 ml of aerated artificial fresh water for 6 h in darkness in order to purge their gut. Worms were then quickly dried for 2 seconds on a paper towel. Total worm wet weight of all recovered individuals was determined on a microbalance (4503 micro, Sartorius GmbH, Göttingen, Germany). After weighing, worms were placed in a 5 ml LSC vial containing 0.5 ml of tissue dissolvent and dissolved overnight at 50°C. On the next day, 5 ml of LSC cocktail (UltimaGold^{TM}) were added and worms were homogenized. Potential quenching effects of chemiluminescence were eliminated by allowing the samples to settle for a minimum of 48 h prior to measurement. Final chemical concentrations were calculated based on the measured ^{14}C-activity and known specific activity. Data were corrected for potential quenching effects by means of the external standards ratio method. Worms were not given any additional nourishment during exposures. At each sampling, pH values of the overlying water were monitored.

4.3.5   Bioaccumulation factor

Because a steady state was not achieved at the end of the total exposure period (14 days), an elimination rate constant (k_e) could not be determined and no bioconcentration factors could be calculated. Instead, the bioaccumulation factors (BAFs) were calculated at each sampling time. Total radioactivity measured in the worms (pmol / g ww) was divided by the total

radioactivity determined in the sediment (pmol / g dw) for each replicate at each sampling point.

4.3.6 Statistical analysis

Statistical evaluation was carried out using SigmaStat 3.5 (Systat Software GmbH, Erkrath, Germany, 2006). Normality of distribution and homogeneity of variances were verified and *t-test* and analyses of variance (ANOVA) were performed. As post hoc test, Student-Newman-Keuls method was used for pairwise comparison of the treatment groups. Datasets which did not fulfil normality and/or variance homogeneity criteria (Kolmogorov-Smirnov/Levene tests) were analysed using non-parametric ANOVA on ranks with Dunn's method for pairwise comparison of the treatment groups. The significance limit used throughout all comparisons was $p \leq 0.05$.

4.4   Results

4.4.1   Sediment characteristics

Sediments varied in their characteristics, especially in terms of their dry weight. Artificial sediment was coarse-grained sediment with lower water content. Natural sediment had higher water content and was sludgy.

Höytiäinen lake sediment assay pH varied between 7.54 at first time point and 5.21 after 336 h, and between 7.99 and 8.62 in the artificial sediment exposures, respectively.

4.4.2   Bioaccumulation

In both sediments, the worms survived until the end of the exposure period of 14 days. The average chemical concentrations in the overlying water were < 1 pmol / ml for both compounds and negligible in comparison to the concentrations in sediments and organisms.

At the termination of NS I, the concentrations of PCP increased from 1400 pmol/g ww worms to 4700 pmol/g ww worms and from 600 pmol/g ww worms to 2400 pmol/g ww worms for NS II, as given in figure 4.1. In both approaches an accumulation of the pollutant in worm tissue could be determined. The increase of PCP was significantly higher in NS I compared to NS II (Fig. 4.2).

FA showed a slight increase in worms and a very low decrease in the respective sediment. Bioaccumulation in worm tissue showed the least increase (up to 50 pmol/g ww worm tissue) of all tests (Figure 4.1). No significant difference could be determined between NS I and NS II for FA. The measured concentrations of FA were 20-40 times lower than for PCP. The accumulated amount of PCP increased between each sampling point, whereas for FA, an increase could only be observed between 0 and 24 h of exposure (Fig. 4.2).

Fig. 4.1 Concentration (pmol / g dw sediment and ww worms; mean ± SD of 3 replicates) of ^{14}C-PCP and ^{14}C-FA in natural sediment and worm tissue after 5 days of spiking (NS I) and 18 days (NS II). Sampling times were after 24, 48, 96, 168 and 336 h.

SD = standard deviation; dw = dry weight; ww = wet weight; NS = natural sediment; PCP = pentachlorophenol, FA = fluoranthene.
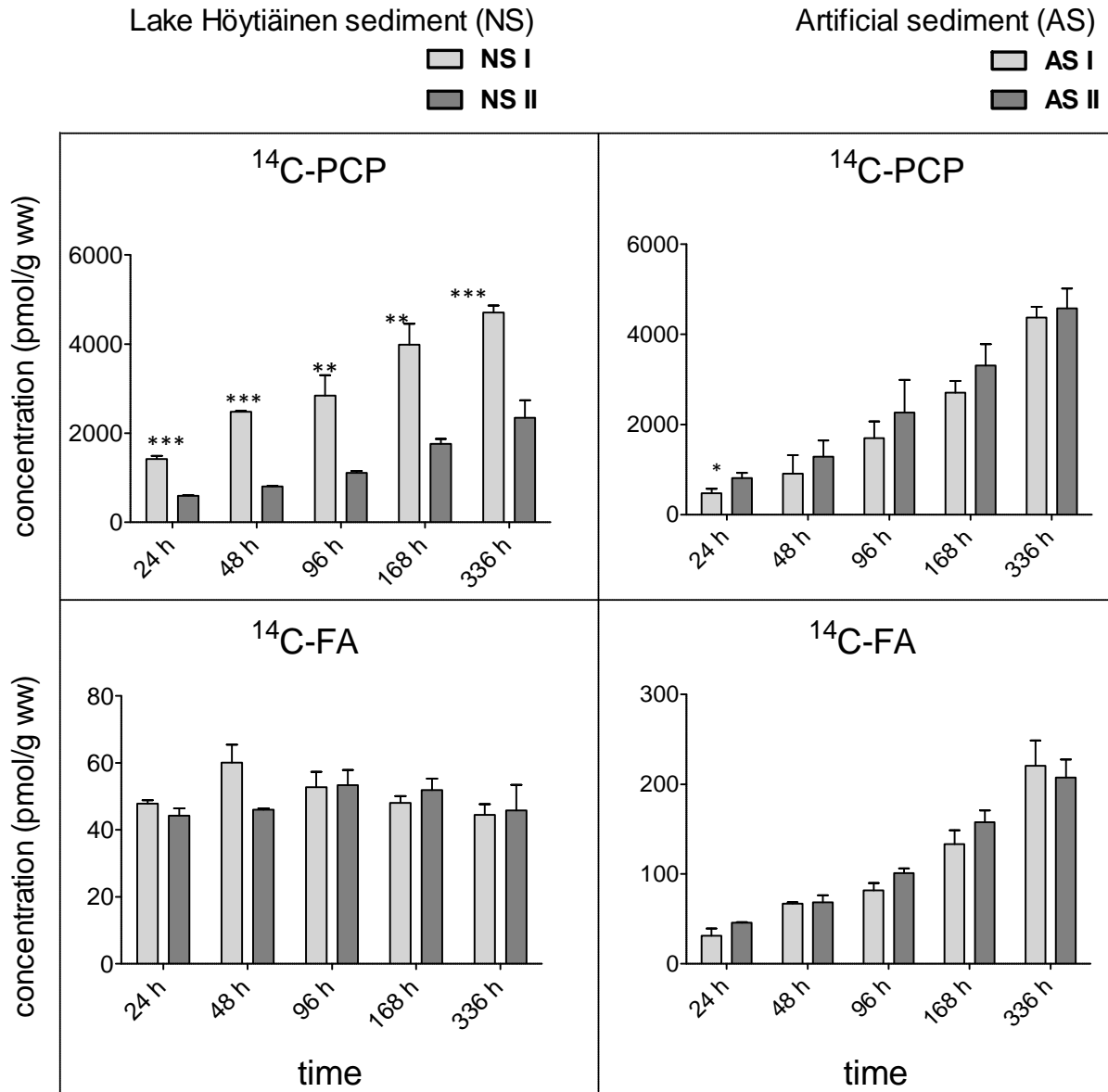
Fig. 4.2 Time-dependence of bioaccumulation in earthworms from both sediments with means ± SD of 3 replicates after 5 days of spiking (NS I, AS I) and 18-21 days (NS II-AS II). Sampling times were after 24, 48, 96, 168 and 336 h. **/*** significant difference (p≤0,01/p≤0,001) after the storage of 13 days (for the Lake Höytiäinen sediment) and 16 days (for artificial sediment) with t-tests.

SD = standard deviation; dw = dry weight; ww = wet weight; NS = natural sediment; AS = artificial sediment; PCP = pentachlorophenol, FA = fluoranthene

Concentrations of PCP increased in the experiments with artificial sediment as well as in NS I and II (Fig. 4.3). An increase of FA in *Lumbriculus variegatus* could be shown only after the exposure to artificial sediment (Figure 4.3). Figure 4.3 shows a great increase of PCP for both AS I and AS II to 5000 pmol/g ww worm during the test period of 336 h. Sediment concentrations stayed nearly constant at 130 pmol/g dw.

In AS I and AS II, the concentrations of PCP and FA increased in the worms at each sampling point, although these changes were not significantly different between consecutive time points. Again, the concentrations of FA remained much lower (approximately 20 times) than for PCP (Fig. 4.2). In both experiments with PCP and FA, no significant difference in bioaccumulation could be determined in either AS I or AS II (Figure 4.4).
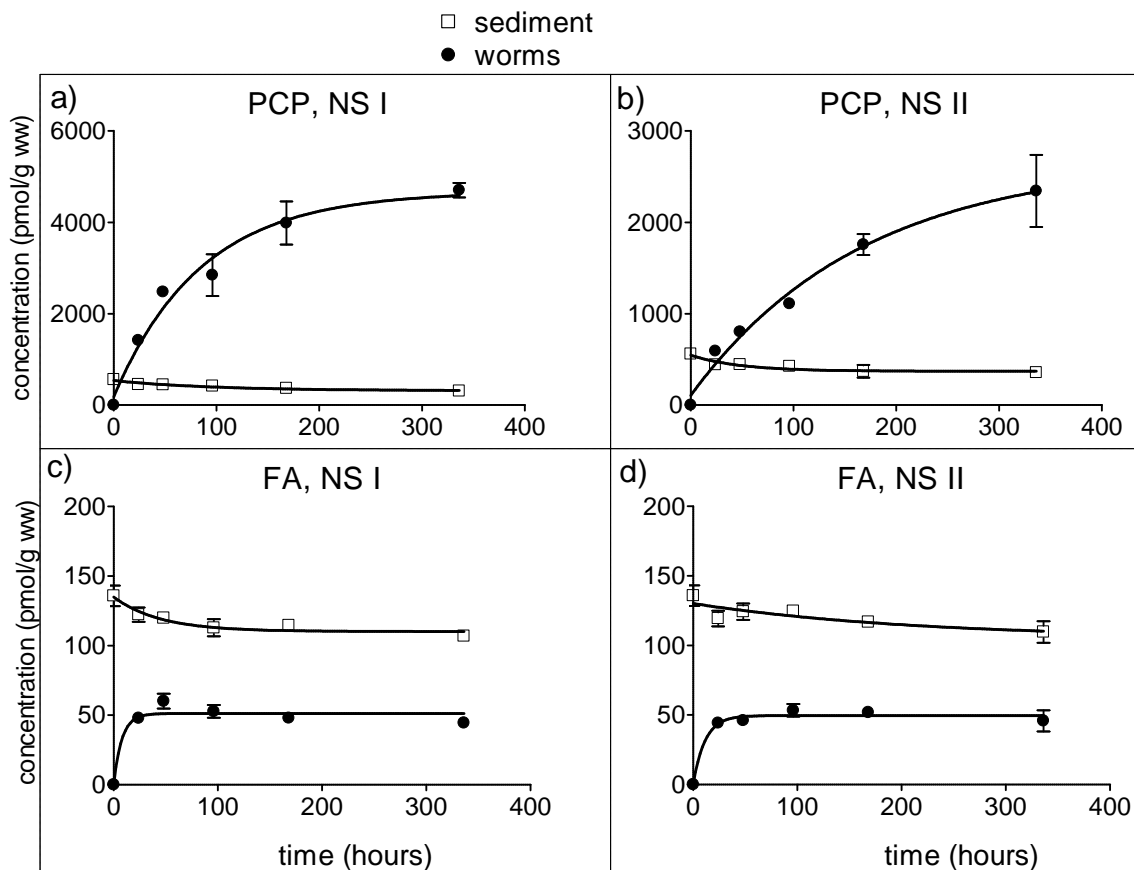

Fig. 4.3 Concentration (pmol / g dw sediment and ww worms; mean ± SD of 3 replicates) of ^{14}C-PCP and ^{14}C-FA in artificial sediment and worm tissue after 5 days of spiking (AS I) and 21 days (AS II). Sampling times were after 24, 48, 96, 168 and 336 h.

SD = standard deviation; dw = dry weight; ww = wet weight; AS = artificial sediment; PCP = pentachlorophenol, FA = fluoranthene.
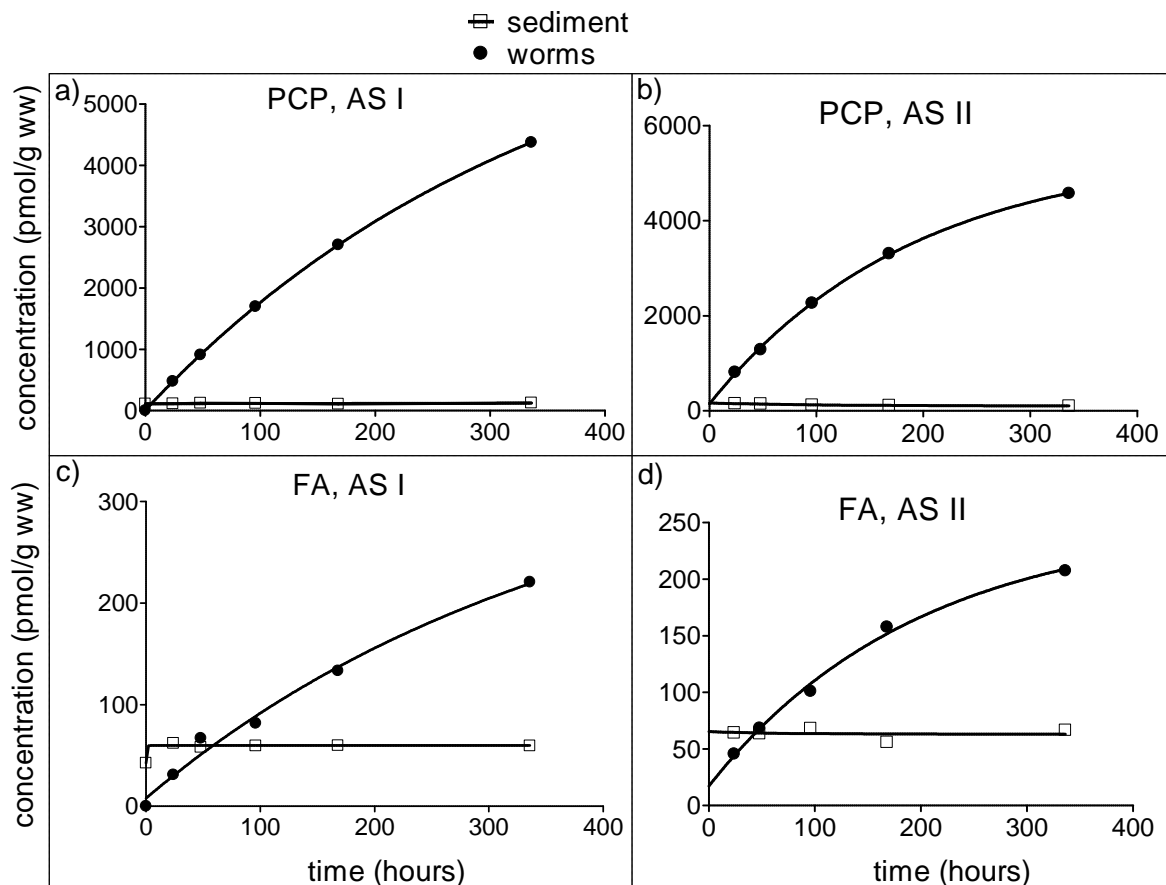
In summary, uptake and bioaccumulation of PCP could be determined from both sediments. A constant uptake of PCP could be shown across all experimental timepoints for NS I and NS II. The increase of PCP in AS I and II occurred only at the last sampling points, when the worms were exposed for 168 h or longer.

No uptake in worm tissue could be shown after 14 days for FA in NS I and II (refer to figure 4.1 while an increase could be detected in AS I and II, as shown in figure 4.3.

Because the steady state was not reached, bioaccumulation factors could only be determined separately for each time point. Figure 4.4 shows the BAF for short-term ageing after 14 days. Calculated BAF was < 1 g sediment /g worm. BAF of PCP decreased from 15 g sediment /g worm (NS I) to 7 g sediment /g worm (NS II). Increasing sediment-pollutant contact time resulted to a significantly decreased bioaccumulation. In contrast, no significant time-dependent change of BAF could be observed for PCP in artificial sediment. FA showed no significant accumulation in both sediments.
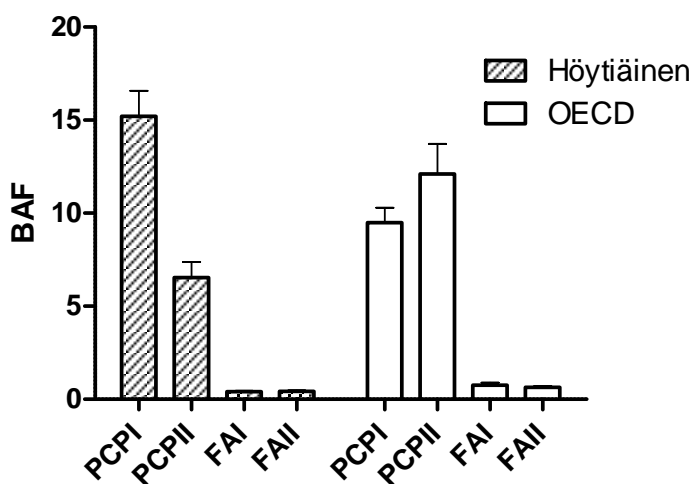

Fig. 4.4 Bioaccumulation factors [g sediment /g worm] after 336 h for both sediments. 3 replicates with mean and SD.

BAF = bioaccumulation factor;
SD = standard deviation;
PCP = pentachlorophenol,
FA = fluoranthene.

4.5 Discussion

4.5.1 Pentachlorophenol

The significantly lower BAF of PCP after 14 days of storage indicated the need to account for the impact of time in bioaccumulation studies. The reason for the decrease very likely was time-dependent sequestration, i.e. ageing, of PCP in the natural sediment and, thus, decrease bioavailability to *Lumbriculus variegatus*. Water as an environmental compartment has properties which favour accumulation of chlorophenols, in particular in bottom sediments and on suspended matter (Boyd 1982, Peuravuori et al. 2002, Schellenberg et al. 1984). The sorption of chlorophenols is a function of their coefficients of partition between water and sediments or suspended particulates. Investigations by Xie et al. (1986) on the distribution of chlorophenols in the marine environment confirmed that sorption of chlorophenols can strongly be influenced by the pH of ambient water. In addition, the accumulation of chlorophenols in sediment depend on the percentage and nature of the organic matter (Xie et al. 1986). PCP is a weak acid (pKa = 4.7-4.9, $\log K_{OW}$ = 3.3-5.86 at pH 4-7 (Czaplicka 2004, Mackay et al. 1995) and, thus, the fate of PCP in the environment depends on ionization of the chemical in the ambient pH. In the case of lake sediments, Paaso et al. (2002) showed that an increase of dissolved humic matter concentration can affect the equilibrium partitioning of PCP between the solid sediment matter and dissolved humic matter.

According to the measured pH, most of PCP was in neutral form with the exception of NS II (pH decreased to 5.21). Whereas the tendency to accumulate in fatty tissue increases with decreasing pH, acute toxicity increases simultaneously because the ionized form of PCP can easily penetrate biological membranes (BUA 1986). However, this effect could not be observed in the current study because no worms died during exposure. PCP could have bound to the organic matter of natural sediment (Shiu et al. 1994) and no toxic amount was available for the organisms. PCP could also have been biodegradated by microbial activity (Puglisi et al. 2009). Furthermore Nikkila et al (2003) showed that PCP can evaporate from liquid even though it is not very volatile.

In addition, it has been suggested that the non-ionized form of PCP is the most toxic to aquatic organisms, due to its superior ability to permeate membranes (Fisher et al. 1999, Kobayashi et al. 1979, Kobayashi & Kishino 1980). However, the affinity of the ionized form of PCP in biological membranes is equal to the octanol-water partition coefficient of unionized PCP (Smejtek et al. 1996). Consequently, both forms of PCP have a similar potential to bioaccumulate in *Lumbriculus variegatus*, which could explain why no major

differences were observed in the bioaccumulation of PCP between NS I and AS I & II, in spite of differences in pH.

With regard to the artificial sediment, particle sizes could explain why there was no difference in bioaccumulation between AS I and II. Organic material in sediments and soils usually resides on the surface of the smallest particles (Kukkonen & Landrum 1996). Almost 75 % of the applied artificial sediment consisted of quartz sand, in contrast to the natural sediment, of which 90 % consisted of particles with a diameter of less than 63 µm. Hence, fewer binding sites were freely available for chemicals in artificial sediment. In contrast, the chance of uptake of a pollutant from sediment by feeding is greater in natural sediment because there are more freely available binding sites for the chemicals.

4.5.2   Fluoranthene

The availability of polycyclic aromatic hydrocarbons (PAHs) to epibenthic and infaunal deposit feeders is less than that of other classes of compounds with similar physico-chemical properties (Landrum & Faust 1991, Ma et al. 1998, Tracey & Hansen 1996). Reduced availability of PAHs has been ascribed to sequestration of a fraction of the total compound, rendering it unavailable to partition into pore water, even over large time scales (Farrington et al. 1983, Landrum 1989, McGroddy & Farrington 1995, Prahl & Carpenter 1983). The decline in bioavailable fractions with increasing contact time is hypothesized to result from slow solute diffusion into interior regions of organic matrices (Brusseau & Rao 1989, Chiou 1989) and/or entrapment within intraparticle micropores or polymeric microvoids (Pignatello 1990, Steinberg et al. 1987). Additionally, for PAHs, reduced bioavailable fractions can result from preferred partitioning to the aromatic components of particulate organic matter (Chiou et al. 1998) or incorporation into ash and soot matrices. Thus, the solute-sorbent contact time and the source of PAHs (i.e. pyrogenic, petrogenic, and diagenetic) will have a significant influence on partitioning, sorption kinetics, and bioavailability.

Furthermore, increasing sediment-pollutant contact time can be expected to reduce bioavailability of non-equilibrated compounds by allowing for the diffusion of PAHs (Van Hoof et al. 2001). This could not be determined in the present study, because bioaccumulation remained constant from Lake Höytiäinen sediment, and neither a reduced nor an increased bioavailability could be shown for the artificial sediment after 16 days of storage. In addition,

the uptake of PAHs by organisms in bioassays should be greater when using freshly-dosed instead of field-contaminated sediment or, alternatively, dosed and subsequently-aged sediment (Landrum 1989, Landrum et al. 1991, Varanasi et al. 1985). Independent studies showed that an increase in the sediment-chemical contact time decreased the bioavailability of PAHs (Harkey et al 1995, Kraaij et al 2002, Leppänen et al. 1999). Cases are reported, however, in which uptake kinetics have increased with increasing equilibration time (Van Hoof et al. 2001).

The present results did not show decreasing bioavailability with increasing sediment-chemical contact time in case of FA. With regard to literature, contact time likely was too short to impact bioaccumulation of FA.

4.6   Conclusion

Time-dependent changes in bioaccumulation occurred only in the case of PCP in natural sediment. However, this impact lead to a two-fold underestimation of the potential bioaccumulation in the applied experimental setup within 14 days, indicated a need to further address the influence of contact time in future studies on bioaccumulation.

4.7   References

Alexander M (2000): Ageing, bioavailability, and overestimation of risk from environmental pollutants. Environmental Science and Technology 34, 4259-4265

Boyd SA (1982): Adsorption of substituted phenols by soil. Soil Science 134, 337-343

Brusseau ML, Rao PSC (1989): Sorption nonideality during organic contaminant transport in porous-media. Critical Reviews in Environmental Control 19, 33-99

BUA 1986: Pentachlorphenol, BUA-Stoffbericht 3, Weinheim

Chapman PM (1990): The sediment quality triad approach to determining pollution-induced degradation. Sci Total Environ 97-8, 815-825.

Chiou C (1989): Theoretical considerations of the partition uptake of nonionic organic compounds by soil organic matter.

Chiou CT, McGroddy SE, Kile DE (1998): Partition characteristics of polycyclic aromatic hydrocarbons on soils and sediments. Environmental Science & Technology 32, 264-269

Cornelissen G, Kukulska Z, Kalaitzidis S, Christanis K, Gustafsson O (2004): Relations between environmental black carbon sorption and geochemical sorbent characteristics. Environmental Science & Technology 38, 3632-3640

Coulston F, Kolbye A (1994): Appendix G: Chlorinated phenolics. Regul Toxicol Pharmacol 20, 895-938

Czaplicka M (2004): Sources and transformations of chlorophenols in the natural environment. Science of the Total Environment 322, 21-39

do Nascimento NR, Nicola SMC, Rezende MOO, Oliveira TA, Oberg G (2004): Pollution by hexachlorobenzene and pentachlorophenol in the coastal plain of Sao Paulo state, Brazil. Geoderma 121, 221-232

Egeler P, Rombke J, Meller M, Knacker T, Nagel R (1999): Bioaccumulation test with Tubificid Sludgeworms in artificial media - development of a standardisable method. Hydrobiologia 406, 271-280

Ehlers LJ, Luthy RG (2003): Contaminant bioavailability in soil and sediment. Environ Sci Technol 37, 295A-302A.

Escher BI, Hermens JLM (2004): Internal exposure: Linking bioavailability to effects. Environmental Science & Technology 38, 455A-462A

Farrington JW, Goldberg ED, Risebrough RW, Martin JH, Bowen VT (1983): United-states mussel watch 1976-1978 - an overview of the trace-metal, DDE, PCB, Hydrocarbon, and artificial radionuclide data. Environmental Science & Technology 17, 490-496

Fisher SW, Hwang H, Atanasoff M, Landrum PF (1999): Lethal Body Residues for Pentachlorophenol in Zebra Mussels (Dreissena polymorpha) under Varying Conditions of Temperature and pH. Ecotoxicology and Environmental Safety 43, 274-283

Harkey GA, van Hoof PL, Landrum PF (1995): Bioavailability of polycyclic aromatic hydrocarbons from a historically contaminated sediment core. Environmental Toxicology and Chemistry 14, 1551-1560

Hatzinger PB, Alexander M (1995): Effect of ageing of chemicals in soil on their biodegradability and extractability. Environmental Science & Technology 29, 537-545

Hong HC, Zhou HY, Luan TG, Lan CY (2005): Residue of pentachlorophenol in freshwater sediments and human breast milk collected from the Pearl River Delta, China. Environment International 31, 643-649

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.

Kelsey JW, Kottler BD, Alexander M (1997): Selective chemical extractants to predict bioavailability of soil-aged organic chemicals. Environmental Science & Technology 31, 214-217

Kobayashi K, Akitake H, Manabe K (1979): Studies on the metabolism of chlorophenols in fish:10. relation between toxicity and accumulation of various chlorophenols in goldfish. Bulletin of the Japanese Society of Scientific Fisheries 45, 173-175

Kobayashi K, Kishino T (1980): Studies on the metabolism of chlorophenols in fish:13. Effect of pH on the toxicity and accumulation of pentachlorphenol in goldfish. Bulletin of the Japanese Society of Scientific Fisheries 46, 167-170

Kraaij RH, Tolls J, Sijm D, Cornelissen G, Heikens A, Belfroid A (2002): Effects of contact time on the sequestration and bioavailability of different classes of hydrophobic organic chemicals to benthic oligochaetes (Tubificidae). Environmental Toxicology and Chemistry 21, 752-759

Kukkonen J, Landrum PF (1994): Toxicokinetics and toxicity of sediment-associated pyrene to lumbriculus variegatus (oligochaeta). Environmental Toxicology and Chemistry 13, 1457-1468

Kukkonen J, Landrum PF (1996): Measuring assimilation efficiencies for sediment-bound PAH and PCB congeners by benthic organisms (vol 32, pg 75, 1995). Aquatic Toxicology 34, 86-86

Kukkonen JVK (2002): Lethal body residue of chlorophenols and mixtures of chlorophenols in benthic organisms. Archives of Environmental Contamination and Toxicology 43, 214-220

Landrum PF (1989): Bioavailability and toxicokinetics of polycyclic aromatic-hydrocarbons sorbed to sediments for the amphipod *Pontoporeia hoyi*. Environmental Science & Technology 23, 588-595

Landrum PF, Eadie BJ, Faust WR (1991): Toxicokinetics and toxicity of a mixture of sediment-associated polycyclic aromatic hydrocarbons to the amphipod *Diporeia sp.* Environmental Toxicology and Chemistry 10, 35-46

Landrum PF, Faust WR (1991): Effect of Variation in Sediment Composition on the Uptake Rate Coefficient for Selected PCB and PAH Congeners by the Amphipod, Diporeia sp. Aquatic toxicology and risk assessment: fourteenth volume, 263

Landrum PF, Gedeon ML, Burton GA, Greenberg MS, Rowland CD (2002): Biological responses of Lumbriculus variegatus exposed to fluoranthene-spiked sediment. Archives of Environmental Contamination and Toxicology 42, 292-302

Leppanen M 1999: Bioaccumulation of sediment-associated polycyclic aromatic hydrocarbons in the freshwater oligochaete *Lumbriculus variegatus* (Müller). PhD thesis, University of Joensuu publications in sciences no. 53.

Leppänen MT, Kukkonen JVK (1998): Relationship between reproduction, sediment type, and feeding activity of Lumbriculus variegatus (Müller): Implications for sediment toxicity testing. Environmental Toxicology and Chemistry 17, 2196-2202

Leppänen MT, Kukkonen JVK (2000): Effect of sediment-chemical contact time on availability of sediment-associated pyrene and benzo a pyrene to oligochaete worms and semi-permeable membrane devices. Aquatic Toxicology 49, 227-241

Leppänen MT, Kukkonen JVK (2004): Toxicokinetics of sediment-associated polybrominated diphenylethers (flame retardants) in benthic invertebrates (Lumbriculus variegatus, oligochaeta). Environmental Toxicology and Chemistry 23, 166-172

Livingstone DR (1998): The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 120, 43-49

Lyytikäinen M, Pehkonen S, Akkanen J, Leppänen M, Kukkonen JVK (2007): Bioaccumulation and biotransformation of polycyclic aromatic hydrocarbons during sediment tests with oligochaetes (Lumbriculus variegatus). Environmental Toxicology and Chemistry 26, 2660-2666

Ma WC, van Kleunen A, Immerzeel J, de Maagd PGJ (1998): Bioaccumulation of polycyclic aromatic hydrocarbons by earthworms: Assessment of equilibrium partitioning theory in in situ studies and water experiments. Environmental Toxicology and Chemistry 17, 1730-1737

Mackay D, Shiu W, Ma K (1995): Illustrated Handbook of Physical-chemical Properties and Environmental Fate for Organic Chemicals. CRC Press Inc, Boca Raton, FL, pp.377-384

Mäenpää K, Sorsa K, Lyytikäinen M, Leppänen MT, Kukkonen JVK (2008): Bioaccumulation, sublethal toxicity, and biotransformation of sediment-associated pentachlorophenol in Lumbriculus variegatus (Oligochaeta). Ecotoxicology and Environmental Safety 69, 121-129

McCarty LS, Mackay D (1993): Enhancing ecotoxicological modeling and assessment. Environmental Science & Technology 27, 1719-1728

McGroddy SE, Farrington JW (1995): Sediment porewater partitioning of polycyclic aromatic hydrocarbons in three cores from Boston Harbor, Massachusetts. Environmental Science & Technology 29, 1542-1550

Mount DR, Dawson TD, Burkhard LP (1999): Implications of gut purging for tissue residues determined in bioaccumulation testing of sediment with Lumbriculus variegatus. Environmental Toxicology and Chemistry 18, 1244-1249

Muir J, Eduljee G (1999): PCP in the freshwater and marine environment of the European Union. The Science of The Total Environment 236, 41-56

Nikkila A, Halme A, Kukkonen JVK (2003): Toxicokinetics, toxicity and lethal body residues of two chlorophenols in the oligochaete worm, Lumbriculus variegatus, in different sediments. Chemosphere 51, 35-46

OECD (2004) OECD guideline 218: Sediment-water chironomid toxicity using spiked sediment. Organisation for Economic Cooperation and Development.

Paaso N, Peuravuori J, Lehtonen T, Pihlaja K (2002): Sediment-dissolved organic matter equilibrium partitioning of pentachlorophenol:: The role of humic matter. Environment International 28, 173-183

Peuravuori J, Paaso N, Pihlaja K (2002): Sorption behaviour of some chlorophenols in lake aquatic humic matter. Talanta 56, 523-538

Pignatello JJ (1990): Slowly reversible sorption of aliphatic halocarbons in soils .1. formation of residual fractions. Environmental Toxicology and Chemistry 9, 1107-1115

Prahl FG, Carpenter R (1983): Polycyclic aromatic hydrocarbon (PAH)-phase associations in washington coastal sediment. Geochimica Et Cosmochimica Acta 47, 1013-1023

Puglisi E, Vernile P, Bari G, Spagnuolo M, Trevisan M, de Lillo E, Ruggiero P (2009): Bioaccessibility, bioavailability and ecotoxicity of pentachlorophenol in compost amended soils. Chemosphere 77, 80-86.

Ristola T, Pellinen J, Van Hoof PL, Leppänen M, Kukkonen J (1996): Characterization of Lake Ladoga sediments. II. Toxic chemicals. Chemosphere 32, 1179-1192

Sandau CD, Ayotte P, Dewailly E, Duffe J, Norstrom RJ (2002): Pentachlorophenol and hydroxylated polychlorinated biphenyl metabolites in umbilical cord plasma of

neonates from coastal populations in Quebec. Environmental Health Perspectives 110, 411-417

Schellenberg K, Leuenberger C, Schwarzenbach RP (1984): Sorption of chlorinated phenols by natural sediments and aquifer materials. Environmental Science & Technology 18, 652-657

Shiu WY, Ma KC, Varhanickova D, Mackay D (1994): Chlorophenols and alkylphenols: a review and correlation of environmentally relevant properties and fate in an evaluative environment. Chemosphere 29, 1155-1224

Smejtek P, Blochel A, Wang S (1996): Hydrophobicity and sorption of chlorophenolates to lipid membranes. Chemosphere 33, 177-201

Sormunen AJ, Leppänen MT, Kukkonen JVK (2008): Influence of sediment ingestion and exposure concentration on the bioavailable fraction of sediment-associated tetrachlorobiphenyl in oligochaetes. Environmental Toxicology and Chemistry 27, 854-863

Steinberg SM, Pignatello JJ, Sawhney BL (1987): Persistence of 1,2-Dibromoethane in soils - entrapment in intraparticle micropores. Environmental Science & Technology 21, 1201-1208

Streit B (1993): Bioaccumulation processes in ecosystems. Experientia 49, 184-184

Tracey GA, Hansen DJ (1996): Use of biota-sediment accumulation factors to assess similarity of nonionic organic chemical exposure to benthically-coupled organisms of differing trophic mode. Archives of Environmental Contamination and Toxicology 30, 467-475

Van Hoof PL, Kukkonen JVK, Landrum PF (2001): Impact of sediment manipulation on the bioaccumulation of polycyclic aromatic hydrocarbons from field-contaminated and laboratory-dosed sediments by an oligochaete. Environmental Toxicology and Chemistry 20, 1752-1761

Varanasi U, Reichert WL, Stein JE, Brown DW, Sanborn HR (1985): Bioavailability and biotransformation of aromatic hydrocarbons in benthic organisms exposed to sediment from an urban estuary. Environmental Science & Technology 19, 836-841

Xie TM, Abrahamsson K, Fogelqvist E, Josefsson B (1986): Distribution of chlorophenolics in a marine environment. Environmental Science & Technology 20, 457-463

Chapter 5

Impact of test vessel material on results obtained in the fish embryo test with zebrafish

Hanno Zielke[1], Carina Adamzyk[1], Nina Rehage[1], Thomas-Benjamin Seiler[1] and Henner Hollert[1]

[1]Department of Ecosystem Analysis, Institute for Environmental Research (Biology V), RWTH Aachen University, Aachen, Germany

5.1   Abstract

Organic compounds can adsorb to test vessels made from plastic materials. The present study investigated the impact of glass and plastic vessels on the effects of 3,4-dichloroaniline (DCA) in the fish embryo tests (FET) with zebrafish (*Danio rerio*). DCA solutions were added to test vessels 1 week, at 16 h, or directly prior to the actual start of exposure. Both the use of plastic vessels as well as longer equilibration times of 16 h and 1 week gave lesser effects in the FET. Conclusively, vessel material and experimental timing can impact biotest results and should be avoided by means of semi-static renewal, passive dosing or flow-through experiments.

5.2   Introduction

The fish embryo test (FET) with zebrafish (*Danio rerio*) has been developed as an alternative to acute toxicity tests with adult fish (Braunbeck et al. 2005, Nagel 2002). Lammer et al. (2009a) reported good correlations between the FET and four test protocols with adult fish of different species, and concluded that the FET is a suitable replacement of acute fish tests. In Germany, the FET has mandatorily replaced the acute fish test with the golden ide (*Leuciscus idus melanotus*) in wastewater testing and the corresponding regulations (DIN 2001, German Federal Ministry of Justice 2005). The FET is also internationally standardised (2007).

According to the current guidelines, the FET is carried out in 24-well plastic plates usually made from polystyrene (DIN 2001, ISO 2007). However, this plastic compound was indicated in several studies to absorb organic chemicals (Dahlstrom et al. 2004, Koutsopoulos et al. 2007, Palmgren et al. 2006). As discussed by Lammer et al. (2009b), this can result in differing test concentrations and affect the outcomes of biotests. The present communication provides results on effects induced by the common positive control in the FET, 3,4-dichloroaniline, after parallel exposure in plastic and glass test vessels, and different vessel-DCA equilibration times prior to the actual start of exposure.

5.3 Material and methods

Zebrafish were maintained according to Braunbeck et al. (2005) in 30 L aquaria at $26 \pm 1°C$ in hatching groups of 12 males and 8 females. Photoperiod was adjusted to 10 h of darkness and 14 h of light, and aquaria were filled with charcoal filtered tap water with a temperature of 26 ± 1 °C, hardness of 11 °dH, oxygen saturation of ≥ 80 %, and a pH of 7.8 ± 0.5. Tank water was exchanged once a week. The fish were daily fed dry flakes (TetraMin™, Tetra GmbH, Melle, Germany) and *Artemia* sp. nauplii (Great Salt Lake Artemia Cysts, Sanders, Ogden, USA). For spawning, glass dishes were transferred into the tanks on evenings before experiments. In order to prevent egg predation, dishes were covered with a stainless steel grid with a mesh opening size of 1 mm through which the eggs could drop. Plastic plant imitates were attached to the mesh in order to stimulate mating. Spawning occurred within 0.5 - 1 h after onset of illumination.

The FET was conducted according to DIN 38415 6 (DIN 2001). Artificial water according to ISO 7346/3 (1996) was used as the test medium (294.0 mg/l $CaCl_2$; 2 H_2O, 123.3 mg/l $MgSO_4 \cdot 7 H_2O$, 63.0 mg/l $NaHCO_3$ and 5.5 mg/l KCl). Purity of DCA (Sigma-Aldrich, Steinheim, Germany) was ≥ 98.0 %. Dilutions applied in the FET were prepared from a stock solution of 100 mg/l DCA. In modification of the protocol given in the guideline, tests were carried out either in polystyrene 6-well-plates (TPP, Zurich, Switzerland) or 20 ml borosilicate glass crystallisation wells, both with a diameter of approximately 4 cm. Per well, five eggs were exposed in 5 ml of test solution. Ten embryos pre-selected for normal development were tested per treatment. As quality criterion, egg fertilisation rate had to be ≥ 70 % in order for a test to be carried out (Lammer et al. 2009b). Negative controls contained only artificial water for both types of vessel material. Applied concentrations of DCA were 1, 2, 3, 4, or 5 mg/l. DCA dilutions and controls were transferred into the test vessels directly, at 16 h, and 1 week before embryos were introduced into the vessels. Plates were covered with self-adhesive foil (Nunc, Roskilde, Denmark) and beakers were covered with parafilm (Pechiny Plastic Packaging, Chicago, USA) in order to prevent evaporation. Embryos were exposed for 48 h at 26 ± 1 °C, and subsequently examined under a microscope to record any adverse effects. Mortality criteria were (a) coagulation, (b) lack of heartbeat, (c) missing somite development, and (d) failure of the tail to detach from the yolk sack (DIN 2001). Negative controls were regarded valid if mortality did not exceed 10 % (DIN 2001, Nagel 2002).

All exposures were repeated independently three times using embryos from randomly selected hatching groups, and DCA dilutions were always prepared freshly. Means and standard deviations were calculated for each concentration using a spreadsheet template (Microsoft Excel 2007). Data were plotted with Graphpad Prism 5.X (Graphpad Inc., San Diego, USA) and fitted by means of a sigmoid regression with variable slope as a model equation for determination of the corresponding LC_{50} values.
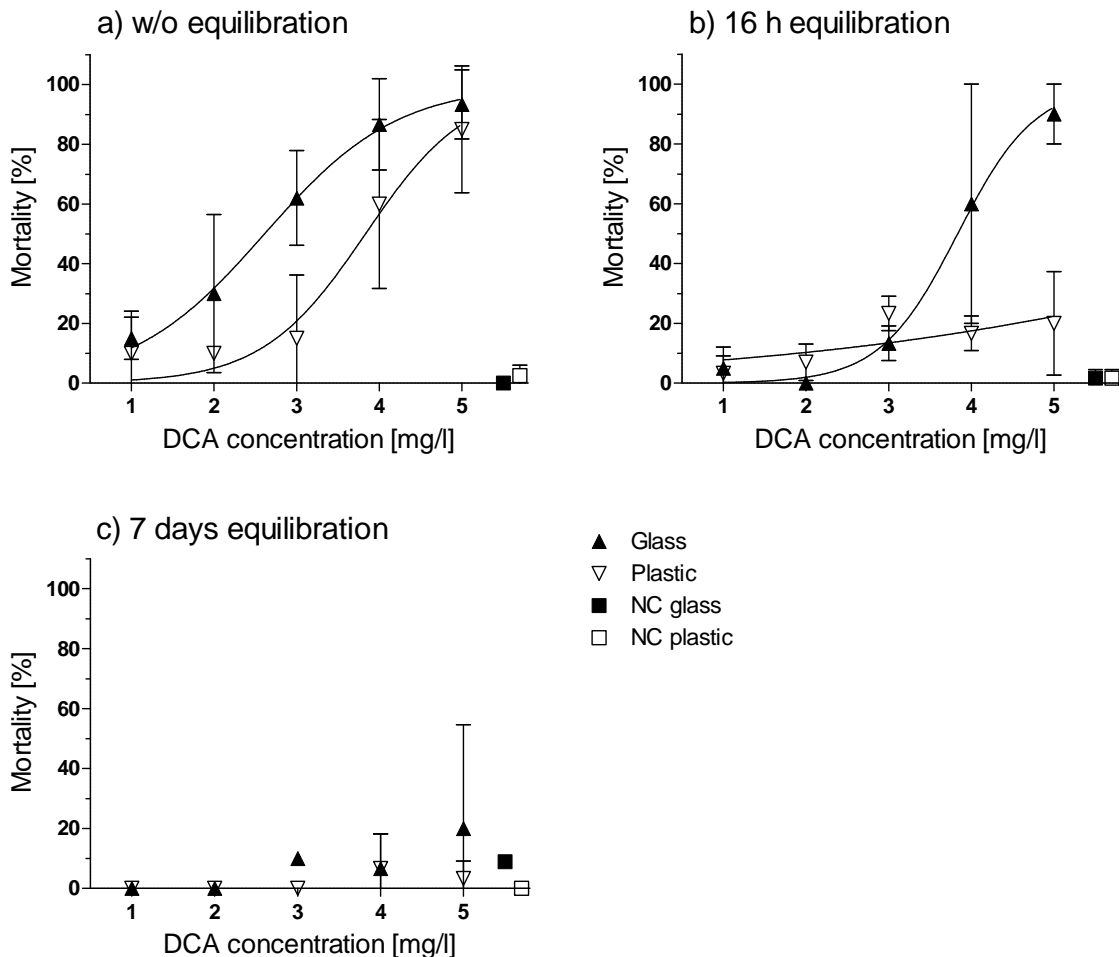

Fig 5.1a-c Mortality of 3,4-dichloroaniline in the fish embryo test after 48 h of exposure. a) without equilibration, b) 16 h of equilibration, and c) 1 week of equilibration prior to exposure. NC = Negative control; Symbols represent means; Error bars give standard deviations; n = 2-3.

5.4   Results

Results shown in figure 5.1a-c represent three independent replicates, i.e. independent experiments on a different day conducted with embryos from randomly selected hatching groups and with freshly prepared test dilutions. Due to occasional experimental difficulties, few data points are based on only two single values. Mean mortality ± standard deviation of the negative controls was $3.5 \pm 4.4\%$ (glass) and $2.8 \pm 5.1\%$ (plastic). DCA exposure returned LC_{50} values of 2.5 mg/L (glass vessels) and 3.8 mg/L (plastic vessels) in the experiments without equilibration prior to the on-set of exposure (Fig. 5.1a). With 16 h of equilibration in the test vessels, no LC_{50} could be determined in plastic 6-well plates within the tested range of concentration, whereas $LC_{50,glass,16 h}$ was 3.8 mg/L (Fig. 5.1b). No LC_{50} values could be determined for both types of vessel after 7 days of equilibration prior to exposure (Fig. 5.1c). Effects after one week of prior substance-vessel equilibration occurred only in the concentration of 5 mg/l DCA in one of three replicates using glass vessels. No effects above 10 % were found at all in tests with exposure in plastic after one week of prior substance-vessel contact.

5.5   Discussion

Vessel-DCA contact time and test exposure in plastic vessels consistently resulted in lesser effectiveness. For a DCA concentration of 3.7 mg/ml, literature reports a mortality of $68 \pm 24\%$ in a 16 interlab-study (DIN 2001). LC_{50} values of DCA were reported to be $3.3 \pm 0.$ mg/l (DIN 2001) and 1.6 to 2.4 mg/l (Lammer et al. 2009a). LC_{50} values obtained in the present study were within this range only in case of exposure directly after introduction of DCA into both vessel types and in case of exposure in glass after 16 h of prior vessel-substance equilibration. LC_{50} of DCA in plastic was greater compared to glass in case of direct exposure, and no LC_{50} could be determined after 16 h of prior equilibration in plastic vessels.

Adsorption of test substances to, in particular, plastic test vessels, which very likely lead to the observed differences, could also impact the effects recorded for other substances in the FET. Because metals have been reported to bind to glass (Catalfamo et al. 2006, Stas et al.

2004), the impact of plastic vessel cannot be addressed by simply altering procedures and substituting glass for plastic. In order to counter potential sorption to vessel materials, Lammer et al. (2009b) proposed a flow-through system for the FET. However, such an approach is time- and cost-intensive (Lammer et al. 2009b). Current efforts of optimisation of the test protocol advocate semi-static approaches with renewal of the test solution at constant intervals, in order to equilibrate tested compounds with vessels (Busquet et al. 2010). Passive-dosing techniques are one very promising strategy to address the identified impact of vessel materials because the test substance is continuously renewed by partitioning from a dominating reservoir loaded in a biocompatible polymer (Kwon et al. 2009, Smith et al. 2010).

The presented results confirm that measures are necessary to minimise the influence of vessel material, and that the exact time test compounds are introduced into test vessels can be crucial for the outcomes of experiments.

5.6   References

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

Busquet FS, Braunbeck T, Belanger SE, Kleensang A, Halder M, Lillicrap A, Amcoff P (2010): OECD validation study on the transferability, intra- and inter-laboratory reproducibility of the zebrafish embryo toxicity test. Proceedings of the 20th Annual SETAC Europe Meeting, May 23rd-27th 2010, Seville, Spain

Catalfamo P, Primerano P, Arrigo I, Corigliano F (2006): Use of a glass residue in the removal of heavy metals from wastewater. Ann Chim-Rome 96, 487-492

Dahlstrom M, Jonsson PR, Lausmaa J, Arnebrant T, Sjogren M, Holmberg K, Martensson LGE, Elwing H (2004): Impact of polymer surface affinity of novel antifouling agents. Biotechnology and Bioengineering 86, 1-8

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

German Federal Ministry of Justice (2005): Gesetz über Abgaben für das Einleiten von Abwasser in Gewässer (Abwasserabgabengesetz - AbwAG)

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 (2007): ISO 15088: Water quality — Determination of the acute toxicity of waste water to zebrafish eggs (*Danio rerio*). Iso Guideline

Koutsopoulos S, Patzsch K, Bosker WTE, Norde W (2007): Adsorption of trypsin on hydrophilic and hydrophobic surfaces. Langmuir 23, 2000-2006

Kwon JH, Wuethrich T, Mayer P, Escher BI (2009): Development of a dynamic delivery method for in vitro bioassays. Chemosphere 76, 83-90

Lammer E, Carr GJ, Wendler K, Rawlings JM, Belanger SE, Braunbeck T (2009a): Is the fish embryo toxicity test (FET) with the zebrafish (Danio rerio) a potential alternative for the fish acute toxicity test? Comp Biochem Phys C 149, 196-209

Lammer E, Kamp HG, Hisgen V, Koch M, Reinhard D, Salinas ER, Wendler K, Zok S, Braunbeck T (2009b): Development of a flow-through system for the fish embryo toxicity test (FET) with the zebrafish (Danio rerio). Toxicol in Vitro 23, 1436-1442

Nagel R (2002): DarT: The embryo test with the zebrafish Danio rerio - a general model in ecotoxicology and toxicology. Altex 19, 38-48

Palmgren JJ, Monkkonen J, Korjamo T, Hassinen A, Auriola S (2006): Drug adsorption to plastic containers and retention of drugs in cultured cells under in vitro conditions. Eur J Pharm Biopharm 64, 369-378

Smith KEC, Dom N, Blust R, Mayer P (2010): Controlling and maintaining exposure of hydrophobic organic compounds in aquatic toxicity tests by passive dosing. Aquatic Toxicology 98, 15-24

Stas IE, Shipunov BP, Pautova IN, Sankina YV (2004): A study of adsorption of lead, cadmium, and zinc ions on the glass surface by stripping voltammetry. Russ J Appl Chem+ 77, 1487-1490

Chapter 6

___

The impact of extraction methodologies on the toxicity of sediments in the zebrafish (Danio rerio) embryo test

Hanno Zielke[1], Thomas-Benjamin Seiler[1], Sabine Niebergall[2], Erik Leist[2], Markus Brinkmann[1], Denise Spira[3], Georg Streck[4], Werner Brack[4], Ute Feiler[3], Thomas Braunbeck[2] and Henner Hollert[1]

[1]Department of Ecosystem Analysis, Institute for Environmental Research (Biology V), RWTH Aachen University, Aachen, Germany

[2]Aquatic Ecology and Toxicology Section, Department of Zoology, University of Heidelberg, Heidelberg, Germany

[3]Department Biochemistry and Ecotoxicology, German Federal Institute of Hydrology, Koblenz, Germany

[4] Department Effect-Directed Analysis, Helmholtz Center of Environmental Research – UFZ, Leipzig, Germany

6.1   Abstract

6.1.1   Purpose

Traditionally, methods for sediment extractions are characterised using chemical analyses. However, in order to evaluate sediment extracts with regard to biological effects and, thus, bioaccessibility, extraction methods have to be compared to effect data obtained from experiments with *in situ* exposure scenarios, i.e., sediment contact tests. This study compares four extraction methods and sediment contact test data from a previous project with respect to predictive power in the fish embryo test (FET) with zebrafish (*Danio rerio*).

6.1.2   Materials and methods

A natural and an artificial sediment spiked with a mixture of six organic pollutants (2,4 dinitrophenol, diuron, fluoranthene, nonylphenol, parathion and pentachlorophenol) were extracted using (a) membrane dialysis extraction (MDE), (b) a Soxhlet procedure, (c) hydroxypropyl-β-cyclodextrin (HPCD) or (d) Tenax®-TA. Whereas the former two are regarded being exhaustive with respect to non-covalently bound contaminants, the latter two are considered to predict bioaccessibility. Resulting extracts were tested in the fish embryo assay with *Danio rerio* for embryotoxic and teratogenic potential.

6.1.3   Results and discussion

Mortalities caused by organic extracts from Soxhlet extraction and MDE were high. However, HPCD extracts turned out to be at least as effective as extracts obtained with these two methods. One possible reason might be short ageing time of the spiked sediments. Only Tenax®-TA extracts gave results comparable to the sediment contact assay for natural sediment, but revealed low reproducibility.

Significant differences between natural and artificial sediment were found for extracts obtained with techniques using native (i.e., non-freeze-dried) sediments, i.e., HPCD and Tenax®-TA. In contrast, MDE and Soxhlet extracts used freeze-dried sediment and did not differentiate between natural and artificial sediment. Therefore, freeze-drying likely altered

and equalised sediment properties that influence accessibility, such as composition of bacterial communities and organic matter quality.

6.1.4   Conclusions

Four extraction methods were successfully characterised with respect to their stringency and predictiveness for bioaccessibility. MDE was confirmed as an alternative to Soxhlet extraction. High mortalities induced by HPCD extracts underline the need to include ageing into consideration when assessing sediments. Although Tenax®-TA may basically be used to predict bioaccessibility in the fish embryo test, the high variability observed warrants further investigation of the relation between effect and extractability. Apparently, freeze-drying can severely affect sediment properties, potentially eliminating individual properties of natural sediments.

6.2   Introduction

Sediments have the property to both bind pollutants (e.g. Alexander 1995, 2000, Burton 1991, Ehlers & Loibner 2006, Hollert et al. 2007, Huang et al. 2003) as well as to release them, e.g., during flood events (Brils et al. 2007, Hollert et al. 2000, Smit et al. 2008, 2009, Wölz et al. 2008). As a consequence, sediment contamination considerably impacts water quality and the ecological status of water bodies. However, although the role of sediments for water protection has been addressed by scientists since the 1970s (Burton 1991), sediment relevance has only recently been fully recognised and acknowledged, e.g., by inclusion of sediments into regulatory frameworks such as the European Water Framework Directive by means of an amendment published in 2008 (EP/EC 2008, Förstner 2009, 2009, Hollert et al. 2007).

Since interactions between sediments, pollutants and organisms are highly complex, the assessment strategy has profound impact on the outcome of a given study. Currently, several different lab-based strategies are used in order to assess sediments easily and rapidly. Extraction is one such approach, and widely used to prepare sediment samples for biotesting (e.g. Arditsoglou & Voutsa 2008, De la Cal et al. 2008, Hallare et al. 2005, Karlsson et al. 2008, Kosmehl et al. 2007, Qiao et al. 2008, Wölz et al. 2008). Furthermore, extractions are

also an important part of integrated approaches such as effect-directed analyses (Brack 2003, Brack et al. 2005, 2009). Although extractions inevitably change the tested materials by transfer and potential alteration of the pollutants from the sediment phase to a solvent (Seiler et al. 2008), they provide a wide variety of possible insights into the contaminant spectrum of the test sample, however, depending on the procedure applied. Extraction procedures range from vigorous methods for exhaustive extraction to procedures that have been specifically developed to predict bioaccessibility and yield only a certain fraction of pollutants (e.g. Cornelissen et al. 2001, Luque de Castro & Garcia-Ayuso 1998, Reid et al. 2000, Seiler et al. 2006, 2008). Researchers may easily adapt experimental setups to the demands of the respective study by choosing an extraction method with appropriate properties. Whereas vigorous extractions simulate a "worst-case scenario", biomimetic extractions can yield insight on bioaccessibility (Hallare et al. 2010). However, in turn, appropriate interpretation of results requires profound knowledge of the specific characteristics of the extraction method applied.

Direct sediment contact tests (SCT) are an alternative tool for sediment assessment, which, by definition, directly represent bioaccessibility (Feiler et al. 2004, Heise & Ahlf 2005, Hollert et al. 2003, Marklevitz et al. 2008, Neumann-Hensel & Melbye 2006, Pane et al. 2008). These test systems simulate natural conditions more accurately than extraction-based assessment strategies by exposing the test organisms to solid test materials (i.e., sediments) with minimal alteration of the sample in comparison to most extraction procedures (Seiler et al. 2008). Thus, SCTs provide species-related data that can be used in organism-based protection strategies. In Germany, the SeKT (Sediment KontaktTest = sediment contact test) joint project framework, funded by the German Federal Ministry of Education and Research (BMBF) was initiated in order to compare recently developed limnic sediment contact tests by addressing reference conditions, control sediments and toxicity thresholds (Feiler et al. 2009a, Feiler et al. 2005, Höss et al. 2010).

When assessing sediments with either extractions or *in situ* contact tests, one factor limits comparability: Each natural sediment has unique characteristics and may vary distinctly from each other with respect to its abiotic and biotic properties. One consequence is an inherent variability of sediment toxicity tests performed with natural sediments. In contrast, artificial sediments lack the bacterial abundance and diversity as well as the complex organic matrix found in natural sediments (Fleming et al. 1998, Goedkoop et al. 2005). Therefore, artificial sediments can be used to investigate basic principles, but often lack the ability to mimic field conditions.

The present study aims at improving the understanding and interpretation of lab-based strategies for sediment assessment, i.e. bioassay results obtained from sediment testing, by relating the stringency of extraction directly to biological bioaccessibility. Semple et al. (2004) established the distinction between bioavailability and bioaccessibility. Using this definition, readily available compounds are termed bioavailable, whereas substances potentially available by desorption are termed bioaccessible (Semple et al. 2004). Since the biotests applied in the present study do not differentiate whether effects are induced by readily available and desorbed compounds, the term "bioaccessibility" is used. Furthermore, bioavailability/bioaccessibility can be defined as a dual concept that integrates accessibility and chemical activity, with accessibility being the mass of a contaminant that is available for uptake into an organism and chemical activity representing the potential for physico-chemical reactions (Reichenberg and Mayer 2006).

However, as Reichenberg and Mayer (2006) point out, this approach focuses on the physicochemical aspects of bioavailability, not its equally important biological aspects. Biotests act as excellent first-line procedure that provide insight into bioaccessibility at the level of biological effect, i.e. mortality or effect in general (Hallare et al. 2010). The present study was designed in order to relate this biologically-expressed bioaccessibility to several extraction methods widely-used in sediment assessment.

An artificial and a natural sediment containing a complex mixture of six organic contaminants (2,4-dinitrophenol, diuron, fluoranthene, nonylphenol, parathion and pentachlorophenol) were extracted using four different extraction methods. These sediments and pollutants had been chosen, spiked and tested for effects in direct sediment contact tests within the SeKT project framework (Feiler et al. 2005, 2009a, Höss et al. 2010, Seiler 2010) and, thus, allowed for a comparison between direct sediment contact test (SCT) and extraction data. Details on the selection of the compounds are given in Feiler et al. (2009a). Untreated control sediments have been used as baseline reference, being applied in the SCT as well as in the extractions.

This approach addresses the following factors:

(a) Four extraction methods were selected. In order to compare different stringencies, two methods represented vigorous extraction, whereas the other two were chosen because they have been developed to predict bioaccessibility.

Soxhlet extraction (SOX; Bjorklund et al. 2002, Luo et al. 2009, Luque de Castro & Garcia-Ayuso 1998, Wölz et al. 2008) is a widely known and commonly applied technique. Several protocols for SOX have been shown to yield extracts containing all leachable contaminant fractions at high recovery rates (Luque de Castro & Garcia-Ayuso 1998, Seiler et al. 2008).

The distribution and acceptance of this method were the reasons for including SOX into the present study. Membrane dialysis extraction (MDE) is a recently introduced procedure by Seiler et al. (2006), based on previous work by Macrae and Hall (1998) and Huckins et al. (1990). This passive leaching technique also provides exhaustive extracts regarding non-covalently bound contaminants without using any auxiliary energy sources and, thus, effectively reduces the risk of loss of volatile or thermally labile substances. Hence, the method can be addressed as an exhaustive passive extraction technique for solid environmental samples (Seiler et al. 2006). Hydroxyl-propyl-β-cyclodextrin extraction (HPCD) and Tenax®-TA extraction (TNX) are both techniques considered to provide extracts which represent the bioaccessible fractions of pollutants (Brack et al. 2009, Cornelissen et al. 2001, De la Cal et al. 2008, Reid et al. 2000, Schwab & Brack 2007, Ten Hulscher et al. 2003, Van der Heijden & Jonker 2009).

Within the present study, SOX was used as a reference and expected to yield highly effective extracts. The novel method MDE was compared to SOX in order to further support its classification as a vigorous extraction procedure. HPCD and TNX were tested to confirm their predictiveness for bioaccessibility.

(b) This comparison was carried out at the biotest level, i.e., with the fish embryo assay with zebrafish (Braunbeck et al. 2005, Nagel 2002). Most extraction methods have so far been characterised in relation to chemically determined uptake and accumulation of pollutants (e.g. Cornelissen et al. 2001, De la Cal et al. 2008, Moermond et al. 2007, Sormunen et al. 2009, Swindell & Reid 2006, Ten Hulscher et al. 2003). To our knowledge, only few studies have been conducted in order to comparatively investigate the direct or observable biological effects of sediment extracts (Andersson et al. 2009, Feiler et al. 2009b, Kosmehl et al. 2007, Seiler et al. 2006). Since directly displayed effects are easily observable endpoints in bioassays, this approach readily provides information about potentially negative impacts of sediment contamination on aquatic organisms. The choice of four different methods in this regard allowed an evaluation whether extraction stringency translates into different observable effects. Furthermore, the zebrafish is a well-established vertebrate model organism with benthic eggs, which provides insight into potential effects on freshwater fish in general (Braunbeck et al. 2005, Lammer et al. 2009, Nagel 2002).

(c) Use of sediments from the SeKT project enabled a direct comparison of extract-induced effects with effects in *in situ* zebrafish sediment contact tests. Thus, a more realistic classification of extract-related effects became possible. In case the two biomimetical methods HPCD and TNX also predicted bioaccessibility at the level of observable biological

effects in a 48 h toxicity assay with zebrafish, their respective extract toxicity was assumed to be in agreement with the contact test results. In contrast, SOX and MDE were expected to give significantly higher toxicities.

(d) Simultaneous testing of natural and artificial sediments covered influences of sediment type and accompanying parameters on biological effects. In accordance with results from the SeKT project framework and known properties of the two sediments used, such as organic matter and organic carbon contents (Feiler et al. 2009a, Seiler 2010) as well as different composition of the bacterial community described for other natural and artificial sediment (Goedkoop et al. 2005, Verrhiest et al. 2002), higher effects were expected for extracts from the artificial OECD 218 sediment.

6.3   Material and methods

6.3.1   Sediments, substances and spiking

Sediments had been sampled and spiked within SeKT framework project (Feiler et al. 2005, 2009a, Höss et al. 2010, Seiler 2010). Natural sediment was sampled at Altrip, a back water of the river Rhine near Worms (Rhineland-Palatinate, Germany) in August 2006. Details on the sampling procedure are given in Feiler et al (2009a). Total organic carbon was 35 g/kg. Sediment composition was 0.8% gravel, 1.3% sand, 74.1% silt and 23.4% clay. Chemical analyses revealed no relevant background contamination. Detailed chemical data on background concentrations in the natural sediment are given in Feiler et al. (2009a). Artificial sediment was prepared according to OECD guideline 218 (OECD 2004) except for a kaolin clay content reduced from 20% to 5% and replaced with quartz sand (F36; Quarzwerke Frechen, Frechen, Germany). In order to exclude any background effects by residual contamination (natural sediment) or formulation (artificial sediment), unspiked sediments were prepared as controls.

Both sediments were spiked with a mixture of 2,4-dinitrophenol, diuron, fluoranthene, nonylphenol, parathion and pentachlorophenol at 100 mg/kg dry weight each. For purposes of comparison and since the observed effects could not be attributed to single compounds, all further calculations are based on the total of 600 mg organic contaminant/kg dry weight. Spiking was carried out according to OECD guideline 218 (OECD 2004). The mixture was applied to a previously dried portion of 10% wet weight of sediment to be spiked. Acetone

(picograde, Sigma Aldrich) was used as solvent for the organic substances and completely evaporated at room temperature before remixing the spiked portion with the remaining 90% sediment. To exclude background effects caused by residual acetone, a solubiliser control was prepared in parallel. After merging the two sediment fractions and thorough stirring, the sediments were equilibrated at 20°C and aerated for 5 - 7 days before further usage. Between experiments, spiked sediments were stored in a darkroom at 4°C.

All results are given as nominal concentrations.

6.3.2   Extractions

Depending on the extraction method, either freeze-dried or native sediments were applied. All glassware was rinsed successively with distilled water, acetone p.a. (Applichem, Darmstadt, Germany) and *n*-hexane p.a. (Merck, Darmstadt, Germany), followed by duplicate rinsing with the solvent applied in the respective extraction method. Each extraction was replicated three or four times, with replications on the extraction level for all methods except for SOX, where the replication was done at biotest level. For the purpose of the present study, a replicate is defined as an independent extraction of a sample taken from the homogenized spiked stock sediment and subsequent testing of the resulting extract in the fish embryo assay with embryos from randomly selected hatching groups. In case of SOX, one replicate is defined as one test of the same extract in an independent fish embryo assay with embryos from randomly selected hatching groups. Each replicate was carried out on a different day. Prior to the main experiments, range-finding tests were carried out to identify relevant concentration ranges.

6.3.3   Soxhlet extraction

Soxhlet extraction was carried out according to the protocol described by Hollert et al. (2000). In brief, 4.5 g of freeze dried sediment were placed in a cellulose extraction thimble (Whatman, Maidstone, England), covered with glass wool (Riedel-de-Haën, Seelze, Germany) and extracted with 400 ml acetone for 14 h at 6 to 8 cycles per hour. All extracts were reduced first using a Laborota 4011-digital rotary evaporator (Heidolph, Kehlheim, Germany) with a vacuum of 450 mbar (CVC 2, Vacubrand, Wertheim, Germany) at 35°C

(acetone) and 48°C (*n*-hexane), respectively, to a volume of 1 - 2 ml and then close to dryness under a continuous nitrogen stream (Hollert et al. 2000). After re-dissolving in 500 µl dimethyl-sulfoxide (DMSO, Mallinckrodt Baker, Deventer, Netherlands), extracts were stored at -20°C until further use. The storage concentration of SOX stock extracts was 9 g dry weight sediment equivalent (DW SEQ)//ml, three times higher than for the three other stock extracts due to experimental necessities.

In addition to sediment controls and solubiliser controls, process controls with extraction thimbles containing no sediment were performed along with each extraction replicate.

6.3.4   Membrane dialysis extraction

Membrane dialysis extraction (MDE) was conducted following the protocol of Seiler et al. (2006). Potentially toxicologically relevant production residues were removed from membranes prior to utilization by means of a newly developed multi-reflux cleaning facility called Prescot (Pre-extraction Soxhlet cold technology; Fig. 6.1). Three 85 cm sections of a 50 µm thick low-density polyethylene membrane (Jencons, Leighton Buzzard, UK) were coiled with tweezers, inserted into the Soxhlet extractor of the PRESCOT facility and pre-extracted with 500 ml *n*-hexane for 24 h at approx. 12 - 14 cycles/h. Once cleaned, membranes were dried by suspending in a fume hood for approximately 15 min and the last 1 cm was cut off from either end. The cleaned and dried membranes were then coiled and stored in a solvent-rinsed vapour-tight stainless steel container at -20°C under nitrogen until required.

For sediment extraction, 1.5 g freeze dried sample were inserted into the membranes which were subsequently transferred to brown glass jars containing 150 ml *n*-hexane and dialysed for 48 h at room temperature. After this period, membranes were carefully removed; extracts were reduced in volume and re-dissolved as described above. Stored stock concentration was 3 g DW SEQ/ml DMSO.

Extractions were replicated four times for each sample. Sediment, solubiliser and process controls with empty membranes as well as solvent controls without membranes were carried out in parallel.
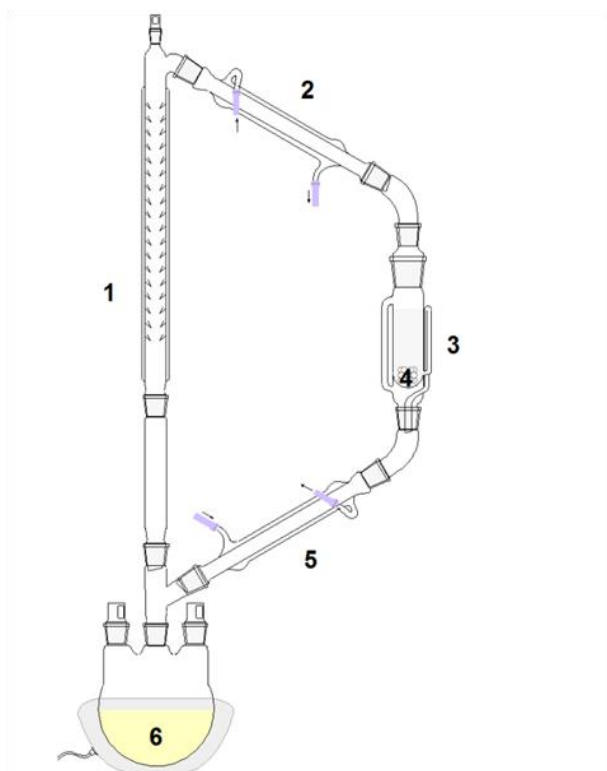
Fig. 6.1 Cold Soxhlet pre-extraction, PRESCOT. A distillation facility and a Soxhlet extractor are connected by two Liebig condensers. The solvent (as a rule, 500 ml *n*-hexane) is evaporated similarly to common distillation procedures (1), cooled by a Liebig condenser (2) and drips into a 150 ml Soxhlet extractor (3) containing the membranes on glass beads (4). Each Soxhlet cycle, hexane is flushed back *via* a second Liebig condenser (5) into the reservoir (6)

6.3.5 Hydroxypropyl-β-cyclodextrin extraction

Hydroxypropyl-β-cyclodextrin extraction (HPCD) was based on the protocol described by Reid et al. (2000) and modified according to the results of preliminary tests.

1.5 g sediment dry weight equivalent of each sample were shaken horizontally together with 30 ml 50 mM HPCD (Sigma-Aldrich) in 100 ml Erlenmeyer flasks for 20 h at room temperature. The flasks were covered with parafilm (Pechiny, Chicago, USA) to prevent sample loss due to volatilisation.

After shaking, samples were transferred to 50 ml centrifuge tubes (Greiner Bio-One, Kremsmünster, Austria) and centrifuged at 1900 g for 20 min. The resulting supernatant was separated and adjusted to pH ≤ 2 with 1 M HCl (Riedel-de-Haën, Seelze, Germany).

Pollutants were recovered from the HPCD by overlaying the aqueous phase with 60 ml *n*-hexane, followed by shaking for 10 h at room temperature on a horizontal shaker. The

hexane phase was then collected with a glass separation funnel and subsequently reduced and re-dissolved like the other extracts types (see above). Stored stock concentration was 3 g DW SEQ/ml DMSO.

Extractions were repeated four times for each sample. As controls, sediment, solubiliser and process controls (HPCD only) were run.

6.3.6 Tenax® TA extraction

Tenax® TA extraction (TNX) was carried out with modifications and adaptations of several protocols (Cornelissen et al. 2001, Cornelissen et al. 1997, Ten Hulscher et al. 2003).

Prior to use, Tenax® TA (Sigma-Aldrich) was pre-extracted three times with 10 ml/g distilled water, three times with 10 ml/g acetone and three times with 10 ml/g *n*-hexane, respectively, to remove potential production residues (Cornelissen et al. 1997). For handling reasons, quantitative removal of any cleaning solvent had to be ensured prior to application of the next solvent. For this, after rinsing, Tenax® TA was dried each time overnight in a 250 ml Erlenmeyer flask at 75 - 90°C.

For extraction, 1.5 g Tenax® TA were added to 1.0 g sediment dry weight equivalent of each sediment sample and control in 100 ml Erlenmeyer flasks. After addition of 70 ml distilled water, the flasks were shaken on a horizontal shaker for 6 h at 20 ± 2°C.

Subsequently, samples were transferred to a 100 ml separation funnel, and the sediment and most of the water was removed. Tenax® TA beads were then extracted for 30 seconds three or four times with a total volume of 30 ml *n*-hexane (Ten Hulscher et al. 2003).

The resulting extracts were finally reduced in volume and re-dissolved like the other extract types (see above). Stored stock extract concentration was 3 g DW SEQ/ml DMSO. Each extraction was independently repeated four times. As controls, sediment (Altrip sediment only), solubiliser and process controls were run.

6.3.7   Fish embryo test with *Danio rerio*

Fish culture

Zebrafish were maintained according to Braunbeck et al. (2005) in flow-through 30 L aquaria at $26 \pm 1°C$ in hatching groups of 6 males and 6 females each at an artificial day/night-rhythm of 14/10 h. The animals were fed dry feeding flakes (TetraMin™, Tetra GmbH, Melle) and *Artemia* sp. nauplii (Great Salt Lake Artemia Cysts, Sanders, Ogden, USA).

Embryo collection

For spawning, the animals were transferred into special breeding aquaria, which contained plastic plants in order to stimulate mating. Spawning occurred within 0.5 - 1 h after the onset of illumination. In order to prevent egg predation, the bottoms of the aquaria had been replaced with a mesh with 1 mm openings, so that eggs fell through and could be easily collected.

Aqueous fish embryo test with *Danio rerio*

The fish embryo assay was conducted according to DIN 38415-6 (DIN 2001) and the methods given in Seiler et al. (2006) and Nagel (2002). Artificial water according to ISO 7346/3 (1996) was used as the test medium (294.0 mg/l $CaCl_2 \cdot 2\ H_2O$, 123.3 mg/l $MgSO4 \cdot 7\ H_2O$, 63.0 mg/l $NaHCO_3$ and 5.5 mg/l KCl).

Dilutions series were carried out with each stored stock extract in order to determine LC_{50} values. Per concentration, 10 embryos preselected for normal development were exposed in 2 ml of test solution each. Positive controls (PC, 3.7 mg/L 3,4-dichloroaniline) and negative controls (NC, artificial water only) were tested using 20 and 40 embryos, respectively. Sediment, solubiliser, process and solvent controls were tested in a concentration equal to the highest tested sample concentration. NCs were carried out with artificial water only, whereas PCs contained a concentration of 3.7 mg/l DCA (DIN 2001, Nagel 2002).

The exposure lasted 48 h at $26 \pm 1°C$ with subsequent microscopic examination and evaluation of the embryos. A test was regarded valid if mortality in the NC was $\leq 10\%$. Mortality criteria were (a) coagulation, (b) lack of heartbeat, (c) missing somite development and (d) failure of tail detachment from the yolk sack (DIN 2001). In addition, sublethal parameters including reduced heart beat (not to be confused with the acute mortality criterion "no heartbeat"), lack

of blood stream, edema formation, complete lack of or reduced pigmentation, delayed development as well as general and spine malformations were recorded (Hollert et al. 2003, Nagel 2002). Measured lethal and sublethal effects are given in Table 6.1.

Table 6.1 Lethal and sublethal effects measured in the fish embryo test after 48 h of exposure (cf. Hollert et al. 2003)

Lethal	Sublethal
No heartbeat	No pigmentation
Tail not detached	No eye pigmentation
No somites developed	No bloodstream
Coagulation	Spine malformation
	Edema
	General developmental aberrations

Sediment contact assay with *Danio rerio* embryos

The protocol was carried out according to the protocol given in Hollert et al. (2003), modified as described by Seiler (2010). This test was carried out in 6-well plates with 3 g test sediment and 5 ml artificial water per 5 fish embryos and well, and 15 embryos (3 wells) per sample. In order to compare LC_{50} values from the sediment contact test (mg pollutants/g sediment dry weight) to those for extracts in the present study (mg pollutants/ml test volume), an adaption became necessary. This was achieved relating contact test data to total water volume. Total water volume consists of artificial water plus pore water (= wet weight minus dry weight). The known dry weight fraction of 3 g sediment in each well yielded the maximal mass of pollutants in the contact test system, which was then divided by the total water volume, resulting in the desired joint concentration unit of µg pollutant/ml test volume.

Validity

As a control for the validity of a given test, the results of the positive control (3,7 mg/l 3,4-dichloroaniline) had to be $\geq 10\ \%$. Literature reports a mortality of $68 \pm 24\ \%$ mortality for

this concentration in a 16 lab-study (DIN 2001) and $LC_{50,DCA}$ of 3.3 ± 0.5 mg/l (DIN 2001) as well as 1.6 to 2.4 mg/l (Lammer et al. 2009).

Negative, sediment, process and solubiliser controls were regarded valid if the mortality did not exceed 10%. (DIN 2001, Nagel 2002).

As further quality criteria, egg fertilisation rate had to be ≥ 90 % and more than 70 % of the embryos were to display normal development prior to selection in order for a test to be carried out (Lammer et al. 2009).

Data processing and statistical analyses

Results were evaluated using an Excel 2007 sheet (Microsoft, Redmond, USA) and plotted in Graphpad Prism 4 (Graphpad, San Diego, USA). A sigmoid dose-response regression and the corresponding LC_{50} values were determined for each replicate. Finally, means and standard deviations for the samples were calculated.

Statistical analyses were carried out with SigmaStat 3.5 (Systat, Erkrath, Germany). It was assumed that statistical samples were not obtained from the same statistical population, since five different methods were applied in sample preparation. Therefore, LC_{50} values of each independent test replicate were used as raw data and individually compared pair-wise with t-tests. If tests for normality distribution or variance homogeneity were negative, a rank-based Kruskal-Wallis-test was performed.

6.4   Results

Mean control mortalities ($\pm$ standard deviation) were 1.6 ± 2.6 % (negative controls, n = 23), 3.2 ± 4.8 % (solvent controls, n = 19), 2.3 ± 4.3 % (process controls, n = 31), 2.7 ± 7.4 % (sediment controls, n = 19), 1.7 ± 3.8 % (solubiliser controls, n = 30) and 85.1 ± 13.2 % (positive controls, n = 23). The applied dilutions gave mortalities of 0 % in the lowest concentrations and of 100 % in the highest concentrations in all performed tests.

Statistical comparison of the results for natural and artificial sediment revealed significant differences between both sediment types in the whole sediment contact test as well as in the hydroxyl-propyl-β-cyclodextrin-extraction (HPCD) and Tenax[®] TA (TNX) extraction, but not for the Soxhlet (SOX) and membrane dialysis extractions (MDE; Fig. 6.2).
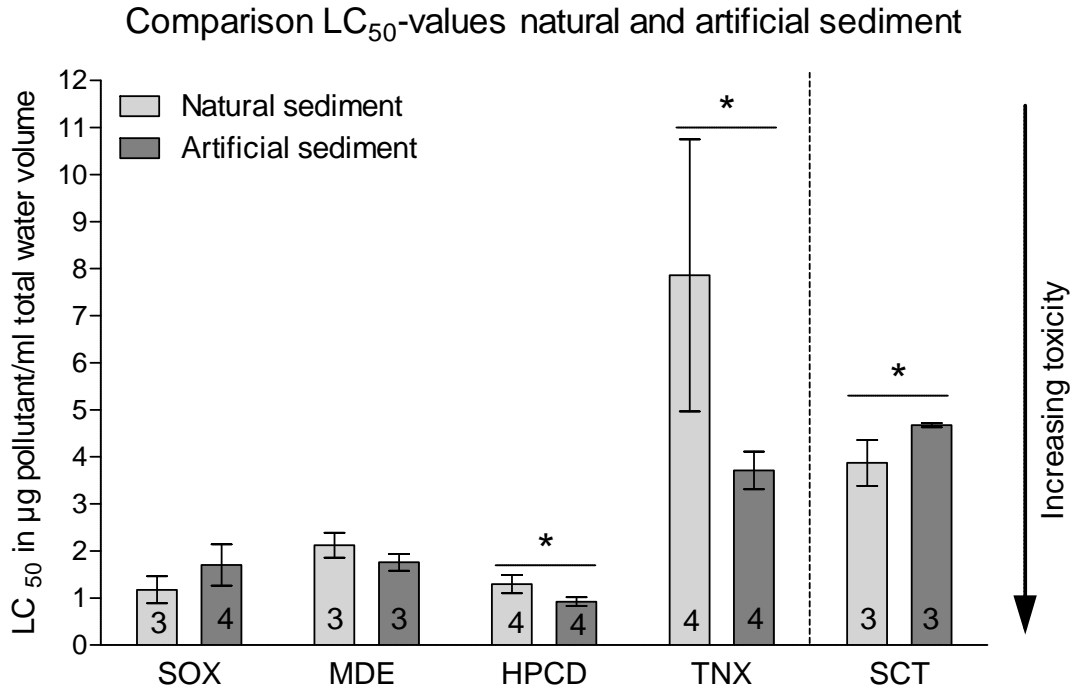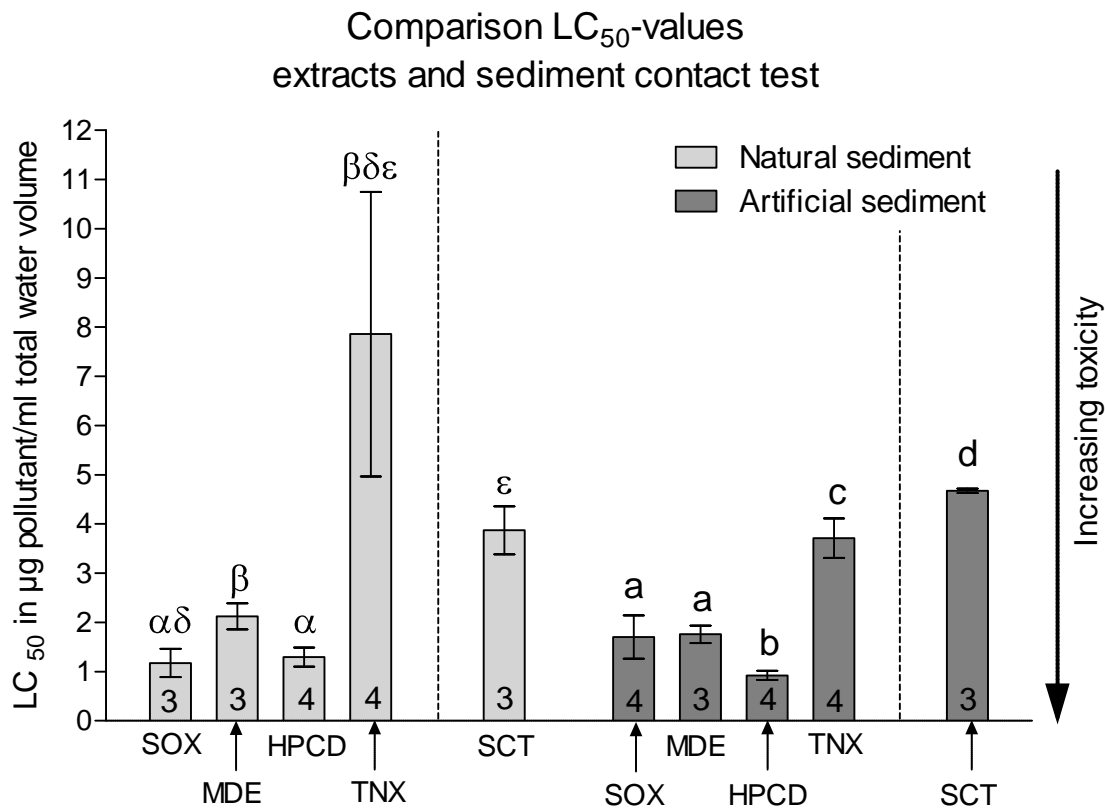
Fig. 6.2 Comparison of 48 h LC_{50} values for all extract types and whole sediment contact test (SCT) in the fish embryo assay. SOX: Soxhlet extraction; MDE: membrane dialysis extraction; HPCD: hydroxy-propyl-β-cyclodextrin extraction; TNX: Tenax[®] TA extraction; SCT: direct sediment contact test; Sediments were spiked with a mixture of 600 mg organic contaminant/kg (2,4-dinitrophenol, diuron, fluoranthene, nonylphenol, parathion-ethyl and pentachlorophenol; 100 mg/kg each). Numbers in columns indicate independent extraction replicates, except Soxhlet (= biotest replicates). Asterisks indicate significant difference (Student t-test, $p \leq 0.05$). Error bars indicate standard deviations

When evaluating the extraction methods separately for Altrip and OECD 218, two groups of effectiveness are distinguishable (Fig. 6.3). Whereas SOX, MDE and HPCD extracts resulted in comparable toxicities to zebrafish embryos, TNX extracts were less toxic. However, it should be noted that data for TNX of natural sediment had the highest standard deviation (36.8% of mean) of all tests with extracts as well as the sediment contact tests (SCT), ranging from 0.9% (artificial sediment in SCT) to 25.7% (artificial sediment in SOX) of respective means.

TNX extracts turned out to be significantly less effective than HPCD when applied on natural sediment and than SOX, MDE and HPCD on artificial sediment. Furthermore, MDE results were significantly less toxic than SOX and HPCD results for the natural sediment. For OECD 218, HPCD extracts were significantly more toxic than SOX and MDE extracts (see Fig. 6.3). Comparing the extractions with the SCT, extracts from all methods except TNX had significantly stronger toxic effects, regardless of the sediment type (see Fig. 6.3). TNX

extracts from artificial sediment were significantly less toxic than sediment directly tested in the SCT, whereas toxicity of extracts from natural sediment was in the same range as SCT. However, the latter displayed a high variability (see Fig. 6.3).

All extracts induced sublethal effects after 48 h, which were highly reproducible and correlated well with respective mortalities (Fig. 6.4). There was a distinct concentration-dependent increase of occurrence and severity of lethal and sublethal effects. Starting at the highest concentrations, the effects showed the following sequence: (a) coagulation, (b) developmental arrest at the epiboly stage, (c) lack of tail detachment and delayed development including no heartbeat and lack of pigmentation, (d) no pigmentation, no heartbeat and edema, and (e) reduced pigmentation and occasional edema in the lowest effect concentrations without mortality.

Fig. 6.3 Comparison of 48 h LC_{50} values for all extract types and whole sediment contact test (SCT) in the fish embryo assay. SOX: Soxhlet extraction; MDE: membrane dialysis extraction; HPCD: hydroxy-propyl-β-cyclodextrin extraction; TNX: Tenax® TA extraction; SCT: direct sediment contact test; Sediments were spiked with a mixture of 600 mg organic contaminant/kg (2,4-dinitrophenol, diuron, fluoranthene, nonylphenol, parathion-ethyl and pentachlorophenol, 100 mg/kg each). Numbers in columns indicate independent extraction replicates, except Soxhlet (= biotest replicates). Different letters indicate statistical difference (Student t-test, $p \leq 0.05$). Error bars indicate standard deviation
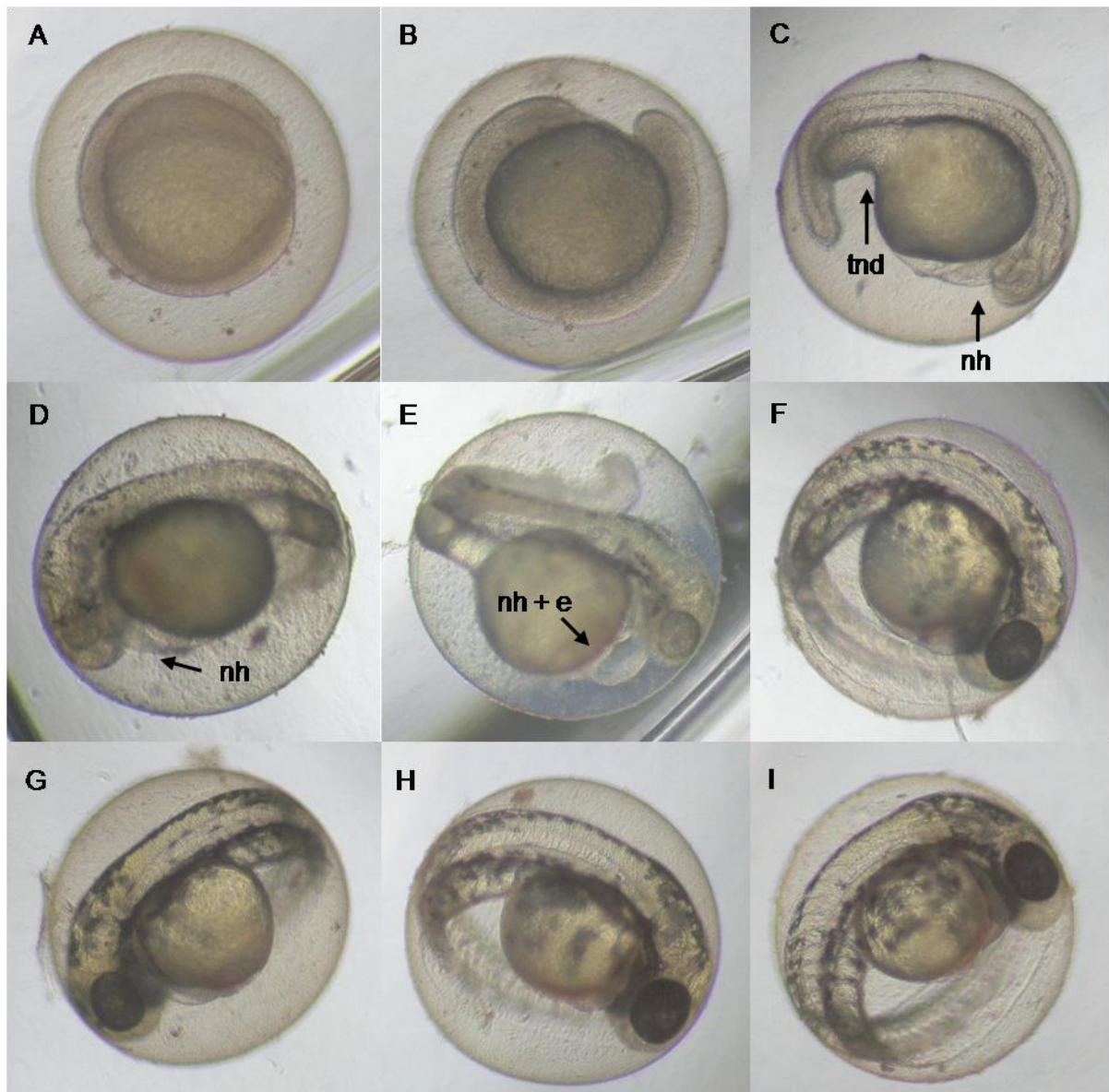
Fig. 6.4 Concentration-dependent sequence of sublethal and lethal effects after 48 h of exposure; natural sediment, MDE, first replicate; nh = no heartbeat, tnd = tail not detached, e = edema. **A:** early somite stage, 11.54 mg dry weight sediment equivalent (DW SEQ)/ml; **B:** late somite stage, 9.23 mg DW SEQ /ml; **C:** tail not detached, no heartbeat, reduced pigmentation, edema, 8.08 mg DW SEQ /ml; **D:** no heartbeat, reduced pigmentation, 4.63 mg DW SEQ /ml; **E:** no heartbeat, reduced pigmentation, edema, 3.46 mg DW SEQ /ml; **F:** normal development, 1.15 mg DW SEQ /ml; **G:** normal development, sediment control; **H:** normal development, solubiliser control; **I:** normal development, negative control

6.5 Discussion

A comparison of the influence of sediment type (natural or artificial) reveals a corresponding behaviour of methods reproducing bioaccessibility (HPCD, TNX and SCT) on the one hand and vigorous methods (SOX, MDE) on the other (see Fig. 6.2), which is likely to be due to the inherent properties of native sediments. Organic matter dominates sorption of organic compounds at levels ≥ 0.1% of total sediment (Northcott & Jones 2000). In general, binding to organic matter reduces bioavailability and, thus, toxicity of pollutants (e.g. Ghosh et al. 2000, Nam et al. 1998, Ramos et al. 1998). The influence of natural organic matter cannot fully be mimicked with artificial sediments, so that natural sediments regularly provide higher binding capacities for organic pollutants (Fleming et al. 1998). Furthermore, whereas artificial sediments have been shown to bear reduced bacterial abundance and diversity (Goedkoop et al. 2005), the higher abundance of bacteria in natural sediments may have lead to a degradation of effective compounds and could, thus, explain the lower mortality observed (see Fig. 6.2).

For SOX and MDE, the sediment samples were freeze-dried prior to extraction. Freeze-drying has most likely eliminated distinct differences deriving from the composition of the bacterial community. In parallel, properties of the organic matter might have been altered and rendered more similar between the two types of sediments (Northcott & Jones 2000, Seiler et al. 2008). Such changes are likely to account for the observed lack of significant difference in effects by extracts from natural and artificial sediment obtained with SOX and MDE. On the other hand, results suggest that freeze-drying may severely affect sediment properties and modify effects observed with natural sediments.

Despite the inherent limitations of artificial sediments discussed above, the results also underline their great advantage of providing stable test conditions. Therefore, we recommend to use artificial sediment in addition to natural field samples in order to provide a stable reference to which natural samples can be related to.

With respect to the differences between extraction methods as well as between extraction methods and sediment contact test, SOX was employed as reference method. The high mortality induced by SOX extracts correlates with expectations, since this method has frequently been documented to exhaustively extract the non-covalently bound fraction of organic compounds from sediments (e.g. Hollert et al. 2000, Reid et al. 2000, Santos et al. 1997, Stokes et al. 2005).

The comparable toxicity of MDE and SOX extracts for artificial sediment underlines the applicability of MDE as alternative vigorous method shown in chemically as well as biologically focused studies (Seiler 2010, Seiler et al. 2006). However, the significant difference between these two techniques found for extracts of natural sediment also indicates the need for further research into this correlation. One possible reason may be retention of organic matter as strong sorption phases in MDE (Seiler et al. 2006, 2008).

HPCD also provided extracts at least as toxic as the vigorous techniques SOX and MDE. This finding clearly contradicts the majority of studies published on HPCD, which reported the method to be predictive of the contaminant fraction bioaccessible for bacteria and earthworms (Chung & Alexander 1999, Cuypers et al. 2002, Hickman & Reid 2005, Tang & Alexander 1999). The higher toxicity of extracts from HPCD may be explained by the short ageing time of the sediments used. For example, HPCD had been shown to recover 100% of phenanthrene one day after initial spiking (Swindell & Reid 2006). However, this extractive capacity was significantly reduced after 40 and 80 days storage (Swindell & Reid 2006). Whereas samples in the study by Swindell and Reid (2006) were kept at 15°C, the sediments in the present extraction study were stored at 4°C. Such a decreased temperature distinctly slows down chemical and biological processes and, thus, most likely delayed also processes of ageing. However, other studies showed that the ageing of fluoranthene, one of the organic contaminants in the mixture applied, can be completed within 75 or 60 days at a temperature of 20°C and 22 ± 2°C, respectively (Moermond et al. 2007, Tang & Alexander 1999). Therefore, other possible explanations have to be taken into account. Particulate organic matter present in SOX extracts may have masked contaminant toxicity (Seiler et al. 2008) in comparison to HPCD, although this does not explain the higher toxicity of HPCD compared to MDE.

For TNX, one hypothesis to be tested was whether the predictability for contaminant bioaccessibility holds true at the level of biological effects in the zebrafish embryo test. For the artificial sediment, bioaccessibility was significantly underestimated with TNX, whereas for the natural sediment the high variability and reduced reproducibility in independent replicates decreased the power of results obtained. To our knowledge, similar variations have not been reported before. They may relate to special properties of the used natural sediment such as the high silt content. As indicated by numerous studies (i.e. Cuypers et al. 2001, Morrison et al. 2000, Ten Hulscher et al. 2003, Van der Heijden & Jonker 2009, You et al. 2006), it is apparent that TNX may be used to predict bioaccessibility, but the relation of

amount of compounds extracted and "bioaccessible" compounds has to be determined individually for each new combination of sample, organism, endpoint and extraction time.

It is important to note that, since the present comparison focuses on biological bioaccessibility, no conclusions can be drawn towards whether single compounds in the mixture had a higher chemical activity or accessibility. Furthermore, aspects and possible influences important in the assessment of physico-chemical bioaccessibility, such as over-proportional sorption of a single compound to sediment matrix, test vessels or different extraction efficiencies, were not covered by the present approach.

In summary, the present study successfully characterised four different extraction methods and the direct sediment contact test with zebrafish based on a complex chemical mixture with regard to biological effectiveness. These results provide valuable information on how to interpret results obtained with either of the applied lab-based assessment tools. TNX was identified as a possibly suitable method for the prediction of bioaccessibility at the biological effect level in the sediment contact test with zebrafish embryos. The other three methods (SOX, MDE and HPCD) overestimated bioaccessibility with regard to this definition, as was expected for SOX and MDE. For HPCD, this result may be explained with insufficient ageing. These data may help to decide which method is best depending on the investigated scientific problem or protection goal. In an approach focused on protection of biota, specific bioaccessibility has to be determined (Brack et al. 2009). In this case, TNX as well as SCTs may provide suitable data, whereas if a "worst-case" scenario is asked for, i.e. protection focuses on the whole sediment phase, the use of either SOX or MDE would be the method of choice. Furthermore, since methods mimicking bioaccessibility may also underestimate risk as they do not account for intra-organismic processes such as gut-fluid extraction, it might even be advisable to use two methods in order to assess and compare basic bioaccessibility as well as total hazard potential.

The relationship between HPCD and biological effect remains to be further examined.

6.6 Outlook

Experiments to elucidate ageing-related changes in the bioaccessibility of pure substances have been initiated with special focus on the relationship between extractability of organic contaminants *via* HPCD and ageing.

Furthermore, an extension of the scope towards additional "mild" extraction methods (e.g., persulfate oxidation (Cuypers et al. 2001, Cuypers et al. 2000), XAD (Carroll et al. 1994), tetrahydrofurane (Tang et al. 2002, Tang et al. 1999), *n*-butanol (Andersson et al. 2009), C18 membranes (Tang et al. 2002, Tang et al. 1999), solid phase microextraction (Hawthorne et al. 1998, Ramos et al. 1998, Zambonin et al. 1998) or other sediment contact test systems (e.g. Feiler et al. 2004, Heise & Ahlf 2005, Moermond et al. 2007) might prove useful to improve our understanding of the reactions of organisms to sediment contamination.

6.7   Acknowledgements

6.8 References

Alexander M (1995) How toxic are toxic chemicals in soil? Environ Sci Technol 29:2713–2717

Alexander M (2000) Aging, bioavailability, and overestimation of risk from environmental pollutants. Environ Sci Technol 34:4259–4265

Andersson E, Rotander A, von 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 Poll Res 16:521–530

Arditsoglou A, Voutsa D (2008) Determination of phenolic and steroid endocrine disrupting compounds in environmental matrices. Environ Sci Poll Res 15:228–236

Bjorklund E, Holst C, Anklam E (2002) Fast extraction, clean-up and detection methods for the rapid analysis and screening of seven indicator PCBs in food matrices. Trends Anal Chem 21:39–52

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) Bioavailability in effect-directed analysis of organic toxicants in sediments. Trends Anal Chem 28:543–549

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

Brils J et al. (2007) Sediment management: An essential element of river basin management plans. J Soils Sediments 7:117–132

Burton GA (1991) Assessing the toxicity of fresh-water sediments. Environ Toxicol Chem 10:1585–1627

Carroll KM, Harkness MR, Bracco AA, Balcarcel RR (1994) Application of a Permeant Polymer Diffusional Model to the Desorption of Polychlorinated-Biphenyls from Hudson River Sediments. Environ Sci Technol 28:253–258

Chung N, Alexander M (1999) Effect of concentration on sequestration and bioavailability of two polycyclic aromatic hydrocarbons. Environ Sci Technol 33:3605–3608

Cornelissen G, Van Noort PCM, Govers HAJ (1997) Desorption kinetics of chlorobenzenes, polycyclic aromatic hydrocarbons, and polychlorinated biphenyls: Sediment extraction with Tenax® and effects of contact time and solute hydrophobicity. Environ Toxicol Chem 16:1351–1357

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

Cuypers C, Clemens R, Grotenhuis T, Rulkens W (2001) Prediction of petroleum hydrocarbon bioavailability in contaminated soils and sediments. Soil Sediment Contam 10:459–482

Cuypers C, Pancras T, Grotenhuis T, Rulkens W (2002) The estimation of PAH bioavailability in contaminated sediments using hydroxypropyl-ß-cyclodextrin and Triton X-100 extraction techniques. Chemosphere 46:1235–1245

Cuypers H, Grotenhuis T, Joziasse J, Rulkens W (2000) Rapid persulfate oxidation predicts PAH bioavailability in soils and sediments. Environ Sci Technol 34:2057–2063

De la Cal A, Eljarrat E, Grotenhuis T, Barcelo D (2008) Tenax (R) extraction as a tool to evaluate the availability of polybrominated diphenyl ethers, DDT, and DDT metabolites in sediments. Environ Toxicol Chem 27:1250–1256

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, 14 S.

EP/EC (2008) Directive 2008/105/EC of the european parliament and of the council of 16 December 2008 on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:348:0084:0097:EN:PDF

Ehlers GAC, Loibner AP (2006) Linking organic pollutant (bio)availability with geosorbent properties and biomimetic methodology: A review of geosorbent characterisation. and (bio)availability prediction. Environ Pollut 141:494–512

Feiler U, Kirchesch I, Heininger P (2004) A new plant-based bioassay for aquatic sediments. J Soils Sediments 4: 261-266

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 Poll Res 12:257–258

Feiler U, Ahlf W, Fahnenstich C, Gilberg D, Hammers-Wirtz M, Höss S, Hollert H, Melbye K, Meller M, Neumann-Hensel H, Ratte HT, Seiler TB, Spira D, Weber J, Heininger (2009a) Definition von Referenzbedingungen, Kontrollsedimenten und Toxizitätsschwellenwerten für limnische Sedimentkontakttetst (SeKT) - Final project report BfG1614, Federal Institute of Hydrology, Koblenz, Germany, ISBN 978-3-940247-01-8, 251 p.

Feiler U, Claus E, Spira D, Heininger P (2009b) Aquatic plant bioassays used in the assessment of sediment quality. Umweltwiss Schadst Forsch21:264–266

Fleming RJ, Holmes D, Nixon SJ (1998) Toxicity of permethrin to Chironomus riparius in artificial and natural sediments. Environ Toxicol Chem 17:1332–1337

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

Ghosh U, Gillette JS, Luthy RG, Zare RN (2000) Microscale location, characterization, and association of polycyclic aromatic hydrocarbons on harbor sediment particles. Environ Sci Technol 34:1729–1736

Goedkoop W, Widenfalk A, Haglund AL, Steger K, Bertilsson S (2005) Microbial characterization of artificial sediment and comparisons with natural sediments – Implications for toxicity testing. Environ Toxicol Chem 24:2725–2733

Hallare AV, Kosmehl T, Schulze T, Hollert H, Kohler HR, Triebskorn R (2005) Assessing contamination levels of Laguna Lake sediments (Philippines) using a contact assay with zebrafish (Danio rerio) embryos. Sci Total Environ 347:254–271

Hallare AV, Seiler TB, Hollert H (2010) The versatile, changing, and advancing roles of fish in sediment toxicity assessment—a review. J Soils Sediments, doi 10.1007/s11368-010-0302-7

Hawthorne SB, Grabanski CB, Hageman KJ, Miller DJ (1998) Simple method for estimating polychlorinated biphenyl concentrations on soils and sediments using subcritical water extraction coupled with solid-phase microextraction. J Chromatogr A 814:151–160

Heise S, Ahlf W (2005) A new microbial contact assay for marine sediments – Dedicated to Prof. Dr. Ulrich Forstner on his 65th birthday. J Soils Sediments 5:9–15

Hickman ZA, Reid BJ (2005) Towards a more appropriate water based extraction for the assessment of organic contaminant availability. Environ Pollut 138:299–306

Hollert H, Durr 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, 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 Soils Sediments 3:197–207

Hollert H, Seiler TB, Blaha L, Young AL (2007) Multiple stressors for the environment: Present and future challenges and perspectives. Environ Sci Poll Res 14:222–222

Hollert H, Ernst M, Ahlf W, Duerr M, Erdinger L, Grund S, Keiter S, Kosmehl T, Seiler T-B, Woelz J, Braunbeck T (2009) Strategies for assessing sediment toxicity – a review. Umweltwissenschaften und Schadstoff-Forschung 21:160–176

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 freshwater sediment contact tests in sediments with low anthropogenic contamination – Determination of toxicity thresholds. Environ Pollut, doi:10.1016/j.envpol.2010.05.013

Huang WL, Ping PA, Yu ZQ, Fu HM (2003) Effects of organic matter heterogeneity on sorption and desorption of organic contaminants by soils and sediments. Appl Geochem 18:955–972

Huckins JN, Tubergen MW, Lebo JA, Gale RW, Schwartz TR (1990) Polymeric film dialysis in organic-solvent media for cleanup of organic contaminants. J Assoc Off Ana Chem 73:290–293

Karlsson J, Sundberg H, Akerman G, Grunder K, Eklund B, Breitholtz M (2008) Hazard identification of contaminated sites – ranking potential toxicity of organic sediment extracts in crustacean and fish. J Soils Sediments 8:263–274

Kosmehl T, Krebs F, Manz W, Braunbeck T, Hollert H (2007) Differentiation between bioavailable and total hazard potential of sediment-induced DNA fragmentation as measured by the comet assay with zebrafish embryos. J Soils Sediments 7:377–387

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 Phys C 149:196–209

Luo JP, Ma M, Liu C, Zha JM, Wang ZJ (2009) Impacts of particulate organic carbon and dissolved organic carbon on removal of polycyclic aromatic hydrocarbons, organochlorine pesticides, and nonylphenols in a wetland. J Soils Sediments 9:180–187

Luque de Castro MD, Garcia-Ayuso LE (1998) Soxhlet extraction of solid materials: an outdated technique with a promising innovative future. Anal Chim Acta 369:1–10

Macrae JD, Hall KJ (1998) Comparison of methods used to determine the availability of polycyclic aromatic hydrocarbons in marine sediment. Environ Sci Technol 32:3809–3815

Marklevitz SAC, Almeida E, Flemming J, Hellou J (2008) Determining the bioavailability of contaminants and assessing the quality of sediments. J Soils Sediments 8:86–91

Moermond CTA, Roessink I, Jonker MTO, Meijer T, Koelmans AA (2007) Impact of polychlorinated biphenyl and polycyclic aromatic hydrocarbon sequestration in sediment on bioaccumulation in aquatic food webs. Environ Toxicol Chem 26:607–615

Morrison DE, Robertson BK, Alexander M (2000) Bioavailability to earthworms of aged DDT, DDE, DDD, and dieldrin in soil. Environ Sci Technol 34:709–713

Nagel R (2002) DarT: The embryo test with the zebrafish Danio rerio – a general model in ecotoxicology and toxicology. Altex 19:38–48

Nam K, Chung N, Alexander M (1998) Relationship between organic matter content of soil and the sequestration of phenanthrene. Environ Sci Technol 32:3785–3788

Neumann-Hensel H, Melbye K (2006) Optimisation of the solid-contact test with Artbrobacter globiformis. J Soils Sediments 6:201–207

Northcott GL, Jones KC (2000) Experimental approaches and analytical techniques for determining organic compound bound residues in soil and sediment. Environ Pollut 108:19–43

OECD (2004) OECD guideline 218: Sediment-water chironomid toxicity using spiked sediment. Organisation for Economic Cooperation and Development, Berlin

Pane L, Giacco E, Corra C, Greco G, Mariottini GL, Varisco F, Faimali M (2008) Ecotoxicological evaluation of harbour sediments using marine organisms from different trophic levels. J Soils Sediments 8:74–79

Qiao M, Huang S, Wang Z (2008) Partitioning characteristics of PAHs between sediment and water in a shallow lake. J Soils Sediments 8:69–73

Ramos EU, Meijer SN, Vaes WHJ, Verhaar HJM, Hermens JLM (1998) Using solid-phase microextraction to determine partition coefficients to humic acids and bioavailable concentrations of hydrophobic chemicals. Environ Sci Technol 32:3430–3435

Reichenberg F, Mayer P (2006) Two complementary sides of bioavailability: Accessibility and chemical activity of organic contaminants in sediments and soils. Environ Sci Technol 25:1239-1245

Reid BJ, Stokes JD, Jones KC, Semple KT (2000) Nonexhaustive cyclodextrin-based extraction technique for the evaluation of PAH bioavailability. Environ Sci Technol 34:3174–3179

Santos FJ, Sarrion MN, Galceran MT (1997) Analysis of chlorobenzenes in soils by headspace solid-phase microextraction and gas chromatography-ion trap mass spectrometry. J Chromatogr A 771:181–189

Schwab K, Brack W (2007) Large volume tenax (R) extraction of the bioaccessible fraction of sediment-associated organic compounds for a subsequent effect-directed analysis. J Soils Sediments 7:178–186

Seiler TB (2010) Total or biomimetic extracts or direct contact exposure? - Comparative research towards a realistic ecotoxicological characterisation of sediments. PhD thesis, Ruprecht-Karls University Heidelberg, 340 p., http://archiv.ub.uni-heidelberg.de/volltextserver/volltexte/2010/11171/pdf/20100701_Dissertation_Seiler_veroeff.pdf

Seiler TB, Rastall AC, Leist E, Erdinger L, Braunbeck T, Hollert H (2006) Membrane dialysis extraction (MDE): A novel approach for extracting toxicologically relevant hydrophobic organic compounds from soils and sediments for assessment in biotests. J Soils Sediments 6:20–29

Seiler TB, 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

Smit MPJ, Grotenhuis T, Bruning H, Rulkens WH (2008) Desorption of dieldrin from field aged sediments: Simulating flood events. J Soils Sediments 8:80–85

Sormunen AJ, Leppanen MT, Kukkonen JVK (2009) Examining the role of temperature and sediment-chemical contact time on desorption and bioavailability of sediment-associated tetrabromo diphenyl ether and benzo(a)pyrene. Ecotox Environ Safe 72:1234–1241

Standardization (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 3: Flow-through method. Iso Guideline

Stokes JD, Wilkinson A, Reid BJ, Jones KC, Semple KT (2005) Prediction of polycyclic aromatic hydrocarbon biodegradation in contaminated soils using an aqueous hydroxypropyl-ß-cyclodextrin extraction technique. Environ Toxicol Chem 24:1325–1330

Streck G, Seiler TB, Schulze T, Schwab K, Braunbeck T, Hollert H, Brack W (2010) On the comparability of procedures for sediment extraction in environmental assessment. Part B: Recovery of target analytes. Environ Toxicol Chem, in prep

Swindell AL, Reid BJ (2006) Comparison of selected non-exhaustive extraction techniques to assess PAH availability in dissimilar soils. Chemosphere 62:1126–1134

Tang J, Robertson BK, Alexander M (1999) Chemical-extraction methods to estimate bioavailability of DDT, DDE, and DDD in soil. Environ Sci Technol 33:4346–4351

Tang J, Liste HH, Alexander M (2002) Chemical assays of availability to earthworms of polycyclic aromatic hydrocarbons in soil. Chemosphere 48:35–42

Tang JX, Alexander M (1999) Mild extractability and bioavailability of polycyclic aromatic hydrocarbons in soil. Environ Toxicol Chem 18:2711–2714

Ten Hulscher TEM, Postma J, Den Besten PJ, Stroomberg GJ, Belfroid A, Wegener JW, Faber JH, Van Der Pol JJC, Jan Hendriks A, Van Noort PCM (2003) Tenax extraction mimics benthic and terrestrial bioavailability of organic compounds. Environ Toxicol Chem 22:2258–2265

Van der Heijden SA, Jonker MTO (2009) PAH bioavailability in field sediments: Comparing different methods for predicting in situ bioaccumulation. Environ Sci Technol 43:3757–3763

Verrhiest GJ, Cortes S, Clement B, Montuelle B (2002) Chemical and bacterial changes during laboratory conditioning of formulated and natural sediments. Chemosphere 46:961–974

Wölz J, Engwall M, Maletz S, Takner HO, 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 Poll Res 15:536–553

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

You J, Landrum PF, Lydy MJ (2006) Comparison of chemical approaches for assessing bioavailability of sediment-associated contaminants. Environ Sci Technol 40:6348–6353

Zambonin CG, Catucci F, Palmisano F (1998) Solid phase microextraction coupled to gas chromatography-mass spectrometry for the determination of the adsorption coefficients of triazines in soil. Analyst 123:2825–2828

Chapter 7

Accumulation of metals from sediments and molecular responses of zebrafish embryos

Hanno Zielke[1*], Regine Redelstein[1*], Denise Spira[2], Ute Feiler[2], Lothar Erdinger[3], Holger Zimmer[4], Steve Wiseman[5], Markus Hecker[5,6], John P. Giesy[5,7,8,9,10], Thomas-Benjamin Seiler[1] and Henner Hollert[1]

[1]Department of Ecosystem Analysis, Institute for Environmental Research, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany

[2]German Federal Institute of Hydrology, Am Mainzer Tor 1, 56068 Koblenz, Germany

[3]Department of Medical Microbiology and Hygiene, University of Heidelberg, Im Neuenheimer Feld 324, 69120 Heidelberg, Germany

[4]University Hospital Heidelberg, Department of Internal Medicine and Clinical Chemistry I, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany

[5]Toxicology Centre, University of Saskatchewan, 44 Campus Drive, Saskatoon, Saskatchewan, S7N 5B3, Canada

[6]School of the Environment & Sustainability, University of Saskatchewan, 44 Campus Drive, Saskatoon, Saskatchewan, S7N 5B3, Canada

[7]Department of Veterinary Biomedical Sciences, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, S7N 5B3.

[8]Department of Zoology, and Center for Integrative Toxicology, Michigan State University, East Lansing, MI, USA, 48895

[9]Zoology Department, College of Science, King Saud University, P. O. Box 2455, Riyadh 11451, Saudi Arabia

[10]Department of Biology & Chemistry, City University of Hong Kong, Kowloon, Hong Kong, SAR, PR China

*Both authors contributed equally to this work.

7.1 Abstract

Bioavailability of metals spiked into riverine sediments to be accumulated by and affect transcript abundance of the metallothioneins MT1 and MT2 and the transcription factor MTF in embryos of zebrafish (*Danio rerio*) was determined. Abundances of sod1, hsp70 and hsp90α1 transcripts were measured as indicators of cellular stress. Accumulation from a natural and an artificial sediment spiked with metals were determined. Embryos were exposed individually to cadmium (Cd), copper (Cu), nickel (Ni) and zinc (Zn) or as a mixture at concentrations ranging from 150 to 3000 mg/kg dw. *Danio rerio* embryos accumulated metals from spiked sediments to concentrations 100-fold greater than in the sediment. The BAF for Cu from artificial sediment was 275 ± 42 (SD), which was significantly greater than the BAF of other metals. BAFs of other metals ranged from 13.8 for Cd in natural sediment to 66.9 for Cu in natural sediment. Accumulation of metals from sediments was greater when embryos were exposed to individual metals than when they were exposed to the mixture spiked with same concentrations as in the individual exposure. Embryos accumulated greater concentrations of all metals from artificial than natural sediment. Exposure of embryos to Zn or the mixture exhibited up to 30-fold greater transcript abundances of MT1, MT2, and hsp70 in comparison to the controls. Transcript abundance of hsp90α1 was significantly greater in embryos exposed to natural sediment spiked with Cd or Cu.

7.2 Introduction

While water quality of many European rivers has significantly improved during the past three decades (Schwarzenbach et al. 2006), however, contaminated sediments in many of these water bodies still represent a legacy of past uncontrolled releases of pollutants and will continue to influence the water quality into the future (Breitholtz et al. 2006, Eklund et al. 2010, Hilscherova et al. 2010). These sediments can become a secondary source of pollution and pose a potential threat to infaunal organisms and their predators. Because sediment-bound contaminants can be re-mobilised by bioturbation, flood events or dredging and relocation of sediments these contaminants can also affect organisms in the water column (Förstner & Westrich 2005, Hilscherova et al. 2010, Hollert et al. 2003, Wölz et al. 2009).

Hence, predicting bioavailability and effects of metals in sediments is of concern due to potential toxic effects on aquatic organisms, including sensitive life stages such as fish larvae. Tests in which organisms are directly exposed to sediments (Sediment Contact Tests; SCTs) are ecologically relevant to assess potential risks associated with contaminated sediments (Ahlf & Förstner 2001, Blaha et al. 2010, e.g. Duft et al. 2003, Eklund et al. 2010, Feiler et al. 2005, Hallare et al. 2011, Höss et al. 2010, Schmitt et al. 2010, Turesson et al. 2007). The SCT with zebrafish (*Danio rerio*) embryos is used to evaluate the potential toxicity of contaminants associated with sediments to vertebrates (Hollert et al. 2003). The zebrafish is a model organism for which the ontogeny and genome have been well characterized. Zebrafish are also easily maintained and bred (Braunbeck et al. 2005, Nagel 2002) and allow the study of specific mechanisms (Kosmehl et al. 2006). The aqueous version of the test is internationally standardized (ISO 2007). The zebrafish embryo test is also one component of a comprehensive SCT battery established by the recent German joint research framework project (Sediment Kontakt Test, SeKT; Feiler et al. 2009, Feiler et al. 2005, Höss et al. 2010). The battery of tests uses test organisms of different trophic levels, which inhabit various microhabitats of freshwater sediments, including bacteria, fungi, nematodes, oligochaetes, higher plants and fish. One approach to assess the utility of the battery of tests used in the SeKT was to test a mixture of cadmium (Cd), copper (Cu), nickel (Ni) and zinc (Zn).

Metals are an ubiquitous class of pollutants which are persistent, can be toxic to organisms, and affect populations and structures of communities (Boyd 2010, Dell'Anno et al. 2003). In sediments, metals are partitioned in various forms and species, which are dependent on the chemical and physical characteristics of the sediment (Burton 1991, Di Toro et al. 1990). Bioavailability of metals from sediments is influenced by geochemistry, activities of organisms and contaminant/particle interactions (Ahlf et al. 2009, Dell'Anno et al. 2003). Thus, various exposure and uptake routes have to be considered for benthic organisms. These species can be exposed to metals associated with ingested particles as well as those dissolved in the porewater and/or overlying water (Simpson & Batley 2006). Predicting the bioavailability of metals in sediments is necessary to assess adverse effects of contaminants in sediments (Ahlf et al. 2009, Simpson & Batley 2006).

One objective of this study was to determine bioaccumulation of four individual transition metals, Cd, Cu, Ni and Zn. Also, since metals can occur in mixtures (Borgmann et al. 2008, Komjarova & Blust 2009b, Rodrigues et al. 2010) effects of a mixture of all four metals on zebrafish embryos were determined. In addition, two different sediments representing artificial and natural sediments were investigated.

Range-finding tests were conducted with both aqueous solutions of metals and sediments spiked with metals to determine LC_{50} values and no effect concentrations (NOEC) that would allow selection of appropriate concentrations for use in the definitive exposures. In the definitive studies, zebrafish were exposed to sediments spiked with individual metals or a mixture of all four metals. Concentrations of metals in embryos were determined by use of inductively-coupled plasma mass spectrometry (ICP-MS). Bioaccumulation factors (BAFs) were calculated for each metal and compared among metals. Effects of the fraction of metals that was biologically available was determined by measuring abundances of mRNA transcripts of selected genes by use of quantitative real-time polymerase chain reaction (Q-RT-PCR). Abundances of mRNA for metallothioneins (MT1 and MT2) and the metallothionein transcription factor (MTF) were used as indication of magnitude of exposure to metals while expression of superoxide dismutase (sod1) and heat shock proteins (hsp70 and hsp90α1) were used as indicators of cellular stress. CYP1A was used as an indicator of exposure to dioxin-like chemicals that could modulate responses through the aromatic hydrocarbon receptor (AhR) and expression of GST was used as an indicator of biotransformation activity (Hollert et al. 2002, Whyte et al. 2000).

7.3   Material and methods

7.3.1   Aqueous range-finding tests

Stock solutions of 2 g/l (ZnCl, NiCl$_2$), 200 mg/l (CdCl) and 20 mg/l (CuCl$_2$, all Sigma-Aldrich Chemie GmbH,) were prepared in doubly distilled water and diluted as required in all subsequent tests. Between experiments, stock solutions were stored in plastic bottles at 4°C. In the aqueous range-finding tests, Zn and Ni were tested at concentrations of 1, 10, 100, 500 and 1000 mg/l. Range-finding concentrations were 0.1, 1, 10, 50 and 100 mg/l for Cd and 0.01, 0.1, 1, 5 and 10 mg/l for Cu.

7.3.2   Sediments

Natural sediments were collected in August 2008 and May 2009 at Altrip, a back water of the river Rhine, (river km 416.9, Rhineland-Palatinate, Germany) by use of a Van Veen grab from a water depth of 5 m (c.f. Höss et al. 2010). Sediments from this location had been

established as a reference within the SeKT framework project (Feiler et al., 2009; Höss et al., 2010). These sediments were predominantly composed of silt (Table 7.1).

An artificial sediment was prepared according to OECD guideline 218 (OECD 2004) except for kaolin clay content (Table 7.2). Since during the SeKT project the clay content of 20 % given in the OECD guideline proved to be unsuitable for use in the nematode contact assay, the clay content was reduced from 20 % to 5 % and replaced with quartz sand (F36; Quarzwerke Frechen, Frechen, Germany; Feiler et al. 2009, Höss et al. 2010).

Sediment dry mass was determined by drying a defined amount of sediment for 14 h at 105 °C and measuring weight loss. Prior to spiking, sediments were stored in a darkroom at 4 °C. In order to account for potential background effects by residual contamination (natural sediment) or formulation (artificial sediment), unspiked samples were tested as sediment controls in all experiments.

Tab. 7.1 Physical-chemical characteristics, particle size distribution[2] and background contamination[2] with the test-relevant metals of the natural sediment (Altrip, Germany).

dw [1] [% ww]				pH [2]			TOC [2] [g/kg dw]	
34				7.5			34	
gravel >2 mm	sand >630 µm	sand >200 µm	sand >125 µm	sand >63 µm	silt >20 µm	silt >6.3 µm	silt >2.0 µm	clay <2.0µm
0.8 %	0.4 %	0.4 %	0.4 %	0.5 %	4.5 %	41.4 %	28.2 %	23.4 %
Cd [mg/kg dw]		Cu [mg/kg dw]		Ni [mg/kg dw]		Zn [mg/kg dw]		
0.49		65		59		213		

dw = dry weight; ww = wet weight; TOC = total organic carbon

[1] own data

[2] determined by BfG (Feiler et al. 2009)

Tab. 7.2 Physical-chemical characteristics and composition of the modified artificial sediment OECD 218.

dw [% ww]		pH	TOC [% dw]	
58		6.7	2 ± 0.5	
quartz sand [% of dw]	kaolinite clay [% of dw]	peat [% of dw]	CaCO₃ [% of dw]	water
90	5	5	0.05 - 1	30 - 50 %

dw = dry weight; ww = wet weight; TOC = Total organic carbon

7.3.3   Sediment spiking

Sediments were spiked according to OECD Guidelines 207 (OECD 1984) and 218 (OECD 2004) with three replicates prepared per sample. Ten percent of sediment total wet weight were dried for 14 h at 105 °C. To each gram of dry sediment 0.2 to 0.25 ml of solubilised metals were applied and water was allowed to completely evaporate at room temperature for three days before remixing the spiked portion with the remaining 90 % of the sediment. To exclude background effects caused by the drying procedure, a process control consisting of 90 % wet and 10 % dry sediment was prepared in parallel. As a modification of the OECD guidelines for metal-spiking of sediments (OECD 1984, 2004), biotests were always conducted immediately after spiking, and sediments were not equilibrated for 5 to 7 d. This approach was chosen in order to determine uptake concentrations directly after a lab-simulated contamination event.

Concentrations of metals in sediment used in the range-finding tests in natural sediment were: 22.06, 2.206 or 0.2206 g/kg dw of Cd or Zn and 196, 19.6, 1.96 or 0.196 mg/kg dw of Cu. In artificial sediment the concentrations tested were: 11.54, 1.154 or 0.1154 g/kg dw of Cd and Zn or 154, 15.4, 1.54 or 0.154 mg Cu/kg dw. Concentrations studied in the definitive studies were selected based on the results of these range-finding tests.

7.3.4   Fish maintenance and collection of embryos

Zebrafish were maintained according to the methods described by Braunbeck et al (2005) in 30 L aquaria containing charcoal-filtered tap water at 26 ± 1 °C in hatching groups of 12 males and 8 females. The photoperiod was adjusted to 10 h of darkness and 14 h of light. Oxygen saturation of ≥ 80 %, pH of 7.8 ± 0.5, and water hardness of 196.33 mg $CaCO_3$/L were maintained in all tanks. Water in tanks was exchanged once a week. Fish were daily fed with dry flakes (TetraMin™, Tetra GmbH, Melle, Germany) and *Artemia* sp. nauplii (Great Salt Lake Artemia Cysts, Sanders, Ogden, USA). For spawning, glass dishes were transferred into the tanks on evenings before experiments. In order to prevent egg predation, dishes were covered with a stainless steel grid with a mesh opening size of 1 mm through which the eggs could drop. Plastic imitation plants were attached to the mesh in order to stimulate mating. Spawning occurred within 0.5 to 1 h after onset of illumination.

7.3.5   Tests with zebrafish embryos in water or sediment

Tests with zebrafish embryos were conducted according to the protocol provided by the German regulation DIN 38415-6 (DIN 2001) and the methods given in Nagel (2002) and Lammer et al. (2009). Modifications made for use in sediment assessment have been described previously (Hollert et al. 2003). Artificial water (294.0 mg/l $CaCl_2 \cdot 2\,H_2O$, 123.3 mg/l $mgSO_4 \cdot 7\,H_2O$, 63.0 mg/l $NaHCO_3$ and 5.5 mg/l KCl) prepared according to ISO 7346/3 (1996) was used as the test medium.

All tests were conducted in 6-well plates (Techno Plastic Products TPP, Zurich, Switzerland). For the tests with aqueous metal solutions, each well was filled with 5 ml of the respective solution. For the sediment contact tests (both range-finding and definitive experiment), wells were prepared with 3 g wet weight of test sediment and 5 ml of artificial water. All sediments were weighed into plates one day before testing, covered with self-adhesive foil (Nunc, Roskilde, Denmark) and placed on a horizontal shaker over night at 50 rpm and 26 ± 1 °C. In all tests, only fertilized and normally developed eggs which were at least in the 8-cell stage were selected for testing using a binocular microscope (SMZ 1500, Nikon, Düsseldorf, Germany). Selected eggs were then transferred into wells containing the prepared solution or sediment, covered with self-adhesive foil and incubated for 48 h on the horizontal shaker at 50 rpm and 26 ± 1 °C.

For each concentration in the aqueous range-finding tests, 5 embryos were tested per well and a total of 10 embryos (2 wells) was used per concentration. Positive controls (PC, 3.7 mg/L 3,4-dichloroaniline) and negative controls (nc, artificial water only) were tested using 20 and 40 embryos, respectively (DIN 2001, Nagel 2002).

In the sediment range-finding tests, 5 embryos were tested per well, and 15-20 embryos (3-4 wells) were used per sample and control. In the definitive experiments, 10 embryos were transferred into each well, and 50 embryos were exposed to each concentration. In addition to sediment, process and solubiliser controls, quartz sand negative controls (20 eggs, 3 g quartz sand F36 + artificial water), quartz sand positive controls (10 eggs, 3.7 mg/l DCA freshly applied to the water phase + 3 g quartz sand F36 + artificial water), aqueous negative controls (40 eggs, artificial water only) and aqueous positive controls (20 eggs, artificial water + 3.7 mg/l freshly applied DCA) were performed in each test. In the sediment contact tests, eggs were collected from the 6-well plates prior to evaluation. In order to simplify re-collection of eggs from the sediment, wells were illuminated with a cold light source (KL 1500 LCD, Schott, Mainz, Germany). After 48 h of incubation, eggs were collected from the sediments

and briefly rinsed in artificial water to remove remaining sediment particles and evaluated for lethal and sublethal effects by means of an inverse microscope (Eclipse TS100, Nikon, Düsseldorf, Germany). Mortality criteria were (a) coagulation, (b) lack of heartbeat, (c) missing somite development and (d) failure of tail detachment from the yolk sack (Braunbeck et al. 2005, DIN 2001, Hollert et al. 2003, Nagel 2002). This evaluation was only conducted in the range-finding tests.

7.3.6   Quantification of Metals

During experiments conducted to measure accumulation of metals by embryos, the embryos were not evaluated according to the FET mortality criteria listed above. Eggs were collected from sediments and anesthetised in a saturated solution of benzocaine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). All non-coagulated eggs were transferred into polypropylene test tubes (Cellstar®, Greiner Bio-One GmbH, Solingen, Germany) in a volume of 2 ml of artificial water. Samples were stored at -20 °C until analyses. After collection of eggs from sediments, the pH in the wells was measured by means of an insertion electrode (FiveGo^{TM}, Mettler Toledo, Schwerzenbach, Switzerland). All samples were prepared in triplicates except for those from the mixture study (n = 1).

Samples were digested by means of an UV digestion device (UV 1000, Kürner Analysentechnik, Rosenheim, Germany). An aliquant of 2 ml of each sample and 0.8 ml HNO_3 (65 %, suprapur, Merck, Darmstadt, Germany) were placed in the UV 1000 quartz glass tubes. After one and four hours, respectively, 0.4 ml H_2O_2 (30 %, suprapur, Merck) were added. Total duration of digestion was 5 h (Schramel 2003). Digested samples were made to a final volume of 10 ml with distilled water and transferred to plastic tubes for ICP-MS measurements.

Concentrations of metals were measured by use of inductively coupled plasma-mass spectrometry with an ELAN 6100 ICP-mass spectrometer (PerkinElmer SCIEX, Waltham, USA) (ICP-MS ;Linge & Jarvis 2009, Schramel et al. 1999).

The ICP-mass spectrometer was calibrated with standard solutions of metals prepared from stock solutions of 1000 mg metal/l (Merck). For each metal, the two isotopes with the greatest natural abundances were used for calibration. Concentrations of metals in samples were calculated by plotting measured intensities against concentrations of the standards by means of the software Elan Instrument Control Utility (Version 2.3.2, PerkinElmer). Each sample

was measured three times and means were calculated. Concentrations of metals expressed on a dry mass basis, was reported after subtracting background concentrations in blanks and controls.

7.3.7   Calculation of bioaccumulation factors

Bioaccumulation factors (BAF) were calculated (Equation 1).

$$\text{BAF} = \frac{c\,[\text{organism}]}{c\,[\text{sediment}]} \qquad (1)$$

c [organism]: Metal concentrations in fish embryos given in nanogramme of metal per gram of fish egg. Average wet weight of one fish egg: 0.37 mg.

c [sediment]: Concentration of metal spiked into sediment given in milligramme metal per kilogram of sediment wet weight.

7.3.8   Molecular analyses

The magnitude of expression of mRNA of selected genes was measured in four replicates per treatment on embryos that had been stored at -80 °C in RNAlater (Qiagen GmbH, Hilden, Germany) until further analysis.

7.3.9   Total RNA isolation and cDNA synthesis

Total RNA was extracted from approximately 25 embryos per treatment using the RNeasy® Plus Mini Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's protocol. Purified RNA was quantified using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Samples were checked for RNA integrity by use of a 1 % denaturing formaldehyde-agarose gel, stained with ethidium bromide and visualised by use of UV light with a VersaDoc® 4000 MP imaging system (Bio-Rad, Hercules, CA, USA).

The purified RNA samples were stored at -80 °C until analysis. First-strand cDNA synthesis was performed using the iScript^{TM} cDNA Synthesis Kit (Bio-Rad). A volume of 2.5 µg total RNA was combined with 10 µl of 5x iScript$^®$ Reaction Mix, 2.5 µl of iScript$^®$ Reverse Transcriptase, and RNase-free water was added to make a final volume of 50 µl. Reaction mixes were incubated at 25 °C for 5 min, 42 °C for 30 min, and, on completion, were inactivated at 85 °C for 5 min. cDNA was stored at -20 C until further analysis.

Tab. 7.3 Nucleotide sequences of primers used for real-time PCR quantification of *Danio rerio* transcript abundance.

Gene		Sequence (5^{I} – 3^{I})	Accession no.
β-Actin	Forward	ACATCCGTAAGGACCTG	AF057040
	Reverse	GGTCGTTCGTTTGAATCTC	
MT1	Forward	CGTCTAACAAAGGCTAAAGAGGGA	AY514790
	Reverse	GCAGCAGTACAAATCAGTGCATC	
MT2	Forward	TGCATCGCATGATTGTCTTT	NM_001131053.2
	Reverse	CAGTGCATCGTTTTCCCTCT	
MTF	Forward	AATCAGAGGGATGCACCAAG	NM_001001942.1
	Reverse	TGCGTCCGTACATGTGTTTT	
sod1	Forward	GTTTCCACGTCCATGCTTTT	NM_131294.1
	Reverse	CGGTCACATTACCCAGGTCT	
hsp70	Forward	AAAGCACTGAGGGACGCTAA	NM_131397.2
	Reverse	TGTTCAGTTCTCTGCCGTTG	
hsp90α1	Forward	GCAAACCGCATCTACAGGAT	NM_131328.1
	Reverse	TCCAGAACGGGCATATCTTC	
CYP1A	Forward	AGGACAACATCAGACACATCACCG	NM_131879
	Reverse	GATAGACAACCGCCCAGGACAGAG	
GST	Forward	AGAGCCCATCAGGACACACT	AB231640.1
	Reverse	TCACCCAGATGGCTCCTAAC	

7.3.10 Real-time PCR

Primers were designed using Primer3$^®$ software (http://frodo.wi.mit.edu/primer3/version 0.4.0). Primer sequences and accession numbers are reported (Tab. 7.3). Quantitative real-time PCR was performed in 96-well PCR plates using an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, USA). A separate 70 µl PCR reaction mixture consisting of

3.5 µl of gene-specific primers (Tab. 7.3), 35 µl of 2x Power SYBR$^{®}$ Green master mix (Applied Biosystems), 3.5 µl cDNA, and 28 µl of nuclease-free water was prepared for each cDNA sample and for each primer pair. A final reaction volume of 20 µl was transferred to each well and reactions were performed in triplicate. ROX was used as reference dye. To account for differences in amplification efficiency among primers, standard curves were constructed for each primer by use of serial dilutions of the cDNA template. Since efficiencies were approximately equal, gene expression data were analysed using the ΔΔCt method (Livak & Schmittgen 2001), with β-Actin used as the reference gene. Melt curves were generated for each primer-pair to ensure amplification of a single PCR product.

7.3.11 Statistical analyses

Statistical evaluation was accomplished by use of SigmaStat$^{®}$ 3.5 (Systat Software GmbH, Erkrath, Germany, 2006). All datasets were tested for statistically significant differences between control groups and each treatment as well as between samples. Data was tested for normality distribution (Kolmogorov-Smirnov test) and on homogeneity of variance (Levene test). The datasets that met both assumptions were analysed using parametric one-way ANOVA. As a post hoc test, the Holm-Sidak method was used for pair-wise comparison of the treatment groups. Data sets which did not meet the assumptions of normality and/or homogeneity of variance were analysed using non-parametric one-way ANOVA based on ranks by use of Dunn's method for pair-wise comparison. The significance limit used throughout all comparisons was $p \leq 0.05$.

In order to compare the LC_{50} values from aqueous tests to those obtained with spiked sediments, the results of the sediment contact assays were converted into concentrations of mg/l. The amount of metal present at the respective LC_{50} concentration was divided by the sum of porewater and overlaying water volumes in one well.

7.4   Results

7.4.1   Sediment properties

Physical-chemical characteristics and composition of the sediments as well as residual concentrations of the four metals studied in the natural sediment are given in tables 7.1 and 7.2. Background concentrations of organic contaminants were not within test-relevant ranges for the fish embryo test (Zielke et al. 2010). Detailed chemical data on background concentrations have been given previously in Feiler et al. (2009) and Höss et al. (2010).

7.4.2   Aqueous and sediment range-finding tests

With the exception of Ni, concentration-dependant greater mortality was observed when *Danio rerio* embryos were exposed to metals. LC_{50} values determined in the aqueous range-finding tests were 14.1 mg Cd/l, 0.4 mg Cu/l and 87.1 mg Zn/l. Ni caused no statistically significant effects up to the greatest tested concentration, which was 1000 mg/l. In the natural sediment, LC_{50} values were 7.0 g Cd/kg dw and 4.0 g Zn/kg dw. Both Cd and Zn when spiked into artificial sediment resulted in LC_{50} values of 3.7 g/kg dw. Copper caused no effects in the fish embryo sediment contact test up to the greatest concentrations of 200 and 150 mg/kg dw in natural or artificial sediments, respectively. Nickel was not tested in range-finding tests of sediments, due to the lack of effects in the aqueous tests. Concentrations selected for the bioaccumulation and molecular biomarker experiments were as follows: 2.94 g of Cd, Ni or Zn per kg dw of sediment were spiked into the natural sediment and 1.53 g/kg were spiked into artificial sediment. In the case of Cu, concentrations were 0.294 g/kg dw (natural sediment) and 0.153 g/kg dw (artificial sediment), respectively.

7.4.3   Validity of embryo tests

As a control for the validity of a given test, embryo mortality in the positive controls and quartz positive controls (3.7 mg/l DCA) had to exceed 10 %. Mortality of 68 ± 24 % (DIN 2001) and an $LC_{50,DCA}$ of 3.3 ± 0.5 mg/l (DIN 2001) and 1.6 to 2.4 mg/l (Lammer et al. 2009) has been reported for this concentration of DCA. The negative, quartz sand negative, sediment, process and solubiliser controls were regarded valid if the mortality did not exceed

10 % (DIN 2001, Nagel 2002). As further quality criterion, egg fertilisation rate had to exceed 70 % in order to carry out a test (Lammer et al. 2009).

Mortalities (mean ± standard deviation) were less than 10 % in all negative controls and greater than 10 % in the positive controls. Mortalities were 3.6 ± 4.3 % (aqueous negative controls, n=8), 58.8 ± 29.0 % (aqueous positive controls, n=4), 0.1 ± 0.3 % (natural sediment controls, n=4), 0.5 ± 0.4 % (artificial sediment controls), 0.0 ± 0.0 % (quartz sand negative controls, n=2), 85.0 ± 21.2 % (quartz sand positive controls, n=2), 5.0 ± 7.1 % (solubiliser controls, natural sediment, n=2), 0.0 ± 0.0 % (process controls, natural sediment, n=2), 2.5 ± 3.5 % (solubiliser controls, artificial sediment, n=2) and 0.0 ± 0.0 % (process controls, artificial sediment, n=2).

7.4.4   Uptake and bioaccumulation

In studies of accumulation of metals and molecular responses, embryos were not evaluated in detail with regard to FET criteria. Only non-coagulated embryos were used in these further experiments. Since the almost exclusively observed mortality criterion in the range-finding tests with metal solutions and spiked sediments was coagulation, it is assumed that total mortalities in the negative controls were less than 10 % in all replicates.

The pH of sediments spiked with individual metals measured after 48 h of exposure were in a range of 7.27 ± 0.15 (SD; Fig. 7.1). These values are within the optimal pH range of 7.8 ± 1 for *Danio rerio* embryos (Braunbeck et al. 2005). Only the pH observed in artificial sediment spiked with all four metals (pH 6.47) was less than the optimum pH of 7.8 ± 1.0 (Fig. 7.1). The pH was correlated with a great number of coagulated eggs in this sample (20 out of 50).
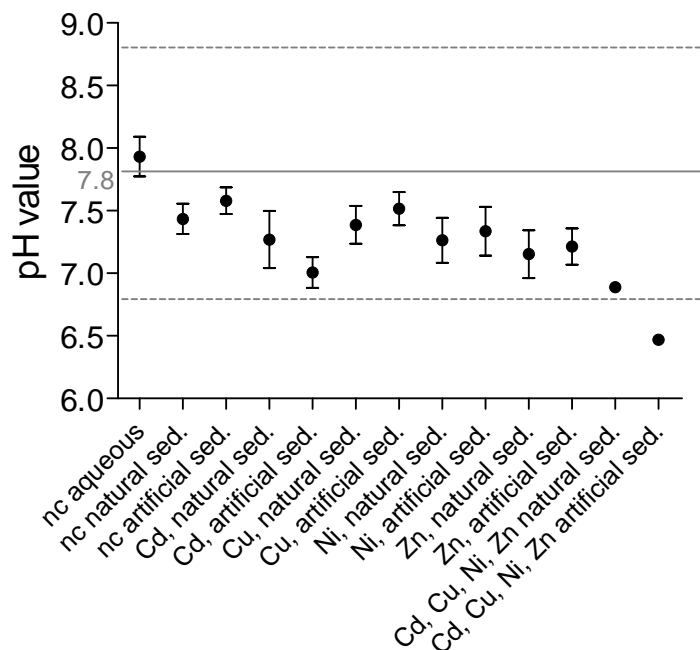
Fig. 7.1 pH of natural and artificial sediments after 16 h of equilibration plus 48 h of fish embryo exposure; dots represent means of three independent replicates, error bars indicate standard deviations. n = 3 for sediments spiked with single metals, n = 1 for sediments spiked with the mixture; n = number of independent replicates. Dotted lines indicate the optimal pH range of 7.8 ± 1 for *Danio rerio* embryos.

Embryos contained greater concentrations of metals after exposure to sediment spiked with individual metals compared to the unspiked sediment controls (Fig. 7.2). Fish embryos exposed to artificial sediment spiked with Cd, Cu or Zn contained significantly greater concentrations of the respective metal than embryos exposed to the aqueous and artificial sediment controls. For Ni, both spiked sediments contained significantly greater concentrations compared to the aqueous negative control and the respective sediment control. Concentrations of all four metals were greater in eggs exposed to spiked artificial sediment compared to the spiked natural sediment. For Cd and Cu, these differences were statistically significant.

Concentrations of all four metals were also greater in zebrafish embryos exposed to sediment spiked with the mixture than unspiked controls (Fig. 3; n = 1). Concentrations of all four metals were greater in eggs exposed to spiked artificial sediment compared to spiked natural sediment. Accumulation of individual metals was always greater from sediments spiked with a single metal than sediments spiked with the mixture of all four metals (Fig. 2 & 3).
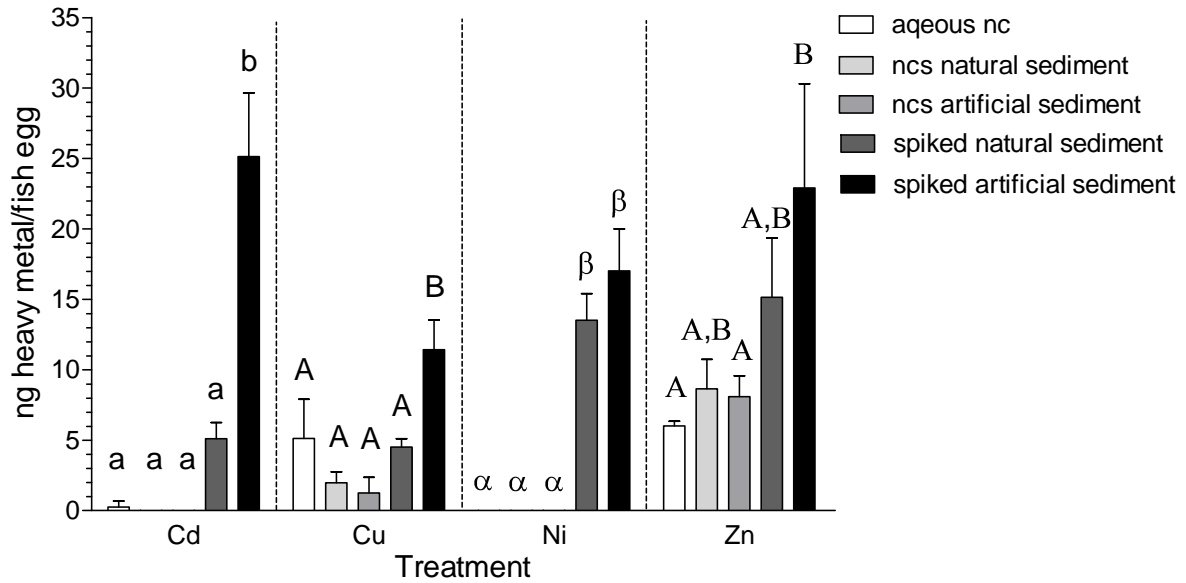
Fig. 7.2 Metal concentrations in zebrafish embryos after exposure to metal-spiked natural and artificial sediments for 48 h compared to unspiked sediment controls (ncs) as well as to the aqueous negative control (nc). Columns represent means of three independent replicates (two independent replicates for Zn aqueous nc and Zn-spiked natural sediment) with 43-50 embryos (coagulated embryos were not included in measurements) per treatment; error bars indicate standard deviations. For a given metal exposure, treatments with different letters are significantly different (One-Way ANOVA with Holm-Sidak method, $p \leq 0.05$).
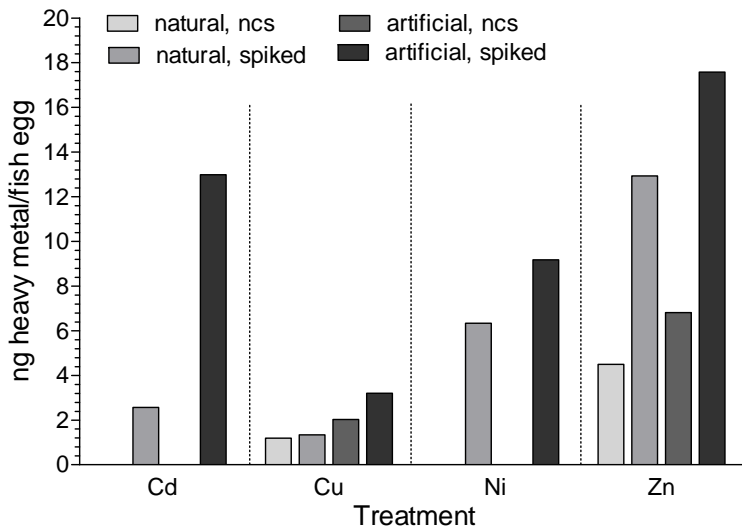
Fig. 7.3 Metal concentrations in zebrafish embryos after exposure to natural and artificial sediments spiked with a mixture of four metals over 48 h compared to unspiked control sediments (ncs = negative control sediment); columns represent means of 50 (natural sediment) and 30 (artificial sediment) measured embryos per treatment, respectively, exposed within one experiment (n = 1).

BAF of each metal was > 10 in all samples (Fig. 7.4). Metals were accumulated to a greater extent from spiked artificial sediment than from natural sediment. The least BAF was for Cd-spiked natural sediment (BAF = 14), whereas the greatest BAF was from artificial sediment spiked with Cu (BAF = 275). The BAF of eggs exposed to artificial sediment spiked with Cu was significantly greater than that of the other three metals as well as for Cu-spiked natural sediment. BAFs of individual metals comprising the mixture were generally lower compared to the respective sediment samples containing only one metal, with the exception of Zn in both sediments (Fig. 7.4).
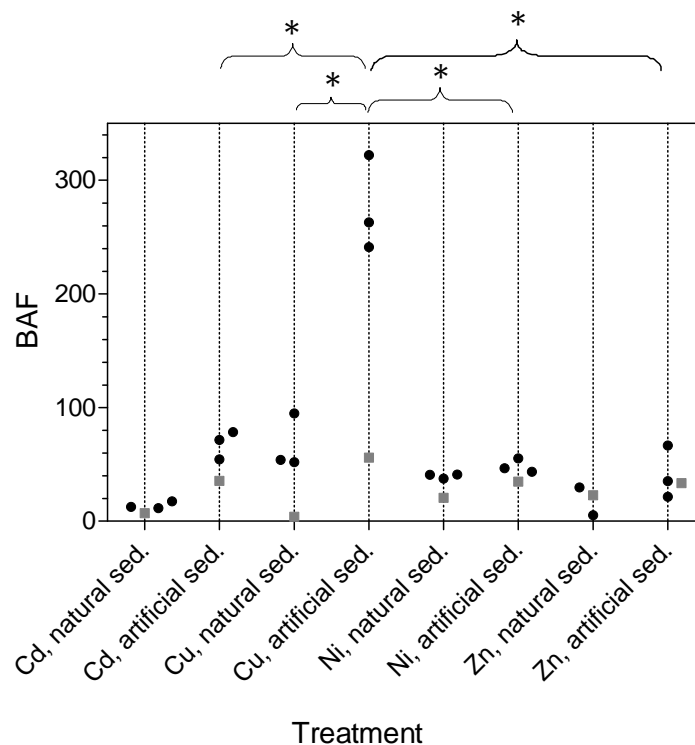
Fig 7.4 Bioaccumulation factors (BAF) of metals in fish eggs after exposure to spiked natural (Altrip, Germany) and artificial (OECD) sediments; black dots represent BAF of three independent replicates with 43-50 embryos (coagulated embryos were not included in measurements) in the main test; grey squares represent BAF of fish eggs in the test with sediments spiked with four metals simultaneously (n = 1, with 50 (natural) and 30 (artificial) measured embryos); * mark statistically significant differences between groups of the main test analysed with one-way ANOVA (Holm-Sidak method, $p \leq 0.05$).
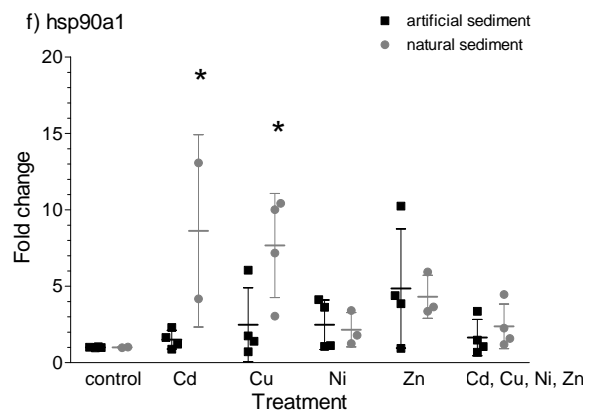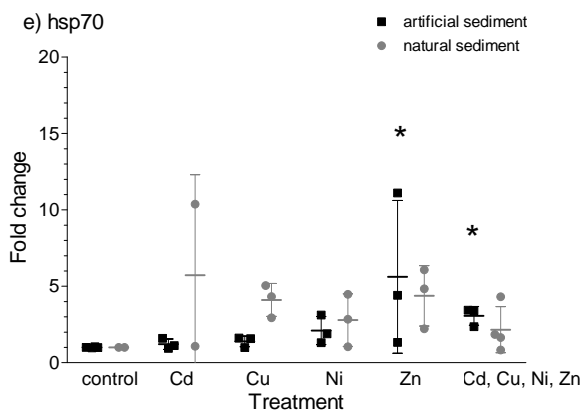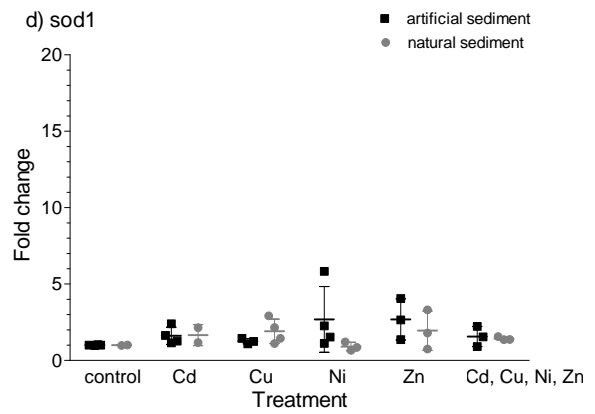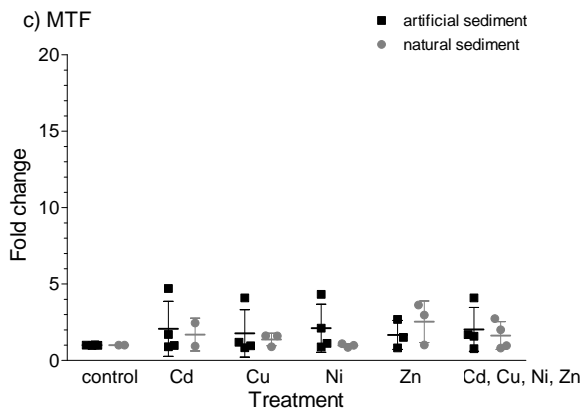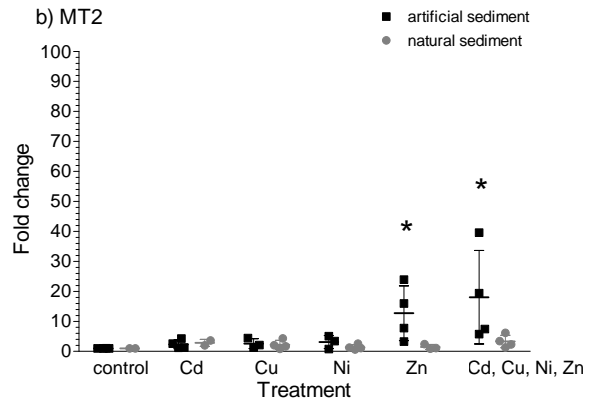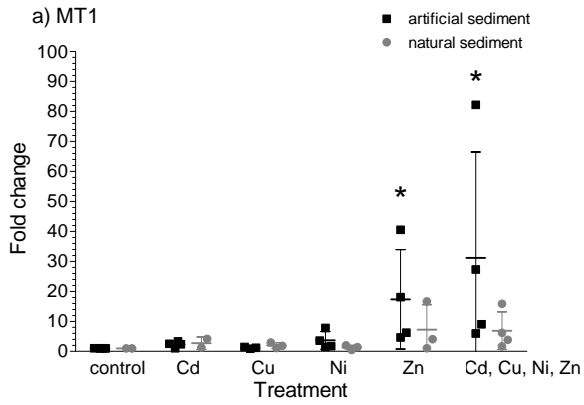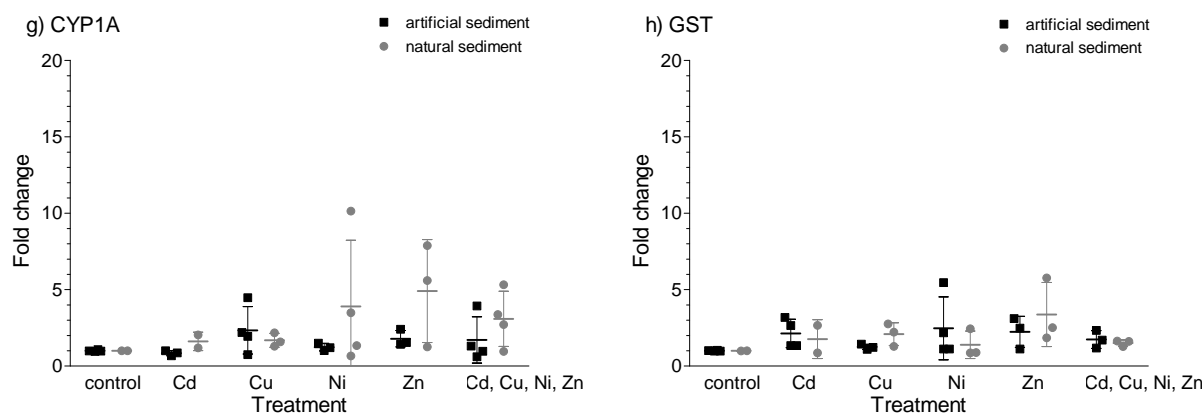
Fig. 7.5 Effects of single metals and a mixture of metals spiked to artificial (OECD) and natural (Altrip) sediment on MT1 (a), MT2 (b), MTF (c), sod1 (d), hsp70 (e), hsp90α1 (f), CYP1A (g) and GST (h) mRNA abundances in *Danio rerio* embryos after exposure to spiked sediments over 48 h. Values represent the fold change in transcript abundance in treatment groups relative to the control group. Statistical analyses used Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Dunn's post-hoc test comparing each treatment versus the negative sediment controls. Data are shown as means ± standard deviations (n = 2-4, with n = number of independent replicates and 25 embryos per replicate and treatment). Significant changes in transcript abundances are indicated by an asterisk (p ≤ 0.05).

7.4.5 Transcriptional responses

The greatest change in transcript abundance after exposure to metal-spiked sediments was for the two metallothionein genes (Fig. 7.5). The mixture of metals spiked to artificial sediment induced a 30-fold increase in expression of MT1 mRNA (Fig. 7.5a) and an 18-fold increase in expression of MT2 mRNA (Fig. 7.5b). Significant increases in expression of MT1 (17-fold) and MT2 (12-fold) mRNA were observed in embryos exposed to artificial sediment spiked with Zn. Changes in transcript abundance of metallothioneins were less than 10-fold in all other treatment groups. Abundances of MTF transcript were slightly but not statistically significantly greater in embryos exposed to metals in sediments. The differences were less than 5 fold in all experiments (Fig. 7.5c).

Transcript abundance of sod1 was less than 5 fold greater in metal-spiked sediments than in the control (Fig. 7.5d), whereas the transcript abundance of hsp70 (Fig. 7.5e) was significantly greater in embryos exposed to artifical sediment spiked with Zn or the mixture of metals than the control sediment. Expression of hsp90α1 mRNA (Fig. 7.5f) was 8.6-fold

greater in sediment spiked with Cd and 7.6-fold greater in natural sediment spiked with copper.

Expression of CYP1A (Fig. 7.5g) and GST (Fig. 7.5h) mRNA were greater for natural sediment than for artificial sediment, with the greatest but not significant changes, a 6.7-fold increase in CYP1A, occurring in response to exposure to Zn-spiked natural sediment. Effects of all of the other treatments were less than 5-fold for these two genes.

7.5   Discussion

7.5.1   Aqueous Pre-tests

LC_{50} values for Cd and Cu (Tab. 7.4) were within the ranges of concentrations reported for zebrafish embryos. The LC_{50} for Cd has been reported to be 30.1 mg/l (Hallare et al. 2005) and 4.2 mg/l (Canton & Slooff 1982). Both results, which were obtained in 48 h tests with zebrafish embryos are consistent with the results of the present study, which was 14.1 mg/l. The LC_{50} of 0.43 mg Cu/l) was also within the range reported in literature (0.21 mg/l, 96 h test; Bellavere & Gorbi 1981). Only the toxicity of Zn, 2.1 mg/l, was less than that reported in another study, which was 87.1 (Nguyen & Janssen 2001). However, Nguyen and Janssen (2001) performed a prolongued test, which likely lead to greater sensitivity of embryos due to loss of the chorion as potential barrier after hatching. In contrast to the other metals tested, Ni was not acutely toxic to fish. The reason for this is that Ni exists primarily as aquo-ion $[Ni(H_2O)_6]^{2+}$. In this form there is little accumulation into organisms (Köck 1996, Komjarova & Blust 2009a). This is supported by the lack of acute effects observed in this study.

Tab. 7.4 Comparison of LC_{50} values determined in the aqueous and in the sediment contact assay (converted data).

LC_{50} [mg/l]	Aqueous fish embryo assay	Sediment contact assay	
		Natural sediment	Artificial sediment
Cd	14.12	1020	1121
Cu	0.43	≥ 28.6	≥ 47.3
Ni	≥ 1000	n.d.	n.d.
Zn	87.10	579	1121

n.d. = not determined

LC_{50} values for early life-stage rainbow trout (*Oncorhynchus mykiss*) reported in the literature range from 10 to 85 µg Cu/l, and from 1.0 to 13.6 µg Cd/l Cd (Besser et al. 2007, Brinkman & Hansen 2007, Marr et al. 1999, Mebane et al. 2008). Since no induction of metallothionein transcript abundance following Cu and Cd exposure was observed in the present study, this difference in sensitivity between both species cannot be attributed to a greater efficiency in metal-detoxification by the metallothioneins in zebrafish embryo. Even though the experiments were not designed to investigate inter-species differences in tolerance, the most likely explanation is that these values were derived by exposing hatched larvae, which due to the loss of the barrier provided by the chorion, exhibit greater sensitivity after hatching (Lammer et al. 2009, Rawson et al. 2000). Determination of LC_{50} values was not the primary function of the present study, and because they are based on one replicate of each aqueous or sediment treatment, LC_{50} values are of limited precision. The precision is further limited because 10-fold dilutions of the metals were used in order to test a wide range of concentrations.

7.5.2   Sediment range-finding tests

Toxicity of metals spiked into the sediments was considerably lower than in aqueous solutions. LC_{50} values of Cd and Cu in the sediment contact assay were higher by a factor of 100 and those of Zn were higher by a factor of 10 compared to the aqueous pre-tests (Tab. 7.4). These findings can be easily explained by the binding of metals to sediments which reduces water concentrations and therefore, the concentration of metals available for uptake (Di Toro et al. 1992, Eimers et al. 2002, Simpson et al. 2004).

7.5.3   Accumulation of metals
Individual metals

The greater concentrations of metals found in all fish eggs after exposure to spiked sediments compared to the aqueous negative control and the sediment controls demonstrates that zebrafish embryos accumulated metals from spiked sediments (Fig. 7.2), and that this accumulation was not accompanied by toxicity under the conditions of the tests. Background concentrations in the sediments (Feiler et al. 2009, Höss et al. 2010) may explain Cu and Zn

measured in the controls and also the relatively great variability of individual concentrations of Zn among replicates.

Metals were more available for uptake from artificial sediment than from natural sediment (statistically significant for Cd and Cu). BAFs confirmed this finding (Fig. 7.4). This was possibly due to different geochemical compositions of the sediments such as organic carbon and clay/silt content. Concentrations of metals in porewater equilibrate faster in sediments with high concentrations of metal-binding sites, such as particulate sulfide, organic matter and iron hydroxide phases and larger surface areas, such as fine, silt sediments, than in sandy sediments with lesser binding capacities (Simpson & Batley 2006). Since the natural sediment used in this study contained greater amounts of organic matter (3.4 % vs. 2.0 % TOC) and particles in the clay fraction (23.4 % vs. 5.0 %) than the artificial sediment, it is possible that steady state of metals with binding sites occurred more rapidly with the natural sediment after spiking occurred and metals were more tightly bound to the sediment. There were, thus, likely greater concentrations of metals in porewater in case of treatments with artificial sediments, which would be consistent with greater bioavailability and accumulation of metals by embryos (Di Toro et al. 2005, Eimers et al. 2002, Simpson & Batley 2006).

Although metals were more available from artificial sediment than from natural sediment, the use of artificial sediment for toxicity testing has several benefits, including the absence of background contamination and indigenous biota (Goedkoop et al. 2005, Verrhiest et al. 2002). Additionally, artificial sediments are well-characterized and have a highly reproducible composition (Goedkoop et al. 2005, Verrhiest et al. 2002). However, there are parameters, such as redox potential, organic matter and biological activity which are important regarding sequestration and bioavailability of contaminants and lead to distinct differences compared to natural sediments (Goedkoop et al. 2005, Verrhiest et al. 2002). Therefore, studies of accumulation from sediments with natural sediment samples can be more suitable for extrapolation to natural situations. In conclusion, the use of artificial sediment in addition to natural sediments can provide a stable reference to which natural samples can be compared.

The results are consistent with the chorion not fully restricting metals from accumulation in the embryo (Li et al. 2004). In this study, concentrations of the entire fish egg, including the embryo, perivitelline fluid and chorion, were measured. Thus it is not possible to know which proportion of the measured concentrations was accumulated directly into the embryo. Jezierska et al. (2009) reported for several fish species, including *Salmo salar*, *Oryzias latipes* and *Clupea harnegus* that most metal accumulates in the egg shell, a certain amount in the perivitelline fluid and little is accumulated into the embryo. Hence, it is likely that most of the

measured metal concentrations observed in eggs were associated with the chorion. The application of a minimal equilibration time of less than 24 h applied in the present study specifically simulated the bioavailability of metals from sediments immediately after an initial "contamination event", such as spiking. This represents a worst-case scenario of maximal potential uptake of metals from sediments. It may be reasonably assumed that with increasing equilibration time of the sediments before exposure of fish eggs, uptake would be less. In oxic sediments, trace metals tend to associate with particular sediment components, such as organic matter as well as iron and manganese oxides (Eimers et al. 2002). In anoxic sediments, added metals are time-dependently associated with sulfides (Di Toro et al. 1992). Thus, equilibration leads to lesser concentrations of metals in porewater and less accumulation, whereas shorter equilibration times result in greater concentrations of metals in porewater, which in turn results in greater accumulations by organisms (Eimers et al. 2002, Simpson et al. 2004).

Influence of pH could have affected accumulation of metals in this study. The pH of all sediments spiked with metals was less than the un-spiked sediments (Fig. 7.1). The observed lesser pH was consistent with hydrolysis of the added metals, the displacement of Fe(II) from particulate material by the applied metals followed by oxidative hydrolysis, as well as the competitive displacement of protons from organic matter and metal-binding sites (Hutchins et al. 2007, Simpson et al. 2004). The greater the amount of metal added, the greater the decrease in pH (Simpson et al. 2004). Lower pH results in greater concentrations of spiked metals in porewater, which would cause more toxicity than metals associated with the solid phase (Di Toro et al. 2005, Simpson & Batley 2006). The pHs of sediments spiked with individual metals were all still within the optimal pH range for fish embryos (Fig. 7.1).

Mixture

Similar to the exposure studies with individual metals, a general trend of greater uptake from the artificial sediment than from the natural sediment was observed when embryos were exposed to a mixture of all four metals (Fig. 7.3). With the exception of natural sediments spiked with Zn, uptake and bioaccumulation was less when embryos were exposed to a mixture of all four metals (Fig. 7.3 & 7.4). This effect might have been masked in Zn-spiked natural sediment by the variability of the measured concentrations and the great background contamination which were determined in the controls. Inhibiting interactions between metals may have occurred and explain this finding, since different metals can interact with each other

affecting individual uptake, bioaccumulation and toxicity (Borgmann et al. 2008, Komjarova & Blust 2009b). In particular, the bioaccumulation of Cu from artificial sediment spiked with the mixture was 5-fold less compared to accumulation in eggs exposed to artificial sediment spiked with Cu as single metal (Fig. 7.4). However, this may have been caused by the lesser initial concentration of Cu. Accumulation of metals from sediments spiked with the mixture may have been limited by uptake and transport across the chorion. Since the primary role of the chorion is that of physical protection of the embryo, while allowing two-way movement of water and solutes, metals can pass through pores due to a gradient of concentrations from an area of greater to lesser concentrations (Rawson et al. 2000). Total metal concentration in fish embryos after exposure to the mixture was greater than when exposed to individual metals. If the total internal metal concentration was sufficiently great to approximate equilibrium with the external concentration, this could have inhibited further metal uptake.

The impact of interaction and inhibition might have been even more pronounced than the actual results indicate, but masked by changes in pH, since greater decreases in pH were observed for samples spiked with all four metals (Fig. 7.1). This might have led to greater concentrations of metals in porewater and resulted in greater accumulation than when exposed to individual metals. Thus, the number of coagulated fish eggs observed in the sediment with the lowest pH could have occurred not only due to direct effects of either low pH or the greater total metal concentration, but also because of greater toxicity of metals due to the lesser pH.

In summary, exposure of embryos to single metals might lead to an overestimation of uptake if transferred to a multi-exposure scenario without appropriate adjustment. Furthermore, the results indicated the need to account not only for more than one metal, but to accordingly monitor pH changes, thus underlining the great relevance of multi-stressor focused approaches in sediment assessment (Hecky et al. 2010, Hollert et al. 2007, Sundback et al. 2010).

7.5.4   Gene expression
Individual metals

Expression of MT1 and MT2 mRNA was not greater in either Cu-spiked sediment (Fig. 7.5a & b), even though metallothioneins are known to bind and thus detoxify metals (Klerks & Weis 1987). These small, metal-binding proteins are responsible for protection against Cu toxicity in many organisms (Roesijadi 1992). This was also the case for zebrafish (Craig et al. 2009a, Craig et al. 2009b). Expression of metallothionein genes occurs during embryogenesis of zebrafish, and the significant increases of MT transcript abundance after Zn-exposure in the present study confirm this finding (Chen et al. 2004). In the 48 h fish embryo range-finding test, the most frequent lethal endpoint caused by metals was coagulation (> 90 %). Coagulation is an "all-or-nothing" criterion that can potentially cause a narrow relationship between concentration and effect. Therefore, even though the concentrations of Cu applied in the present study were determined based on NOECs determined in several range-finding tests, the concentration-spacing might have been too wide. Therefore the applied NOECs could have underestimated effective concentrations and thus been too low to induce increases of MT transcript abundance.

In embryos exposed to artificial sediment spiked with Zn, expression of MT1 and MT2 mRNA was significantly greater than that in the controls. Greater expression of MT mRNAs in response to exposure to Zn has been previously reported (Chen et al. 2007). However, no significant changes in MT transcript abundance were observed in embryos exposed to natural sediment that had been spiked with Zn. This observation is consistent with lesser bioavailability and subsequent accumulation of metals from the natural sediment.

The reason for the lack of MT induction after exposure to Cd or Ni likely was that there was insufficient accumulation of these metals. Although Chen et al. (2007) reported induction of MT genes in *Danio rerio* embryos by Cd, no significant changes in MT transcript abundance were observed for Cd-spiked sediments in the present study. These results are consistent with the report that expression of MT genes in adult zebrafish is "late-onset biomarker" and that sufficient accumulation of Cd in the target tissue is necessary before up-regulation of the MT gene is observed (Gonzalez et al. 2006). The MT induction by Cd is consistent with the exposure period of 48 h. The lack of changes when exposed to Ni is consistent with the fact that Ni exhibits lesser potency.

The non-significant, maximal 5-fold increase of MTF transcript abundance in all embryos exposed to metal-spiked artificial sediment indicates a poor induction of the transcription factor by metal exposure in general (Fig. 7.5c).

Up-regulation of expression of MT1, MT2 and MTF was indicative of exposure to metals. Accumulation of metals does not necessarily increase expression of MT mRNA during acute exposures. Chronic exposure plays a key role in terms of the adverse impacts of metals on fish in the environment (e.g. Besser et al. 2007, Besser et al. 2005, Brix et al. 2004, Craig et al. 2009b, Guadagnolo et al. 2001, Kusch et al. 2008, Mebane et al. 2008). Since the present study only focused on acute effects, it cannot be excluded that the applied sublethal metal concentrations could be sufficient to induce chronic effects. In conclusion, MT transcript abundances were confirmed as applicable biomarkers indicating metal uptake, but a lack of induction does not exclude the possibility of uptake or chronic effects. More research on this interrelationship is necessary.

Metals can induce oxidative stress subsequently leading to cell damage, including alteration of DNA and membranes (Worms et al. 2006). For this reason genes related to oxidative stress, including mitochondrial superoxide dismutase (sod1) and the heat shock proteins hsp70 and hsp90α1 were investigated in this study. Since expression of the hsp gene is induced not only in response to heat shock, but also after exposure to chemicals that exhibit proteo-toxicity and particularly metals such as Cd (Blechinger et al. 2002, Gonzalez et al. 2006, Krone et al. 2003, Pierron et al. 2009), observed increases in transcript abundance of hsp70 and hsp90α1 in comparison to the controls can be attributed to the metal exposure, since presence of metals was the only differing parameter between control and exposure treatments (Fig. 7.5e & f). There was no difference in the amount of mRNA of the hsp genes in embryos exposed to natural sediment or artificial sediments.

Expression of sod1 mRNA displays different responses to Cu and Zn (Pierron et al. 2009). Exposure to Cu resulted in greater expression of sod1 in perch liver, whereas exposure to Zn resulted in lesser expression of mRNA. In the present study, transcript abundance did not increase decisively or statistically significant for all four metals, except for an outlier in case of Ni-spiked artificial sediment.

The reason for the slightly greater expression of CYP1A and GST mRNA is not known (Fig. 7.5g & h), however, the lesser transcript abundances of CYP1A and GST in the unspiked sediments exclude potential influences of residual contaminations of the natural sediment.

Mixture

The responses of the genes studied were the same in the mixture as they were in the sediments containing individual metals. This could indicate that molecular biomarkers react less sensitive to impacts that confound results, such as the difference between exposures with individual contaminants or complex mixtures.

7.6   Conclusion and outlook

Copper can cause adverse effects on zebrafish embryos at environmentally relevant concentrations in sediments. The fact that metals were accumulated differently when they occurred individually or in a mixture is relevant for evaluation of metals in sediments. This effect should be addressed in future research, especially in combination with other potentially confounding factors, such as pH and time-dependence. Eventually, it appears crucial that risk assessment accounts for the potential impact of single- vs. multi-substance exposures.

7.7   Acknowledgements

7.8 References

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

Ahlf W, Drost W, Heise S (2009): Incorporation of metal bioavailability into regulatory frameworks-metal exposure in water and sediment. J Soil Sediment 9, 411-419

Bellavere C, Gorbi J (1981): A Comparative-Analysis of Acute Toxicity of Chromium, Copper and Cadmium to Daphnia-Magna, Biomphalaria-Glabrata, and Brachydanio-Rerio. Environ Technol Lett 2, 119-128

Besser JM, Wang N, Dwyer FJ, Mayer FL, Ingersoll CG (2005): Assessing contaminant sensitivity of endangered and threatened aquatic species: Part II. Chronic toxicity of copper and pentachlorophenol to two endangered species and two surrogate species. Arch Environ Con Tox 48, 155-165

Besser JM, Mebane CA, Mount DR, Ivey CD, Kunz JL, Greer IE, May TW, Ingersoll CG (2007): Sensitivity of mottled sculpins (Cottus bairdi) and rainbow trout (Onchorhynchus mykiss) to acute and chronic toxicity of cadmium, copper, and zinc. Environ Toxicol Chem 26, 1657-1665

Blaha L, Hilscherova K, Cap T, Klanova J, Machat J, Zeman J, Holoubek I (2010): Kinetic bacterial bioluminescence assay for contact sediment toxicity testing: relationships with the matrix composition and contamination. Environ Toxicol Chem 29, 507-514

Blechinger SR, Warren JT, Kuwada JY, Krone PH (2002): Developmental toxicology of cadmium in living embryos of a stable transgenic zebrafish line. Environ Health Persp 110, 1041-1046

Borgmann U, Norwood WP, Dixon DG (2008): Modelling bioaccumulation and toxicity of metal mixtures. Hum Ecol Risk Assess 14, 266-289

Boyd RS (2010): Heavy Metal Pollutants and Chemical Ecology: Exploring New Frontiers. J Chem Ecol 36, 46-58

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

Breitholtz M, Eriksson J, Green N, Ruden C (2006): Testing and risk assessment of persistent and bioaccumulating chemical substances - Improvements within REACH? Hum Ecol Risk Assess 12, 782-805

Brinkman SF, Hansen DL (2007): Toxicity of cadmium to early life stages of brown trout (Salmo trutta) at multiple water hardnesses. Environ Toxicol Chem 26, 1666-1671

Brix KV, Keithly J, DeForest DK, Laughlin J (2004): Acute and chronic toxicity of nickel to rainbow trout (Oncorhynchus mykiss). Environ Toxicol Chem 23, 2221-2228

Burton GA (1991): Assessing the toxicity of fresh-water sediments. Environ Toxicol Chem 10, 1585-1627

Canton JH, Slooff W (1982): Toxicity and Accumulation Studies of Cadmium (Cd-2+) with Fresh-Water Organisms of Different Trophic Levels. Ecotox Environ Safe 6, 113-128

Chen WY, John JAC, Lin CH, Lin HF, Wu SC, Chang CY (2004): Expression of metallothionein gene during embryonic and early larval development in zebrafish. Aquat Toxicol 69, 215-227

Chen WY, John JAC, Lin CH, Chang CY (2007): Expression pattern of metallothionein, MTF-1 nuclear translocation, and its DNA-binding activity in zebrafish (Danio rerio) induced by zinc and cadmium. Environ Toxicol Chem 26, 110-117

Craig PM, Galus M, Wood CM, McClelland GB (2009a): Dietary iron alters waterborne copper-induced gene expression in soft water acclimated zebrafish (Danio rerio). Am J Physiol-Reg I 296, R362-R373

Craig PM, Hogstrand C, Wood CM, McClelland GB (2009b): Gene expression endpoints following chronic waterborne copper exposure in a genomic model organism, the zebrafish, Danio rerio. Physiol Genomics 40, 23-33

Dell'Anno A, Mei ML, Ianni C, Danovaro R (2003): Impact of bioavailable heavy metals on bacterial activities in coastal marine sediments. World J Microb Biot 19, 93-100

Di Toro D, McGrath J, Hansen D, Berry W, Paquin P, Mathew R, Wu KB, Sanatore RC (2005): Predicting sediment toxicity using a sediment Biotic Ligand Model: Methodology and initial application. Environ Toxicol Chem 24, 2410-2427

Di Toro DM, Mahony JD, Hansen DJ, Scott KJ, Hicks MB, Mayr SM, Redmond MS (1990): Toxicity of Cadmium in Sediments - the Role of Acid Volatile Sulfide. Environ Toxicol Chem 9, 1487-1502

Di Toro DM, Mahony JD, Hansen DJ, Scott CJ, Carlson AR, Ankley GT (1992): Acid Volatile Sulfide Predicts the Acute Toxicity of Cadmium and Nickel in Sediments. Environ Sci Technol 26, 101-

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

Duft M, Schulte-Oehlmann U, Tillmann M, Markert B, Oehlmann J (2003): Toxicity of triphenyltin and tributyltin to the freshwater mudsnail Potamopyrgus antipodarum in a new sediment biotest. Environ Toxicol Chem 22, 145-152

Eimers MC, Evans RD, Welbourn PM (2002): Partitioning and bioaccumulation of cadmium in artificial sediment systems: application of a stable isotope tracer technique. Chemosphere 46, 543-551

Eklund B, Elfstrom M, Gallego I, Bengtsson B-E, Breitholtz M (2010): Biological and chemical characterization of harbour sediments from the Stockholm area. J Soil Sediment 10, 127-141

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 R 12, 257-258

Feiler U, Ahlf W, Fahnenstich C, Gilberg D, Hammers-Wirtz M, Höss S, Hollert H, Melbye K, Meller M, Neumann-Hensel H, Ratte HT, Seiler TB, Spira D, Weber J, Heininger P 2009: Final report SeKT framework project - Definition von Referenzbedingungen, Kontrollsedimenten und Toxizitätsschwellenwerten für limnische Sedimentkontakttests - SeKT

Förstner U, Westrich B (2005): BMBF coordinated research project SEDYMO (2002-2006) - Sediment dynamics and pollutant mobility in river basins. J Soil Sediment 5, 134-138

Goedkoop W, Widenfalk A, Haglund AL, Steger K, Bertilsson S (2005): Microbial characterization of artificial sediment and comparisons with natural sediments - Implications for toxicity testing. Environ Toxicol Chem 24, 2725-2733

Gonzalez P, Baudrimont M, Boudou A, Bourdineaud JP (2006): Comparative effects of direct cadmium contamination on gene expression in gills, liver, skeletal muscles and brain of the zebrafish (Danio rerio). Biometals 19, 225-235

Guadagnolo CM, Brauner CJ, Wood CM (2001): Chronic effects of silver exposure on ion levels, survival, and silver distribution within developing rainbow trout (Oncorhynchus mykiss) embryos. Environ Toxicol Chem 20, 553-560

Hallare AV, Schirling M, Luckenbach T, Kohler HR, Triebskorn R (2005): Combined effects of temperature and cadmium on developmental parameters and biomarker responses in zebrafish (Danio rerio) embryos. J Therm Biol 30, 7-17

Hallare AV, Seiler TB, Hollert H (2011): The versatile, changing, and advancing roles of fish in sediment toxicity assessment-a review. J Soil Sediment 11, 141-173

Hecky RE, Mugidde R, Ramlal PS, Talbot MR, Kling GW (2010): Multiple stressors cause rapid ecosystem change in Lake Victoria. Freshwater Biol 55, 19-42

Hilscherova K, Dusek L, Sidlova T, Jalova V, Cupr P, Giesy JP, Nehyba S, Jarkovsky J, Klanova J, Holoubek I (2010): Seasonally and regionally determined indication potential of bioassays in contaminated river sediments. Environ Toxicol Chem 29, 522-534

Hollert H, Duerr 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. Ecotoxicology 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, Seiler TB, Blaha L, Young AL (2007): Multiple stressors for the environment: Present and future challenges and perspectives. Environ Sci Pollut R 14, 222-222

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

Hutchins CM, Teasdale PR, Lee J, Simpson SL (2007): The effect of manipulating sediment pH on the porewater chemistry of copper- and zinc-spiked sediments. Chemosphere 69, 1089-1099

ISO (2007): ISO 15088: Water quality — Determination of the acute toxicity of waste water to zebrafish eggs (Danio rerio). Iso Guideline

Jezierska B, Lugowska K, Witeska M (2009): The effects of heavy metals on embryonic development of fish (a review). Fish Physiol Biochem 35, 625-640

Klerks PL, Weis JS (1987): Genetic adaption to heavy metals in aquatic organisms - a review. Environ. Pollut. 45, 173-205

Köck MG (1996): Die toxische Wirkung von Schwermetallen auf Fische - Beiträge zur Festlegung von Immissionsbereichen für Kupfer, Cadmium, Quecksilber, Chrom, Nickel, Blei und Zink aus fischbiologischer Sicht. Handbuch Angewandte Limnologie - Teil: Aquatische Ökotoxikologie. ecomed

Komjarova I, Blust R (2009a): Effects of Na, Ca, and pH on the Simultaneous Uptake of Cd, Cu, Ni, Pb, and Zn in the Zebrafish Danio rerio: A Stable Isotope Experiment. Environ Sci Technol 43, 7958-7963

Komjarova I, Blust R (2009b): Multimetal Interactions between Cd, Cull, Ni, Pb, and Zn Uptake from Water in the Zebrafish Danio rerio. Environ Sci Technol 43, 7225-7229

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

Krone PH, Evans TG, Blechinger SR (2003): Heat shock gene expression and function during zebrafish embryogenesis. Semin Cell Dev Biol 14, 267-274

Kusch RC, Krone PH, Chiverst DP (2008): Chronic exposure to low concentrations of waterborne cadmium during embryonic and larval development results in the long-

term hindrance of antipredator behavior in zebrafish. Environ Toxicol Chem 27, 705-710

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 Phys C 149, 196-209

Li WH, Chan PCY, Chan KM (2004): Metal uptake in zebrafish embryo-larvae exposed to metal-contaminated sediments. Mar Environ Res 58, 829-832

Linge KL, Jarvis KE (2009): Quadrupole ICP-MS: Introduction to Instrumentation, Measurement Techniques and Analytical Capabilities. Geostand Geoanal Res 33, 445-467

Livak KJ, Schmittgen TD (2001): Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25, 402-408

Marr JCA, Lipton J, Cacela D, Hansen JA, Meyer JS, Bergman HL (1999): Bioavailability and acute toxicity of copper to rainbow trout (Oncorhynchus mykiss) in the presence of organic acids simulating natural dissolved organic carbon. Can J Fish Aquat Sci 56, 1471-1483

Mebane CA, Hennessy DP, Dillon FS (2008): Developing acute-to-chronic toxicity ratios for lead, cadmium, and zinc using rainbow trout, a mayfly, and a midge. Water Air Soil Poll 188, 41-66

Nagel R (2002): DarT: The embryo test with the zebrafish Danio rerio - a general model in ecotoxicology and toxicology. Altex 19, 38-48

Nguyen LTH, Janssen CR (2001): Comparative sensitivity of embryo-larval toxicity assays with African catfish (Clarias gariepinus) and zebra fish (Danio rerio). Environ Toxicol 16, 566-571

OECD (1984): Guideline for Testing of Chemicals 207: Earthworm, Acute Toxicity Tests. Organisation for Economic Cooperation and Developement

OECD (2004): OECD guideline 218: Sediment-water chironomid toxicity using spiked sediment. Organisation for Economic Cooperation and Development

Pierron F, Bourret V, St-Cyr J, Campbell PGC, Bernatchez L, Couture P (2009): Transcriptional responses to environmental metal exposure in wild yellow perch (Perca flavescens) collected in lakes with differing environmental metal concentrations (Cd, Cu, Ni). Ecotoxicology 18, 620-631

Rawson DM, Zhang T, Kalicharan D, Jongebloed WL (2000): Field emission scanning electron microscopy and transmission electron microscopy studies of the chorion, plasma membrane and syncytial layers of the gastrula-stage embryo of the zebrafish Brachydanio rerio: A consideration of the structural and functional relationships with respect to cryoprotectant penetration. Aquac Res 31, 325-336

Rodrigues SM, Henriques B, Coimbra J, da Silva EF, Pereira ME, Duarte AC (2010): Water-soluble fraction of mercury, arsenic and other potentially toxic elements in highly contaminated sediments and soils. Chemosphere 78, 1301-1312

Roesijadi G (1992): Metallothioneins in metal regulation and toxicity in aquatic animals. Aquat Toxicol 22, 81-114

Schmitt C, Balaam J, Leonards P, Brix R, Streck G, Tuikka A, Bervoets L, Brack W, van Hattum B, Meire P, de Deckere E (2010): Characterizing field sediments from three European river basins with special emphasis on endocrine effects - A recommendation for Potamopyrgus antipodarum as test organism. Chemosphere 80, 13-19

Schramel P, Begerow J, Emons H (1999): The use of ICP-MS for human biomonitoring. Analyses of Hazardous Substances in Biological Materials, 6. Wiley VCH, Weinheim

Schramel P (2003): Digestion procedures for the determination of metals in biological materials. Analyses of Hazardous Substances in Biological Materials, 8. Wiley VCH, Weinheim

Schwarzenbach RP, Escher BI, Fenner K, Hofstetter TB, Johnson CA, von Gunten U, Wehrli B (2006): The challenge of micropollutants in aquatic systems. Science 313, 1072-1077

Simpson SL, Angel BM, Jolley DF (2004): Metal equilibration in laboratory-contaminated (spiked) sediments used for the development of whole-sediment toxicity tests. Chemosphere 54, 597-609

Simpson SL, Batley GE (2006): Predicting Metal Toxicity in Sediments: A Critique of Current Approaches. Integrated Environmental Assessment and Management 3, 18-31

Standardization IOf (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 3: Flow-through method. Iso Guideline

Sundback K, Alsterberg C, Larson F (2010): Effects of multiple stressors on marine shallow-water sediments: Response of microalgae and meiofauna to nutrient-toxicant exposure. Journal of Experimental Marine Biology and Ecology 388, 39-50

Turesson EU, Stiernstrom S, Minten J, Adolfsson-Erici M, Bengtsson BE, Breitholtz M (2007): Development and reproduction of the freshwater harpacticoid copepod Attheyella crassa for assessing sediment-associated toxicity. Aquat Toxicol 83, 180-189

Verrhiest GJ, Cortes S, Clement B, Montuelle B (2002): Chemical and bacterial changes during laboratory conditioning of formulated and natural sediments. Chemosphere 46, 961-974

Whyte JJ, Jung RE, Schmitt CJ, Tillitt DE (2000): Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. Crit Rev Toxicol 30, 347-570

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

Worms I, Simon DF, Hassler CS, Wilkinson KJ (2006): Bioavailability of trace metals to aquatic microorganisms: importance of chemical, biological and physical processes on biouptake. Biochimie 88, 1721-1731

Chapter 8

_____

Discussion

8 General discussion

The preceding chapters illustrated that each of the investigated impacts can change the results of biotests. This chapter discusses and evaluates the relevance of these changes for the assessment of contamination in sediments.

8.1 Time-dependence

The studies reported in chapters 2-4 demonstrated the relevance of time-dependence for the interpretation of results obtained in direct sediment contact tests, by showing that effects which are originally recorded after an initial contamination or spiking can be significantly changed or even completely lost within several weeks to a few months. Such alterations can either be decreases or increases. In the experimental studies, impacts of time varied and depended on the specific combination of substance, species and sediment. Inhibition of dehydrogenase by pentachlorophenol (PCP) remained unchanged in the bacterial contact test with *Arthrobacter globiformis* over the course of 44 weeks (chapter 3). This could indicate that bacteria react less sensitively than other species to temporal impacts, but this hypothesis needs to be tested in further investigations. In contrast, results in the zebrafish embryo sediment contact test demonstrated great sensitivity to temporal impact. A complete loss of initial toxicity of all three applied substances to zebrafish embryos was observed within two to sixteen weeks. Falling between these extremes of complete loss of effects or no temporal changes, results were significantly altered in the nematode contact test (chapter 3) and the bioaccumulation assay with *Lumbriculus variegatus* (chapter 4), but not for all combinations of sediment and substances. Number of juveniles reached control level after only six weeks in natural sediment spiked with fluoranthene (FA) in the nematode contact test, whereas no increase in juveniles was reported in the artificial sediment spiked with either FA or PCP, and PCP-spiked natural sediment. In the case of bioaccumulation in earthworms, a significant decrease after 13 days of contact between sediment and pollutant was observed only in PCP-spiked natural sediment.

The given studies systematically reviewed and established the relationship between time and effects in the case of direct sediment contact tests. The uncovered potential alterations lead to two conclusions:

a) Time can significantly change the results obtained in direct sediment contact tests, and altered results on the toxicity of substances in all applied assays in the given studies with the exception of only the bacterial contact test.

b) The direction, speed and numerical value of temporal changes depend on the specific combination of substance, species and sediment and are, thus, different for every such combination.

These conclusions in turn have direct consequence for the assessment of particle-bound chemicals. The results of a spiking-based assessment in the laboratory can be completely different, depending on the point of time after spiking that is selected as the beginning of exposure, and on the applied contact test. Furthermore, if several tests with different organisms are conducted, all can be temporally influenced to a different degree. Therefore, ignoring the potential impact of time on results can lead to an overestimation of risk just as likely as an underestimation. Whereas the latter is simply inacceptable in terms of public and environmental security, the former can easily result in unnecessary and costly measures, and is therefore economically undesirable.

This risk of errors in assessment induced by temporal impact also affects the testing of field samples. However, a given water body is rarely affected by a single impact, and these stressors may have synergistic, additive, or antagonistic effects (Solimini et al. 2009). Hence, solutions are needed that not only account for complex temporal changes, but rather evaluate all impacts within a holistic context.

8.2 Vessel material

Plastic as material of test vessels was shown to reduce the effects of DCA in the aqueous fish embryo test with zebrafish in comparison to glass vessels (chapter 5). This is consistent with literature which indicated binding of organic chemicals to plastic (Dahlstrom et al. 2004, Koutsopoulos et al. 2007, Palmgren et al. 2006). Flow-through tests, passive dosing techniques or constant renewal of the test solutions have been proposed and are promising solutions to counter the changes caused by pollutants binding to vessels (Lammer et al. 2009b, Kwon et al. 2009, Smith et al. 2010). Flow-through systems need to be optimised

regarding efficiency of costs, whereas approaches utilising semi-static renewal or passive dosing are cheaper and can be more easily included into existing guidelines.

The importance of experimental timing was a second crucial result of the investigation on the impact of vessel material. Effects after exposure in either material decreased with time, showing that results can be significantly changed by prolonged contact between substance and vessel prior the beginning of the definite exposure. This negative impact could result in severe underestimation of potential effects. It can be avoided if renewal, passive-dosing or flow-through is started at least a day prior to the definite exposure in order to saturate vessels with the test substance (Lammer et al. 2009a, Lammer et al. 2009b).

In summary, the reduction of the LC_{50} of DCA recorded in the present study clearly showed the need to address the potential impact of vessel material in any investigation that utilises such vessels. Appropriate measures to counter this impact exist, need to be conducted and should also be mandatorily included into respective guidelines.

8.3   Methodology

Different methodologies are applied because they give different results, and can thus be utilised to address different scientific questions. The study on the impact of extraction methodology identified Tenax TA® as a possibly suitable method for the prediction of toxicological bioavailability in the sediment contact test with zebrafish embryos. The other three methods (Soxhlet extraction, membrane dialysis extraction (MDE) and Hydroxy-propyl-ß-cyclodextrin extraction (HPCD) overestimated bioaccessibility in regard to this definition, as was expected for SOX and MDE. However, results for HPCD on the level of biological effect were not consistent with expectations derived from chemical studies (Chung & Alexander 1999, Cuypers et al. 2002, Hickman &Reid 2005, Tang & Alexander 1999). HPCD was expected to predict bioaccessibility, but induced effects that were at least as high as effects of the applied vigorous extraction methodologies, Soxhlet and MDE. It was hypothesised that this observation can be attributed to short contact time between sediment and pollutant in comparison with other studies that investigated HPCD. This, in turn, shows that the interrelationship and differences between extraction methods can be altered by time in a way that can cause severe misinterpretations of results. Hence, besides the general characterisation of methodologies with regard to biological effects, these results further underline the need to address time-dependence as crucial confounding impact on biotest results.

8.4   Multi- vs. single-metal exposure

Exposure to Cd, Cu, Ni and Zn together as compared to individual exposure did not change the transcript abundances of the analysed genes, but uptake was less from sediments spiked with the mixture than from individually spiked sediments (chapter 7). This correlation underlines the need to be aware of potential differences between the testing of single substances in the laboratory and the assessment of field samples, because metals – as well as organic contaminants – are often present together in the environment (Borgmann et al. 2008, Brack 2003, Komjarova & Blust 2009, Rodrigues et al. 2010).

Currently, risk-assessment of metals in the environment focuses on the free ion concentration (Di Toro et al. 2005, Di Toro et al. 2001). However, metal complexes, dietary uptake, and particle-bound metals are also relevant for metal toxicity (Ahlf et al. 2009). Ahlf et al. (2009) proposed to apply studies on bioavailability in the risk assessment of metals because bioavailability integrates all relevant exposure pathways for metals to organisms. Data obtained in such bioaccumulation studies should then be used to develop predictive models on the basis of the existing biotic ligand modelling approaches (Di Toro et al. 2005, Di Toro et al. 2001).

Because the confounding impact resulting from differences between exposures with individual metals or a mixture significantly altered uptake, developing predictive models on the basis of individual exposure could overestimate bioaccumulation and result in incorrect predictions. Hence, this impact needs to be addressed and included into long-term strategies aiming at the development of predictive modelling tools for risk assessment.

8.5   Sediment type

Table 8.1 gives an overview on the differences between artificial and natural sediments both as investigated in the present thesis and as indicated in previous literature. This comparison indicated no preference for either type. In terms of time-dependence, for instance, no clear pattern could be identified, meaning that no sediment type consistently exhibited more rapid changes in effects than the other. Accordingly, no type of sediment was better suited to assess sediments in combination with a given methodology or exposure with either individual substances or mixtures. In conclusion, results confirmed that both artificial and natural

sediments are useful tools for assessing sediment contamination and complement each other well. Thus, a parallel application should be considered rather than advocating either type. However, a parallel application might not always be possible due to economical and temporal considerations. Furthermore, direct comparability between both types was shown to be limited because each combination between species and substance can result in a different correlation of the obtained results. For instance, whereas temporal decreases of effects of DCA and FA occurred more rapidly in natural sediment in the fish embryo sediment contact test, effects of PCP were lost faster in artificial sediment. It can be reasonably assumed that this correlation is further confounded by testing additional sediments of both types. Hence, research data obtained with either type of sediment should be correlated by means of mathematical tools capable of integrating complex factors.

Tab. 8.1 Differences between artificial and natural sediments addressed in the previous chapters.

	Artificial sediment	**Natural sediment**
Time-dependence	Reaction, rate and time factor depends on specific substance-species-sediment combination	
Methodology	No significant differences between sediment types in methodologies that utilised freeze-dried samples Significant differences in methodologies based on native samples	
Reproducibility of extract-based results	Good with each tested method	Great variability in case of Tenax TA
Heavy metal accumulation	Greater accumulation in artificial sediment	
Heavy metal transcript abundance	No difference	
Volume/weight ratio	Constant	Variable
Handling and sampling/preparation	Easy	More complex
Biofilm, humic substances, organic matter	Reduced/not fully reproducible	Naturally complex
Residual contamination	Irrelevant	Possible

8.6   Conclusion and Outlook

Both time and the difference between individual or mixture exposure were identified as greatly relevant confounding impacts on results of biotests using sediments. Eventually, these influences could, in a worst-case scenario, result in wrong decisions in assessment and regulation because of either over- or underestimation of the adverse potential of a given pollutant. In contrast, vessel material and methodology were identified as relevant, but less important impacts. The impact of vessel material can and will be addressed by passive dosing or other promising strategies (Kwon et al. 2009, Smith et al. 2010). Challenges identified in combination with methodology were related to temporal impacts, whereas methodology itself can be termed a "targeted influence". Similarly, sediment type is an impact inherent to assessment, although it should be addressed in the following concepts in order to properly interpret results obtained with either artificial or natural sediment.

The two relevant confounding impacts which were identified as greatly relevant, in particular time, seemingly result in a need for more tests and more results. However, as stated by Breitholtz (2006), even though more information makes it possible to make better decisions, information is not all that is needed. Eventually, simpler and more operational tools to assess contaminations are called for. Considerations of economic feasibility, temporal limitations and animal welfare indicate a reduction of tests and parameters rather than an increase (Hartung & Rovida 2009).

Hence, a long-term strategy was proposed (chapter 2):

a) Pilot-studies on one crucial factor, i.e. time-dependence, conducted in order to provide the necessary background data, such as the identification of time as severe impact on results of direct sediment contact tests conducted in the present thesis.

b) Integrative studies linking different focal points within assessment in order to combine and intercompare the obtained results on different organisational levels with regard to the addressed factor.

c) Transfer of pilot- and integrative results into predictive risk assessment models.

Modelling, in particular, represents a potential long-term solution to the contradiction between the need to address confounding impacts and, at the same time, limit necessary tests.

Integrative strategies were designed to combine and intercompare different levels and methodical approaches of assessment (Brack 2003, Chapman 1990). The triad approach is one common integrative strategy. It directly combines chemical analyses, *in vitro* biotests, and ecological assessments (Chapman 1990, 1996, 2000). Because new environmental and ecotoxicological methodologies have been designed since the original introduction of the triad, researchers suggested expanding the concept to include additional techniques (Chapman & Hollert 2006, Hamers et al. 2010). Accordingly, the triad could be expanded to account for temporal confounding by systematically testing two or three points of time. However, a strategy limited to integrative approaches would not address the problems of costs and animal usage that are associated with increasing demands for tests.

Hence, the third proposed step is to use data obtained in pilot studies and integrative assessment to design predictive mathematical models. Modelling is an excellent approach to address the complex confounding impact of time and reduce costs at the same time, because it aims to predict environmental impacts by applying mathematical models calibrated on the basis of empirical data (Jager et al. 2006, Miller et al. 2007, Murphy et al. 2008, Pastorok et al. 2003). In terms of the confounding impacts, the AQUATOX model, for instance, was specifically developed to integrate additional factors like temperature, flow rates, and multiple-substance exposures (Park et al. 2008).

Integrative approaches such as the triad can also account for additional factors not addressed in the present thesis, such as storage and test temperature (briefly addressed in combination with time-dependence in chapter 2), sampling procedures, freeze-drying (mentioned in chapter 6), pH (briefly discussed in chapter 7), pulsed exposure patterns, and chronic exposure (e.g. Bearr et al. 2006, Beiras et al. 1998, Besser et al. 2005, Burton 1991, Burton et al. 2000, Diamond et al. 2005, Kusch et al. 2008, Leweke 1999, Northcott &Jones 2000, Seiler et al. 2008, Simpson & Batley 2007).

Burton et al. (2000) pointed out the importance of including additional factors into assessment. However, it is also crucial that impacts are not addressed separately, but in parallel and with regard to interactions. Researchers have increasingly realised that the joint consideration of all information is crucial to eliminate confounding factors, as the co-occurrence of natural stressors that may mask the adverse effects of anthropogenic stressors (Liess & von der Ohe 2005, Schäfer et al. 2007). Eventually, tools are needed that address all of the previously listed confounding factors as well as different focal points (molecular, chemical, biological, ecological etc.).
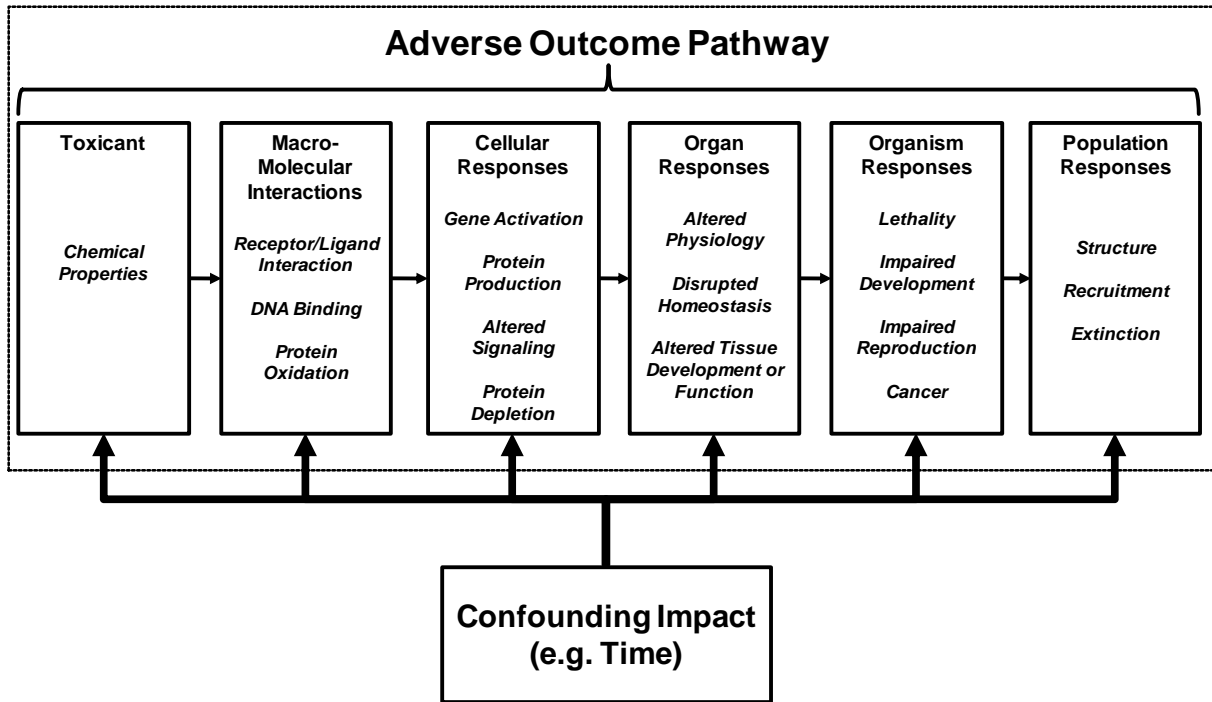
Fig. 8.1 Adverse outcome pathways can be applied to assess and link the impact of confounding factors on multiple organisational levels. Figure adapted from Ankley et al. (2010).

Adverse outcome pathways (AOPs) represent a very recent idea in terms of integrative approaches and a powerful tool to eventually understand and fully characterise confounding impacts. Introduced by Ankley et al. (2010), the concept takes all levels of biological organisation into account and tries to empirically understand the pathways a given compound "follows" through the individual levels (Ankley et al. 2010, Kramer et al. 2011). A fully-developed adverse outcome pathway is synonymous with a complete and detailed understanding of each and every step on every organisational level from molecule to ecosystem in the sequence of events leading to a toxic outcome (Ankley et al. 2010). AOPs represent a powerful and promising approach because they interlink the multiple levels from molecules to population effects that can be targeted by potential impacts (Ankley et al. 2010). AOPs also have great potential for developing and validating predictive models (Kramer et al. 2011).

The concept of AOPs could be adapted and applied to similarly "follow" a given confounding impact, in order to assess how responses on the different organisational levels are influenced (Fig. 8.1). For instance, uptake of metals was impacted by exposure to the individual metals

or the mixture (chapter 7), but transcript abundances of metallothionein genes were not changed. This information could be integrated into a metal-related AOP, and could, in combination with data obtained on other levels of the pathway, greatly increase the understanding of how this impact exerts its influence. Accordingly, integrating time-dependence on the level of observable results in direct sediment contact tests with time-dependence on the level of bioaccumulation and the level of molecular response in an AOP could help uncover new relationships, which in turn could allow predicting the reactions of individual combinations of substance and species.

However, such an application of AOPs to investigate confounding factors also represents an organisational effort. Strategies such as the previously proposed three-step approach, and even more complex multi-level concepts such as AOPs, automatically need to be evaluated with regard to realistic applicability. Interdisciplinary concentrated framework approaches will be needed to address all potential impacts at multiple organisational levels at the same time. Such efforts by scientific consortia aimed at confounding impacts should:

a) Firstly, gather data on sediment-associated pollutants, both by laboratory-based studies and field monitoring with regard to the addressed confounding impact. This should involve the levels of chemical fate, bioaccessibility, environmental bioavailability and toxicological bioavailability.

b) Secondly, collect these data in a comprehensive, publicly-available database. Such a comprehensive catalogue database that includes data on external factors in relation to species and sediment could greatly improve assessment and regulatory decision processes.

c) Thirdly, apply and validate integrative models in order to sustainably address the targeted impact.

d) Fourthly, utilise these models to sustainably assess and regulate sediment-associated environmental pollution with regard to the addressed impact.

The MODELKEY framework project is an excellent example for such a project framework. It aims to develop interlinked tools for an enhanced understanding of cause-effect-relationships between insufficient ecological status and environmental pollution as causative factor and for the assessment and forecasting of the risks of key pollutants on fresh water and marine ecosystems at a river basin and adjacent marine environment scale (Brack et al. 2005). Eventually, new modelling tools and corresponding user-friendly decision support systems for

environmental risk assessment will be developed (Brack et al. 2005). Results given in von der Ohe et al. (2009) lead the authors to conclude that integrated risk assessment such as MODELKEY may not only permit assigning an ecological status, but will also help to unravel potential confounding factors. So far, sediments have not been covered in the MODELKEY-related publications (Von der Ohe et al. 2009), but are explicitly accounted for in the project plan (Brack et al. 2005). Accordingly, the potential of contact time between sediments and chemicals to induce changes in the results of biotests that was identified in the present thesis should be either addressed in existing frameworks such as MODELKEY or in future frameworks.

Even though such comprehensive frameworks are time-intensive at first, they will eventually save time and money compared to independent research due to their long-term orientation. Hence, such concentrated approaches are greatly beneficial in terms of environmental protection as well as economic considerations. Interdisciplinary scientific consortia that fully cooperate and also involve industry and regulatory agencies should be preferred at the organisational level.

In summary, the paradox of increasing recognition and realisation of confounding factors while necessarily minimising test demands is the current and crucial challenge in environmental research and protection efforts. Integrative approaches are the appropriate and most promising strategy to address this challenge, but there is a clear need to broaden and improve these concepts in order to include and account for all potentially confounding factors, as well as to organise and systemise assessment. Projects like MODELKEY provide examples how such frameworks should be conceived. In the long term, results of such framework projects can provide the necessary information in order to adequately address confounding factors such as time and exposure type (mixtures vs. individual substances) in regulatory guidelines.

8.7   References

Ahlf W, Drost W, Heise S (2009): Incorporation of metal bioavailability into regulatory frameworks-metal exposure in water and sediment. J Soil Sediment 9, 411-419

Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung MW, Johnson RD, Mount DR, Nichols JW, Russom CL, Schmieder PK, Serrrano JA, Tietge JE, Villeneuve DL (2010): Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environ Toxicol Chem 29, 730-741

Bearr JS, Diamond J, Latimer H, Bowersox M (2006): Effects of pulsed copper exposures on early life-stage Pimephales promelas. Environ Toxicol Chem 25, 1376-1382

Beiras R, His E, Seaman MNL (1998): Effects of storage temperature and duration on toxicity of sediments assessed by Crassostrea gigas oyster embryo bioassay. Environ Toxicol Chem 17, 2100-2105

Besser JM, Wang N, Dwyer FJ, Mayer FL, Ingersoll CG (2005): Assessing contaminant sensitivity of endangered and threatened aquatic species: Part II. Chronic toxicity of copper and pentachlorophenol to two endangered species and two surrogate species. Arch Environ Con Tox 48, 155-165

Borgmann U, Norwood WP, Dixon DG (2008): Modelling bioaccumulation and toxicity of metal mixtures. Hum Ecol Risk Assess 14, 266-289

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 et al. (2005): MODELKEY - Models for assessing and forecasting the impact of environmental key pollutants on freshwater and marine ecosystems and biodiversity. Environ Sci Pollut R 12, 252-256

Breitholtz M, Eriksson J, Green N, Ruden C (2006): Testing and risk assessment of persistent and bioaccumulating chemical substances - Improvements within REACH? Hum Ecol Risk Assess 12, 782-805

Burton GA (1991): Assessing the toxicity of fresh-water sediments. Environ Toxicol Chem 10, 1585-1627

Burton GA, Pitt R, Clark S (2000): The role of traditional and novel toxicity test methods in assessing stormwater and sediment contamination. Crit Rev Env Sci Tec 30, 413-447

Chapman PM (1990): The sediment quality triad approach to determining pollution-induced degradation. Sci Total Environ 97-8, 815-825

Chapman PM (1996): Presentation and interpretation of sediment quality triad data. Ecotoxicology 5, 327-339

Chapman PM (2000): The Sediment Quality Triad: then, now and tomorrow. Int J Environ Pollut 13, 351-356

Chapman PM, Hollert H (2006): Should the sediment quality triad become a tetrad, a pentad, or possibly even a hexad? J Soil Sediment 6, 4-8

Chung N, Alexander M (1999): Effect of concentration on sequestration and bioavailability of two polycyclic aromatic hydrocarbons. Environ Sci Technol 33, 3605-3608

Cuypers C, Pancras T, Grotenhuis T, Rulkens W (2002): The estimation of PAH bioavailability in contaminated sediments using hydroxypropyl-ß-cyclodextrin and Triton X-100 extraction techniques. Chemosphere 46, 1235-1245

Dahlstrom M, Jonsson PR, Lausmaa J, Arnebrant T, Sjogren M, Holmberg K, Martensson LGE, Elwing H (2004): Impact of polymer surface affinity of novel antifouling agents. Biotechnol Bioeng 86, 1-8

Di Toro D, McGrath J, Hansen D, Berry W, Paquin P, Mathew R, Wu KB, Sanatore RC (2005): Predicting sediment toxicity using a sediment Biotic Ligand Model: Methodology and initial application. Environ Toxicol Chem 24, 2410-2427

Di Toro DM, Allen HE, Bergman HL, Meyer JS, Paquin PR, Sanatore RC (2001): Biotic Ligand Model of the Acute Toxicity of Metals. 1. Technical Basis. Environ Toxicol Chem 20, 2383-2396

Diamond J, Bowersox M, Latimer H, Barbour C, Bearr J, Butcher J (2005): Effects of pulsed contaminant exposures on early life stages of the fathead minnow. Arch Environ Con Tox 49, 511-519

Hamers T, Leonards PEG, Legler J, Vethaak AD, Schipper CA (2010): Toxicity Profiling: An Integrated Effect-Based Tool for Site-Specific Sediment Quality Assessment. Integrated Environmental Assessment and Management 6, 761-773

Hartung T, Rovida C (2009): Chemical regulators have overreached. Nature 460, 1080-1081

Hickman ZA, Reid BJ (2005): Towards a more appropriate water based extraction for the assessment of organic contaminant availability. Environ. Pollut. 138, 299-306

Jager T, Heugens EHW, Kooijman S (2006): Making sense of ecotoxicological test results: Towards application of process-based models. Ecotoxicology 15, 305-314

Komjarova I, Blust R (2009): Multimetal Interactions between Cd, Cull, Ni, Pb, and Zn Uptake from Water in the Zebrafish Danio rerio. Environ Sci Technol 43, 7225-7229

Koutsopoulos S, Patzsch K, Bosker WTE, Norde W (2007): Adsorption of trypsin on hydrophilic and hydrophobic surfaces. Langmuir 23, 2000-2006

Kramer VJ, Etterson MA, Hecker M, Murphy CA, Roesijadi G, Spade DJ, Spromberg JA, Wang M, Ankley GT (2011): Adverse outcome pathways and ecological risk assessment - bridging to population-level effects. Environ Toxicol Chem 30, 64-76

Kusch RC, Krone PH, Chiverst DP (2008): Chronic exposure to low concentrations of waterborne cadmium during embryonic and larval development results in the long-term hindrance of antipredator behavior in zebrafish. Environ Toxicol Chem 27, 705-710

Kwon JH, Wuethrich T, Mayer P, Escher BI (2009): Development of a dynamic delivery method for in vitro bioassays. Chemosphere 76, 83-90

Lammer E, Carr GJ, Wendler K, Rawlings JM, Belanger SE, Braunbeck T (2009a): Is the fish embryo toxicity test (FET) with the zebrafish (Danio rerio) a potential alternative for the fish acute toxicity test? Comp Biochem Phys C 149, 196-209

Lammer E, Kamp HG, Hisgen V, Koch M, Reinhard D, Salinas ER, Wendler K, Zok S, Braunbeck T (2009b): Development of a flow-through system for the fish embryo toxicity test (FET) with the zebrafish (Danio rerio). Toxicol in Vitro 23, 1436-1442

Leweke EC (1999): Einfluss verschiedener Lagerungsbedingungen auf die ökotoxikologische Wirkung von Kunst- und Süßwassersedimenten - Untersuchungen mit *Daphnia magna*, *Enchytraeus albidus*, *Chironomus riparius* und *Tubifex tubifex*. Cuvillier Verlag Göttingen, 194 pp

Liess M, von der Ohe PC (2005): Analyzing effects of pesticides on invertebrate communities in streams. Environ Toxicol Chem 24, 954-965

Miller DH, Jensen KM, Villeneuve DL, Kahl MD, Makynen EA, Durhan EJ, Ankley GT (2007): Linkage of biochemical responses to population-level effects: A case study with vitellogenin in the fathead minnow (Pimephales promelas). Environ Toxicol Chem 26, 521-527

Murphy CA, Rose KA, Alvarez MD, Fuiman LA (2008): Modeling larval fish behavior: Scaling the sublethal effects of methylmercury to population-relevant endpoints. Aquat Toxicol 86, 470-484

Northcott GL, Jones KC (2000): Developing a standard spiking procedure for the introduction of hydrophobic organic compounds into field-wet soil. Environ Toxicol Chem 19, 2409-2417

Palmgren JJ, Monkkonen J, Korjamo T, Hassinen A, Auriola S (2006): Drug adsorption to plastic containers and retention of drugs in cultured cells under in vitro conditions. Eur J Pharm Biopharm 64, 369-378

Park RA, Clough JS, Wellman MC (2008): AQUATOX: Modeling environmental fate and ecological effects in aquatic ecosystems. Ecol Model 213, 1-15

Pastorok RA, Akcakaya HR, Regan H, Ferson S, Bartell SM (2003): Role of ecological modeling in risk assessment. Hum Ecol Risk Assess 9, 939-972

Rodrigues SM, Henriques B, Coimbra J, da Silva EF, Pereira ME, Duarte AC (2010): Water-soluble fraction of mercury, arsenic and other potentially toxic elements in highly contaminated sediments and soils. Chemosphere 78, 1301-1312

Schäfer RB, Caquet T, Siimes K, Mueller R, Lagadic L, Liess M (2007): Effects of pesticides on community structure and ecosystem functions in agricultural streams of three biogeographical regions in Europe. Sci Total Environ 382, 272-285

Seiler TB, 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

Simpson SL, Batley GE (2007): Predicting metal toxicity in sediments: a critique of current approaches. Integr Environ Assess Manag 3, 18-31

Smith KEC, Dom N, Blust R, Mayer P (2010): Controlling and maintaining exposure of hydrophobic organic compounds in aquatic toxicity tests by passive dosing. Aquatic Toxicology 98, 15-24

Solimini AG, Ptacnik R, Cardoso AC (2009): Towards holistic assessment of the functioning of ecosystems under the Water Framework Directive. TRAC-Trend Anal Chem 28, 143-149

Tang JX, Alexander M (1999): Mild extractability and bioavailability of polycyclic aromatic hydrocarbons in soil. Environ Toxicol Chem 18, 2711-2714

Von der Ohe PC, De Deckere E, Pruess A, Munoz I, Wolfram G, Villagrasa M, Ginebreda A, Hein M, Brack W (2009): Toward an Integrated Assessment of the Ecological and Chemical Status of European River Basins. Integrated Environmental Assessment and Management 5, 50-61

Curriculum vitae and scientific contributions

Curriculum vitae

	Dipl.-Biol. Hanno Zielke
Date of Birth:	October 7th, 1980

Since 05/2011	Scientific employee/Coordinator of the Exploratory Teaches Spaces Project "FILM-Förderung Individueller Live-Medienkompetenz" at the Department of Ecosystem Analysis, Institute for Environmental Research, RWTH Aachen University
Since 05/2008	PhD student at the Department of Ecosystem Analysis, Institute for Environmental Research, RWTH Aachen University
05/2008-04/2011	Coordinator of the students lab „Fascinating Environment" at the Department of Ecosystem Analysis, Institute for Environmental Research, RWTH Aachen University
05/2008-04/2011	PhD-Scholarship holder of the German National Academic Foundation (Studienstiftung des Deutschen Volkes e.V.)
10/2007-04/2008	Scientific employee at RWTH Aachen
09/2007	Diploma in Biology (Diplom Biologie)
04/2004 – 09/2007	Scholarship holder of the German National Academic Foundation (Studienstiftung des Deutschen Volkes e.V.)
10/2001 – 09/2007	Studies of Biology at the Ruprecht-Karls-University Heidelberg,
07/2000-06/2001	Community service
06/2000	Abitur
1991-2000	Dietrich-Bonhoeffer-Gymnasiums in Hilden (Secondary School)
1987-1991	Gemeinschaftsgrundschule Elbsee in Hilden (Elementary School)

Scientific Contributions

Research articles in international peer-reviewed journals

Zielke, H., Seiler, T.-B., Niebergall, S., Leist, E., Streck, G., Brack, W., Braunbeck, T. and Hollert, H. (2011) The impact of extraction methodologies on the toxicity of sediments in the zebrafish (*Danio rerio*) embryo test (FET). J Soils Sediments 11:352–363

Research articles to be submitted to international peer-reviewed journals

Zielke, H., Adamzyk, C., Berens, M., Conrad, R., Gerringer, M., Oellers, J., Ottermanns, R., Rehage, N., Schneider, A.J., Simon, A., Spira D., Weigel, E., Ahlf, W., Feiler U., Seiler T.-B. and Hollert, H. (2011) A question of timing - Time-dependence of results in three sediment contact test systems using fish embryos, bacteria and nematodes. To be submitted to Aquatic Toxicology

Zielke, H., Adamzyk, C., Rehage, N., Seiler, T.-B. and Hollert, H. (2011) Impact of test vessel material on results obtained in the fish embryo test with zebrafish. Manuscript in preparation

Zielke, H., Redelstein, R., Spira, D., Feiler, U., Erdinger, L., Zimmer, H., Weisman, S., Hecker, M., Giesy J.P., Seiler, T.-B., and Hollert, H. (2011) Accumulation of metals from sediments and molecular responses of zebrafish embryos. To be submitted to Chemosphere.

Zielke, H., Seiler, T.-B. and Hollert, H. (2011) Time-dependence of results in direct sediment contact tests. To be submitted to Environmental Sciences Europe by invitation.

Simon, A., Zielke, H., Schmidt, B., Schäffer, A., Seiler, T.-B., Kukkonen, J.V.K. and Hollert, H. (2011) Impact of storage time on bioaccumulation of sediment-bound pollutants in the sediment contact test with *Lumbriculus variegatus*. Manuscript in preparation

Seiler, T.-B., Schulze, T., Streck, G., Schwab, K., Zielke, H., Brinkmann, M., Bernecker, C., Brack, W., Braunbeck, T. and Hollert, H. (2011) Passive membrane dialysis is a promising approach for the exhaustive extraction of PAHs from dried sediment samples. To be submitted to Environmental Science and Pollution Research

Invited presentations

Zielke, H. and Hollert, H. (2010) Sediment als Schadstoffsenke und Schadstoffquelle - Bedeutung für die Wasserrahmenrichtlinie. Hydrobiologial Colloquium, Technical University Dresden, 28.01.2010

Zielke, H., Seiler, T.-B., Niebergall, S., Streck, G., Spira, D., Leist, E., Zimmer, H., Erdinger, L., Brack, W., Feiler, U., Braunbeck, T. and Hollert, H. (2008) Vergleich verschiedener Methoden zur Sedimentextraktion im Fischeitest mit dem Zebrabärbling (*Danio rerio*). Plenary presentation best diploma thesis 2008, 3rd Annual Meeting SETAC-GLB/GDCh, Frankfurt, 23.- 26.09.2008

Platform presentations

Zielke, H., Adamzyk, C., Oellers, J., Schneider, A. J., Spira, D., Feiler, U., Seiler, T.-B. and Hollert, H. (2009) Einfluss von Alterung auf den Effekt sedimentgebundener Schadstoffe. PhD Colloquium of the German National Academic Foundation, Koppelsberg, 13.-16.11.2009

Zielke, H., Adamzyk, C., Rehage, N., Spira, D., Preuss, T. G., Schmidt, B., Feiler, U., Seiler, T.-B. and Hollert, H. (2009) Effects of ageing on bioavailability and uptake of pollutants from spiked sediments. Young Environmental Scientist Meeting, Landau, 16.3.-18.03.2009

Zielke, H., Adamzyk, C., Rehage, N., Spira, D., Preuss, T. G., Schmidt, B., Feiler, U., Seiler, T.-B. and Hollert, H. (2010) Alterung, Bioverfügbarkeit and Aufnahme sedimentassoziierter Schadstoffe im Sedimentkontakttest mit *Danio rerio* and in bioverfügbarkeitsabbildenden Extraktionsmethoden. PhD Colloquium of the German National Academic Foundation., Köln, 07.-10.12.2008

Zielke, H., Seiler, T.-B., Niebergall, S., Streck, G., Leist, E., Zimmer, H., Erdinger, L., Brack, W., Braunbeck, T. and Hollert, H. (2008) Comparison of different methods for sediment extraction using the fish egg assay with zebrafish (*Danio rerio*). 5. SETAC World Meeting, Sydney, Australia, 03.-07.08.2008

Simon, A., Zielke, H., Spira, D., Feiler, U., Schmidt, B., Seiler, T.-B., Schäffer, A., Kukkonen, J.V.K. and Hollert, H. (2010) Bioanalytische and radioaktive Untersuchungsmethoden zum Verteilungsverhalten von Monosubstanzen im Sediment-Wasser-Organismus-System. 4th Annual Meeting SETAC-GLB/GDCh, Dessau, 06.- 09.09.2010

Seiler, T.-B., Schulze, T., Streck G., Schwab, K., Zielke, H., Brinkmann, M., Bernecker, C., Brack, W., Braunbeck, T. and Hollert, H. (2008) Evaluation of the leaching power of passive membrane dialysis compared to conventional extraction procedures. 18th Annual Meeting SETAC Europe, Warsaw, Poland, 25.-29.05.2008

Poster proceedings

Conrad, R., Zielke, H., Spira, D., Feiler, U., Seiler, T.-B. and Hollert, H. (2011) Impact of ageing on bioaccessibility of pentachlorophenol in the bacterial contact assay with *Arthrobacter globiformis*. 21st Annual Meeting SETAC Europe, Milano, Italy, 15.-19.05.2011

Redelstein, R., Zielke, H., Spira, D., Feiler, U., Erdinger, L., Zimmer, H., Seiler, T.-B. and Hollert, H. (2011) Uptake of sediment-bound heavy metals and molecular response in zebrafish (*Danio rerio*) embryos. 21st Annual Meeting SETAC Europe, Milano, Italy, 15.-19.05.2011

Simon, A., Zielke, H., Schmidt, B., Seiler, T.-B., Schäffer, A., Kukkonen, J.V.K. and Hollert, H. (2011) Chemo- and bioanalyses of the partitioning of radiolabelled organic chemicals in sediment-water-organism-systems. 21st Annual Meeting SETAC Europe, Milano, Italy, 15.-19.05.2011

Redelstein, R., Zielke, H., Spira, D., Feiler, U., Erdinger, L., Zimmer, H., Seiler, T.-B. and Hollert, H. (2010) Uptake and effects of heavy metals from spiked sediments in zebrafish embryos. 37th Aquatic Toxicity Workshop, Toronto, Canada, 03.-06.10.2010

Conrad, R., Zielke, H., Spira, D., Feiler, U., Seiler, T.-B. and Hollert, H. (2010) Einfluss von Alterung auf die Biozugänglichkeit von Pentachlorphenol im Bakterienkontakttest mit Arthrobacter globiformis. 4th Annual Meeting SETAC-GLB/GDCh, Dessau, 06.-09.09.2010

Zielke, H., Adamzyk, C., Oellers, J., Schneider, A. J., Spira D., Feiler U., Seiler T.-B. and Hollert, H. (2010) Effects of ageing on the biological effectiveness of spiked sediments. 20th Annual Meeting SETAC Europe, Seville, Spain, 23.-27.05.2010

Adamzyk, C., Zielke, H., Gerringer, M., Spira, D., Feiler, U., Seiler, T.-B., Ahlf, H.-W. and Hollert, H. (2010) Challenges of the miniaturized Solid Contact Assay with Arthrobacter globiformis, 20th Annual Meeting SETAC Europe, Seville, Spain, 23.-27.05.2010

Simon, A., Zielke, H., Spira, D., Feiler, U., Schmidt, B., Seiler, T.-B., Schäffer, A., Kukkonen, J.V.K. and Hollert, H. (2010) Chemical and radioactive analyses of the partitioning of organic chemicals in sediment-water-organism-systems. 20th Annual Meeting SETAC Europe, Seville, Spain, 23.-27.05.2010

Kaiser, D., Zielke, H., Oetken, M., Hollert, H. and Oehlmann, J. (2010) Chemical UV-filters – A threat to aquatic wildlife?. 20th Annual Meeting SETAC Europe, Seville, Spain, 23.-27.05.2010

Zielke, H., Adamzyk, C., Rehage, N., Gerringer, M., Weigel, E,. Schneider, A. J., Spira D, Preuss, T.G., Schmidt, B., Feiler U., Seiler T.-B. and Hollert, H. (2009) Einfluss von Alterung auf die Bioverfügbarkeit und Aufnahme sedimentassoziierter Schadstoffe. 14th Annual Meeting SETAC-GLB, Weihenstephan, 05.-07.10.2009

Zielke, H., Brinkmann, M., Ottermanns, R., Preuss, T.G., Roß-Nickoll, M., Schmidt, B., Ratte, H.-T., Schäffer, A., Seiler, T.-B. and Hollert, H. (2009) Das Studentenlabor „Faszination Umwelt" – Universität and Industrie Hand in Hand für eine exzellente Ausbildung in der Ökotoxikologie. 14th Annual Meeting SETAC-GLB, Weihenstephan, 05.-07.10.2009

Adamzyk, C., Gerringer, M., Zielke, H., Spira, D., Feiler, U., Seiler, T.-B., Ahlf, H.-W. and Hollert, H. (2009) Der miniaturisierte Bakterienkontakttest mit Arthrobacter globiformis. 14th Annual Meeting SETAC-GLB, Weihenstephan, 05.-07.10.2009

Winkens, K., Otte, J.C., Brinkmann, M., Zielke, H., Wölz, J., Seiler, T.-B. and Hollert, H. (2009) Weiterentwicklung eines biologischen Testverfahrens zur Messung der Aktivität von Ah-Rezeptor-Agonisten in *Danio rerio* Fischeiern. 14th Annual Meeting SETAC-GLB, Weihenstephan, 05.-07.10.2009

Zielke, H., Adamzyk, C., Rehage, N., Spira, D., Preuss, T. G., Schmidt, B., Feiler, U., Seiler, T.-B. and Hollert, H. (2009) Effects of ageing on bioavailability and uptake of pollutants from spiked sediments. 19th Annual Meeting SETAC Europe, Göteborg, Sweden, 31.5.-04.06.2009

Zielke, H., Seiler, T.-B., Niebergall, S., Leist, E., Zimmer, H., Erdinger, L., Braunbeck, T. and Hollert, H. (2008) Comparison of different methods for sediment extraction using the fish egg assay with zebrafish (*Danio rerio*). 19th Annual Meeting SETAC Europe, Warsaw, Poland, 25.-29.05.2008

Mariani, C.; Pompêo, M., Rocha, P.; Zimmer, H.; Zielke, H., Wölz, J., Erdinger, L. and Hollert, H. (2008) Integrated assessment of sediments from Rio Grande Reservoir: Biotests preliminary results. 18th Annual Meeting SETAC Europe, Warsaw, Poland, 25.-29.05.2008

Seiler, T.-B., Strecker, R., Niebergall, S., Zielke, H., Lammer, E. Leist, E. Zimmer, H., Erdinger, L. Braunbeck, T. and Hollert, H. (2007) Der Sedimentkontakttest mit *Danio rerio* im SeKT-Verbundprojekt. 13th Annual Meeting SETAC-GLB, 12.-14.09.2007

Seiler., T.-B., Ricking, M., Rastall, A., Zielke, H., Kosmehl, T., Braunbeck, T. and Hollert, H. (2007): Expanded possibilities: Optimization and new applications for membrane dialysis extraction (MDE). 17th SETAC Europe Annual Meeting, Porto, Portugal, May 20-24

Seiler, T.-B., Niebergall, S., Zielke, H., Lammer, E., Leist, E., Zimmer, H., Erdinger, L., Braunbeck, T. and Hollert, H. (2007) Sediment contact assay with *Danio rerio* in the SeKT joint research project. 17th SETAC Europe Annual Meeting, Porto, Portugal, May 20.-24.05.2007

Seiler, T-B., Niebergall, S., Zielke, H., Lammer, E., Leist, E., Braunbeck, T,. and Hollert, H (2006) Der Embryotoxizitätsassay mit *Danio rerio* im SeKT-Verbundprojekt. 11th Annual Meeting SETAC-GLB, Landau, 03.-05.09.2006

Zielke, H. and Brandis, D. (2005): Untersuchungen zur Morphologie von *Ebalia tumefacta* (Leucosiidae, Brachyura). 12th Annual Crustaceologists Meeting, Wilhelmshafen, 17.-20.02.2005