Environ Sci Pollut Res (2015) 22:16290–16304
DOI 10.1007/s11356-015-5328-3

Bioaccumulation and molecular effects of sediment-bound metals in zebrafish embryos

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Received: 30 July 2014 / Accepted: 27 August 2015 / Published online: 10 September 2015

Abstract Predicting the bioavailability and effects of metals in sediments is of major concern in context with sediment risk assessment. This study aimed to investigate the bioavailability and molecular effects of metals spiked into riverine sediments to zebrafish (*Danio rerio*) embryos. Embryos were exposed to a natural and an artificial sediment spiked with cadmium (Cd), copper (Cu), nickel (Ni) and zinc (Zn) individually or as a mixture at concentrations ranging from 150 to 3000 mg/kg dry weight (dw) over 48 h, and uptake of metals was determined. Furthermore, transcript abundances of the metallothioneins MT1 and MT2, the metal-responsive element-binding transcription factor (MTF) and the genes *sod1*, *hsp70* and *hsp90α1* were measured as indicators of metal-induced or general cellular stress. *D. rerio* embryos accumulated metals from sediments at concentrations up to 100 times greater than those spiked to the sediment with the greatest bioaccumulation factor (BAF) for Cu from artificial sediment (275.4±41.9 (SD)). Embryos accumulated greater concentrations of all metals from artificial than from natural sediment, and accumulation was greater when embryos were exposed to individual metals than when they were exposed to the mixture. Exposure of embryos to Zn or the mixture exhibited up to 30-fold greater transcript abundances of MT1, MT2 and hsp70 compared to controls which is related to significant uptake of Zn from the sediment. Further changes in transcript abundances could not be related to a significant uptake of metals from sediments. These studies reveal that metals from

Responsible editor: Philippe Garrigues

Electronic supplementary material The online version of this article (doi:10.1007/s11356-015-5328-3) contains supplementary material, which is available to authorized users.

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spiked sediments are bioavailable to *D. rerio* embryos directly exposed to sediments and that the induction of specific genes can be used as biomarkers for the exposure of early life stages of zebrafish to metal-contaminated sediments.

Keywords Bioavailability · Metals · Zebrafish · Metallothioneins · Bioaccumulation · Sediment risk assessment

Introduction

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 water quality into the future (Breitholtz et al. 2006; Eklund et al. 2010; Hilscherova et al. 2010; Cousin and Cachot 2014). Specifically, there is concern about these sediments becoming a secondary source of pollution and the potential threat posed to infaunal organisms, their predators and other organisms living in close association with sediments. Because sediment-bound contaminants can be re-mobilized by bioturbation, flood events or dredging and relocation of sediments, contaminants can also affect organisms in the water column (Hollert et al. 2003; Förstner and Westrich 2005; Wölz et al. 2009; Hilscherova et al. 2010).

Tests in which organisms are directly exposed to sediments (sediment contact tests, SCTs) allow assessment of potential risks associated with contaminated sediments in an ecologically relevant manner (e.g. Ahlf and Förstner 2001; Duft et al. 2003; Feiler et al. 2013; Turesson et al. 2007; Blaha et al. 2010; Eklund et al. 2010; Höss et al. 2010; Schmitt et al. 2010; Hallare et al. 2011).

In the last decades, several groups developed and applied sediment contact tests with various fish species such as the zebrafish *Danio rerio* (Hollert et al. 2003; Kosmehl et al. 2006), the Japanese medaka *Oryzias latipes* (Barjhoux et al. 2012; Vicquelin et al. 2011; Liebl et al. 2008) or the marine species *Galaxias maculatus* (Barbee et al. 2014)

One test, the SCT with zebrafish (*D. rerio*) embryos, has been shown to be a useful tool for the evaluation of 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 (Nagel 2002; Braunbeck et al. 2005; Strähle et al. 2012; Vignet et al. 2014) and have been proven as a useful model organism to study the specific mechanisms of contaminants (Kosmehl et al. 2006). The aqueous version of the test is internationally standardized (ISO 2007), and a test guideline has recently been published by the Organisation for Economic Cooperation and Development (OECD 2013).

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. 2005, 2009, 2013; Höss et al. 2010). The test battery uses organisms at different trophic levels, which inhabit various microhabitats of freshwater sediments, including bacteria, fungi, nematodes, oligochaetes, higher plants and fish.

Metals are a ubiquitous class of pollutants that are persistent, can be toxic to organisms, and have been shown to affect populations and structures of communities (Dell'Anno et al. 2003; Boyd 2010). In sediments, metals are partitioned in various forms and speciations, which are dependent on the chemical and physical characteristics (Di Toro et al. 1990; Burton 1991). Bioavailability of metals from sediments is influenced by geochemistry, activities of organisms and contaminant/particle interactions (Dell'Anno et al. 2003; Ahlf et al. 2009). Thus, various exposure and uptake routes have to be considered for benthic organisms. These species can be exposed to metals through ingested particles and dissolved elements in pore water and/or overlaying water (Simpson and Batley 2006). Predicting the bioavailability of metals in sediments represents a critical step to enable assessment of potentially adverse effects of contaminants in sediments (Simpson and Batley 2006; Ahlf et al. 2009).

One objective of this study was to determine bioaccumulation of four individual transition metals spiked to sediments, namely, Cd, Cu, Ni and Zn, in zebrafish embryos to further our understanding of metal bioavailability from sediments. This life stage is of particular interest because early life stages of many species are often residing on or are closely associated with sediments. Since metals typically occur in mixtures (Borgmann et al. 2008; Komjarova and Blust 2009b; Rodrigues et al. 2010), effects of a mixture of all four metals on zebrafish embryos were also determined. Two different sediments representing artificial and natural sediments were investigated. The use of natural sediment is of greater ecological relevance while use of artificial sediment provides a stable reference and excludes several confounding factors such as background contamination.

In addition to the quantification of concentrations of metals that were taken up by embryos, abundances of messenger RNA (mRNA) for metallothioneins (MT1 and MT2) were explored as potentially sensitive biomarkers for the assessment of exposure of fish embryos to metals. Furthermore, expression of superoxide dismutase (sod1) and heat shock proteins (hsp70 and hsp90α1) was explored as potential indicators of cellular stress after metal exposure. CYP1A was applied as marker of exposure to dioxin-like compounds and glutathione S-transferase (GST) as general marker for biotransformation, to account for potential background effects,

e.g. residual contamination of the natural sediment with dioxin-like compounds (Whyte et al. 2000; Hollert et al. 2002).

Material and methods

Sediments

Natural sediments were collected in July 2008 and May 2009 at Altrip, a backwater of the river Rhine, (river km 416.9, Germany) by use of a Van Veen grab from a water depth of 5 m (cf. Höss et al. 2010). Sediments from this location had been previously established as a reference within the SeKT framework project due to their low residual concentrations of contaminants of concern such as Cd=0.04 mg/kg, Cu=58 mg/kg and total polycyclic aromatic hydrocarbons (ΣPAHs)=2.9 mg/kg (Feiler et al. 2005, 2009; Höss et al. 2010). Sediments were predominantly composed of silt (Table 1). In addition, an artificial sediment was prepared according to OECD guideline 218 (OECD 2004). Since during the SeKT project the clay content 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 (Feiler et al. 2009; Höss et al. 2010; Table 2). Sediment dry mass was determined according to OECD 218 by drying a defined amount of sediment for 14 h at 105 °C and measuring weight loss.

Sediment spiking

Sediments were spiked according to OECD Guidelines 207 (OECD 1984) and 218 (OECD 2004). Ten percent of sediment total wet weight was dried for 14 h at 105 °C. To each gram of dry sediment, 0.2 to 0.25 ml of metals solubilized in distilled water (ZnCl, $NiCl_2$, CdCl and $CuCl_2$, all Sigma-Aldrich Chemie GmbH) was applied and water was allowed to completely evaporate at room temperature for 3 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 conducted immediately after spiking, and sediments were not equilibrated for 5 to 7 days as recommended by the guidelines. This approach was chosen in order to determine uptake concentrations directly after a lab-simulated contamination event.

Fish maintenance and collection of embryos

Zebrafish were maintained according to the methods described by Braunbeck et al. (2005). For spawning, glass dishes were transferred into the tanks on evenings before experiments. Dishes were covered with a stainless steel grid with a mesh opening size of 1 mm through which the embryos 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. For testing, fertilized and normally developing embryos which were at least in the eight-cell stage were selected by use of a binocular microscope.

Fish embryo toxicity test and sediment contact assay with *D. rerio*

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 (NOECs) that would allow selection of appropriate concentrations for use in the definitive exposures. In the definitive studies, zebrafish were exposed to sediments spiked with sub-lethal concentrations of individual metals or a mixture of all four metals.

Tests with zebrafish embryos were conducted according to the methods described by the German regulation DIN 38415-6 (DIN 2001), Nagel (2002) and Lammer et al. (2009) with modifications for use in sediment assessments as described by

Table 1 Physical-chemical characteristics, particle size distribution, and background contamination with the test-relevant metals of the natural sediment (Altrip, Germany)

dw[a] (% ww)			pH[b]			TOC[b] (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 <2.0 μm
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

[a] Own data

[b] pH and TOC, as well as particle size distribution and background contamination were determined by BfG (Feiler et al. 2009)

Table 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 125–250 µm (% of dw)	Kaolinite clay <2.0 µm (% of dw)	Peat ≤0.5 mm (% of dw)	$CaCO_3$ (% of dw)	Water
90	5	5	0.05–1	30–50 %

dw dry weight, *ww* wet weight, *TOC* total organic carbon

Hollert et al. (2003). Artificial water (294.0 mg/l $CaCl_2 \cdot 2H_2O$, 123.3 mg/l $MgSO_4 \cdot 7H_2O$, 63.0 mg/l $NaHCO_3$ and 5.5 mg/l KCl, pH 7.8±0.2) prepared according to ISO 7346/3 (1996) was used as test medium.

All tests were conducted in six-well plates (Techno Plastic Products TPP, Zurich, Switzerland). For the range-finding tests with aqueous metal solutions, each well was filled with 5 ml of metal solution. Each metal was tested in five concentrations (Table 3). Gram atomic weight concentrations are presented to compare relative potencies.

For the sediment contact tests, wells were prepared with 3 g wet weight of test sediment and 5 ml of artificial water. All sediments were weighed into plates 1 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. Nominal concentrations (refers to spiked concentrations; background concentrations of metals in natural sediment are not included) of metals in sediments used in the range-finding tests are given in Table 3. Concentrations studied in the definitive studies were selected based on the results of these range-finding tests (Table 3). 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.

Selected embryos were transferred into wells containing the prepared solution or sediment, covered with self-adhesive foil and incubated for 48 h on a horizontal shaker at 50 rpm and 26±1 °C in the dark. For each concentration in the aqueous tests, 5 embryos were tested per well and two replicate wells were used per concentration (10 embryos per concentration). Positive controls (pc; 3.7 mg/L 3,4-dichloroaniline (DCA)) 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 were used per sample and control. In addition to process and solubilizer controls for both sediments, quartz sand negative controls (20 embryos, 3 g quartz sand F36+artificial water), quartz sand positive controls (10 embryos, 3.7 mg/l DCA freshly applied to the water phase+3 g quartz sand F36), aqueous negative controls

Table 3 Nominal metal concentrations (conc.) and gram atomic weight concentrations (GAW) for exposure of zebrafish embryos to aqueous solutions and spiked sediments in range-finding tests and the definitive studies (accumulation and molecular biomarker investigations)

	Aqueous range-finding test		Sediment range-finding test Natural sediment		Sediment range-finding test Artificial sediment		Definitive studies Natural sediment		Definitive studies Artificial sediment	
	Conc. (mg/L)	GAW (mol/L)	Conc. (g/kg dw)	GAW (mol/kg dw)	Conc. (g/kg dw)	GAW (mol/kg dw)	Conc. (g/kg dw)	GAW (mol/kg dw)	Conc. (g/kg dw)	GAW (mol/kg dw)
Cd	0.1	8.9E-7	0.2206	1.96E-3	0.1154	1E-3	2.94	2.6E-2	1.53	1.4E-2
	1	8.9E-6	2.206	1.96E-2	1.154	1E-2				
	10	8.9E-5	22.06	1.96E-1	11.54	1E-1				
	50	4.4E-4								
	100	8.9E-4								
Cu	0.01	1.6E-7	196E-6	3.1E-6	154E-6	2.4E-6	0.294	4.6E-3	0.153	2.4E-3
	0.1	1.6E-6	196E-5	3.1E-5	154E-5	2.4E-5				
	1	1.6E-5	196E-4	3.1E-4	154E-4	2.4E-4				
	5	7.9E-5	0.196	3.1E-3	0.154	2.4E-3				
	10	1.6E-4								
Ni	1	1.7E-5	–	–	–	–	2.94	5.0E-2	1.53	2.6E-2
	10	1.7E-4								
	100	1.7E-3								
	500	8.5E-3								
	1000	1.7E-2								
Zn	1	1.5E-5	0.2206	3.4E-3	0.1154	1.8E-3	2.94	4.5E-2	1.53	2.3E-2
	10	1.5E-4	2.206	3.4E-2	1.154	1.8E-2				
	100	1.5E-3	22.06	3.4E-1	11.54	1.8E-1				
	500	7.6E-3								
	1000	1.5E-2								

dw sediment dry weight

(40 embryos, artificial water only) and aqueous positive controls (20 embryos, 3.7 mg/l freshly applied DCA) were performed in each test.

In the definitive experiments, 10 embryos were transferred into each well, and 50 embryos were exposed to each concentration. Concentrations are given in Table 3. For quantification of metal uptake, 50 embryos were exposed to each concentration per test and every test was repeated three times, except for those from the mixture study where only one replicate could be conducted. For molecular analyses, 25 embryos were exposed to each concentration per test and each test was repeated four times.

Since it was shown previously that dissolved oxygen is a key factor for embryo development (Küster and Altenburger 2008; Strecker et al 2011), controls with unspiked sediments were conducted for each experiment in order to make sure that lack of oxygen during exposure was not the reason for embryo mortality.

Evaluation of range-finding tests

After 48 h of incubation, embryos were collected from the sediments, briefly rinsed in artificial water and evaluated for lethal and sub-lethal 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 (DIN 2001; Nagel 2002; Hollert et al. 2003; Braunbeck et al. 2005). This evaluation was only conducted in the range-finding tests. LC_{50} values were determined using sigmoidal dose-response curves with variable slope as regression model.

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 pore water and overlaying water volumes in one well.

Quantification of metals

For measurement of metal accumulation by embryos, all non-coagulated embryos were collected from sediments after 48 h of incubation, anaesthetized in a saturated solution of benzocaine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and stored at −20 °C in 2 ml of artificial water until analyses. The total amount of embryos plus water was analysed. After collection of embryos from sediments, the pH in the wells was measured by means of an insertion electrode (FiveGo™, Mettler Toledo, Schwerzenbach, Switzerland).

Samples were digested by means of an UV digestion device (UV 1000, Kürner Analysentechnik, Rosenheim, Germany). The total amount (2 ml) of each sample and 0.8 ml HNO_3 (65 %, Suprapur, Merck, Darmstadt, Germany) were placed in UV 1000 quartz glass tubes. After 1 and 4 h, respectively, 0.4 ml H_2O_2 (30 %, Suprapur, Merck) was added (Schramel 2003). Digested samples were made up to a final volume of 10 ml with distilled water.

Concentrations of metals were measured by use of inductively coupled plasma–mass spectrometry (ICP-MS) with an ELAN 6100 ICP-mass spectrometer (PerkinElmer SCIEX, Waltham, USA) (Schramel et al. 1999; Linge and Jarvis 2009). 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 were reported after subtracting background concentrations in blanks and controls.

Bioaccumulation factors (BAFs) were calculated (Eq. 1):

$$BAF = \frac{c[organism]}{c[sediment]} \quad (1)$$

where $c[organism]$ is the metal concentrations in fish embryos given in nanogram of metal per gram of fish embryo. Average wet weight of one fish embryo is 0.37 mg. $c[sediment]$ is the concentration of metal spiked into sediment given in milligram metal per kilogram of sediment wet weight.

Molecular analyses

After termination of the exposure experiments, embryos were stored at −80 °C in RNAlater (Qiagen GmbH, Hilden, Germany) before measuring the magnitude of expression of mRNA of selected genes.

Total RNA was extracted from embryos 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 visualized 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 complementary DNA (cDNA) synthesis was performed using the iScript™ cDNA Synthesis Kit (Bio-Rad). A volume of 2.5 μg total RNA was combined with 10 μl of 5× iScript® Reaction Mix and 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 and 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.

Primers were designed using Primer3® software (http://frodo.wi.mit.edu/primer3/version 0.4.0). Primer sequences and accession numbers are reported (Table 4). 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 (100 μM), 35 μl of 2× 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. The thermal cycling program included incubation at 50 °C for 2 min, an initial denaturing step at 95 °C for 10 min, followed by 40 cycles of denaturation (15 sec at 95 °C) and annealing (1 min at 60 °C). ROX was used as the 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 and 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.

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 treatments. Data was tested for normality distribution (Kolmogorv–Smirnov test) and homogeneity of variance (Levene test). The datasets that met both assumptions were analysed by use of parametric one-way ANOVA. As a post hoc test, the Holm–Sidak method was used for pair-wise comparison of the treatment groups. Datasets 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. Statistical significance was accepted when $p \leq 0.05$.

Results

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 %. The negative, quartz sand negative, sediment, process and solubilizer controls were regarded valid if the mortality did not exceed 10 % (DIN 2001; Nagel 2002). As further quality criterion, egg fertilization 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 controls except the positive controls. Mortality in the positive controls was greater than 10 %.

Aqueous and sediment range-finding tests

With the exception of Ni, exposure of *D. rerio* embryos to aqueous metal solutions resulted in concentration-dependent

Table 4 Nucleotide sequences of primers used for real-time PCR quantification of *Danio rerio* transcript abundance

Gene		Sequence (5′–3′)	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	

mortalities. 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 effects up to the greatest concentration, which was 1000 mg/l. In the natural sediment, LC_{50} values were 7.0 g Cd/kg dry weight (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. Cu caused no acute effects in the fish embryo sediment contact assay up to the greatest tested concentrations of 200 and 150 mg/kg dw in natural or artificial sediments, respectively. Ni was not tested in range-finding tests of sediments due to lack of effects in the aqueous tests. In addition, LC_{50} values from aqueous and sediment contact assays were expressed as gram atomic weight concentrations to compare relative potencies among metals (Table 5). In the aqueous assay, Cu was 20 times more potent than Cd and 200 times more potent than Zn. In sediment contact assays with both sediments, Cd and Zn were similar in potency.

Uptake and bioaccumulation

All pH values were within the optimal pH range of 7.8±1 for *D. rerio* embryos (Braunbeck et al. 2005) except for the mixture of metals spiked to artificial sediment (pH 6.47). The lesser pH in this sample coincided with a great number of coagulated embryos (20 out of 50) compared to mortality in the other treatment groups. Mean mortality in the uptake experiments was 3.5±8.2 %.

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 (Fig. 1). For Ni, embryos from both spiked sediments contained significantly greater concentrations compared to the aqueous negative control and the respective sediment control. Concentrations of Cd and Cu were significantly greater in embryos exposed to spiked artificial sediment compared to the spiked natural sediment.

Concentrations of all four metals were also greater in zebrafish embryos exposed to sediment spiked with the metal mixture than unspiked controls (Fig. 2; $n=1$). Concentrations of all four metals were greater in embryos exposed to spiked artificial sediment compared to those with spiked natural sediment. Although only one replicate was conducted for the mixture of metals, it is allusively recognizable that accumulation of metals from sediments spiked with a single metal was always greater than that from sediments spiked with the mixture of all four metals (Figs. 1 and 2).

The BAF of each metal was >10 in all samples (Fig. 3). Metals were accumulated to a greater extent from spiked artificial sediment than from spiked natural sediment. The least BAF was for Cd-spiked natural sediment (BAF=14), whereas the greatest BAF was for artificial sediment spiked with Cu (BAF=275). The BAF of embryos exposed to both sediment types spiked with Cu was significantly greater than that of the other metals. 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.

Transcriptional responses

There were statistically significant changes in the transcript abundances for four out of the eight genes measured after exposure to certain treatment groups (Fig. 4). The greatest change in transcript abundance was observed for the two metallothionein genes. The mixture of metals spiked to artificial sediment induced a 30-fold increase in expression of MT1 mRNA (Fig. 4a) and an 18-fold increase in expression of MT2 mRNA (Fig. 4b). Significant increases in expression of MT1 (17-fold) and MT2 (12-fold) mRNA were also 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. Transcript abundance of hsp70 (Fig. 4e) was significantly greater in embryos exposed to artificial sediment spiked with Zn or the mixture of metals than the control sediment. Transcript abundance of hsp90α1 mRNA (Fig. 4f) was significantly greater in natural

Table 5 Comparison of LC_{50} values determined in the aqueous and in the sediment contact assay (converted data) presented as spiked concentrations (conc.) and gram atomic weight concentrations (GAW)

LC_{50}	Aqueous fish embryo assay		Sediment contact assay			
			Natural sediment		Artificial sediment	
	Conc. (mg/l)	GAW (mol/l)	Conc. (mg/l)	GAW (mol/l)	Conc. (mg/l)	GAW (mol/l)
Cd	14.12	1.3E-4	1020	9.1E-3	1121	1.0E-2
Cu	0.43	6.8E-6	≥28.6	≥4.5E-4	≥47.3	≥7.4E-4
Ni	≥1000	≥1.7E-2	n.d.	–	n.d.	–
Zn	87.10	1.3E-3	579	8.9E-3	1121	1.7E-2

n.d. not determined

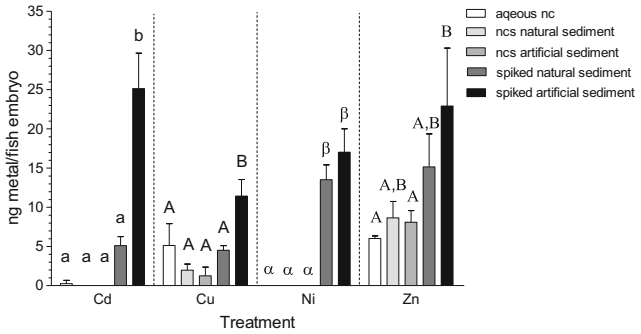


Fig. 1 Metal concentrations in zebrafish embryos (ng/embryo) exposed to metal-spiked natural and artificial sediments, unspiked sediment controls (ncs) and an aqueous negative control (nc) for 48 h. *Columns* represent means of three independent replicates (two independent replicates for Zn nc and Zn-spiked natural sediment) each containing ~50 embryos 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, $p \leq 0.05$)

sediment spiked with Cd (8.6-fold) or for Cu (7.6-fold). No statistically significant differences were observed for any of the other genes regardless of treatment.

Discussion

Metal toxicity in range finding tests

Results of the range-finding tests demonstrated that the four metals tested in this study have different toxic potencies in *D. rerio* embryos. LC_{50} value for Cd (14.1 mg/l) was comparable to those previously reported for zebrafish embryos. Hallare et al. (2005) and Canton and Slooff (1982) reported LC_{50} values of 30.1 and 4.2 mg/l, respectively. The LC_{50} of Cu (0.43 mg/l) was also within the range reported in literature (0.21 mg/l, 96-h test; Bellavere and Gorbi 1981). In contrast,

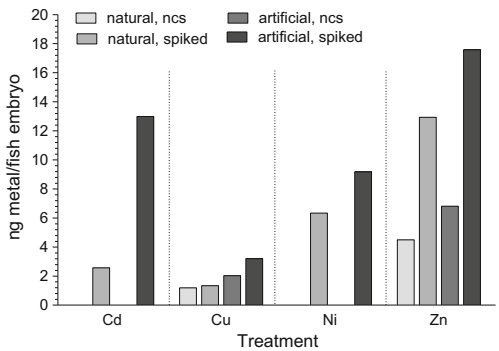


Fig. 2 Metal concentrations in zebrafish embryos (ng/embryo) exposed to natural and artificial sediments spiked with a mixture of four metals over 48 h compared to unspiked control sediments (ncs=negative control sediment); *columns* represent individual measurements of single composite samples containing 50 (natural sediment) and 30 (artificial sediment) measured embryos per treatment (one replicate)

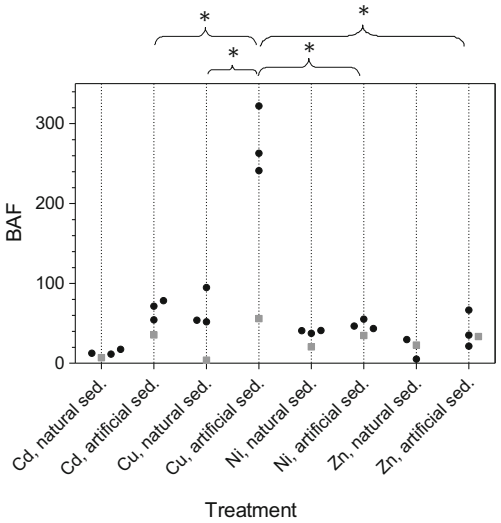


Fig. 3 Bioaccumulation factors (BAFs) of metals in fish embryos after exposure to spiked natural (Altrip, Germany) and artificial (OECD) sediment; *black dots* represent BAFs of the three independent replicates (each representing a composite sample containing approx. embryos) in the individual element tests; *grey squares* represent BAFs of fish embryos in the tests with sediments spiked with four metals simultaneously (individual measurements of single composite samples containing 50 (natural sediments) and 30 (artificial sediments) measured embryos); *asterisk* indicates statistically significant differences between groups of the individual element tests analysed with one-way ANOVA (Holm–Sidak method, $p \leq 0.05$)

the LC_{50} for Zn (87.1 mg/l) was approximately 45-fold greater than that reported by a different study (2.1 mg/l; Nguyen and Janssen 2001). However, Nguyen and Janssen (2001) performed a prolonged test, which likely led to greater sensitivity of embryos due to the 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 an aquo-ion $[Ni(H_2O)_6]^{2+}$. In this form, there is little accumulation into organisms (Köck 1996; Komjarova and Blust 2009a) which is supported by the lack of acute effects observed in this study.

Toxicity of metals spiked into the sediments was considerably less 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 (Table 5). 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). It should be noted, however, that characterization of precise LC_{50} values was not the primary aim of the present study. Since these values are based on only one replicate for each aqueous or sediment treatment, and because a wide range of concentrations was used, they are of limited precision.

Bioavailability and accumulation of metals

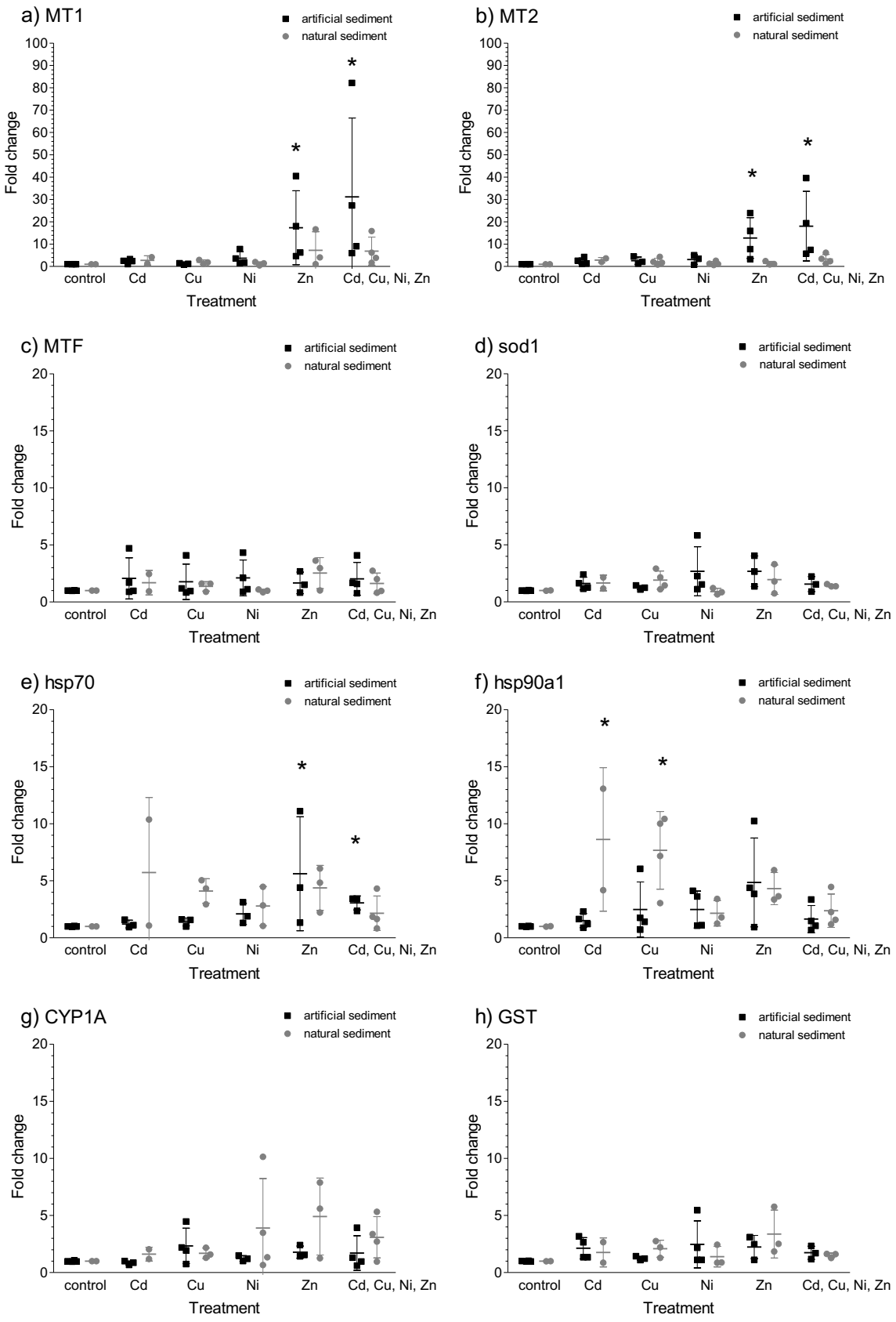
The greater concentrations of metals found in all fish embryos after exposure to spiked sediments compared to the aqueous negative control and the sediment controls demonstrate that zebrafish embryos accumulated metals (Fig. 1). Residual concentrations of Cd, Cu, Ni and Zn measured in the unspiked natural sediments as well as the variability of individual concentrations of Zn among replicates may be explained by background concentrations of these metals previously reported in these sediments (Feiler et al. 2009; Höss et al. 2010). Since the calculation of BAFs was based on concentrations spiked into the sediment (and not to concentrations measured in the sediment), it cannot be distinguished between uptake pathways to the embryos. Possible uptake pathways are (a) directly from the sediment, (b) via pore water and (c) via the overlaying water phase, and it must be assumed that the bulk of accumulated metals was derived from the overlaying water. However, this proportion of metals is still part of the concentration spiked to the sediment and, therefore, the calculated BAFs include metals accumulated from all three compartments.

Metals were more available for uptake from artificial sediment than from natural sediment (statistically significant for Cd and Cu) which is confirmed by BAFs (Fig. 3). This was possibly due to different geochemical compositions of the sediments such as organic carbon and clay/silt content. 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, there were more binding sites for metals in the natural sediment, and thus, metals were likely bound more rapidly and tightly to this sediment after spiking. As a consequence, it can be assumed that there were greater concentrations of metals in the water overlaying the artificial sediments, which would be consistent with greater bioavailability and accumulation of metals by embryos in these treatments (Eimers et al. 2002; Di Toro et al. 2005; Simpson and Batley 2006).

Although, in the present study, 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. However, there are parameters, such as redox potential, organic matter and biological activity, which are important factors influencing sequestration and bioavailability of contaminants, and that limit comparison between artificial and natural sediments (Verrhiest et al. 2002). Therefore, studies of accumulation with natural sediments can be more suitable for extrapolation to natural situations. Use of artificial sediment in addition to natural sediments can provide a stable reference to which natural samples can be compared. 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 accidental spills. This represented a worst-case scenario of maximal bioavailability of metals from sediments. Since shorter equilibration times result in greater concentrations of metals bound to the sediments and greater fluxes of metals from sediments to overlaying waters (Eimers et al. 2002; Simpson et al. 2004), it may be reasonably assumed that with increasing equilibration time of the sediments before initiation of exposure studies, uptake would be less.

Results for the bioaccumulation of a mixture of metals have to be interpreted with caution as they are based on only one replicate due to experimental constraints. However, the finding that accumulation of individual metals was less when embryos were exposed to the mixture of metals is consistent with previously reported findings that different metals can interact with each other affecting individual uptake, bioaccumulation and toxicity (Borgmann et al. 2008; Komjarova and Blust 2009b). Decreases in pH in samples spiked with all four metals could also have affected uptake and accumulation by embryos exposed to these sediments. The observed lesser pH was likely a consequence of hydrolysis of the added metals, including 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 (Simpson et al. 2004; Hutchins et al. 2007). It was previously hypothesized that lower pH results in greater concentrations of spiked metals in pore water, which would cause greater toxicity than metals associated with the solid phase (Di Toro et al. 2005; Simpson and Batley 2006) and thus leads to greater accumulation in embryos compared to those exposed to individual metals. Since this effect of increased accumulation was not observed in the present study, it is likely that accumulation of metals from sediments spiked with the mixture may have been limited by uptake and transport across the chorion. 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, and the total internal metal concentration was possibly sufficiently great to approximate equilibrium with the external concentration and inhibit further metal uptake.

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 not only account for more than one metal but also accordingly monitor pH


a) MT1
b) MT2
c) MTF
d) sod1
e) hsp70
f) hsp90a1
g) CYP1A
h) GST
artificial sediment
natural sediment
Fold change
Treatment
control
Cd
Cu
Ni
Zn
Cd, Cu, Ni, Zn

◀ **Fig. 4** 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* ($p \leq 0.05$)

changes, thus underlining the great relevance of multi-stressor-focused approaches in sediment assessment (Hollert et al. 2007; Hecky et al. 2010; Sundback et al. 2010).

Gene expression

Since chemical metal analyses were only conducted for the embryos and not for the spiked sediments, transcriptional responses are related to the proportion of accumulated metal in the embryos. Only for Zn, significant uptake from artificial sediment was accompanied by an induction of three of the assessed genes (MT1, MT2 and hsp70). Therefore, it is assumed that here effects on transcript abundances are caused by metal uptake. For all other metal treatments, there was no significant change in transcript abundances in combination with significant metal uptake from the spiked sediment. Changes in gene expression are in these cases (hsp90α1 for Cd and Cu from natural sediment) likely induced by interactions with other components in the sediments. Further, no significant changes in transcript abundances of any of the investigated genes could be found for the sediment controls (Fig. 4) which excludes possible effects of background concentrations in the natural sediment.

Metallothioneins are known to bind and thus detoxify metals (Klerks and Weis 1987). It has been shown that expression of metallothionein genes occurs during embryogenesis of zebrafish from one-cell stage onwards (Chen et al. 2004). Significantly greater expression of MT mRNAs in the early larval period of zebrafish (48 to 120 hpf) in response to exposure to a 100 μM $ZnCl_2$ has been previously reported (Chen et al. 2007). The significant increases of MT1 and MT2 transcript abundance after exposure to artificial sediment spiked with Zn in the present study confirm this finding. However, no significant changes in MT transcript abundance were observed in embryos exposed to natural sediment spiked with Zn. The observation that significant induction of MTs occurred in artificial but not natural sediments is consistent with lesser bioavailability and subsequent accumulation of metals from the natural sediment.

Metallothioneins are, among others, responsible for protection against Cu toxicity in many organisms (Roesijadi 1992) including the zebrafish (Craig et al. 2009a, b). However, expression of MT1 and MT2 mRNA was not greater in either of the Cu-spiked sediments (Fig. 4a, b). Even though the concentrations of Cu applied in the present study were determined based on NOECs from range-finding tests, the concentration spacing might have been too wide considering the sometimes steep concentration–response relationships for metals such as Cu. Therefore, the applied NOECs could have underestimated effective concentrations and thus been too low to induce MT transcript abundance.

Although Chen et al. (2007) reported induction of MT genes in *D. rerio* embryos by a 5-μM $CdCl_2$ solution, 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 after exposure to Cd is a "late-onset biomarker" because sufficient accumulation of Cd in the target tissue is necessary before upregulation of the MT gene occurs (Gonzalez et al. 2006). The exposure period of 48 h may not have been long enough to lead to sufficient accumulation of Cd at the active binding sites required for upregulation of MT genes. The lack of changes in MT gene expression after exposure to Ni is consistent with Ni exhibiting lesser toxicity than other metals (Köck 1996; Komjarova and Blust 2009a).

The binding of metal-responsive element-binding transcription factor-1 (MTF-1) plays an essential role in MT induction by metals, particularly Zn and Cd (Smirnova et al. 2000; Chen et al. 2007). However, in neither treatment, significantly increased induction of MTF transcript abundance could be observed (Fig. 4c). Also, Chen et al. (2007) found that MTF-1 expression is not induced by Zn or Cd although it is essential for normal embryonic development of zebrafish and expresses during zebrafish embryonic stages. These results are consistent with the observation that MTF-1 is a pre-existing cellular protein which is present in unstressed cells and is activated to bind to DNA by metal ions (Smirnova et al. 2000). MT gene expression is not dependent on de novo synthesis of MTF-1 (Smirnova et al. 2000); thus, the spiked metals may have activated DNA binding of MTF-1 without increasing MTF-1 transcript abundance.

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. Expression of the hsp gene is induced not only in response to heat shock but also after exposure to chemicals that exhibit proteotoxicity and particularly metals such as Cd (Blechinger et al. 2002; Krone et al. 2003; Gonzalez et al. 2006; Pierron et al. 2009). Results by Kosmehl et al. (2012) also indicate a link between the induction of Hsp70 expression

and exposure to metal-contaminated sediment. Hence, observed increases in transcript abundance of hsp70 and hsp90α1 in comparison to the controls were likely a function of metal exposure, since presence of metals was the only differing parameter between control and exposure treatments (Fig. 4e, f).

Hsp70 is involved in initial folding after protein biosynthesis and many other cellular processes related to protein stabilization, whereas hsp90α1 is involved in the stabilization of inactive protein states (Hartl 1996; Roberts et al. 2010). However, it is unknown how the mRNA levels of both hsps are impacted by different metals in zebrafish. Whereas hsp70 was induced after exposure to Zn and the mixture in artificial sediment in zebrafish embryos, Micovic et al. (2009) reported hsp70 to be induced by exposure to Cd-contaminated natural sediments in the teleost fish *Solea solea*. Short-term exposures to sub-lethal concentrations of Cu also induced hsp70 proteins in midge larvae (Karouna-Renier and Zehr 2003). In contrast, hsp90α1 was induced in zebrafish embryos after exposure to Cd and Cu in natural sediments in the present study although uptake of these metals was only significant from artificial sediment. This leads to the assumption that effects on hsp90α1 could have been induced by very small concentrations of Cd and Cu which were, although not significantly accumulated, present in the embryos at higher concentrations than in the controls. While it can be assumed that Cd, Cu and Zn exhibit different modes of action in zebrafish embryos and, thus, induce hsp70 and hsp90α1 differently, the pattern of hsp induction in relation to different metals, sediment types and species requires further investigation.

Although previous studies have reported significant effects on the expression of sod1 mRNA after exposure of perch to Cu and Zn (Pierron et al. 2009), sod1 was not confirmed as applicable biomarker for metal exposure of *D. rerio* embryos since there was no statistically significant change in transcript abundance after exposure to the four metals as single elements or in mixture.

The lack of transcript abundances of CYP1A and GST in the unspiked sediments exclude potential influences of residual contaminations with dioxin-like chemicals (e.g. PCBs, PAHs or dioxins/dibenzofurans) of the natural sediment.

The significant induction of MT1, MT2 and hsp70 after exposure to the mixture was likely caused by Zn, since Zn was the only metal that induced an increase of these three mRNA abundances after a single exposure. Therefore, increases in mRNA abundance of MTs and hsp70 after exposure to complex samples could be primarily caused by only a single metal, rather than being an integrative biomarker of all contaminants. Overall, it was shown that induction of MTs and hsp70 in zebrafish embryos can be indicative of Zn contamination.

Conclusion

The present study demonstrated that sediment-bound metals are bioavailable and accumulative to *D. rerio* embryos leading to the induction of several transcript abundances. Especially, mRNA abundances of MT1 and MT2 were shown to be applicable as biomarkers for exposure to sediment-bound Zn at sub-lethal concentrations. Furthermore, hsp70 mRNA abundance was also shown to be increased in response to Zn exposure of zebrafish embryos. More research on the induction of MT mRNA and further transcript abundances by metals is necessary to further establish the investigation of gene expression levels in zebrafish embryos as a biomarker for risk assessment of sediment contamination caused by exposure to metals.

Acknowledgments The RWTH Aachen University Undergraduate Research Opportunity Program (UROP) provided funding for a research stay in Saskatoon. Dr. Zielke was supported by the German National Academic Foundation. The authors wish to acknowledge the support of an instrumentation grant from the Canada Foundation for Infrastructure. Profs. Giesy and Hecker were supported by the Canada Research Chair program. In addition, Prof. Giesy was supported by an at large Chair Professorship at the Department of Biology and Chemistry and State Key Laboratory in Marine Pollution, City University of Hong Kong, The Einstein Professor Program of the Chinese Academy of Sciences and the Visiting Professor Program of King Saud University.

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Supplemental material

Paper # ESPR-D-14-02375

Redelstein et al. "Bio-accumulation and molecular effects of sediment-bound metals in zebrafish embryos"

Toxicological data (Results of range-finding tests)

Fish embryo toxicity test

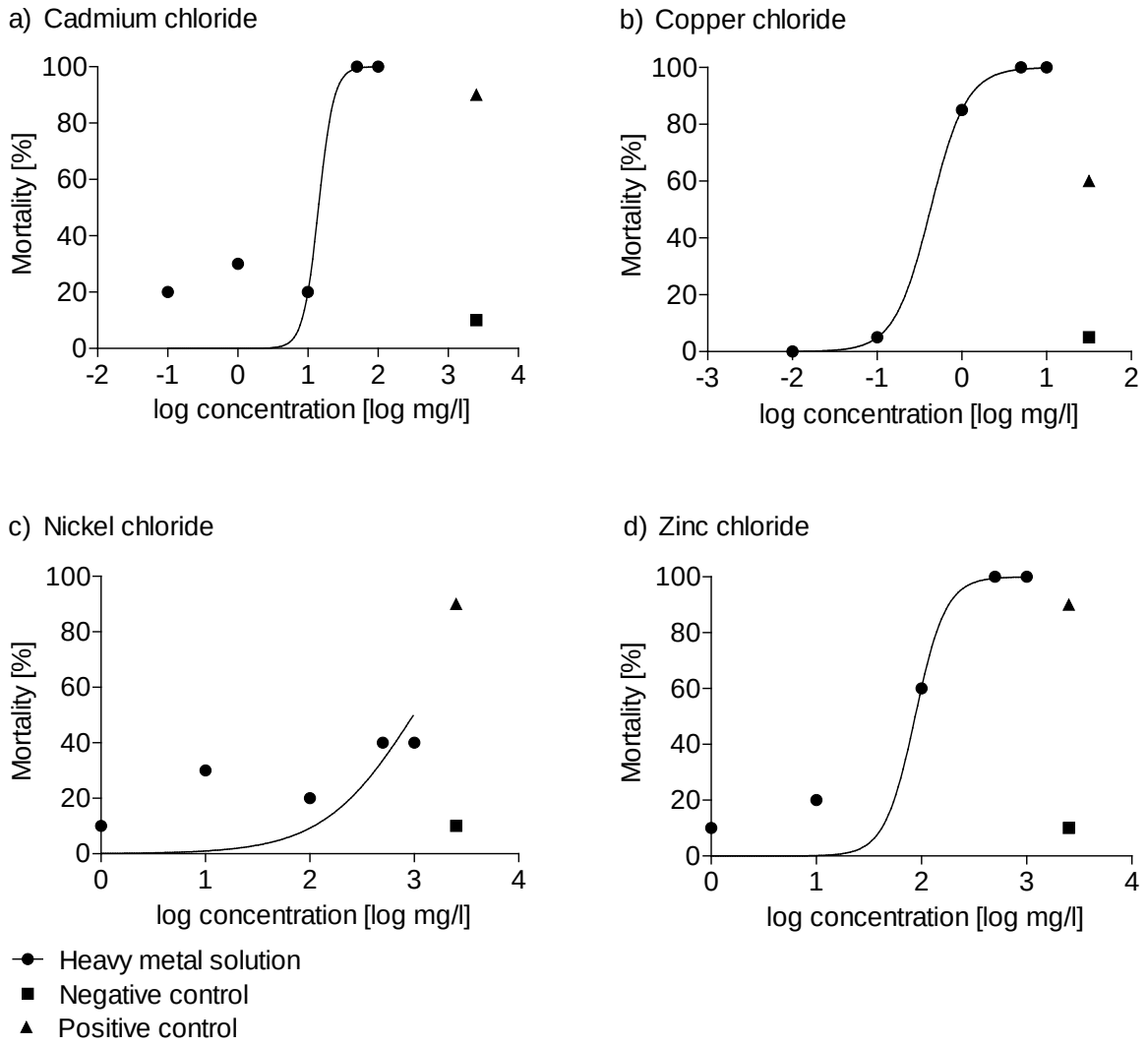

Fig. SI.1: Mortalities in the fish embryo toxicity assay with *Danio rerio* after 48 h of exposure to solutions of different heavy metal chlorides (n = 1). Applied concentrations of heavy metals:

a) 100, 50, 10, 1, 0.1 mg/l CdCl
b) 10, 5, 1, 0.1, 0.01 mg/l CuCl
c) 1000, 500, 100, 10, 1 mg/l NiCl
d) 1000, 500, 100, 10, 1 mg/l ZnCl

Sediment contact assay

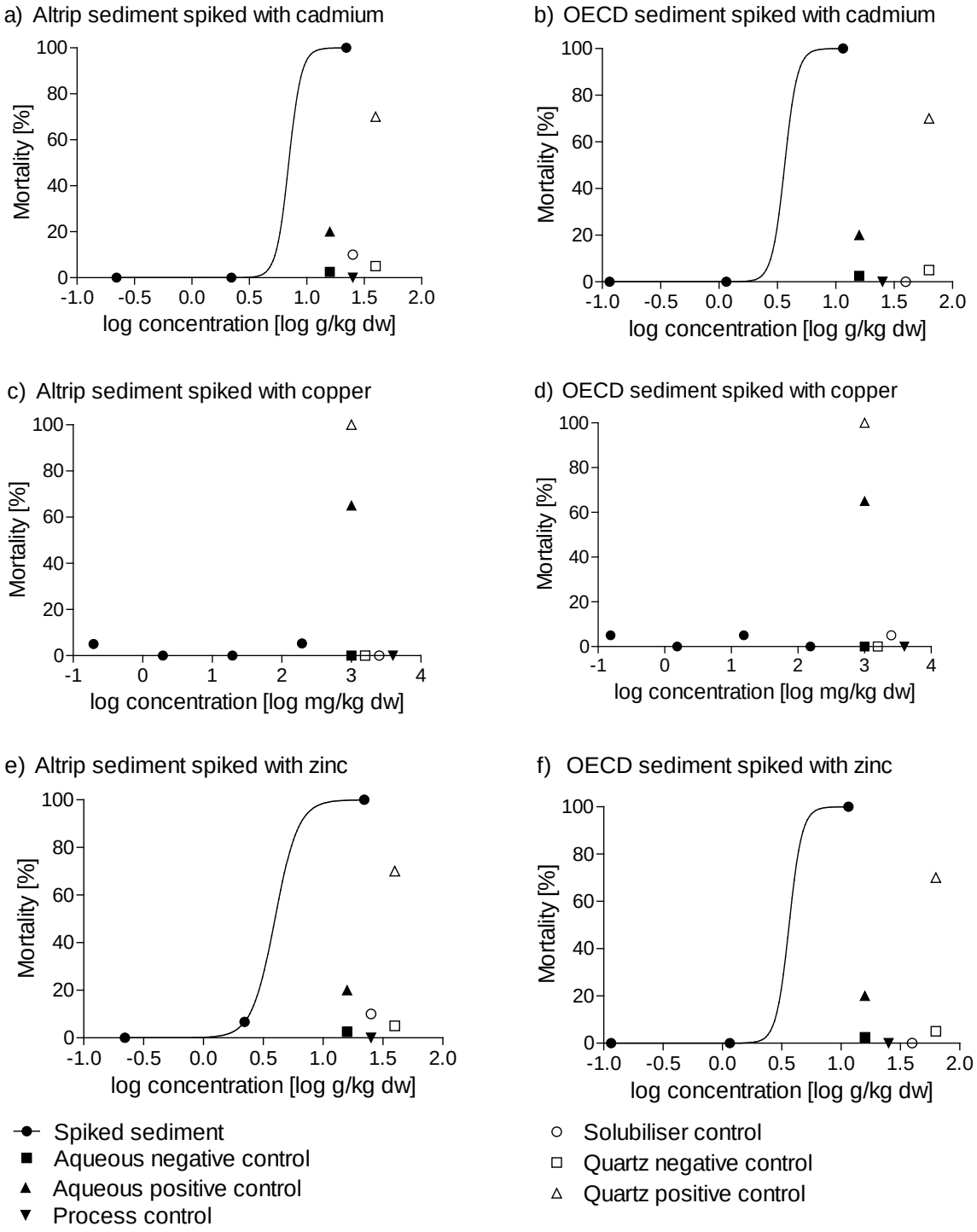

Fig. SI.2: Mortalities in the fish embryo assay with *Danio rerio* after 48 h of exposure to sediments spiked with heavy metals (n = 1); dw = dry weight. Applied concentrations:
a) Altrip sediment (22.06 g Cd/kg dw; 2.206 g Cd/kg dw; 0.2206 g Cd/kg dw)
b) OECD sediment (11.54 g Cd/kg dw; 1.154 g Cd/kg dw; 0.1154 g Cd/kg dw)
c) Altrip sediment (196 mg Cu/kg dw; 19.6 mg Cu/kg dw; 1.96 mg Cu/kg dw; 0.196 mg Cu/kg dw)
d) OECD sediment (154 mg Cu/kg dw; 15.4 mg Cu/kg dw; 1.54 mg Cu/kg dw; 0.154 mg Cu/kg dw)
e) Altrip sediment (22.06 g Zn/kg dw; 2.206 g Zn/kg dw; 0.2206 g Zn/kg dw)
f) OECD sediment (11.54 g Zn/kg dw; 1.154 g Zn/kg dw; 0.1154 g Zn/kg dw)

pH values

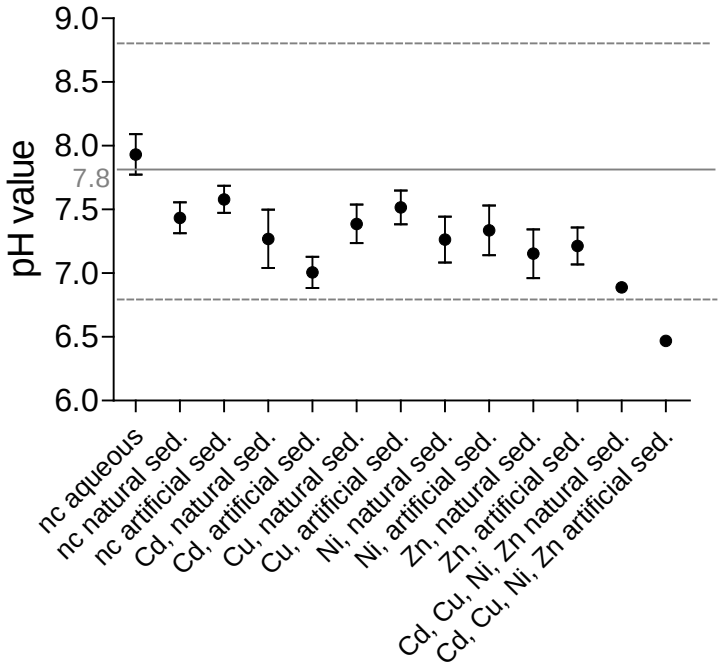

Fig. SI.3: pH values measured in artificial (OECD) and natural (Altrip) sediments after 16 h of equilibration plus 48 h of zebrafish embryo exposure; dots represent means of triplicate pH measurements, bars the standard deviation (n = 3); n = 1 for sediments spiked with all four metals simultaneously; the continuous line indicates the optimal pH value of 7.8 for *Danio rerio* embryos; dotted lines: optimal pH range of 7.8 ± 1; nc = negative control.