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Metallothionein modulation in relation to cadmium bioaccumulation and age-dependent sensitivity of *Chironomus riparius* larvae

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Abstract The goal of this study was to contribute to understanding of the mechanisms behind sensitivity differences between early and late instar larvae of *Chironomus riparius* and to address the influence of the differences in standard testing approaches on the toxicity evaluation. A 10-day contact sediment toxicity test was carried out to assess sensitivity to cadmium exposure in relation to different age and laboratory culture line origin of test organisms. Chironomid larvae of early (OECD 218 method) and late instar (US-EPA600/R-99/064 method) differed substantially in sensitivity of traditional endpoints (OECD: LOEC 50 and 10 µg Cd/g dry weight (dw); US-EPA: LOEC > 1000 and 100 µg Cd/g dw for survival and growth, respectively). Bioaccumulated cadmium and metallothioneins (MTs) concentrations were analyzed to investigate the role of MTs in reduced sensitivity to cadmium in late instar larvae. Metallothioneins were induced after treatment to greater Cd concentrations, but their levels in relation to cadmium body burdens did not fully explain low sensitivity of late instars to cadmium, which indicates some other effective way of detoxification in late instars. This study brings new information related to the role of MTs in age-dependent toxicant sensitivity and discusses the implications of divergence in data generated by chironomid sediment toxicity tests by standardized methods using different instars.

Keywords *Chironomus* · Artificial sediment · Cadmium · Bioaccumulation · Metallothionein

Introduction

Frequent occurrence of anthropogenic pollution in freshwater environments emphasizes the urgent need for simple and reliable methods of bottom sediment toxicity testing. Sediments can serve as a sink for contaminants from the overlaying water on one hand, and as a potential source of secondary contamination from sediment particles re-suspended to the water column on the other (Harrahy and Clements 1997). The complex nature of bottom sediments presents a great challenge addressed by researchers trying to describe and explain the behavior of contaminants in this heterogeneous matrix (Hilscherova et al. 2010).

Chironomus species are commonly used as a model organism in sediment toxicity tests, in which sediment dwelling larvae are exposed to toxicants. Chironomids were selected as a model species due to many advantages for laboratory bioassays such as easy laboratory culture, tolerance to a wide range of physico-chemical characteristics, relatively short generation time and red color of larvae, which makes them easily visible. Chironomid larvae, dwelling in the sediment and feeding on its fine particles, may also serve as a good model organism for bioaccumulation experiments (Desrosiers et al. 2008). Both subchronic (10-day) and chronic (life-cycle) bioassays were standardized by organizations such as OECD (OECD 2004) and US-EPA (US-EPA 2000). However, these test guidelines deviate in certain details of the test procedures, leading to problems in comparing and

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interpreting toxicity data (Péry et al. 2003). The OECD guideline 218 works with more sensitive newly hatched larvae corresponding to the first instar, while US-EPA method is based on less sensitive 10-day-old larvae corresponding to the third or fourth instar. The difference in sensitivity between instars should always be carefully considered when interpreting the chironomid toxicity data from tests carried out according to different standardized guidelines. Moreover, the OECD and US EPA guidelines work with 20 and 12 individuals per test vessel respectively, which may lead to density-dependent effects or different statistical power in testing for significant effects (Ristola et al. 1999).

Variability in the results of sediment toxicity testing may be also associated with using various chironomid species (*C. riparius*, *C dilutus*, *C. yoshiatsui*, or *C. xanthus*) and various laboratory strains. Intraspecies variability between distinct laboratory strains of other well established model test organisms was addressed by several studies (Abd-El-Monem et al. 1998; Varó et al. 1998; Cotman et al. 2007; Toumi et al. 2013), e.g. the differences in sensitivity of distinct laboratory strains of *Daphnia* reached more than an order of magnitude, depending on the endpoint and toxicant (Oda et al. 2007; Toumi et al. 2015).

Cadmium ions have been widely used as a model toxicant since cadmium is one of the most toxic metals, affecting aquatic life even at very low concentrations (Dallinger 1995). The naturally occurring levels of cadmium are severely increased in areas impacted by coal combustion, mine wastes, electroplating processes, iron and steel production, production of pigments, fertilizers, pesticides and zinc refining (Eaton et al. 2001). Cadmium present in sediment accumulates in the benthic biota and consequently may reach the consumers at the higher levels of the food chain (Oskarsson et al. 2004). Exposure to cadmium was reported to cause adverse effects by means of oxidative stress, membrane disruption and chromosomal aberrations in freshwater invertebrates (Hodl et al. 2010).

Apart from the traditional endpoints such as lethality or growth, various biochemical markers have also been investigated as early warning signs of toxicity in bioassays. Metallothioneins (MTs) are low molecular metal-binding proteins, which play an important role in intracellular regulation of certain metals and their detoxification (Adam et al. 2010; Ryvolova et al. 2011). These cysteine-rich proteins allow adaptation of benthic organisms to tolerate these metals naturally occurring in higher concentrations in bed sediments. Toxic metals associated with MTs or metal-rich granules (MRGs) are removed from the toxic pool and do not cause adverse effects (Wallace et al. 2003). The induction of MTs has been used as a biomarker of metal exposure in numerous organisms including gastropods, bivalves, oligochaetes, echinoderms, and fish (Amiard et al. 2006). Isolation, separation, detection, and/or quantification of MTs are a great challenge for modern analytical chemistry. Because of their low molecular mass and unique primary structure, common methods for detection of proteins fail due to insufficient specificity and sensitivity. The methods most frequently used for detection of these proteins are indirect and based on quantification of heavy metal ions occurring in their structure or on high content of sulfhydryl groups. However, these methods are influenced by various affinities of MTs to particular metal ions. Electrochemical methods like differential pulse voltammetry (DPV) with Brdicka reaction belong to the most sensitive ones for determination of MTs and their results are comparable with immune-based methods (Krizkova et al. 2009).

The purpose of the present research is to contribute to understanding of the mechanisms behind sensitivity differences between early and late instar larvae of *Chironomus riparius*, which are model organisms for routine contact sediment toxicity testing according to OECD (218) and US EPA (600/R-99/064) guidelines, respectively. We have studied cadmium toxicity, cadmium bioaccumulation and MTs modulation in chironomid larvae exposed to spiked artificial sediment, so as to investigate the role of MTs in relation to bioaccumulated cadmium concentration in early and late larval instars. This study provides a closer look at differences in responses of chironomid-based bioassays with respect to larval age and original culture line, which maybe a significant source of intraspecific variability.

The specific objective of this paper was the comparison of the effects of cadmium in artificial sediment on classic endpoints, bioaccumulation and MTs modulation between (i) early and late instar larvae and between (ii) larvae originating from two distinct culture lines, in order to identify possible differences explaining distinct sensitivity.

Materials and methods

Test conditions

Toxicity of cadmium was tested in a sediment-water system using artificial sediment and standard water. Artificial sediment was formulated according to OECD guideline 218: 5 % dried peat—Agro CS, Czech Republic; 20 % kaolin clay—Sigma-Aldrich 18672; 75 % silica sand 0.05–0.2 mm—Filtrační písky s.r.o., Chlum, Hornbach, Czech Republic, and 0.3 % $CaCO_3$ were mixed with distilled water (equivalent to 30 % of dry weight of sediment). 52 g of wet weight was added to each 400-mL glass test vessel. Cadmium chloride ($CdCl_2 . 2.5 H_2O$) was dissolved in distilled water to prepare stock solutions to reach the nominal concentrations of 1, 10, 25, 50, 100, and 1000 µg Cd/g dry weight of sediment. Artificial sediment was spiked with 1 mL of stock solution of cadmium individually in each test vessel and thoroughly homogenized. Clean artificial sediment was used as

the control treatment. Artificial sediment was then overlaid up to 200 mL with test media—Dutch Standard Water consisting of 100 mg NaHCO₃, 20 mg KHCO₃, 150 mg CaCl₂, 176 mg MgSO₄. 7H₂O per 1 L. Test vessels were placed in an air-conditioned laboratory (22 ± 2 °C) with 16:8 h photoperiod and provided with a smooth continuous aeration (aquarium aeration pumps—TetraWerke, Melle, Germany). The test system was allowed to condition for 20 h before introduction of test organisms. Each concentration treatment was tested in five replicates.

Test organisms

Chironomid egg masses were taken from a laboratory culture line maintained under the same conditions as the test. Three distinct experiments with larvae of distinct age and culture line origin were performed to test for variability between them (for experimental overview see Table 1). Specific design of each experiment (Test 1–3) is described below. In all tests, twenty larvae were introduced under the water surface into each test vessel. The aeration system was switched off for 24 h to let the larvae settle onto the substrate. One mL of feed suspension (1 g of ground commercial fish feed Tetramin [Sera GmbH, Heinsberg, Germany] in 100 mL distilled water), corresponding to 0.5 mg Tetramin per larva and day, was added daily to each test vessel. Water quality (temperature, pH, conductivity, and dissolved oxygen) was assessed at the start and the end of each test.

Test design

Test 1

Twenty newly hatched larvae (1 day old, collected from a compact egg rope—corresponding to the 1st instar) originating from the first laboratory culture line were introduced to the test system on day 0. Laboratory culture line 1 was obtained from Institute of Ecology, Evolution and Diversity, J.W. Goethe-University, Frankfurt am Main, Germany in 2005. On day 10, the test was terminated and surviving 10-day-old

larvae (corresponding to the 3rd–4th instar) were extracted from the sediment with forceps and placed individually into the wells of 96-well plate with clean test medium and allowed to depurate for 24 h (depuration period). The larvae were then killed and fixed in 70 % ethanol and their body length was measured (binocular with built-in camera—Zeiss Stemi 2000-C and QuickPHOTO MICRO image processing software, Promicra, Czech Republic). Larval biomass was then freeze-dried for dry weight and cadmium content measurements. In Test 1, we applied nominal concentrations of cadmium 0, 1, 10, 100, and 1000 µg Cd/g dw sediment and assessed larvae survival, body length, larval dry weight along with tissue and sediment cadmium contents. For the determination of cadmium concentration in larvae, all larval tissue from five replicates had to be pooled to a single sample per concentration treatment to obtain sufficient biomass for analysis.

Test 2

The design of Test 2 was identical to Test 1 except for the origin of the test organisms and concentration range tested. The chironomid culture line 2 was obtained from the Department of Animal Physiological Ecology, University in Tübingen, Germany in 2010. In Test 2, nominal exposure concentrations of cadmium were 0, 1, 10, 25, 50, and 100 µg Cd /g dw sediment and assessment included survival, body length, larval dry weight, content of MTs and cadmium in tissue and sediment at the end of exposure. Lower cadmium concentrations were included in Test 2, based on the results of Test 1, in order to better describe dose-response relationships. The biomass of larvae from each replicate was split in two halves, where one half served for cadmium analysis and the other for the determination of MTs. The larvae designated for MTs analysis were deep-frozen (−80 °C) immediately after their extraction from the sediment.

Test 3

The test was initiated with 10-day-old larvae obtained from preincubation in clean silica sand. This preincubation was

Table 1 Summary of experimental design and studied endpoints (Tests 1–3)

TEST 1	TEST 2	TEST 3
Early instar—line 1	Early instar—line 2	Late instar—line 1
Cd (µg /g dw):	Cd (µg /g dw):	Cd (µg /g dw):
0, 1, 10, 100, and 1000	0, 1, 10, 25, 50, and 100	0, 1, 10, 100, and 1000
Survival	Survival	Survival
Length	Length	Length
Weight	Weight	Weight
Cd conc. tissue/sediment	Cd conc. tissue/sediment	Cd conc. tissue/sediment
	MTs	MTs

started with newly hatched larvae (1 day-old, collected from a compact egg rope—corresponding to 1st instar), originating from laboratory culture line 1. Larvae were put into vessels with clean silica sand overlaid with Dutch Standard Water on day 0 and allowed to grow for 10 days (fed daily by 0.5 mg Tetramin per larva and day). On day 10, they were gently extracted from the sand with a plastic dropper and 20 individuals of these 10-day-old larvae (corresponding to 3rd–4th instar) were transferred into each exposure replicate with contaminated artificial sediment (0, 1, 10, 100, and 1000 μg Cd/g dw). The larvae were exposed to cadmium in sediment for the following 10 days. The test was finished on day 20 and the 20-day-old surviving larvae (corresponding to the 4th instar) were counted. Survival, body length and dry weight of larvae were determined along with tissue and sediment cadmium concentrations in Test 3 similarly to Test 1. The biomass of larvae from each replicate was split in two halves, where one half served for cadmium analysis and the other for the determination of MTs. The larvae designated for MTs analysis were frozen such as in Test 2.

Analyses of cadmium

Cadmium was extracted from sediments by leaching with aqua-regia according to ISO 11466 adapted to our analytical instrumentation. The mixture of 1 g of sediment sample with 2.3 mL of nitric acid (p.a., Merck, Germany) and 7 mL of hydrochloric acid (p.a., Merck, Germany) was left to react overnight under room temperature followed by 2 h heating under reflux. After cooling, the mixture was transferred to 200 mL volumetric flask and filled up with deionized water (18.2 MΩ.cm^{-1}, Simplicity 185, Millipore, USA). The analysis of all constituents of artificial sediment and food was performed to identify the cadmium source in control treatments.

Individual larvae from each replicate were pooled and their biomass was freeze-dried and then mineralized by mixture of concentrated nitric acid and hydrogen peroxide in microwave digestion system MWS3+ (Berghof, Germany). The mixture was then heated in a closed vessel microwave system under the temperature of 190 °C (1450 W) for 15 min. After mineralization, the solution was transferred to 50 mL volumetric flask and filled up with deionized water.

The final analyses of both mineralizates were performed using 7500ce ICP-MS (Agilent Technologies, Japan). ICP operates at 1500 W and with a flow of 0.4 and 0.125 mL.min^{-1} for sample and internal standard, respectively. Calibration curve (isotope ^{111}Cd) with internal standard correction (^{115}In) was used for calculation of concentration of cadmium in solution. Estimated limits of detection were established by means of three standard deviations of cadmium concentration in analytical blanks and were 0.004 mg.kg^{-1} for sediment samples and 0.005–0.05 mg.kg^{-1} (mass dependent) for larvae samples.

Determination of metallothioneins (MTs)

MTs content was determined in deep-frozen larvae biomass according to Fabrik et al. (2008). The biomass was homogenized by liquid nitrogen, diluted in 1 mL phosphate buffer (pH 6.8), further homogenized using Schütt-Homogen^{plus} (Schütt-biotec, Germany), ultrasound Sonoplus, 5 min, 200 W (Bandeline Electronic, Germany) and vortex, 30 min, 1000 rpm (Vortex-2 Genie, Scientific industries, USA). The samples were denatured in a thermoblock at 99 °C for 15 min (Eppendorf 5430, USA), cooled and centrifuged at 4 °C and 16,400 rpm for 20 min (Eppendorf 5402, USA). Supernatant was analyzed by differential pulse voltammetry (DPV) Brdicka reaction. DPV measurements were performed with 747 VA Stand instrument connected to 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and cooled sample holder (4 °C). A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3 M KCl electrode was the reference and glass carbon electrode was the auxiliary. GPES 4.9 supplied by EcoChemie was employed for data processing. The analyzed samples were deoxygenated prior to measurements by purging with argon (99.999 %) and saturated with water for 120 s. Brdicka supporting electrolyte containing 1 mM Co(NH$_3$)$_6$Cl$_3$ and 1 M ammonia buffer (NH$_3$(aq) + NH$_4$Cl, pH = 9.6) was used. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement were as follows: initial potential of −0.7 V, end potential of −1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude −250 mV, Eads = 0 V, volume of injected sample: 5 μl. All experiments were carried out at temperature 4 °C employing thermostat Julabo F25 (Labortechnik GmbH, Germany).

Determination of total protein content

Spectrometric measurements were carried out using an automated chemical analyzer BS-200 (Mindray, China). Reagents and samples were placed on cooled sample holder (4 °C) and automatically pipetted directly into plastic cuvettes. Incubation proceeded at 37 °C. Mixture was consequently stirred. The washing steps by distilled water (18 mΩ) were done in the midst of the pipetting. Apparatus was operated using software BS-200 (Mindray, China). The total protein content was determined using the photometric pyrogallol red technique (Kleckerova et al. 2011).

Statistical analyses

The data were analyzed with STATISTICA 8 (StatSoft Inc., Tulsa, OK, USA) using parametric one-way ANOVA— Tukey honest significant difference (HSD) test, which was

applied for MTs concentrations comparison. Non-parametric Kruskal-Wallis ANOVA with post hoc multiple comparisons of mean ranks with Bonferroni adjustment were used for all other variables. Shapiro-Wilk test was used for testing of normality and Levene's test for testing of homogeneity of variance. Correlations between cadmium concentrations in sediment and larvae, MTs and cadmium concentrations in larvae were evaluated using Pearson's correlation coefficient. Lowest observable effect concentrations (LOECs) for all measured endpoints were determined as the lowest concentrations causing effect significantly different from the control groups.

Results

Survival

The test validity criteria for survival in control treatment (>80 %) were met in all experiments. The overlaying water quality parameters fell within the range of the OECD guideline 218 criteria—pH value 6–9, dissolved oxygen >60 % air saturation value and temperature 20 ± 2 °C, when measured at the start and end of the experiments. Survival data clearly showed great differences between the sensitivity of early and late instars. The older larvae were more than 20 times less sensitive to cadmium in artificial sediment compared to their young counterparts (see Fig. 1). The lowest observable effect concentration (LOEC) for survival of early instars was 50 μg Cd/g dw (Fig. 1—Test 2), whereas no effects on survival of late instars were observed over the whole tested concentration range up to 1000 μg Cd/g dw (Kruskal-Wallis test, $p < 0.05$). No differences in survival were observed between the two culture lines (compare Fig. 1—Tests 1 and 2) (Kruskal-Wallis test, $p > 0.05$). In the case of late instar larvae (Test 3), incidence of pupation was observed in the control and the two lowest concentration treatments. Pupae were counted as surviving larvae and excluded from further analyses. Interestingly, several cases of pupation were observed also in Test 2, where early instar larvae from culture line 2 were used. Pupation was observed in 0 and 1 μg Cd/g dw treatments, and this may be attributed to an accelerated development rate in line 2, which is further discussed below. No pupae were observed above 10 μg Cd/g dw in either test, where their development was restrained by higher concentrations of Cd. In the original study design it was planned to test age- and origin-dependent differences on both culture lines. However, it was impossible to carry out the test with late instar larvae of culture line 2 due to their accelerated development and the mentioned pupation after 10 days.

Growth

Growth was assessed as body length and dry weight of freeze-dried biomass (Fig. 2). The body length measurements again confirmed lower sensitivity of the late instar larvae with LOEC values 10 and 100 μg Cd/g dw for early and late instars, respectively (Kruskal-Wallis test, $p < 0.05$). Per capita dry weight of freeze-dried biomass corresponded with the body length (Pearson's $r = 0.89$), but dry weight showed greater relative differences among treatments. The decrease of larval dry weight by 23 % at 10 μg Cd/g dw treatment in Test 3 with late instar (Fig. 2, bottom—Test 3) indicates there is an effect but due to high variability this growth inhibition was statistically non-significant (Kruskal-Wallis test, $p > 0.05$). Similar non-significant trend towards lower dry weight can be also seen at 1 μg Cd/g dw treatment in Test 2 and 3. The same LOEC values (10 μg Cd/g dw) for growth were observed for early instar larvae from the two distinct culture lines, however, despite equal sensitivity the absolute values of both determined growth parameters differed substantially between the culture lines (Kruskal-Wallis test, $p < 0.05$).

Fig. 1 Survival data for Tests 1–3. Tests 1 and 2 conducted with 1st instar of culture line 1 and 2, respectively, Test 3 with 4th instar of culture line 1. *Error bars* show standard deviation; *asterisks* mark groups significantly different from control (Kruskal-Wallis test, $n = 5$, $p < 0.05$)
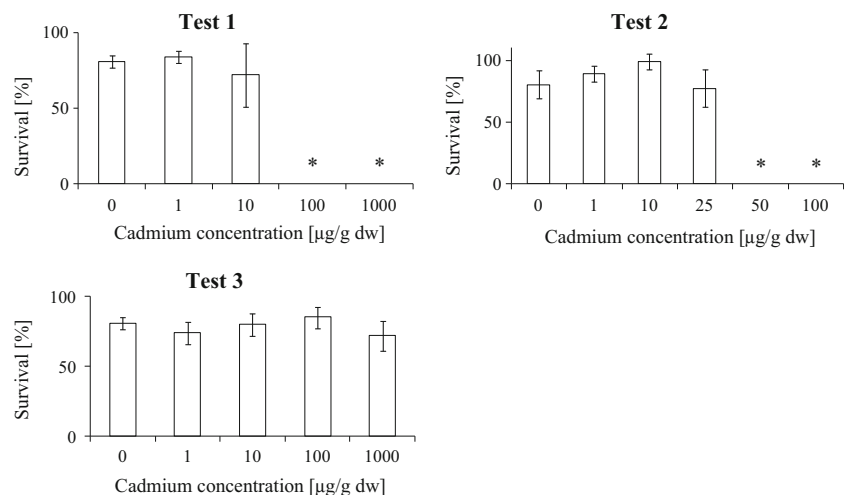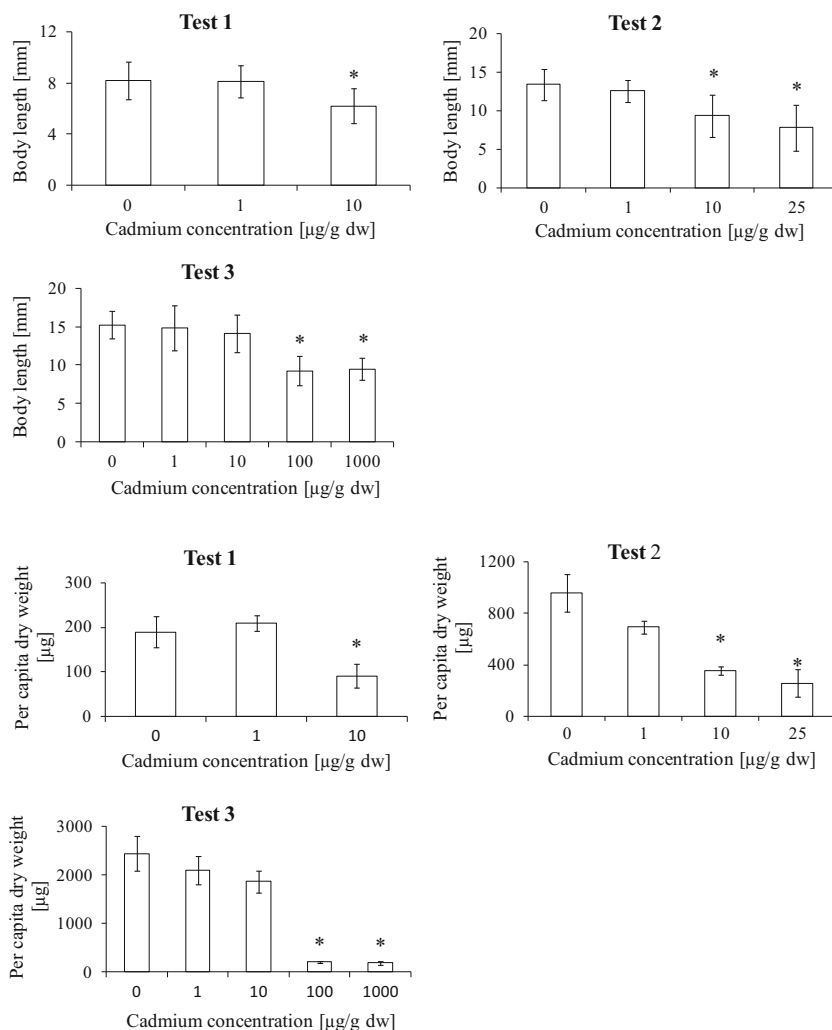
Fig. 2 Growth data for tests 1–3 (Tests 1 and 2 conducted with 1st instar of culture line 1 and 2 respectively, Test 3 with 4th instar of culture line 1): *top*—body length in mm, *bottom*—per capita dry weight of larval biomass in μg. *Error bars* show standard deviation; *asterisks* mark groups significantly different from control (Kruskal-Wallis test, $n = 5$, $p < 0.05$)

Analyses of cadmium

The results presented in Table 2 provide information about the overall success of the spiking process according to the OECD guideline 218 and cadmium recovery after 10 days in the test system as well as the cadmium concentrations in larvae. The measured concentrations in sediment were in most cases lower than their nominal values, which may be caused by transfer of cadmium ions to the water phase; we do not assume any substantial loss of cadmium from the sediment via the larvae since the amount of bioaccumulated cadmium is negligible compared to the overall content in the test vessels. Traces of cadmium were found in sediments and larvae from control groups and spiking of the highest concentration 1000 μg Cd/g dw resulted in the biggest difference from its nominal value (see Table 2, Tests 1 and 3). Analyses of artificial sediment components and food revealed that the added food, peat and calcium carbonate, containing 0.181, 0.071 and 0.164 μg Cd/g dw respectively, may bring some cadmium into the system. The silica sand and kaolin clay are unlikely to contaminate the controls as they did not

contain any Cd above the detection limit of 0.004 μg Cd/g dw. The levels of cadmium bioaccumulated by larvae differed between Tests 1–3 in the respective concentration treatments, however, no trends could be observed and the differences result namely from high variability between individual replicates. As shown in Table 2, only minor non-significant difference was observed between the values of tissue cadmium in late instar larvae (Test 3) exposed to 10 and 100 μg Cd/g dw (tissue values 189 and 222 μg Cd/g dw, respectively) for 10 days. Despite this, there was a significant difference in toxic effects between these two treatments (Fig. 2—Test 3). At the same time, similar effects were observed in 100 μg Cd/g dw and 1000 μg/g dw treatments (Fig. 2—Test 3), even though Cd tissue concentration was approximately five times higher in the 1000 μg/g dw treatment (1022 μg Cd/g dw). The early instar larvae showed effects at lower (107 μg Cd/g dw in Test 1) Cd tissue concentration than their late instar counterparts from the same culture line 1 (222 μg Cd/g dw in Test 3). However, the early instar larvae did not survive above this cadmium dose as did the late ones. The ratio of cadmium concentration in larvae after 10 days

Table 2 Cadmium concentrations in spiked sediment and larval tissues in µg/g dw for Tests 1–3 after 10 days of exposure

nominal cadmium conc. [µg/g dw]	TEST 1			TEST 2			TEST 3		
	sediment	larvae*	larvae to sediment ratio	sediment	larvae	larvae to sediment ratio	sediment	larvae	larvae to sediment ratio
0	0.07 ± 0.017	7.3	104.29	0.023 ± 0.003	2.6 ± 0.80	113.04	0.04 ± 0.01	17.1 ± 7.8	427.50
1	1.3 ± 0.05	17.8	13.69	0.77 ± 0.03	45 ± 6.3	58.44	0.86 ± 0.14	20 ± 11.7	23.26
10	10.2 ± 1.1	107	10.49	7.4 ± 1.2	223 ± 49.4	30.14	6.9 ± 1.9	189 ± 13.2	27.39
25	–	–	–	17.1 ± 3.9	265 ± 34.7	15.50	–	–	–
50	–	–	–	41 ± 8.4	m	–	–	–	–
100	102 ± 6	m	–	82 ± 10.1	m	–	74 ± 8.3	222 ± 38	3.00
1000	736 ± 261	m	–	–	–	–	522 ± 155	1022 ± 296	1.96

Presented are the average values and standard deviations for $n = 5$. The average ratio of cadmium content in larval biomass and spiked sediment for each treatment is listed to illustrate the rate of cadmium bioaccumulation. Limit of detection for cadmium content in sediment is 0.004 µg/g dw and in larval tissue 0.005–0.05 µg/g dw, depending on the sample mass

Asterisk marks a group where no variability data were available $n = 1$—larval biomass from all five replicates of each concentration treatment was analyzed as a single pooled sample

m denotes treatments where 100 % mortality did not allow any analysis

"–" denotes treatments not included in the particular test

exposure and sediment was decreasing with the growing concentration treatment.

Determination of metallothioneins (MTs)

MTs concentrations in wet tissue of larvae were determined in Tests 2 and 3 and the results are expressed in µg MTs per 1 g of protein. The larvae from Test 1 were not analyzed for MTs as their low biomass did not allow such measurement. We observed significantly elevated relative concentration of MTs at 10 and 25 µg Cd/g dw treatments for early instar larvae and at 100 and 1000 µg/g dw treatment for the late instar (Tukey honest significant difference (HSD), $p < 0.05$); (Fig. 3). In case of the late instar larvae (Fig. 3—Test 3), MTs concentration in treatments with the greatest exposure and without any lethality reached approximately 4-times the control level, while in case of the early instar (Fig. 3—Test 2) these relative increases were lower with maxima around 1.5-times. The control levels of MTs for both early and late instar larvae were similar (Fig. 3).

Figure 4 shows the relationship between the tissue Cd concentrations and MTs levels in Test 2 (early instar, culture line

2). Bioaccumulated cadmium concentration correlated significantly with MTs concentration with Pearson's $r = 0.86$, $p < 0.05$.

Discussion

Our study shows that substantial differences in sensitivity between early and late instar larvae may lead to serious ambiguities in standard toxicity data for *Chironomus* species. Today's use of both standardized guidelines with either early (OECD) or late (US-EPA) instar larvae in 10-day contact sediment toxicity test is likely to generate two distinct data sets, where the same chemicals tested by OECD method may show at least an order of magnitude higher toxicity than if tested by US-EPA procedure. Therefore, more detailed specification should be listed along with entries in toxicological databases for chironomid toxicity data, including which instar of larvae and test method were used. Our results confirmed considerable age-dependent differences in sensitivity of chironomids to cadmium on traditional endpoints like survival and growth, which had been described earlier (Ristola et al. 1999). We

Fig. 3 Concentration of metallothioneins (MTs) in µg/g of protein for Tests 2 (1st instar line 2) and 3 (4th instar line 1). *Error bars* show the standard deviation; *asterisks* mark groups significantly different from control. (Tukey honest significant difference (HSD), $n = 3$ for Test 2, $n = 5$ for Test 3; $p < 0.05$)
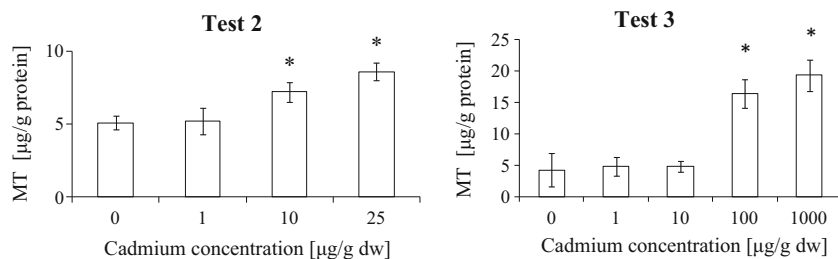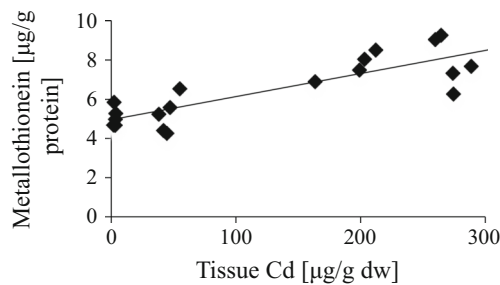
Fig. 4 Significant linear relationship of cadmium and metallothionein (MTs) content in larval tissue in Test 2 (Pearson's $r = 0.86$, $n = 20$, $p < 0.05$)

report LOEC values, which are in agreement with earlier published data (Gillis et al. 2002).

The late instar larvae survived even in very high cadmium concentrations (1000 μg Cd/g dw), which speaks for existence of some effective mechanisms of high resistance to cadmium especially pronounced in this later larval stage. There are several known physiological responses towards the toxicological challenge of metal exposure: detoxification by increased levels of metallothioneins—MTs (Amiard et al. 2006), antioxidants like glutathione—GSH (Kafel et al. 2012), metal-rich granules—MRGs (Wallace et al. 2003), or high molecular proteins like ABCC2 (Long et al. 2011); detoxification by increased retention of metal ions in midgut gland and excretion when molting; detoxification by increased cellular turnover and cell proliferation of digestive tissue, increased production of lipofuscin granules, vesiculation of endoplasmatic reticulum (Hodl et al. 2010); or adaptation by downregulation of heat shock proteins—Hsp70 (Haap and Kohler 2009). Age-dependent differences in sensitivity to toxic metal ions may be attributed to intensified employment of the above mentioned mechanisms in the late instar larvae compared to early instars or to size-related effects and allometry. Small larvae have relatively larger permeable surface area susceptible to ion uptake inhibition caused by toxic metals and are therefore unable to maintain ionoregulatory equilibrium (Brix et al. 2011). Body size also seems to play an important role in subcellular compartmentalization of metals into metal-sensitive fractions like organelles and heat-sensitive proteins, and into biologically detoxified fractions like MTs and MRGs, which are increasingly represented in older and bigger individuals (Wallace et al. 2003).

The central role of MTs in cadmium detoxification, the molecular bases of which were described in detail (Adam et al. 2008), was assessed in relation to cadmium bioaccumulation. Even though MTs were upregulated in early instar larvae at lower concentration, the late instar larvae were able to upregulate MTs to higher extent than early instars. MTs in the late instars were induced up to 3.9- and 4.6-fold in 100 and 1000 μg Cd/g dw treatment, respectively, while the maximal upregulation in early instars was 1.7-fold in 25 μg Cd/g dw

treatment. In both early and late instar larvae, the significant upregulation of MTs corresponded well to the significant effects in the whole-body endpoints. The late instar larvae surviving in the two highest cadmium concentration treatments showed similar level of MTs modulation despite substantial difference in cadmium body burdens (222 and 1022 μg Cd/g dw in 100 and 1000 μg Cd/g dw treatment, respectively). This may be due to increased MTs turnover in the higher concentration treatment or MTs saturation when the highest MTs gene expression and protein translation levels are reached (Gillis et al. 2002, Ng et al. 2007). MTs saturation level was reported in snails *Helix pomatia* at 90 μg Cd/g dw in midgut gland (Hodl et al. 2010). In case of MTs saturation we would expect transition from stress resistance to exhaustion of resistance capacity leading to severe or lethal effects in larvae from the highest concentration treatment. Nevertheless, even despite extremely high cadmium content in tissues, the larvae survived and showed the same effects on all whole-body endpoints as the larvae from 100 μg Cd/g dw treatment with body burdens at least 4-times lower. This fact strongly suggests that late instar larvae use some other dominant detoxification mechanism alongside with MTs induction to cope with stress presented by high cadmium concentrations. Further research is needed to identify this dominant detoxification mechanism or certain combination of synergistic mechanisms responsible for chironomids' resistance to metal exposures.

In general, we have observed lower MTs content if compared to earlier studies with chironomids or other freshwater invertebrates (Gillis et al. 2002; Amiard et al. 2006). Many different ways on how to express MT content have been published, which causes difficulties in making comparisons. In this study, re-calculation of MTs on total protein content was selected. This re-calculation enables to observe changes in MTs content as one of the proteins. It correlated well with the total MTs content in our previously published study (Fabrik et al. 2008). According to our results, sensitivity of MTs modulation as biomarker of exposure corresponds to the sensitivity of the whole-body endpoints (growth) and therefore can hardly serve as an early warning sign of toxicity in our study. On the other hand, Gillis et al. (2002) demonstrated induction of metallothionein-like proteins (MTLP) in early instars with LOEC of 0.43 μg Cd/g dw, which is slightly lower than NOEC of 1 μg Cd/g dw in our study, and suggested its use as a sensitive subcellular endpoint in chironomids and tubificids.

Divergences among studies analyzing MTs content may be related to the application of different methods for MTs determination that usually vary in their very principle and also specificity. In the case of field MTs measurements, results demonstrate even greater variability and less pronounced relationship to heavy metal concentrations (Gillis et al. 2006). It must be also taken into account that there are great differences in MTs contents between species and even tissues (Mieiro et al. 2011). The influence of environmental or laboratory conditions should always be considered.

In attempt to assess the variability of results of 10-day chironomid bioassay between distinct larval developmental stages and culture lines origin, we arrived to some interesting findings: Growth (especially dry weight) was found to be the most sensitive endpoint in all experiments, which is consistent with the findings of earlier studies (De Haas et al. 2004; Milani et al. 2003). Substantial difference was observed between the values of growth parameters and development rate between the early instar larvae originating from two distinct culture lines. High variability of growth as an endpoint was reported also in a previously published inter-laboratory comparison (Norberg-King et al. 2006). Marked variability between distinct culture lines may possibly be explained by rapid deterioration of genetic diversity due to inbreeding, which is common in laboratory culture populations (Nowak et al. 2007). The genetic diversity of the laboratory populations may be difficult to maintain, however, it seems to be one of the crucial assumptions for the proper organism's response to stress factors, especially if they combine (Haap and Kohler 2009).

Bioaccumulation data from our experiments showed a clear trend towards decreasing ratio of bioaccumulated and sediment cadmium concentrations with the increasing cadmium concentration treatment, which complies with the results of De Haas et al. (2004). According to our results, the adverse effects may not be simply attributed to a certain cadmium body burden level as for example suggested previously by Pery et al. (2007), since the potency of bioaccumulated cadmium to cause toxic effects depends on cellular compartmentalization between detoxified and metal-sensitive fractions constituting the toxic pool (Wallace et al. 2003). Our results indicate that age-related sensitivity can play role in the reaction of the larvae to certain body burdens.

Concentrations of cadmium used in our experiments were selected according to literature and preliminary experiments in our laboratory. It is important to note here that cadmium concentrations in natural sediments usually do not exceed 10 µg/g dw, which is a value reported only at highly polluted sites (Desrosiers et al. 2008). Sediment quality guidelines (SQGs), derived by regulatory forces in various countries, determine the level of cadmium which is considered safe as 2.3, 1.5, 0.6, and 0.9 µg/g dw for EU, Australia, Canada, and the USA, respectively (Burton 2002; Smolders et al. 2007). Chironomids have been routinely used for sediment toxicity testing thanks to all the advantages and ease of the bioassay, however, it is necessary for risk assessors to be aware of the fact that chironomids are relatively tolerant to pollution and that the development stage of test larvae matters. Use of distinct original culture lines may lead to variability of test results among and/or within laboratories, especially due to differences in development rates, and it is therefore crucial to assess relative rather than absolute data and refer to a relevant control. Despite this, chironomids are still among the most sensitive in vivo methods for contact sediment toxicity testing if compared to other bioassays such as with *Hyalella azteca*, *Hexagenia*, or *Tubifex tubifex* (Milani et al. 2003). Modifications of test procedure or new endpoints leading to increased sensitivity of chironomid bioassay should be investigated by future research.

In addition to the standard sediment toxicity testing chironomid larvae seem to be a promising model for benthic bioaccumulation assessment as they demonstrate comparable or greater bioaccumulation rates than other benthic macroinvertebrates routinely used in bioaccumulation studies such as *Tubifex tubifex* and *Lumbriculus variegatus* (*Oligochaeta*) (Gillis et al. 2002).

Conclusions

Our study proved age-dependent differences in sensitivity of chironomid larvae to cadmium exposure in artificial sediment. According to survival data, late instar larvae are at least 20-times less sensitive and therefore are likely to use efficient detoxification mechanisms. The principles of detoxification of cadmium have been studied intensely and there are several possible explanations to chironomids' high tolerance to cadmium exposure. Our results support the hypothesis that late instar larvae use some further mechanism of detoxification along with MTs. This mechanism or combination of mechanisms is, however, yet unknown and therefore should be addressed by further research.

Based on our results, we recommend following the OECD guideline preferably, since it covers the more sensitive early larval stage unlike the US-EPA method. Our study also stresses the need to renew chironomid laboratory cultures frequently in order to avoid results biased by use of inbred individuals. Use of chironomid larvae for bioaccumulation assessment satisfies the need of a quick procedure with relevant benthic organism with high bioaccumulation potential.

References

Abd-El-Monem HM, Corradi MG, Gorbi G (1998) Toxicity of copper and zinc to two strains of Scenedesmus acutus having different sensitivity to chromium. Environ Exp Bot 40:59–66. doi:10.1016/S0098-8472(98)00021-5

Adam V, Baloun J, Fabrik I et al (2008) An electrochemical detection of metallothioneins at the zeptomole level in nanolitre volumes. Sensors 8:2293–2305. doi:10.3390/s8042293

Adam V, Fabrik I, Eckschlager T et al (2010) Vertebrate metallothioneins as target molecules for analytical techniques. Trac-Trends Anal Chem 29:409–418. doi:10.1016/j.trac.2010.02.004

Amiard JC, Amiard-Triquet C, Barka S et al (2006) Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. Aquat Toxicol 76:160–202. doi:10.1016/j.aquatox.2005.08.015

Brix KV, DeForest DK, Adams WJ (2011) The sensitivity of aquatic insects to divalent metals: a comparative analysis of laboratory and field data. Sci Total Environ 409:4187–4197. doi:10.1016/j.scitotenv.2011.06.061

Burton GA (2002) Sediment quality criteria in use around the world. Limnology 3:65–75

Cotman M, Drolc A, Ros M, Tisler T (2007) Daphnia magna wastewater toxicity assays: an interlaboratory study. Int J Environ Pollut 31

Dallinger R (1995) Metabolism and toxicity of metals: metallothioneins and metal elimination. In: Cajaraville MP (ed) Cell Biol. Environ. Toxicol. University of the Basque Country Press service, Bilbao, pp 171–190

de Haas EM, Paumen ML, Koelmans AA, Kraak MHS (2004) Combined effects of copper and food on the midge Chironomus riparius in whole-sediment bioassays. Environ Pollut 127:99–107. doi:10.1016/s0269-7491(03)00252-5

Desrosiers M, Gagnon C, Masson S et al (2008) Relationships among total recoverable and reactive metals and metalloid in St. Lawrence River sediment: bioaccumulation by chironomids and implications for ecological risk assessment. Sci Total Environ 389:101–114. doi:10.1016/j.scitotenv.2007.08.019

Eaton JG, Gentile JH, Stephan CE, Hansen DJ (2001) Update of ambient water quality criteria for cadmium. Office of Water, Office of Science and Technology, Washington DC

Fabrik I, Ruferova Z, Hilscherova K et al (2008) A determination of metallothionein in larvae of freshwater midges (Chironomus riparius) using Brdicka reaction. Sensors 8:4081–4094. doi:10.3390/s8074081

Gillis PL, Diener LC, Reynoldson TB, Dixon DG (2002) Cadmium-induced production of a metallothioneinlike protein in Tubifex tubifex (Oligochaeta) and Chironomus riparius (Diptera): correlation with reproduction and growth. Environ Toxicol Chem 21:1836–1844

Gillis PL, Reynoldson TB, Dixon DG (2006) Metallothionein-like protein and tissue metal concentrations in invertebrates (Oligochaetes and Chironomids) collected from reference and metal contaminated field sediments. J Great Lakes Res 32:565–577

Haap T, Kohler HR (2009) Cadmium tolerance in seven Daphnia magna clones is associated with reduced hsp70 baseline levels and induction. Aquat Toxicol 94:131–137. doi:10.1016/j.aquatox.2009.06.006

Harrahy EA, Clements WH (1997) Toxicity and bioaccumulation of a mixture of heavy metals in Chironomus tentans (Diptera: Chironomidae) in synthetic sediment. Environ Toxicol Chem 16:317–327

Hilscherova K, Dusek L, Sidlova T et al (2010) Seasonally and regionally determined indication potential of bioassays in contaminated river sediments. Environ Toxicol Chem 29:522–534. doi:10.1002/etc.83

Hodl E, Felder E, Chabicovsky M, Dallinger R (2010) Cadmium stress stimulates tissue turnover in Helix pomatia: increasing cell proliferation from metal tolerance to exhaustion in molluscan midgut gland. Cell Tissue Res 341:159–171. doi:10.1007/s00441-010-0980-x

Kafel A, Zawisza-Raszka A, Szulinska E (2012) Effects of multigenerational cadmium exposure of insects (Spodoptera exigua larvae) on anti-oxidant response in haemolymph and developmental parameters. Environ Pollut 162:8–14. doi:10.1016/j.envpol.2011.09.034

Kleckerova A, Sobrova P, Krystofova O et al (2011) Cadmium(II) and zinc(II) ions effects on maize plants revealed by spectroscopy and electrochemistry. Int J Electrochem Sci 6:6011–6031

Krizkova S, Blahova P, Nakielna J et al (2009) Comparison of metallothionein detection by using Brdicka reaction and enzyme-linked immunosorbent assay employing chicken yolk antibodies. Electroanalysis 21:2575–2583. doi:10.1002/elan.200900243

Long Y, Li Q, Zhong S et al (2011) Molecular characterization and functions of zebrafish ABCC2 in cellular efflux of heavy metals. Comp Biochem Physiol - C Toxicol Pharmacol 153:381–391. doi:10.1016/j.cbpc.2011.01.002

Mieiro CL, Bervoets L, Joosen S et al (2011) Metallothioneins failed to reflect mercury external levels of exposure and bioaccumulation in marine fish—considerations on tissue and species specific responses. Chemosphere 85:114–121. doi:10.1016/j.chemosphere.2011.05.034

Milani D, Reynoldson TB, Borgmann U, Kolasa J (2003) The relative sensitivity of four benthic invertebrates to metals in spiked-sediment exposures and application to contaminated field sediment. Environ Toxicol Chem 22:845–854

Ng TYT, Rainbow PS, Amiard-Triquet C et al (2007) Metallothionein turnover, cytosolic distribution and the uptake of Cd by the green mussel Perna viridis. Aquat Toxicol 84:153–161

Norberg-King TJ, Sibley PK, Burton GA et al (2006) Interlaboratory evaluation of Hyalella azteca and Chironomus tentans short-term and long-term sediment toxicity tests. Environ Toxicol Chem 25:2662–2674

Nowak C, Vogt C, Diogo JB, Schwenk K (2007) Genetic impoverishment in laboratory cultures of the test organism Chironomus riparius. Environ Toxicol Chem 26:1018–1022

Oda S, Tatarazako N, Dorgerloh M et al (2007) Strain difference in sensitivity to 3,4-dichloroaniline and insect growth regulator, fenoxycarb, in Daphnia magna. Ecotoxicol Environ Saf 67:399–405. doi:10.1016/j.ecoenv.2006.12.010

OECD (2004) OECD Guideline 218: sediment-water chironomid toxicity using spiked sediment. 1–21. doi: 10.1787/9789264070264-en

Oskarsson A, Widell A, Olsson IM, Grawe KP (2004) Cadmium in food chain and health effects in sensitive population groups. Biometals 17:531–534

Péry ARR, Sulmon V, Mons R et al (2003) A model to understand the confounding effects of natural. Environ Toxicol Chem 22:2476–2481

Pery ARR, Ducrot V, Geffard A, Garric J (2007) Do differences between metal body residues reflect the differences between effects for Chironomus riparius exposed to different sediments? Chemosphere 66:397–403. doi:10.1016/j.chemosphere.2006.06.040

Ristola T, Pellinen J, Ruokolainen M et al (1999) Effect of sediment type, feeding level, and larval density on growth and development of a midge (Chironomus riparius). Environ Toxicol Chem 18:756–764

Ryvolova M, Krizkova S, Adam V et al (2011) Analytical methods for metallothionein detection. Curr Anal Chem 7:243–261

Smolders E, Schoeters I, Waegeneers N et al (2007) European Union risk assessment report: cadmium oxide and cadmium metal part 1 environment, institute. Office for Official Publications of the European Communities, Luxembourg

Toumi H, Boumaiza M, Millet M et al (2013) Effects of deltamethrin (pyrethroid insecticide) on growth, reproduction, embryonic development and sex differentiation in two strains of Daphnia magna (Crustacea, Cladocera). Sci Total Environ 458–460:47–53. doi:10.1016/j.scitotenv.2013.03.085

Toumi H, Boumaiza M, Millet M et al (2015) Investigation of differences in sensitivity between 3 strains of Daphnia magna (crustacean Cladocera) exposed to malathion (organophosphorous pesticide). J Environ Sci Heal Part B 50:34–44. doi:10.1080/03601234.2015.965617

US-EPA (2000) Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates. 192

Varó I, Serrano R, Navarro JC et al (1998) Acute lethal toxicity of the organophosphorus pesticide chlorpyrifos to different species and strains of Artemia. Bull Env Contam Toxicol 61:778. doi:10.1007/s001289900828

Wallace WG, Lee BG, Luoma SN (2003) Subcellular compartmentalization of Cd and Zn in two bivalves. I. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). Mar Ecol Ser 249:183–197. doi:10.3354/meps249183