Ecotoxicology and Environmental Safety 133 (2016) 82–89

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Subcellular partitioning of cadmium and zinc in mealworm beetle (*Tenebrio molitor*) larvae exposed to metal-contaminated flour

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ARTICLE INFO

Article history:
Received 11 April 2016
Received in revised form
14 June 2016
Accepted 20 June 2016

Keywords:
Metal
Invertebrates
Sequestration
Granules
Kinetic

ABSTRACT

By studying the internal compartmentalization of metals in different subcellular fractions we are able to better understand the mechanisms of metal accumulation in organisms and the transfer of metals through trophic chains. We investigated the internal compartmentalization of cadmium (Cd) and zinc (Zn) in mealworm beetle (*Tenebrio molitor*) larvae by breeding them in flour contaminated with either Cd at 100, 300 and 600 mg kg^{-1}, or Zn at 1000 and 2000 mg kg^{-1}. We separated the cellular components of the larvae into 3 fractions: the S1 or cytosolic fraction containing organelles, heat-sensitive and heat-stable proteins, the S2 or cellular debris fraction and the G or metal-rich granule fraction. The concentration of Cd and Zn in each fraction was measured at 0, 7, 14 and 21 days of being fed the flour.

The concentration of Cd in the flour affected the concentration of Cd measured in each larval subcellular fraction ($p \leq 0.0001$), while the concentration of Zn in the flour only affected the Zn concentration in the S2 and G fractions ($p \leq 0.02$). Both Cd and Zn concentrations in mealworms remained relatively constant during the exposure (days 7, 14 and 21) in all three fractions, but the Cd concentrations were much higher than those found in larvae before the exposure (day 0). The concentration of Cd in the flour, however, did not affect the percentage of Cd in the S1 fraction. The contribution of Cd in the G fraction to the total Cd amount was similar (30–40%) in all Cd treatments. The percentage of Zn in all three fractions was not affected by the concentration of Zn in the flour and the relative contributions of each subcellular fraction to the total burden of Zn remained generally constant for both control and treated larvae. In general, larvae sequestered approximately 30% of Cd and Zn in the S1 fraction, which is important for the transport of metals to higher trophic levels in a food web.

1. Introduction

Metals assimilated by organisms are sequestered in various subcellular compartments and some types of sequestration are more stable than other. The sequestration mechanisms used by invertebrates to detoxify metals and prevent their interaction with important biomolecules include binding the metal to heat-stable and heat-denatured proteins, lysosomes, mitochondria and other ligands, and storing the metal in inorganic granules (Wallace et al., 2003; Rosabal et al., 2012; Ding et al., 2013). The internal compartmentalization of metals within an invertebrate allows for the determination of the fraction of a particular metal that is present in the body in a metabolically available form, which is more useful for understanding and predicting the potential toxic effects of the metal than its total concentration (Rainbow, 2002; Wallace et al., 2003). There is no many studies on metal sequestration mechanisms in terrestrial invertebrates, and those available were done mostly on earthworms (Beaumelle et al., 2015; Conder et al., 2002; Li et al., 2009; Vijver et al., 2004, 2006, 2007; Yu and Lanno, 2010). For example, it has been shown that the subcellular distribution of Cd in the earthworm *Aporrectodea caliginosa* responded more strongly to increasing Cd concentrations in the soil than the total internal Cd content (Beaumelle et al., 2015). Moreover differences between metals were found, with Cd accumulating in the debris and granular fractions of the earthworms exposed to the most contaminated soils, while subcellular distribution of Zn remained unchanged regardless of the level of soil contamination (Beaumelle et al., 2015). It was also suggested that toxic effect of metals on earthworms is linked with specific internal pools of metals assumed to be biologically active (Vijver et al., 2004).

Subcellular fractionation is a procedure that separates the total metal content into operationally defined subcellular compartments following different centrifugations (e.g., Wallace et al., 2003). It allows for the comparison of how different metals are sequestered within a given species, or of how a specific metal is

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http://dx.doi.org/10.1016/j.ecoenv.2016.06.033
0147-6513/

sequestered in different species. This procedure has been used to isolate at least three metal fractions in terrestrial invertebrates: metal-rich granules, cytosolic fraction and cellular debris (a fraction consisting of cell membranes, tissue fragments and intact cells) (Beaumelle et al., 2015; Gimbert et al., 2008; Vijver et al., 2007). The main mechanisms of cellular sequestration of metals that also affect the bioavailability of metals to consumers from higher trophic levels are the binding of metals to heat-stable proteins located mainly in the cytosol and the formation of distinct inclusion bodies (granules) (Vijver et al., 2004; Wallace et al., 1998).

Different durations of exposure to a metal may be needed to initiate specific physiological processes of metal regulation in an organism. The physiological process of metal regulation in an organism may additionally change over the duration of the exposure, and depend on the biological role of metal (essential vs non-essential), the chemical speciation of the metal and the concentration of the metal. For example, both the concentration and the duration of exposure to Cd had significant effects on the subcellular distribution of the metal in the deposit-feeding worm *L. hoffmeisteri* (Wallace and Lopez, 1996); both the Cd concentration and proportion of Cd bound to the soluble fraction increased with exposure time and Cd concentration, whereas the Cd concentration in cellular debris and granules remained at a relatively stable level. Differences in subcellular partitioning between non-essential and essential metals were found for the earthworm *Aporrectodea caliginosa*, in which subcellular distribution of Zn, in contrast to Cd and Pb, remained unchanged along the gradient of metal pollution (Beaumelle et al., 2015). As far as the influence of metal speciation on its bioavailability and compartmentalization, Calhôa et al. (2011) showed that the terrestrial isopods *Porcellio dilatatus* exposed to a diet contaminated with $Cd(NO_3)_2$ assimilated more Cd than those fed with $Cd(Cys)_2$, and the subcellular distribution also depended on the Cd species provided.

The aim of the present study was to compare the sequestration processes of cadmium (nonessential metal) and zinc (essential metal) in *Tenebrio molitor* larvae and to assess the potential availability of the metals to higher trophic levels. *Tenebrio molitor*, a cosmopolitan pest of stored grains, was chosen in this study to represent a soil-dwelling epigeic organisms feeding on various types of organic matter (Vijver et al., 2003a). The main uptake route of metals for mealworm larvae is via the ingestion of contaminated food (Vijver et al., 2003a) which is in contrast to earthworms, for which the additional route of entry for metals is via the skin (Vijver et al., 2003b). *T. molitor* has been previously used to study metal uptake from different soils and soil-sediment mixtures (Vijver et al., 2003a), excretion of different metal (including Cd and Zn) during moulting and metamorphosis (Lindqvist and Block, 1995, 1997) or to isolate Cd-binding protein (Pedersen et al., 2008). In this study, mealworm larvae were exposed to Cd (nonessential metal) or Zn (essential metal) at different concentrations, and the subcellular distribution of the metals was followed for three weeks. Similar to studies on earthworms (Beaumelle et al., 2015; Vijver et al., 2007) and snails (Gimbert et al., 2008), we isolated three subcellular fractions from the larvae: (1) metal-rich granules; (2) a fraction combining microsomes and lysosomes, mitochondria, metallothioneins and heat sensitive proteins (cytosolic fraction); and (3) a fraction consisting of cell membranes, tissue fragments and intact cells (cellular debris). This was done by using a relatively simple method based on a number of centrifugation steps (Vijver et al., 2004; Wallace et al., 2003) to allow for determination of metals present in the soluble fraction of the larvae considered more bioavailable to its predators than metals bound to the insoluble fractions (Wallace et al., 2003).

2. Materials and methods

2.1. Experimental design

Tenebrio molitor larvae were obtained from a pet shop in Kraków, Poland. They were kept in plastic boxes at 20 °C and 75% relative humidity (RH) under 16:8 (L:D) h. For the experiment, the mealworms were placed in 500-ml plastic boxes with perforated lids that had been filled with 50 g of either: uncontaminated, Zn-contaminated, or Cd-contaminated wheat flour. The wheat flour was used as a medium and food for the mealworm larvae and was contaminated by adding either water (control) or an aqueous solution of metal salts ($CdCl_2 \times 2.5H_2O$, POCH Poland, or $ZnCl_2$, Merck, Germany) at the desired concentration: 100, 300, or 600 mg Cd kg^{-1} flour, and 1000 or 2000 mg Zn kg^{-1} flour. Then the flour was dried at 105 °C for 24 h, ground and sieved. The nominal and actual concentrations of the metals in the flour are listed in Table 1.

The mealworm larvae, approximately 100 per box, were randomly allocated to treatments and fed with a new portion of flour ad libitum twice a week for 21 days. Ten larvae were sampled before the start of the exposure and five larvae were sampled on days 7, 14 and 21 from each treatment based on their body mass to ensure that the whole range of sizes which might influence the results were covered. The sampled mealworms were kept in empty boxes for 24 h to empty their gut contents, washed in deionized water to remove all remnants of flour from their body surface, weighed to the nearest 0.0001 g (Radwag AS/C/2, Poland) and killed by freezing at −20 °C.

2.2. Subcellular fractionation

We used the procedure of subcellular partitioning to separate larvae bodies into three fractions: S1 (cytosolic) – containing microsomal and cytosolic components, e.g., metallothioneins and heat-sensitive proteins; S2 (cellular debris) – comprising tissue and cell membranes; and G – containing granules. All three fractions together constitute the whole organism. Subcellular partitioning was based on a series of differential centrifugations as well as NaOH digestion and heat treatment steps as described by Vijver et al. (2004), but optimized for *T. molitor* larvae. Briefly, frozen larvae were homogenized with a tissue homogenizer (Gen-Bio PRO200) in 500 μL of ice-cold 0.01 M Tris-HCl buffer (pH 7.5, Sigma-Aldrich, USA). Homogenates were centrifuged at 10,000*g* for 30 min at 4 °C to separate the supernatants (S1 fraction) from the pellets. The pellets were heated at 100 °C for 2 min, then 250 μL of 1 M NaOH (POCH, Poland) was added, and the fraction was hydrolysed for 1 h at 70 °C. After centrifuging at 10,000*g* for 10 min at 20 °C, the supernatant consisting of metals bound to cellular debris (S2 fraction) was separated from the pellets (granular fraction, G). Pellets were resuspended in 600 μL of 0.2% HNO_3

Table 1
Nominal and actual concentrations of metals (Cd, Zn) in treated and control flours (mean ± SD, n=3).

Metal	Treatment	Metal concentration [mg kg^{-1}]	
		Nominal	Actual
Cd	Control	0	0.22 ± 0.01
	100	100	131 ± 2.0
	300	300	380 ± 36
	600	600	669 ± 48
Zn	Control	0	291 ± 8
	1000	1000	990 ± 24
	2000	2000	1836 ± 156

(69.0–70.0%, INSTRA-Analysed, Baker, Germany). Because of the large number of individuals, the subcellular partitioning was done at 5 series. Three blanks were conducted in each series using 500 μL of 0.01 M Tris-HCl buffer to ensure the absence of contamination during the homogenization and fractionation procedures. All samples (345 in total) were stored at 4 °C until they were analysed for metal concentrations.

2.3. Chemical analysis

To analyse metal concentrations in subcellular fractions, samples were dried at 105 °C for 12 h before digesting in 300 μL of boiling HNO_3 (69.0–70.0%, INSTRA-Analysed, Baker, Germany) and then diluting to 1 or 3 ml with 0.2% HNO_3. Three samples of flour per treatment group were dried at 105 °C for 24 h and weighed to the nearest 0.001 g (Radwag AS/C/2, Poland) before digesting in 1.5 ml boiling HNO_3 and then diluting to 5 ml with 0.2% HNO_3. Cd concentrations in subcellular fractions and the flour were measured using either a graphite furnace atomic absorption spectrophotometer (Perkin-Elmer AAnalyst 800; detection limit 0.024 $\mu g\,L^{-1}$) or a flame atomic absorption spectrophotometer (Perkin-Elmer AAnalyst 200; detection limit: 0.011 $mg\,L^{-1}$), depending on the metal concentration in the samples. Zn concentrations were measured using a flame AAS (Perkin-Elmer AAnalyst 200). The quality control sample was run every 10 samples and each sample was analysed in duplicate. To check the analytical precision three blanks and three samples of reference material (fish liver – *Certified Reference Material Dolt-4 Dogfish Liver*, National Research Council of Canada) were run with the samples. The results of the reference material indicated that the chemical analyses were within ± one standard deviation of the respective certified values. The results were not corrected for recovery. The amount of metal found in each fraction was normalized by dividing them by fresh larvae body weight and the results were expressed in $mg\,kg^{-1}$.

2.4. Statistical analysis

The effect of metal concentration in flour and the duration of exposure on both the metal concentrations and the metal proportion (in percentage) in each subcellular fraction were tested separately for Cd and Zn treatments using a two-way ANOVA ($p \leq 0.05$) with body mass as a covariate. If the interaction between metal concentration and exposure time appeared nonsignificant, it was removed from the model. Statistically significant differences were further analysed using Fisher's least significant difference (LSD) test for the post hoc comparison of means. Metal concentrations in each fraction were rank-transformed, and the arc sine of the square root transformation was applied to percentages of a metal in each fraction prior to ANOVA (Zar, 1999). Day 0, which was common for all treatments, was excluded from the ANOVA, as it would not be possible to test for interactions between the factors if it was included. The total metal concentration was obtained by summing up amounts of metal in all subcellular fractions divided by fresh weight of larvae and the percentage of metal in a fraction was calculated by relating the amount of metal retrieved from the particular fraction to the total amount of the metal (i.e., sum of the metal amounts in all studied fractions) in the organism.

All statistical analyses were performed using the Statgraphics Centurion XVI program (StatPoint Technologies, Inc., USA).

3. Results

The body mass of unexposed larvae was in the range 30–190 mg, for Cd-treated ones 27–190 mg, and for Zn-treated 29–195 mg. Individual larvae that had Cd or Zn concentration below the detection limit in at least one of the studied fractions (S1, S2 or G) were excluded from statistical analysis. This was the case for four (all sampled at day 0) out of the 70 larvae analysed for Cd and four (two control sampled at day 7 and two from the 1000 mg Zn kg^{-1} group sampled at days 14 and 21) out of 55 larvae analysed for Zn. We excluded the individuals rather than particular sample (fraction) because unprecise measurement of metal concentration in one fraction would affect the calculation of the metal proportion (percentage) in each subcellular fraction for this individual.

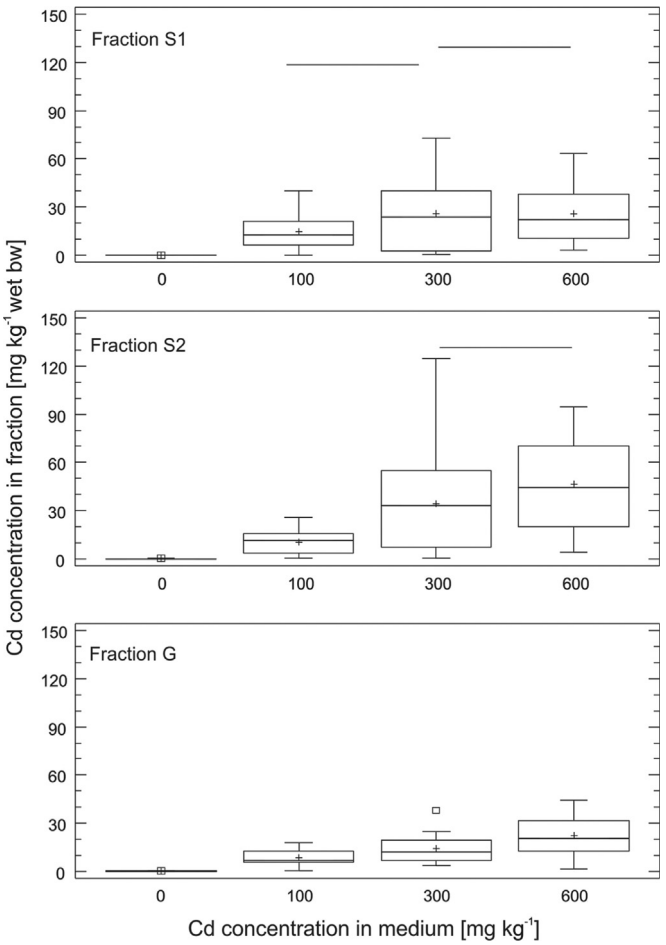

Fig. 1. Box-and-whisker plots for cadmium concentrations measured in subcellular fractions (S1 fraction – cytosolic, S2 fraction – cellular debris and G fraction– metal-rich granules) of mealworm larvae (*Tenebrio molitor*) fed flour with different Cd concentrations. The *plus sign* on each box represents the mean value, the *centre line* shows the sample median, the *boxes* contain second and third quartiles, and the *whiskers* extend to the minimum and maximum values. The most extreme values outside this range are show as *small squares*. The horizontal lines above boxes indicate no significant differences ($p > 0.05$) between treatments in a two-way ANOVA on ranks.

3.1. Internal metal concentrations in mealworms

The concentration of Cd in the flour affected both the total Cd concentration and the Cd concentration in each fraction extracted from the mealworm larvae ($p \leq 0.0001$). The Cd concentration in fraction S1 of the control group larvae was significantly lower than that of the Cd treatment groups and larvae from the 100 mg Cd kg^{-1} group had significantly lower Cd concentrations in the S1 than larvae in the 600 mg Cd kg^{-1} group (Fig. 1). With increasing Cd concentrations in the flour (100, 300 and 600 mg Cd kg^{-1}), the mean concentration of Cd in the S2 and G fractions increased: from 10.4 to 34.1 and 46.3 $mg\,kg^{-1}$ in the S2 fraction and from 8.5 to 14.1 and 22.0 $mg\,kg^{-1}$ in the G fraction, respectively.

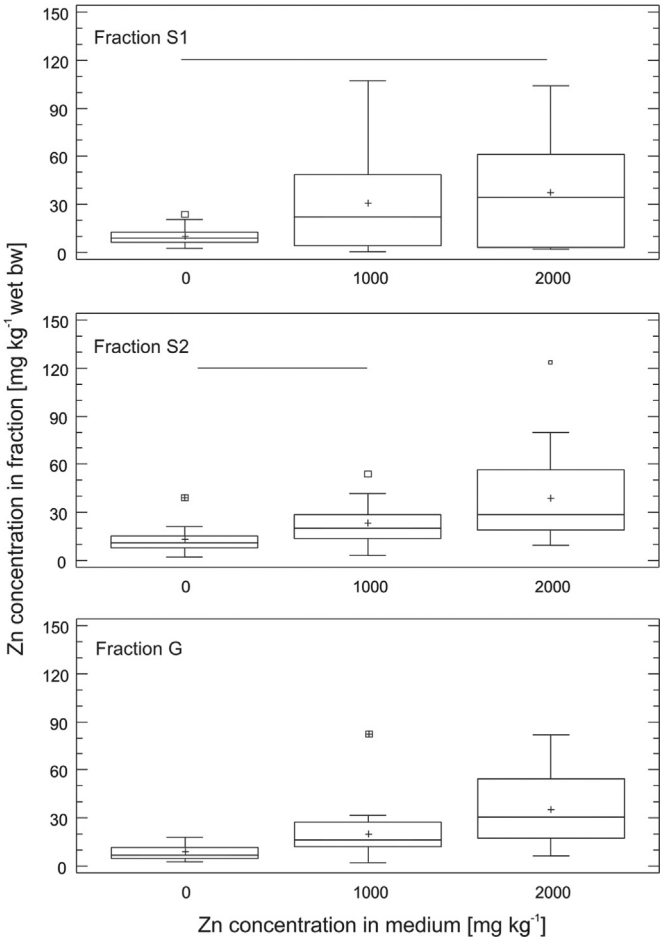

Fig. 2. Box-and-whisker plots for zinc concentrations measured in subcellular fractions (S1 fraction – cytosolic, S2 fraction – cellular debris and G fraction– metal-rich granules) of mealworm larvae (*Tenebrio molitor*) fed flour with different Zn concentrations. The *plus sign* on each box represents the mean value, the *centre line* shows the sample median, the *boxes* contain second and third quartiles, and the *whiskers* extend to the minimum and maximum values. The most extreme values outside this range are show as *small squares*. The horizontal lines above boxes indicate no significant differences ($p > 0.05$) between treatments in a two-way ANOVA on ranks.

Already at the first sampling day since the exposure (day 7), Cd concentration in Cd-treated larvae was much higher than in control beetles in all studied fractions and remained constant until day 21, as the sampling time point (7, 14 or 21 days) was found to be a non-significant factor in ANOVA analysis.

The total Zn concentration in larvae at the highest Zn concentration in flour (2000 mg Zn kg^{-1}) was significantly higher than in both the control larvae and the larvae exposed to 1000 mg Zn kg^{-1} ($p=0.02$), and body mass was a significant covariate $p=0.0001$. Neither exposure concentration nor duration of exposure affected the Zn concentration in fraction S1. However, in fraction S2, a significantly higher Zn concentration was found in larvae in the 2000 mg Zn kg^{-1} group than in other treatment groups ($p=0.002$), and the Zn concentration in fraction G increased with increasing Zn concentration in the flour ($p=0.0001$; Fig. 2). The sampling time point (7, 14 or 21 days) was found to be a non-significant, but, body mass was a significant covariate at $p \leq 0.01$ for all fractions.

3.2. Metal distribution among the three subcellular fractions

The concentration of Cd in the flour did not affect the percentage of Cd found in the S1 fraction ($p=0.1$), but a higher percentage of Cd was found in this fraction at days 14 and 21 compared with day 7 ($p=0.03$). The percentage of Cd in fraction S2 was affected by the Cd concentration in the flour ($p=0.002$) and the interaction between concentration in the flour and the duration of exposure ($p=0.04$). In larvae exposed to 300 mg Cd kg^{-1}, the S2 fraction had a much lower percentage of Cd at day 14 in comparison to days 7 and 21. The percentage of Cd in fraction G was significantly higher in larvae from the control group than in other treatment groups ($p=0.03$). Regardless of the high percentage of Cd in G fraction in control animals, the quantities of metal in fraction G were generally low in this group as the total Cd concentration for control individuals did not exceed 0.35 mg kg^{-1}. The average percentages of Cd in different fractions according to the treatment and duration of exposure are shown in Fig. 3.

The percentage of Zn in the S1, S2 and G fractions was affected neither by the concentration of Zn in the flour nor by the duration of Zn exposure ($p \geq 0.5$ and $p \geq 0.26$ for all fractions). Moreover, Zn was divided fairly equally between the fractions, regardless of the exposure concentration and time (Fig. 4). The mean percentage of the total Zn content that was found in each fraction for all Zn concentration and durations of exposure >0 were as follows: S1 = 30.8%, S2 = 38%, and G = 31.2%.

4. Discussion

Both the present study and the one by Lindqvist and Block (1995) showed that the internal body Cd concentrations in *T. molitor* is positively correlated to the concentration of Cd in food. Just because the metal is taken up by the organism does not necessarily mean that it will be harmful. Even if accumulated at high levels, metals can be isolated metabolically and prevented from becoming toxicologically bioavailable. Thus, only a portion of total body burden is bioavailable for interaction at receptor sites on an organism (Vijver et al., 2004).

Previous studies indicated that both the metal concentration and the duration of exposure had a significant effect on metal distribution in different species. For example, the amounts of Cd bound to the soluble fraction (S1) in the deposit-feeding worm *L. hoffmeisteri* increased with time and metal concentration, whereas Cd concentration in the cellular debris fraction remained at a relatively stable level (Wallace and Lopez, 1996). Similarly, Cd concentration found in the S1 fraction in the earthworm *Eisenia fetida* exposed to a high Cd concentration (1575 mg kg^{-1}) added to the artificial soil as a solution of $Cd(NO_3)_2$ increased linearly over a 14-day exposure, whereas Cd bound to the pellet fraction containing tissue debris and metal-rich granules (S2+G fractions in our experiment) remained stable over time (Conder et al., 2002). Conder et al. (2002) suggest that upon short-term exposure, the granules have a limited capacity for storing the incoming metal. In our study, however, time of exposure longer than 7 days appeared to not be a significant factor, but metal concentration did affect sequestration mechanisms differently for Cd and Zn. For example, in fraction S1, the concentration of Cd was affected by Cd concentration in the flour fed to the larvae, whereas this was not the case for Zn. On the other hand, exposure to both Cd and Zn affected metal concentrations in fractions S2 and G. The increase of Cd concentrations in the S1 fraction with increasing Cd concentrations in the flour presumably reflects a progressive Cd detoxification by low cysteine-type Cd-binding proteins produced in the guts of mealworms (Pedersen et al., 2007, 2008). Low cysteine-type Cd-binding proteins, i.e., with non-metallothionein like characteristics, similar to rich cysteine-type Cd-binding proteins, i.e., metallothionein-like proteins, have a high Cd-affinity and low molecular mass and heat stability and can therefore be included in the S1 fraction. Production of low cysteine-type Cd-binding

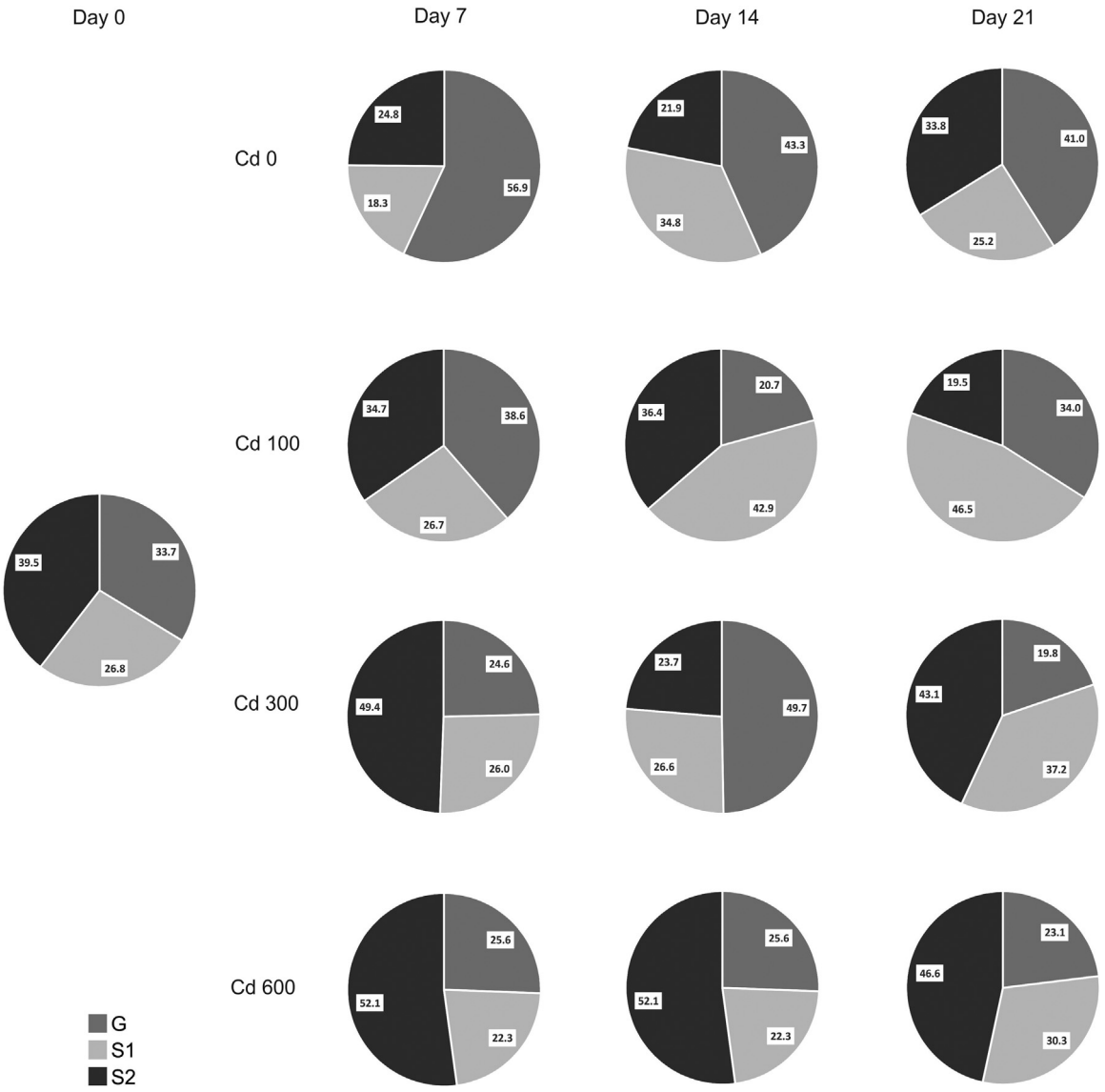

Fig. 3. Overall patterns of Cd distribution and the relative percentage of Cd retrieved from the different fractions in mealworm larvae (*Tenebrio molitor*) sampled before Cd exposure (day 0) and after 7, 14 and 21 days of exposure to 0, 100, 300 or 600 mg Cd kg^{-1} in flour; S1 – cytosolic fraction, S2 – cellular debris, G - metal-rich granules. The percentage of Cd in each fraction is calculated by relating Cd retrieved from the subcellular fraction concerned to the total amount of Cd (sum of all fractions).

proteins was also observed in the stonefly *Pteronarcys californica* (Clubb et al., 1975) and the woodlouse *Porcellio scaber* (Dallinger, 1993).

The background levels of Cd in soils usually range between 0.06 and 1.1 mg kg^{-1}, but in metal polluted areas this range can increase to 40–325 mg kg^{-1} (Spurgeon and Hopkin, 1999; Giska et al., 2014) and the highest reported Cd concentration in soil was recorded around metal smelters and reached over 1700 mg kg^{-1} (Kabata-Pendias and Mukherjee, 2007). In uncontaminated soils, concentrations of Zn range between 10 and 300 mg kg^{-1}, but over 10,000 mg kg^{-1} have been recorded around metal smelters (Kabata-Pendias and Mukherjee, 2007). Thus, the concentrations used in our study can be considered realistic for some most polluted soils. The goal of the study was to test whether biochemical affinity of metals for different biochemical fractions in organism depends on a metal type and its concentration. Because *T. molitor* was shown to accumulate high Cd levels in their body when exposed to this metal as $CdCl_2$ added to the food (Lindqvist and Block, 1995), we used high exposure concentrations to exert the sequestration mechanisms. Different exposure concentrations were used for Cd (nonessential metal) and Zn (essential metal) as they differ in their intrinsic toxicity to many organisms: Cd is usually more toxic than Zn even at low concentrations (Wallace et al., 2003; Gimbert et al., 2008).

In general, regardless of Cd concentrations in the flour, the highest Cd concentration was found in the S2 fraction followed by the S1 fraction, and finally the G fraction of *T. molitor* larvae, whereas Zn concentrations were similar in all fractions. These results are different from those found for the earthworm *A. caliginosa* exposed to a high Cd concentration (325 mg kg^{-1}) in the soil (Vijver et al., 2006). The earthworms accumulated the highest Cd concentrations in the cytosolic fraction that corresponds to the S1 fraction in our study (Vijver et al., 2006). In addition, the earthworm *E. fetida* accumulated Cd mostly in fraction S1 in two different studies; one where it was exposed for a week to a solution containing 0.5 mg Cd L^{-1} (Li et al., 2008), and another one where it was exposed for 21 days to soil containing 1 mg Cd kg^{-1} dry weight (Li et al., 2009). One of the differences in Cd sequestration in S1 fraction between mealworms and earthworms may be due to differences in the types of protein that is predominant in each species. For earthworms, the predominant protein in the S1 fraction is metallothionein, a protein with a high affinity to Cd (Yu

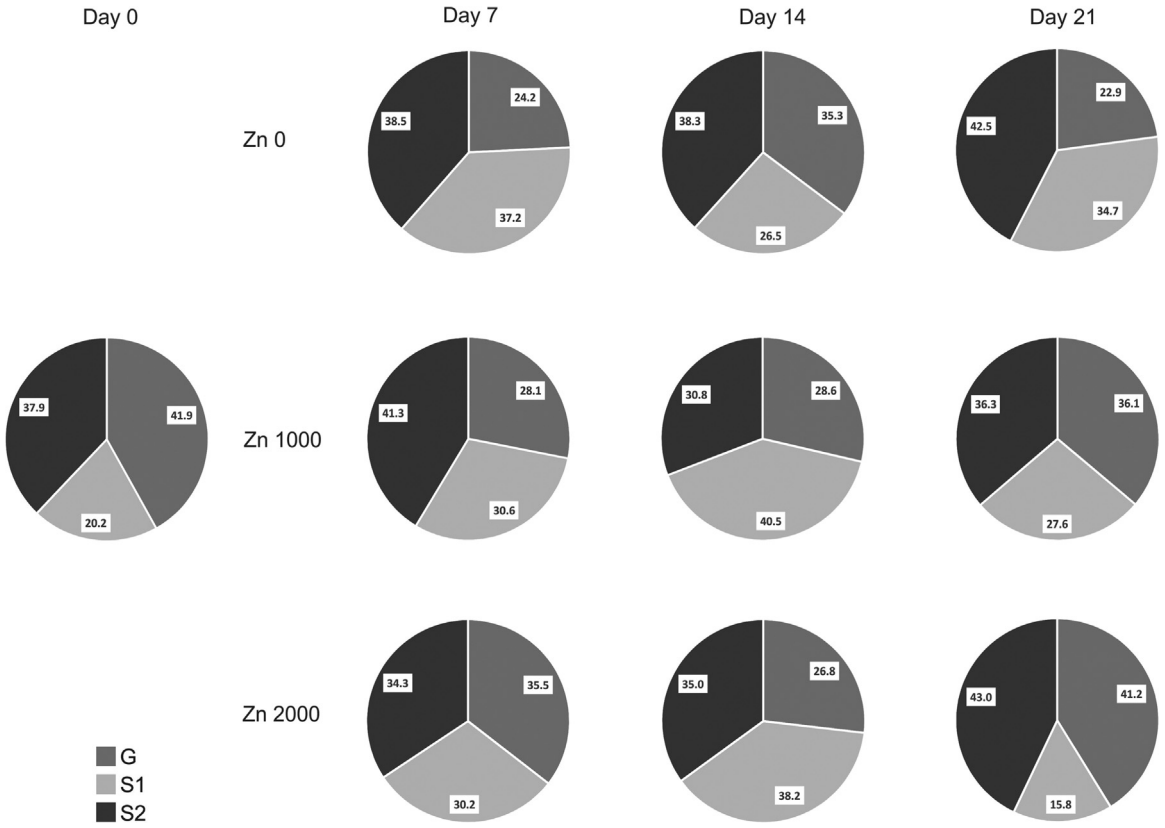

Fig. 4. Overall patterns of Zn distribution and the relative percentage of Zn retrieved from the different fractions in mealworm larvae (*Tenebrio molitor*) sampled before Zn exposure (day 0) and after 7, 14 and 21 days of exposure to 0, 1000 or 2000 mg Zn kg^{-1} in flour; S1 – cytosolic fraction, S2 – cellular debris, G - metal-rich granules. The percentage of Zn in each fraction is calculated by relating Zn retrieved from the fraction concerned to the total amount of Zn (sum of all fractions).

and Lanno, 2010), whereas in mealworms the predominant proteins in the S1 fraction are non-metallothionein proteins (Pedersen et al., 2007). For both *T. molitor* (this study) and abovementioned species of earthworms (Li et al., 2009; Pan and Wang, 2008; Vijver et al., 2006; Yu and Lanno, 2010) the lowest Cd concentrations were found in the G or granular fraction, regardless of the chemical form of Cd in the medium, which was either $CdCl_2$ (this study, Li et al., 2009; Pan and Wang, 2008), cadmium acetate (Vijver et al., 2006) or $Cd(NO_3)_2$ (Yu and Lanno, 2010). However, the form in which Cd was offered to isopods *Porcellio dilatatus* (Cd $(Cys)_2$ or $Cd(NO_3)_2$) altered both the bioavailability and the internal sequestration of the assimilated metal (Calhôa et al., 2011). The authors showed that isopods which were offered food contaminated with Cd-cysteinate had significantly more Cd distributed in the cell debris and organelles at the expense of allocation to granules.

Although Cd concentrations in the S1 fraction were affected by the concentration of Cd in the flour, the concentration of Cd in the flour did not affect significantly the percentage of Cd in this fraction. In the S1 fraction, the percentage of Cd ranged from 18 to 27% at day 7, from 27 to 43% at day 14 and from 25 to 46.5% at day 21. Similarly, although the Cd concentration in fraction G did increase linearly with the concentration of Cd in the flour, the proportion of Cd in the G fraction was not affected by the concentration of Cd in the flour. Cd percentage in the G fraction for all treatment groups ranged from 20% to 39%, with the exception of larvae treated with 300 mg kg^{-1} of Cd for 14 days, in which the percentage of Cd in the G fraction was 50% of the total Cd level in the body (Fig. 3). Such results are in contrast with the study on *E. fetida* exposed to Cd-contaminated soil, in which more than 80% of the total Cd was distributed in the cytosolic and organelle fraction (the S1 fraction in our study), and less than 1% of the total Cd existed in the granular fraction (Li et al., 2008). This may be explained by the fact that the authors exposed the earthworms to much lower Cd concentrations (1 mg kg^{-1} dry soil) than used in our study (100–300 mg kg^{-1} flour). The concentration of Cd used by Li et al. (2008) is within the range of 0.06 and 1.1 mg kg^{-1} typical for uncontaminated soils (Kabata-Pendias and Mukherjee, 2007). The allocation of Cd to different subcellular fractions of *T. molitor* and *E. fetida* can be also explained, at least partly, by the different uptake routes of Cd in these species. The main uptake route of Cd in *T. molitor* larvae is via food, while in earthworms the additional route of entry for Cd and other metals is via the skin. In contrast to earthworms, mealworm larvae have a water-impermeable integument and wax-coating (Vijver et al., 2003a) and live in dry medium. The proportion of Cd found in granules of *T. molitor* larvae is comparable with that for *Prodenia litura* larvae fed with Cd-enriched amaranth leaves (i.e., leaves obtained from plant reared on the soil spiked with aqueous solutions of $CdCl_2$) for 7 days (Ding et al., 2013). In both studies, there was a decrease in the percentage of Cd accumulated in the G fraction with increasing Cd concentration in food observed after 7 days of exposure: from 25 to 19% in *P. litura* (Ding et al., 2013) and from 38.6 to 25.6% in *T. molitor* (this study). These results indicate that transferring Cd into granules is not an effective detoxification strategy for either species.

In the control group of *T. molitor* larvae, granules were the main accumulation compartment for Cd. The control larvae maintained their internal Cd concentration at 0.16–0.36 mg kg^{-1} fresh weight, and the concentration of Cd in granules did not exceed 0.13 mg kg^{-1} after 21 days of exposure. However, the percentage of Cd in fraction G was significantly higher in control larvae compared to those exposed to Cd-contaminated flour. Unlike Cd, the distribution of Zn among the three fractions in Zn-exposed

larvae was close to that observed in control larvae. Even if the total internal Zn concentration increased with increasing Zn concentration in the flour, the metal was divided fairly equally between fractions regardless of the Zn concentration in the flour. This difference in sequestration of different metals over various subcellular compartments was also observed in the earthworm *A. caliginosa* by Beaumelle et al. (2015). The authors measured Cd and Zn concentrations in three subcellular compartments – cytosolic, granular and debris in *A. caliginosa* exposed to a range of field-collected metal contaminated soils and found that only partitioning of Cd was modified along the pollution gradient whereas Zn partitioning remained unchanged; the latter was explained by an effective regulation process (Beaumelle et al., 2015). Due to the biological essentiality of Zn, invertebrates not only sequester and detoxify any excess but, when needed, release Zn to meet the cells physiological requirements (Andre et al., 2010). Beaumelle et al. (2015) indicated also that both the total metal contents in soil and the free ion concentrations predicted by a geochemical speciation model better described the subcellular distribution of Cd and Pb in *A. caliginosa* than $CaCl_2$-extractable and dissolved metal concentrations in soil.

The variation in larval development time, the number of instars and the pattern of sequential instar development time of *T. molitor* depend on many factors, including temperature, humidity, photoperiod, parental age and food quality (see e.g., Morales-Ramos et al., 2010). This made impossible precise determination of the stage of larvae obtained from the pet shop for our study. However, even using a cohort would not let us overcome the problem of potentially different impacts of particular Cd or Zn treatments on the number of instars, as the number of instars in *T. molitor* increases in response to adverse conditions (Esperk et al., 2007). Therefore, we focused on collecting the wide range of masses of the larvae at each sampling date, and by using body mass as a covariate in ANOVA, the results were controlled for potential effects of body mass. Eventually, body mass appeared a significant covariate only in Zn-treated larvae. The previous study by Lindqvist and Block (1995) support our hypothesis that the size of individuals rather than moulting itself influenced the results, as only a small Cd loss during moulting was observed in *T. molitor* larvae, mostly due to the lack renewal of the midgut and a small degree of the cuticule reabsorbing (Lindqvist and Block, 1995). Also Vijver et al. (2003a), who analysed *T. molitor* larvae and their exuviae separately, confirmed similar or lower concentrations of different metals in the exuviae than body concentrations, and the mass of the exuviae was much lower than the body mass. This means that metal concentrations in the exuvium have a low impact on total body concentrations and therefore moulting does not form an important elimination route for metals in *T. molitor* (Vijver et al., 2003a).

The compartmentalization of metals in insects is important for the transport of metals between trophic levels in food webs and thus determines the distribution of metals in ecosystems. Not all metal fractions are likely to be transferred along the food chain, and the fraction stored in granules is hypothesized to be unavailable for assimilation by a predator (Vijver et al., 2004). The bioavailability of metals and, thus, their potential transfer in the trophic web vary according to the physico-chemical form (speciation) of a metal in a prey and the potential breakdown of metal complexes or granules during the digestion by a predator. Generally, it is accepted that metals present in the soluble form in a prey, i.e., cytosol and proteins, can be considered more bioavailable to a predator than metals bound to the insoluble fractions, i.e., cell walls, exoskeleton, and metal granules (Wallace et al., 2003). Wallace et al. (2003) postulated that Cd associated with the organelles, heat-denatured and heat-stable proteins of the prey (oligochaete *L. hoffmeisteri*) was trophically available and assimilated at high efficiency by the predator (grass shrimp *Palaemonetes pugio*), while Cd sequestrated to metal-rich granules was much less bioavailable to the predator (Wallace et al., 2003, 1998). Other authors showed, however, that metals bound to the insoluble fraction of a prey can be mobilized via enzymes and/or digestion at acidic pH (Bragigand et al., 2004; Wallace and Luoma, 2003). Because gut pH may play an important role in dissolving metal-rich granules, the bioavailability of metals stored in granules by *T. molitor* is likely to be higher for a predator with a low gut pH. How metal sequestration in *T. molitor* influences the sequestration mechanisms in its predators will be studied in a toxicokinetic experiment where *T. molitor* larvae previously exposed to Cd and Zn will be fed to the ground beetle *Pterostichus oblongopunctatus* (Coleoptera: Carabidae).

Acknowledgments

Financial support was provided by the Foundation for Polish Science (Project No. HOMING PLUS/2013-8/1) and by Jagiellonian University in Kraków (DS-758).

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