

Indication of metal homeostasis disturbance in earthworm *Eisenia fetida* after exposure to semi-solid depot sludge

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Abstract

Treated sewage sludge is commonly used in agriculture as fertilizer. It is, therefore, necessary to determine possible detrimental influences of sludge application on soil contamination and accumulation of contaminants in tissues of terrestrial animals, which in the long run could also have undesirable effects on humans. With that aim, the study was performed using earthworm *Eisenia fetida* as test organism and semi-solid depot sludge from wastewater treatment plant as exposure media. The concentrations of 26 metals/metalloids were determined in depot sludge, and their bioaccumulation was estimated in whole tissue of *E. fetida*, and for the first time in the soluble tissue fraction, which represents metal fraction available for metabolic requirements and toxic effects. Obtained results have revealed acceptable levels of several elements (Cd, Cr, Cu, Ni, Pb, Zn) in depot sludge, when compared to currently valid regulations, and only moderate accumulation of some elements (e.g. As, Ba, Cd, Co, Fe, Tl, V, and Zn) in earthworms, as a consequence of exposure to depot sludge. However, concentration increase after exposure to depot sludge was observed in *E. fetida* for several elements (Cd, Mo, and Zn), which were present in lower concentrations in the exposure mixtures than in soil. Contrary, concentration decrease was observed for Cs, Mn, and Rb, although they were present in higher concentrations in depot sludge than in soil. It was an indication of disturbance in metal homeostasis in earthworms, possibly caused by exposure to complex mixture of contaminants present in depot sludge. The cumulative effect of exposure to a number of various contaminants (inorganic, organic, microbiological and pharmaceutical), even if each of them was not present in very high concentrations, could have caused distress in earthworms exposed to depot sludge.

Keywords: earthworms, metal bioaccumulation, semi-solid depot sludge, soil metal contamination, metals in soluble tissue fraction

1. Introduction

Biosolids, such as active and depot sludge, are often used in agriculture as fertilizers, due to their ability to enhance growth of agricultural crops (Scheifler et al., 2003; Carpené et al., 2006; Braguglia et al., 2015). However, besides the obvious benefits of agricultural use of sludge, possible disadvantages should also be considered. As is well known, sludge can contain various organic, inorganic and microbiological contaminants, which could be transferred to soil or accumulated in crops, and consequently could have toxic effects on animal and human health (Scheifler et al., 2003; Carpené et al., 2006; Braguglia et al., 2015). Although the use of sewage sludge in agriculture is regulated by national and international directives (Minister of Environmental Protection, Physical Planning and Construction, 2008; Council of the European Communities, 1986), these regulations are unfortunately constrained on providing recommendations only for few selected contaminants. In addition to the fact that total content of contaminant in the soil is less important for risk assessment than its bioavailable amount (Nahmani et al., 2007a), the regulations also do not take into consideration the possibility of contaminant interactions, as well as the enhanced toxicity of complex mixtures of contaminants. Therefore, bioavailability and possible toxicity of multicontaminated soil could only be determined by exposure and effect assessment using various bioindicator organisms (Kamitani and Kaneko, 2007; Calisi et al., 2013).

According to the available literature, earthworms are one of the most adequate organisms for testing toxicity of soil (Nahmani et al., 2007a). They can accumulate high levels of contaminants due to their feeding habits (Morgan and Morgan, 1998; Carpené et al., 2006; Hsu et al., 2006), as well as due to dermal exposure of their whole body (Lanno et al., 2004; Kamitani and Kaneko, 2007). The most commonly used earthworm species for biomonitoring is *Eisenia fetida* (Nahmani et al., 2007a), which is recommended for use in several international standard toxicity tests (OECD, 2004). The reasons for its frequent use are related with its fast maturation, higher reproductive rate and a shorter generation time compared to other species, as well as responsiveness to a wide range of toxicants (Paoletti, 1999; Nahmani et al., 2007a). It can be easily cultured in large quantities in the laboratory, and is readily available from commercial suppliers (Nahmani et al., 2007a).

A large number of studies using soils prepared and contaminated with selected contaminants in the laboratory have been performed using earthworms as bioindicator organisms (Leveque et al., 2014; Irizar et al., 2015). However, Nahmani et al. (2007a) have pointed out that there is a need for further accumulation studies which use real contaminated soils with a variety of properties. In that way, synergistic effects of various contaminants present in the soil would be better understood and more useful data for risk assessment would be collected. Therefore, to follow the recommendations and cognitions of current scientific trends, we have aimed to perform a study of toxicity of real semi-solid depot sludge obtained from a wastewater treatment plant (WWTP) which purifies municipal, hospital

and sugar factory wastewaters. Special emphasis was directed on metal/metalloid contamination. The specific aims of the study were: (1) to define the level of metal/metalloid contamination of depot sludge; (2) to establish a baseline for metal/metalloid elements in whole earthworm tissues, as well as for the first time in the soluble tissue fraction, based on the measured concentrations in non-exposed organisms; and (3) to study the patterns of metal/metalloid variability in earthworms after exposure to depot sludge. Commonly, only few selected elements are monitored in the earthworms, such as Cd, Cu, Fe, Pb, and Zn (Carpené et al., 2006; Kamitani and Kaneko, 2007; Suthar and Singh, 2009; Li et al., 2010; Giska et al., 2014). Only seldom studies extend on larger number of elements (Nahmani et al., 2007b; Nahmani et al., 2009; Nannoni et al., 2014). In our study, 26 trace and macro elements were analysed, thus providing extensive database for future soil monitoring programmes. Our study was primarily focused on the analyses of contamination of the depot sludge with metals and metalloids and their accumulation in the earthworms. However, we have further estimated the overall effect that exposure to complex mixture of contaminants, such as the one present within the depot sludge, could have on earthworm general well-being.

2. Materials and methods

2.1. Experimental organisms

Adult *E. fetida* (Savigny, 1826) earthworms (Oligochaeta, Lumbricidae) were randomly collected from earthworm farm Eršek in Donja Bistra (Croatia). In the laboratory, they were maintained in the dark in glass terrarium filled with soil from the earthworm farm, under controlled temperature ($20\pm 1^\circ\text{C}$). Soil in glass terrarium was periodically moistened and pH was monitored. Since metal concentrations may vary considerably according to the stage of earthworm development (Ma, 2004), only adult specimens with well-developed clitellum and weighing 170.0 ± 14.8 mg were selected for this study.

2.2. Sewage sludge sampling

Semi-solid depot sewage sludge was sampled in April 2014 from depot of the municipal WWTP in Virovitica (Croatia), serving an area of about 20,000 population estimate. Depot is located near an agricultural area, approximately 1.5 km away from WWTP, and the sludge has been disposed there over the years. By primary and secondary processes, WWTP purifies municipal, hospital and sugar factory wastewaters. Basic properties of soil from the earthworm farm and of depot sludge are given in Table 1. Values of pH were determined by use of 3200P Benchtop pH Meter (Agilent Technologies, USA) and conductivity by use of Datalogging Conductivity TDS Meter – 850039 (Sper Scientific, USA).

2.3. Experimental design

For the present study, collected depot sludge was mixed with soil from the earthworm farm in order to achieve two exposure concentrations, 30% and 70% of depot sludge, which are much higher than concentrations usually applied for fertilization, but interesting for toxicity testing. The soil from earthworm farm itself was used as a control. The mixtures were manually homogenized and placed into two 3L glass jars, one containing 30% of depot sludge and the other one containing 70% of depot sludge. In each jar, 70 adult earthworms were inoculated and covered with a perforated lid, for requirements of this study and parallel studies of acute, semi-acute and chronic toxicity. During exposure period, jars were kept in the dark at $20\pm 1^\circ\text{C}$ and moisture was maintained at $\sim 30\%$. At the end of each exposure period, earthworms were randomly selected (five earthworms after 14 days and 3 earthworms after 28 days from each jar), rinsed with distilled water and placed on moist filter paper to remove gut content. After depuration which lasted 24 hours, earthworms were frozen at -80°C until further analyses. As a control group, five earthworms were taken directly from farm soil. Altogether, 21 earthworms were analyzed in this study. During the experiment, significant mortality of earthworms was not observed.

2.4. Digestion of soil and depot sludge

Dry samples of soil and depot sludge were ground and homogenized using an agate mill. Subsamples of ground and homogenized soils (0.1 g) were subjected to total digestion in the microwave oven (Multiwave 3000, Anton Paar, Graz, Austria) in two step procedure. The first step consisted of digestion with a mixture of HNO_3 (4 mL), HCl (1 mL) and HF (1 mL). It was followed by second digestion after addition of 6 mL of H_3BO_3 . Resulting solutions were transferred to a 100 mL flask and diluted with Milli-Q water. Prior to measurement, aliquots of these solutions were further diluted with Milli-Q water, 10 times for trace element analyses and 100 times for macro element analyses, and acidified with HNO_3 (Suprapur, Merck, Germany; final concentration in the samples 1.3%).

2.5. Isolation of soluble tissue fraction from earthworms

Homogenization of frozen earthworms was performed in five volumes of cooled homogenization buffer [100 mM Tris-HCl/Base (Sigma, pH 8.1 at 4°C) supplemented with reducing agent (1 mM dithiothreitol, Sigma) and inhibitors of proteolytic activity (0.5 mM phenylmethylsulfonylfluoride and 0.006 mM leupeptine)] by ten strokes of Potter-Elvehjem homogenizer (Glas-Col, USA) in an ice-cooled tube at 6,000 rpm. An aliquot of each homogenate was separated for digestion and determination of total metal concentrations. The remaining volumes of homogenates were centrifuged (Avanti J-E centrifuge; Beckman Coulter) at $50,000\times g$ for 2 h at 4°C . The obtained supernatants, which correspond to water-soluble tissue fraction, were separated and stored at -80°C for subsequent metal/metalloid analyses. Prior to measurement, supernatants were diluted with Milli-Q water, 10

times for trace element analyses, and 100 times for macro element analyses, and acidified with HNO₃ (Suprapur, Merck, Germany; final concentration in the samples: 0.65%).

2.6. Digestion of earthworm tissue homogenate

Digestion of earthworm tissue homogenate (0.3 mL) was performed in laboratory drying oven (ST-05, Instrumentaria, Croatia) for 3.5 h at 85°C in PFA (perfluoroalkoxy polymer resin) vials, by use of digestion mixture containing HNO₃ (1.35 mL; 65%, Suprapur, Merck, Germany) and H₂O₂ (0.45 mL; 30%, Suprapur, Merck, Germany). After digestion, obtained solutions were diluted with Milli-Q water to lower the acid level in the samples below 10%, 5 times for trace element analyses, and 20 times for macro element analyses.

2.7. Determination of metal and metalloid concentrations in soluble tissue fractions, digested tissue and digested soil and depot sludge

Measurements of 26 elements were performed on a high-resolution inductively coupled plasma mass spectrometer (HR ICPMS Element 2, Thermo Finnigan, Germany) equipped with an autosampler ASX 510 (CETAC Technologies, USA). Typical instrumental conditions and measurement parameters used throughout the work were reported previously (Fiket et al., 2007). Measurements of ⁷Li, ⁸²Se, ⁸⁵Rb, ⁹⁸Mo, ¹¹¹Cd, ¹³³Cs, ²⁰⁵Tl, ²⁰⁸Pb, and ²³⁸U were operated in low-resolution mode; of ²³Na, ²⁴Mg, ²⁷Al, ⁴²Ca, ⁴⁷Ti, ⁵¹V, ⁵²Cr, ⁵⁵Mn, ⁵⁶Fe, ⁵⁹Co, ⁶⁰Ni, ⁶³Cu, ⁶⁶Zn, ⁸⁶Sr, and ¹³⁸Ba in medium resolution mode; and of ³⁹K and ⁷⁵As in high resolution mode. External calibration was performed using standard solutions prepared from multielement stock standard solution for trace elements (Analytika, Czech Republic) in which single element standard solution of U (Aldrich, USA) was added. Separate external calibration was performed for macro elements using standard containing Ca, Mg, Na, and K (Fluka, Germany). All standards were prepared in 1.3% HNO₃ (Suprapur; Merck, Germany). Prior to measurement, In (1 µg L⁻¹; indium atomic spectroscopy standard solution, Fluka, Germany) was added to all samples and standards as internal standard (Fiket et al., 2007). Quality control of measurements on HR ICP-MS was performed by simultaneous analysis of the blanks and quality control (QC) samples for trace metals (QC trace metals, catalog no. 8072, lot no. 146142-146143; UNEP GEMS, Burlington, Canada) and for macro elements (QC minerals, catalog no. 8052, lot no. 146138-146139; UNEP GEMS). The results of quality control are presented as supplementary information (Table SI-1). Additional control of measurements in digested soil samples was performed by analysis of certified reference material for soil (NCS DC 73302, also known as GBW 07410, China National Analysis Center for Iron and Steel, Beijing, China). Good agreement was obtained between analysed and certified concentrations in the certified reference material for soil, within analytical uncertainties for all measured elements (±10%). Limits of quantification (LOQ) for measurements of trace and macro elements in the soluble tissue fractions and in the acid digested tissues of earthworms were calculated

based on three standard deviations of five measurements performed in adequate blank samples (Table SI-1).

2.8. Data processing and statistical analyses

Statistical program SigmaPlot 11.0 for Windows was applied for graph creation and statistical analyses. Comparison of metal/metalloid concentrations in soil and depot sludge was performed by t-test. Since data for metal/metalloid concentrations in earthworms were not normally distributed, and sample size was small and unequal (n=3-5), nonparametric statistical analysis was applied. Kruskal-Wallis one way analysis of variance on ranks with *post hoc* Dunn's test was used for comparison of metal/metalloid concentrations measured in different exposure groups, for both soluble fraction and acid digested tissue. By use of Spearman correlation coefficient we have defined which soluble metals and metalloids in earthworms exhibit similar pattern of variability (n=21). The same analysis was performed to compare the variability of each metal/metalloid in soluble and total fraction of earthworms (n=18).

3. Results

3.1. Metal and metalloid concentrations in soil and depot sludge

Concentrations in depot sludge were found to be significantly higher than in soil for several metals/metalloids: Al, As, Ba, Ca, Co, Cr, Cs, Fe, Li, Na, Ni, Pb, Rb, Sr, Tl, U, and V (Table 2). Metals/metalloids that were significantly less present in the sludge compared to control were Cd, Cu, K, Mo and Zn (Table 2). For earthworm exposure, mixtures containing 30% and 70% of depot sludge were prepared and concentrations of above listed metals/metalloids were approximately 1.5-2 times higher in 30% mixture and 2.5-3 times higher in 70% mixtures compared to soil. Contrary, concentrations of few metals were lower in 30% and 70% mixtures than in soil: Cu (~10%), Cd, K and Zn (10% and 30%, respectively), whereas the most pronounced decrease was observed for Mo (20% and 60%, respectively).

3.2. Soluble metals and metalloids in earthworms

Concentrations measured in the earthworms taken from farm soil, considered as a control group, for 25 analysed elements can be regarded as the baseline values for soluble tissue fraction of *E. fetida* (Fig. 1). Changes of soluble metal/metalloid concentrations in earthworms after exposure to depot sludge referred at the most to 1.5-3 fold concentration increase of some metals/metalloids or 2-2.5 fold decrease of the others (Fig. 1). The most pronounced increase (3-fold) was observed for As, Ba, Cr, Li, Ni, Se, and V, whereas the least variability was observed for four macro elements (Ca, K, Mg, and Na) and for Sr. Furthermore, three common patterns of variability of metal/metalloid concentrations were observed after exposure to two mixtures of depot sludge (30% and 70%) lasting 14 and 28 days:

- 1) First pattern referred to continuous concentration increase during exposure period, and included eight elements: As, Ba, Cd, Co, Fe, Tl, V, and Zn. This was confirmed by high statistically significant positive inter-correlations between four of these elements (As, Cd, Co, and Fe; $r=0.742-0.906$, $p<0.001$), as well as their somewhat weaker correlations with V and Zn ($0.499-0.669$, $p<0.05$). However, only for Ba and Zn comparable increase was observed after exposure to both mixtures. For Tl and V increase was more pronounced after exposure to 70% mixture ($r_{Tl-V}=0.705$, $p<0.001$) and for Cd and Co it was more pronounced after exposure to 30% mixture. An increase in accumulation of As and Fe was only observed after exposure to 30% mixture.
- 2) Second pattern referred to opposite trend of continuous concentration decrease during exposure period, and included only four elements: Cs, Mn, Na, and Rb. Although continuous decrease was observed after exposure to both mixtures, it was more pronounced after exposure to 70% mixture for Cs, Mn, and Rb (inter-correlations $r=0.583-0.639$, $p<0.01$). For Na, merely slight decrease was observed after exposure to 70% mixture.
- 3) Third pattern referred to concentration increase after 14 days of exposure, and subsequent decrease to initial concentrations after 28 days of exposure, and included 10 elements: Ca, Cr, Cu, Li, Mo, Ni, Pb, Se, Ti, and U. The concentration increase of Ca, Pb, Ti, and U after 14 days was comparable after exposure to 30% and 70% mixture. For all the other elements (Cr, Cu, Li, Mo, Ni, and Se), the concentration increase observed after 14 days was more pronounced after exposure to 70% mixture. The inter-correlations between all these elements were positive, and mostly statistically significant, and especially within the group of five elements (Cr, Cu, Mo, Ni, and Se; $r=0.471-0.946$; $p<0.05$ to $p<0.0001$).

3.3. Total metals and metalloids in earthworms

Total metal/metalloid concentrations in the whole tissue of earthworms were also measured, with the exception of the group exposed to 30% mixture for 28 days, due to small available quantity of samples. Same as stated for metal/metalloid concentrations in the soluble tissue fractions, total tissue concentrations measured in the control group can be regarded as the baseline values of *E. fetida* (Table 3). Changes of total metal/metalloid concentrations in earthworms after exposure to depot sludge were mainly even less pronounced than changes of their soluble concentrations, and referred for the most elements to either 1.1-2 fold concentration increase or 20-60% concentration decrease (Table 3). Somewhat higher increase (2.5-6-fold) was observed for Al, Ba, Ca, Li, Mn, Pb, Sr, Ti, and U. The patterns of total and soluble concentration variability were compared, indicating that although several elements followed the same trend as soluble metals/metalloids, some of them changed in a different way.

- 1) Concentrations of total As, Cd, Co, Fe, Tl, V, and Zn showed the increasing trend, Na and Rb decreasing trend, whereas total Ca, Cu, Pb, Se, and U increased after 14 days of exposure and

then decreased again, which was comparable to the pattern of changes of their soluble concentrations. This was confirmed by high positive correlations between soluble and total concentrations of As, Cd, Co, Cu, Na, Pb, Rb, Se, Tl, and U ($r=0.637-0.962$; $p<0.05$ to $p<0.0001$), as well as of Fe and V ($r=0.533$; $p=0.06$). However, the variability of total Ca during exposure period was much more observable than of soluble Ca, and referred to 6-fold concentration increase after 14 days of exposure compared to only 10% increase of soluble Ca. It also resulted with lower correlation between soluble and total Ca concentrations ($r=0.379$; $p>0.05$). Similarly to total Ca, total Sr increased 3-fold after 14 days of exposure and then decreased to its initial values, whereas its soluble form exhibited only slight concentration variability.

- 2) Total concentrations of following elements changed in different way than their soluble concentrations after exposure to depot sludge: total Ba increased after 14 days and then decreased again, contrary to continuous increase of its soluble form; total Cs continuously increased during the exposure to depot sludge, whereas total Mn increased after 14 days and then decreased again, contrary to continuous decrease of their soluble forms; total Cr, Li, Mo, Ni, and Ti continuously increased after exposure to depot sludge, contrary to increase of their soluble forms after 14 days of exposure and subsequent decrease after 28 days.
- 3) Total concentration of Al was continuously increasing during the exposure period, and the increase was more pronounced after exposure to 30% sludge mixture, whereas soluble form of Al was at the level of LOQ, and thus could not be discussed.

It was interesting to observe that increase of total metal/metalloid concentrations was generally more pronounced after exposure to 30% than 70% mixture of depot sludge. This was opposite to observations for soluble metal/metalloid concentrations, which mainly exhibited more intense changes after exposure to 70% mixture.

3.4. Percentage contribution of soluble to total metal and metalloid levels in earthworms

Comparison of metal/metalloid concentrations in soluble tissue fraction with their total concentrations in whole earthworm tissue enabled grouping of metals/metalloids according to their portion in the soluble form (Table 4). Elements which presence in the soluble form was almost negligible were Al, Cr, and Ti (less than 5%), whereas elements completely present in the soluble form were As, Cd, Na, Rb, Se, and Zn (100-200%). Even higher concentrations obtained in the soluble fraction than in total tissue for few elements (As, Cd, Se, and Zn) were possibly the consequence of uneven metal/metalloid distribution in tissue homogenate, a part of which was separated for digestion and subsequent determination of total metal/metalloid concentrations. The other possible cause for the observed discrepancy is the effect of matrix on measurements in soluble tissue fraction, since it was only diluted prior to analyses, and not digested like whole earthworm tissue. As could be expected the best

matching of soluble and total metal/metalloid concentrations was obtained for the elements which were mainly present in the soluble fraction (As, Cd, Na, Rb, and Se; $r_{\text{sol-tot}}=0.802-0.962$; $p<0.0001$).

4. Discussion

4.1. Characterization of depot sludge

Since depot sludge is commonly used in agriculture for soil enrichment and fertilization, it is of utmost importance to define possible negative effects of its application on terrestrial organisms. In this study, soil mixtures containing 30% and 70% of depot sludge were used, and they were characterized by following metal/metalloid composition (on wet mass basis, Table 2): the concentrations above $1,000 \mu\text{g g}^{-1}$ were found for $\text{Ca}>\text{Al}>\text{K}>\text{Fe}>\text{Mg}>\text{Na}>\text{Ti}$, from $100-1000 \mu\text{g g}^{-1}$ for $\text{Mn}>\text{Ba}>\text{Sr}$, from $10-99 \mu\text{g g}^{-1}$ for $\text{Zn}>\text{Rb}>\text{V}>\text{Cr}>\text{Li}>\text{Cu}>\text{Ni}>\text{Pb}$, from $1-9 \mu\text{g g}^{-1}$ for $\text{Co}>\text{As}>\text{Cs}>\text{U}>\text{Mo}$ and the lowest concentrations below $1 \mu\text{g g}^{-1}$ for $\text{Cd}>\text{Tl}>\text{Se}$. Among analysed elements in this study, only five metals are regulated in depot sludge intended for agricultural use. Their permitted levels ($\mu\text{g g}^{-1}$, expressed on dry mass basis) defined by Croatian (Minister of Environmental Protection, Physical Planning and Construction, 2008) and European provisions (Council of the European Communities, 1986) are, respectively, the following: Cd 5 and 20-40; Cu 600 and 1000-1750; Ni 80 and 300-400; Pb 500 and 750-1200; Zn 2000 and 2500-4000 (Minister of Environmental Protection, Physical Planning and Construction, 2008; and Council of the European Communities, 1986; respectively); and Cr 500 (Minister of Environmental Protection, Physical Planning and Construction, 2008). The concentrations of these elements in depot sludge, after multiplication with fresh to dry tissue mass ratio (1.42), were still manifold lower compared to regulations. In addition, based on the comparison with previously published concentrations of several metals (Cd, Cu, Fe, and Zn), depot sludge used in our study could be categorized as uncontaminated or weakly contaminated soil (Carpené et al., 2006). However, increased concentrations of ammonia, nitrate, phosphate, total nitrogen and phosphorus, as well as high total bacteria count were found in treated wastewater discharged from the same WWTP from which depot sludge used in this study was acquired (unpublished results). It was an indication that depot sludge probably contained other inorganic and organic contaminants in addition to metals and metalloids. A pharmaceutical contamination could also be presumed, based on high resistance profiles towards tested antimicrobial compounds which were established for treated wastewater (unpublished results). It was probably a consequence of hospital discharge contribution, which is treated at that same municipal WWTP.

4.2. Patterns of variability of metal/metalloid concentrations in soluble tissue fractions

It has often been pointed out that soil chemical analysis is not sufficient to assess the risk of deleterious effects of contaminants present in the soil on the biota (Sanchez-Hernandez, 2006). It does

not take into account toxicity of non-analysed chemicals or the interactive effects of contaminants (Calisi et al., 2013). Taking that in consideration, as well as the fact that only moderately higher metal/metalloid concentrations were measured in depot sludge than in soil, it had to be considered that contaminants and factors other than metal exposure could have brought about at least a part of the observed variability in metal/metalloid accumulation in the soluble tissue fractions of earthworms in this study. Nahmani et al. (2007b) also concluded that factors other than metal concentrations had impact on life cycle parameters of metal exposed earthworms. Accordingly, it is possible that toxicological effects on earthworm metabolism, as well as on ion transport and excretion systems, could have occurred as a consequence of exposure of earthworms to complex mixture of contaminants in depot sludge. It could have resulted in various changes of metal concentrations in earthworm tissues. This assumption is in accordance with significant inhibition of multixenobiotic resistance transport activity, histopathological alterations of body wall and intestine, significant increase of oxidative stress, as well as behavioural changes which were found in *E. fetida* exposed to same depot sludge as used in this study (unpublished results).

Many studies have reported that concentrations of metals (such as Cd, Cu, Fe, Pb, and Zn) in whole worms are directly related to availability of metals in soil (Morgan and Morgan, 1998; Suthar and Singh, 2009; Nannoni et al., 2014). The uptake of Cd and Pb in earthworms was even sometimes characterized by linear pattern, due to their commonly very slow or non-existent elimination (Giska et al., 2014). The continuous concentration increase during the exposure period which was observed for As, Ba, Cd, Co, Fe, Tl, V, and Zn in this study, therefore, would not be surprising, if only the concentrations of all eight elements were also increased in the exposure mixtures. However, the concentrations of Cd and Zn were lower in the depot sludge than in soil. And, still, slight accumulation of both Cd and Zn was recorded. The ability of earthworms for high Cd accumulation is probably related to the induction of Cd-binding proteins which have characteristics of metallothioneins (MT) (Suzuki et al., 1980; Morgan et al., 1989; Morgan and Morgan, 1998; Calisi et al., 2009; Calisi et al., 2011). The accumulation of some other metals, like Cu, Pb and Zn, in earthworms was also described as a result of their binding to MTs (Kagi and Kojima, 1987; Maity et al., 2011). But, as is well known, the synthesis of MTs can be induced by other compounds, such as antibiotics, vitamins and herbicides, and not only metals (Amiard et al., 2006). Exposure to such contaminants in the depot sludge was plausible, and could have resulted with increased MT level, which in return could have bound additional quantity of Cd and Zn.

Contrary to Cd and Zn, which demonstrated accumulation in the earthworms exposed to depot sludge, despite of lower metal exposure, the concentrations of several other elements decreased in the earthworms exposed to depot sludge, although their concentrations in the exposure mixtures were either higher (Cs, Na, and Rb) or equal (Mn) to concentrations in soil. It is possible that they were

displaced as a consequence of enhanced accumulation of several other metals, since some metal cations may be sufficiently similar to replace other cations. Due to existence of transporters of low specificity, different cations can use the same transport system (Barton, 2005): the cation which is present in higher concentration in the environment, or has a higher affinity for the transport protein, will enter the cell in higher concentration or even cause removal of the other cation. For example, in bacteria Mn^{2+} , Ni^{2+} and Co^{2+} use the same non-specific transport system for Mg^{2+} , whereas Cd^{2+} uptake by bacteria takes place by either Zn^{2+} or Mn^{2+} transport system (Barton, 2005). Therefore, observed decrease of Mn concentrations in earthworms could be hypothetically associated to concurrent concentration increase of Co, Cd and Zn. Similarly, in bacteria Tl^{2+} , Rb^{2+} and Cs^{2+} , as chemical analogues of K^+ , could be transported into the cell through the same nonspecific K^+ transport system (Barton, 2005). Thus, observed concentration decrease of Rb and Cs in earthworms could be possibly connected to simultaneous concentration increase of Tl. It was already reported that very strong toxicity of Tl on growth and reproduction of *E. fetida* may be related to its competition with the physiologically essential element K (Fischer and Molnár, 1997).

Third pattern of variability, observed for Ca, Cr, Cu, Li, Mo, Ni, Pb, Se, Ti, and U, referred to initial concentration increase after 14 days of exposure, which was followed by decrease to original concentrations after 28 days. It is, however, interesting to point out that among these 10 elements, Cu and especially Mo were present in the exposure mixtures in lower concentrations than in soil. Similarly to previously mentioned Zn increase, initial increase of their concentrations could not be the result of increased metal exposure. In addition, earthworms very efficiently physiologically regulate body concentrations of essential elements until a certain threshold (Spurgeon and Hopkin, 1999) and many authors have reported lack of concentration increase after exposure of earthworms to Cu and Zn in soil (Nahmani et al., 2007b; Giska et al., 2014; Nannoni et al., 2014). Concentration increase of these essential elements in our study, therefore, was possibly a consequence of some disturbance in their homeostasis. For example, it was previously reported that, next to high-affinity transport systems, molybdate anions are also cotransported by sulphate and phosphate transporters (Mendel and Schwarz, 2002). Consequently, any factor that stimulates the activity of those transporters to intensify sulphate and phosphate uptake would probably also result in enhanced Mo uptake. On the other hand, metal concentration decrease which has occurred after 28 days of exposure to mixtures of depot sludge was possibly a consequence of an increase in elimination rate or a toxicological effect on earthworm metabolic rate (Nahmani et al. 2007a), due to prolonged exposure to complex mixture of contaminants. It was previously described that many metals show high affinity for accumulation and immobilization in earthworm alimentary canal (Morgan and Morgan, 1998), which inner coelomic wall is abundantly covered by vesicular cells (Sharma, 2010). These cells, known as chloragogen or yellow cells, absorb nitrogenous waste from the blood vessels and deposit it in a form of granules of chloragosome, which is excretory substance within their cytoplasm (Sharma, 2010). In time,

chloragogen cells increase in size, get detached from the coelomic epithelium and break up in the coelomic fluid, from where they are removed through the dorsal pores or septal nephridia (Sharma, 2010). In the animals from uncontaminated sites, the major elemental components of the chloragosomes were P, Ca, S and Zn (Morgan and Morgan, 1998). However, chloragosomes possess significant cation-exchange capacities (Fischer, 1977). They are, therefore, able to accumulate and immobilize many metals via a mechanism of cationic displacement of Ca, resulting in diminished chloragosomal Ca (Morgan and Morgan, 1998). It is, therefore, possible that decrease of soluble Ca, which was even more observable for total Ca concentration after 28 days of exposure, was a consequence of Ca displacement due to accumulation of several other metals in chloragosome. In addition, due to toxic effect of prolonged exposure to depot sludge, it is also possible that removal of chloragogen cells from coelomic cavity was accelerated. It was in agreement with previously described increased turnover rate of chloragocytes after exposure of earthworms to organic chemicals (Sforzini et al., 2015). Several other studies have also shown that chloragocyte depletion may occur in worms as a way of eliminating toxic chemicals (Fischer and Molnár, 1992; Cancio et al., 1995). Consequently, depletion of chloragocytes would cause loss of the metals which possess affinity for accumulation in chloragosome. For example, the loss of Pb was observed after 28 days of exposure, but not of Cd, which is in accordance with reports on Pb accumulation in chloragosome of *Aporrectodea caliginosa*, but not Cd (Morgan and Morgan, 1998). The hypothesis on involvement of chloragocytes in metal loss after 28 days of exposure to depot sludge can be further confirmed by much more severe damages on chloragogenous tissue observed in *E. fetida* after 28 days than after 14 days of exposure to the same depot sludge as used in this study (severe necrosis vs. atrophy and aggregation) (unpublished results).

4.3. Total metal/metalloid concentrations in earthworms

As expected, the highest total metal concentrations in the earthworms were measured for four macro elements (Na, K, Ca, Mg). Among trace elements, the highest concentrations were measured for Fe, as previously observed for the earthworm *Allolobophora caliginosa* (Carpené et al., 2006). For many elements, total concentrations in the tissues of earthworms were much higher than their soluble concentrations, indicating that only small metal/metalloid percentage is present in soluble, metabolically and toxicologically available form. Partly it could be attributed to the fact that portions of metals which entered the cells were detoxified and immobilized in a form of insoluble granules (Morgan and Morgan, 1998). Additional explanation is based on the fact that earthworms extract their nutrients, including heavy metals, by the ingestion of large amounts of soil (Carpené et al., 2006). Therefore, to remove the soil, they have to be depurated prior to metal analyses. However, depuration should not last too long to prevent tissue detoxification and excretion of metals (Nahmani et al., 2007a). Consequently, it was also possible that some soil remained within earthworm alimentary canal or even on its body surface. Thus, the influence of residual soil content on total metal content should

also be considered. In that case, higher presence in insoluble fraction could be expected for elements which were present in the soil in high concentrations, such as Al, Ti, Fe ($>1000 \mu\text{g g}^{-1}$) and Mn ($400\text{-}500 \mu\text{g g}^{-1}$). And, indeed, more than 85% of these metals were present in the insoluble form. This was especially evident for Al, which soluble portion was extremely low (0.5%), and could not be reliably detected ($<1 \mu\text{g g}^{-1}$), contrary to its total concentrations in earthworms, which were rather high ($\sim 20\text{-}60 \mu\text{g g}^{-1}$) and continuously increased during the exposure period. For many elements, the pattern of variability of soluble and total concentrations was comparable, but for some of them it was evidently different. Since the variability of total metal concentrations reflects both soluble metals and metals immobilized in granules or contained in the residual soil, it was not surprising that the most pronounced differences between pattern of variability of soluble and total metal concentrations was observed for eight elements largely present in the insoluble form, i.e. Ba (40%), Cs (60%), Tl (65%), Mo (74%), Mn (85%), Ni (86%), Li (87%), and Cr (97%).

In addition, contrary to observations made for the soluble metal concentrations, increase of total metal/metalloid concentrations was more pronounced after exposure to mixture containing 30% than after exposure to mixture containing 70% of depot sludge. Depot sludge was abundant in Al and Fe, and as is well known, metal sorption on particles like Al and Fe oxyhydroxides, when they are present in increased concentrations in soil, causes reduction of soluble metal concentrations in soil and consequently decrease of metal accumulation (Janssen et al., 1997; Peijnenburg et al., 1999; Nahmani et al., 2007a). Furthermore, previous studies have reported decrease of contaminant bioaccumulation by earthworms as their concentrations in soil increase due to reduced activity of earthworms (Sample et al., 1998). It is possible that earthworms have ingested less soil under the conditions of higher toxicity of mixture containing 70% of depot sludge, which consequently influenced more insoluble fraction, and thus also total concentrations, than soluble metal/metalloid fraction in earthworm tissue.

5. Conclusion

Analyses of metal/metalloid concentrations in soil and depot sludge from wastewater treatment plant which purifies municipal, hospital and sugar factory wastewaters revealed metal contamination which was within permitted limits of currently valid regulations. In addition, analyses of metal/metalloid concentrations in earthworm *E. fetida* after exposure to semi-solid depot sludge indicated continuous moderate accumulation during entire exposure period only for As, Ba, Cd, Co, Fe, Tl, V, and Zn. However, despite the fact that only weak increase in metal/metalloid concentrations was observed in earthworms after exposure to depot sludge, some disturbances in metal homeostasis have probably occurred. Continuous or temporary concentration increase was observed for several metals, such as Cd, Mo and Zn, which were present in lower concentrations in depot sludge than in soil. Contrary, the loss of several other metals, such as Cs, Mn and Rb was recorded, although they were present in increased concentration in depot sludge. Obviously, exposure to depot sludge, which presents complex

mixture of different contaminants, could cause nonspecific disturbances in metal transport. They could potentially result in diverse toxic effects, either as a consequence of enhanced uptake of toxic elements or due to loss of essential elements. Although depot sludge was only weakly contaminated by metals and metalloids and, therefore, each particular contaminant present in it did not pose a risk for earthworm health by itself, the complex combination of numerous contaminants which constitutes depot sludge obviously could cause distress for terrestrial organisms. Additional benefit of the study was identification of soluble tissue fraction of earthworms as a more reliable and relevant compartment for metal determination in process of metal exposure evaluation than commonly used total acid digested tissue. Metal concentrations determined in soluble tissue fraction of earthworms refer mainly to metals which are accessible for both metabolic requirements and possible toxic effects, contrary to total metal concentrations, which comprise both potentially toxic metals and metals which are immobilized and thus detoxified. In addition, specific difficulty of working with earthworms as test organisms is probability that some soil will remain within their body after exposure experiments. Our study demonstrated that residual soil had the main impact on insoluble metal levels and thus ultimately affected total metal concentrations, whereas soluble metal concentrations more realistically reflected actual metal exposure in soil.

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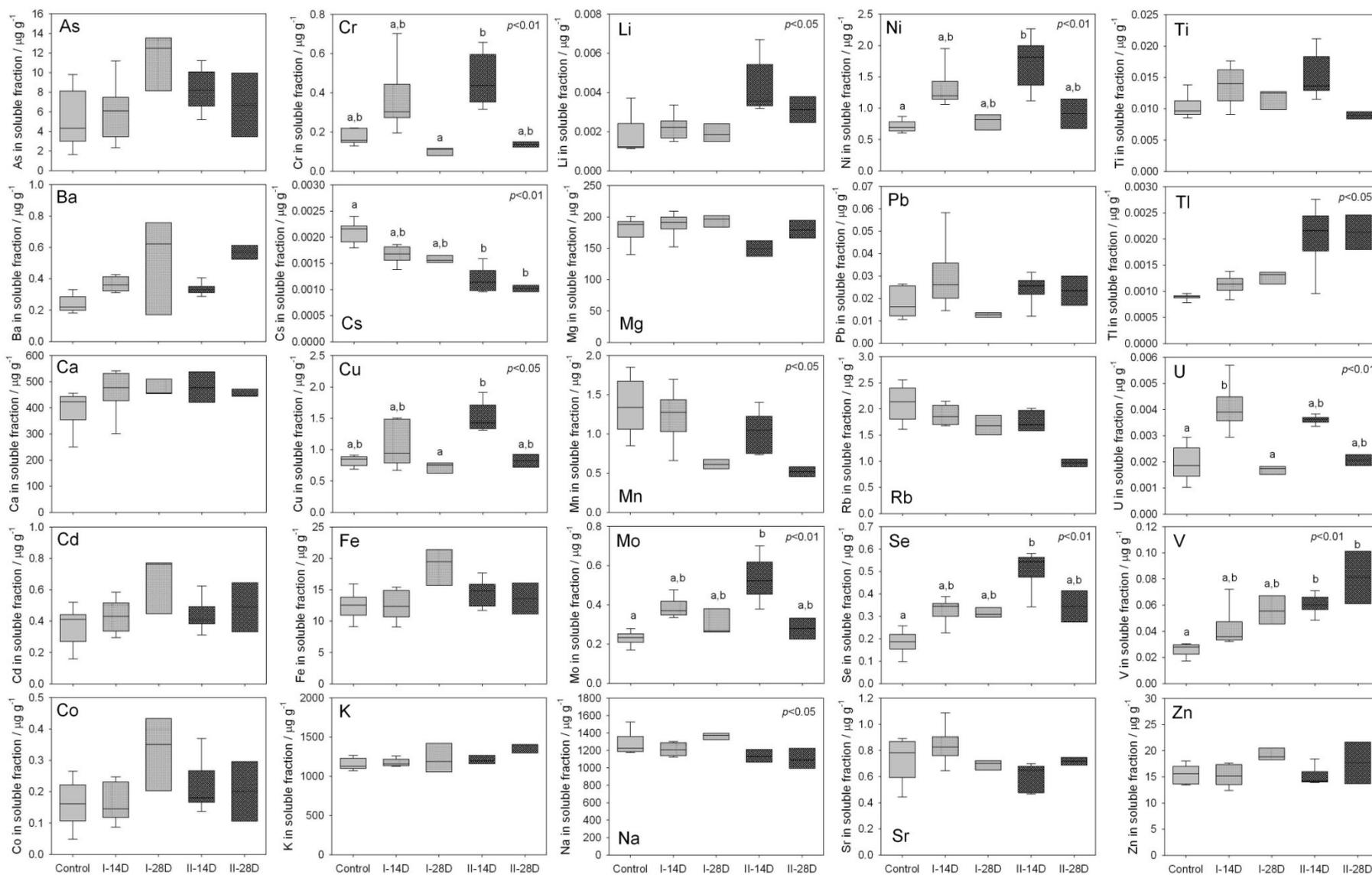
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Figure legends

Figure 1. The concentrations ($\mu\text{g g}^{-1}$; on wet mass basis) of 25 metals/metalloids (As, Ba, Ca, Cd, Co, Cr, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, Pb, Rb, Se, Sr, Ti, Tl, U, V, Zn) in the soluble tissue fractions of the earthworm *Eisenia fetida* from soil and after 14 days (14D) and 28 days (28D) of exposure to mixtures containing 30% (I) and 70% (II) of depot sludge. The results are presented as box-plots. The boundaries of box-plot indicate 25th and 75th percentiles; a line within the box marks the median value; whiskers above and below the box indicate 10th and 90th percentiles. Differences among exposure groups are indicated with different letters (a, b), based on Kruskal-Wallis one way analysis of variance on ranks (p values indicated in figures) and *post hoc* Dunn's test ($p < 0.05$). Number of samples per each group was the following: control $n=5$; I-14D $n=5$; I-28D $n=3$; II-14D $n=5$; II-28D $n=3$.

Figure 1.



Highlights

- Exposure of earthworm *Eisenia fetida* to depot sludge - complex contaminant mixture
- Bioaccumulation of 26 metals/metalloids in whole earthworm tissue
- First data for metal/metalloid levels in soluble tissue fraction of *E. fetida*
- Continuous mild increase of As, Ba, Cd, Co, Fe, Tl, V, Zn during exposure period
- Disturbance of metal homeostasis in earthworms due to exposure to depot sludge

Table 1. Basic properties of soil from earthworm farm and depot sludge from wastewater treatment plant.

	Soil	Depot sludge
Texture	solid	semi-solid
Composition	mixture of loam, compost and peat in equal proportions	mixture of liquid active sludge removed after secondary WWTP treatment and semi-solid sludge from the sugar factory
pH (1:2.5 H₂O)	7.80 ± 0.3	7.61 ± 0.4
Conductivity (1:2.5 H₂O)	7.94-8.08 μScm ⁻¹	383.0-385.2 μScm ⁻¹
Moisture	75%	30%

Table 2. Measured metal/metalloid concentrations ($\mu\text{g g}^{-1}$, on wet mass basis) in soil and in depot sludge (mean \pm standard deviation), and calculated concentrations for exposure mixtures containing 30% and 70% of depot sludge.

	Soil	Depot sludge	30% mixture	70% mixture
Al*	11,626 \pm 586	49,594 \pm 1,770	23,016	38,204
As*	1.96 \pm 0.07	6.59 \pm 0.96	3.35	5.20
Ba*	142.4 \pm 2.43	311.2 \pm 18.7	193.0	260.5
Ca*	24,197 \pm 272	80,165 \pm 10,418	40,988	63,375
Cd*	0.423 \pm 0.007	0.219 \pm 0.023	0.362	0.280
Co*	2.82 \pm 0.06	8.57 \pm 0.58	4.55	6.84
Cr*	16.38 \pm 0.86	58.94 \pm 3.34	29.15	46.17
Cs*	1.02 \pm 0.03	3.28 \pm 0.17	1.69	2.60
Cu*	21.10 \pm 0.63	17.35 \pm 0.39	19.97	18.47
Fe*	6,092 \pm 268	24,349 \pm 2,002	11,569	18,872
K*	23,623 \pm 1,766	12,569 \pm 389	20,307	15,885
Li*	8.38 \pm 0.26	29.63 \pm 1.24	14.75	23.25
Mg	7,529 \pm 124	7,612 \pm 219	7,554	7,587
Mn	436.0 \pm 10.9	517.7 \pm 140.0	460.5	493.2
Mo*	1.86 \pm 0.40	0.321 \pm 0.025	1.40	0.78
Na*	2,816 \pm 86	10,185 \pm 397	5,027	7,974
Ni*	9.16 \pm 0.18	23.57 \pm 1.44	13.48	19.25
Pb*	5.88 \pm 0.10	16.20 \pm 0.60	8.97	13.10
Rb*	45.01 \pm 1.03	71.74 \pm 3.21	53.03	63.72
Se	ND	ND	-	-
Sr*	63.08 \pm 1.80	172.5 \pm 24.2	95.90	139.65
Ti	997.6 \pm 4.4	4319 \pm 178	1,994	3,322
Tl*	0.131 \pm 0.005	0.395 \pm 0.008	0.210	0.316
U*	0.515 \pm 0.012	1.80 \pm 0.10	0.899	1.411
V*	19.00 \pm 0.56	71.43 \pm 2.83	34.73	55.70
Zn*	95.20 \pm 13.86	53.37 \pm 2.13	82.65	65.91

* statistically significant difference between concentrations in soil and in depot sludge, according to t-test ($p < 0.01$)

ND – nondetectable.

Table 3.Total metal/metalloid concentrations ($\mu\text{g g}^{-1}$; on wet mass basis)in acid digested tissue of earthworm *Eisenia fetida*(median, with minimum and maximum in brackets). Legend: exp I - exposure to mixture containing 30% of depot sludge; exp II - exposure to mixture containing 70% of depot sludge.

	Control	Exp I / 14 days	**ExpI / 28 days	Exp II / 14 days	Exp II / 28 days
Al	17.22 (3.60-21.61)	63.38 (20.15-63.57)	-	34.17 (6.77-36.79)	45.23 (16.24-53.82)
As	3.00 (0.81-5.89)	3.92 (2.32-4.38)	-	4.49 (2.33-5.70)	2.09 (1.76-5.13)
Ba	0.72 (0.24-0.98)	2.21 (0.87-2.52)	-	2.04 (0.57-2.13)	0.56 (0.17-0.59)
Ca*	651 (416-807) ^a	3506 (1650-3639)	-	4141 (1730-5066) ^b	811 (623-827)
Cd	0.223 (0.102-0.326)	0.270 (0.230-0.308)	-	0.236 (0.186-0.244)	0.221 (0.198-0.356)
Co	0.365 (0.132-0.579)	0.561 (0.370-0.795)	-	0.446 (0.371-0.492)	0.412 (0.307-0.653)
Cr	11.67 (6.02-15.34)	19.76 (8.26-28.81)	-	5.24 (3.65-7.72)	24.32 (8.02-25.20)
Cs	0.005 (0.002-0.006)	0.010 (0.004-0.010)	-	0.005 (0.002-0.006)	0.007 (0.003-0.007)
Cu	1.36 (0.87-1.52)	1.70 (1.23-2.37)	-	1.80 (1.67-1.96)	1.22 (1.00-1.46)
Fe	134.5 (51.8-135.6)	301.7 (129.3-316.8)	-	183.6 (108.8-232.5)	202.4 (99.1-243.4)
K	1106 (925-1248)	1193 (756-1292)	-	1036 (903-1037)	1171 (1147-1336)
Li	0.025 (0.006-0.031)	0.072 (0.028-0.073)	-	0.040 (0.009-0.042)	0.062 (0.021-0.074)
Mg	259 (162-273)	320 (218-329)	-	214 (149-249)	214 (184-223)
Mn*	6.26 (3.09-7.88)	22.96 (10.04-25.49)	-	23.92 (9.73-27.94)	4.70 (1.87-4.93)
Mo	1.64 (0.83-2.03)	2.71 (1.12-3.59)	-	0.812 (0.694-1.141)	2.76 (1.00-2.98)
Na	1108 (913-1527)	1079 (827-1231)	-	915 (809-1101)	891 (797-1009)
Ni	8.25 (4.89-10.60)	14.01 (6.18-19.57)	-	6.04 (3.68-8.42)	14.70 (5.01-15.09)
Pb	0.053 (0.021-0.082)	0.145 (0.071-0.168)	-	0.152 (0.044-0.206)	0.106 (0.053-0.194)
Rb*	1.98 (1.33-2.26) ^a	1.90 (1.19-2.02)	-	1.48 (1.42-1.53)	0.83 (0.82-1.06) ^b
Se	0.116 (0.055-0.163)	0.185 (0.146-0.209)	-	0.206 (0.205-0.264)	0.148 (0.125-0.215)
Sr*	1.17 (0.72-1.61)	3.43 (1.83-4.05)	-	3.29 (1.46-4.07)	1.23 (1.06-1.40)
Ti	0.878 (0.232-1.024)	2.27 (0.78-2.47)	-	1.03 (0.30-1.16)	1.86 (0.60-1.97)
Tl*	0.003 (0.002-0.003)	0.005 (0.004-0.005)	-	0.004 (0.003-0.004)	0.005 (0.004-0.006)
U*	0.006 (0.003-0.008)	0.018 (0.011-0.023)	-	0.020 (0.010-0.020)	0.007 (0.005-0.010)
V	0.179 (0.073-0.199)	0.356 (0.204-0.415)	-	0.215 (0.114-0.226)	0.322 (0.169-0.425)
Zn	9.88 (7.77-12.40)	10.60 (8.31-11.85)	-	9.38 (8.71-9.50)	10.17 (9.34-13.04)

* statistically significant difference according to Kruskal-Wallis one way analysis of variance on ranks ($p < 0.05$); different groups according to pairwise comparison performed by Dunn's test ($p < 0.05$) were assigned different letters

** there is no data for 28 days exposure to mixture containing 30% of depot sludge, due to small sample quantity

Table 4. The portion (%) of soluble intotal metal/metalloid quantity in whole tissue of earthworm *Eisenia fetida*, based on pooled data from all exposure groups.

		Median	Minimum	Maximum
<u><5%</u>	Al	0.5	0.0	2.9
	Ti	1.9	0.5	5.0
	Cr	3.2	0.6	8.6
<u>10-20%</u>	Fe	10.0	3.5	17.8
	Li	12.8	2.4	35.6
	Ni	14.1	6.4	30.3
	Mn	15.4	3.1	34.5
<u>21-50%</u>	V	23.9	8.1	42.5
	Mo	26.5	11.1	59.1
	U	29.7	17.2	52.8
	Pb	30.7	12.9	72.3
	Tl	35.2	17.4	54.1
	Cs	39.7	15.8	93.5
	Co	40.9	23.0	61.3
	Ca	43.3	10.0	74.4
	Sr	44.7	14.2	75.1
<u>51-80%</u>	Ba	59.5	12.4	302.3
	Cu	72.2	48.8	120.3
	Mg	77.1	57.9	92.2
<u>100-120%</u>	K	111.9	85.9	150.4
	Rb	113.3	97.8	141.7
	Na	119.5	99.9	146.1
<u>150-200%</u>	Zn	158.6	131.4	212.3
	Cd	168.7	150.7	216.9
	Se	181.6	137.3	273.0
	As	186.6	144.4	256.0

Table SI-1.Limits of quantification (LOQ; $\mu\text{g g}^{-1}$ on wet mass basis) for metal/metalloid concentrations in soluble tissue fraction and in whole tissue of earthworm *Eisenia fetida*, and the results of quality control ($\mu\text{g L}^{-1}$) for metal/metalloid measurements in *E. fetida* (control samples: QC for trace metals, catalog no. 8072, lot no. 146142-146143; and QC for minerals, catalog no. 8052, lot no. 146138-146139; UNEPGEMS, Burlington, Canada).

	LOQ _{soluble} / $\mu\text{g g}^{-1}$	LOQ _{total} / $\mu\text{g g}^{-1}$	Control sample		
			Assigned value / $\mu\text{g L}^{-1}$	Measured value / $\mu\text{g L}^{-1}$	Recovery / %
Al	0.078	0.288	1.47	1.70±0.12	116±8
As	0.036	0.112	1.81	1.78±0.13	98±7
Ba	0.005	0.264	2.93	2.84±0.14	97±5
Ca	20.02	53.31	1000	1055±19	105±2
Cd	0.006	0.001	0.788	0.773±0.007	98±1
Co	0.000	0.001	1.16	1.14±0.01	98±1
Cr	0.002	0.034	2.04	1.96±0.09	96±5
Cs	0.000	0.001	-	-	-
Cu	0.005	0.025	3.63	3.58±0.15	99±4
Fe	0.026	0.204	3.13	2.86±0.14	91±5
K	4.91	18.83	986	965±3	98±0
Li	0.000	0.002	-	-	-
Mg	3.07	6.68	776	745±5	96±1
Mn	0.001	0.011	3.07	3.00±0.08	98±3
Mo	0.011	0.030	1.49	1.40±0.01	94±1
Na	18.31	77.53	5770	6131±18	106±0
Ni	0.040	0.058	1.42	1.29±0.09	91±7
Pb	0.008	0.033	1.56	1.55±0.02	100±1
Rb	0.009	0.023	-	-	-
Se	0.003	0.015	0.854	0.829±0.015	97±2
Sr	0.009	0.037	1.09	1.06±0.04	98±3
Ti	0.005	0.021	0.844	0.780±0.057	92±7
Tl	0.000	0.000	2.14	2.22±0.04	104±2
U	0.000	0.001	-	-	-
V	0.000	0.001	1.54	1.51±0.01	98±1
Zn	0.438	2.336	2.06	1.64±0.67	80±32