The effect of earthworms on the fractionation and bioavailability of heavy metals before and after soil remediation

Metka Udovic, Domen Lestan*

Agronomy Department, Centre for Soil and Environmental Science, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana; Slovenia

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Abstract

Earthworm activity increases heavy metal bioavailability in soil before and after remediation.

The effect of two earthworm species, *Lumbricus rubellus* and *Eisenia fetida*, on the fractionation/bioavailability of Pb and Zn before and after soil leaching with EDTA was studied. Four leaching steps with total 12.5 mmol kg$^{-1}$ EDTA removed 39.8% and 6.1% of Pb and Zn, respectively. EDTA removed Pb from all soil fractions fairly uniformly (assessed using sequential extractions). Zn was mostly present in the chemically inert residual soil fraction, which explains its poor removal. Analysis of earthworm casts and the remainder of the soil indicated that *L. rubellus* and *E. fetida* actively regulated soil pH, but did not significantly change Pb and Zn fractionation in non-remediated and remediated soil. However, the bioavailability of Pb (assessed using Ruby's physiologically based extraction test) in *E. fetida* casts was significantly higher than in the bulk of the soil. In remediated soil the Pb bioavailability in the simulated stomach phase increased by 5.1 times.

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1. Introduction

The contamination of soils with toxic heavy metals is ubiquitous. Contaminated soils often present an unacceptable risk to human and ecological health and need to be remediated. One of the most promising remediation methods is soil washing with solutions containing chelating agents (chelants). Chelants desorb heavy metals from soil solid phases by forming strong water-soluble complexes, which can be removed by batch extraction of soil slurry in a reactor, in situ soil flushing, or soil heap or column leaching.

Heavy metals are present in soil in various chemical forms and bound to different soil fractions. They are usually not entirely accessible to chelants, especially since the use of chelants in high concentration is constrained by their relatively high price. Consequently, only part of the total soil heavy metals content is typically removed by soil washing, especially from soils rich in organic matter or clay. Peters and Shem (1992), for example, reported that a maximum 64.2% and 19.1% Pb (compared with initial Pb concentration) was removed with ethylenediamine tetraacetate (EDTA) and nitrilotriacetic acid (NTA) as chelants, respectively, from contaminated soil of high clay and silt content. Similarly, Pichtel et al. (2001) reported that various concentrations of EDTA and pyridine-2,6-dicarboxylic acid (PDA) removed up to 58% and 56% of Pb, respectively, from soil material from a battery recycling/smelting site. Borona and Romero (1996) extracted Pb contaminated soil with EDTA and observed that the amount of Pb removed was correlated with the amount of Pb associated with the Fe and Mn-oxide and organic matter soil fractions. Finzgar et al. (2005) reported that using 40 mmol kg$^{-1}$ of [(S,S)-ethylenediamine disuccinate (EDDS) extracted 31.1% of Pb from vegetable garden soil, rich in...
organic matter. Pb was removed proportionally from carbonate and organic matter soil fractions.

When soils are returned to the site and put into use after remediation, they become exposed to various abiotic and biotic factors, which could redistribute residual heavy metals among soil fractions. Of the biotic factors, earthworms are perhaps the most important soil organisms in terms of their influence on soil properties (Boyle et al., 1997). They ingest soil particles and egest them as surface or subsurface casts. Aristotle called earthworms the “intestines of the earth”. By ingesting organic debris, earthworms have been shown to enhance the bioavailability of soil nutrients such as C, N and P (Devliegher and Verstraete, 1996). Similarly, gut-related processes in earthworms may also increase metal availability. Indeed it has been reported that, after treatment with earthworms, the distribution of heavy metals in soil fractions was changed significantly, presumably increasing their bioavailability (Cheng and Wong, 2002; Ma et al., 2002; Wen et al., 2004).

The possibility that earthworm activity may raise heavy metal bioavailability is of considerable relevance for the success of soil remediation, especially when the methods that are used (i.e. soil washing, phytoextraction) remove only part of the (presumably labile and bioavailable) heavy metals, or heavy metals even remain in the soil immobilized by the addition of various chemicals (solidification/stabilisation). It has been shown that earthworms can rapidly invade remediated soil (Spurgeon and Hopkin, 1999; Langdon et al., 2001). With estimated annual rates of earthworm cast production ranging from 5 to more than 250 tons ha⁻¹ (Bohlen, 2002), earthworm activities might therefore, even in the short term, affect metal fractionation and bioavailability. Even so, there are no reports in the current scientific literature on earthworms or other soil biotic factors and their possible effects on the fractionation/bioavailability of heavy metals residual in soil after remediation with soil washing technologies.

In the present study, we investigate the effect of two earthworm species Lumbricus rubellus and Eisenia fetida on the fractionation (assessed using sequential extractions) and bioavailability (assessed using Ruby’s physiologically based extraction test [PBET]) of Pb and Zn before and after leaching of contaminated soil using EDTA as a chelant.

2. Materials and methods

2.1. Soil and soil analysis

Soil was collected from the 0–30 cm surface layer of an abandoned vegetable garden in the vicinity of a former industrial site in Mežica Valley in Slovenia. The Mežica Valley has been exposed to more than three hundred years of active Pb mining and smelting. For standard pedological analysis soil (and earthworm cast) pH was measured in a 1/2.5 (w/v) ratio of soil and 0.01 M CaCl₂ water solution suspension. Soil samples were analysed for organic matter by Walkley–Black titrations, cation exchange capacity by the ammonium acetate method, soil texture by the pipette method, easily extractable P was determined colorimetrically, soil moisture by the pipette method, and carbonates manometrically after soil reaction with HCl (Kalra and Maynard, 1991). A single collected sample was used for pedological analysis of non-remediated soil, while the analysis of remediated soil was performed in triplicate (Table 1).

2.2. Soil remediation

For soil remediation we employed a recently introduced chelant-based soil leaching method, using ozone/UV for treatment and reuse of the washing solution in a closed process loop. The schematic flowsheet of the method and the detailed description of the process are available in Finzag and Lestan (2006). In short: air-dried soil (4.6 kg) was sieved through a 5-mm mesh sieve and placed, in triplicates, in 15-cm diameter soil columns 27 cm high. Plastic mesh (D = 0.2 mm) at the bottom of the column retained the soil. The soil was treated in a four-step leaching experiment, first with 3 × 2.5 mmol kg⁻¹, followed by 1 × 5 mmol kg⁻¹ EDTA in 2.4 L tap water (washing solution). In each leaching step the washing solution (2.4 L) was first circulated solely through the soil column for 48 h using a peristaltic pump (flow rate 12 ml min⁻¹) to remove Pb and Zn. The washing solution (2.4 L) was then circulated in a closed loop through the soil column to remove soil residual heavy metals-EDTA complexes and through a washing solution treatment unit. This unit consisted of an ozonator, UV-light and a heavy metal adsorption filter. Ozone and UV irradiation produced hydroxyl radicals for oxidative decomposition of EDTA complexes in the washing solution; the released heavy metals were removed by absorption into the commercial absorbent Slovakite (IPRES, Bratislava, Slovakia). When Pb and Zn concentration in the washing solution fell below 5 and 10 mg L⁻¹, respectively (after 18–24 h of treatment), we started a new leaching step with a new dosage of (2.5 or 5 mmol kg⁻¹) EDTA. During the experiment, the washing solution was kept at 2.4 L. It was supplemented with tap water to compensate for the water lost during the process (approx. 10% in each leaching step). After the final, 4th leaching step, the soil was air-dried and homogenized before further use.

2.3. Pot experiments with earthworms

For the experiment, clean plastic pots (height 9 cm, diameter 12.5 cm) were filled with 250 g of air-dried non-remediated and remediated soil, in three replicates. The soil was wetted with deionized water to approximately 80% soil field water capacity and left to stabilize for a week at 20°C and 80% relative air humidity.

Two earthworm species, Lumbricus rubellus Hoffmeister and Eisenia fetida Savigny, were obtained from Biobrazda (Dragomer, Slovenia) and Regenwurmfarme Tacke GmbH (Borken, Germany), respectively. E. fetida and L. rubellus species are litter dwelling epigeic earthworms feeding mainly on organic particles (Mršič, 1997).

The earthworms were kept in the dark at a constant T and relative air humidity (20°C, 80%). Fully clittulated adult specimens and subadult specimens with clear signs of developing tubercula pubertas, of both species were selected, rinsed clean of adhering soil particles and kept on moist paper towel disks in plastic Petri dishes (5 animals per dish) at 20°C for 2 days to permit complete egestion of gut contents. To prevent coprophagy, the paper towels were changed at least daily. The animals were then washed, blotted with paper towel to remove adhering water and weighed.

Ten earthworms of each species of 0.12–0.29 g fresh weight (L. rubellus) and 0.16–1.42 g (E. fetida) were introduced in each pot and kept in the dark at a constant temperature and relative air humidity (20°C, 80%) for 7 (L. rubellus) and 4 weeks (E. fetida) until they produced enough casts to perform the analysis. The pot contents were regularly moistened with deionized water to maintain soil field water capacity (80%). Casts produced on top of the soil, as well as within the soil, were carefully collected. Their structure was clearly distinguishable from the bulk of the soil using a stereomicroscope (Fig. 1). The casts were air-dried before further analysis.

2.4. Six-step sequential extraction

A sequential extraction procedure (Lestan et al., 2003) was used to determine the fractionation of Pb and Zn in non-remediated and remediated soil and in earthworm casts, into six fractions: soluble in soil solution, exchangeable from soil colloids, bound to carbonates, bound to Fe and Mn oxides, bound to organic matter and residual fraction soluble in aqua regia. Three determinations of Pb and Zn concentration were made for each fractionation sequence. The final fractional recovery of Pb and Zn was calculated after summing the
recoveries of all six steps of sequential extractions, against aqua regia Pb and Zn concentration.

2.5. Lead bioavailability

Lead bioavailability in non-remediated and remediated soil and in earthworm casts was determined as oral bioavailability in simulated stomach and intestinal phases of the human gastrointestinal tract using PBET (Ruby et al., 1996). The stomach phase of PBET was simulated by adding 1.25 g pepsin, 0.50 g citrate, 0.50 g malate, 420 ml lactic acid and 500 ml acetic acid to 1 L water and pH adjusted to 2.50 ± 0.05 using 12 N HCl. A 0.4 g soil sample (sieved through a 250 µm sieve) was mixed in a 250 ml polypropylene vessel with 40 ml of the simulated stomach solution. The contents of the vessel were agitated by bubbling water-saturated argon gas at a flow rate of 20 L h⁻¹. The vessel was suspended in a constant temperature bath at 37 ± 1°C. Samples (2 ml each) were collected after 60 min, centrifuged and decanted. After 1 h, the flask contents were titrated to pH 7 using a dialysis bag (8000 MWCO, Spectra/Por cellulose ester tubing) containing 1 g of NaHCO₃ and 2 ml of water. Twenty milligrams of Pancreatin and 70 mg bile extract were added. Samples (2 ml) were obtained from small intestinal incubation for 1 h after the reaction flask had reached equilibrium at pH 7, been centrifuged, decanted and stored in cold storage (5 ± 1°C) for further analysis. The PBET was conducted in triplicate.

2.6. Heavy metal determination

Air-dried samples of non-remediated and remediated soil and earthworm casts (3 g) were ground in an agate mill, digested in aqua regia (28 ml), diluted with deionized water up to 100 ml, and Pb and Zn analysed by AAS (Perkin-Elmer 1100-B, Norwalk, CT, USA). Pb and Zn in extracts (washing solution, sequential extractions, PBET) were determined by AAS directly. A standard reference material used in inter-laboratory comparisons (ALV A Boden 1) from the HBLFA Raumberg-Gumpenstein, Irdning, Austria, was used in the digestion and analysis as part of the QA/QC protocol. The recovery percentage was 109 ± 7% for Pb and 112 ± 7% for Zn. The detection limits were 0.5 and 0.2 mg L⁻¹ for Pb and Zn, respectively. Reagent blank and analytical duplicates were also used where appropriate to ensure accuracy and precision in the analysis.

2.7. Statistical analysis

Student’s t-test for paired data sets and Duncan’s multiple range test (p < 0.05) were performed using Statgraphics 4.0 for Windows.

3. Results and discussion

3.1. Soil remediation

Soil properties, determined by standard pedological analysis, and Pb and Zn concentrations before and after soil remediation, are presented in Table 1. The soil pH remained within the neutral range, although the change from 6.6 before remediation to an average value of 7.2 after remediation was significant. The differences between other pedological properties before and after soil remediation lay more or less within the standard deviation of data.

Four-step soil leaching with a total 12.5 mmol kg⁻¹ EDTA used removed 39.8 ± 2.2% and 6.1 ± 3% of total initial Pb and Zn. The novel remediation method (closed loop treatment of soil washing solution using ozone/UV and heavy metal removal by absorption) enabled effective removal of residual EDTA-heavy metal complexes from the soil. This was important, since chelant mobilized heavy metals are water soluble and easily bioavailable in soil. Evidence that almost no mobilized Pb and Zn remained in the soil was their very low concentration in the washing solution at the end of each leaching step. For example: the Pb and Zn concentration in the final

Table 1

<table>
<thead>
<tr>
<th>Pedological analysis</th>
<th>Heavy metals</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>Organic</td>
</tr>
<tr>
<td>Before remediation</td>
<td>6.64</td>
</tr>
<tr>
<td>After remediation</td>
<td>7.23 ± 0.06</td>
</tr>
</tbody>
</table>

Results from remediated soil are presented as means of three replicates ± s.d.

Fig. 1. Lumbricus rubellus (A) and Eisenia fetida (B) casts.
measured in casts produced by both earthworm species in remediated soil were significantly lower (6.86 ± 0.13 and 7.03 ± 0.06 for *L. rubellus* and *E. fetida*, respectively) compared to the pH of the rest of the soil (7.23 ± 0.06).

To our knowledge, no other data for the pH of earthworm casts produced in remediated soil are available in the scientific literature. However, the significant change of pH towards neutral values in *E. fetida* casts in both remediated and non-remediated soil is congruent with the results of Wen et al. (2004) for soils with *E. fetida* activity. Changes in pH value could be attributed to the activity of the earthworm’s calciferous glands or to their alkaline urine (Salmon, 2001). However, the exact mechanisms are still unclear.

Our results indicate the active role of earthworms in regulation of soil pH. This should be considered in after-remediation risk assessments. It has already been reported that earthworm gut conditions modify the mobility of metals due to pH change and thus favour their assimilation (Weltje, 1998).

### 3.3. Effect of earthworms on Pb and Zn fractionation

Bioavailability, entrance to the food chain, toxicity, mobility and transport of metals presumably depend on their chemical forms and fractionation in the soil. It is known that earthworms actively alter soil chemical and physical properties responsible for heavy metal fractionation (Edwards and Bohlen, 1996; Cheng and Wong, 2002; Ma et al., 2002). Casts of both earthworm species produced in non-remediated and in remediated soil were therefore subjected to sequential extractions to compare the patterns of heavy metal fractionation and detect possible shifts of their binding sites.

In non-remediated soil, an 11-fold increase in the water soluble and 3-fold decrease in the exchangeable Pb fraction was observed in *E. fetida* casts (Table 2). However, the changes in the major Pb bearing fractions were insignificant (*p < 0.05*). No significant changes were found in the Pb fractionation pattern in *L. rubellus* casts. In remediated soil, a slight but statistically significant decrease (*p < 0.05*) in the Pb bound to Fe and Mn oxides was determined in casts of both earthworm species. The share of water soluble Pb increased (5-times) in *E. fetida* casts only. Similarly as in non-remediated soils, we

<table>
<thead>
<tr>
<th>Fractionation (%)</th>
<th>Before remediation</th>
<th>After remediation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soil</td>
<td><em>L. rubellus</em> casts</td>
</tr>
<tr>
<td>In soil solution</td>
<td>8.02 ± 0.00</td>
<td>8.04 ± 0.01</td>
</tr>
<tr>
<td>Exchangeable</td>
<td>0.13 ± 0.01</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>Bound to carbonate</td>
<td>21.8 ± 1.0</td>
<td>20.4 ± 1.5</td>
</tr>
<tr>
<td>Bound to Fe and Mn oxides</td>
<td>0.43 ± 0.00</td>
<td>0.47 ± 0.03</td>
</tr>
<tr>
<td>Bound to organic matter</td>
<td>6.26 ± 1.0</td>
<td>7.14 ± 6.7</td>
</tr>
<tr>
<td>Residual fraction</td>
<td>14.9 ± 0.9</td>
<td>13.8 ± 1.9</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>86.7</td>
<td>89.5</td>
</tr>
</tbody>
</table>

Results are presented as means of three replicates ± s.d. The superscript letters (a, b) denote statistically different fractionation within categories before and after remediation according to the Duncan test (*p < 0.05*).
found no significant fractionation shift in the major Pb bearing fractions (Table 2).

Zn was predominantly present in the (chemically and biologically) least accessible residual soil fraction. Consequently, no significant shift in Zn fractionation due to earthworm activity was even expected. Worth mentioning is the increase (by a factor of 1.5) of Zn bound to organic matter in casts of *L. rubellus* produced in remediated soil (Table 3). Some small changes in the share of Zn bound to carbonate and oxides were also observed. As with Pb, a very small part of the total Zn was present in soil solution or exchangeable from soil colloids. Changes in these two fractions, although some statistically significant, could therefore not shift the entire Zn fractionation pattern (Table 3). Overall, Pb and Zn fractionation in earthworm casts was not very different from the fractionation in the soils in which they were produced.

Our results are not directly comparable with results reported by others. We considered Pb and Zn fractionation in earthworm casts while others have analysed the whole soil. There are also no previous studies concerned with remediated soil. Nevertheless, Wen et al. (2004) reported an increase of heavy metals (Cr, Co, Ni, Zn, Cu, Cd and Pb) in the water soluble, exchangeable and carbonate soil fraction, due to the presence of earthworms (*E. fetida*). These fractions represent the most available form of heavy metals to plants. On the other hand, Cheng and Wong (2002) reported that the addition of earthworms (*Pheretima* sp.) in soil decreased the concentration of exchangeable Zn and of Zn bound to carbonates, although the significance of the results varied according to the soil type used in the experiment. These diverse results can be explained by different soil characteristics and by physiological and ecological differences among earthworm species, which are known to be selective consumers (Edwards and Bohlen, 1996; Morgan and Morgan, 1999). This selectivity, for example, is reflected in different tissue metal accumulation in different earthworm species (Dai et al., 2004).

### 3.4. Effect of earthworms on Pb bioavailability

Incidental ingestion is the major pathway of exposure to heavy metals in soils and dust. Oral bioavailability of Pb was determined *in vitro* using Ruby’s tests (PBET) for predicting the bioavailability of metals from a solid matrix. Using animal models, Ruby et al. (1996) have validated results from the PBET model only for Pb and As. The assessment of Zn bioavailability was therefore not attempted.

It is clearly evident from PBET results that the effect of earthworm activity on the bioavailability of heavy metals in soil is species specific. The bioavailability of Pb in *E. fetida* casts was thus significantly higher than in *L. rubellus* casts and higher than in the bulk of non-remediated and remediated soil (Fig. 2). In casts of *E. fetida* produced in non-remediated soil, the Pb bioavailability in the stomach and intestinal phases was 4.1- and 2.3-times higher, respectively, than in the rest of the soil. In casts of *E. fetida* produced in remediated soil, Pb bioavailability in the stomach phase increased by 5.1 times, while Pb bioavailability in the small intestine phase showed no statistically significant difference (*p* < 0.05). Pb bioavailability in non-remediated and remediated soil and in *L. rubellus* casts was not statistically different (*p* < 0.05).

For the correct interpretation of these results, we should take into consideration that the PBET does not mimic the entire physiological process controlling the uptake of Pb,
because it does not simulate transport of Pb across the intestinal epithelium. From this point of view, the concentrations achieved with PBET represent the bioaccessible concentrations, which are usually higher than the bioavailable concentrations, due to the incomplete uptake of solubilized Pb in the small intestine (Ruby et al., 1996). Nevertheless, our results clearly indicate that some earthworm species could significantly increase heavy metal bioavailability, not just in polluted but also in remediated (washed) soils, from which only a part of heavy metals has been stripped.

4. Conclusions

In our study, leaching with EDTA removed only part of the total Pb and Zn from the contaminated soil, rich with organic matter. After ingestion of non-remediated and remediated soil by L. rubellus and E. fetida, the fractionation of Pb and Zn in earthworm casts did not change very much. However, the ingested soil passed through the earthworm’s alimentary canal only once. In a real situation and over a longer period, earthworms process the same soil several times, which could magnify their effect on heavy metal fractionation. Although the fractionation remained more or less the same, Pb oral bioavailability in E. fetida casts increased significantly. The fact that Pb bioavailability increased while the Pb fractionation pattern remained relatively unchanged indicates that the relation between fractionation (determined by sequential extractions) and heavy metal bioavailability is not self-evident.

While Pb bioavailability in E. fetida casts increased, in L. rubellus casts it remained the same. It is well known that earthworm species differ considerably in respect to their selectivity in feeding, which probably caused the observed difference. This calls for other earthworm species to be tested for their ability to change heavy metal bioavailability in non-remediated and remediated soil.

The important aspect of increased Pb oral bioavailability is the risk that earthworm activity may pose in soil after remediation. In soil washing (and phytoextraction) labile, bioavailable forms of heavy metals are expected to be removed first (selectively), while heavy metals strongly bound to solid soil fractions and chemically less available to chelants could remain in the soil even after remediation (heavy metals bio-stripping concept). However, our results clearly indicate that some earthworm species can considerably increase the bioavailability of residual heavy metals in remediated soil.

Acknowledgements

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