



## Bioaccumulation in *Porcellio scaber* (Crustacea, Isopoda) as a measure of the EDTA remediation efficiency of metal-polluted soil

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Bioaccumulation tests with *Porcellio scaber* isopods are proposed as a supplement to chemical extraction in assessing metal bioavailability before and after soil remediation.

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### ABSTRACT

Leaching using EDTA applied to a Pb, Zn and Cd polluted soil significantly reduced soil metal concentrations and the pool of metals in labile soil fractions. Metal mobility (Toxicity Characteristic Leaching Procedure), phytoavailability (diethylenetriaminepentaacetic acid extraction) and human oral-bioavailability (Physiologically Based Extraction Test) were reduced by 85–92%, 68–91% and 88–95%, respectively. The metal accumulation capacity of the terrestrial isopod *Porcellio scaber* (Crustacea) was used as in vivo assay of metal bioavailability, before and after soil remediation. After feeding on metal contaminated soil for two weeks, *P. scaber* accumulated Pb, Zn and Cd in a concentration dependent manner. The amounts of accumulated metals were, however, higher than expected on the basis of extraction (in vitro) tests. The combined results of chemical extractions and the in vivo test with *P. scaber* provide a more relevant picture of the availability stripping of metals after soil remediation.

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### 1. Introduction

Remediation techniques for polluted soil can be divided into two main groups. Immobilization technologies leave metals in the soil, but they change their speciation and fractionation in soil solid phases, thus minimizing their availability and mobility (Guo et al., 2006). Removal technologies such as phytoextraction, electrokinetic extraction and washing/leaching methods, remove metals from the soil, thus being preferred (Peters, 1999). Soil washing/leaching involves the separation of metals from soil solid phases by solubilizing them in a washing/leaching solution. In addition to acids, chelating agents (chelants) are the most prevalent and effective removal agents used in soil washing/leaching (Lestan et al., 2008; Peters, 1999). Chelants desorb metals from the soil surfaces by forming strong and water-soluble metal-chelant coordination compounds (complexes) that can be removed (washed/leached) from the soil. Among the chelants tested, ethylenediamine tetraacetic acid (EDTA) has shown to be highly effective in decreasing the concentration of metals (Finžgar and Leštan, 2007).

Metals in soil are speciated in different ways, which results in different solubilities (Ure, 1996). They are not entirely accessible to chelating agents and they are consequently only partially removed.

Residual metals left in the soil after remediation remain present in chemically stable species bound to non-labile soil fractions (Finžgar and Leštan, 2007; Nowack et al., 2006). The efficiency and success of soil remediation is often judged on the reduced availability (availability stripping) of contaminants for organisms, assessed by chemical extraction tests.

Different extraction techniques are used to assess metals. Sequential extractions are used to assess different metal fractionations in soils, discriminating metals according to their physico-chemical associations with other soil components, mobility and bioavailability (McGrath, 1996; Mulligan et al., 2001; Sabienė and Brazauskienė, 2004). The mobility and potential leaching of metals into the groundwater system are assessed according to the standardized Toxicity Characteristic Leaching Procedure (TCLP) (US EPA, 1995). Several in vitro digestion models based on certain physiological parameters in humans have been developed and used to estimate the oral-bioavailability of soil contaminants to humans (Oomen et al., 2002, 2003). The Physiologically Based Extraction Test (PBET) was designed to assess the oral-bioavailability of metals in the human stomach, where the pH is low (pH 2.5), and in the small intestine, where the pH is neutral (pH 7.0). The latter method estimates the amount of metals ready to be absorbed from the intestine into the blood (Ruby et al., 1996; Turner and Ip, 2007).

Moreover, a variety of biotests are used to examine the efficiency of the soil remediation process in terms of reduced toxicity.

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However, when the bioavailability of chemicals and potential trophic transfer is of interest, such bioassays must be employed where the accumulated contaminants are determined. Some plants and animals, especially invertebrates have already been used for assessing the metal availability in soils (Meier et al., 1997). These organisms accumulate metals in proportion to the environmental concentrations and are therefore used as bioindicators/biomonitoring of environmental metal pollution.

Among invertebrates, the most popular organisms for metals accumulation research are mollusks, earthworms, crustaceans, insects, myriapods and arachnids (Hopkin, 1989), among which isopods appear to be the most efficient of them as assimilators of metals. They accumulate the highest concentrations of metals such as zinc, cadmium, lead and copper so far recorded in any soft tissue of terrestrial animals (Hopkin, 1989; Hopkin et al., 1993; Vijver et al., 2006; Witzel, 1998). Efficient metal assimilation, the wealth of knowledge on their metal physiology, and ease of handling in the laboratory, were the main reasons for choosing terrestrial isopods as the experimental animal in our study. Because terrestrial isopods accumulate metals from their environment in proportion to their concentration in the soil (Heikens et al., 2001; Hopkin et al., 1993), they appeared very suitable as indicators of the metal bioavailable fraction in polluted soil and leaf litter (Gál et al., 2008).

Isopods are omnivorous animals, but they have clear feeding preferences. Many species prefer feeding on decaying leaf litter rather than fresh with high microbial density (Zimmer, 2002). Some other species are soil dwelling with differing food preferences. Terrestrial isopod *Porcellio scaber* inhabits a wide range of habitats. They chew dead plants or plant material mixed with soil into small fragments. Terrestrial isopods must have evolved efficient ways of assimilating essential elements from the food, because unlike their marine ancestors, they could no longer obtain them directly from the external medium across the respiratory surfaces (Warburg, 1993).

The main metal storage organ in isopods is the hepatopancreas. Metals are bound here to specific low molecular weight peptides, or stored in insoluble granules (Hopkin, 1989). The metal uptake from food depends on many factors, of which the most important are the bioavailability of metals in the ingested material, gut microflora, the rate of food consumption and pH inside the gut, metal concentration, the duration of exposure and the combination of factors to which the metals are exposed (Hopkin, 1989; Hopkin et al., 1993; Odendaal and Reinecke, 2004). Earlier data suggested that isopods accumulate metals for a life time, but more recent researches provide evidence also of loss of metals once transferred to uncontaminated food sources (Bibič et al., 1997; Odendaal and Reinecke, 2004; Witzel, 1998). Nevertheless, a correlation between metal body burden and metal concentrations in food/substratum can be demonstrated (Hopkin, 1989).

The aim of our study was to investigate whether chemical extraction tests provide (include) information on availability of metals for soil fauna. The hypothesis was that isopods accumulate metals from the soil in proportion to the bioavailable metal fraction. We expected the bioavailable fraction in remediated soil to be lower and consequently, less metals to be accumulated by isopods than from the soil before remediation. We discuss the benefits of in vivo assessment of soil remediation efficiency by terrestrial organisms in addition to established chemical extraction tests based on chemical extraction.

## 2. Materials and methods

### 2.1. Soil and soil analysis

In 1990, Pb mining and smelting in Žerjav (Mežica Valley, Slovenia;  $x = 489,300$  m and  $y = 152,300$  m, Gauß-Krüger coordinate system) ceased after having been active

for more than three centuries. Soil was collected from the upper 30 cm layer of a regularly managed vegetable garden near the abandoned Pb smelter in this valley. Soil pH was measured in a 1/2.5 (w/v) ratio of soil and 0.01 M CaCl<sub>2</sub> suspension. Soil samples were analyzed for organic matter by modified Walkley–Black titrations (ISO 14235, 1998), cation exchange capacity (CEC) by the ammonium acetate method (Rhoades, 1982), soil texture by the pipette method (Fiedler et al., 1964), total N content after dry combustion (ISO 13878, 1995), and carbonates manometrically after soil reaction with HCl (ISO 10693, 1995). Pedological analysis of the non-leached soil was performed in triplicate and the results are presented as means  $\pm$  SD.

### 2.2. Soil leaching

Five-mm sieved air-dried soil (4.6 kg per column) was placed 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 first leached with EDTA solution circulating through the soil columns in a closed loop for 24 h using a peristaltic pump (flow rate 15 mL min<sup>-1</sup>) to mobilize and remove Pb, Zn and Cd as water-soluble metal-EDTA complexes. Different EDTA concentrations; 2.5, 10, 40, and four batches of 40 mmol kg<sup>-1</sup> EDTA ( $4 \times 40$  mmol kg<sup>-1</sup> EDTA, each batch lasting 24 h) in 3.1 L tap water were used. Afterwards, each column was rinsed with 80 L of pure tap water in an open loop (flow rate 15 mL min<sup>-1</sup>) to remove mobilized metal species and EDTA. EDTA in the rinsing solutions from all treatments was determined spectrophotometrically according to the procedure of Hamano et al. (1993) and metals were determined directly with atomic absorption spectrophotometry (AAS) (Perkin-Elmer 1100-B, Norwalk, USA) to ensure that no mobilized metals and/or EDTA were left in the soil after rinsing with tap water.

### 2.3. Six-step sequential extraction

A modified Tessier's sequential extraction procedure (Lestan et al., 2003) was used to determine the fractionation of Pb, Zn and Cd in the soil into six fractions. The water extractable fraction was obtained by extraction of 1 g of air-dry soil, 2-mm sieved, with 10 mL of deionized water for 1 h. The fraction exchangeable from soil colloids was extracted from the residual soil sample with 10 mL of 1 M Mg(NO<sub>3</sub>)<sub>2</sub> for 2 h. The fraction bound to carbonates was extracted with 10 mL of 1 M NH<sub>4</sub>OAc (pH 5) for 5 h. The fraction bound to Fe and Mn-oxides was extracted with 20 mL of 0.1 M NH<sub>2</sub>OH  $\times$  HCl (pH 2) for 12 h. The fraction bound to organic matter was obtained after heating the soil suspension in 3 mL of 0.02 M HNO<sub>3</sub> and 5 mL of 30% H<sub>2</sub>O<sub>2</sub> for 3 h at 85 °C, followed by extraction with 15 mL of 1 M NH<sub>4</sub>OAc for 30 min. The extraction solution was centrifuged after each extraction step and the supernatant stored at 4 °C for analysis. The residual soil sample was shaken in 10 mL of deionized water for 10 min, centrifuged and the supernatant discarded. The last, residual fraction was obtained after the digestion of the samples with aqua regia. Three replicates were performed for each fractionation sequence. The final fractional recovery of Pb, Zn and Cd was calculated after summing the concentrations of all six-steps of sequential extractions and comparing them with the total amount of Pb, Zn and Cd in the respective soil.

### 2.4. Toxicity Characteristic Leaching Procedure (TCLP)

The mobility (leachability) of Pb, Zn and Cd in the soil was determined using TCLP analysis (US EPA, 1995). Analyses were conducted in triplicate. The procedure involved shaking a 1 g soil sample, sieved to 2 mm, in 20 mL of 0.0992 M acetic acid and 0.0643 M NaOH extraction solution (1:20 ratio) with a pH of  $4.93 \pm 0.05$ , for 18 h on a rotary shaker at about 300 rpm. After the reaction period, the contents were filtered (Whatman No. 4 filter paper), the filtrate acidified with 65% HNO<sub>3</sub> to pH < 2 and stored at 5 °C for metal determination.

### 2.5. Assessment of metal availability to plants

Metal availability to plants (phytoavailability) was assessed by diethylenetriamine pentaacetic acid (DTPA) extraction, initially designed to extract simultaneously plant available Zn, Fe, Mn and Cu in near-neutral and calcareous soil (Lindsay and Norvell, 1978) and used as a predictor of metal bioavailability and ecotoxicity (Conder et al., 2001; Ma et al., 2002). The DTPA extraction solution was prepared to contain 0.005 M DTPA, 0.01 M CaCl<sub>2</sub>, and 0.1 M triethanolamine (TEA) and was adjusted to pH  $7.30 \pm 0.05$ . The procedure involved shaking a 5 g soil sample, sieved to 2 mm, in 10 mL of DTPA extracting solution for 2 h on a horizontal shaker at about 120 rpm. After the extraction period, the contents were filtered (Whatman No. 42 filter paper) and the filtrates analyzed for Pb, Zn and Cd. The extractions were conducted in triplicate.

### 2.6. Pb, Zn and Cd oral-bioavailability assessment

The PBET used is designed around the pediatric gastrointestinal tract parameters for a child 2–3-years-old (Turner and Ip, 2007), since children ingest more soil and dust particles than adults, due to their mouthing behavior, and are thus more exposed to soil pollutants (Davis and Mirick, 2006). The PBET includes two phases. Firstly, 0.5 g of sieved soil sample (250  $\mu$ m) is digested in a reaction flask for 2 h at constant

temperature (37 °C) in simulated gastric fluid (50 mL) prepared by adjusting 1 L of deionized water to pH  $2.50 \pm 0.05$  with diluted HCl and adding 1.25 g of pepsin (porcine, Sigma), 0.50 g of citrate, 0.50 g of malate, 420  $\mu\text{L}$  of lactic acid and 500  $\mu\text{L}$  of acetic acid. The pH of the reaction mixture was measured every 10 min and adjusted with HCl as necessary to keep it at a value of  $2.50 \pm 0.05$ . Samples (3 mL each) were collected after 2 h and centrifuged at 1600 g for 25 min. The liquid fraction was decanted for further analysis. The 3 mL sample volume was replaced with gastric solution to maintain a constant volume in the reaction flask. After 2 h, the reaction was titrated to pH 7 with saturated  $\text{NaHCO}_3$  solution. When the reaction vessel reached equilibrium at pH 7, 175 mg of bile salts (porcine, Sigma) and 50 mg of pancreatin (porcine, Sigma) were added, thus simulating small intestine conditions. After 2 h, the reaction solutions were centrifuged at 1600 g for 25 min. The liquid fraction was decanted and analyzed as the small intestine fraction. During both phases, a constant moistened argon flow (1 L  $\text{min}^{-1}$ ) at 37 °C was conducted through the reaction mixture in order to simulate peristalsis. The PBET was conducted in triplicate.

### 2.7. Metal analyses

Air-dried samples of soil (1 g) were ground in an agate mill, digested in aqua regia (28 mL), diluted with deionized water up to 100 mL, and Pb, Zn and Cd analyzed by flame (acetylene/air) AAS with a deuterium background correction (Perkin-Elmer 1100-B, Norwalk, USA and Varian AA240FS). Pb, Zn and Cd in extracts (leaching solutions, rinsing water, solutions from sequential extractions, PBET, TCLP and DTPA extracts) were determined by AAS directly. A standard reference material used in inter-laboratory comparisons (WEPAL 2003.1.1) from HBLFA Raumberg-Gumpenstein, Irnding, Austria, was used in the digestion and analyses as part of the QA/QC protocol. The recovery percentages were  $106 \pm 5.9$ ,  $108 \pm 12.7$  and  $105 \pm 30.1\%$ , and the limits of quantification (LOQ) were 0.25, 0.02 and 0.03  $\text{mg L}^{-1}$  for Pb, Zn and Cd, respectively. Reagent blank and analytical duplicates were also used where appropriate to ensure accuracy and precision in the analysis.

### 2.8. Metal accumulation in Porcellio scaber

Adult specimens of a terrestrial isopod *P. scaber* were collected in July 2007 in two non-polluted managed gardens in Celje and Krško, Slovenia, kept in laboratory at constant room temperature (24 °C) and fed with common hazel leaves (*Corylus avellana*) collected from an unpolluted site in Tivoli Regional Park, Rožnik and Šišenski hrib (Ljubljana, Slovenia). Metal concentrations in the leaves were in the range within which leaves are classified as non-metal polluted ( $64.5 \pm 11.4$ ,  $48.3 \pm 9.5$ ,  $4.50 \pm 0.44$   $\mu\text{g}$  of Pb, Zn and Cd per g of leaf dry weight, respectively). These are the usual conditions in our laboratory for maintaining animals prior to experiments. During the experiment, the animals were exposed only to soil. No leaf litter was added to the experimental containers.

For the experiment, 15 adult specimen of 30–60 mg fresh weight were exposed for 14 days to approximately 600 mL of air-dried experimental soil (polluted and remediated with 2.5, 10, 40 and  $4 \times 40$   $\text{mmol kg}^{-1}$  EDTA, respectively) in plastic vessels ( $17 \times 13 \times 7$  cm) with plastic covers. The covers were drawn back to allow circulation of air. In order to achieve a soil moisture gradient, one corner in the vessel was daily moistened with tap water (5–10 mL). The experimental vessels were kept at room temperature and checked daily to regulate the soil moisture content. After 14 days of exposure, the animals were removed from the vessels and fed with uncontaminated hazel leaves for 24 h to remove metals from the digestive system. They were then transferred separately into plastic tubes, placed for a 4 h at 5 °C to obtain an anesthetic-like effect and then frozen. Before the analyses, samples were lyophilized, weighed and completely digested in a nitric/perchloric acid mixture (7:1). After evaporation of the acid, the residue was taken up in 0.1%  $\text{HNO}_3$ . Total Pb, Zn and Cd concentrations in whole animals were determined by flame atomic absorption spectrometry (Perkin-Elmer Analyst 100). Reagent blanks and standard solutions (Merck) were used to ensure accuracy and precision in the analysis. We expressed the metal accumulation in animals with BAFs by calculating the ratio of metal concentration in the isopods to the total soil metal concentration.

The duration of exposure was determined on the basis of preliminary results, where different stress parameters were assessed. We concluded that 14 days of exposure under suboptimal condition do not severely affect animals. On the other hand this is long enough for them to accumulate substantial amount of bioavailable metals (Bibič et al., 1997).

For the initial metal concentrations present in *P. scaber* prior to exposure to polluted and remediated soil, body Pb, Zn and Cd concentrations were determined in 15 specimens, as described above. The initial body concentrations  $30.2 \pm 22.7$ ,  $350.2 \pm 95.5$  and  $4.9 \pm 1.6$   $\mu\text{g g}^{-1}$  of Pb, Zn and Cd, respectively, were subtracted from the measured accumulated concentrations in the test animals exposed to polluted and remediated soil.

### 2.9. Statistical analysis

The differences between the Pb, Zn and Cd concentrations in isopods exposed to differently remediated and non-remediated soils as well as the differences between BAFs for remediated and non-remediated soil were determined by Duncan test at 95% confidence level ( $P < 0.05$ ) using Statgraphics software (Statgraphics Plus for

**Table 1**

Total concentration of Pb, Zn and Cd in soil before and after remediation with leaching with different EDTA concentrations. Results are presented as means of three replicates  $\pm$ SD.

	Pb ( $\text{mg kg}^{-1}$ )	Zn ( $\text{mg kg}^{-1}$ )	Cd ( $\text{mg kg}^{-1}$ )
Before leaching	4603 $\pm$ 94.4	1826 $\pm$ 60.7	30.4 $\pm$ 0.3
After leaching			
2.5 $\text{mmol kg}^{-1}$ EDTA	4323 $\pm$ 169	1774 $\pm$ 102	25.3 $\pm$ 1.9
10 $\text{mmol kg}^{-1}$ EDTA	2712 $\pm$ 207	1599 $\pm$ 118	14.1 $\pm$ 0.7
40 $\text{mmol kg}^{-1}$ EDTA	2112 $\pm$ 122	1464 $\pm$ 120	10.2 $\pm$ 1.5
40 ( $4 \times$ ) $\text{mmol kg}^{-1}$ EDTA	1239 $\pm$ 9.0	1402 $\pm$ 94.1	7.8 $\pm$ 0.3

Windows 4.0, Statistical Graphics, Herndon, VA, USA). Linear regression analysis was performed to detect correlations among concentrations of metals in isopods, metals in soil, and in vitro metal mobility and bioavailability, using the computer program Microsoft Office Excel 2003.

## 3. Results and discussion

### 3.1. Soil remediation

The following pedological properties of the soil used in the experiment were assessed: pH  $6.57 \pm 0.02$ , organic matter  $11.3 \pm 0.4\%$ , CEC  $27 \pm 1$   $\text{cmol}_{(c)} \text{kg}^{-1}$  of soil, sand  $42 \pm 1\%$ , silt  $47.5 \pm 1.4\%$ , clay  $10.5 \pm 0.4\%$ , N  $4.8 \pm 0.1$   $\text{g kg}^{-1}$ , carbonates  $202 \pm 15$   $\text{g kg}^{-1}$ . Soil remediation with EDTA leaching significantly reduced the soil metal burden. By using leaching solutions containing four different EDTA concentrations (2.5, 10, 40 and  $4 \times 40$   $\text{mmol kg}^{-1}$  EDTA), we achieved an anticipated gradient removal of Pb, Zn and Cd with a slight increase in soil pH in each treatment ( $6.89 \pm 0.04$ ,  $6.9 \pm 0.01$ ,  $6.88 \pm 0.1$  and  $6.98 \pm 0.02$ , respectively, significant at  $P < 0.05$ ). Respective treatments removed 6, 41, 54 and 73% of initial Pb, 3, 13, 20 and 23% of initial Zn and 17, 54, 66 and 74% of initial Cd (Table 1). The amount of Pb, Zn and Cd removed with EDTA leaching is congruent with the metal concentrations in the major metal bearing fractions, as resolved by sequential extractions (Table 2). The majority of Pb in the soil before remediation was bound to carbonates and to organic matter. Leaching with EDTA removed up to 85% of the Pb initially bound to

**Table 2**

Fractionation of Pb, Zn and Cd in soil before and after remediation with EDTA expressed as percentage of the sum of all fractions. Results are presented as means of three replicates  $\pm$ SD. LOQ, below the limit of quantification.

	I <sup>a</sup>	II	III	IV	V	VI
<b>Before leaching</b>						
Pb (%) LOQ		0.3 $\pm$ 0.0	29.2 $\pm$ 2.9	0.4 $\pm$ 0.0	63.5 $\pm$ 1.3	6.6 $\pm$ 0.2
Zn (%) 0.1 $\pm$ 0.0		2.0 $\pm$ 0.0	19.0 $\pm$ 0.8	3.1 $\pm$ 0.3	30.8 $\pm$ 0.8	45.1 $\pm$ 3.9
Cd (%) LOQ		7.6 $\pm$ 0.1	48.0 $\pm$ 0.9	9.4 $\pm$ 0.2	30.4 $\pm$ 0.4	4.2 $\pm$ 2.6
<b>After leaching</b>						
<b>2.5 <math>\text{mmol kg}^{-1}</math> EDTA</b>						
Pb (%) LOQ		LOQ	24.3 $\pm$ 0.5	0.5 $\pm$ 0.0	64.9 $\pm$ 9.0	10.3 $\pm$ 1.8
Zn (%) 0.04 $\pm$ 0.00		1.0 $\pm$ 0.0	11.2 $\pm$ 0.2	3.2 $\pm$ 0.3	31.4 $\pm$ 1.3	53.3 $\pm$ 2.8
Cd (%) LOQ		3.9 $\pm$ 0.2	38.7 $\pm$ 0.4	9.2 $\pm$ 0.6	34.6 $\pm$ 2.7	13.1 $\pm$ 2.1
<b>10.0 <math>\text{mmol kg}^{-1}</math> EDTA</b>						
Pb (%) 0.2 $\pm$ 0.1		0.1 $\pm$ 0.0	23.0 $\pm$ 0.5	0.5 $\pm$ 0.1	67.8 $\pm$ 6.5	14.4 $\pm$ 4.1
Zn (%) 0.04 $\pm$ 0.00		0.4 $\pm$ 0.0	8.3 $\pm$ 0.9	2.2 $\pm$ 0.0	31.8 $\pm$ 2.1	57.3 $\pm$ 2.4
Cd (%) LOQ		2.7 $\pm$ 0.3	34.6 $\pm$ 0.9	8.5 $\pm$ 0.6	41.3 $\pm$ 2.5	15.2 $\pm$ 3.8
<b>40.0 <math>\text{mmol kg}^{-1}</math> EDTA</b>						
Pb (%) 0.2 $\pm$ 0.1		LOQ	18.1 $\pm$ 1.5	0.5 $\pm$ 0.3	64.7 $\pm$ 9.2	16.4 $\pm$ 3.5
Zn (%) 0.1 $\pm$ 0.0		0.2 $\pm$ 0.0	4.3 $\pm$ 0.1	1.2 $\pm$ 0.1	30.5 $\pm$ 3.5	63.7 $\pm$ 3.9
Cd (%) LOQ		LOQ	24.8 $\pm$ 1.8	5.8 $\pm$ 0.2	43.2 $\pm$ 6.2	23.2 $\pm$ 2.2
<b><math>4 \times 40.0</math> <math>\text{mmol kg}^{-1}</math> EDTA</b>						
Pb (%) LOQ		LOQ	18.5 $\pm$ 0.4	LOQ	65.7 $\pm$ 4.8	15.6 $\pm$ 2.1
Zn (%) 0.1 $\pm$ 0.0		0.1 $\pm$ 0.0	2.4 $\pm$ 0.1	0.6 $\pm$ 0.0	30.9 $\pm$ 2.1	66.0 $\pm$ 4.9
Cd (%) LOQ		LOQ	16.9 $\pm$ 0.4	4.3 $\pm$ 0.7	46.7 $\pm$ 2.5	33.2 $\pm$ 0.1

<sup>a</sup> I: water extractable; II: exchangeable; III: bound to carbonates; IV: bound to Fe and Mn-oxides; V: bound to organic matter; VI: residual fraction.

carbonates and up to 75% of the Pb initially bound to organic matter. The Cd present in the soil was mostly bound to carbonates and organic matter, but its removal was achieved in all fractions, except the residual fraction (Table 2). Most of the Zn was bound to organic matter and in the residual fraction and was apparently inaccessible for EDTA. Only 2.1% of total Zn was detected in the most accessible, water extractable and exchangeable fraction (Table 2). After leaching with EDTA, Zn was successfully removed from the carbonate fraction (up to 91% of initially bound) and to Fe and Mn-oxides (up to 87% of initially bound). However, altogether only 23% of total Zn could be removed from the soil with the highest EDTA concentration treatment ( $4 \times 40 \text{ mmol kg}^{-1}$  EDTA). The effectiveness of EDTA for Zn removal from this soil seems to be limited. This could be explained by differences in the kinetics of Zn desorption/dissolution compared with other metals and by the fact that the majority of Zn in soils is present in the residual fraction (Sun et al., 2001; Finžgar and Leštan, 2006). EDTA is capable of metal extraction mostly from the water extractable, exchangeable and carbonate fractions, partly from organic matter, but not from the residual fraction (Peters, 1999; Tandy et al., 2004; Ure, 1996). The different extraction efficiencies for different metals obtained by the chelant are therefore simply due to different distributions of the metal fractions (Table 2). Consequently, EDTA was very successful in Pb and Cd removal from soil, but was not appropriate for Zn removal.

The mobility of Pb, Zn and Cd in the soil before and after remediation was determined by TCLP. Leaching with EDTA considerably reduced Pb, Zn and Cd mobility in the soil below the limit values set by US EPA (1995), i.e.  $5 \text{ mg L}^{-1}$  for Pb,  $250 \text{ mg L}^{-1}$  for Zn and  $1 \text{ mg L}^{-1}$  for Cd (Table 3). Concentrations of mobile Zn in soil treated with  $4 \times 40 \text{ mmol kg}^{-1}$  EDTA decreased by 88% compared to the concentration of mobile Zn in non-remediated soil, while in that same soil treatment the concentrations of mobile Pb and Cd decreased below the limit of detection.

EDTA remediation reduced also the phytoavailable fraction as determined by DTPA extraction of metals by up to 94, 91 and 95% of the initially phytoavailable Pb, Zn and Cd, respectively (Table 3).

In the treatment with  $4 \times 40 \text{ mmol EDTA kg}^{-1}$  the amount of oral-bioavailable Pb and Zn in the stomach fraction as assessed using the PBET decreased by 88 and 68%, respectively (Table 3). The

same treatment reduced the oral-bioavailability of Pb in the small intestine fraction below the limit of quantification and of Zn by 70% (Table 3). The concentration of orally bioavailable Cd was already below the limit of detection in the non-remediated soil (Table 3).

### 3.2. Pb, Zn and Cd accumulation in *P. scaber*

Our results showed that the gradient of metal removal by increasingly higher EDTA concentration of the leaching solution was generally reflected in the amounts of metal accumulated by isopods (Fig. 1A). After 14 days of exposure to non-remediated and remediated soil, *P. scaber* accumulated Pb, Zn and Cd in a concentration dependent manner. This confirms literature reports that the internal concentrations of (accumulated) metals in isopods increase with the metal concentration in soil (Gräff et al., 1997; Cortet et al., 1999; Paoletti and Hassall, 1999; Heikens et al., 2001; Gál et al., 2008).

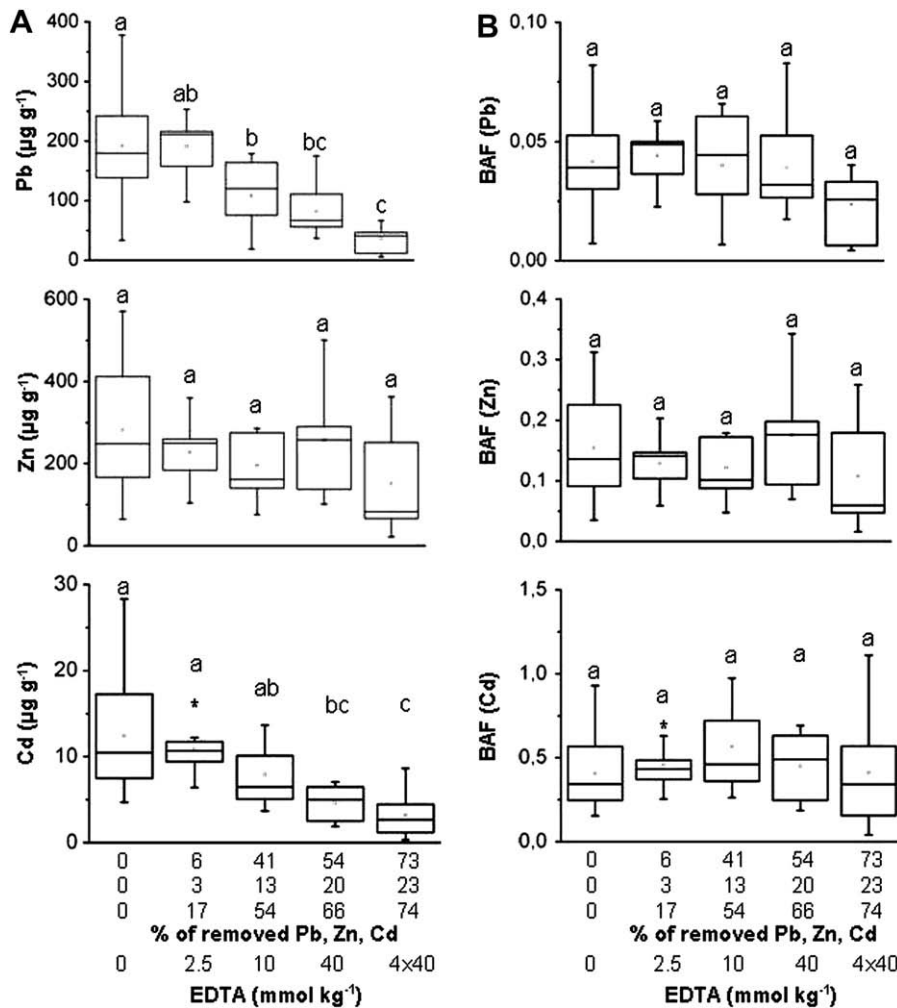
Specifically, significant differences ( $P < 0.05$ ) were observed among Pb accumulations in animals exposed to non-remediated soil, soil treated with 10 and soil treated with  $4 \times 40 \text{ mmol kg}^{-1}$  EDTA (Fig. 1A). In the case of Cd accumulation, significant differences ( $P < 0.05$ ) appeared between animals exposed to non-remediated soil and soil leached with  $10 \text{ mmol kg}^{-1}$  EDTA or higher (Fig. 1A). The differences in Zn accumulation by *P. scaber* among groups were not significant ( $P < 0.05$ ) (Fig. 1A). The smaller differences in Zn accumulation by *P. scaber* were probably due to a lower efficiency of Zn removal from the soil by EDTA extraction (Table 1). In contrast to Zn accumulation in *P. scaber*, Zn mobility (TCLP), phytoavailability (DTPA) and oral availability (PBET) decreased significantly with more stringent EDTA soil treatment (Table 3).

As shown in Fig. 1B, there were no apparent decreasing or increasing trends and no significant differences ( $P < 0.05$ ) between bioaccumulation factors (BAFs) for Pb, Zn and Cd in soils treated with increasing EDTA concentrations. This indicates a constant ratio between non-bioavailable and bioavailable (to isopods) concentrations of Pb, Zn and Cd in polluted (non-remediated) and remediated soils, even in the most EDTA processed soils. Contrary to indications given by chemical extraction tests (Table 3), EDTA treatment did not preferentially remove fractions of Pb, Zn and Cd

**Table 3**

Pb, Zn and Cd mobility (assessed with Toxicity Characteristic Leaching Procedure, TCLP), accessibility to plants (diethylenetriamine pentaacetic acid (DTPA) extraction) and oral-bioavailability (Physiologically Based Extraction Test, PBET) in soil before and after remediation with leaching with different EDTA concentrations. Results are presented as means of three replicates  $\pm$ SD. LOQ, below limit of quantification.

	TCLP ( $\text{mg L}^{-1}$ )	DTPA ( $\text{mg kg}^{-1}$ )	PBET	
			Stomach phase ( $\text{mg kg}^{-1}$ )	Small intestine phase ( $\text{mg kg}^{-1}$ )
<b>Soil before leaching</b>				
Pb	$22.2 \pm 0.7$	$952 \pm 3$	$519 \pm 72$	$264 \pm 52$
Zn	$42.4 \pm 2.3$	$230 \pm 16$	$249 \pm 31$	$146 \pm 27$
Cd	$1.7 \pm 0.1$	$12 \pm 1$	$4.1 \pm 1.2$	LOQ
<b>Soil after leaching</b>				
<i>2.5 mmol kg<sup>-1</sup> EDTA</i>				
Pb	$19.0 \pm 0.2$	$987 \pm 5$	$515 \pm 57$	$247 \pm 37$
Zn	$31.0 \pm 0.7$	$189 \pm 4$	$208 \pm 22$	$129 \pm 16$
Cd	$1.3 \pm 0.1$	$9.3 \pm 0.1$	$1.6 \pm 0.3$	LOQ
<i>10.0 mmol kg<sup>-1</sup> EDTA</i>				
Pb	$13.2 \pm 0.5$	$646 \pm 46$	$336 \pm 166$	$161 \pm 21$
Zn	$20.7 \pm 1.3$	$108 \pm 7$	$146 \pm 55$	$88 \pm 12$
Cd	$0.7 \pm 0.0$	$3.8 \pm 0.3$	LOQ	LOQ
<i>40.0 mmol kg<sup>-1</sup> EDTA</i>				
Pb	$7.7 \pm 0.6$	$212 \pm 1$	$136 \pm 9$	$62 \pm 18$
Zn	$8.4 \pm 0.2$	$41 \pm 2$	$93 \pm 4$	$48 \pm 6$
Cd	LOQ	$1.3 \pm 0.0$	LOQ	LOQ
<i>40.0 (4<math>\times</math>) mmol kg<sup>-1</sup> EDTA</i>				
Pb	LOQ	$78 \pm 5$	$63 \pm 21$	LOQ
Zn	$4.9 \pm 0.6$	$22 \pm 2$	$80 \pm 13$	$44 \pm 3$
Cd	LOQ	$0.6 \pm 0.0$	LOQ	LOQ



**Fig. 1.** Pb, Zn and Cd concentrations in *Porcellio scaber* (A) and respective bioaccumulation factors, BAFs (B). *P. scaber* were fed for 14 days with non-remediated and remediated soils. Box and Whisker plots represent minimum, 1st quartile, average value (■), median ( $n = 3$ ), 3rd quartile and maximum values of accumulated Pb, Zn and Cd concentrations in the animals' whole body. Asterisks (\*) denote outliers. Data followed by the same letters are not significantly different, according to the Duncan test ( $P < 0.05$ ).

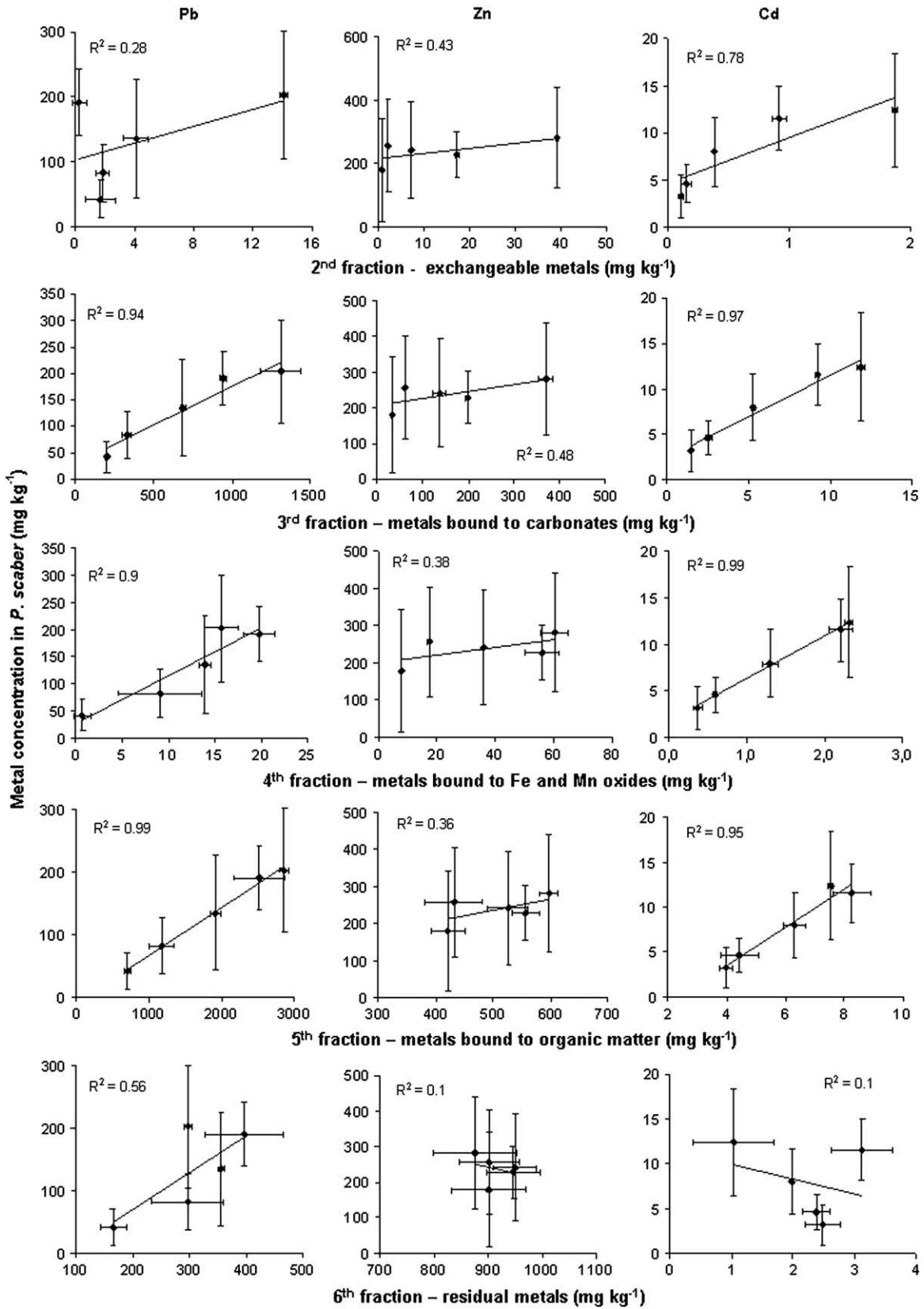
considered bioavailable to isopods. If bioavailable fractions had been preferentially or completely removed, BAFs would be higher in non-remediated than in EDTA treated soils.

The results presented in Fig. 1A imply a linear correlation between Pb and Cd accumulation in *P. scaber* and the total metal concentration in non-remediated and EDTA treated soil (this correlation is explicitly presented in Fig. 3). Lead, Zn and Cd concentrations in *P. scaber* and in the water extractable fraction were not linearly correlated ( $R^2$  were  $< 0.1$ , data are not shown). However, the proportion of metals in this fraction was much lower than in other soil fractions (Table 2). Apparent linear correlations with  $R^2 > 0.5$  existed between Pb concentrations in *P. scaber* and Pb bound to carbonate, oxide, organic and residual soil fractions (Fig. 2). Cadmium accumulation in *P. scaber* and Cd bound to exchangeable, carbonate, oxide and organic matter soil fractions were also correlated with  $R^2 > 0.5$  (Fig. 2). Linear correlations between Zn bioaccumulated in *P. scaber* and Zn bound to specific soil fractions (Fig. 2), as well as the total Zn concentration (Fig. 3), were less obvious than for Pb and Cd (for Zn  $R^2$  were  $< 0.4$ ).

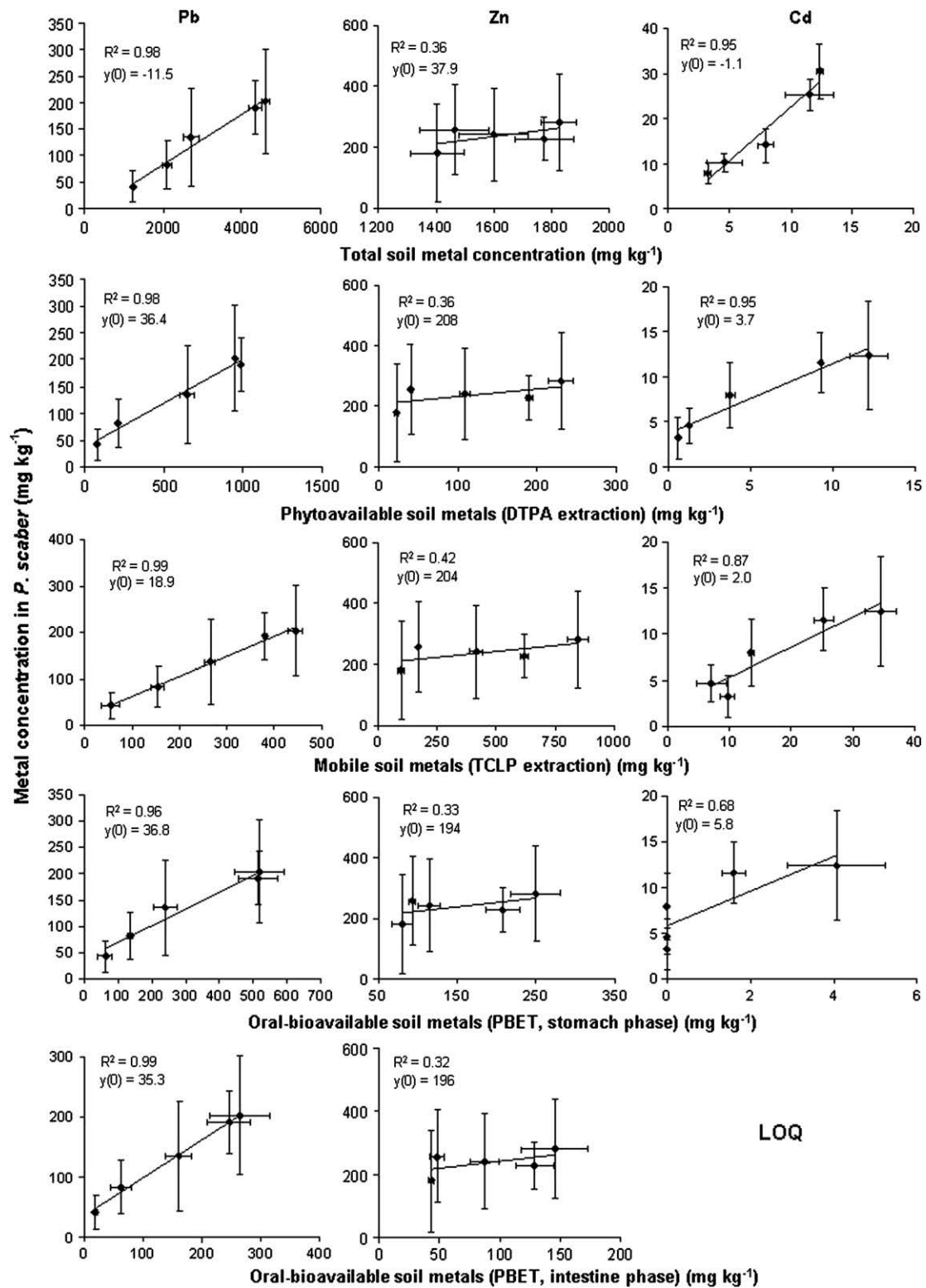
Overall, our data indicate that the metal uptake (especially of Pb and Cd) by isopods linearly correlates with the total metal concentration and also with metal concentrations in specific soil fractions in non-remediated and remediated soils (Figs. 2 and 3).

*P. scaber* is probably able to acquire metals from several soil fractions, not just the water-soluble one as expected.

Various tests based on chemical extractions are used today to assess the mobility and bioavailability of heavy metals in soil, as a part of a risk assessment procedure or as tools to measure soil remediation efficiency. In Fig. 3, we correlated the concentrations of metals accumulated in *P. scaber* with the concentrations of mobile metals (assessed using TCLP), phytoavailable metals (assessed by DTPA extraction), and metals orally bioavailable from stomach and intestinal phases (assessed with the PBET). Significant linear correlations, particularly for Pb and Cd, indicate that the pool of available metals decreased with the remediation (EDTA concentration) intensity, as assessed both by accumulation in *P. scaber* and by chemical extractions (in the small intestine phase, the concentrations of Cd were below LOQ, Table 3). However, positive intercept values (extrapolation of metal concentration in *P. scaber* to the null concentration of chemically extracted metals, given by  $y(0)$  values in Fig. 3) indicate that metals that were otherwise unavailable for chemical extractions were available and were accumulated by *P. scaber*. For a more relevant picture of the availability of metals in soil both before and after remediation, therefore, chemical extractions should be supplemented by in vivo accumulation tests.



**Fig. 2.** Pb, Zn and Cd concentrations in *Porcellio scaber* after two weeks of exposure as a function of metals present in different soil fractions determined using six-step sequential extraction (except for metals in the 1st – soil solution fraction). Linear regressions are plotted and respective  $R^2$  given. Error bars represent standard deviation from the mean value ( $n = 3$ ).



**Fig. 3.** Pb, Zn and Cd concentrations in *Porcellio scaber* after two weeks of exposure as a function of total metals concentration in soil (aqua regia extraction), phytoavailable metals (diethylenetriamine pentaacetic acid extraction), leachable metals (Toxicity Characteristic Leaching Procedure extraction), oral-bioavailable metals in simulated stomach and intestine solution (Physiologically Based Extraction Test). Linear regressions are plotted and respective  $R^2$  and intercept values  $y(0)$  are given. Error bars represent standard deviation from the mean value ( $n = 3$ ).

#### 4. Conclusions

The efficiency of remediation of polluted soil is usually assessed by determining the bioavailable metal fraction by selective chemical extractions. However, chemical soil extraction cannot fully comprise the bioavailable fraction, due to the dynamic and complex nature of metal–soil and metal–organism interactions.

Chemical extraction tests have shown that leaching the contaminated soil with EDTA successfully decreased metal mobility, phytoavailability and oral-bioavailability.

When exposed to non-remediated and remediated soil, *P. scaber* accumulated Pb and Cd in a concentration dependent manner from different soil fractions. Contrary to expectations, we observed nearly constant BAFs in remediated soil when compared to soil before treatment. When the bioavailable fraction is assessed solely on the basis of the results of chemical extraction tests, one would expect much lower amounts of bioavailable metals than indicated by BAFs.

Based on our results we concluded that combined results of chemical extractions and the accumulation test with *P. scaber* provide a more complete and relevant picture of the availability stripping of metals after soil remediation. We suggest that bioaccumulation tests with isopods *P. scaber* could be used as a supplement to chemical extractions in assessing the efficiency of remediation and the metal fraction bioavailable to soil fauna.

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