



An *in vivo* invertebrate bioassay of Pb, Zn and Cd stabilization in contaminated soil



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HIGHLIGHTS

- ▶ Biotest with isopods is suited for testing soil metal stabilization efficiency.
- ▶ *In vivo* bioaccumulation tests confirmed the results of chemical bioaccessibility.
- ▶ The isopod biotest is more sensitive and discriminatory as chemical one.
- ▶ It is suited to fast screening of bioavailable metals in soil.

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ABSTRACT

The terrestrial isopod (*Porcellio scaber*) was used to assess the remediation efficiency of limestone and a mixture of gravel sludge and red mud as stabilizing agents of Pb, Zn and Cd in industrially polluted soil, which contains 800, 540 and 7 mg kg⁻¹ of Pb, Zn and Cd, respectively. The aim of our study was to compare and evaluate the results of the biological and non-biological assessment of metal bioavailability after soil remediation. Results of a 14 d bioaccumulation test with *P. scaber* showed that that Pb and Zn stabilization were more successful with gravel sludge and red mud, while Cd was better stabilized and thus less bioavailable following limestone treatment. *In vivo* bioaccumulation tests confirmed the results of chemical bioaccessibility, however it was more sensitive. Biotesting with isopods is a relevant approach for fast screening of bioavailability of metals in soils which includes temporal and spatial components. **Capsule:** Bioavailability assessed by *P. scaber* is a more relevant and sensitive measure of metal bioavailability than chemical bioaccessibility testing in remediated industrially polluted soil.

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1. Introduction

Contamination of soils with metals is a worldwide environmental problem and soil clean-up operations have become among priority research activities with the aim to bring new applications and remediation efficiency control strategies (Manta et al., 2002; Contin et al., 2008; Legind et al., 2012).

Stabilization of polluted soils with amendments of potentially toxic metals (PTMs) like Zn, Cd, and Pb is an effective, feasible and inexpensive remedial option (Mench et al., 2000). It involves the reduction of the bioavailability and the solubility of PTMs by elevation of the soil pH by application of lime, mineral carbonates or red mud (Lombi et al., 2002; Friesl et al., 2004), by adsorption of PTMs on solid phases, such as zeolites, clay minerals and organic

matter (e.g. peat) (Boruvka and Vacha, 2006) or by precipitation of PTMs as insoluble salts (Laperche et al., 1997). Lime is widely regarded as a key ameliorant which can decrease the bioavailability, and thus the toxicity of PTMs in soils. Recently, various industrial residues such as beringite and red mud have also been considered. These are alkaline and contain solid phases capable of adsorbing metals.

After stabilization, PTMs remain in the soil as stable chemical species bound to non-labile soil fractions, and they are thus expected to be significantly less likely to affect organisms (Maenpaa et al., 2002). Nevertheless, reliable biological tests for monitoring remediation efficiency are particularly important when remediation with stabilization technologies is attempted.

Stabilization efficiency is reported to have been measured directly by quantification *in situ* of metals in food crops with the aim of assessing reduction of the entry of metals into the food chain after remediation (Friesl et al., 2006), or indirectly by assessing the bioavailability of PTMs in soil using selective chemical extraction tests (Guo et al., 2006). More frequently, various

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chemical extraction tests with specific chemical properties, such as sequential extractions, have been designed for this purpose. (McGrath, 1996; Kim and McBride, 2006). Examples include ligand-assisted extractions (Kosson et al., 2002) and other simple one-step extractions, such as extraction with CaCl_2 combined with NH_4OAc (Kalra and Maynard, 1991; Novozamsky et al., 1993).

Two terms “bioavailability” and “bioaccessibility” are used to refer to bioavailable metal fraction in substratum. These are used to distinguish between the biological and non-biological measurements of bioavailability, respectively (e.g. Oomen et al., 2003; Semple et al., 2004). The term “bioaccessibility” is used to describe the chemically measured fraction of metals which has a potential to interact with an organism. However, the term “bioavailability” is used to indicate the share of a metal taken up by an organism and accumulated during the bioaccumulation assay. Term “bioavailability” has also been used for that fraction of a metals extracted by a specific chemical assay designed for the “bioavailable” fraction (Ure, 1996). Different fast and relatively inexpensive selective chemical extractions are used to assess operationally defined pools of metals with the purpose of representing the maximum amount of available metals for absorption into organisms (Sizmur and Hodson, 2009). The aim of the current study was to compare the results of the biological and non-biological tests of bioavailability.

Arguably, the ultimate measure of contaminant bioavailability is the bioavailability to organisms which live in such environments. There is an abundance of studies on the roles played by different soil animals, especially invertebrates, as potential bio-indicators of metal pollution (Heikens et al., 2001; Han et al., 2012).

The aims of bioaccumulation studies include estimation of the bioavailable metal fraction and examination of the effects of bioavailable metals. Terrestrial isopods, which are known to accumulate the highest recorded amounts of metals in their soft tissue (Hopkin et al., 1993) are among favorable groups of invertebrates used in biomonitoring of metal pollution. From the toxicological point of view, an efficient metal accumulation strategy is actually an efficient metal detoxification mechanism, which could at the same time be used to evaluate the bioavailable metal fraction (Hopkin, 1989). The correlation between metal body burdens in isopods and available metal concentrations in the food/substratum has been often demonstrated (Heikens et al., 2001; Godet et al., 2011), and it has also been shown that the metal accumulation has no adverse effects in terms of toxicity (Hopkin, 1989).

In the study presented here an *ex situ* approach was selected in which contaminated soil from the field is tested in the laboratory. We have recently used the terrestrial isopod *Porcellio scaber* (Crustacea, Isopoda) for assessment of the bioavailability of PTMs remaining in soil after extraction with ethylenediaminetetraacetic acid (EDTA) in the laboratory (Udovic et al., 2009). Animals consumed soil for 14 d and as a result of efficient metal assimilation, significant amounts of metals were detected in the digestive glands, one of whose functions is metal storage. In the present study, we used the same experimental arrangement to assess the soil metal stabilization efficiency achieved with limestone or with a mixture of gravel sludge and red mud. This study is part of a long-term field experiment, begun in 2004, on stabilized soil in the historically smelter-polluted rural land in the vicinity of Arnoldstein in Austria (Friesl-Hanl et al., 2009). We compared the PTM bioavailability data obtained by chemical extraction tests with the bioavailability data from a *P. scaber* bioaccumulation assay. The effect of the two treatments on PTM uptake assessed by plants has been reported by Friesl et al. (2004, 2006) and Friesl-Hanl et al. (2009), and consequently, plant assays were not included in the current study. Our aim was assessment of whether the addition of limestone and of a mixture of gravel sludge (a waste product

of the gravel industry) and red mud (a bauxite residue) actually reduces the availability of metals for a selected soil invertebrate in a long-term field-experiment environment. The most popular invertebrate organisms for PTM accumulation research are molluscs, earthworms, crustaceans, insects, myriapods and arachnids (Hopkin, 1989). Because terrestrial isopods accumulate PTMs from their environment in proportion to bioavailable concentration in the soil (Hopkin et al., 1993; Heikens et al., 2001), they appeared to be very suitable as indicators of the PTM bioavailability in polluted soil and leaf litter (Gál et al., 2008). To our knowledge, this is the first report on the use of the common woodlouse *P. scaber* in a bioaccumulation assay for the assessment of the efficiency of long-term soil PTM stabilization as an approach to PTM remediation.

2. Materials and methods

2.1. Soil analysis

Soil was collected from the upper 30 cm layer of a regularly managed experimental plot near an abandoned Pb smelter in Arnoldstein (Stossau) in the southern part of Austria (46°33'16"N 13°40'57"E, 646 m above sea level), where Pb/Zn smelting activity ended in 1992, after 500 years of activity and emissions. It has been reported by Friesl et al. (2006) that local garden soils contain high amounts of Zn, Cd, Cu and As. As part of a previous study (Friesl-Hanl et al., 2009), experimental plots at this site were treated with different applications of gravel sludge (GS), red mud (RM) and limestone (CA). GS (pH 8.2) is a fine-grained waste product composed of 40–65% SiO_2 , 10–14% Al_2O_3 , 3–7% Fe_2O_3 , 5–12% CaO and 4–6% MgO. RM (pH 10.4) is a by-product of bauxite processing and consists of 15–17% Al_2O_3 , 12–14% SiO_2 , 39–43% Fe_2O_3 and 8–10% Na_2O and CA consists of 97% CaCO_3 . Analytical data and a detailed description of the experimental plots have been provided by Friesl-Hanl et al. (2009). Different treatments were applied to replicate ($n = 4$) plots using a randomized block design. For purposes of our study, we used untreated control soil (CO), a GS + RM treatment (2.5% of GS and 0.5% of RM w/w) and a CA treatment (0.5% w/w CA). We sampled soil from all four replicate plots and then pooled the samples for further use.

Soil pH was measured in a 1/2.5 (w/v) suspension of soil in 0.01 M CaCl_2 solution. Soil samples were analyzed for organic matter with modified Walkley–Black titrations (ISO, 14235), for cation exchange capacity (CEC) by the ammonium acetate method (Rhoades, 1982), and for soil texture by the pipette method (Fiedler et al., 1964). Samples were further analyzed for total N content after dry combustion (ISO, 13878), and manometrically for carbonates after reaction of the soil with HCl (ISO, 10693). Analyses were performed in triplicate and the results are presented as mean \pm SD.

2.2. Six-step sequential extraction

A modified Tessier's sequential extraction procedure (Lestan et al., 2003) was used to assess the fractionation of Pb, Zn and Cd in the soil into six fractions. The water extractable fraction (I) was obtained by extraction of 1 g of air-dried soil, 2-mm sieved, with 10 mL of deionized water for 1 h. The fraction exchangeable from soil colloids (II) was extracted from the residual soil sample with 10 mL of 1 M $\text{Mg}(\text{NO}_3)_2$ for 2 h. The fraction bound to carbonates (III) was extracted with 10 mL of 1 M NH_4OAc (pH 5) for 5 h. Fraction IV, bound to Fe and Mn oxides was extracted with 20 mL of 0.1 M $\text{NH}_2\text{OH}\cdot\text{HCl}$ (pH 2) for 12 h. The fraction bound to organic matter (V) was obtained after heating the soil suspension in 3 mL of 0.02 M HNO_3 and 5 mL of 30% H_2O_2 for 3 h at 85 °C, followed by extraction with 15 mL of 1 M NH_4OAc for 30 min. After each extraction step, the extraction solution was centrifuged and

the supernatant was 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 (VI) was obtained after the digestion of the samples with *aqua regia*. Three replicates were performed for each fractionation step. The final fractional recovery of Pb, Zn and Cd ($92.8 \pm 2.2\%$, $66.6 \pm 1.9\%$ and $88.9 \pm 8.2\%$, respectively) was calculated by summing the concentrations of all six sequentially contained fractions and expressing them as a proportion of the pseudototal amount of Pb, Zn and Cd in the particular soil, assessed as described in Section 2.4. Since PTMs may be re-adsorbed and non-selectively affected during the extraction (Kim and McBride, 2006), a fraction of the Zn could have been lost by adsorption onto the residual fraction, but most probably, a portion of Zn could have been lost with the supernatant, which was discarded after shaking residual samples in deionized water after each sequence.

2.3. Bioavailability of Pb, Zn and Cd

Different non-exhaustive extraction tests were used to assess Pb, Zn and Cd bioavailability in soil. Extraction with diethylenetriaminepentaacetic acid (DTPA), originally employed to assess plant accessible micronutrients (Zn, Fe, Mn and Cu) in near-neutral and calcareous soil (Lindsay and Norvell, 1978), is now also used extensively as a plant accessibility index in studies on PTMs (Soriano-Disla et al., 2010). The DTPA extraction solution contains 0.005 M DTPA, 0.01 M CaCl₂ and 0.1 M triethanolamine and was adjusted to pH 7.3 ± 0.05 . Air-dried soil (10 g), sieved to 2 mm, was shaken in 20 mL of DTPA extraction solution for 2 h at about 150 cycles per min on an orbital shaker. After extraction, the contents were filtered (Whatman no. 42 filter papers) and the filtrates analyzed for Pb, Zn and Cd. The extractions were conducted in triplicate.

Extraction with ethylenediaminetetraacetic acid (EDTA) disodium salt is used to assess the mobile and easily mobilizable fraction of PTMs from soil, which can be taken up by soil organisms together with soil water (Kosson et al., 2002). Sieved (2 mm) air-dried soil (0.8 g) was extracted with 80 mL of 0.05 M EDTA solution at pH 7.5 ± 0.05 for 48 h on an end-over-end shaker. After the extraction period, the contents were filtered and the filtrates analyzed for Pb, Zn and Cd. The extractions were conducted in triplicate.

One-step extractions with CaCl₂ and NH₄OAc were developed to assess the bioavailability of PTMs, especially in plants (Kalra and Maynard, 1991; Novozamsky et al., 1993). For extraction with CaCl₂, air-dried, sieved (2 mm) triplicate samples of soil (10 g) were extracted with 100 mL of 0.01 M CaCl₂ solution for 3 h at room temperature on an orbital shaker operated at approximately 150 cycles per minute. After the extraction, the extract was filtered and the filtrates analyzed for Pb, Zn and Cd. For the NH₄OAc extraction, air-dried sieved (2 mm) samples of soil (10 g) were extracted in 100 mL of 1 M NH₄OAc at pH 7 for 1 h on an orbital shaker set at 150 cycles per minute. The extract was then filtered and the filtrate analyzed for Pb, Zn and Cd.

2.4. Metal pseudototal concentration analyses in soil samples

Air-dried samples of soil (1 g) were ground in an agate mill, digested for 2 h in boiling *aqua regia* (28 mL), and diluted with deionized water to 100 mL. Pb, Zn and Cd were analyzed by flame (acetylene/air) atomic absorption spectroscopy (AAS) with a deuterium background correction (AAAnalyst 100, Perkin–Elmer and Varian AA240FS). Pb, Zn and Cd in the solutions from sequential extractions, and the DTPA, CaCl₂, EDTA and NH₄OAc extracts were analyzed directly by AAS. The total concentration of metals was assessed after *aqua regia* digestion. A standard reference material

(WEPAL 2003.1.1) used in inter-laboratory comparisons from HBLFA (Raumberg-Gumpenstein, Irdning, Austria), was used in the digestion and analyses as part of the QA/QC protocol. The limits of quantification of Varian AA240FS for Pb, Zn and Cd were 0.25, 0.02 and 0.03 mg L⁻¹ respectively. In case of animal samples, reagent blanks and standard solutions (Merck) were used to ensure analytical accuracy and precision. Certified reference material (TORT-2, National Research Council of Canada) was used to check the accuracy of the analytical procedure. The sensitivity of AAAnalyst 100, Perkin–Elmer for Pb, Zn and Cd were 0.45, 0.018 and 0.028 mg L⁻¹ respectively. The percentage recoveries of Pb, Zn and Cd were $127 \pm 3\%$, $106 \pm 5\%$ and $137 \pm 2\%$, respectively. Where appropriate, reagent blank and analytical duplicates were used to ensure accuracy and precision in the analyses.

2.5. Experimental design with *P. scaber*

Adult specimens of a terrestrial isopod *P. scaber* collected in September 2009 at a farm near Litija, Slovenia, were kept in the 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). Since at the end of the exposure period, the isopods are fed uncontaminated hazel leaves to remove soil and metal from their digestive systems, the uncontaminated hazel leaves were chemically analyzed. Metal concentrations in the leaves were in the range within which leaves are classified as non-metal polluted and were $64.5 \pm 11.4\%$, $48.3 \pm 9.5\%$, $4.50 \pm 0.44 \mu\text{g g}^{-1}$ of leaf dry weight of Pb, Zn and Cd, respectively. During the experiment, the animals were exposed only to soil. No leaf litter was added to the experimental containers.

In each experimental group, 15 adult specimens of 30–60 mg fresh weight were each exposed for 14 d to approximately 400 mL of air-dried experimental soil (CO, GC + RM and CA) in rectangular 1 L plastic vessels with plastic covers perforated to allow circulation of air. Before the exposure, approximately 400 animals were selected from the stock culture and divided into three weight classes (20–30 mg, 30–40 mg, 40–50 mg). Females with marsupium were not included in the experiment. Five vessels per treatment (CO, GC + RM or CA) were filled with the corresponding soil. Then 15 animals (five from each weight class) were placed in each experimental container. All together 225 animals were selected out of initial population of 400 animals. In order to achieve a soil moisture gradient, one corner of the vessel was kept moistened with tap water. The experimental vessels were maintained at room temperature and checked daily to regulate the soil moisture content. No organic matter which could serve as an additional food source was added to the container, in addition to the soil, which consequently served both as a substratum in which the animals burrowed and as a food source. After 14 d of exposure, the animals were removed from the vessels and fed with uncontaminated hazel leaves for 24 h to remove soil and metals from their digestive systems. This is a commonly used approach to replace the contaminated food with uncontaminated one when bioassimilation is studied (Pipan-Tkalec et al., 2010). Presence of chemicals in the gut lumen would affect the final result concerning the amount of measured chemical (Udovic et al., 2009). After transfer to clean food, the animals were moved separately into plastic tubes, anaesthetised by cooling, frozen and stored, then lyophilized. Prior to analysis, dry samples were weighed and completely digested in a nitric acid/perchloric acid mixture (7:1). Samples were evaporated in a dry block heater for 30 min at 40 °C, followed by 30 min at 65 °C, 1 h at 85 °C, 1 h at 105 °C, 1 h at 125 °C and 12 h at 185 °C. After evaporation of the acid, the residue was taken up in 0.1% HNO₃ and total Pb, Zn and Cd concentrations in whole animals were analyzed by flame atomic absorption spectrometry (Perkin

Elmer AAnalyst 100). Reagent blanks and standard solutions (Merck) were used to ensure accuracy and precision in the analysis.

We calculated metal accumulation in animals with bioaccumulation factors (BAFs) as the ratio of metal concentration in the isopods to that measured in soil.

The initial body concentrations of Pb, Zn and Cd in *P. scaber* prior to exposure to polluted and remediated soil assessed in 15 specimens, as described above, were $30.2 \pm 22.7\%$, $350.2 \pm 95.5\%$ and $4.9 \pm 1.6 \mu\text{g g}^{-1}$ respectively.

2.6. Statistical analysis

Statistical significance of differences between the Pb, Zn and Cd concentrations in isopods exposed to variously remediated and non-remediated soils was assessed by Mann–Whitney *U* test ($p < 0.05$) using Statgraphics software (Statgraphics Plus for Windows 4.0, Statistical Graphics, Herndon, VA, USA).

3. Results and discussion

3.1. Soil properties and PTM bioavailability

The properties of non-treated control soil (CO) and soils treated with CA and with GS + RM are presented in Table 1. Treatment of the CO soil with CA and with GS + RM significantly increased the soil pH from 4.7 to 6.4. Similarly, in CA and GS + RM, the C/N ratio and the cation exchange capacity increased, while the percentage of organic matter was significantly decreased. The CO soil contained almost no carbonates and the amendments failed to alter their percentage significantly. The pseudototal concentrations of Pb, Zn and Cd in CA and GS + RM soils were lower than in CO soil, which can be attributed to “dilution” of the metals by the applied amendments.

All the chemical extraction tests showed significantly lower PTM bioavailability in treated soils compared to the untreated soils, but only NH_4OAc extraction showed significant differences between CA and GS + RM (Table 2). CA treatment resulted in a 65%, 84% and 59% decrease in Pb, Zn and Cd bioavailability, respectively, while the more efficient GS + RM treatment resulted in a 78%, 93% and 77% decrease in Pb, Zn and Cd bioavailability, when assessed after NH_4OAc extraction. These results are in agreement with those reported by Friesl-Hanl et al. (2009) who measured the accumulation of Pb, Zn and Cd in barley (*Hordeum vulgare*), velvet grass (*Holcus lanata*) and plantain (*Plantago lanceolata*) grown on the same untreated (CO) and treated soils (CA and GS + RM) used in our study. They reported lower PTM plant uptake in plots

Table 1

Selected physical and chemical soil properties and pseudototal metal concentrations in untreated control soil (CO), soil treated with gravel sludge and red mud (GS + RM) and soil treated with limestone (CA) from Stossau, Arnoldstein (Austria). Different letters denote significant difference (Duncan, $p < 0.05$) between CO, CA and GS + RM.

	CO	CA	GS + RM
<i>Physical and chemical properties</i>			
pH	4.66 ± 0.03^a	6.37 ± 0.3^b	6.43 ± 0.2^b
Organic matter (%)	4.6 ± 0.1^a	3.4 ± 0.1^c	3.9 ± 0.1^b
C/N	8.1 ± 0.2^a	10.5 ± 0.0^b	11.4 ± 0.3^c
CEC (mmol $\text{C}^+ 100 \text{g}^{-1}$)	15.2 ± 0.2^a	16 ± 0.3^b	16.8 ± 0.3^c
Sand (%)	41.6 ± 1.1^a	41.7 ± 0.2^a	40.9 ± 1.8^a
Silt (%)	44.5 ± 2.6^a	44.1 ± 0.7^a	46.4 ± 2.3^a
Clay (%)	13.9 ± 1.6^a	14.2 ± 0.7^a	12.7 ± 1.3^a
<i>Pseudototal metal concentrations</i>			
Pb (mg kg^{-1})	802.5 ± 13.4^a	706.4 ± 12^b	716.5 ± 11.1^b
Zn (mg kg^{-1})	543.2 ± 2.9^a	474.1 ± 3.6^b	469 ± 12.4^b
Cd (mg kg^{-1})	7.44 ± 0.5^a	6 ± 0.1^b	5.8 ± 0.3^b

Table 2

Pb, Zn and Cd bioaccessibility in untreated control soil (CO), soil treated with limestone (CA) and soil treated with gravel sludge and red mud (GS + RM), assessed with 0.005 M diethanoltriaminepentaacetic acid (DTPA), 1 M ammonium acetate (NH_4OAc), 0.05 M ethylenediaminetetraacetate (EDTA) and 0.01 M CaCl_2 extractions. Results are presented as the means of three replicates \pm SD. LOQ, below the limit of quantification, i.e. 0.25, 0.02 and 0.03 mg L^{-1} for Pb, Zn and Cd, respectively. Different letters denote a significant difference (Duncan, $p < 0.05$) among CO, CA and GS + RM.

	CO	CA	GS + RM
<i>Pb (mg kg^{-1})</i>			
DTPA	125.5 ± 14.3^a	98.9 ± 5.9^b	91.7 ± 10.7^b
NH_4OAc	40.8 ± 1.1^a	14.1 ± 0.8^b	9 ± 0.1^c
EDTA	306 ± 11^a	249 ± 22^b	227 ± 9.3^b
CaCl_2	3 ± 0.3^a	1.9 ± 0.1^b	2 ± 0.5^b
<i>Zn (mg kg^{-1})</i>			
DTPA	38.6 ± 3.7^a	13.8 ± 0.9^b	10.7 ± 1.2^b
NH_4OAc	21.4 ± 0.4^a	3.4 ± 0.1^b	1.6 ± 0.2^c
EDTA	88 ± 6.3^a	39.5 ± 2.7^b	33.8 ± 1.2^b
CaCl_2	31.7 ± 1.6^a	1.5 ± 0.1^b	0.6 ± 0.2^b
<i>Cd (mg kg^{-1})</i>			
DTPA	1.5 ± 0.2^a	1.1 ± 0.1^b	0.9 ± 0.1^b
NH_4OAc	1.7 ± 0.1^a	0.7 ± 0.1^b	0.4 ± 0.0^c
EDTA	2.6 ± 0.2^a	1.9 ± 0.2^b	1.7 ± 0.1^b
CaCl_2	1.1 ± 0.1	LOQ	LOQ

remediated with GS + RM even 5 years after remediation, when compared to CO soil. The results of the sequential extraction also indicated that both CA and GS + RM treatments resulted in significantly lowered fractions of labile Pb, Zn and Cd chemical species (measured in water extractable and exchangeable soils) when compared to CO soil (Table 3). In general, the proportions of Zn and Cd in the less labile fractions, those bound to carbonates, to Fe and Mn oxides, to organic matter, and the residual fraction, increased significantly. The concentration of Pb decreased in fractions bound to carbonates and to Fe and Mn oxides while it increased in the organic matter and residual fractions.

Applications of CA, GS and RM have been reported to be effective in reducing the mobility of PTMs (Lothenbach et al., 1998; Krebs et al., 1999; Udeigwe et al., 2009). In our study, both amend-

Table 3

Results of six step sequential extractions: Fractionation of Pb, Zn and Cd in untreated control soil (CO), soil treated with limestone (CA) and soil treated with gravel sludge and red mud (GS + RM). Results are presented as percentage of the sum of all fractions; data are given as means of three replicates \pm SD. LOQ, below the limit of quantification, i.e. 0.25, 0.02 and 0.03 mg L^{-1} for Pb, Zn and Cd, respectively. Different letters denote significant difference (Duncan, $p < 0.05$) between CO, CA and GS + RM. I: water extractable; II: exchangeable; III: bound to carbonates; IV: bound to Fe and Mn-oxides; V: bound to organic matter; and VI: residual fraction.

	CO	CA	GS + RM
<i>Pb (%)</i>			
I	LOQ	LOQ	LOQ
II	3.5 ± 0.2^a	2.2 ± 1.9^b	2 ± 0.1^b
III	24.2 ± 0.4^a	21.6 ± 0.4^b	20.6 ± 0.7^b
IV	26.3 ± 2.4^a	$21.6 \pm 2.1^{a,b}$	17.9 ± 3.3^b
V	41.1 ± 3.9^a	48.7 ± 3.9^b	53.3 ± 2.7^b
VI	4.8 ± 0.1^a	5.9 ± 0.2^b	6.2 ± 0.1^b
<i>Zn (%)</i>			
I	0.7 ± 0.1^a	0.1 ± 0.0^b	0.1 ± 0.0^b
II	21.9 ± 0.9^a	3.2 ± 0.0^b	1.5 ± 0.1^c
III	9.5 ± 0.4^a	10.4 ± 0.1^b	9.5 ± 0.3^a
IV	13.7 ± 0.6^a	16.9 ± 0.9^b	16.3 ± 0.7^b
V	21.9 ± 2.3^a	28.3 ± 2^b	29.8 ± 1.2^b
VI	32.4 ± 0.1^a	41 ± 0.1^b	42.7 ± 1.2^b
<i>Cd (%)</i>			
I	LOQ	LOQ	LOQ
II	36.7 ± 1.9^a	16.4 ± 0.4^b	11.4 ± 0.0^c
III	25.4 ± 0.1^a	34 ± 0.5^c	32.7 ± 0.8^b
IV	16.7 ± 0.3^a	24.3 ± 1.6^b	24.5 ± 2.4^b
V	8.9 ± 0.5^a	11.3 ± 0.5^b	13.5 ± 1.4^c
VI	12.2 ± 1^a	14.1 ± 1.4^a	17.9 ± 1.8^b

ments caused a significant increase in soil pH (Table 1), largely responsible for the immobilising effect, which may include the conversion of PTMs to less labile chemical forms by adsorption and desorption and co-precipitation processes in soil (Lombi et al., 2002; Friesl-Hanl et al., 2009). As proposed by Kumpiene et al. (2008), the data presented in Table 3 could indicate, that the rise in pH lead to the formation of Zn and Cd carbonates and oxides, which precipitated as less soluble, and less bioavailable chemical forms. In the case of GS + RM application, adsorption on clay, clay-organic matter complexes and oxides, constituents of gravel sludge and red mud, could contribute to PTM stabilization (Arias et al., 2002; Friesl et al., 2006), but a more focused study on the topic would be necessary to recognize the stabilization mechanisms involved.

Animals were exposed to soils containing similar PTM pseudo-total amounts (Table 1), but with different bioaccessibility (Table 2) due to the applied amendments. After 14 d of exposure to the CO, CA or GS + RM treated soils, the isopods accumulated the highest amount of PTMs in CO soil, compared to CA and GS + RM. The two amendments had different effects on metal bioavailability. Only the GS + RM treatment significantly reduced the accumulation of Pb and Zn in *P. scaber*, by 52.7% and 17.8%, respectively; the CA treatment had no significant effect on Pb and Zn accumulation. For Cd, both CA and GS + RM treatments resulted in a signifi-

cantly lower body burden than in the animals exposed to CO soil. Compared to animals exposed to non-treated CO soil, the CA treatment was more effective than the GS + RM treatment, as the Cd body burdens were lowered in CA soil by 64.2% and in GS + RM soil by 10.1%. These results indicate that clay minerals, constituents of gravel sludge and red mud (GS + RM), are effective stabilizing agents for Pb and Zn, but are less effective for Cd. On the other hand, in CA soil, the high content of calcite (CaCO_3) in limestone (95%), as compared to the smaller (5–12%) amounts of CaO in GS + RM soil, favors Cd stabilization. Similarly, Lothenbach et al. (1998) reported that calcite is more effective than clay minerals in stabilizing Cd in soil.

Our results on metal accumulation are consistent with those of Heikens et al. (2001) who also provided evidence that the accumulation of Cd and Pb, in isopods is not correlated with the total soil concentrations. In our study, the BAF was unchanged while the soil metal concentrations were different (Fig. 1). Godet et al. (2011) emphasized that the chemical forms of metals as well as their mutual effects may govern tissue accumulation. Zidar et al. (2009) and Godet et al. (2011) provided evidence on the mutual effect of Cd and Zn on accumulation of each of these two metals and their results were confirmed by Witzel (2000) and by Odendaal and Reinecke (2004). In addition to mutual effects, the availability of ingested metals in the digestive system may be altered and this cannot be predicted by chemical tests. The bioavailability of metals to *P. scaber* is influenced by a variety of chemical and/or physical factors. There may be a correlation between biotests using *P. scaber* and chemical tests however such a correlation is not always evidenced. Consequently, although chemical and biological tests are expected to give similar results on remediation efficiency, it is not possible to compare chemical and biological tests directly. However, chemical and biological tests are in agreement as demonstrated by our results. The purpose of biotests is to confirm the conclusions of chemical tests on the bioavailability, especially when the data are to be used in decision making.

4. Conclusion

In this study, we tested the stabilization efficiency by chemical methods and a bioassay of soil contaminated with Pb, Zn and Cd. Since metal bioavailability depends on many factors including those of the digestive systems of animals, chemical analyses and biotests may give different results. Our results showed there to be agreement between biotests and chemical tests. Chemical extractions however proved to be less sensitive. In particular, the chemical method failed to discriminate between the efficiency the two stabilization amendments examined (Table 2), and the biotest with *P. scaber* showed that limestone (CA) was more effective than gravel sludge combined with red mud (GS + RM) (Fig. 1). Based on these results we can conclude that stabilization of the metals was successful and even ingestion of stabilized soil did not render metals into more available forms before ingestion. We suggest that the chemical tests may even be validated with biotests especially when the safety of remediated soil is discussed or efficiency of metal remediation is calculated. For such purposes, a selection of available biotests is needed. We recommend a *P. scaber* biotest for the study of metal bioavailability. It is capable of accumulating metals relative to available concentrations and the animals are tolerant to high metal concentrations and large enough to allow metal analyses in individual animals.

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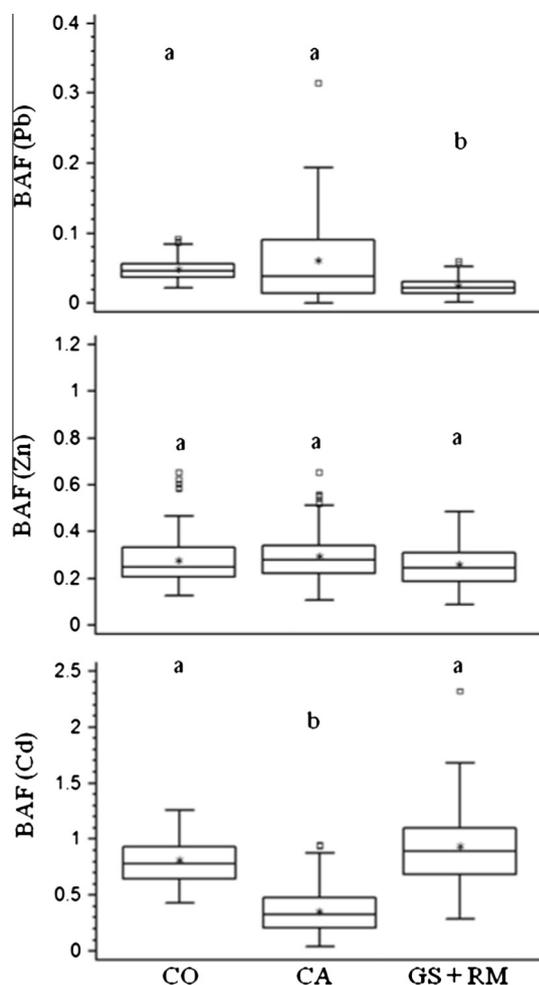


Fig. 1. Bioaccumulation factors (BAFs) for Pb, Zn and Cd accumulated in *Porcellio scaber*. Box and Whiskers plots represent minimum, 1st quartile, average value (*), median, 3rd quartile and maximum values. □ denote outliers. Different letters denote significant differences among CO, CA and GS + RM (Mann–Whitney test, $p < 0.05$).

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