



## Evaluation of the effectiveness of various amendments on trace metals stabilization by chemical and biological methods

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### ABSTRACT

We evaluated the effects of five different kinds of amendments on heavy metals stabilization. The five amendments were: zero valent iron, limestone, acid mine drainage treatment sludge, bone mill, and bottom ash. To determine bioavailability of the heavy metals, different chemical extraction procedures were used such as, extraction with  $(\text{Ca}(\text{NO}_3)_2)$ , DTPA; toxic characteristic leaching procedure (TCLP), physiologically based extraction test (PBET) that simulates gastric juice, and sequential extraction test. Bioavailability was also determined by measuring uptake of the heavy metals by lettuce (*Lactuca sativa* L.) and earthworms (*Eisenia fetida*). In addition, dehydrogenase activity was measured to determine microbial activity in the soil with the different amendments. The addition of amendments, especially limestone and bottom ash, resulted in a significant reduction in extractable metal contents. Biological assays using lettuce, earthworm, and enzyme activity were found as appropriate indicators of available metal fraction after *in situ* stabilization of heavy metals. In conclusion, TCLP and sequential extraction test appear to be promising surrogate measure of metal bioavailability in soils for several environment endpoints.

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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 [1]. Commonly used cleanup methods involve excavation and landfilling of the contaminated soil; however, in large areas with high levels of heavy-metal contamination, such as agricultural fields adjacent to abandoned mines, conventional remedial options are too expensive, and alternative technologies have been recently favored. These include *in situ* stabilization to reduce the toxic effects of heavy metals present in the soil and thus the major threat that they pose to agricultural and other ecosystems. Stabilization techniques can improve soil physico-chemical and biological properties, do not generate by-products, are less expensive and therefore are more suitable for remediation of extensive areas of low-value land [2].

Most environmental guidelines for remediation of soils contaminated with trace elements are based on total concentrations of these elements. However, knowledge of the total concentrations of trace elements in soil provides very limited information about their chemical behavior and potential fate [3]. The (bio)available

fraction is the fraction of the total amount of a chemical present in a specific environmental compartment that, within a given time span, is either available or can be made available for uptake by (micro)organisms from either the direct surrounding of the organism or by ingestion of food [4]. Although the readily soluble fraction of trace elements is generally considered to be bioavailable, current methods used to assess soluble and bioavailable fractions need re-evaluation due to their variability in space and over time [5]. These variations arise from fluctuations in total trace-element concentrations and are not directly related to soil organism toxicity due to a number of modifying factors, such as pH, organic matter content, and clay content.

Different types of solutions are used as extraction methods for bioavailability evaluation including (i) mineral acids (e.g., HCl,  $\text{HNO}_3$ ), (ii) chelating agents (e.g., EDTA, DTPA), (iii) buffered salts (e.g.,  $\text{NH}_4\text{OAc}$ ), and (iv) neutral salts (e.g.,  $\text{CaCl}_2$ ,  $\text{NH}_4\text{NO}_3$ ,  $\text{Ca}(\text{NO}_3)_2$ ). While chemical extraction procedures for available trace elements have been reported in the literature, none is considered universally applicable to assessing the bioavailable metal fraction in soils [6]. Indeed, the bioavailability of toxic elements cannot be measured directly using chemical analyses, as bioavailability can only be determined in living organisms [7]. Thus, a method of measuring metal availability not involving organisms (reduce expense and variability) that is well related to bioavailability would be an extremely useful screening tool for evaluating metal

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**Table 1**  
Physico-chemical properties and total metal concentrations of the soils and amendments used in this experiment<sup>a</sup>.

Parameter	Soil	LS <sup>b</sup>	AMD	BM	BA
pH <sup>c</sup>	5.78 ± 0.12	9.50 ± 1.24	8.21 ± 0.56	6.69 ± 0.52	9.23 ± 0.49
Clay <sup>d</sup>	10.25 ± 0.25	– <sup>g</sup>	–	–	–
Silt	25.31 ± 0.33	–	–	–	–
Sand	64.44 ± 0.22	–	–	–	–
CEC <sup>e</sup>	12.53 ± 0.41	–	–	–	–
Total carbon (%)	2.72 ± 0.17	–	–	13.7 ± 1.23	–
Total nitrogen (%)	0.14 ± 0.10	–	–	4.27 ± 0.72	–
Cd (mg kg <sup>-1</sup> ) <sup>f</sup>	4.23 ± 0.18	1.18 ± 0.32	<0.01	0.08 ± 0.02	0.14 ± 0.03
Cu (mg kg <sup>-1</sup> )	30.12 ± 0.26	23.24 ± 0.21	0.16 ± 0.11	1.88 ± 0.28	11.61 ± 1.22
Pb (mg kg <sup>-1</sup> )	214.41 ± 5.32	30.99 ± 1.42	0.04 ± 0.09	5.81 ± 0.12	2.82 ± 0.46
Zn (mg kg <sup>-1</sup> )	259.83 ± 1.29	808.45 ± 10.25	0.15 ± 0.23	115.11 ± 9.21	11.00 ± 1.45

<sup>a</sup> Mean values and standard deviations of three replicates.

<sup>b</sup> LS, limestone; AMDTS, acid mine drainage treatment sludge; BM, bone mill; BA, bottom ash.

<sup>c</sup> Soil pH measured at the ratio of soil to H<sub>2</sub>O as 1:5 (mass:volume).

<sup>d</sup> Particle size (%) was analyzed by pipetting method.

<sup>e</sup> Cation exchange capacity (cmol kg<sup>-1</sup>).

<sup>f</sup> Aqua regia extractable metal concentration.

<sup>g</sup> “–” means not determined.

contaminated soils [8]. However, only a few bioavailability studies using a range of ecological receptors that engage the relevant pathways have been reported thus far [3,9].

The objectives of the present research were to (i) determine the efficacy of chemical immobilization amendments in reducing bioavailability and ecotoxicity in toxic, metal-contaminated abandoned mine soil, and (ii) evaluate the efficacy of amendments as reflected in the results of tests with different targets: water, plants, soil fauna, and humans. This was carried out using Ca(NO<sub>3</sub>)<sub>2</sub>, DTPA, TCLP (toxic characteristic leaching procedure), sequential soil extraction, plant and earthworm uptake of heavy metals, and a physiologically based extraction test (PBET) for estimating heavy metals bioavailability in the human gastrointestinal (GI) tract.

## 2. Materials and methods

### 2.1. Experimental set-up

Topsoil (<20 cm depth) from contaminated sites in the Suseong gold mining area, Chungnam Province, Korea, was collected. Soil subsamples were air-dried, passed through a 2 mm sieve, and subjected to chemical characterization. Soil texture was analyzed using the pipetting method of Gee and Bauder [10]. Soil pH was determined using 1:5 soil:H<sub>2</sub>O. Total nitrogen was determined by the Kjeldahl method [11], total carbon (C) by the Turin method [12], and cation exchange capacity using the ammonium-saturation and distillation method [13]. Soil used in this experiment was a saline alluvial sandy loam containing 63% sand, 27% silt and 10% clay (Table 1).

Effects of five amendments were examined; zero valence iron (ZVI), limestone (LS), acid mining drainage sludge (AMDTS), bone mill (BM), and bottom ash (BA). Limestone (pH 9.50) (Table 1), collected from limestone mine, consists of 58% CaCO<sub>3</sub>. AMDTS (pH 8.21), obtained from AMD treatment facility, consists of 22.8% Fe<sub>2</sub>O<sub>3</sub>, 30.5% CaO, 1.88% Al<sub>2</sub>O<sub>3</sub>, 0.67% MgO. BA (pH 9.23), obtained from coal power plant, consists of 52.8% SiO<sub>2</sub>, 22.3% Al<sub>2</sub>O<sub>3</sub>, 9.28% Fe<sub>2</sub>O<sub>3</sub>. ZVI and BM were obtained from Aldrich Chemical Supplies and commercial fertilizer vendor, respectively. Other amendments, LS, AMDTS, BM, and BA are all industrial waste or by-products which have been shown to have great potential to stabilize heavy metals in soils. Application of those materials also achieves reduction of waste disposal through revalorization of industrial wastes into industrial co-products.

Pseudo-total trace element (Cd, Cu, Pb, and Zn) concentrations in soil and amendments were measured by inductively

coupled plasma optical emission spectrometry (ICP-OES) after digestion of the samples with aqua regia according to ISO 11466 [14].

Each amendment was thoroughly incorporated into soil to a final amount of 2% (w/w). The amendments were thoroughly mixed with the soil to obtain homogeneity, after which the soils were equilibrated for 40 days.

### 2.2. Chemical assessment

The effect of each amendment on contaminant extractability was evaluated by extraction of the sample with a 0.1 M Ca(NO<sub>3</sub>)<sub>2</sub> solution according to the procedure of Conder et al. [8]. Briefly, 1 g of soil was combined with 0.1 M Ca(NO<sub>3</sub>)<sub>2</sub>, mixed in a shaker, centrifuged for 20 min at 5100 × g, and then filtered through a Whatman GF/F 0.7 μm borosilicate glass filter.

Metals were DTPA-extracted using the method of Lindsay and Norvell [15]. Briefly, 1.5 g of soil was extracted with 15 ml of a solution containing 5 mM DTPA and 10 mM CaCl<sub>2</sub> in a 40 ml polypropylene centrifuge tube. The tube was shaken on a wrist-action shaker for 2 h, centrifuged for 20 min at 5100 × g, and then filtered through Whatman GF/F 0.7 μm borosilicate glass filters.

Contaminants were measured in soils by TCLP extraction according to the USEPA method 1311 [16]. The TCLP extracting agent was acetic acid (pH 2.88). The solid/liquid ratio TCLP was kept at 1:20. After 18 h of mixing in a TCLP tumbler, the leachate was filtered using Whatman GF/F 0.7 μm borosilicate glass filters. The filtrate was acidified by adding a small amount of nitric acid to a pH < 2, followed by ICP analysis.

The bioaccessibility of heavy metals in the GI tract was measured using the modified PBET described by Geebelen et al. [17]. Briefly, 0.35 g soil was shaken (30 rpm) with synthetic gastric solution (0.4 M glycine; pH 2.2) for 1 h at 37 °C and then filtered through a Whatman GF/F 0.7 μm borosilicate glass filter.

Changes in the fractions of heavy metals were determined as modified version of Tessier et al. [18]. Briefly, the heavy metals were separated into six operationally defined fractions: extractable with distilled water (water soluble, F1), extractable with 1 M NH<sub>4</sub>OAc at pH 7 (exchangeable, F2), extractable with 1 M NH<sub>4</sub>OAc at pH 5 (carbonate bound, F3), extractable with hydroxylamine (associated mainly with Fe–Mn oxides, F4), extractable with H<sub>2</sub>O<sub>2</sub> in 1 M HNO<sub>3</sub> (strongly complexed with organic matter, F5), and extractable with HClO<sub>4</sub> and HF (residual, F6).

All chemicals were of analytical grade or better. Centrifuge tubes were acid-washed (5% HNO<sub>3</sub>) before each use, and disposable

**Table 2**  
pH and extractable heavy metals (mg kg<sup>-1</sup>) of the soils with different amendment addition<sup>a</sup>.

Treatments	pH	Ca(NO <sub>3</sub> ) <sub>2</sub> -extractable			DTPA <sup>b</sup> -extractable			PBET			TCLP		
		Cd	Pb	Zn	Cd	Pb	Zn	Cd	Pb	Zn	Cd	Pb	Zn
Control	7.20c	0.30a	0.11a	5.05a	0.49b	120.04b	27.17a	1.31a	186.37ab	76.03a	0.07a	2.36a	3.08a
ZVI <sup>c</sup>	7.22c	0.19c	0.10a	1.72c	0.47b	117.98bc	17.33d	1.11a	139.51cd	51.02c	0.06bc	2.20ab	2.05bc
LS	8.85a	0.01d	0.02c	0.00d	0.40c	103.59c	17.64d	1.09a	170.02bc	62.98b	0.06ab	1.90b	2.18b
AMDS	7.42b	0.03d	0.03c	0.00d	0.53a	140.70a	25.42b	1.31a	224.85a	71.30ab	0.06ab	2.43a	2.38b
BM	7.02d	0.24b	0.07b	2.41b	0.56a	137.39a	25.58b	1.22a	188.48ab	61.24b	0.05cd	0.72c	1.63c
BA	7.26 bc	0.16c	0.04c	1.96bc	0.32d	74.98d	20.26c	0.78b	102.03d	49.50c	0.04d	1.08c	1.97bc

<sup>a</sup> Means ( $n = 3$ ) followed by same letter within a column are not significantly different ( $p > 0.05$ ).

<sup>b</sup> DTPA, diethylenetriamine pentaacetate; PBET, physiologically based extraction test; TCLP, toxic characteristic leaching test.

<sup>c</sup> ZVI, zero valent iron; LS, limestone, AMDS, acid mine drainage treatment sludge; BM, bone mill; BA, bottom ash.

labware was used for all other analyses. Heavy metal analyses were conducted using ICP-OES; blanks were run for background correction and to identify other sources of error. Multi-element standards were run every 20 samples, with recovery within  $100 \pm 10\%$ . The detection limits of Cd, Pb, and Zn were 0.001, 0.01, and 0.001 mg L<sup>-1</sup>, respectively.

### 2.3. Biological assessment

To examine the effect of the addition of amendments on the phytoavailability of contaminants, lettuce (*Lactuca sativa* L.) was grown from seed in plastic pots (1 kg capacity, 15 cm diameter, 25 cm height) filled with contaminated soils prepared as described above. The seeds were initially sown in peat-based horticultural compost. Two weeks after germination, the seedlings were transplanted into contaminated soils (five seedlings/pot, three pots/treatment). The experiment was conducted under controlled greenhouse conditions (temperature 15–25 °C, relative humidity 60–70%) with daily watering. After 45 days, lettuce was harvested, rinsed with distilled water, and dried at 60 °C for 48 h. The edible parts of harvested plants were digested with HNO<sub>3</sub> by hot-block digestion procedure at 140. The resultant was diluted with 1% (v/v) nitric acid and the levels of heavy metals in the prepared leaves were determined by ICP-OES. Spinach leaves (SRM 1570a) were used as certified plant material (i.e., NIST 1570a) to ensure the quality of plant digestion analyses. Average recoveries were 93%, 107%, and 98% for Cd, Pb, and Zn, respectively.

Earthworms, *Eisenia fetida*, were obtained from the National Institute of Agricultural Science and Technology, Korea, and maintained in control soil in a plastic container. The container was placed in a dark incubator set at 20 °C, with 80% relative humidity, for an acclimation period of 2 weeks. Each of the control and amended soils (250 g wet weight) was placed in a 500 ml polypropylene jar together with five earthworms, and the jars were then covered with perforated laboratory film. The moisture content of the soils was maintained at 70% of the water holding capacity. Incubation experiments were conducted for 28 days. At the end of the exposure period, the earthworms were removed and placed on moist tissue for 24 h to trap the material emptied from their guts. Each earthworm was then dried at 40 °C for 24 h in a pre-weighed conical flask. Following drying and the addition of concentrated nitric acid, the flasks were agitated for 24 h.

Immediately following equilibrium periods, soils were immediately subsampled to determine dehydrogenase activity which was used as the microbial endpoint. Soil subsamples were kept moist at <4 °C. Prior to the analyses, the samples were sieved through a 2-mm sieve, and their water content was then determined to express soil enzyme activity on a dry-matter basis. Dehydrogenase activity was assayed by the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to triphenyl formazan [19].

### 2.4. Data analysis

All determinations were performed in triplicate with each independent soil and plant sample. The means from the different treatments were compared using one-way analysis of variance (one-way ANOVA). When significant  $p$ -values ( $p < 0.05$ ) were obtained, the differences between individual means were compared using Tukey's HSD post-hoc test ( $p < 0.05$ ) or Student's  $t$  test, when suitable. Pearson's correlation coefficients were calculated between soil metal contents and soil enzyme activity or plant metal contents. Statistical analyses were conducted using SPSS 11.5 for Windows.

## 3. Results

### 3.1. Contaminant extractability

The soluble and extractable fractions of heavy metals in soil were significantly decreased by amendment additions (Table 2). The Ca(NO<sub>3</sub>)<sub>2</sub>-extractable metal content was in the range of 0.2–7.3%, 0.01–0.06%, and 0.7–2.0% of the total concentrations of Cd, Pb, and Zn, respectively. LS was the most effective in reducing Ca(NO<sub>3</sub>)<sub>2</sub> extractability, with decreases in Ca(NO<sub>3</sub>)<sub>2</sub>-extractable Cd, Pb, and Zn of 19.30%, 37.43%, and 52.21%, respectively, compared to control soil.

DTPA extracted considerably more heavy metals than Ca(NO<sub>3</sub>)<sub>2</sub>, with 8–14%, 38–70%, and 7–11% of the total concentrations of Cd, Pb, and Zn, respectively, extracted by the DTPA method. Of the examined amendments, BA was the most effective in reducing DTPA extractability, with decreases in DTPA-extractable Cd, Pb, and Zn of 35%, 38%, and 25%, respectively, compared to control soil.

The mobility (leachability) of the heavy metals present in the samples was determined using the TCLP method and was found to be lowered by amendment additions. Notable decreases in the leachability by 31%, 70%, and 47% compared to control soil were observed for Cd, Pb, and Zn, respectively.

The PBET mimics the biochemical conditions of, and estimates the trace element bioaccessibility in, the human GI tract [17]. In control soil, 85% of total Pb was bioaccessible, as determined by the PBET, and its bioaccessibility was decreased significantly, by 65% and 48%, respectively, following ZVI and BA additions. However, this was not the case for the other amendments, which did not significantly affect the proportion of bioaccessible Pb ( $p > 0.05$ ).

Sequential extraction was used to investigate the distribution of metals among different soil pools (Tables 3–5). Initially, Pb existed in a more bioavailable and mobile form than the other metals, with 48% of total Pb in the F1–F3 fraction, whereas Cd and Zn were mainly in the residual fraction, with very low mobile fractions (20% and 14% of total content, respectively). Soil amendments shifted the metal distributions from the mobile and/or bioavailable fractions to the residual fractions. The shift from the exchangeable to the carbonate fraction of Cd and Pb was significant in the LS treatment, and the

**Table 3**  
Distribution of cadmium fractions in different amendment addition soils<sup>a</sup>.

Treatment	Fraction (mg kg <sup>-1</sup> )						MF (%)
	F1 <sup>d</sup>	F2	F3	F4	F5	F6	
Control	<0.001b (0.06) <sup>b</sup>	0.38a (8.4)	0.51b (11.1)	1.23b (26.8)	0.50a (10.9)	1.97b (42.8)	19.53b
ZVI <sup>c</sup>	0.01ab (0.13)	0.12c (2.6)	0.59ab (12.3)	1.60a (33.2)	0.23b (4.8)	2.27a (47.1)	14.95c
LS	<0.001b (0.016)	0.25b (6.7)	0.66a (17.7)	0.83de (22.2)	0.17b (4.6)	1.83b (48.8)	24.46a
AMDS	0.02ab (0.39)	0.40a (10.1)	0.57b (14.4)	1.02cd (25.9)	0.22b (5.6)	1.73b (44.0)	24.76a
BM	0.02a (0.58)	0.38a (9.4)	0.52b (13.1)	1.07bc (26.8)	0.21b (5.2)	1.83b (45.6)	22.91a
BA	0.01ab (0.33)	0.25b (7.5)	0.39c (11.4)	0.79e (23.2)	0.16b (4.6)	1.81b (53.3)	19.17b

<sup>a</sup> Means ( $n = 3$ ) followed by same letter within a column are not significantly different ( $p > 0.05$ ).

<sup>b</sup> Number in parenthesis means % of each fraction.

<sup>c</sup> ZVI, zero valent iron; LS, limestone, AMDS, acid mine drainage treatment sludge; BM, bone mill; BA, bottom ash.

<sup>d</sup> F1, 1st fraction (water soluble); F2, 2nd fraction (exchangeable); F3, 3rd fraction (carbonate bound); F4, 4th fraction (Fe–Mn oxides associated); F5, 5th fraction (organic matter bounded); F6, 6th fraction (residual).

**Table 4**  
Distribution of lead fractions in different amendment addition soils<sup>a</sup>.

Treatment	Fraction(mg kg <sup>-1</sup> )						MF (%)
	F1 <sup>d</sup>	F2	F3	F4	F5	F6	
Control	0.59c (0.27) <sup>b</sup>	7.50a (8.4)	97.23 bc (11.1)	8.61ab (26.8)	21.91a (10.9)	82.90ab (42.8)	48.16cd
ZVI <sup>c</sup>	1.02ab (0.52)	1.66c (2.6)	82.50c (12.3)	7.41bc (33.2)	26.29a (4.8)	75.59abc (47.1)	43.82e
LS	0.04d (0.02)	1.30c (6.7)	109.99ab (17.7)	7.42bc (22.2)	22.52a (4.6)	60.30cd (48.8)	55.26a
AMDS	0.93b (0.40)	7.70a (10.1)	116.79a (14.4)	9.66a (25.9)	25.95a (5.6)	69.54bc (44.0)	54.48ab
BM	1.22a (0.54)	7.85a (9.4)	90.83c (13.1)	8.40ab (26.8)	27.99a (5.2)	90.42a (45.6)	44.09de
BA	0.13d (0.10)	3.88b (7.5)	60.87d (11.4)	5.66c (23.2)	13.81 (4.6)	43.86d (53.3)	50.56bc

<sup>a</sup> Means ( $n = 3$ ) followed by same letter within a column are not significantly different ( $p > 0.05$ ).

<sup>b</sup> Number in parenthesis means % of each fraction.

<sup>c</sup> ZVI, zero valent iron; LS, limestone, AMDS, acid mine drainage treatment sludge; BM, bone mill; BA, bottom ash.

<sup>d</sup> F1, 1st fraction (water soluble); F2, 2nd fraction (exchangeable); F3, 3rd fraction (carbonate bound); F4, 4th fraction (Fe–Mn oxides associated); F5, 5th fraction (organic matter bounded); F6, 6th fraction (residual).

shift to the Fe–Mn oxide fraction of Cd was most notable in the ZVI treatment. The mobility and/or bioavailability of metals in soil may be assessed on the basis of absolute and relative contents of fractions weakly bound to soil components. The relative index of metal mobility was calculated as the “mobility factor” [20] using the following equation:

$$MF = \frac{(F1 + F2 + F3)}{(F1 + F2 + F3 + F4 + F5 + F6)} \times 100$$

where MF is the relative content of the mobile fraction (F1–F3) and for Cd, Pb, and Zn was in the range of 15–25%, 44–54%, and 7–14%, respectively. The lowest MF value of Cd, Pb, and Zn was observed in ZVI-amended soil.

The amounts of Cd extracted by Ca(NO<sub>3</sub>)<sub>2</sub>, DTPA, and PBET correlated with the total Cd content in soils (Table 6), with  $r^2$  values of 0.512, 0.496, and 0.505 for Ca(NO<sub>3</sub>)<sub>2</sub>, DTPA, and PBET, respectively. For Pb, the amounts extracted by DTPA and PBET correlated with

the total Pb content in soils, with  $r^2$  values of 0.876 and 0.816 for DTPA and PBET, respectively. Finally, DTPA-extractable and PBET-extractable Zn correlated with the total Zn content, with  $r^2$  values of 0.646 and 0.817 for DTPA and PBET, respectively.

### 3.2. Plant (lettuce) uptake

With the exception of the BM treatment, lettuce shoot yields did not differ significantly ( $p > 0.05$ ) among treatments. Although some treatments appeared to increase lettuce biomass relative to the control, the differences were not statistically significant (Fig. 1).

By contrast, compared with the control soil, the ZVI, AMD, BM, and BA amendments significantly ( $p < 0.05$ ) decreased the concentrations of heavy metals, except in the cases involving BA treatment for Cd. Compared with non-amended soil, 32–125%, 34–97%, and 38–62% of Cd, Pb, and Zn, respectively, were translocated to lettuce shoots in amended soils.

**Table 5**  
Distribution of zinc fractions in different amendment addition soils<sup>a</sup>.

Treatment	Fraction(mg kg <sup>-1</sup> )						MF (%)
	F1 <sup>d</sup>	F2	F3	F4	F5	F6	
Control	0.67c (0.27) <sup>b</sup>	5.85a (2.2)	32.06ab (11.9)	4.71a (1.7)	52.41a (19.5)	174.05bc (64.7)	14.35a
ZVI <sup>c</sup>	1.00bc (0.52)	1.30e (0.6)	15.21d (6.7)	2.93c (1.3)	9.91d (4.4)	198.07ab (87.1)	7.66c
LS	0.06d (0.02)	2.62d (1.0)	32.76a (12.9)	3.17bc (1.2)	24.30bc (9.5)	191.93abc (75.3)	13.93a
AMDS	1.12ab (0.40)	4.62b (1.7)	29.48b (10.7)	3.84b (1.4)	29.62b (10.7)	208.10a (75.5)	12.75ab
BM	1.50a (0.54)	4.54b (1.7)	25.25c (9.4)	3.81b (1.4)	25.62bc (9.6)	208.64a (77.9)	11.63b
BA	1.30ab (0.10)	3.86c (1.8)	24.24c (11.4)	3.40c (1.6)	17.69cd (8.3)	162.84c (76.8)	13.77a

<sup>a</sup> Means ( $n = 3$ ) followed by same letter within a column are not significantly different ( $p > 0.05$ ).

<sup>b</sup> Number in parenthesis means % of each fraction.

<sup>c</sup> ZVI, zero valent iron; LS, limestone, AMDS, acid mine drainage treatment sludge; BM, bone mill; BA, bottom ash.

<sup>d</sup> F1, 1st fraction (water soluble); F2, 2nd fraction (exchangeable); F3, 3rd fraction (carbonate bound); F4, 4th fraction (Fe–Mn oxides associated); F5, 5th fraction (organic matter bounded); F6, 6th fraction (residual).

**Table 6**  
Correlation coefficients between total metal contents and extractable metal contents.

Element	Parameter	Correlation				
		Ca(NO <sub>3</sub> ) <sub>2</sub>	DTPA <sup>a</sup>	PBET	TCLP	pH
Cd	Total	0.512*	0.496*	0.505*	0.464	-0.338
	Ca(NO <sub>3</sub> ) <sub>2</sub>		0.245	0.132	-0.143	-0.369
	DTPA			0.809**	0.382	-0.709**
	PBET				0.635**	-0.111
	TCLP					0.260
Pb	Total	0.172	0.876**	0.816**	0.361	0.011
	Ca(NO <sub>3</sub> ) <sub>2</sub>		0.195	-0.073	0.18	-0.248
	DTPA			0.828**	0.286	-0.577**
	PBET				0.349	0.049
	TCLP					0.206
Zn	Total	0.045	0.646**	0.817**	0.354	0.061
	Ca(NO <sub>3</sub> ) <sub>2</sub>		0.499*	0.245	0.466	-0.521*
	DTPA			0.720**	0.398	-0.556*
	PBET				0.692**	0.064
	TCLP					0.049

<sup>a</sup> DTPA, diethylenetriamine pentaacetic acid; PBET, physiologically based extraction test; TCLP, toxic characteristic leaching procedure.

\*  $p < 0.05$ .

\*\*  $p < 0.01$ .

### 3.3. Earthworm uptake

Because the survival of earthworms in remediated soil may have ecological consequences for their consumers, tissues of depurated earthworms were analyzed (Fig. 2). The concentrations of Cd, Pb, and Zn in earthworm tissues were not lowered by amendment additions, although earthworms exposed to AMD- and BM-amended soils had higher Cd and Pb concentrations compared with control earthworms. There were obvious differences in cast production among treatments (data not shown), with more casts produced in AMD- and BM-amended soils than in the other soils.

### 3.4. Soil enzyme activities

Dehydrogenase activity in the soil samples is shown in Fig. 3. With the exception of BM treatment, activity was significantly higher (1.1–15 times) in the amended soils than in control soil ( $p < 0.05$ ).

### 3.5. Correlations

Strong relationships were found between heavy metals extracted using the TCLP method and the phytoavailability of Cd ( $p < 0.05$ ), Pb ( $p < 0.01$ ), and Zn ( $p < 0.01$ ) (Table 7). The relationship between PBET extraction and lettuce uptake was also significant for Pb and Zn ( $p < 0.05$ ). Lettuce uptake of Zn was significantly cor-

related with Ca(NO<sub>3</sub>)<sub>2</sub>-extractable metal ( $p < 0.01$ ). Although the summed F1–F3 fraction obtained from sequential extraction correlated with Cd and Pb content in lettuce, it was not predictive of Zn phytoavailability.

Strong relationships were found between Ca(NO<sub>3</sub>)<sub>2</sub>-extractable metals and the water-soluble (F1) fraction in sequential extraction vs. earthworm uptake ( $p < 0.01$ ). Ca(NO<sub>3</sub>)<sub>2</sub>-extractable Pb and Zn negatively correlated with earthworm content, whereas the water-soluble fractions of Cd and Zn were positively correlated with their concentrations in earthworms. Distinctively, concentrations of Zn in earthworms were negatively correlated with various Zn extraction methods in soil.

Soil dehydrogenase activity was negatively correlated with metal content. Strong negative relationships were found between F2 and the summed F1–F2 fraction obtained from sequential extraction and dehydrogenase activity ( $p < 0.01$ ). The relationship between PBET extraction and dehydrogenase activity was significant for Pb and Zn.

## 4. Discussion

In this study, the efficacy of *in situ* stabilization of heavy metals was evaluated using several extraction methods, lettuce uptake, earthworm uptake, and soil enzyme activity. *In situ* stabilization of heavy metals, defined as a decrease in their bioavailability, must be determined based on a comparative risk assessment that takes

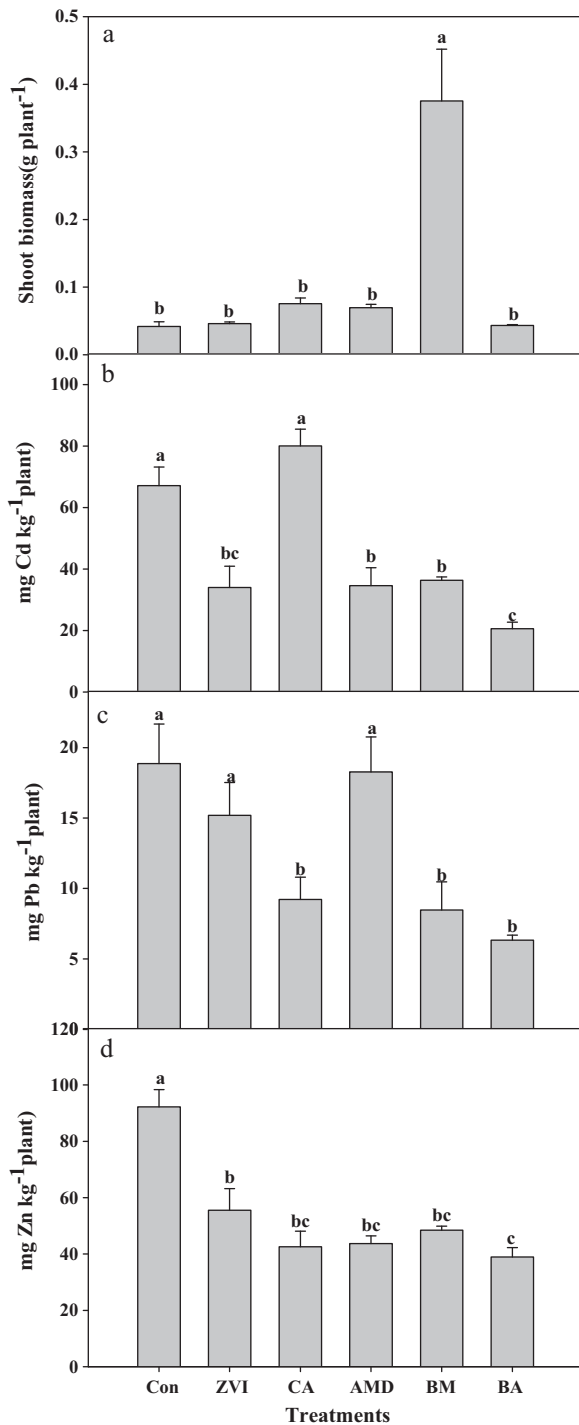
**Table 7**  
Correlation coefficients between heavy metal concentrations in soil and lettuce, earthworm concentration and dehydrogenase activity.

	Heavy metals in lettuce			Heavy metals in <i>E. fetida</i>			Dehydrogenase		
	Cd	Pb	Zn	Cd	Pb	Zn	Cd	Pb	Zn
Total	0.131	0.562*	0.219	-0.165	0.336	-0.242	0.234	-0.39	-0.652**
Ca(NO <sub>3</sub> ) <sub>2</sub>	-0.138	0.436	0.833**	-0.374	0.604**	0.529*	-0.239	0.044	-0.38
DTPA <sup>a</sup>	0.077	0.539*	0.465	0.615**	0.437	-0.156	-0.362	-0.303	-0.829**
PBET	0.367	0.510*	0.503*	0.358	0.472*	-0.469*	-0.387	-0.514*	-0.694**
TCLP	0.575*	0.828**	0.718**	0.015	-0.374	-0.599**	-0.175	0.188	-0.307
F1	-0.446	0.527*	-0.185	0.607**	0.287	0.496*	-0.252	-0.033	-0.063
F2	0.149	0.437	0.428	0.309	0.261	-0.191	-0.904**	-0.760**	-0.886**
F3	0.626**	0.448	0.182	0.308	0.383	-0.299	0.201	-0.339	-0.746**
F1 + F2	0.103	0.478*	0.328	0.348	0.282	-0.025	-0.890**	-0.703**	-0.810**
F1 + F2 + F3	0.529**	0.499*	0.238	0.511**	0.407	-0.267	-0.301	-0.430	-0.845**

<sup>a</sup> DTPA, diethylenetriamine pentaacetic acid; PBET, physiologically based extraction test; TCLP, toxic characteristic leaching procedure.

\*  $p < 0.05$ .

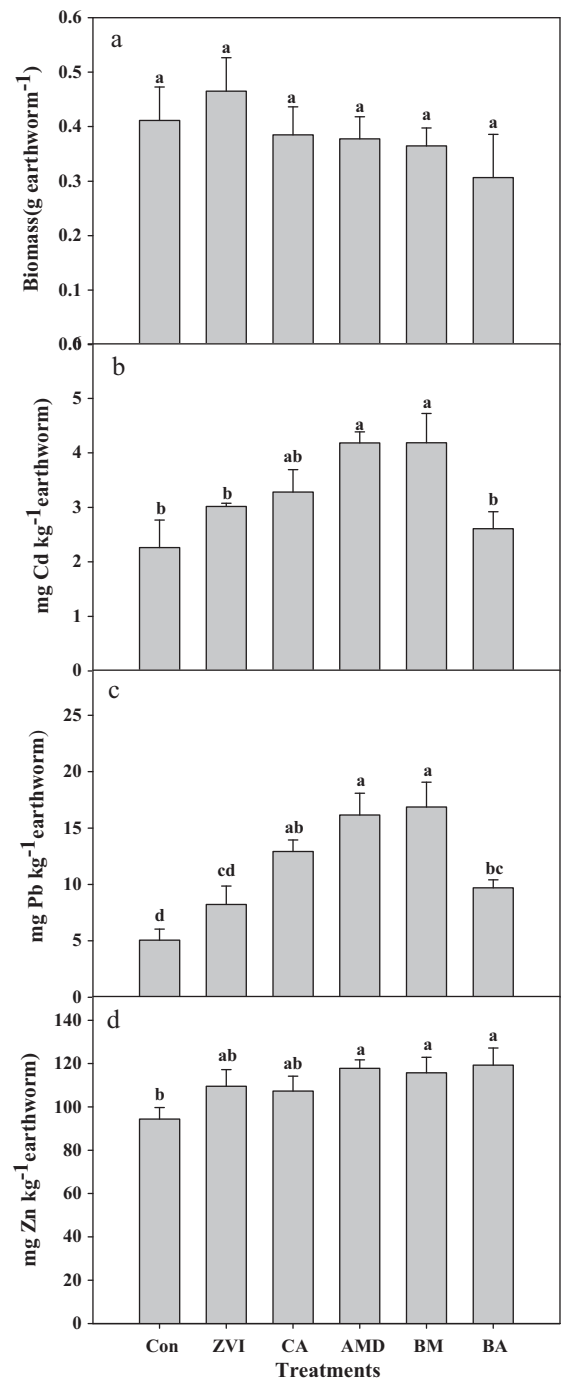
\*\*  $p < 0.01$ .



**Fig. 1.** Shoot dry biomass of lettuce and metals concentrations in dry shoot of lettuce grown in non-amended control soil and in the amended soils. (a) Biomass; (b) Cd; (c) Pb and (d) Zn. Error bars represent ± SE of three replicates. Con, control; ZVI, zero valent iron; LS, limestone; AMDS, acid mine drainage treatment sludge; BM, bone mill; BA, bottom ash.

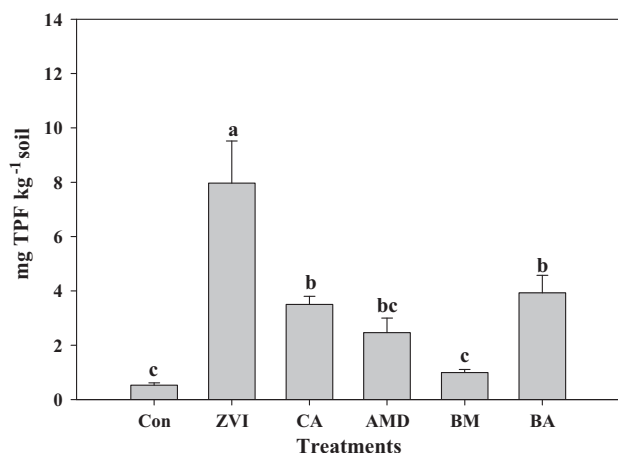
into account relevant pathways of exposure and different environmental endpoints.

In these experiments, the goal of remediation was not to remove the metals, but to stabilize the available species by changing chemical properties of the soil. The observation that total metal content is a poor indicator of bioavailability was not unexpected (Table 7); the solubility and bioavailability of heavy metals are more important in *in situ* stabilization, as both represent the most labile fractions sub-



**Fig. 2.** Biomass and metals concentrations in earthworm grown in non-amended control soil and in the amended soils. (a) Biomass; (b) Cd; (c) Pb and (d) Zn. Error bars represent ± SE of three replicates. Con, control; ZVI, zero valent iron; LS, limestone; AMDS, acid mine drainage treatment sludge; BM, bone mill; BA, bottom ash.

ject to leaching and thus have important effects on soil organisms [21]. Of the tested chemical methods, TCLP and sequential extraction better demonstrated the relationship between soil enzyme activity and soil-extractable metal content and plant or earthworm uptake. Remediation investigations of contaminated sites typically include TCLP and total metal data, but little information is available on the relationship between metal determined by TCLP and bioavailability. Among the extraction methods evaluated in this study, the TCLP method was the best predictor of lettuce uptake of Cd, Pb, and Zn. The results of Basta and Gradwohl [22] were similar to our finding of a significant correlation between lettuce uptake



**Fig. 3.** Soil dehydrogenase activity in non-amended control soil and in the amended soils. Error bars represent  $\pm$ SE of three replicates. Con, control; ZVI, zero valent iron; LS, limestone, AMDS, acid mine drainage treatment sludge; BM, bone mill; BA, bottom ash; TPF, triphenyl formazan.

of Cd and Pb and TCLP extractable metal ( $p < 0.01$  and  $p < 0.05$ , respectively). Strictly speaking, the TCLP method consists of a low-molecular-weight organic acid (acetic acid,  $\text{CH}_3\text{COOH}$ ) extraction. The use of organic acids to extract bioavailable metals from soils is a well-accepted approach, as these compounds are able to release particulate bound metals into soil solutions; the effects of organic acids on metal release from soil and on uptake of metals are significant [23].

Studies on the speciation of heavy metals in polluted soils rely increasingly on extraction methods because these techniques provide information regarding the affinity of the different metals for the various soil components and the strength with which they bind to the matrix [20]. Unlike single extraction, sequential extraction yields information about the mobile and stable fractions of metals in soil, and thus about the actual and potential mobility of the metals. Furthermore, the MF values obtained from sequential extraction can be used to describe heavy-metal mobility and/or bioavailability. According to the sequential extraction results of this study, adsorption and/or precipitation of Cd, Pb, and Zn were increased by amendment addition. The carbonate, Fe-oxide, and organic matter components of the amendments tested are known to be very effective in metal sorption. The metals present in fractions F1 (water soluble) to F3 (carbonate bound) obtained in sequential extraction correlated well with the bioassay results. In the study of Ahumada et al., the amount of metals extracted with  $\text{NH}_4\text{OAC}$  (at pH 7) correlated well with plant uptake [24], with results comparable to our experiment using the neutral salt  $\text{Ca}(\text{NO}_3)_2$ .

Electrolyte extractions using  $\text{Ca}(\text{NO}_3)_2$  solution have shown promise, as demonstrated here in toxicity-related measures of Zn availability to lettuce and of Pb and Zn availability to *E. fetida* (Table 5). Conder and Lanno [25] and Basta and Gradwohl [22] reported similar results, showing that the  $\text{Ca}(\text{NO}_3)_2$  fraction was well related to earthworm toxicity in metal-spiked artificial soil and to phytotoxicity in lettuce.

Although determinations of metal contents in shoots as a phytoavailability indicator have been criticized, lettuce has been used to assess heavy-metal bioavailability in contaminated soil and food-chain risk to humans [26,27]. In this study, lettuce was sensitively responsive to change in metal species, and in most cases, lettuce-shoot concentrations correlated well with the chemical extraction methods tested.

Contaminant bioavailability has been assessed in earthworms, as these organisms ingest large quantities of soil and are in full

contact with the substrate as it passes through their digestive system. Moreover, earthworms are a constituent of many food chains, thereby providing a route through which contaminants may be transferred to higher trophic levels [28]. Earthworms are less able to perform their essential functions in soils when they are exposed to harmful concentrations of heavy metals [29], but they are also capable of decreasing the toxic effects of heavy metals by regulating internal concentrations and by avoiding contaminated soils. Observations from laboratory studies have shown that *E. fetida* and *L. terrestris* avoid contaminated soil layers [30]. In this study, extractable metal concentrations negatively correlated with earthworm concentrations, most likely due to earthworm avoidance. Usually, acute toxicity tests are effective in screening highly contaminated soils, whereas avoidance tests provide essential data regarding the sublethal effects of low-level exposures and the long-term ecological risks of soil contamination.

In this study, as in several others [31–33], strong negative correlations were found between extractable heavy-metal content and dehydrogenase activity. Soil enzymes are highly sensitive to heavy metals, and their use as standard biochemical indicators has been recommended to assess the quality of soils polluted with heavy metals [34,35]. Thus, dehydrogenases (or other microbial properties) are useful indicators of the re-establishment of biotic connections and the restoration of related functions in remediated systems.

In conclusion, *in situ* stabilization strategies must take into account both the sustainability of the soil treatment and its effect in reducing the magnitude of exposure [3]. Thus, regular biological and chemical monitoring, using a range of ecological receptors and relevant pathways, is a prerequisite for assessing the ongoing processes that contribute to the restoration of ecosystem function [3]. The addition of amendments, especially limestone and bottom ash, resulted in a significant decrease in extractability and mobility of heavy metals. Biological assays using lettuce, earthworm, and enzyme activity were well correlated with chemical monitoring results. Especially, TCLP together with sequential extraction appears to be a promising surrogate measure of metal bioavailability for several environmental endpoints (plant, earthworm, and microorganisms).

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