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Metal availability in heavy metal-contaminated open burning and open detonation soil: Assessment using soil enzymes, earthworms, and chemical extractions

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ABSTRACT

The effects of heavy metal contamination on soil enzyme activity and earthworm health (bioaccumulation and condition) were studied in contaminated soils collected from an formerly open burning and open detonation (OBOD) site. Soil extraction methods were also evaluated using $CaCl_2$ and DTPA solutions as surrogate measures of metal bioavailability and ecotoxicity. Total heavy metal content of the soils ranged from 0.45 to 9.68 mg Cd kg^{-1}, 8.96 to 5103 mg Cu kg^{-1}, 40.21 to 328 mg Pb kg^{-1}, and 56.61 to 10,890 mg Zn kg^{-1}. Elevated metal concentrations are assumed to be primarily responsible for the reduction in enzyme activities and earthworm health indices. We found significant negative relationships between $CaCl_2$- and DTPA-extractable metal content (Cd, Cu, and Zn) and soil enzyme activity ($P < 0.01$). Therefore, it could be concluded that soil enzyme activity and metal bioaccumulation by earthworms can be used as an ecological indicator of metal availability. Furthermore, $CaCl_2$ and DTPA extraction methods are proved as promising, precise, and inexpensive surrogate measures of Cd, Cu, Pb, and Zn bioavailability from heavy metal-contaminated soils.

1. Introduction

Environmental hazard assessment of metal-polluted soils and sediments is usually based on soil metal concentrations as quantified after digestion of soils with strong acids. Although useful for indicating the level of contamination, total soil analysis does not reflect the relative bioavailability and toxic potential of heavy metals. Total heavy metal concentrations may not be directly related to soil organism toxicity due to a number of modifying factors such as pH, organic matter content, and clay content [1–4].

Bioavailability is defined as the extent to which living receptors are exposed to contaminants in the soil [5]. Bioavailability depends on a specific target organism, the specific contaminants, and the following factors: exposure time, transfer of contaminants from soil to organisms, and their accumulation in the target organisms and the subsequent effects [6]. The bioavailability of metals cannot be measured directly using chemical analyses; only living organisms can actually determine bioavailability [2].

To assess the bioavailability of metals in soils, approaches involving total metal content of soils alone must be abandoned in favor of approaches relating some biological response to the available fraction of metals [1]. Soil enzyme activities have been used as bioindicators for soil quality and health in environmental soil monitoring due to their high sensitivity to soil changes [7,8]. Soil enzyme activity measurements are highly sensitive to metals and the methodology for their determination is rapid, simple, and inexpensive [9–11].

Soil invertebrate testing has also been proposed as an alternative bioassay in which a soil-dwelling organism is used to determine the potential biological availability of a given soil metal load [12]. Because earthworms are key indicators of ecosystem health and their responses to metals have been used as markers for soil health or toxicity, the influence of heavy metals in soils and their bioaccumulation in earthworms has been the subject of many studies for a long period [3,12,13].

A method of measuring metal availability not involving organisms that relates well to bioavailability would be an extremely useful tool for evaluating metal-contaminated soils [1]. Various one-step extraction methods are frequently used for bioavailability evaluation because of simplicity and ease of operation. Chelating agents such as diethylene triamine pentaacetic acid (DTPA) [14], and ethylene diamine tetra acetic acid (EDTA) [15] have been widely used due to their availability to form very stable, water-soluble, and well-defined complexes with metal cations. Extractions using

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weak (<1 M) $CaCl_2$ or $Ca(NO_3)_2$ solutions have also been frequently adopted for toxicity-related measures of metal availability in soils [1,16].

This study aimed to (i) assess the impact of elevated levels of heavy metals on soil enzyme activities (urease, alkaline phosphomonoesterase, and dehydrogenase) and the bioaccumulation of heavy metals in the earthworm *Eisenia fetida* in heavy metal-contaminated open burning and open detonation (OBOD) site soil, and (ii) evaluate soil extraction methods using $CaCl_2$, and DTPA as surrogate measures of metal bioavailability and ecotoxicity.

2. Materials and methods

2.1. Site description and soil sampling

The soils investigated in this study were obtained from TBP (Toxic Burning Pit) site located in Gi-Jang, southeastern Korea. Between 1970 and 2003, this pit was used for the disposal of explosive wastes, including OBOD.

Soils were collected from 11 sampling point across the formerly OBOD site by grid method. Each sample point was about 25 m^2 (5 m × 5 m). Three soil samples of the surface horizons (0–20 cm) were collected from each sampling point. Soil cores were placed in a cooler as collected, and stored at 4 °C until shipped for analyses. For all subsequent chemical and biological analyses soil from each core were conducted separately and were not pooled. Each soil sample was characterized for following physical and chemical properties: pH by glass electrode method (1:5 water suspension); organic matter(OM) content by Tyurin method [17]; texture by pipetting method [18]; cation exchange capacity (CEC) by the ammonium-saturation and distillation method [19]. The main characteristics of the soil samples are presented in Table 1.

2.2. Soil enzyme activity

Urease activity was measured using the method of Kandeler [20]. After the addition of 79.9 mM urea solution, the soil was incubated for 2 h at 37 °C. The released ammonia was extracted with 2 M KCl and determined spectrophotometrically at 690 nm. Phosphatase activity was determined using *p*-nitrophenyl phosphate disodium as a substrate [21]. A 2 ml of aliquot, containing 0.1 M modified universal buffer (MUB, pH 11) and 0.5 ml of 0.115 M *p*-nitrophenyl phosphate disodium, was added to 0.5 g of soil and incubated at 37 °C for 90 min. The reaction was stopped by the addition of 0.5 ml 0.5 M $CaCl_2$ and 2 ml 0.5 M NaOH. The released *p*-nitrophenol was determined spectrophotometrically at 410 nm. Dehydrogenase activity was determined by the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to triphenyl formazan (TPF) [22]. A 1 ml aliquot of TTC solution (1%) and 1.5 ml of distilled water were added to 10 g of soil that had been mixed with 0.1 g of $CaCO_3$. After 24 h at 25 °C, the reaction product, TPF was extracted with methanol, with the absorbance measured at 485 nm.

2.3. Earthworm incubations

The earthworms, *E. 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. Prepared soils (250 g wet weight) were placed in a 500 ml polypropylene jar, with five earthworms introduced to each jar. The beakers 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.

Individually incubated earthworms were monitored every 7 days, with their condition graded in accordance with a condition index (CI) taken from Langdon et al. [23]; Earthworms graded as '2' had good muscle colour and rapidly responded to stimulation. A grade of '1.5' indicated fair muscle colour and stimulation responsiveness. The lower grades of '1' and '0.5' indicated fair muscle colour and poor response to stimulation and poor muscle colour and very poor stimulation responsiveness, respectively. A grade of '0' indicated that the earthworm was dead.

2.4. Treatment of earthworm for heavy metal determination

At the end of the exposure period, the earthworms were removed and placed on moist tissue for 24 h to empty their guts. Each earthworm was then dried at 40 °C for 24 h in a pre-weighed conical flask. Following drying, concentrated nitric acid was added to the conical flasks and agitated for 24 h.

2.5. Determination of heavy metals for soils

The total heavy metal content of the soils was determined by extraction with aqua regia (10:1 extractant to soil ratio) at 120 °C for 2 h [24]. The digests were then further heated until dry, resolubilized in a 15 ml of 0.5 M HNO_3, filtered, and made up to a volume of 50 ml with 0.5 M HNO_3.

Extraction with 0.5 M $CaCl_2$ solution was performed according to the procedure of Esnaola and Millan [25]; 3 g of soil was added of 30 ml of DW in 40-ml polypropylene centrifuge tubes. The tubes were 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. DTPA extraction of metals was performed using the method of Lindsay and Norvell [14]; 1.5 g of soil was extracted with 15 ml of a solution containing 5 mM DTPA and 10 mM $CaCl_2$ in 40-ml polypropylene centrifuge tubes. The tubes were 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. All measurements of heavy metals were performed with an inductively coupled plasma optical emission spectrometry (ICP-OES).

All chemicals used in this study were of analytical grade or better. Centrifuge tubes used metal extraction were washed (5% HNO_3) before use. All other analysis were done using disposable plastic lab ware. Multi-element standards were run for every 20 sample and recovery was found to be within 100 ± 10%.

2.6. Data analysis

The bioaccumulation factors (BAFs) were calculated according to Eq. (1)

$$\mathrm{BAF} = \frac{M_{\mathrm{worm}}}{M_{\mathrm{soil}}} \quad (1)$$

where BAF is the bioaccumulation factor, M_{worm} is the metal concentration in the earthworm tissue (mg kg^{-1} dry weight), M_{soil} is the metal concentration in the soil (mg kg^{-1} dry weight).

Log linear correlation coefficients between metal concentrations in worm and the soils were calculated according to Eq. (2)

$$\log M_{\mathrm{worm}} = a \times \log M_{\mathrm{soil}} + b \quad (2)$$

where *a* and *b* are correlation constants.

All the determinations were performed in triplicate on each independent soil sample. One-way analysis of variance (one-way ANOVA) was carried out to compare all the means from different soils except for the values of condition index and mortality. When significant *P*-values ($P < 0.05$) were obtained, differences between

Table 1
Characteristics of the soils used in this study[a].

Soil ID	pH[b]	Particle size distribution[c]			OM[d]	CEC[e]
		Clay	Silt	Sand		
A	5.88 ± 0.10	35.83 ± 0.65	30.71 ± 0.03	33.46 ± 0.62	1.04 ± 0.06	3.07 ± 0.62
B	6.98 ± 0.06	21.70 ± 0.44	14.16 ± 0.66	64.14 ± 1.10	1.22 ± 0.12	3.73 ± 0.20
C	6.68 ± 0.07	20.46 ± 0.39	14.28 ± 1.44	65.26 ± 1.10	0.74 ± 0.24	1.46 ± 0.31
D	6.82 ± 0.05	25.77 ± 0.65	18.56 ± 0.17	55.67 ± 0.83	0.17 ± 0.01	1.00 ± 0.23
E	6.39 ± 0.09	25.32 ± 0.06	20.61 ± 0.17	54.07 ± 0.11	0.33 ± 0.03	1.31 ± 0.32
F	5.42 ± 0.01	39.96 ± 0.23	27.32 ± 1.32	32.71 ± 1.55	1.50 ± 0.11	3.25 ± 0.20
G	5.31 ± 0.02	28.52 ± 0.91	23.51 ± 3.19	47.97 ± 4.09	1.94 ± 0.13	3.79 ± 0.24
H	4.98 ± 0.01	41.11 ± 0.56	34.06 ± 1.01	24.83 ± 0.44	1.97 ± 0.08	4.19 ± 0.37
I	4.85 ± 0.01	39.24 ± 0.39	41.45 ± 0.34	19.31 ± 0.05	1.27 ± 0.05	2.87 ± 0.10
J	4.97 ± 0.01	28.30 ± 0.93	24.73 ± 1.09	46.97 ± 0.16	0.98 ± 0.04	1.76 ± 0.14
K	5.53 ± 0.05	32.74 ± 1.01	10.76 ± 0.42	57.09 ± 0.59	1.50 ± 0.03	2.61 ± 0.43

[a] Mean values and standard deviations of three replicates.
[b] Soil pH measured at the ratio of soil to H_2O as 1:5 (mass:volume).
[c] Particle size (%) was analyzed by pipetting method and classified according to USDA classification system.
[d] Organic matter (%) by Tyurin method.
[e] Cation exchange capacity (cmol kg^{-1}).

individual means were compared using a Tukey's HSD test. Correlations between the parameters were analyzed using Pearson product moment correlation. Because earthworm CI was not normally distributed, Kruskall–Wallis test was conducted to determine significant differences of the CI values among the soils. All statistical analyses were carried out with SPSS 11.5 for Windows.

3. Results and discussion

3.1. Soil contamination (total and extractable metal content of the soils)

The examined soils were severely contaminated with Cd, Cu, Pb, and Zn from OBOD ammunition disposal (Table 2). The total metal concentrations ranged from 0.45 to 9.68 mg Cd kg^{-1}, 8.96 to 5103 mg Cu kg^{-1}, 40.21 to 328 mg Pb kg^{-1}, and 56.61 to 10,890 mg Zn kg^{-1}.

Soil organisms are exposed mainly via the soil–water pathway, and the bioavailable fraction of contaminants can be roughly determined by the chemical analysis of aqueous soil extracts simulating soil pore concentrations [26]. We used two extractants, $CaCl_2$ and DTPA, to study the extractability of heavy metal fractions in soils (Table 3). The $CaCl_2$-extractable metal content was in the range of 5.7–56%, 0.1–2.6%, 0.1–3.1%, and 0.2–11% of the total concentrations of Cd, Cu, Pb, and Zn, respectively. DTPA extracted considerably more heavy metals than $CaCl_2$; 0.8–84%, 5.6–34%, 1.6–15%, and 2.0–19% of the total concentrations of Cd, Cu, Pb, and Zn, respectively.

$CaCl_2$-extractable metals are thought to represent the labile metal fraction that may potentially enter the body [27]. Moreover, $CaCl_2$ extraction has been recommended for the assessment of the bioavailable fractions of various nutrients and micronutrients, largely because it has a similar salinity to the soil solution and no influence on the pH of the extracts [28]. DTPA extraction, which is a widely used soil testing method [29], provides an operationally defined soil compartment that is characterized by its solubility. DTPA can release soluble, exchangeable, adsorbed, and organically-bound metals and could release some metals fixed by oxides [14].

The amounts of Cd, Cu, and Zn extracted by DTPA were well correlated with the total content of these metals in the soils: the r^2 values were 0.726, 0.938, and 0.778 for Cd, Cu, and Zn, respectively (Fig. 1). The correlations between total and DTPA-extractable contents of metals were clearer in the lower concentrations of heavy metals.

The relationship between total and DTPA-extractable contents of metals indicates that the available metals should come from the same mineralogical sources and that pedological processes occurring in the soil sampling points should be similar [30].

3.2. Soil enzyme activities

Enzyme activities in the soil samples are shown in Fig. 2. Urease, phosphatase, and dehydrogenase activities were significantly lower in the heavily contaminated soils (B–E soil) ($P < 0.01$). Simple linear correlation coefficients for the relationships between enzyme activities and heavy metal concentrations are shown in Table 4. Soil enzyme activities (urease, phosphatase, and dehydrogenase) were negatively correlated with metal content. Our results were consistent with several other studies demonstrating that soil enzyme activities are inversely related to metal concentrations [31–33].

Table 2
Total heavy metals concentrations (mg kg^{-1})[a,b] of the soils used in this study.

Soil ID	Cd	Cu	Pb	Zn
A	2.02 ± 0.09de	548.39 ± 15.80c	76.19 ± 6.56cde	824.47 ± 4.26c
B	1.89 ± 0.11ef	5103.56 ± 628.80a	328.62 ± 49.36a	10890.81 ± 17.24a
C	9.68 ± 1.48a	391.76 ± 32.30c	104.5 ± 5.31bcd	947.03 ± 15.20bc
D	4.79 ± 0.36c	313.41 ± 192.18d	105.86 ± 3.47bcd	403.6 ± 0.92cd
E	6.45 ± 0.27d	771.96 ± 89.26b	124.89 ± 14.47b	1651.28 ± 7.19b
F	5.06 ± 0.19c	222.62 ± 9.15d	42.77 ± 0.84ef	349.68 ± 2.07cd
G	3.34 ± 0.02d	454.39 ± 24.27c	110.81 ± 6.43bc	685.46 ± 3.78cd
H	1.55 ± 0.14ef	31.83 ± 7.22e	77.3 ± 10.90cde	87.1 ± 0.02d
I	1.33 ± 0.01ef	14.75 ± 2.06e	71.48 ± 10.64def	64.39 ± 1.10d
J	1.21 ± 0.04ef	9.38 ± 1.53e	40.21 ± 3.48f	69.34 ± 0.09d
K	0.45 ± 0.04f	8.96 ± 0.40e	42.75 ± 2.03ef	56.61 ± 0.40d

[a] Aqua regia extractable metal concentration.
[b] Concentrations are the mean values and standard deviations of three replicates; those followed by the same letter are not significantly different ($P > 0.05$).

Table 3
Extractable heavy metal concentrations (mg kg^{-1})[a] of the soils.

Soil ID	$CaCl_2$-extractable				DTPA-extractable			
	Cd	Cu	Pb	Zn	Cd	Cu	Pb	Zn
A	1.77d (56)[b]	4.80c (0.9)	1.15c (1.5)	29.18c (3.5)	1.16e (58)	85.17d (15)	6.41d (8.4)	102.4 (12)
B	0.58f (31)	3.25d (0.1)	0.26e (0.1)	18.21e (0.2)	0.79f (42)	492.6a (10)	5.18de (1.6)	488.5a (4.5)
C	1.55c (16)	3.90d (1.0)	0.35e (0.3)	25.06d (2.6)	4.52b (48)	130.9bc (34)	27.0a (26)	205.2 (22)
D	0.71e (15)	1.84e (0.8)	0.28e (0.3)	9.36f (2.2)	1.70d (36)	53.80e (24)	21.5 (20)	59.93f (14)
E	2.96a (46)	4.76c (0.6)	0.62d (0.5)	48.15b (2.9)	5.42a (84)	142.5 (18)	17.0c (14)	263.15b (16)
F	1.25d (25)	5.81b (2.6)	1.32b (3.1)	28.74c (8.2)	1.09e (22)	67.07de (30)	6.42d (15)	66.64f (19)
G	1.87b (56)	11.83a (2.6)	3.04a (2.7)	78.09a (11)	2.28c (68)	118.61c (26)	15.4c (14)	154.2 (23)
H	0.16g (10)	0.55f (1.9)	0.66d (0.9)	3.69g (4.5)	0.05g (3.5)	8.00f (28)	1.36g (1.8)	7.39g (9.0)
I	0.06g (4.8)	0.10g (0.6)	1.33b (1.8)	0.75g (1.2)	0.01g (0.8)	0.57f (5.6)	1.65fg (2.2)	1.31g (2.0)
J	0.07g (5.7)	0.09g (1.1)	0.68d (1.7)	1.18g (1.7)	0.06g (5.0)	1.57f (17)	2.80fg (7.0)	3.11g (4.5)
K	0.13g (30)	0.16fg (1.6)	1.13c (2.7)	2.76g (4.9)	0.19g (44)	1.60f (18)	3.62ef (8.5)	7.93g (14)

[a] Concentrations are the average of three replicates; those followed by the same letter are not significantly different ($P > 0.05$).
[b] Numbers in parenthesis are percent of each fraction relative to total concentration which is reported in Table 2.

The inhibition of enzyme activities by metals in soil is principally attributed to an indirect effect (suppression of the microbial population and cellular activities) and/or a direct effect (inactivation of extracellular enzymes due to binding with the metals) [34]. This indicates that soil enzyme activities are good indicators of heavy metal soil contamination and could be useful for monitoring changes in soil health.

Table 5 shows the correlation coefficients between enzyme activities and soil properties. All soil enzyme activities were negatively correlated with soil pH ($P < 0.01$) and positively correlated with OM content, clay content, and CEC ($P < 0.01$).

Significant correlations between OM content, clay content, and CEC, and enzyme activity may be due to adsorption capacity; the extracellular enzymes (e.g., urease, phosphatase) could thus have

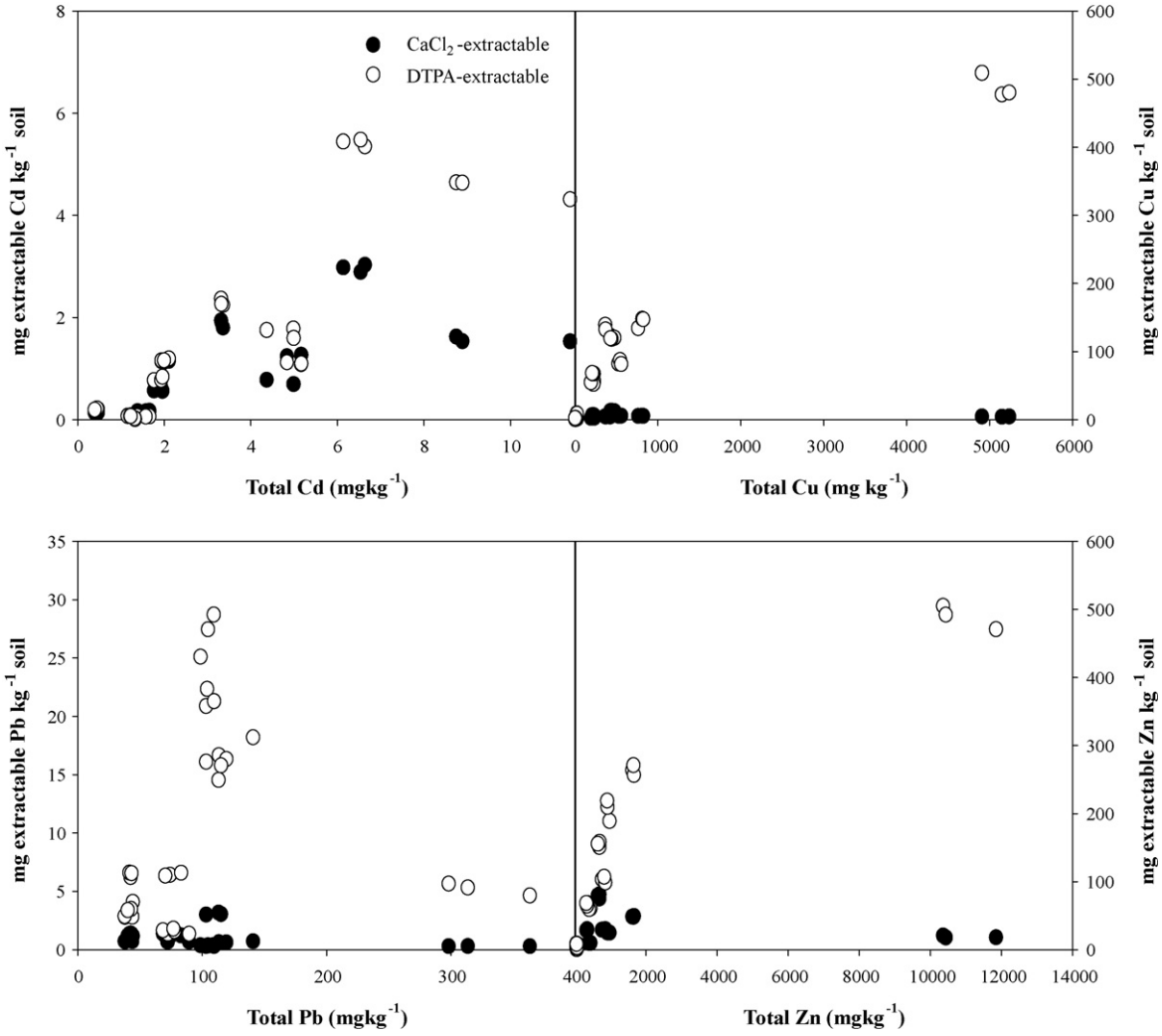

Fig. 1. Extractability of Cd, Cu, Pb, and Zn in soils. $CaCl_2$ and DTPA were used as extractants. Total versus DTPA-extractable metal: Cd, $y = 0.5538x - 0.3294$ ($r^2 = 0.7260$); Cu, $y = 0.0914x + 34.774$ ($r^2 = 0.9378$); Pb, $y = 0.0279x + 6.60$ ($r^2 = 0.0086$); Zn, $y = 0.0416x + 62.89$ ($r^2 = 0.7775$).

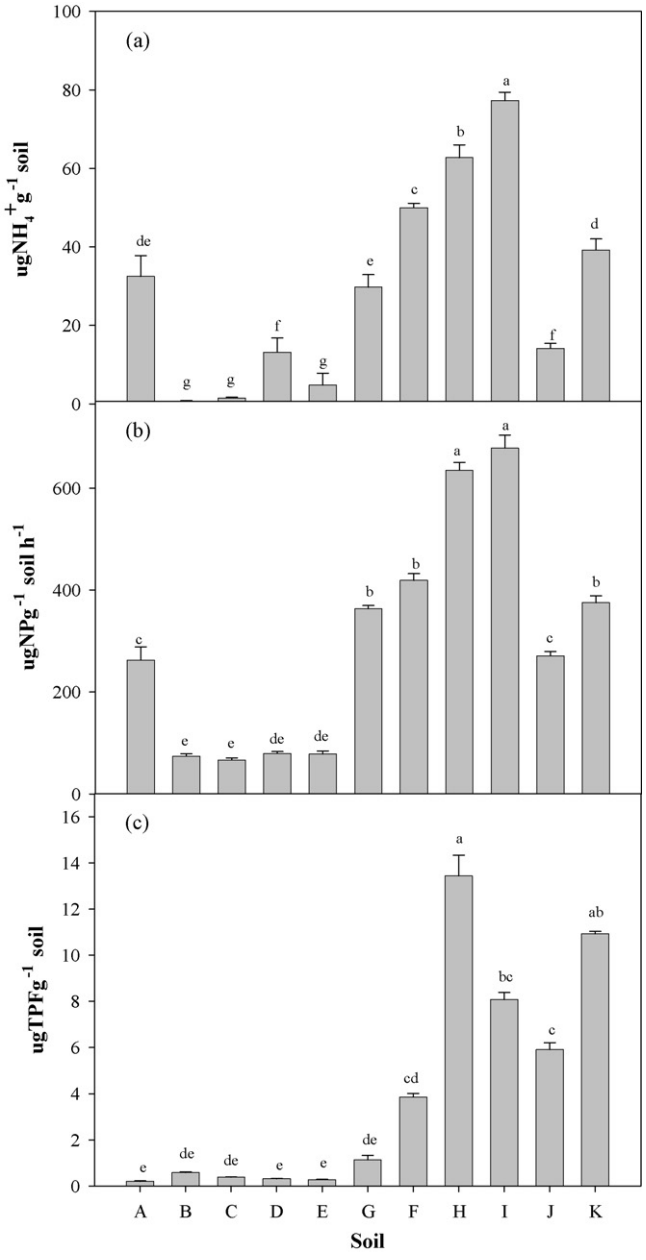

Fig. 2. Soil enzyme activities in examined soils (a) urease, (b) phosphomonoesterase, (c) dehydrogenase. Error bars indicate the standard deviation of the means ($n = 3$). Activities by the same letter are not significantly different ($P > 0.05$).

been absorbed by these soil constituents. Similar evidence supporting this conjecture was reported by Turner et al. [35]; other investigations have also shown that the clay in soils can retain and protect extracellular hydrolases [36].

3.3. Earthworm health and bioaccumulation of heavy metals

Throughout the experiments, earthworms exhibited different condition indices and mortalities (Table 6). Decreases in the CI and increased lethality occurred shortly after the commencement of the experiments. This could be attributed to the initial uptake of heavy metals by the earthworms, resulting in acute toxicity. This notion is supported by the studies of Spurgeon and Hopkin [37], who reported that a rapid uptake of heavy metals was followed by equilibrium after a few days of exposure.

Table 4
Simple linear correlation coefficient (r)[a] for the relationships between the soil enzyme activities and $CaCl_2$ and DTPA-extractable, as well as the total metal concentrations.

Metal fraction	Heavy metal	Soil enzyme[b]		
		URE	PME	DHA
$CaCl_2$ extractable	Cd	−0.455**	−0.516**	−0.646**
	Cu	−0.229	−0.270	−0.582**
	Pb	0.384*	0.384*	0.003
	Zn	−0.279	−0.296	−0.570**
DTPA extractable	Cd	−0.593**	−0.628**	−0.618**
	Cu	−0.563**	−0.559**	−0.511**
	Pb	−0.616**	−0.661**	−0.655**
	Zn	−0.657**	−0.652**	−0.599**
Total	Cd	−0.484**	−0.569**	−0.600**
	Cu	−0.457**	−0.439**	−0.357*
	Pb	−0.492**	−0.482**	−0.420*
	Zn	−0.458**	−0.436**	−0.338

[a] The probability values (P-value) of regression are shown after r^2 values. *$P < 0.05$; **$P < 0.01$.
[b] URE for urease; PME for alkaline phosphomonoesterase (phosphatase); DHA for dehydrogenase.

Simple linear correlation coefficients for the relationship between the earthworm bioassays and soil properties were determined (Table 7). The conditions of the earthworms were negatively correlated only with the internal Cd ($r = -0.417$, $P < 0.05$) and Cu ($r = -0.541$, $P < 0.01$) levels; earthworm mortalities were not correlated with any of the internal metal concentrations or soil parameters. Significant relationships were found between internal Cu and OM contents ($r = -0.446$, $P < 0.01$), CEC ($r = -0.413$, $P < 0.05$), clay content ($r = -0.634$, $P < 0.01$), and pH ($r = 0.791$, $P < 0.01$). Zn content was correlated with soil pH ($r = 0.656$, $P < 0.01$) and clay content ($r = -0.526$, $P < 0.01$), but no significant correlation was found between the internal Pb content and the soil parameters. A significant negative correlation between concentration in earthworms and OM content, CEC, and pH could be explained by a decreased availability of metals due to adsorption or decreasing solubility of metals. The metal concentrations in earthworm tissue reflected the level of soil contamination (Table 8) and varied from 3.94 to 19.76 mg Cd kg^{-1}, 4.66 to 82.03 mg Cu kg^{-1}, 1.30 to 45.00 mg Pb kg^{-1}, and 69.08 to 293.77 mg Zn kg^{-1}.

The BAFs showed large variability between the soils (Table 8); from 0.58 to 9.33 for Cd, 0.09 to 0.60 for Cu, 0.01 to 0.41 for Pb, and 0.10 to 1.88 for Zn, indicating large BAF variability between soils for all metals and among the metals themselves. It is known that the affinity of metals for soil constituents has a crucial role in earthworm metal accumulation. Morera et al. [38] showed for various soils that the relative affinity of metals was consistent with the value of the first hydrolysis constant (pK) of the

Table 5
Correlation matrix of the soil enzyme activity and soil properties.

Parameter[a]	Correlation coefficient (r)[b]					
	PME	DHA	pH	OM	CEC	Clay
URE	0.959**	0.682**	−0.784**	0.603**	0.790**	0.930**
PME		0.804**	−0.875**	0.698**	0.810**	0.873**
DHA			−0.704**	0.580**	0.536**	0.636**
pH				−0.665**	−0.741**	0.768**
OM					0.855**	0.563**
CEC						0.819**

[a] URE for urease; PME for alkaline phosphomonoesterase (phosphatase); DHA for dehydrogenase; OM for organic matter; CEC for cation exchange capacity.
[b] The probability values (P-value) of regression are shown after r^2 values. * is for $P < 0.05$; ** is for $P < 0.01$.

Table 6
Temporal changes in condition index (CI) and mortality (*M*, %) of earthworms during the experiment[a].

Soil ID	Days							
	7		14		21		28	
	CI	M	CI	M	CI	M	CI	M
A	1.13 ± 0.25	15ab	1.25 ± 0.87	15abc	1.25 ± 0.87	15bc	1.25 ± 0.87	15bc
B	1.38 ± 0.48	0b	1.13 ± 0.25	0c	1.00 ± 0.00	0c	0.75 ± 0.21	0c
C	1.75 ± 0.50	10ab	1.75 ± 0.50	10abc	1.38 ± 0.95	10bc	1.38 ± 0.95	10bc
D	0.75 ± 0.50	25a	0.75 ± 0.50	35a	0.13 ± 0.25	50a	0.13 ± 0.25	50a
E	1.63 ± 0.75	5ab	1.50 ± 1.00	5bc	1.13 ± 0.75	5c	1.13 ± 0.75	5c
F	1.00 ± 0.71	15ab	0.88 ± 0.75	25abc	0.75 ± 0.87	35ab	0.50 ± 0.41	35ab
G	1.13 ± 0.25	10ab	0.88 ± 0.25	10abc	0.63 ± 0.48	10bc	0.50 ± 0.58	10bc
H	1.50 ± 0.58	0b	1.13 ± 0.75	30ab	0.75 ± 0.65	35ab	0.75 ± 0.29	35ab
I	2.00 ± 0.00	0b	2.00 ± 0.00	0b	2.00 ± 0.00	0b	2.00 ± 0.00	0b
J	2.00 ± 0.00	0b	2.00 ± 0.00	0b	2.00 ± 0.00	0b	1.50 ± 1.00	0b
K	2.00 ± 0.00	0b	2.00 ± 0.00	0b	2.00 ± 0.00	0b	2.00 ± 0.00	0b
	K = 24.110, d.f. = 10 P = 0.0073		K = 21.9906, d.f. = 10 P = 0.0152		K = 26.4109, d.f. = 10 P = 0.0032		K = 24.2601, d.f. = 10 P = 0.0069	

[a] For the statistical comparison of the CI value, Kruskal–Wallis test was conducted (α = 0.05). Mortality values followed by the same alphabetical letters are not significantly different (P > 0.05).

Table 7
Correlation matrix of the earthworm bioassay endpoints and soil properties.

Parameter	Correlation coefficient (r)[a]								
	CI	Cd	Cu	Pb	Zn	pH	OM	CEC	Clay
Mortality	−0.693**	0.147	0.241	−0.041	−0.129	0.121	−0.099	0.197	0.212
CI		−0.417*	−0.541**	−0.201	−0.329	−0.277	0.056	−0.158	0.028
Cd			0.541**	0.234	0.414*	0.130	0.146	0.254	0.103
Cu				0.231	0.667**	0.791**	−0.446**	−0.413*	−0.634**
Pb					0.105	−0.238	0.401	0.393	0.006
Zn						0.656**	−0.101	−0.299	−0.526**

[a] The probability values (P-value) of regression are shown after r^2 values. * is for P < 0.05; ** is for P < 0.01.

cations proposed by Basta and Tabatabai [39]; Cu (pK = 7.7) ≥ Pb (pK = 7.7) > Zn (pK = 9.0) > Cd (pK = 10.1). These data were also fairly consistent with the order of BAF values obtained in our experiments; Pb < Cu ≪ Zn ≪ Cd. Unlike with other studies, very low internal concentrations and BAF values were obtained for Pb in this experiment. The low internal concentrations and BAF value for Pb can be attributed to the previously mentioned inherent hydrolysis constant, i.e., a metal's affinity for soil and the form in which it exists as described by sequential extraction. Pb was mainly in the form of Fe–Mn oxides (70–80% of the total concentration); whereas the

Table 8
Internal concentrations (IC, mg kg^{-1}) of heavy metals and the bioaccumulation factor (BAF) for *Eisenia fetida*.

Soil ID	Cd		Cu		Pb		Zn	
	IC[a]	BAF[b]	IC	BAF	IC	BAF	IC	BAF
A	18.83a	9.33a	49.51d	0.09de	1.30e	0.02f	113.17d	0.14ef
B	12.37c	3.08b	76.66ab	0.25e	5.99d	0.10ef	293.77a	0.54f
C	5.48e	0.58g	66.24c	0.17cd	2.33e	0.02ef	93.32e	0.1ef
D	5.83e	1.22fg	82.03 a	0.32b	11.64c	0.11c	127.74c	0.32d
E	9.52d	1.48f	73.98bc	0.1de	1.61e	0.01f	147.91b	0.09ef
F	19.76a	3.9d	48.64d	0.22c	2.49e	0.06d	110.53d	0.32d
G	16.82b	5.04c	74.59ab	0.16cd	45.00a	0.41a	144.32b	0.21de
H	3.01fg	1.96f	6.38e	0.21c	5.06d	0.07d	76.42fg	0.89c
I	3.77f	2.83e	5.42e	0.37b	16.67b	0.24b	69.08fg	1.07b
J	3.94f	3.26de	5.53e	0.60a	1.87e	0.05de	97.80g	1.41c
K	6.67g	3.95e	4.66e	0.51a	2.88e	0.07d	104.98f	1.88a

[a] Concentrations are the average of three replicates; those followed by the same letter are not significantly different (P > 0.05).
[b] BAF represents the ratio of tissue concentration to soil concentration Eq. (1).

Table 9
The regression coefficients[a] for the earthworm tissue metal concentrations of Cd, Cu, Pb, and Zn after 28 days incubation.

Fraction	Cd			Cu			Pb			Zn		
	a	b	r^2	a	b	r^2	a	b	r^2	a	b	r^2
Total	0.5254	0.6337	0.3215**	0.5622	0.2089	0.8420***	−0.027	1.926	0.043 NS	0.2457	1.4005	0.8493***
$CaCl_2$-extractable	0.4469	0.9760	0.5508***	0.6615	1.3371	0.8604***	0.009	−0.059	0.013 NS	0.1932	1.8415	0.4594***
DTPA-extractable	0.2269	0.9101	0.3439**	0.5503	0.6402	0.8981***	−0.191	0.898	0.197 NS	0.1618	1.7820	0.6349***

[a] The coefficients are given as a function of soil metal contents ($\log_{10}$), according Eq. (2) $\log M_{worm} = a \times \log M_s + b$: M_{worm} = metal in the earthworm (mg kg^{-1}), M_s = metal in the soil (mg kg^{-1}), a and b are constant. The probability values (P-value) of regression are shown after r^2 values. NS is for no significant; * is for P < 0.05; ** is for P < 0.01; *** is for P < 0.001.

remaining metals (Cd, Cu, and Zn) were mainly in carbonate-bound forms (data not shown).

The tissue concentrations of Cd, Cu, Pb, and Zn were log-linearly related to the soil metal concentrations. Simple linear correlation coefficients for the relationship between tissue concentration and heavy metal concentrations are shown in Table 9. Significant correlations were found for the metals (Cd, Cu, and Zn) with respect to one or several of the measured external concentrations. Collectively, these correlations indicated that increased external heavy metal concentrations were accompanied by increased metal concentrations in the earthworms. The significant correlations between the internal Cd, Cu, and Zn of *E. fetida* and the $CaCl_2$- and DTPA-extractable fractions of soil metals agreed with literature reports showing that extractable metals best describe the accumulation of heavy metals in earthworms [40,41].

Significant correlations were observed between the bioavailable metal fractions ($CaCl_2$- and DTPA-extractable) and biological parameters (enzyme activities and earthworm's health indices). Subsequently, by combining different monitoring approaches, i.e., chemical and biological monitoring, a better insight into the effects of heavy metals on soil ecosystems can be gained.

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