CHEMICAL AND BIOLOGICAL MEASURES OF HEAVY METAL BIOAVAILABILITY AND TOXICITY IN SOIL

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CHEMICAL AND BIOLOGICAL MEASURES OF

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TOXICITY IN SOIL

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PREFACE

This study was conducted to better understand the bioavailability and toxicity of cadmium (Cd), lead (Pb), and zinc (Zn) to soil organisms, both as individual contaminants and as mixtures. To adequately protect or restore soil ecosystems there is a great need to characterize soils presumed to be contaminated with heavy metals. Typical chemical analyses of soils, which determine total heavy metal concentrations of soils, are not well correlated with soil organism toxicity due to a host of modifying factors such as pH, organic matter content, and clay content. Due to these modifying factors, no soil will have 100% of its metal content bioavailable to organisms. Only bioavailable metals in soil are able to exert toxic action. Bioavailable metals cannot be directly measured using chemical analyses- only living organisms determine bioavailability. Laboratory toxicity tests, in which soil organisms are exposed to contaminated soils, are routinely used to evaluate toxicity and/or contaminant bioavailability, but are time consuming, expensive, and often difficult to interpret. The goal of this research was to investigate surrogate measures of metal bioavailability that would not only be inexpensive and precise, but also relate directly to toxicity.

Three methods of measuring metal bioavailability in soils were investigated: i) earthworm metal residues; ii) weak electrolyte soil extractions; and iii) ion-exchange membrane uptake. Single- and multiple-metal toxicity tests using the earthworm *Eisenia fetida* and ion-exchange membrane exposures were conducted in artificial soil spiked

with metal salts and remediated/unremediated Zn-smelter contaminated and reference field soils. Toxic units were calculated from the single-metal tests in artificial soil in order to evaluate mixture toxicity of the multiple-metal test. During all artificial soil toxicity tests, dead earthworms were analyzed to determine critical body residues (CBRs) for lethality for each metal. CBRs are concentrations of toxicants in an organism associated with a toxic endpoint, providing a link between the measure of bioavailability (the residue) and toxicity. CBRs were also used to further investigate mixture toxicity. All soils were extracted with a weak electrolyte (0.1M $Ca(NO_3)_2$) hypothesized to extract exchangeable or weakly bound "available" metals in soil. Plant Root Simulators™ (PRS™, Western Ag Innovations, Saskatoon, SK, Canada), ion-exchange membranes coated with disodium-diethylenetriaminepentaacetic acid (DTPA), were exposed to artificial soils at the same concentrations as the earthworm toxicity tests and remediated field soils. $Ca(NO_3)_2$-extractable metals and PRS uptake were compared to toxicity (mortality) and/or earthworm metal uptake to assess their use as surrogate measures of bioavailability.

The acute mixture toxicity of Cd, Pb, and Zn was additive (LC_{50} of approximately 1 toxic unit) regardless of the manner in which toxicity was quantitatively expressed. Earthworm metal concentrations were well related to lethality, and enabled the estimation of LC_{50}s using critical body residues (CBRs, $LCBR_{50}$s), which were 5.72 (3.54-7.91), 3.33 (2.97-3.69), and 8.19 (4.78-11.6) mmol/kg (±95% confidence interval) for Cd, Pb, and Zn, respectively. Zn concentrations of dead earthworms exposed to a lethal remediated Zn-smelter soil were 3-fold above the $LCBR_{50}$ for Zn and comparable to earthworm concentrations in lethal Zn-spiked artificial soils, despite a 14-fold difference in total soil Zn concentration between the lethal field and artificial soils. Metal concentrations in different methodological fractions of the earthworm tissue showed promise as measures of bioavailability at sublethal exposure levels. Weak-electrolyte (0.1M $Ca(NO_3)_2$) extractable metals in soil were precisely related to toxicity in all artificial toxicity tests. $Ca(NO_3)_2$-extractable Zn was comparable between the lethal Zn-spiked artificial and lethal remediated Zn-smelter soils, despite the aforementioned differences in soil parameters. Zn uptake in both live and dead earthworms was also well related to $Ca(NO_3)_2$-extractable Zn regardless of soil type ($R^2 = 0.96$), but poorly related to total soil Zn. Ion-exchange membrane metal uptake in soil was not well related to toxicity or earthworm metal uptake in metal-spiked artificial or remediated Zn-smelter soil.

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NOMENCLATURE

ANOVA	analysis of variance
CBR	critical body residue
CI	confidence interval
$Ca(NO_3)_2$	calcium nitrate
Cd	cadmium
DTPA	diethylenetriaminepentaacetic acid
LC_{50}	median lethal concentration
$LCBR_{50}$	median lethal critical body residue
LSB	lime stabilized municipal sewage sludge biosolids
LSD	least significant difference
pH	$-\log_{10}$ [hydrogen ion activity]
PRS	Plant Root Simulator™ ion-exchange membrane
Pb	lead
RP	North Carolina rock phosphate
SS	anaerobically digested municipal sewage sludge biosolids
TU	Toxic Unit
USEPA	United States Environmental Protection Agency
Zn	zinc

INTRODUCTION

This document consists of four chapters, each reporting separate studies conducted during my Master's program. Each chapter is presented in formats suitable for publication in professional journals.

CHAPTER I

EVALUATION OF SURROGATE MEASURES OF CADMIUM, LEAD, AND ZINC BIOAVAILABILITY TO *EISENIA FETIDA*

Abstract

We evaluated weak-electrolyte (0.1M $Ca(NO_3)_2$) soil extractions and ion-exchange membranes coated with a metal chelator as measures of Cd, Pb, and Zn bioavailability in spiked artificial soil by comparing their metal availability estimates to acute lethal toxicity in the earthworm *Eisenia fetida*. $Ca(NO_3)_2$ extractions were precisely related to toxicity in all toxicity tests, and enabled the development of time-independent LC_{50}s (incipient lethal-levels, ILLs) calculated using exposure levels based on extraction data. ILLs with 95% CIs for the Cd, Pb, and Zn toxicity tests were 9.81 (9.40-10.3), 1.16 (1.11-1.22), and 6.33 (6.18-6.49) $Ca(NO_3)_2$-extractable mmol metal/kg soil, respectively. Mixture toxicity of Cd, Pb, and Zn, assessed using the Toxic Unit (TU) approach, was 1.35 TU, suggesting additivity. Chelating ion-exchange membrane uptake was variable, and not well related to toxicity. Weak electrolyte extractions show promise as precise, inexpensive surrogate measures of Cd, Pb, and Zn bioavailability in soil.

Introduction

To adequately protect or restore soil ecosystems there is a great need to characterize soils suspected or presumed to be contaminated with heavy metals. Not only does metal contamination need to be characterized on varying temporal and spatial scales, but also in situations where interactions among different metals may occur. Soil assessment methods which only determine total heavy metal concentrations are often not accurate in predicting soil organism toxicity due to a number of modifying factors such as pH, organic matter content, and clay content (Beyer et al., 1987; Hopkin, 1989; Lanno and McCarty, 1997; Ma, 1982; Marinussen et al., 1997; McLean and Bledsoe, 1992; Morgan and Morgan, 1988; Spurgeon et al., 1997). Due to these modifying factors, soil metals will rarely (if ever) be 100% bioavailable to organisms. The bioavailability of metals cannot be measured directly using chemical analyses; only living organisms can determine bioavailability (Lanno and McCarty, 1997). Laboratory toxicity tests, in which soil organisms are exposed to contaminated soils, are routinely used to evaluate toxicity and/or metal bioavailability, but are time consuming, expensive, and often difficult to interpret (Lanno and McCarty, 1997). In order to assess the bioavailability, and hence toxicity, of metals in soils, approaches involving only total metal content of soils must be abandoned in favor of approaches relating some biological response to the available fraction of metal. A method measuring metal availability not involving organisms that relates well to bioavailability would be extremely useful as a screening tool in this pursuit.

Two surrogate methods of measuring metal bioavailability in soils that are easy, quick to perform, and relatively inexpensive include: i) weak-electrolyte soil extractions, and ii) ion-exchange membrane metal uptake. Electrolyte extractions using weak (< 1M) $CaCl_2$ or $Ca(NO_3)_2$ solutions have shown promise as toxicity-related measures of metal availability in soils (Basta and Gradwohl, 2000; Gradwohl, 1998; Marinussen et al., 1997; Peijnenburg et al, 1997; Peijnenburg et al, 1999; Posthuma et al., 1997; Sloan et al., 1997; Weljte, 1998). These solutions are hypothesized to extract exchangeable or weakly bound "available" metals in soil (Sloan et al., 1997), which are believed to be available for uptake by earthworms (Peijnenburg et al, 1999; Posthuma et al., 1997). In contrast to weak-electrolyte extractions, ion-exchange membranes have the added advantage of deployment in soils with a minimum of soil physicochemical alteration (Liang and Schoenau, 1995, 1996; Quian and Schoenau, 1997). Cation-exchange membranes complex divalent metal ions such as Cd^{2+}, Pb^{2+}, and Zn^{2+} directly from the soil solution, but suffer from interference from Ca^{2+} cations, which compete with heavy metals for membrane uptake (Liang, 1994). Anion-exchange membranes can be coated with a metal chelator, such as disodium-diethylenetriaminepentaacetic acid (DTPA), which chelates available metals and avoid Ca saturation because DTPA preferentially binds cation transition metals (Liang and Schoenau, 1995; Evangelou, 1998). Liang and Schoenau (1995) found a strong correlation between metal uptake of these chelating membranes and Cd, Cr, Pb, Ni, and Zn uptake for lettuce in soil.

Proper evaluation of surrogate measures of bioavailability must initially involve a comparison with organism responses in traditional toxicity tests. Toxicity testing conducted in artificial soil with the earthworm *Eisenia fetida* is a common standardized approach to evaluating toxicity of metals (ASTM, 1997; Edwards and Bohlen, 1992).

Using lethality data from these toxicity tests, a one-compartment, first-order kinetics (1CFOK) model can be employed to estimate toxicity kinetics and incipient lethal levels (ILL) (Fitzgerald et al., 1997; Lanno et al., 1997). Using ILLs of single-metal toxicity tests, mixture toxicity can be assessed in soils containing metal mixtures using the toxic unit (TU) approach (Sprague, 1970). Once metal toxicity has been assessed, comparisons can be made between surrogate measures of bioavailability and total metal levels.

The objectives of this research were to: i) assess lethal toxicity of Cd, Pb, and Zn (individually and as a mixture) to *E. fetida*, and ii) evaluate $Ca(NO_3)_2$ extractions and ion-exchange membranes coated with DTPA as surrogate measures of bioavailability to *E. fetida*. We hypothesized that i) mixture toxicity of Cd, Pb, and Zn would be additive and ii) metal availability estimates obtained with $Ca(NO_3)_2$ extractions and ion-exchange membranes coated with DTPA would be better related to toxicity than total metal levels.

Materials and Methods

Soil toxicity tests

Four acute lethality tests were conducted with earthworms (*E. fetida*) in artificial soil according to a standard protocol (ASTM, 1997), with noted exceptions. Testing was conducted in environmental chambers maintained at 20 ±1°C under constant light. Single-metal tests consisted of artificial soil spiked with aliquots of stock solutions of $CdSO_4$, $Pb(NO_3)_2$, or $ZnSO_4$ dissolved in deionized water. For each test, soils were spiked in an increasing log-scale exposure series, with three replicates at each concentration. The Cd-Pb-Zn mixture test comprised six exposure levels in triplicate, but was spiked with Cd, Pb, and Zn nitrate salts to achieve a geometrically increasing exposure series based on TU calculated from toxicity data from the single-metal tests. All replicates contained 200g (dry weight) artificial soil, consisting of 69.5% silica sand, 20% kaolin clay, 10% 2-mm sieved *Sphagnum* peat moss, and approximately 0.5% $CaCO_3$, which was added to adjust the initial pH to 6.5 ±0.5. Deionized water was added to hydrate artificial soil to 38% water (w/w). Twenty-four hours prior to the start of each test, test soils were prepared and mature (clitellate) earthworms weighing approximately 0.2-0.4 g were obtained from in-house cultures and placed on moist filter paper to depurate culture bedding from their gastrointestinal tracts. Each replicate received ten earthworms at the beginning of the test. Earthworm mortality was monitored according to a geometric time scale (e.g., 2, 4, 8, 16, 24, 36, 48 h) for the first 48 h (when most mortality occurred), then daily for the remainder of the test. Earthworms were judged to be dead when no movement was observed after gentle stimulation with a blunt probe.

Ion-exchange membrane exposures

Ion-exchange membranes were simultaneously exposed under the same conditions as the earthworm toxicity tests. Plant Root Simulator™ (PRS™, Western Ag Innovations Inc., Saskatoon, SK, Canada, www.westernag.ca) anion-exchange membranes were the ion-exchange membrane design employed throughout the experiment. PRSs were converted before use to chelating ion-exchange membranes by complexation of the anionic membrane surface with disodium-DTPA (Liang, 1994). Individual PRSs were buried in 100 g (dry weight) soil replicates that had been moistened and spiked with metal salts as described for soils used in earthworm exposures. After a

1-h exposure period, PRSs were removed from soil, rinsed thoroughly with deionized water to remove soil, and eluted with 20 ml 0.5M trace metal-grade HNO_3 (Fisher Scientific) in separate, self-sealing plastic bags for 1 h. The eluent was then analyzed for Cd, Pb, and/or Zn using flame and/or graphite furnace atomic absorption (AA) spectroscopy, while the PRSs were regenerated for reuse. Two PRS replicates per soil were exposed alongside the earthworm toxicity tests in the same environmental chamber on the first day of the earthworm exposure periods. After regeneration, the PRSs were exposed again on the seventh day to the same soil replicates to assess changes in metal availability over time. Quality assurance/quality control (QA/QC) procedures included PRS blanks and spikes, conducted with water-rinsed PRSs during the acid elution step.

Soil analyses

Upon termination of toxicity tests and PRS exposures, individual soil replicates from all experiments were stored at -40°C in self-sealing plastic bags until physical and chemical parameters could be measured. Soil pH for all earthworm and PRS soil replicates was measured in the supernatant of a settled 0.01M $CaCl_2$ soil slurry (10 g dry weight soil/20 ml solution) according to Hendershot et al. (1993). Total metal concentrations of soils were obtained by wet digestion of 1 g (dry weight) soil using 5 ml concentrated trace metal-grade HNO_3. The digests were then heated to dryness, resolubilized in 15 ml 0.5N trace metal-grade HNO_3, filtered with a hardened, ashless, paper filter (Whatman 540), and brought to a 50-ml volume with 0.5N trace metal-grade HNO_3. To measure weak electrolyte extractable metals, 1 g (dry weight) of each soil replicate from the earthworm tests was combined with 20 ml 0.1M $Ca(NO_3)_2$, mixed in a rotary mixer for 4 h at 23°C, and centrifuged at 2500 *g* for 15 min. The resulting supernatant was then filtered with a 0.45 µm membrane filter (Millipore HAWP) and acidified with 0.5 ml concentrated trace metal-grade HNO_3. Total metal digests and $Ca(NO_3)_2$ extractions were analyzed by AA spectroscopy as described above. Quality assurance/quality control measures included duplicate analyses, metal spikes, blanks, and analyses of standard reference soil "Sandy Soil B" (CRM-SA-B, Environmental Express, Mt. Pleasant, SC, USA). Total metal analyses of the standard reference soil were within performance acceptance limits determined by USEPA 3050 digestion procedures (USEPA, 1995).

Data analysis

Soils were classified as lethal if the 14-d cumulative mortality rate was $\geq$15%. LC_{50} values were estimated using the Trimmed Spearman-Karber method (Hamilton et al., 1977) for the Cd, Pb, and Zn artificial soil toxicity tests at observation times where any replicate exhibited $\geq$ 50% mortality. All LC_{50} estimates were based on the same biological data using exposure levels determined by total metals digestions or $Ca(NO_3)_2$-extractions. The means of inverse LC_{50} estimates for each replicate were plotted against time to generate a 1CFOK curve using non-linear regression techniques (Lanno et al., 1997). The ILL was estimated from the asymptotic value of the 1CFOK curve. Toxicity kinetics was evaluated using toxicity half-life ($t_{1/2}$), the time taken to reach half of the ILL, which was also calculated from the 1CFOK curve. Toxic units were calculated for the mixture toxicity test using soil analysis data and the ILLs from the Cd, Pb, and Zn single-metal toxicity tests, which were based on total (Equation 1) or $Ca(NO_3)_2$-

extractable metal levels (Equation 2):

$$\mathrm{TU} = \frac{[\text{Total soil Cd}]}{\mathrm{ILL}_{\text{Total Cd}}} + \frac{[\text{Total soil Pb}]}{\mathrm{ILL}_{\text{Total Pb}}} + \frac{[\text{Total soil Zn}]}{\mathrm{ILL}_{\text{Total Zn}}} \tag{1}$$

$$\mathrm{TU} = \frac{[\mathrm{Ca(NO_3)_2}\text{-extractable Cd}]}{\mathrm{ILL}_{\mathrm{Ca(NO_3)_2}\text{-extractable Cd}}} + \frac{[\mathrm{Ca(NO_3)_2}\text{-extractable Pb}]}{\mathrm{ILL}_{\mathrm{Ca(NO_3)_2}\text{-extractable Pb}}} + \frac{[\mathrm{Ca(NO_3)_2}\text{-extractable Zn}]}{\mathrm{ILL}_{\mathrm{Ca(NO_3)_2}\text{-extractable Zn}}} \tag{2}$$

All data were checked for homogeneity of variance and normality and transformed as appropriate to meet requirements for ANOVA (Sokal and Rohlf, 1995). PRS data were analyzed according to a split-plot (repeated measures) test design (Steel et al., 1997). Data not satisfying assumptions for ANOVA were analyzed nonparametrically using the Kruskal-Wallis test. Fisher's Protected Least Significant Difference (LSD) multiple comparison procedure was also employed to further elucidate differences between means ($\alpha = 0.05$).

Results

Single metal experiments

Results of physicochemical analysis of the single-metal artificial soil toxicity tests are presented in Table 1. In all three tests, pH declined markedly with increasing exposure level. All total and $Ca(NO_3)_2$-extractable Cd, Pb, and Zn levels were significantly different among exposure levels. In general, $Ca(NO_3)_2$-extractable Cd and Zn levels were > 50% of total levels, while $Ca(NO_3)_2$-extractable Pb levels, in contrast, were generally 0.4-3% of total Pb levels.

The 1CFOK model provides a good fit to the toxicity data for the individual metals expressed as total metal or $Ca(NO_3)_2$-extractable metal (Fig. 1, 2a). Incipient lethal levels based upon total metal levels indicate that Zn was most toxic to earthworms, followed by Cd, then Pb, with Zn about three-fold more toxic than Pb (Table 2). However, when toxicity was expressed on a $Ca(NO_3)_2$-extractable metal basis, Pb was most toxic, followed by Zn, then Cd, with about a nine-fold difference in toxicity between Pb and Cd. Toxicity half-life data indicated that the Cd and Zn toxicity kinetics were similar and did not differ whether expressed in terms of total metal or $Ca(NO_3)_2$-extractable metal (range – 37-50 h; Table 2). The toxicity kinetics of Pb was faster than Cd and Zn, and was similar whether expressed as total metal or $Ca(NO_3)_2$-extractable metal (range – 7-12 h). Uptake of Cd, Pb, and Zn by PRSs was highly variable and did not appear to be related to toxicity in the single metal tests (Fig. 3a-c). No differences in PRS uptake of metals were observed between day 1 and day 7 of exposure ($P > 0.05$).

Cd-Pb-Zn mixture experiment

As in the single metal tests, pH declined markedly with increasing exposure level (Table 3). All $Ca(NO_3)_2$-extractable Cd, Pb, and Zn levels were significantly different among exposure levels. In general, $Ca(NO_3)_2$-extractable Cd and Zn levels were > 50% of total levels. $Ca(NO_3)_2$-extractable Pb levels were again low compared to $Ca(NO_3)_2$-extractable Cd and Zn, but were slightly more available (1-14% of total Pb levels) than in the Pb single metal test.

The 1CFOK curves provide a good fit to the toxicity data (Fig. 2b). Based on TU calculated from the ILLs estimated using total metal levels in soil, as well as the subsequent 1CFOK model-generated estimates (Table 2), the ILL based on total Cd, Pb, and Zn was 1.35 TU, approximating additivity. The $Ca(NO_3)_2$-based ILL was not significantly different from this estimate. Toxicity half-lives calculated with both approaches were also very similar, and indicated that the mixture toxicity kinetics were similar to toxicity kinetics of the Pb test.

As with the single metal tests, PRS uptake was also poorly related to toxicity. In contrast to PRSs exposed to single metals, Cd and Zn uptake in the mixture test did not approximate increases in exposure level. Instead, PRS uptake for all three metals followed a threshold response, remaining relatively constant among the lower exposure levels and dramatically increasing at the highest exposure level (Fig. 3d-f). No differences in PRS uptake of Cd and Pb were observed between day 1 and day 7 of exposure ($P > 0.05$), although there was a slight increase in PRS Zn uptake on day 7 ($P = 0.038$).

Discussion

Single metal experiments

Incipient lethal levels based upon total metal levels indicate that Zn was the most toxic to earthworms, followed by Cd, then Pb (Table 2). However, when toxicity was expressed on a $Ca(NO_3)_2$-extractable metal basis, Pb was most toxic, followed by Zn, then Cd. These results are not startling since there are a number of studies that show differences in the chemical availability (bioavailability?) of metals using various extraction techniques in different soil types (Aten and Gupta, 1996; Lebourg et al., 1998; Pichtel and Salt, 1998; Qian et al., 1996). However, what is interesting is that the metal that is most toxic changes with the method by which soil toxicity is assessed, supporting the findings of Posthuma et al. (1997) with regards to sublethal Cu and Zn toxicity in the earthworm *Enchytraeus crypticus*. The order of toxicity of these metals, when based on a $Ca(NO_3)_2$-extractable metal basis, is in line with general toxicological theory, in contrast to total-metal based approximations, which rank Pb toxicity very low. This implies that basing toxicity estimates and soil protection guidelines on total levels of metals in soil is incorrect and it may be necessary to abandon this approach in favor of chemical measures that more accurately reflect the bioavailability of metals. The results of our study support the theory that ILLs based on $Ca(NO_3)_2$-extractable metal are a more realistic approximation of metal bioavailability and thus may provide a more accurate representation of the toxicity of metals in soil than total metal levels.

Despite the standardization of artificial soil toxicity tests with earthworms, the measurement of routine physicochemical parameters in test soils is necessary to identify unavoidable changes which occur due to the addition of metal salts. Soil pH is usually the main factor controlling the solubility and bioavailability of metals (Basta et al., 1993; McLean and Bledsoe, 1992; Peijnenburg et al., 1997, 1999; Posthuma et al., 1997; Smit et al., 1998) , but the addition of free metal ions can alter pH. Upon addition of metal salts to artificial soil, metal ions undergo hydrolytic reaction in soil solution to form metal-hydroxide complexes and protons (Basta and Tabatabai, 1992; Evangelou, 1998), resulting in a decrease in soil pH. Thus, soil pH is inversely related to the amount of metal salt added to the soil (Basta and Tabatabai, 1992). Metal hydroxides are relatively insoluble and unavailable for uptake by earthworms, PRSs, or extraction by weak $Ca(NO_3)_2$ solution. Among the metals tested, Pb has the greatest tendency to form hydroxides, as well as other insoluble complexes (Evangelou, 1998; Van Gestel et al., 1998), possibly resulting in the rather low proportion of Pb that was extractable by the $Ca(NO_3)_2$ solution. In this way, metal complexation presents problems for toxicity evaluations based on total metal levels since even in artificial soil, a significant percentage of the metals are not bioavailable.

The toxicity kinetics of Cd and Zn were similar, reflecting their toxicological similarities. Since Cd and Zn are hypothesized to enter earthworms via passive and/or facilitated diffusion (Dallinger, 1993) and both metals are either regulated (Zn) or detoxified (Cd) intracellularly with the aid of separate metal binding proteins (Dallinger, 1993; Marino et al., 1998; Morgan et al., 1993; Morgan and Morgan, 1998), it is likely that these metals would exhibit similar toxicity kinetics. The toxicity kinetics of Pb were much faster, suggesting that different mechanisms were present in the earthworm to regulate Pb toxicity. Because Pb often competes with Ca for uptake and in metabolic reactions (Simkiss and Taylor, 1981), and thus may involve different receptors than for Cd and Zn, the toxicity kinetics of Pb may be quite different from Cd and Zn.

Metal uptake by PRSs was not a good predictor as $Ca(NO_3)_2$ extractions in identifying levels of metals lethal to earthworms in artificial soil. Liang (1994) tested uncontaminated soils spiked with metal salts and observed excellent relationships between PRS uptake and both the amount of metal added and lettuce uptake. However, there was no clear relationship between PRS uptake and total added metal or earthworm toxicity in this study. Assuming that metal bioavailability increased with increasing exposure level in artificial soil, a factor unrelated to metal availability may be affecting PRS uptake of metals. The high salt or differing pH levels in the artificial soil may affect the chelating ability of the membrane-bound DTPA. This would explain why PRS uptake did not increase in a concentration-dependent manner in the Pb test, which had high salt concentrations and low pH. The chelating ability of DTPA decreases with decreasing pH (Evangelou, 1998), but not to the degree that would explain the results of this study. The effect of pH or other chemical factors is uncertain since little is known about the chemical activity of DTPA when it is bound to an anion-exchange membrane. DTPA may not be the ideal chelant to use with PRSs, however, because the conventional DTPA soil extraction is specifically designed for use in soil fertility evaluations, and is not recommended for estimating the bioavailability of metals present at toxic levels (O'Connor, 1988). Differences in metal uptake mechanisms between plants and earthworms may also explain the poor relationship between PRS metal uptake and earthworm toxicity. Further work is needed to characterize and validate the use of PRSs, perhaps in soils in which pH can be controlled or with the use of a different PRS-chelant.

Cd-Pb-Zn mixture experiment

The toxicity of a mixture of Cd, Pb, and Zn in artificial soil suggests that these metals act in an approximately additive manner to produce lethality in *E. fetida*. This result concurs with other metal mixture toxicity studies with earthworms (Khalil et al., 1996a,1996b; Weljte, 1998) and supports the theory that metals have similar modes of toxic action, which has been hypothesized to be non-specific binding to proteins within the organism (Di Giulio et al., 1995; Simkiss and Taylor, 1981; Weljte, 1998). It is not surprising that mixture ILLs expressed as toxic units based on both total metals and $Ca(NO_3)_2$-extractable metals were similar. If the toxicological interaction of Cd, Pb, and

Zn is truly additive, the ILL should always approximate additivity regardless of which method is used to express exposure as long as the results of exposure level quantification for each metal (soil extractions) retain precision between the single metal and mixture experiments. This is the case in this study, for the proportions of $Ca(NO_3)_2$-extractable Cd, Pb, and Zn among the higher exposure levels remained relatively constant between the single metal tests and the mixture test (Tables 1, 3).

Toxicity half-lives indicate that mixture toxicity kinetics were similar to that of the Pb toxicity test. Osmotic stress caused by high salt additions necessary for testing with Pb may have made the earthworms more sensitive to metal toxicity. Also, the similar half-lives may suggest that Pb may be the major cause of toxicity in the mixture experiment. Assuming that the $Ca(NO_3)_2$ extraction is a reliable surrogate measure of metal bioavailability, this may be the case because the lowest lethal exposure level in the mixture test had a higher $Ca(NO_3)_2$-extractable Pb level than the highest nonlethal exposure level in the Pb-only test.

PRS uptake in the mixture test indicated that there was no relationship between the amount of metal added or toxicity. Although slight preferences for DTPA chelation among Cd, Pb, and Zn exist (Evangelou, 1998), this does not explain the data in this mixture experiment. The proportion of metal available for PRS uptake seems to be generally lower in the mixture test, despite the fact that DTPA chelation activity does not change in the presence of single versus multiple metal levels. As with the Pb test, there seems to be another factor involved in the ability of membrane-bound DTPA to chelate available metals.

Conclusions

In light of the improper nature of total metals-based expressions of exposure, even in uniformly prepared, homogeneous artificial soils, the need for a surrogate measure of metal bioavailability in soil has never been greater. Considering the formation of hydroxides and other possible unavailable metal species, it is invalid to assume that 100% of the metal added to artificial soil is bioavailable, making total metal-based expressions of exposure inappropriate. From the results of this study, weak-electrolyte extractions seem to be very promising in this regard. $Ca(NO_3)_2$-extractable Cd, Pb, and Zn in the artificial soil tests not only follow trends in chemical availability according to metal-specific preferences for the formation of insoluble metal complexes, but also relate well to toxic responses in the earthworms. Testing with field soils contaminated with metals is needed to further validate the use of weak-electrolyte extractions as surrogate measures of metal bioavailability in soil. Correlation with metal residues in soil organisms is also necessary. Exploration of the relationship between weak-electrolyte extractable metals and bioavailability has already begun with other soil organisms at sublethal Zn levels (Marinussen et al., 1997; Posthuma et al., 1997; Van Gestel et al., 1998; Weljte, 1998). PRSs are not as precise surrogate measures of bioavailability as $Ca(NO_3)_2$ extractions, but still may be useful because of their ability to measure *in situ* metal availability with a minimum of physicochemical alteration to soil.

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Table 1. Total and $Ca(NO_3)_2$-extractable metal concentrations and pH of artificial soil spiked with Cd, Pb, or Zn (mean, n = 3, 95% CI). Means in columns with the same superscript were not significantly different ($P > 0.05$, Fisher's protected LSD).

	Cd toxicity test				Pb toxicity test				Zn toxicity test			
Exposure level	Total Cd (mmol/kg)	$Ca(NO_3)_2$-extractable Cd (mmol/kg)	%[1]	pH	Total Pb (mmol/kg)	$Ca(NO_3)_2$-extractable Pb (mmol/kg)	%[1]	pH	Total Zn (mmol/kg)	$Ca(NO_3)_2$-extractable Zn (mmol/kg)	%[1]	pH
Control	0.028^{a}	0.000^{a}	0.0	6.95^{a}	0.023^{a}	0.000^{a}	0.0	6.94^{a}	0.078^{a}	0.000^{a}	0.0	7.21^{a}
	(0.000-0.106)	(0.000-0.000)		(6.42-7.48)	(0.015-0.036)	(-0.001-0.002)		(6.74-7.14)	(0.054-0.113)	(0.000-0.000)		(6.74-7.68)
1	3.67^{b}	0.971^{b}	26.5	6.78^{a}	1.89^{b}	0.007^{b}	0.4	$6.85^{a,b}$	3.27^{b}	0.415^{b}	12.7	$6.86^{a,b}$
	(2.64-4.87)	(0.858-1.10)		(6.32-7.24)	(1.59-2.24)	(0.003-0.016)		(6.72-6.98)	(2.32-4.59)	(0.206-0.834)		(6.27-7.45)
2	8.04^{c}	2.77^{c}	34.5	6.80^{a}	4.00^{c}	0.027^{c}	0.7	$6.79^{a,b}$	6.59^{c}	3.37^{c}	51.1	$5.72^{b,c}$
	(6.71-9.48)	(2.29-3.34)		(6.14-7.46)	(3.48-4.59)	(0.011-0.065)		(6.66-6.92)	(6.03-7.21)	(1.24-9.16)		(3.99-7.45)
3	17.1^{d}	8.67^{d}	50.7	6.35^{b}	9.75^{d}	0.058^{d}	0.6	6.72^{b}	15.6^{d}	13.3^{d}	85.3	4.99^{c}
	(14.8-19.6)	(6.79-11.1)		(5.88-6.82)	(9.42-10.1)	(0.016-0.202)		(6.52-6.92)	(12.9-18.7)	(11.2-15.9)		(2.12-7.86)
4	28.7^{e}	15.7^{e}	54.7	5.99^{c}	19.6^{e}	0.379^{e}	1.9	6.36^{c}	35.2^{e}	20.3^{e}	57.7	5.61^{c}
	(25.0-32.7)	(13.7-18.1)		(5.70-6.28)	(15.1-25.3)	(0.140-1.02)		(6.07-6.65)	(33.8-35.6)	(13.0-31.7)		(3.74-7.48)
5	41.3^{f}	24.5^{f}	59.3	5.83^{c}	43.2^{f}	4.20^{f}	9.7	5.61^{d}	66.1^{f}	47.6^{f}	72.0	5.44^{c}
	(40.1-42.6)	(19.7-30.4)		(5.45-6.21)	(37.4-49.9)	(3.43-5.16)		(5.28-5.94)	(41.1-106)	(37.7-60.2)		(4.33-6.55)

[1] Percent of total metal that was $Ca(NO_3)_2$-extractable

Table 2. Incipient lethal levels (ILL), toxicity half-lives ($t_{1/2}$), and coefficients of determination calculated from a one-compartment, first-order kinetics (1CFOK) model applied to toxicity data for *Eisenia fetida* exposed to artificial soils spiked with Cd, Pb, Zn, and a Cd-Pb-Zn mixture. Toxicity data are based on both measured total metal and $Ca(NO_3)_2$-extractable metal levels. Goodness of 1CFOK model fit given by r^2.

	Total metal based			$Ca(NO_3)_2$-extractable metal based		
Toxicity test	ILL (mmol/kg soil)	$t_{1/2}$(h)	r^2	ILL (mmol/kg soil)	$t_{1/2}$(h)	r^2
Cd	19.9	37.2	0.92	9.81	50.3	0.90
	(19.3-20.6)	(31.4-45.8)		(9.40-10.3)	(41.6-63.8)	
Pb	28.1	7.00	0.96	1.16	11.8	0.79
	(27.6-28.7)	(5.9-8.5)		(1.11-1.22)	(9.00-17.1)	
Zn	9.65	45.2	0.92	6.33	47.4	0.96
	(9.12-9.82)	(39.2-53.4)		(6.49-6.17)	(43.0-52.8)	
Mixture	1.35[1]	6.25	0.94	1.38[1]	9.70	0.94
	(1.31-1.39)	(5.30-7.61)		(1.34-1.43)	(8.40-11.6)	

[1] ILL in TU

Table 3. Total and $Ca(NO_3)_2$-extractable metal concentrations and pH of the Cd-Pb-Zn mixture artificial soil toxicity test (mean, n = 3, 95% CI). Means in columns with the same superscript were not significantly different ($P > 0.05$, Fisher's protected LSD).

Exposure level	Total Cd (mmol/kg)	$Ca(NO_3)_2$-extractable Cd (mmol/kg)	%[1]	Total Pb (mmol/kg)	$Ca(NO_3)_2$-extractable Pb (mmol/kg)	%[1]	Total Zn (mmol/kg)	$Ca(NO_3)_2$-extractable Zn (mmol/kg)	%[1]	pH
Control	0.005^a	0.000^a	0.0	0.020^a	0.000^a	0.0	0.088^a	0.000^a	0.0	6.69^a
	(-0.052-0.135)	(0.000-0.000)		(0.017-0.023)	(-0.001-0.003)		(0.061-0.127)	(0.000-0.000)		(6.31-7.07)
1	1.62^b	0.520^b	32.1	2.34^b	0.039^b	1.7	0.847^b	0.155^b	18.3	6.37^b
	(3.22-6.68)	(0.434-0.623)		(1.91-2.86)	(0.011-0.139)		(0.687-1.04)	(0.105-0.227)		(5.92-6.82)
2	3.22^c	1.38^c	42.9	4.71^c	0.090^c	1.9	1.63^c	$.482^c$	29.6	6.00^c
	(3.08-3.36)	(1.19-1.59)		(4.39-5.05)	(0.031-0.258)		(1.42-1.86)	(0.376-0.619)		(5.87-6.13)
3	6.67^d	3.75^d	56.2	10.1^d	0.195^d	1.9	3.25^d	1.65^d	50.8	5.56^d
	(6.06-7.32)	(3.71-3.79)		(9.58-10.6)	(0.158-0.239)		(1.42-1.86)	(1.49-1.84)		(5.39-5.73)
4	12.1^e	9.87^e	81.6	16.1^e	0.995^e	6.2	6.48^e	4.75^e	73.3	5.14^e
	(10.9-13.3)	(9.42-10.3)		(11.9-21.9)	(0.669-1.48)		(2.96-3.57)	(4.30-5.25)		(4.81-5.47)
5	28.6^f	16.5^f	57.7	37.0^f	5.43^f	14.7	14.0^f	7.90^f	56.4	4.70^f
	(18.0-41.6)	(10.7-25.4)		(29.4-46.7)	(4.48-6.58)		(4.96-8.48)	(4.83-12.9)		(4.65-4.75)

[1] Percent of total metal that was $Ca(NO_3)_2$-extractable

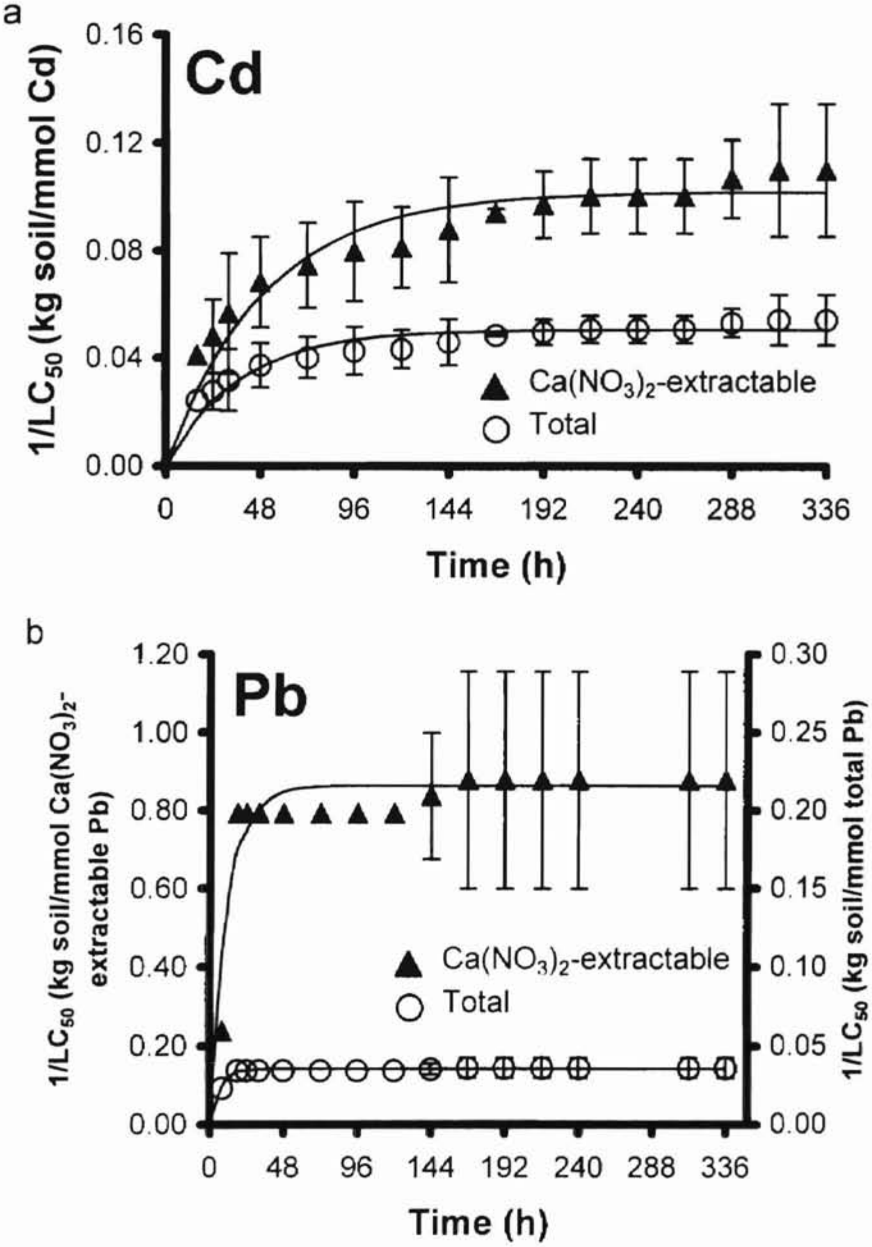

Figure 1. Toxicity curves (1/LC$_{50}$s based on total and Ca(NO$_3$)$_2$-extractable metals vs. exposure time) for *Eisenia fetida* exposed to artificial soils spiked with Cd (**a**) and Pb (**b**). Data points (mean 1/LC$_{50}$, n=3) were used to generate non-linear 1CFOK curves which were used to calculate incipient lethal-levels (ILL) and toxicity half-lives ($t_{1/2}$) for each test. Note that the total-Pb based toxicity data is plotted on a secondary y axis. All error estimates are 95% CIs.

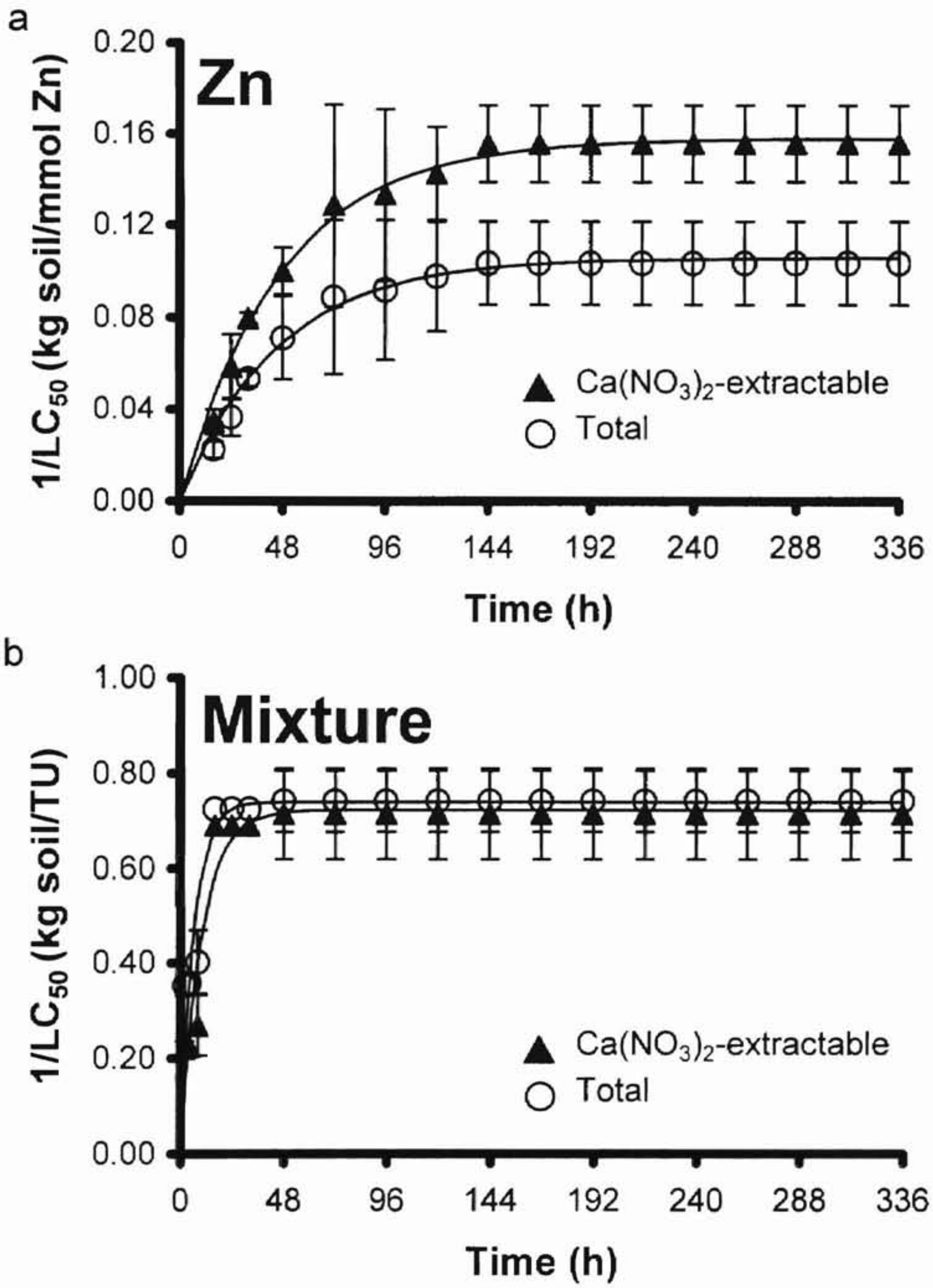

Figure 2. Toxicity curves ($1/LC_{50}$s based on total and $Ca(NO_3)_2$-extractable metals vs. exposure time) for *Eisenia fetida* exposed to artificial soils spiked with Zn (**a**) and a Cd-Pb-Zn mixture (**b**). Data points (mean $1/LC_{50}$, n=3) were used to generate non-linear 1CFOK curves which were used to calculate incipient lethal-levels (ILL) and toxicity half-lives ($t_{1/2}$) for each test. All error estimates are 95% CIs.

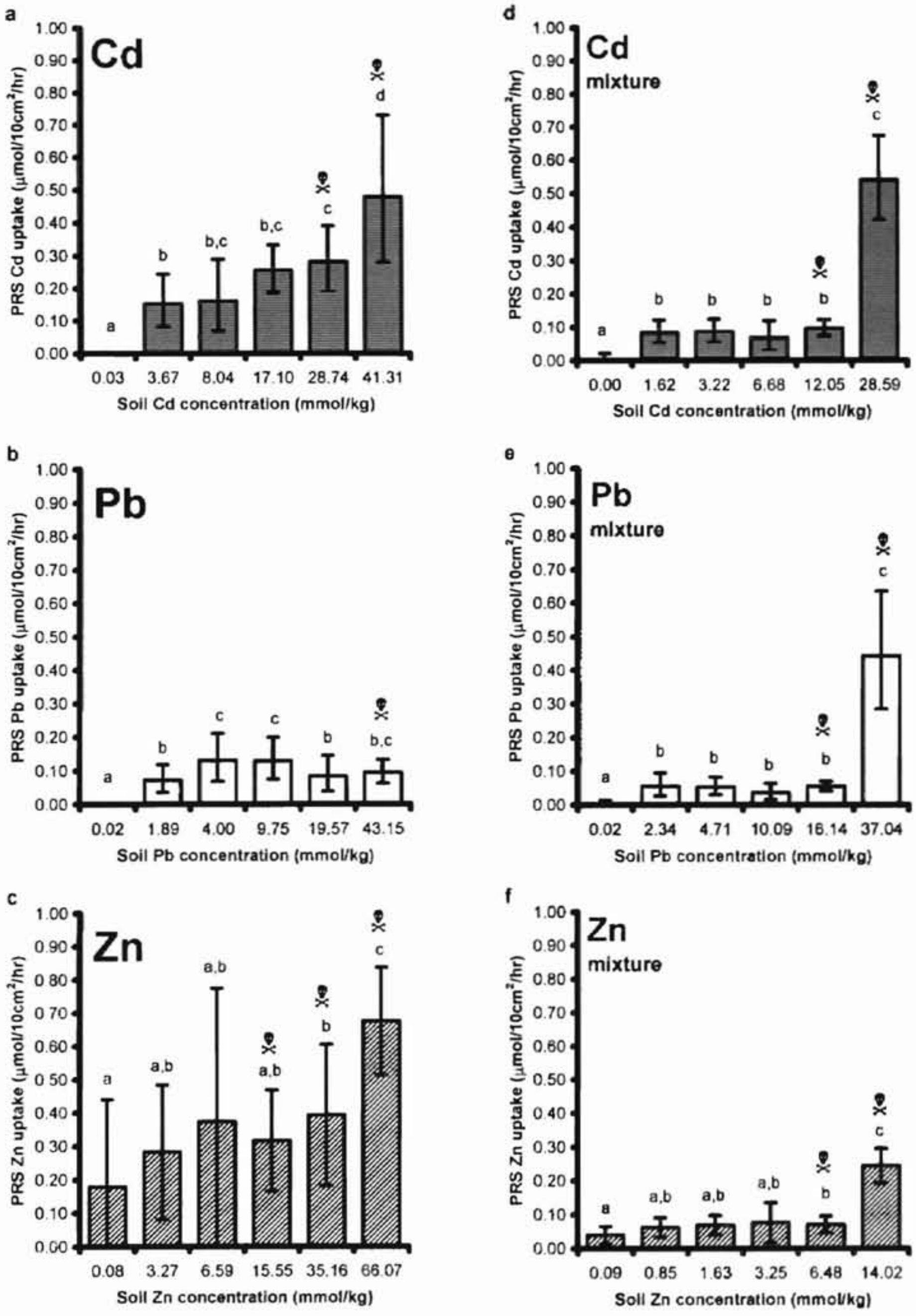

Figure 3. Plant Root Simulator™ (PRS) chelating ion-exchange membrane uptake of Cd, Pb, and Zn in artificial soils spiked with the same metal concentrations as Cd (**a**), Pb (**b**), Zn (**c**), and Cd-Pb-Zn mixture (**d-f**) toxicity tests with *Eisenia fetida*. Columns (mean, n = 4, ± 95% CI) with the same letter are not significantly different ($\alpha = 0.05$). Symbols (☠) indicate lethal soil concentrations.

CHAPTER II

ASSESSMENT OF METAL AVAILABILITY IN A CHEMICALLY REMEDIATED SMELTER SOIL BY *EISENIA FETIDA* AND CHEMICAL EXTRACTIONS

Abstract

Chemical immobilization is a relatively inexpensive *in situ* remediation method that reduces soil contaminant solubility, but the ability of this remediation treatment to reduce heavy metal bioavailability and ecotoxicity to soil organisms has not been evaluated. The objectives of this research were to: i) assess the ability of chemical immobilization treatments (municipal sewage sludge biosolids and rock phosphate) to reduce metal bioavailability and toxicity in a toxic metal-contaminated smelter soil, and; ii) evaluate soil extraction methods using $Ca(NO_3)_2$ solution or ion-exchange membranes coated with DTPA as surrogate measures of metal bioavailability and ecotoxicity. A soil contaminated by Zn and Pb milling and smelting operations and an uncontaminated control soil were treated with lime stabilized municipal biosolids (LSB), rock phosphate (RP), or anaerobically digested municipal biosolids (SS). Soil treatments were evaluated by bioassay using the earthworm *Eisenia fetida*.. Metal availability estimates measured with weak-electrolyte (0.1 M $Ca(NO_3)_2$) soil extractions and ion-exchange membranes coated with DTPA were compared to lethality in earthworms. LSB was the only remediation treatment to successfully immobilize lethal levels of Zn in the smelter soil (14-d cumulative mortality $\leq 15\%$), although RP slightly reduced toxicity. $Ca(NO_3)_2$

extractions were highly related to toxicity. $Ca(NO_3)_2$-extractable Zn in the lethal Zn smelter soil/treatment combinations were 11.5-18.2 mmol/kg, compared with the nonlethal LSB amended soil (0.62 mmol/kg). The $Ca(NO_3)_2$-extractable Zn-based LC_{50} of 6.33 mmol/kg previously developed in Zn-spiked artificial soils was applicable in the remediated smelter soils despite a 14-fold difference in total Zn concentration. Chelating ion-exchange membrane uptake among the field and artificial soils was highly variable (mean CV = 39%) compared to the $Ca(NO_3)_2$-extraction (mean CV = 1.9%) and not well related to toxicity. With further validation with other metals, soils, and organisms, weak-electrolyte extractions may be useful surrogate measures of metal bioavailability in contaminated and/or remediated soils.

Introduction

The mining and smelting of lead (Pb) and zinc (Zn) ore often results in contamination of soil with cadmium (Cd), Pb, and Zn. Metal-contaminated soil often presents an unacceptable risk to human and ecological health and must be remediated (Adriano et al., 1997; Pierzynski, 1997). Commonly used cleanup methods involved excavation and landfilling of smelter-contaminated soil. However, more permanent and less expensive *in situ* solutions have been favored during the last decade (Iskandar and Adriano, 1997). Chemical immobilization is a relatively inexpensive *in situ* remediation method where soil is treated with chemical amendments that reduce metal solubility. Many studies using chemical amendments, including organic matter (Pierzynski and Schwab, 1993), alkaline materials (Mench et al., 1994), and phosphates (Boisson et al., 1999; Ma et al., 1994, 1995), have been used for chemical remediation of metal-contaminated soil. A recent study comparing biosolids, alkaline cement kiln dust, and rock phosphate (carbonated apatite) found many of these treatments reduced phytoavailability and extractability of heavy metals (Basta and Gradwohl, 2000; Gradwohl, 1998). However, the effect of these treatments on metal bioavailability and toxicity to soil invertebrates was not evaluated.

Toxicity testing using earthworms is a well-developed means of studying the bioavailability and acute toxicity of soil contaminants (Edwards and Bohlen, 1992) and contaminated site soils (Marinussen et al., 1997a). 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 (Beyer et al., 1987; Conder and Lanno, 2000; Hopkin, 1989; Lanno and McCarty, 1997; Ma, 1982; Marinussen et al., 1997b; McLean and Bledsoe, 1992; Morgan and Morgan, 1988; Spurgeon et al., 1997). Clean-up levels are often based on total heavy metal content of soil and do not consider biologically available or "bioavailable" metal levels, even though environmental risk is related to the bioavailability of heavy metals in soil. The bioavailability of metals cannot be measured directly using chemical analyses; only living organisms can actually determine bioavailability (Lanno and McCarty 1997). A method of measuring metal availability not involving organisms (reducing expense and variability) that is well related to bioavailability would be an extremely useful screening tool for evaluating metal-contaminated soils.

Two surrogate methods of measuring metal bioavailability in soils that are easy, quick to perform, and relatively inexpensive include: i) weak-electrolyte soil extractions, and; ii) ion-exchange membrane metal uptake. Extractions using weak (< 1M) $CaCl_2$ or $Ca(NO_3)_2$ solutions have shown promise as toxicity-related measures of metal availability in soils (Basta and Gradwohl, 2000; Conder and Lanno, 2000; Gradwohl, 1998; Marinussen et al., 1997b; Peijnenburg et al, 1997; Peijnenburg et al, 1999; Posthuma et al., 1997; Weljte, 1998). These solutions are hypothesized to extract exchangeable or weakly bound "available" metals in soil (Sloan et al., 1997) which are believed to be available for uptake by soil organisms (Peijnenburg et al. 1999; Posthuma et al. 1997). Conder and Lanno (2000) demonstrated that metal levels in weak $Ca(NO_3)_2$ extractions relate well to lethal Cd, Pb, and Zn toxicity in the earthworm *Eisenia fetida* exposed to metal-spiked artificial soil. In contrast, ion-exchange membranes have a definite advantage over weak electrolyte extractions because they can be deployed in soils with a minimum of soil physicochemical alteration, even under *in situ* conditions (Liang and Schoenau, 1995, 1996; Quian and Schoenau, 1997). Cation-exchange membranes complex divalent metal ions such as Cd, Pb, and Zn directly from the soil solution, but suffer interference from Ca cations, which compete with heavy metals for membrane uptake (Liang, 1994). Anion-exchange membranes can be coated with a metal chelator, such as disodium-diethylenetriaminepentaacetic acid (DTPA), which can chelate available metals while avoiding Ca saturation due to preferential binding of cationic transition metals (Evangelou, 1998; Liang and Schoenau, 1995). While Liang and Schoenau (1995) found a strong correlation between metal uptake of these chelating membranes and Cd, Cr, Pb, Ni, and Zn uptake for lettuce in soil, Conder and Lanno (2000) found only a weak relationship between ion-exchange membrane uptake and toxicity in *E. fetida* exposed to Cd, Pb, and Zn in spiked artificial soils.

The objectives of this research were to: i) assess the ability of chemical immobilization treatments (municipal sewage sludge biosolids and rock phosphate) to reduce metal bioavailability and ecotoxicity in a toxic metal-contaminated smelter soil, and; ii) evaluate soil extraction methods using $Ca(NO_3)_2$ solution or ion-exchange membranes coated with DTPA as surrogate measures of metal bioavailability and ecotoxicity.

Materials and Methods

Soil toxicity testing

The soil used in this experiment was collected from a site contaminated by Zn and Pb milling and smelting operations near the city of Blackwell in north-central Oklahoma. The soil contained 2.63 mmol Cd/kg (296 mg Cd/kg), 2.39 mmol Pb/kg (497 mg Pb/kg), and 195 mmol Zn/kg (12740 mg Zn/kg). Clay content and pH of the study soil were 240 g/kg and 6.4, respectively. Three chemical immobilization amendments were examined: a lime stabilized municipal biosolid (LSB); North Carolina rock phosphate (RP); and an anaerobically digested municipal biosolid (SS). Physical and chemical characteristics of the amendments are previously described (Gradwohl, 1998). Each amendment was thoroughly incorporated into soil (100 g/kg soil) in plastic tubs. All soil treatments were performed in triplicate. Soil moisture was adjusted to field capacity (0.33 bar, ca. 25% water) and the soils were incubated at 27 °C for 90 d. Soil moisture was maintained and the soils were thoroughly mixed at weekly intervals. An uncontaminated Taloka soil (fine mixed thermic Mollic Albaqualt) was used as a reference soil. The Taloka reference soil received the same three chemical immobilization treatments as the metal-contaminated smelter soil.

A 14-d toxicity test using *E. fetida* was conducted according to a standard protocol (ASTM 1997), with noted exceptions, to assess the ability of chemical immobilization treatments to reduce metal bioavailability. For each soil/treatment combination, three 100-g soil replicates were moistened to approximately 80% water holding capacity 24 h prior to the addition of five earthworms per replicate. Mature (clitellate) earthworms weighing approximately 0.2-0.4 g were obtained from in-house cultures and allowed to depurate culture bedding from their gastrointestinal tracts for 24 h before exposure to the soil/treatment combinations. Testing was conducted in environmental chambers maintained at 20 ±1 °C under constant light. Earthworm mortality was monitored on a geometric time scale (e.g., 2, 4, 8, 16, 24, 36, 48 h) for the first 48 h (when most mortality occurred), then daily for the remainder of the test. Earthworms were judged to be dead upon observing no movement after gentle stimulation with a blunt probe.

Metal extraction using ion-exchange membranes

Earthworms and ion-exchange membranes were simultaneously exposed to separate soil/treatment combination replicates under the same conditions as in the earthworm toxicity test. Plant Root Simulator™ (PRS™, Western Ag Innovations Inc., Saskatoon, SK, Canada, www.westernag.ca) anion-exchange membranes were the ion-exchange membranes used. PRSs were converted before use to chelating ion-exchange membranes by complexation of the anionic membrane surface with disodium-DTPA (Liang and Schoenau 1996). Individual PRSs were then buried in 100 g (dry weight) soil replicates, moistened as described above for soil/treatment combinations for earthworm exposure. After a 1-h exposure period, PRSs were removed from soil, rinsed thoroughly with deionized water to remove soil, and eluted for 1 h with 20 ml of 0.5M trace metal-grade HNO_3 in separate, self-sealing plastic bags. The eluent was analyzed for Cd, Pb, and/or Zn using flame and/or graphite furnace atomic absorption (AA) spectroscopy, and the PRSs were regenerated for reuse. Two PRS replicates per soil were exposed alongside the earthworm toxicity tests in the same environmental chamber on the first day of the earthworm exposure periods. After regeneration, the PRSs were exposed again on the seventh day. Quality assurance/quality control (QA/QC) procedures included PRS blanks and spikes, conducted with water-rinsed PRSs during the acid elution step.

Soil analyses

Upon the termination of the toxicity tests and PRS exposures, individual soil/treatment combination replicates were stored at -40 °C in self-sealing plastic bags until physical and chemical parameters could be measured. Soil pH for all earthworm and PRS soil replicates was measured in the supernatant of a settled 0.01M $CaCl_2$ soil slurry (10 g dry weight soil/20 ml solution) according to Hendershot et al. (1993). Organic mater content was measured by wet digestion with chromic acid (Yeomans and Bremner, 1988) followed by colorimetric determination (Heanes, 1984). Total metal concentrations of soils were obtained by wet digestion of 1 g (dry weight) soil using 5 ml concentrated trace metal-grade HNO_3. The digests were then heated to dryness, resolubilized in 15 ml 0.5N trace metal-grade HNO_3, filtered with a hardened, ashless, paper filter (Whatman 540), and brought to a 50-ml volume with 0.5N trace metal-grade HNO_3. To measure weak electrolyte extractable metals, 1 g (dry weight) of each soil replicate from the earthworm tests was combined with 20 ml 0.1M $Ca(NO_3)_2$, mixed in a rotary mixer for 4 h at 23 °C, and centrifuged at 2500 *g* for 15 min. The resulting supernatant was then filtered with a 0.45 μm membrane filter and acidified with 0.5 ml concentrated trace metal-grade HNO_3. Total metal digests and $Ca(NO_3)_2$ extractions were analyzed by AA spectroscopy. QA/QC measures included duplicate analyses, metal spikes, blanks, and analyses of standard reference soil "Sandy Soil B" (CRM-SA-B, Environmental Express, Mt. Pleasant, SC, USA). Total metal analyses of the standard reference soil were within performance acceptance limits determined by USEPA 3050 digestion procedures (USEPA, 1995).

Data analysis

All data were checked for homogeneity of variance and normality and transformed as appropriate to meet requirements for ANOVA (Sokal and Rolhf, 1995). Soils were classified as nonlethal if the 14-d cumulative mortality rate was $\leq 15\%$. Toxicity of the Blackwell smelter soil/treatment combinations was assessed by comparing Arcsine Square Root-transformed percent cumulative mortality (Newman, 1995) at each mortality observation. PRS data were analyzed according to a split-plot (repeated measures) test design (Steele et al., 1997). Data not satisfying assumptions for ANOVA were analyzed nonparametrically using the Kruskal-Wallis test. Fisher's Protected Least Significant Difference (LSD) multiple comparison procedure was also employed to further elucidate differences between means ($\alpha = 0.05$).

Results and Discussion

Remediation effectiveness

Results of physicochemical analyses of the remediated soils toxicity test are presented in Table 4. Blackwell smelter soil is contaminated predominantly with Zn, although Cd and Pb levels are elevated compared to Taloka soil. The addition of remediation amendments did not change total metal levels for Cd, Pb, and Zn in Blackwell soil; the remediation goal was not to remove metals from the soil, but immobilize available metal species via changes in soil physicochemical properties. The metals present in the remediation amendments (Gradwohl, 1998) elevated total Cd, Pb, and/or Zn levels in Taloka soil. The remediation treatments significantly increased organic matter (SS and LSB) and pH (LSB).

Municipal sewage sludge biosolids amended with lime (LSB) significantly reduced the toxicity of the Blackwell smelter soil to the earthworms. All Blackwell smelter soil treatment/combinations were lethal to *E. fetida* except for LSB (Figure 4). Applying Fisher's Protected LSD tests to transformed mean cumulative mortality at 16, 24, 48, and 72 h, we concluded that the SS treatment was the most toxic Blackwell soil/treatment combination, followed by NONE and RP. The relative toxicity of the soil/treatment combinations to earthworms was very similar compared to that of lettuce (Gradwohl, 1998), although earthworms were more sensitive since lettuce was able to survive in the Blackwell RP soil/treatment combination. Soil pH appeared to be more important than organic matter in immobilizing bioavailable metals, since LSB reduced toxicity while SS seemed to increase toxicity. Since Fang and Wong (1999) found that the addition of lime to sewage sludge biosolids reduced DTPA- and water-extractable metals in the sewage sludge biosolids, it follows that LSB-amended soils would also have reduced metal availability compared to SS-amended soils (Basta and Gradwohl, 2000; Sloan and Basta, 1995). The RP amendment did reduce toxicity slightly, but not as effectively as LSB. The main component of RP is carbonated fluorapatite, a mineral that is very effective in immobilizing available Pb, not Zn (Ma et al., 1993). Unfortunately, in soil acidification experiments designed to mimic aging of the remediated soils, the ability of LSB to immobilize available metals was not sustained, with only RP maintaining its capacity for metal immobilization with time and decreased pH (Gradwohl, 1998). Although RP did not reduce the lethality of the Blackwell soil to earthworms, it was least toxic among the lethal soil/treatment combinations and may be useful in reducing long-term bioavailability of metals in some site-contaminated soils, especially where Pb is of concern. There was no lethality associated with the three remediation treatments themselves since no mortality rates >15% were observed in the Taloka soil/treatment combinations.

$Ca(NO_3)_2$-extractions as surrogate measures of metal bioavailability

Metal availability as determined by the $Ca(NO_3)_2$ extraction was significantly different for Cd, Pb, and Zn among the Blackwell soil/treatment combinations and depicted very large differences between the lethal Blackwell soil/treatment combinations and the nonlethal Blackwell LSB and Taloka combinations (Table 4). Zinc had the highest availability of all three metals, on a molar basis, with $Ca(NO_3)_2$-extractable levels more than 10-fold greater than Cd and Pb in the lethal Blackwell soil/treatment combinations. Since $Ca(NO_3)_2$-extractable metals were found to be well related to earthworm toxicity in metal-spiked artificial soil (Conder and Lanno, 2000), we compared $Ca(NO_3)_2$-extractable metal levels in the Blackwell soil/treatment combinations to the incipient lethal levels (time-independent LC_{50}s, ILLs) based on $Ca(NO_3)_2$-extractable metals in the artificial soil toxicity tests. $Ca(NO_3)_2$-extractable ILLs (±95% CI) for Cd, Pb, and Zn were 9.8 (9.4-10.3), 1.16 (1.11-1.22), and 6.33 (6.17-6.49) mmol $Ca(NO_3)_2$-extractable metal/kg soil, respectively (Conder and Lanno, 2000). $Ca(NO_3)_2$-extractable Zn levels in the lethal Blackwell soil/treatment combinations were 2-3 times higher than the ILL for $Ca(NO_3)_2$-extractable Zn in artificial soil while $Ca(NO_3)_2$-extractable Cd and Pb were present at only 1% of ILLs. Thus, Zn was the most likely source of toxicity. The two lowest lethal exposure levels in the artificial soil Zn test (13.3 and 20.3 mmol $Ca(NO_3)_2$-extractable Zn/kg soil) (Conder and Lanno, 2000) were very similar to the $Ca(NO_3)_2$-extractable Zn levels found in the lethal Blackwell soil/treatment combinations. Time at which 100% cumulative mortality was reached was also very similar (36-120 h for lethal field soils, 72-144 h for Zn-spiked artificial soils (Conder and Lanno, 2000)). However, $Ca(NO_3)_2$-extractable Zn levels in the nonlethal Blackwell LSB combination were far below the ILL for $Ca(NO_3)_2$-extractable Zn in artificial soil and the $Ca(NO_3)_2$-extractable Zn levels in the Blackwell soil/treatment combinations which were lethal to the earthworms. Compared with Blackwell soil receiving no treatment (NONE), $Ca(NO_3)_2$-extractable Zn in the LSB treatment decreased by a factor of 25, Cd by 10, and Pb by 7. In the other Blackwell soil/treatment combinations, SS and RP, $Ca(NO_3)_2$-extractable Cd and Pb decreased by approximately half, while $Ca(NO_3)_2$-extractable Zn remained the same in SS, and decreased slightly in

RP. The large differences in the $Ca(NO_3)_2$-extractable metal concentrations of the lethal and nonlethal soils, as well as the similarity in lethal $Ca(NO_3)_2$-extractable Zn levels of the smelter soil and Zn-spiked artificial soil suggests the possibility of developing universal, soil-independent ILLs based on $Ca(NO_3)_2$-extractable metal levels. Further testing with a wide range of $Ca(NO_3)_2$-extractable metal levels in metal-contaminated field soils is necessary to validate this concept.

PRSs as surrogate measures of metal bioavailability

Cadmium, Pb, and Zn uptake by PRSs exposed in the Blackwell and Taloka soil/treatment combinations is shown in Figure 5. The mean CV (±95% CI) for PRS Zn uptake was 39% (22-56) compared to 1.9% (0.5-3.2) for $Ca(NO_3)_2$-extractable Zn, suggesting that Zn availability determined by PRSs was not as precise as $Ca(NO_3)_2$-extraction determinations. Due to this reduced precision, PRS measurements were not able to discriminate soil/treatment combinations lethal to earthworms, supporting previous results of PRS deployment in Cd, Pb, and Zn-spiked artificial soils (Conder and Lanno, 2000). However, PRSs did indicate high levels of available Zn in all of the Blackwell soil/remediation treatments, despite the very distinct differences in toxicity of these combinations to earthworms. The PRS-chelate, DTPA, may not be suitable for estimating high levels of available metals in soils (Conder and Lanno, 2000). The use of the conventional DTPA soil extraction to measure high levels of available heavy metals may not even be appropriate (O'Connor, 1988), since it was designed for soil fertility measurements in soils deficient in Zn, iron (Fe), manganese (Mn), or copper (Cu). Differences in soil pH may alter the chelating tendencies for individual metals, and when used for metals other than Zn, Fe, Mn, and Cu, the DTPA extraction tends to overestimate plant available metals (O'Connor, 1988). Its application is also discouraged for estimating plant available heavy metals in sludge or sludge-amended soils (Hooda and Alloway, 1993; O'Connor, 1988). Regardless, PRSs were able to indicate large differences (two orders of magnitude) in metal bioavailability between the Taloka reference and Blackwell smelter soils and may still serve as an *in situ* screening tool for detecting large differences in metal availability.

Conclusions

The earthworm bioassay was able to assess reductions in metal bioavailability and evaluate chemical immobilization treatment effectiveness. Using this approach, we concluded that LSB is an effective treatment for reducing metal bioavailability to earthworms in contaminated soil. Because treatment effectiveness of LSB in reducing metal bioavailability is lost upon soil acidification due to soil weathering (Gradwohl, 1998), RP treatment may be necessary to keep bioavailable metals from returning to pre-treatment levels of bioavailability and toxicity.

$Ca(NO_3)_2$-extractable metals appear to be a very promising surrogate measure of metal bioavailability for earthworms in soil. The large differences in the $Ca(NO_3)_2$-extractable metal concentrations of the lethal and nonlethal soils, as well as the similarity in lethal $Ca(NO_3)_2$-extractable Zn levels of the smelter soil and Zn-spiked artificial soil (Conder and Lanno, 2000) indicates the possibility of developing universal, soil-independent ILLs based on $Ca(NO_3)_2$-extractable metal levels. Testing with site soils contaminated with metals other than predominantly Zn is needed to further validate the use of weak-electrolyte extractions as surrogate measures of metal bioavailability in soil. Correlation with metal residues in soil organisms (underway for earthworms used in this study) is also necessary.

Although $Ca(NO_3)_2$ extractions are easier to perform and better related to earthworm toxicity in soil, PRSs may be useful as an *in situ* screening tool, and may avoid possible physicochemical alterations of soil involved in weak electrolyte sampling techniques.

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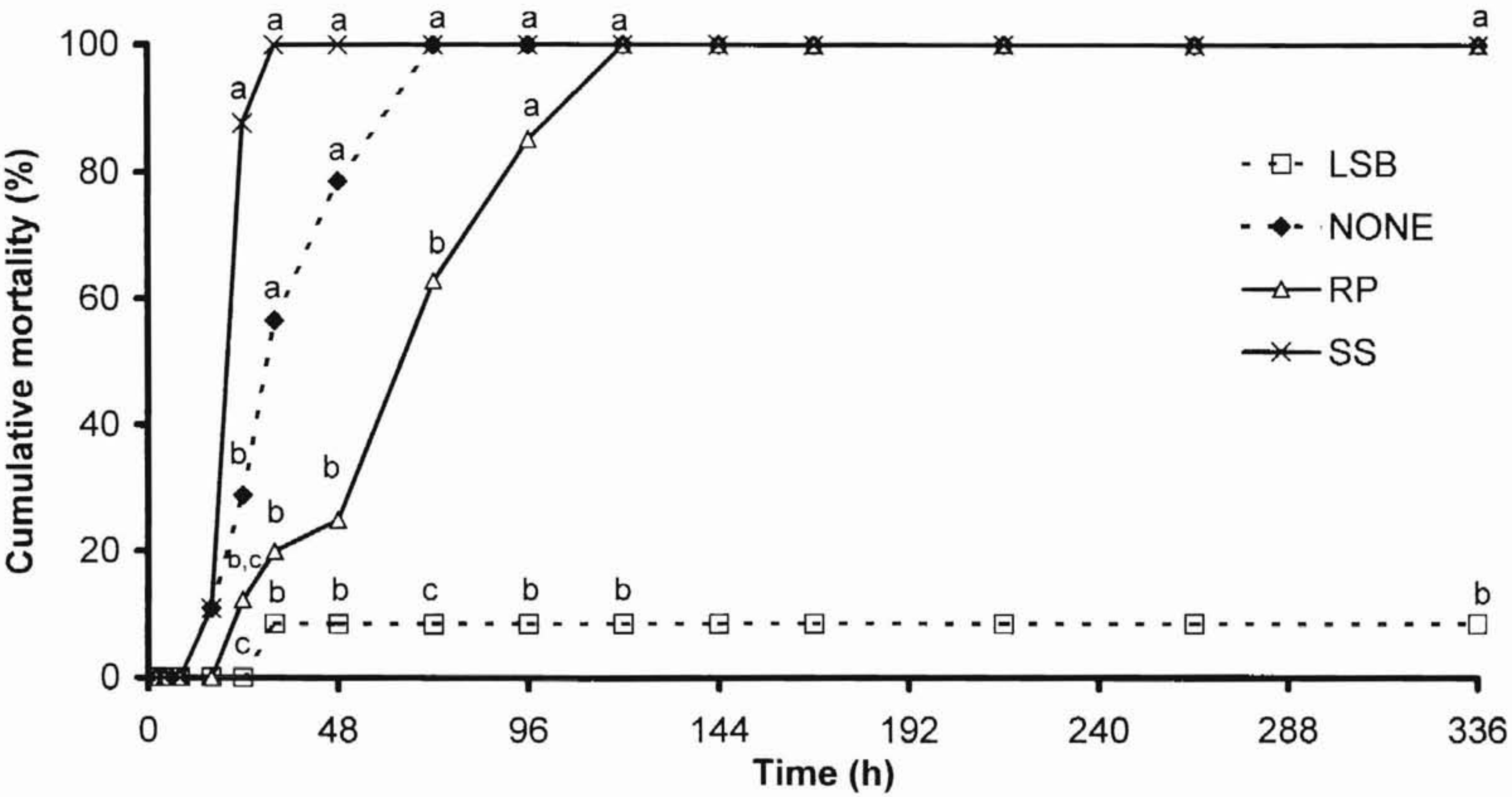

Figure 4. Mean percent cumulative mortality (n = 3) of *Eisenia fetida* exposed to Blackwell smelter soil receiving no treatment (NONE) or amendments of municipal sewage sludge biosolids (SS), municipal sewage sludge biosolids amended with lime (LSB), or rock phosphate (RP). Data points with the same letter at the same mortality observation time are not significantly different (Fisher's Protected LSD, $P > 0.05$). No mortality was observed in the Taloka reference soil (not shown).

Table 4. Total and $Ca(NO_3)_2$-extractable metal concentrations and pH of soil/remediation treatment combinations (mean, n = 3, 95% CI). Within each soil type, means in columns with the same superscript were not significantly different ($P > 0.05$, Fisher's protected LSD).

	Cd			Pb			Zn				
Soil Remediation	**Total (mmol/kg)**	**$Ca(NO_3)_2$-extractable (mmol/kg)**	**%[†]**	**Total (mmol/kg)**	**$Ca(NO_3)_2$-extractable (mmol/kg)**	**%[†]**	**Total (mmol/kg)**	**$Ca(NO_3)_2$-extractable (mmol/kg)**	**%[†]**	**pH**	**Organic matter (g C/kg)**
Blackwell smelter soil											
NONE	3.02^{a}	1.39^{a}	46.1	2.62^{a}	0.007^{a}	0.3	229^{a}	15.8^{a}	6.9	6.24^{b,c}	14.6^{c}
	(2.95-3.09)	(1.29-1.51)		(2.54-2.71)	(0.004-0.13)		(191-266)	(11.6-21.5)		(6.04-6.44)	(13.8-15.5)
LSB	3.043^{a}	0.121^{c}	4.0	2.57^{a}	0.001^{c}	0.0	210^{a}	0.620^{c}	0.3	7.25^{a}	25.0^{b}
	(2.54-3.56)	(0.107-0.135)		(1.97-3.17)	(0.001-0.002)		(187-233)	(0.490-0.786)		(6.90-7.60)	(22.7-27.5)
RP	2.93^{a}	0.601^{b}	20.5	2.50^{a}	0.003^{b}	0.1	218^{a}	11.5^{b}	5.3	6.32^{b}	14.5^{c}
	(2.29-3.58)	(0.566-0.639)		(2.32-2.68)	(0.002-0.004)		(209-228)	(10.4-12.7)		(6.27-6.38)	(14.2-14.9)
SS	3.23^{a}	0.632^{b}	19.6	2.69^{a}	0.002^{b}	0.1	217^{a}	18.2^{a}	8.4	6.14^{c}	31.3^{a}
	(2.54-3.93)	(0.576-0.693)		(2.58-2.80)	(0.001-0.004)		(194-241)	(16.3-20.4)		(6.09-6.19)	(28.3-34.8)
Taloka reference soil											
NONE	BDL[§]	BDL[¶]	-	0.050^{b}	BDL[¶]	-	0.457^{c}	BDL[¶]	-	7.15^{b}	7.5^{d}
				(0.027-0.073)			(0.446-0.468)			(6.95-7.36)	(6.8-8.3)
LSB	0.038^{b}	BDL[¶]	-	0.096^{b}	BDL[¶]	-	2.74^{a}	0.007^{b}	0.3	7.61^{a}	18.1^{b}
	(0.034-0.042)			(0.070-0.121)			(2.49-3.05)	(0.001-0.012)		(7.24-7.99)	(16.1-20.3)
RP	0.050^{a}	BDL[¶]	-	0.050^{b}	BDL[¶]	-	1.29^{b}	0.003^{b}	0.2	7.06^{b,c}	10.4^{c}
	(0.039-0.060)			(0.005-0.096)			(1.15-1.47)	(-0.004-0.009)		(6.58-7.54)	(9.5-11.5)
SS	0.049^{a}	BDL[¶]	-	0.209^{a}	BDL[¶]	-	2.63^{a}	0.026^{a}	1.0	6.85^{c}	29.7^{a}
	(0.025-0.073)			(0.055-0.364)			(1.84-4.60)	(0.014-0.038)		(6.42-7.28)	(25.8-34.2)

[†] Percent of total metal that was $Ca(NO_3)_2$-extractable

[§] Below method detection limit for total Cd analysis of 0.012 mmol/kg soil

[¶] Below method detection limit for $Ca(NO_3)_2$-extractable Cd, Pb, or Zn analysis of 0.005, 0.0002, or 0.002 mmol/kg soil, respectively

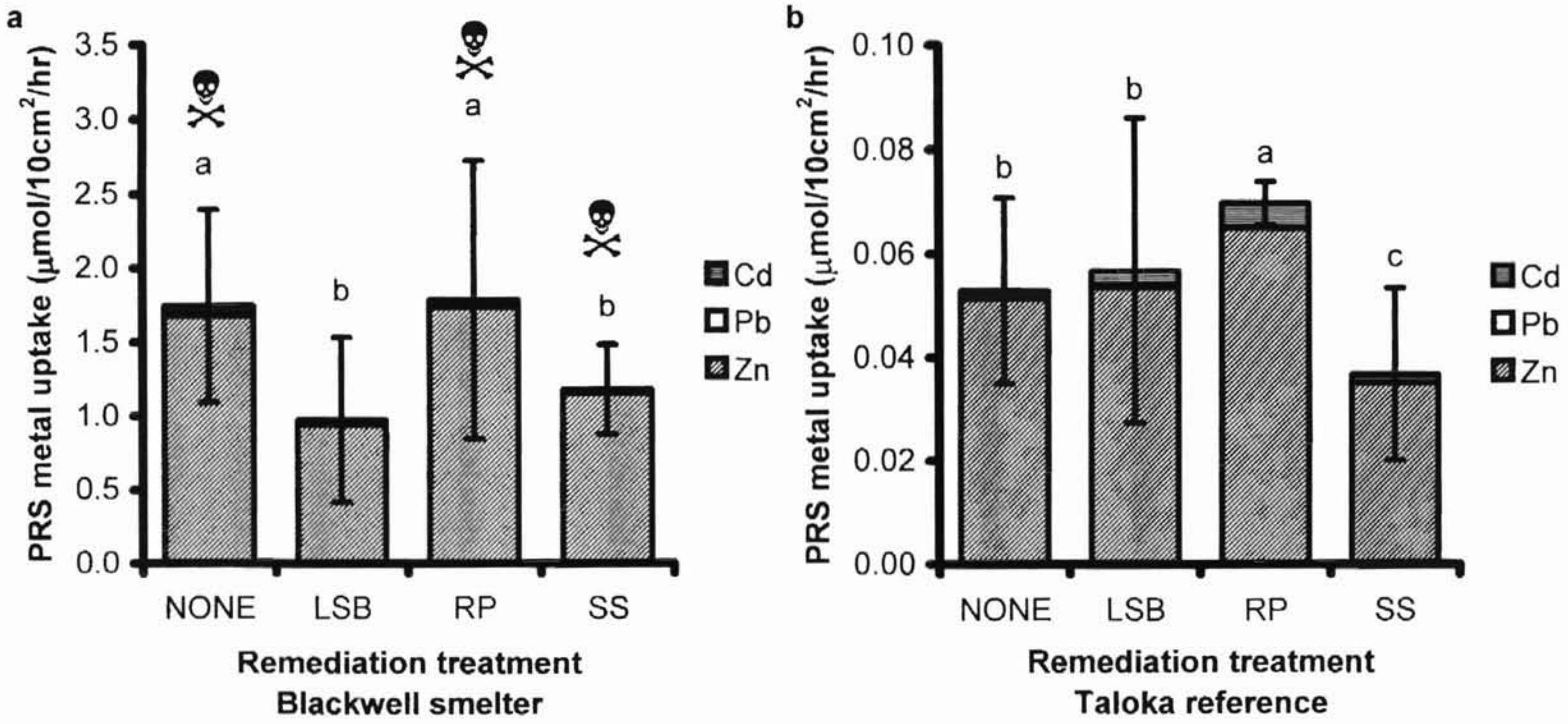

Figure 5. Plant Root Simulator^{TM} (PRS) chelating ion-exchange membrane uptake of Cd, Pb, and Zn in Blackwell smelter (**a**) and Taloka reference (**b**) soils receiving no treatment (NONE) or amendments of municipal sewage sludge biosolids (SS), municipal sewage sludge biosolids amended with lime (LSB), or rock phosphate (RP). Columns (mean, n = 4, ± 95% CI) with the same letter are not significantly different (Fisher's Protected LSD, $P > 0.05$). Soil/treatment combinations were used in a toxicity test with *Eisenia fetida*; symbols (☠) indicate lethal soil/treatment combinations.

CHAPTER III

CRITICAL BODY RESIDUES AS MEASURES OF CD, PB, AND ZN BIOAVAILABILITY AND TOXICITY IN THE EARTHWORM *EISENIA FETIDA*

Abstract

Earthworm heavy metal concentrations may be the most relevant measures of heavy metal bioavailability in soils and may be linkable to toxic effects in order to better assess soil ecotoxicity. Lethal critical body residues (CBRs) for Cd, Pb, and Zn were developed for the earthworm *Eisenia fetida* exposed to spiked artificial soils. Lethal CBRs inducing 50% mortality ($LCBR_{50}$, ±95% CI) were calculated to be 5.72 (3.54-7.91), 3.33 (2.97-3.69), and 8.19 (4.78-11.6) mmol/kg for Cd, Pb, and Zn, respectively. Zn concentrations of dead earthworms exposed to a lethal remediated Zn-smelter soil were 3-fold above the $LCBR_{50}$ for Zn and comparable to earthworm concentrations in lethal Zn-spiked artificial soils, despite a 14-fold difference in total soil Zn concentration between the lethal field and artificial soils. An evaluation of the acute mixture toxicity of Cd, Pb, and Zn in artificial soils using the Toxic Unit (TU) approach revealed an $LCBR_{50}$ of 0.99 (0.57-1.41) TU, indicating additive toxicity. Fractionation of earthworm tissues into cytosolic and pellet digests yielded mixed results for detecting differences in exposure at the sublethal level. CBRs are useful in describing acute Cd, Pb, and Zn toxicity in earthworms, but linking sublethal exposure to total and/or fractionated residues may be more problematic.

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Hopkin 1999a). Metallothionein-bound Cd is slowly accumulated in the chloragogenous tissue where the protein is degraded, leaving inert, granulated Cd which is then slowly excreted (Dallinger 1993, Morgan and Morris 1982, Morgan et al. 1989, 1993). The invertebrate detoxification strategies of Pb and Zn are not as well studied as that of Cd. It is thought that incoming Zn is bound temporarily by small proteins until quickly being bound in the matrix of pyrophosphate granules in the chloragogenous tissue (Morgan and Morgan 1989b). Detoxified Pb also is bound within these same granules, although it is unknown whether there are specific biochemical systems which temporarily detoxify Pb (Dallinger 1993, Ireland 1975, Ireland and Richards 1977, Morgan 1985, Morgan and Morgan 1989a, Morgan and Morris 1982, Morgan et al. 1989, 1993).

Numerous attempts have been made to isolate toxicologically available Cd from detoxified, metallothionein-bound Cd using relatively simple tissue fractionation methods (Conder et al. submitted-b, Honeycutt et al. 1995, Jenkins and Mason 1988). Although these methods are not as accurate or precise as electron microscopy or protein chromatography at the identification of detoxified metal species in tissues, they may provide a simpler, cost-effective method to roughly quantify pools of detoxified/toxicologically active metals in organisms. The method developed by Conder et al. (submitted-b) is tailored for metal-exposed, undepurated earthworms exposed in a soil matrix, and was successful in describing the accumulation of detoxified and toxicologically active Cd during a Cd bioaccumulation test in artificial soil. The hypothesized fractionation of toxicologically unavailable/available metals among the methodological digest fractions is shown in Figure 6.

The objectives of this research were to: i) develop $LCBR_{50}$s (total earthworm toxicant concentration associated with 50% mortality) for Cd, Pb, and Zn; ii) evaluate the $LCBR_{50}$ for Zn in a lethally toxic Zn-smelter soil; iii) evaluate the lethal mixture toxicity of Cd, Pb, and Zn using earthworm metal concentrations and the toxic unit (TU) approach (Sprague 1970); and iv) evaluate total and fractionated earthworm concentrations as indicators of sublethal exposure.

Materials and Methods

Earthworm and soil analyses

Earthworms (*E. fetida*) analyzed in this study were obtained from 5 previous toxicity tests in metal-spiked artificial soil (Conder and Lanno 2000), and unremediated and remediated Zn-smelter and reference soils (Conder et al. submitted-a). Tests were conducted for 14 d in environmental chambers maintained at 20 ±1°C under constant light. Artificial soil tests consisted of three single-metal tests in artificial soil spiked with aliquots of $CdSO_4$, $Pb(NO_3)_2$, or $ZnSO_4$, followed by a Cd-Pb-Zn mixture test spiked with Cd, Pb, and Zn nitrate salts to achieve a geometrically increasing exposure series based on TU calculated from toxicity data obtained from the single-metal tests (Conder and Lanno 2000). Two field soils were tested: a Zn-contaminated soil obtained from a Zn smelter in Blackwell, Oklahoma, USA and a reference soil obtained from an agricultural site near Taloka, Oklahoma, USA. The Blackwell soil was heavily contaminated with Zn (229 mmol/kg) and mildly contaminated with Cd (3 mmol/kg) and Pb (3 mmol/kg). Three remediation materials (chemical immobilization amendments) were added at a rate of 100 g/kg to both soils: a lime stabilized municipal sewage sludge biosolid (LSB), North Carolina rock phosphate (RP), and an anaerobically digested municipal sewage sludge biosolid (SS). See Conder et al. (submitted-a) for more information on the amendment process.

Live earthworms were collected at the at the termination of each test. Dead earthworms were collected upon discovery during mortality observations, which were scheduled according to a geometric time scale (e.g., 2, 4, 8, 16, 24, 36, 48 h) for the first 48 h (when most mortality occurred), then daily for the remainder of the test. Prior to analysis, earthworms were removed from the soil, rinsed, and stored at –20°C. Individual soil replicates from both experiments were stored at -40°C in self-sealing plastic bags until analysis. Earthworms were analyzed for cytosolic and pellet fraction Cd, Pb, Zn, total Cd, Pb, and Zn, and ingested soil (% soil / unpurged body weight) using the methods of Conder et al. (submitted-b). Briefly, earthworms were thawed and individually homogenized using an Omni TH115 tissue homogenizer fitted with a 7-mm saw-tooth blade (Omni International Inc., Warrenton, VA) in 5 ml ice-cold 0.01M Tris-HCl buffer (pH 7.5, Fisher Scientific, Houston, TX). Homogenates were then centrifuged at 10000 *g* for 30 min at 5°C. Homogenate supernatants (cytosolic fractions) were then decanted into separate preweighed 10-ml beakers. The remaining pellet fractions were resuspended in 5 ml 0.01M Tris-HCL and poured into separate preweighed beakers. Beakers containing the earthworm cytosolic/pellet fractions were then dried at 105°C for 24 h and weighed. Fractions were then wet digested with 2 ml concentrated trace metal-grade HNO_3. Digests were heated slowly to dryness and resolubilized in 3 ml 0.5N trace metal-grade HNO_3. Cytosolic fraction digests were then brought to 10 ml with 0.5N trace metal-grade HNO_3. Pellet fraction digests were filtered with hardened, ashless filter paper (Whatman 540) and brought to a 10-ml volume with 0.5N trace metal-grade HNO_3. Filter papers were then ashed in preweighed crucibles at 450°C for 12-18 h and weighed to determine pellet fraction AIA weight. Cytosolic and pellet fraction digests were analyzed for Cd, Pb, or Zn using flame atomic absorption spectroscopy. Method detection limits for earthworm tissue concentrations for Cd, Pb, and Zn were 0.09, 0.002, and 0.035 mmol/kg, respectively. Quality assurance/quality control (QA/QC) procedures included analysis of procedural blanks, spikes, and certified reference material (lobster hepatopancreas, TORT-2, National Research Council, Canada). All soil data presented were previously reported in Conder and Lanno (2000) and Conder et al. (submitted-a). Acid insoluble ash (AIA) content of soils (needed for earthworm tissue analysis) was obtained during total metals digestion (Conder and Lanno 2000, Conder et al. submitted-a) using methods given in Conder et al. (submitted-b).

Data analysis

Metal residues were investigated in earthworms obtained from 4 of the 6 exposure levels in each artificial soil test: the control (unspiked) level, a relatively low level corresponding to approximately 5-10% of $Ca(NO_3)_2$-based LC_{50}s, the highest nonlethal exposure level, and the lowest lethal exposure level (dead earthworms). For field soils, earthworm residues were investigated in the unspiked artificial soil (negative contol), unremediated Taloka (control), Taloka SS (remediated control), Blackwell LSB (only nonlethal Blackwell soil/treatment combination), and Blackwell RP (lowest $Ca(NO_3)_2$-extractable Zn of the lethal Blackwell soil/treatment combinations, longest time to reach 100% earthworm mortality). The time periods at which dead earthworms selected for analysis were removed from the lethal soils were very similar (32-120 h), with the exception of the Pb and Cd-Pb-Zn mixture tests (removal at the 18 h mortality observation), which had very rapid toxicity kinetics (Conder and Lanno 2000).

Two to three earthworms were selected from each soil replicate (3 replicates per soil) for the soils studied. Differences in total, cytosolic, and pellet Cd, Pb, and Zn concentrations, and ingested soil were investigated using a nested, one-way ANOVA. Data not satisfying assumptions for ANOVA were analyzed nonparametrically using the Kruskal-Wallis test on the means of each soil replicate's earthworm data. Fisher's Protected Least Significant Difference (LSD) multiple comparison procedure was also employed to further elucidate differences between means ($\alpha = 0.05$). Means and 95% CIs were calculated using the means of each soil replicate's earthworm data, not each earthworm datum (subsample). LCBR_{50}s were estimated using the Trimmed Spearman-Karber method (Hamilton et al. 1977) and the total metal concentrations of earthworms exposed to the highest nonlethal and lowest lethal exposure levels in the artificial soil tests. Mixture toxicity was evaluated by calculating a TU-based LCBR_{50} using the means of total earthworm Cd, Pb, and Zn concentrations based in TU (Equation 1):

$$\text{Earthworm TU} = \frac{[\text{Earthworm Cd}]}{\text{Cd LCBR}_{50}} + \frac{[\text{Earthworm Pb}]}{\text{Pb LCBR}_{50}} + \frac{[\text{Earthworm Zn}]}{\text{Zn LCBR}_{50}} \qquad (1)$$

Results and Discussion

Total earthworm concentrations as measures of acute toxicity

Total earthworm Cd, Pb, and Zn concentrations of live and dead animals exposed to artificial soils spiked with Cd, Pb, and Zn individually are shown in Figure 7. In all three toxicity tests, the total concentration in dead earthworms was significantly higher than all live earthworms. Using the concentrations of dead earthworms and live earthworms exposed to the next highest exposure level, $LCBR_{50}$s (±95% CI) of 5.72 (3.54-7.91), 3.33 (2.97-3.69), and 8.19 (4.78-11.6) mmol/kg were calculated for Cd, Pb, and Zn, respectively. In contrast to other measures of toxicity such as total metal- or $Ca(NO_3)_2$-extractable metal-based LC_{50}s, total earthworm $LCBR_{50}$s suggest that the order of toxicity among these 3 metals to *E. fetida* is Pb>Cd>Zn. Although there are no previously measured earthworm Cd and Pb concentrations associated with lethality, the $LCBR_{50}$ for Zn is very similar to the earthworm Zn concentration (approximately 8.6 mmol/kg) hypothesized to be the upper threshold limit for Zn regulation in *E. andrei* (van Gestel et al. 1993).

Total Cd, Pb, and Zn concentrations in dead earthworms exposed to artificial soils spiked with the Cd-Pb-Zn mixture (Fig. 8) were 80, 19, and 57% of $LCBR_{50}$s for each metal, respectively, indicating that lethality was probably not due to any single metal. For Cd and Zn, uptake by dead earthworms was significantly greater than live earthworms. In contrast, Pb uptake in dead earthworms was not different from live earthworms exposed to spiked soils. Using Equation 1, earthworm TU (Fig. 9) were calculated using single metal $LCBR_{50}$s and earthworm Cd, Pb, and Zn concentrations in the highest sublethal and lowest lethal exposure levels. Pb TU made up a smaller proportion of the total earthworm TU of dead earthworms, but Cd, Pb, and Zn TU in the live earthworms exposed to spiked soils were more equally proportionate. Uptake kinetics for Cd and Zn may have been slightly faster than that of Pb in the lethal mixture soil during the short (< 24 h) exposure. The TU-based $LCBR_{50}$ was 0.99 (0.57-1.41) TU, indicating additive toxicity. This further confirms previous findings based on soil total and $Ca(NO_3)_2$-extractable metal concentrations to assess Cd, Pb, and Zn acute mixture toxicity (Conder and Lanno 2000).

Total Zn uptake in earthworms exposed to the Blackwell RP soil/treatment combination was significantly greater than that of live earthworms exposed to other soil/treatment combinations (Fig. 10c). Zn concentrations of dead earthworms were 3-fold above the $LCBR_{50}$ for Zn, but only 1.5 times that of dead earthworms exposed to the lowest lethal exposure level in the Zn-spiked artificial soil test, despite a 14-fold difference in total soil Zn concentrations. Total earthworm Cd and Pb (Fig. 10a, b) in dead earthworms were only present at 28 and 1%, respectively, of the individual Cd and Pb single-metal $LCBR_{50}$s, suggesting that acute toxicity was almost entirely due to Zn.

It is notable that in the lethal Cd- and Zn-spiked artificial soils, soil ingestion was extremely low (Fig 11a, c). With the possible exception of the earthworms in the Pb test, all lethally-exposed earthworms had reduced soil ingestion rates compared to controls (Fig 11b, d, e). Acute metal toxicity appears to suppress feeding, probably though the simple reduction in burrowing activity which occurs before death. Thus, the spiked artificial soil-derived $LCBR_{50}$s reported above do not integrate the intestinal exposure route, and are products of dermal metal uptake in dead earthworms and dermal and/or intestinal uptake in live earthworms at the next highest exposure level. Regardless of this discrepancy, the Zn $LCBR_{50}$ seems applicable to toxicity in the Zn-smelter soil/treatment combinations, despite a slightly higher soil ingestion rate in dead earthworms from the Blackwell RP soil/treatment combination compared to dead earthworms from the Zn-spiked artificial soil (t-test, $P = 0.0112$).

Total and fractionated residues as measures of sublethal exposure

It was surprising that there was a significant difference in total metal uptake between the low and high sublethal exposures in the Zn test, and between control/reference soils and Blackwell LSB in the remediated soils test, for Zn is extremely well-regulated, often displaying little difference in total body concentration between large presumed differences in exposure (Posthuma and Baerselman 1998, Spurgeon and Hopkin 1996). However, Spurgeon and Hopkin (1999b) also observed differences in earthworm Zn uptake between low and high sublethal exposure levels. It is only when the mechanisms which detoxify and excrete potentially toxic Zn cannot compete with uptake that total body residues are expected to rise (Marinussen 1997b, van Gestel et al. 1993). If this exposure threshold for net Zn accumulation lies below exposure levels causing sublethal effects in earthworms, then total body Zn concentrations may be useful in assessing sublethal risk.

There was no clear relationship between assumed differences in Zn exposure and Zn concentration in either the cytosolic or pellet fraction. Instead, changes in either fraction may be a signal of increased sublethal exposure. In the Zn test, exposures in the low sublethal and control levels were below the threshold for net Zn accumulation, for there was no difference in total earthworm Zn concentrations. Cytosolic Zn, however, was slightly higher in the low sublethal exposure while pellet Zn remained comparable to that of the controls (Fig. 12a). However, there was no difference in cytosolic Zn between the low and high sublethal exposures in the Zn-spiked artificial soil. Assuming the Zn detoxification mechanisms were fully functional, excess cytosolic Zn in the earthworms exposed to the low sublethal exposure should have been quickly converted to inert granule storage sites, which would be reflected in an increase in pellet Zn. This was not seen at the low sublethal exposure level. Cytosolic Zn, but not pellet Zn, was also indicative of higher Zn exposure in the Blackwell LSB soil/treatment combination in the remediated soils test (Fig. 12c). In contrast, there was no difference in cytosolic or pellet Zn among the live earthworms exposed to the mixture test (Fig. 12b). We do not believe that the lack of difference among total, cytosolic, or pellet Zn was due to uptake or storage antagonism caused by simultaneous exposure to Cd and Pb, as observed by Weltje (1998) and Morgan and Morris (1982). Earthworms were able to maintain total Zn concentrations at approximately 2 mmol/kg in the Cd (Fig. 13a) and Pb (Fig. 13b) tests. This level is very comparable to the regulated Zn levels of approximately 1.5 and 1.5-2.0 mmol/kg in *E. andrei* observed by Janssen et al. (1997) and Posthuma and Baerselman (1998), respectively. There was a slight decrease and increase, respectively, in cytosolic and pellet Zn in dead earthworms obtained from the Cd and Pb tests. Since the tissue fractionation method used separates small organelles, proteins, and other low density cytosolic components from the remaining tissues, it is difficult to comment on this finding considering the physiological and chemical changes (including slight decomposition) which occur in post mortem tissue before collection. Thus, this change in Zn distribution may not be due to a toxicological effect. Zn exposure to earthworms from the mixture test may have been too low to cause the changes in the distribution of cytosolic and pellet Zn observed in other exposure levels too low to cause net Zn accumulation. Thus, changes in cytosolic and pellet Zn may indicate a subtle physiological condition intermediate between total regulation via efficient excretion and the total loss of Zn regulation which results in net accumulation.

In both the Cd and mixture tests, there were no significant differences in total Cd uptake between the low and high exposures. Assuming there were differences in bioavailability (dose) between the low and high sublethal exposure levels, this finding is exactly opposite of what would be expected. Cd has been found to accumulate in earthworms, with little, if any, excretion during or after exposure (Neuhauser 1995, Reinecke et al. 1999, Spurgeon and Hopkin 1999a). The lack of a significant difference between these exposure levels may be explained by an examination of soil ingestion in the earthworms. In both the Cd and mixture tests, soil ingestion of earthworms exposed to the high sublethal exposure levels was significantly lower than earthworms exposed to the low sublethal exposure levels. Earthworms in the higher exposure levels reduced their activity levels and stopped ingesting soil, enabling them to effectively block the intestinal route of exposure. Thus, no differences were observed between total Cd burdens obtained via dermal-only exposure and Cd burdens obtained via dermal and intestinal exposure in earthworms from a lower exposure level.

As found by Conder et al. (submitted-b), most of the Cd in earthworms exposed to the spiked artificial (Fig. 14a, b) and remediated field soils (Fig. 14c) was located in the cytosolic fraction, indicating the presence of metallothionein-bound Cd. Since it is thought that excretion of detoxified Cd in invertebrates occurs only after metallothionein-bound Cd is converted to inert cadmosomes (Dallinger 1993, Morgan and Morris 1982, Morgan et al. 1989, 1993), which would appear in the pellet fraction, it was surprising that neither cytosolic nor pellet Cd concentrations differed between low and high sublethal exposure levels in the artificial soil tests. As with the lack of differences in total earthworm Cd between these exposure levels, this can be best explained by the absence of soil ingestion by the earthworms exposed to the higher metal levels in the Cd and mixture tests.

There were no differences in total earthworm Pb in earthworms exposed to the low and high sublethal exposure levels in the Pb test or mixture tests, or among the remediated soils. Only trace amounts of Pb were found in the earthworms exposed to the field soils, therefore cytosolic and pellet Pb concentrations were of little additional use in assessing the apparent lack of difference in Pb exposure (Fig. 15c). An investigation of Pb distribution within the earthworms exposed to the artificial soils yielded no differences among exposure levels. Most Pb was located in the pellet fraction of the earthworm digests, as was expected, but there were no differences between low and high sublethal exposures in the artificial soils (Fig. 15a, b), except for a slightly higher pellet Pb concentration in the high sublethal exposure in the mixture test. Assuming differences in exposure between the Pb and mixture tests in artificial soil, an increase in pellet Pb was expected, as incoming toxic Pb was bound in inert granules which would be located in the pellet fraction. There were also no differences in soil ingestion, as seen for Cd, so we assumed that all earthworms were exposed to Pb at similar rates via both dermal and intestinal exposure routes. Assuming true differences in exposure in the Pb-spiked artificial soils, this is very puzzling. Unfortunately, the understanding of accumulation and excretion of Pb in earthworms is very coarse. Pb may be able to be regulated in earthworms, for Marinussen et al. (1997b) and Neuhauser (1995) found rapid initial excretion kinetics for Pb in post exposure earthworms *Dendrobaena veneta* and *Allolobophora tuberculata*, respectively. Neuhauser (1995) even observed a decrease in Pb concentrations during exposure. In contrast, Spurgeon and Hopkin (1999a) observed no post-exposure Pb elimination in *E. fetida*. It is quite possible, due to the unavailability of Pb, even in Pb-salt-spiked artificial soil (Conder and Lanno 2000), that the assumption of a biologically significant difference in exposure (bioavailability) between the low and high sublethal exposure levels in these tests is invalid. Although huge differences in total soil Pb were seen, differences in soil $Ca(NO_3)_2$-extractable Pb concentrations, although significant, were on a much smaller scale (Conder and Lanno 2000). True biological exposure (relating directly to uptake) may have even been on a smaller scale. Although exposure may have increased slightly, at this level the difference may have been biologically inconsequential to Pb uptake.

Conclusions

Total Cd, Pb, and Zn concentrations in earthworms were good indicators of lethal metal exposure, and enabled the calculation of $LCBR_{50}$s for lethality. The Zn-$LCBR_{50}$ developed in artificial soil was applicable to earthworms exposed to remediated Zn-smelter soil. Mixture toxicity evaluated using $LCBR_{50}$s from each of the single metal tests indicated additive mixture toxicity among Cd, Pb, and Zn.

Evaluation of total and fractionated Cd, Pb, and Zn earthworm residues as indicators of exposure produced mixed results. Total earthworm Zn concentrations may be useful indicators of exposure and toxicity, but total earthworm Zn concentrations do not increase until Zn exposure is very high. Cytosolic and pellet fraction Zn may be useful in assessing exposure and toxicity below this threshold for net Zn accumulation. Total and fractionated Cd of live earthworms exposed to low and high sublethal Cd levels in artificial soils did not appear to be indicative of assumed differences in exposure, although this difference was thought to have been due to significantly reduced soil ingestion by earthworms exposed to higher metal levels. Total and fractionated Pb residues of live earthworms exposed to low and high sublethal Pb levels in artificial soils were not indicative of assumed differences in exposure, possibly due to the unavailability of Pb in the artificial soil. More research on the detoxification and regulation of heavy metals in earthworms and other invertebrates is needed to establish the link between body residue and sublethal exposure and toxicity.

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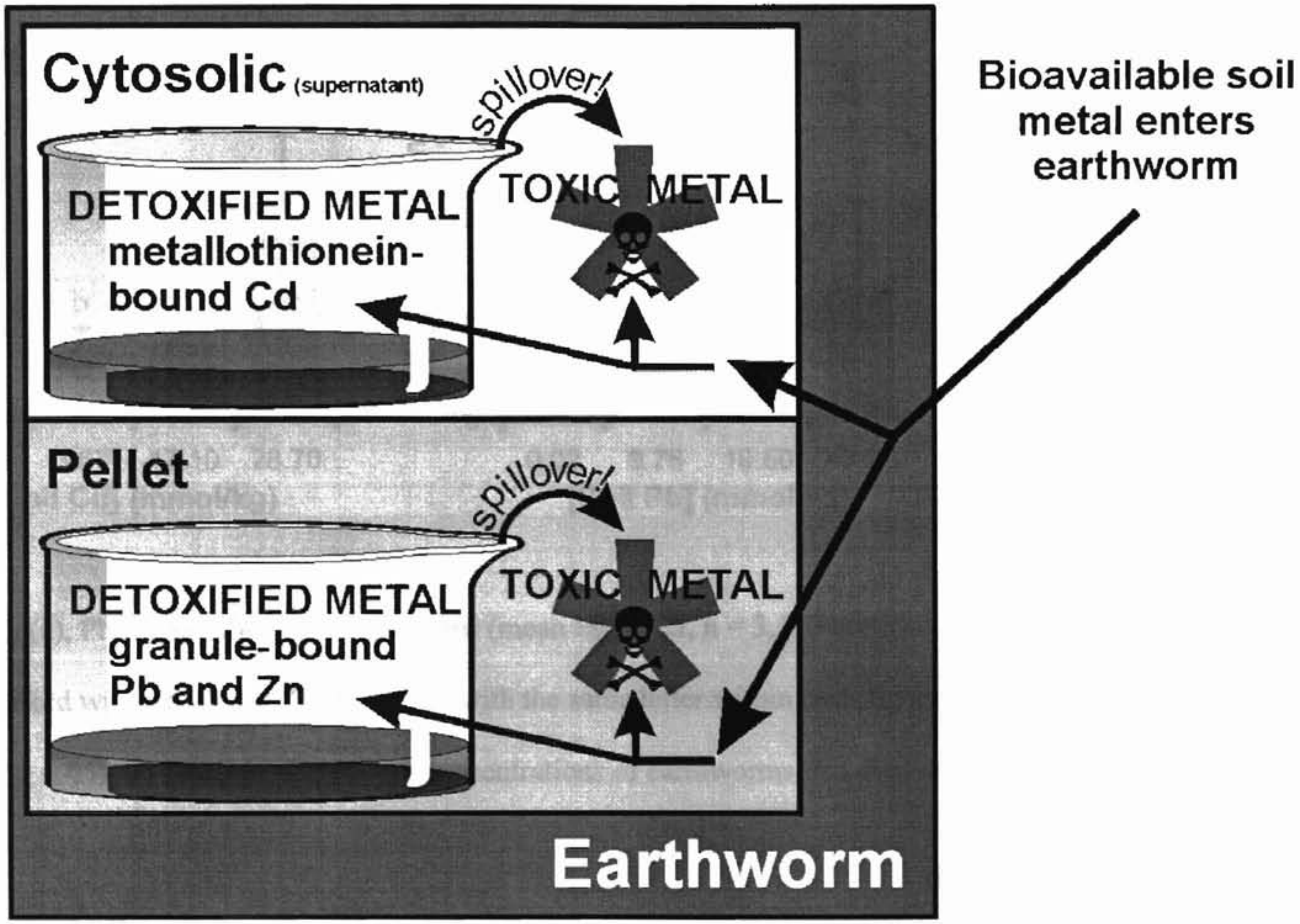

Figure 6. Hypothesized fractionation of toxicologically available/unavailable metals in the earthworm digest procedures used in this experiment.

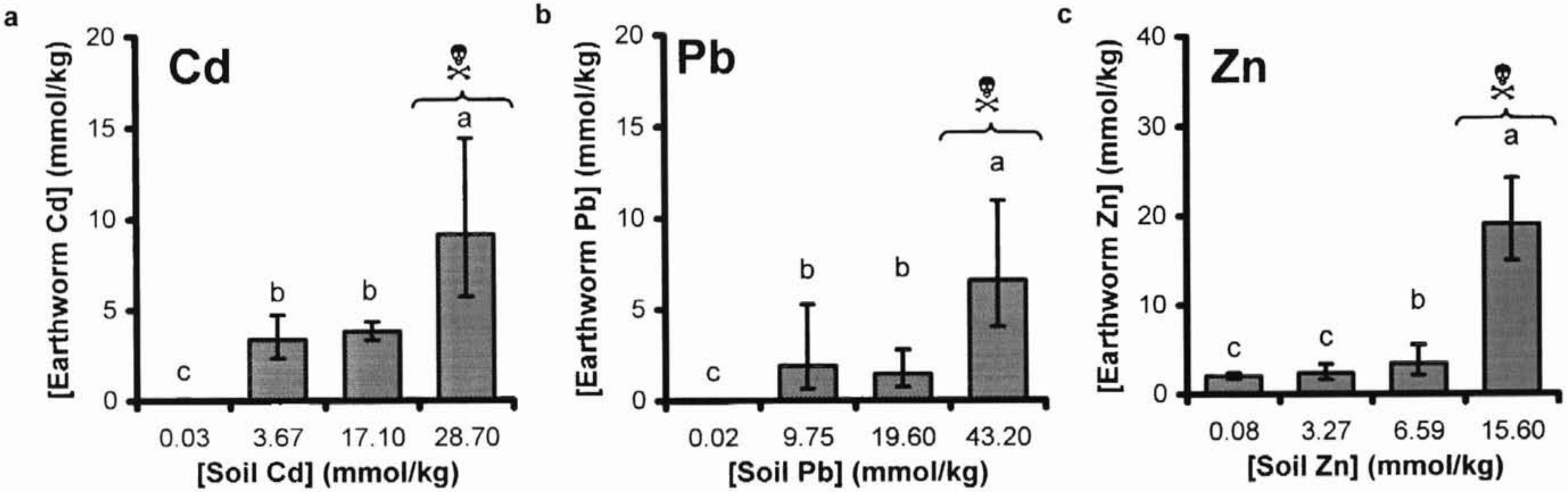

Figure 7. Total Cd(a), Pb(b), and Zn(c) concentrations (mean ±95% CI, n = 3, 2-3 earthworm subsamples/n) of *Eisenia fetida* exposed to artificial soil spiked with Cd, Pb, or Zn. Columns with the same letter within each figure are not significantly different (Fisher's protected LSD, $P > 0.05$). ☠-labeled columns are concentrations of earthworms that died during exposure.

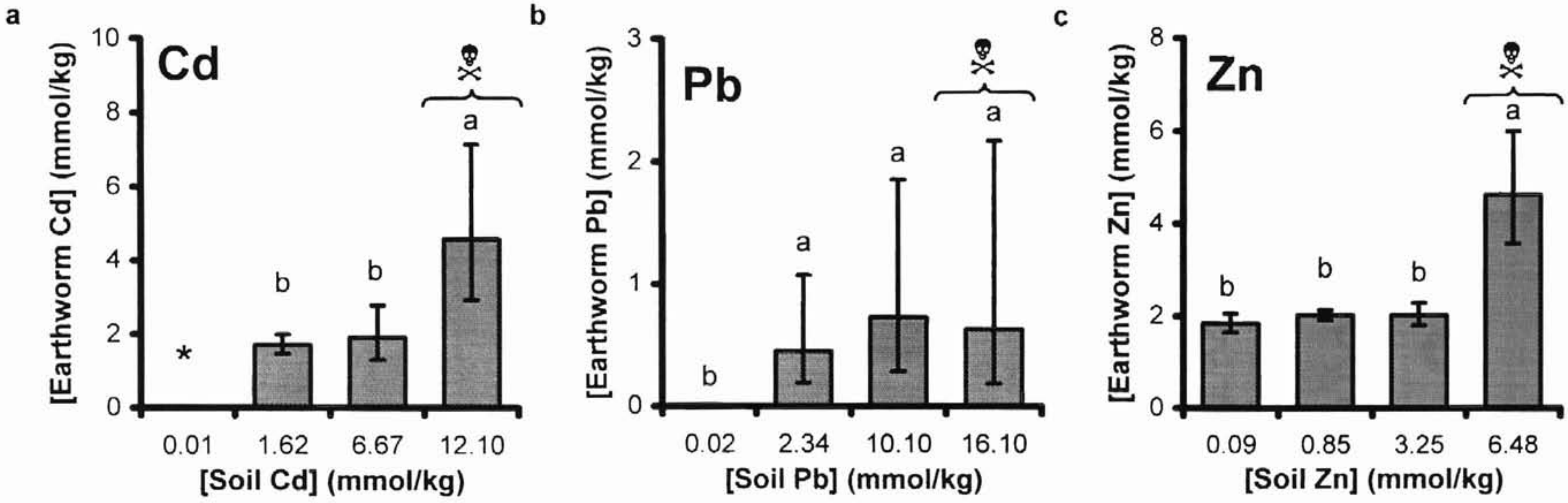

Figure 8. Total Cd(a), Pb(b), and Zn(c) concentrations (mean ±95% CI, n = 3, 2-3 earthworm subsamples/n) of *Eisenia fetida* exposed to artificial soil spiked with an equitoxic mixture of Cd, Pb, and Zn. Columns with the same letter within each figure are not significantly different (Fisher's protected LSD, $P > 0.05$). ☠-labeled columns are concentrations of earthworms that died during exposure. "*" indicates data below detection limit.

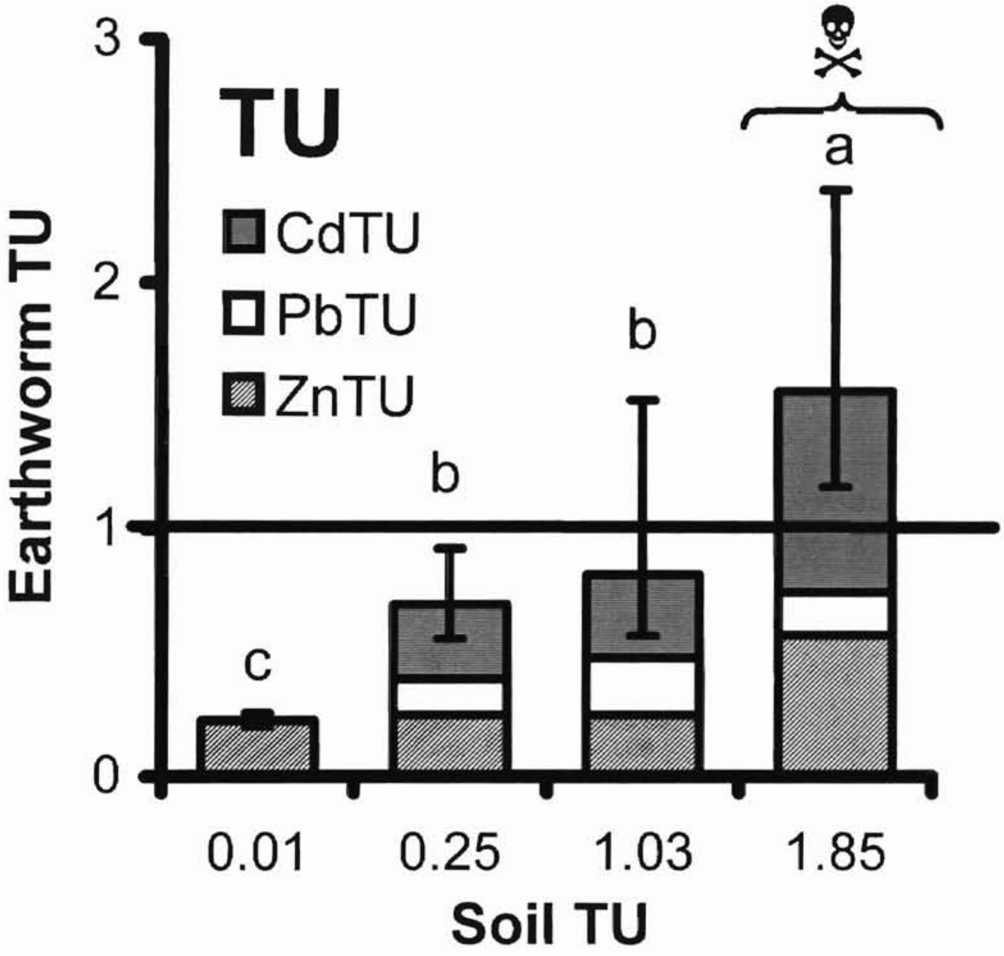

Figure 9. Earthworm total Cd, Pb, and Zn-based toxic units (TU, mean ±95% CI, n = 3, 2-3 earthworm subsamples/n) of *Eisenia fetida* exposed to artificial soil spiked with an equitoxic mixture of Cd, Pb, and Zn. Columns with the same letter within each figure are not significantly different (Fisher's protected LSD, $P > 0.05$). ☠-labeled columns are concentrations of earthworms that died during exposure.

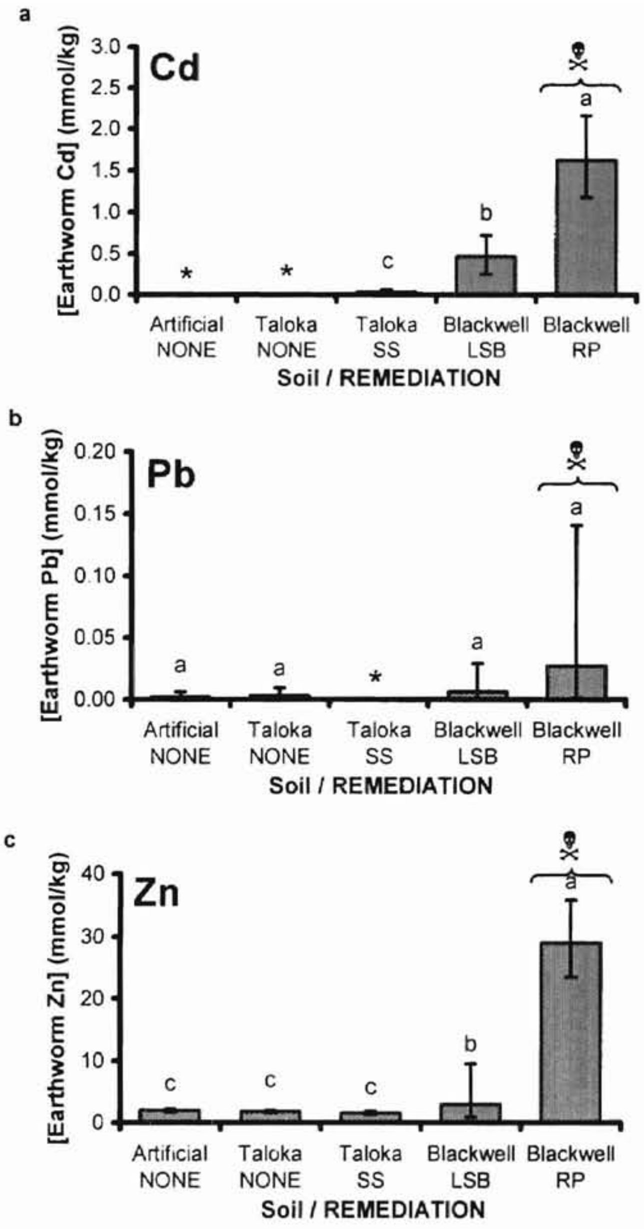

Figure 10. Total Cd(a), Pb(b), and Zn(c) concentrations (mean ±95% CI, n = 3, 2-3 earthworm subsamples/n) of *Eisenia fetida* exposed to artificial or field soils amended with remediation treatments. Columns with the same letter within each figure are not significantly different (Fisher's protected LSD, $P > 0.05$). ☠-labeled columns are concentrations of earthworms that died during exposure. "*" indicates data below detection limit.

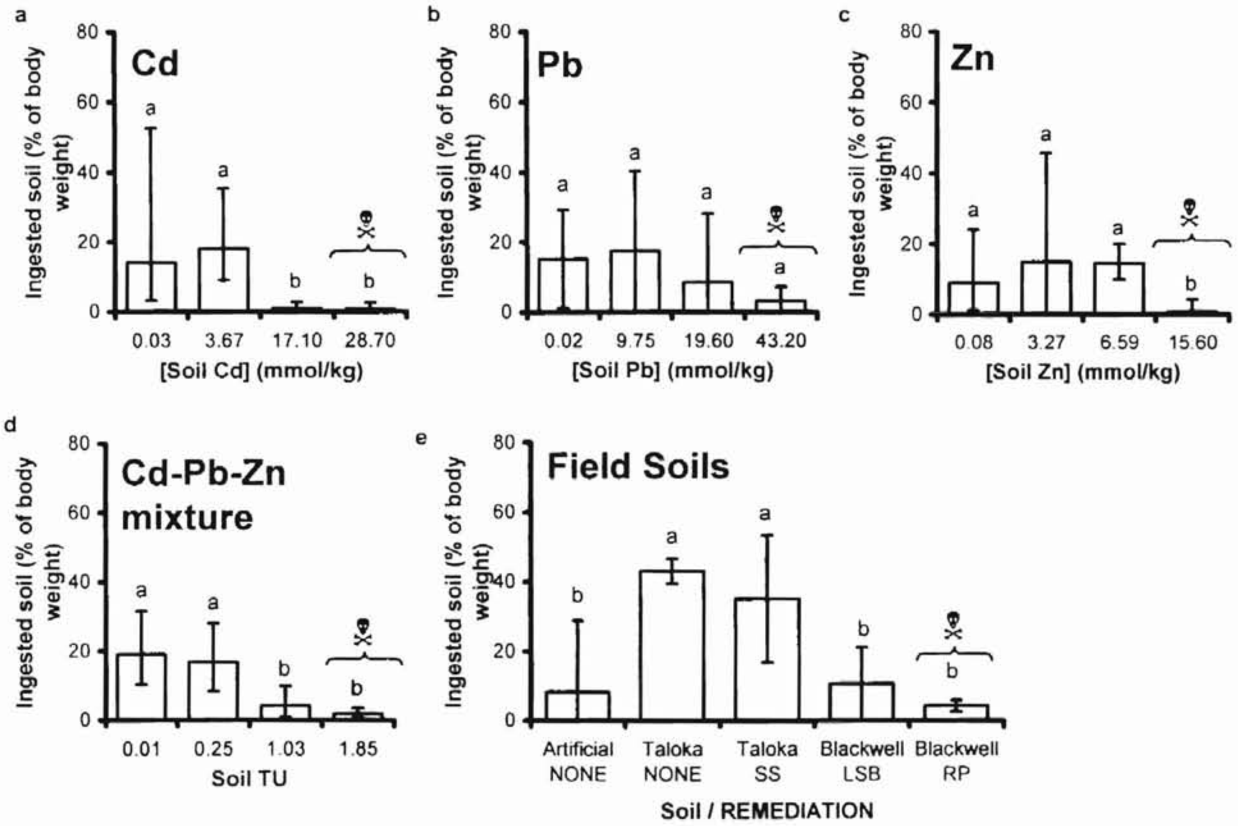

Figure 11. Ingested soil (mean ±95% CI, n = 3, 2-3 earthworm subsamples/n) of *Eisenia fetida* exposed to artificial soil spiked with Cd (a), Pb(b), Zn (c), artificial soil spiked with an equitoxic mixture of Cd, Pb, and Zn (d), or unspiked artificial and field soils amended with remediation treatments (e). Columns with the same letter within each figure are not significantly different (Fisher's protected LSD, $P > 0.05$). ☠-labeled columns are soil ingestion of earthworms that died during exposure.

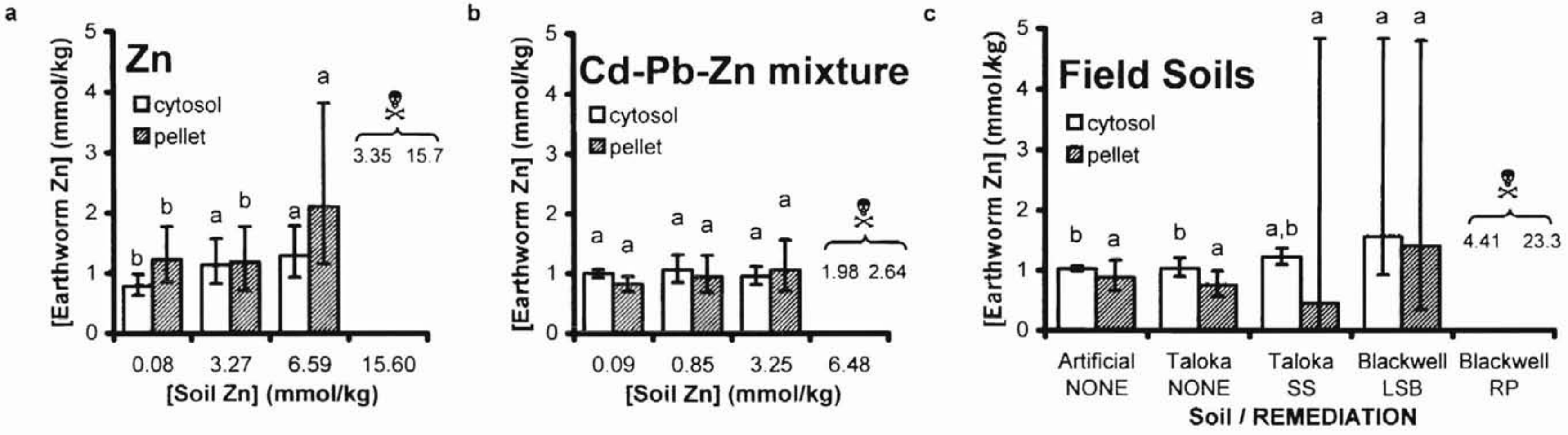

Figure 12. Cytosolic and pellet fraction Zn concentrations (mean ±95% CI, n = 3, 2-3 earthworm subsamples/n) of *Eisenia fetida* exposed to artificial soil spiked with Zn (a), artificial soil spiked with an equitoxic mixture of Cd, Pb, and Zn (b), and artificial or field soils amended with remediation treatments (c). Cytosolic concentrations were compared among soils in each figure; columns with the same letter are not significantly different (Fisher's protected LSD, $P > 0.05$). Pellet concentrations were compared statistically in the same manner. ☠-labeled numbers are cytosolic and pellet concentrations of earthworms that died during exposure, were not included in statistical analysis, and are given for reference only.

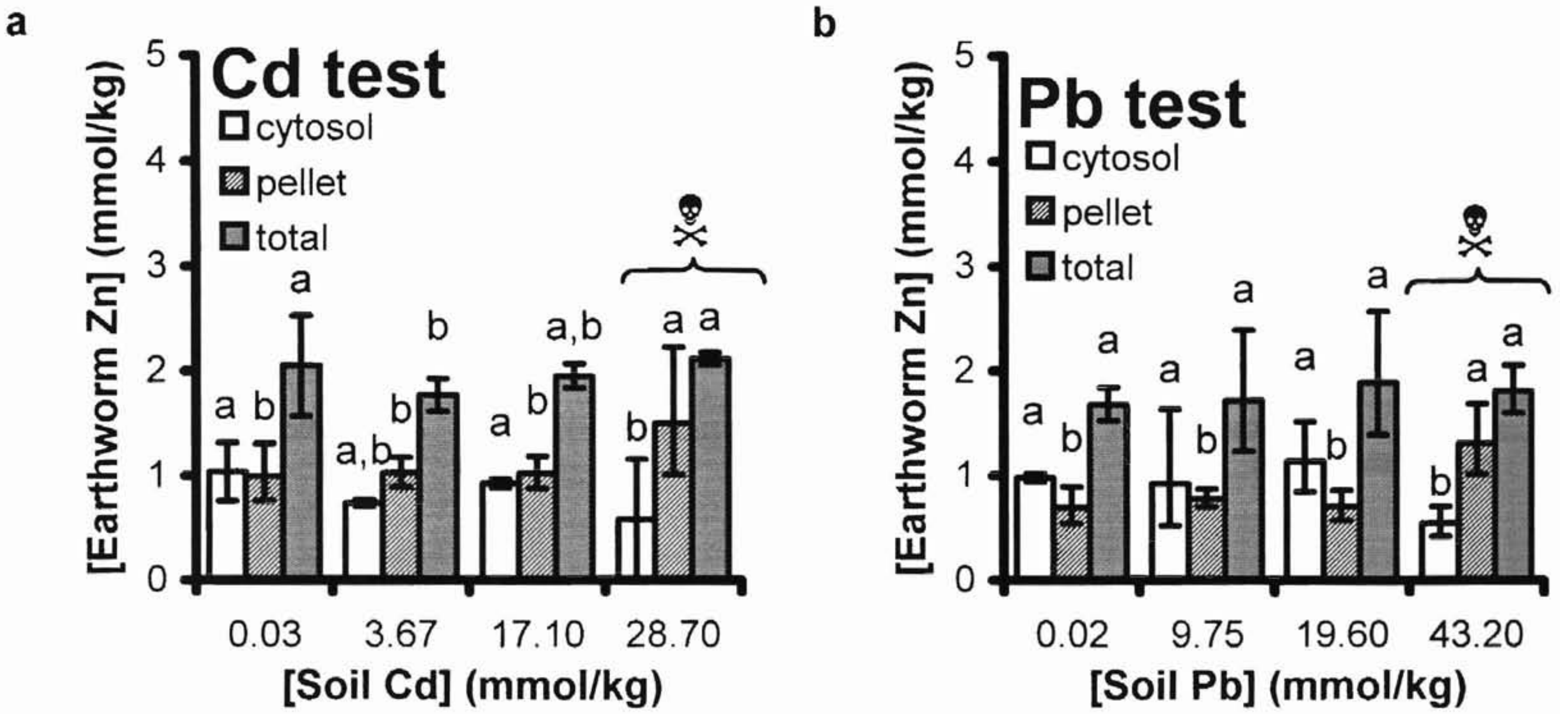

Figure 13. Cytosolic and pellet fraction, and total Zn concentrations (mean ±95% CI, n = 3, 2-3 earthworm subsamples/n) of *Eisenia fetida* exposed to artificial soil spiked with Cd(a) or Pb(b). Cytosolic concentrations were compared among soils in each figure; columns with the same letter are not significantly different (Fisher's protected LSD, $P > 0.05$). Pellet and total concentrations were compared statistically in the same manner. ☠-labeled numbers are cytosolic, pellet, and total concentrations of earthworms that died during exposure.

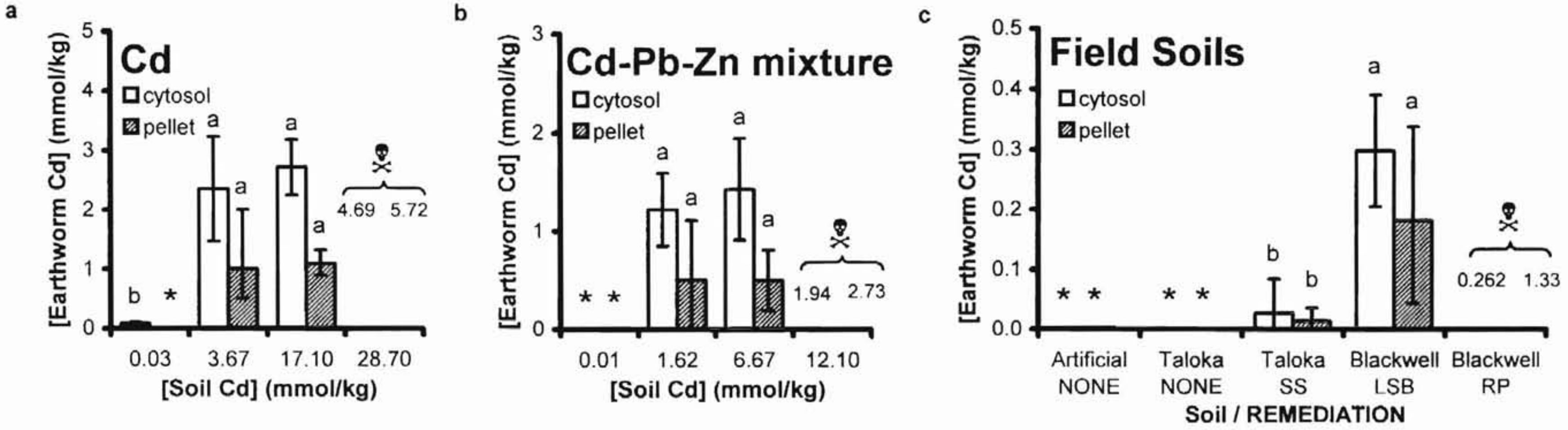

Figure 14. Cytosolic and pellet fraction Cd concentrations (mean ±95% CI, n = 3, 2-3 earthworm subsamples/n) of *Eisenia fetida* exposed to artificial soil spiked with Cd (a), artificial soil spiked with an equitoxic mixture of Cd, Pb, and Zn (b), and artificial or field soils amended with remediation treatments (c). Cytosolic concentrations were compared among soils in each figure; columns with the same letter are not significantly different (Fisher's protected LSD, $P > 0.05$). Pellet concentrations were compared statistically in the same manner. ☠-labeled numbers are cytosolic and pellet concentrations of earthworms that died during exposure, were not included in statistical analysis, and are given for reference only. "*" indicates data below detection limit.

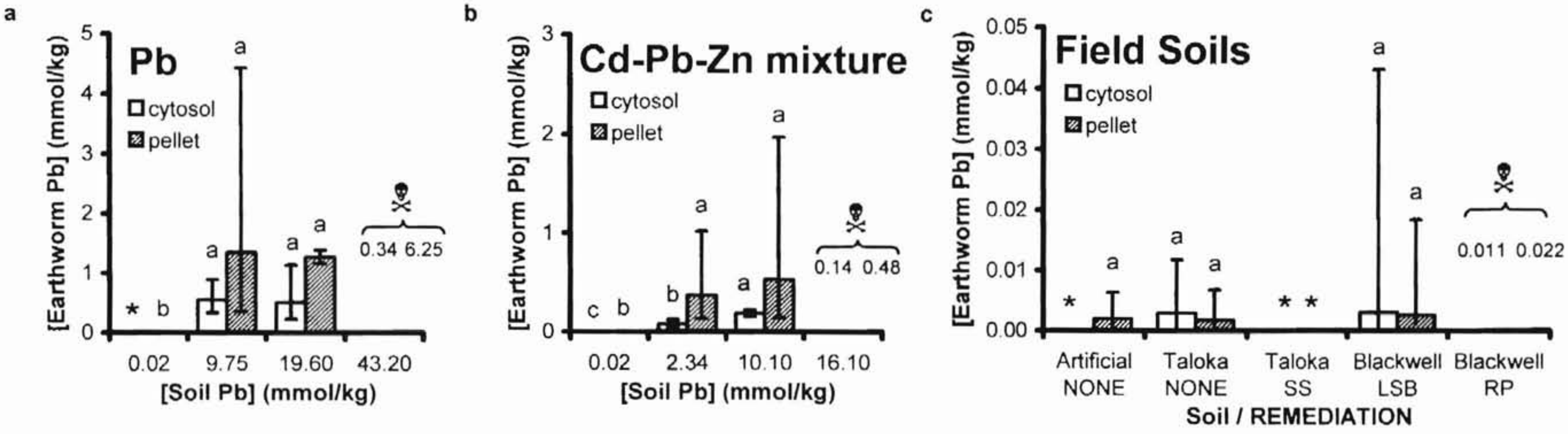

Figure 15. Cytosolic and pellet fraction Pb concentrations (mean ±95% CI, n = 3, 2-3 earthworm subsamples/n) of *Eisenia fetida* exposed to artificial soil spiked with Pb (a), artificial soil spiked with an equitoxic mixture of Cd, Pb, and Zn (b), and artificial or field soils amended with remediation treatments (c). Cytosolic concentrations were compared among soils in each figure; columns with the same letter are not significantly different (Fisher's protected LSD, $P > 0.05$). Pellet concentrations were compared statistically in the same manner. ☠-labeled numbers are cytosolic and pellet concentrations of earthworms that died during exposure, were not included in statistical analysis, and are given for reference only. "*" indicates data below detection limit.

CHAPTER IV

CALCIUM NITRATE EXTRACTABLE SOIL METALS AS A PREDICTOR OF CD, PB, AND ZN UPTAKE IN THE EARTHWORM *EISENIA FETIDA*

Abstract

Weak electrolyte-extractable metals have shown promise as inexpensive, toxicity-related measures of metal availability in soil. The ability of $Ca(NO_3)_2$-extractable soil Cd, Pb, and Zn to predict earthworm (*Eisenia fetida*) uptake in metal-spiked artificial and contaminated field soils was assessed. $Ca(NO_3)_2$-extractable Zn related very well to earthworm Zn uptake ($R^2 = 0.96$), and this relationship was unaffected by differences in soil type. $Ca(NO_3)_2$-extractable soil Cd was slightly better related to earthworm Cd uptake than total soil Cd, but low $Ca(NO_3)_2$-extractable soil Cd levels in the field soils made conclusions difficult. Due to low $Ca(NO_3)_2$-extractable soil Pb levels in the field soils, uptake models could only be derived for Pb uptake in artificial soil. $Ca(NO_3)_2$-extractable soil Cd, Pb, and Zn concentrations show promise as toxicity- and uptake-related surrogate measures of bioavailability.

Introduction

Total concentrations of Cd, Pb, and Zn in earthworms are relevant measures of heavy metal bioavailability in soils (Conder and Lanno submitted, Lanno and McCarty

1997, Lanno et al. 1999, Morgan et al. 1993, van Gestel et al. 1998-030). Earthworm toxicity tests, in which soil organisms are exposed to contaminated soils, are routinely used to evaluate toxicity and/or metal bioavailability, but are time consuming, expensive, and often difficult to interpret (Lanno and McCarty 1997). There is a great need for an inexpensive, rapid, and simple abiotic screening tool for estimating soil metal bioavailability.

Weak electrolyte extractions using weak (< 1M) $CaCl_2$ or $Ca(NO_3)_2$ solutions have shown promise as toxicity-related measures of metal availability in soils (Basta and Gradwohl 2000, Conder and Lanno 2000, Gradwohl 1998, Marinussen et al. 1997b, Peijnenburg et al 1997, Peijnenburg et al 1999, Posthuma et al. 1997, Sloan et al. 1997, van Gestel et al. 1998, Weljte 1998). These solutions are hypothesized to extract exchangeable or weakly bound "available" metals in soil (Sloan et al. 1997), which are believed to be available for uptake by earthworms (Peijnenburg et al 1999; Posthuma et al. 1997). Conder and Lanno (2000) recently found that 0.1M $Ca(NO_3)_2$-extractable soil metal concentrations were precisely related to acute toxicity in the earthworm *Eisenia fetida* exposed to Cd-, Pb-, and Zn-spiked artificial soils and remediated Zn-smelter soils. Cd, Pb, and Zn concentrations in the exposed earthworms also related well to acute toxicity, but were less reliable in detecting assumed differences in exposure at sublethal exposure levels (Conder and Lanno submitted). The objective of this research was to investigate the relationship between 2 toxicity-related measures of Cd, Pb, and Zn availability in soils: earthworm metal concentrations (a true measure of bioavailability) and $Ca(NO_3)_2$-extractable soil metal concentrations (a surrogate measure of bioavailability). This relationship was hypothesized to be better than the relationship between total soil metals and earthworm metal concentrations, and relatively constant among metal-spiked artificial and contaminated field soils.

Materials and Methods

$Ca(NO_3)_2$-extractable and total metals in soil

To measure weak electrolyte extractable metals, 1g (dry weight) of each soil replicate from the earthworm tests was combined with 20 ml 0.1M $Ca(NO_3)_2$, mixed in a rotary mixer for 4 hours at 23°C, and centrifuged at 2500*g* for 15 min. The resulting supernatant was then filtered with a 0.45 μm membrane filter (Millipore HAWP) and acidified with 0.5 ml concentrated trace metal-grade HNO_3. Total metal digestions using concentrated trace metal-grade HNO_3 were also conducted (Conder and Lanno 2000, Conder et al. submitted-a). Total metal digests and $Ca(NO_3)_2$ extractions were analyzed by atomic absorption (AA) spectroscopy. Quality assurance/quality control (QA/QC) measures included duplicate analyses, metal spikes, blanks, and analyses of standard reference soil "Sandy Soil B" (CRM-SA-B, Environmental Express, Mt. Pleasant, SC). Total metal analyses of the standard reference soil were within performance acceptance limits determined by USEPA 3050 digestion procedures (USEPA 1995).

Earthworm residue analysis

Earthworms (*E. fetida*) analyzed in this study were obtained from 5 previous toxicity tests in metal-spiked artificial soil (Conder and Lanno 2000) and unremediated and remediated Zn-smelter and reference soils (Conder et al. submitted-a). Tests were conducted for 14 d in environmental chambers maintained at 20 ±1°C under constant light. Artificial soil tests consisted of three single-metal tests in artificial soil spiked with aliquots of $CdSO_4$, $Pb(NO_3)_2$, or $ZnSO_4$, followed by a Cd-Pb-Zn mixture test spiked with Cd, Pb, and Zn nitrate salts to achieve a geometrically increasing exposure series based on toxic units (TU) calculated from toxicity data from the single-metal tests (Conder and Lanno 2000). Two field soils were tested: a Zn-contaminated soil obtained from a Zn smelter in Blackwell, Oklahoma, USA and a reference soil obtained from an agricultural site near Taloka, Oklahoma, USA. The Blackwell soil was heavily contaminated with Zn (229 mmol/kg) and mildly contaminated with Cd (3 mmol/kg) and Pb (3 mmol/kg). Three remediation materials (chemical immobilization amendments) were added at a rate of 100 g/kg to both soils: a lime stabilized municipal sewage sludge biosolid, North Carolina rock phosphate, and an anaerobically digested municipal sewage sludge biosolid. See Conder et al. (submitted-a) for more information on the amendment process.

Live earthworms were collected at the at the termination of each test. Dead earthworms were collected upon discovery during mortality observations, which were scheduled according to a geometric time scale (e.g., 2, 4, 8, 16, 24, 36, 48 h) for the first 48 h (when most mortality occurred), then daily for the remainder of the test. Prior to analysis, earthworms were removed from the soil, rinsed, and stored at –20°C. Individual soil replicates from both experiments were stored at -40°C in self-sealing plastic bags until analysis. Earthworms were analyzed for total Cd, Pb, and Zn and ingested soil (% soil / unpurged body weight) using the methods of Conder et al. (submitted-b). Method detection limits for earthworm tissue concentrations for Cd, Pb, and Zn were 0.09, 0.002, and 0.035 mmol/kg, respectively. Quality assurance/quality control (QA/QC) procedures included analysis of procedural blanks, spikes, and certified reference material (lobster hepatopancreas, TORT-2, National Research Council, Canada). All soil data presented were previously reported in Conder and Lanno (2000) and Conder et al. (submitted-a). Acid insoluble ash (AIA) content of soils (needed for earthworm tissue analysis) was obtained during total metals digestion (Conder and Lanno 2000, Conder et al. submitted-a) using methods given in Conder et al. (submitted-b).

Data analysis

Statistical analysis was performed using SAS/LAB® guided data analysis software (Version 6.12, SAS Institute Inc., Cary, NC). Transformations of earthworm residue data, suggested by SAS/LAB® (square root for Cd and Pb exposed earthworms, base-10 log for Zn exposed earthworms), were made prior to model analysis. Significant effects of soil type on regression models were interpreted to be failures of the independent variable ($Ca(NO_3)_2$-extractable or total soil metals) to adequately predict earthworm metal uptake among the single- and multiple metal-spiked artificial and field soils with the underlying assumption that the uptake of any single metal is not influenced by the presence of other bioavailable metals. This assumption is supported by previous conclusions about this data (Conder and Lanno submitted) as well as work by Marinussen et al. (1997a).

Results and Discussion

The relationship between total soil Zn and earthworm Zn uptake was very poor (Fig. 16a) and was statistically indescribable using parametric regression. Earthworm concentrations related very well to $Ca(NO_3)_2$-extractable Zn (Fig. 16b, 2-way ANOVA, soil type effect: $P = 0.3813$, interaction: $P = 0.0008$, overall fit: $R^2 = 0.96$, $P < 0.0001$). More importantly, this relationship was unaffected by soil type. Zn residues in earthworms are not often predictable by soil chemical characteristics, especially at the sublethal level, where differences in residues are slight or undetectable (Conder and Lanno submitted). The good fit of this data may be due to the residues of dead organisms at the high soil Zn levels (Conder and Lanno submitted). When the 9 data points representing the total Zn concentrations of dead earthworms in Figure 16b are removed (revised figure not shown), analysis of the small data set still reveals a good overall fit, although the relationships differ by soil type (2-way ANOVA, soil type effect: $P = 0.0072$, interaction: $P < 0.0001$, overall fit: $R^2 = 0.89$, $P < 0.0001$). A good relationship between Zn uptake in the earthworm *Enchytraeus cryptus* was also found for weak-electrolyte-($CaCl_2$) extractable and total soil Zn (Posthuma et al. 1997-037). In our experiments, however, Zn was much less bioavailable in the field soils, causing uptake to be dramatically overestimated by total soil Zn concentrations. More field soils with varying sublethal levels of $Ca(NO_3)_2$-extractable Zn levels must be tested to further investigate this relationship.

Total soil Cd was a good predictor of earthworm Cd uptake, although the effect of soil type was marginally insignificant (Fig. 17a, ANCOVA, soil type effect: $P = 0.0556$, overall fit: $R^2 = 0.80$, $P < 0.0001$). Soil type did affect the relationship between earthworm Cd uptake and $Ca(NO_3)_2$-extractable soil Cd (Fig. 17b, 2-way ANOVA, soil type effect: $P = 0.0129$, interaction: $P = 0.0156$, overall fit: $R^2 = 0.83$, $P < 0.0001$). Despite the effect of soil type on the relationship, we believe $Ca(NO_3)_2$-extractable soil Cd may be slightly better related to earthworm Cd uptake. The effect of soil type on the $Ca(NO_3)_2$-extractable soil Cd versus earthworm Cd model may be due to the field soils, which have very low $Ca(NO_3)_2$-extractable soil Cd (only 2 of 4 site soils tested were above the $Ca(NO_3)_2$-extractable soil Cd method detection limit of 0.005 mmol/kg). Testing in field soils with higher $Ca(NO_3)_2$-extractable soil Cd is necessary to assess any possible advantage over using total soil Cd to predict earthworm Cd uptake. Also, the model fit may have been affected by lower than expected total earthworm Cd burdens of live earthworms exposed to high sublethal Cd exposures. These earthworms had soil ingestion rates near 0%, thereby eliminating the intestinal exposure route. This led to reduced exposure for these earthworms compared to earthworms at lower sublethal exposure levels, which were actively consuming soil (Conder and Lanno submitted). However, the addition of earthworm soil ingestion as an independent variable (multiple regression) did not yield a better model for describing the relationship between total Cd concentrations in live earthworms and total or $Ca(NO_3)_2$-extractable soil Cd concentrations.

Earthworm Pb uptake in the field soils was not significantly different than the artificial soil negative control (Conder and Lanno submitted), therefore only artificial soil data was investigated (Fig. 18a, b). Total soil Pb was a better predictor of earthworm Pb uptake (ANCOVA, soil type effect: $P = 0.2922$, overall fit: $R^2 = 0.84$, $P < 0.0001$) than

$Ca(NO_3)_2$-extractable Pb (ANCOVA, soil type effect: $P = 0.0847$, overall fit: $R^2 = 0.70$, $P < 0.0001$). Mixed results have been seen with regards to the relationship between earthworm Pb uptake and $Ca(NO_3)_2$-extractable or total soil Pb concentrations (Ireland and Wooton 1976-004, Marinussen et al. 1997a, Peijnenburg 1999-015, and Weilgus-Serafinska and Kawa 1976-001). It is generally accepted that the percentage of total Pb levels in fields soils that is bioavailable to earthworms is extremely low, and related to pH and/or soil Ca content (Corp and Morgan 1991-010, Morgan 1985-006, and van Straalen and Bergema 1995-027). More research with field soils containing higher $Ca(NO_3)_2$-extractable Pb levels is needed to evaluate the usefulness of $Ca(NO_3)_2$-extractable soil Pb as a predictor of earthworm uptake.

Conclusions

$Ca(NO_3)_2$-extractable metals in soil are well related to Zn toxicity and uptake in earthworms, regardless of soil type. However, further research is needed to explore this relationship at levels where sublethal toxicity is of concern. $Ca(NO_3)_2$-extractable Cd was slightly better related to earthworm uptake than total Cd levels, while the opposite was true for Pb. Testing with field soils containing higher $Ca(NO_3)_2$-extractable Cd and Pb levels is needed to further explore the possible advantages of $Ca(NO_3)_2$-extractable soil metal levels as surrogate measures of bioavailability.

Acknowledgements

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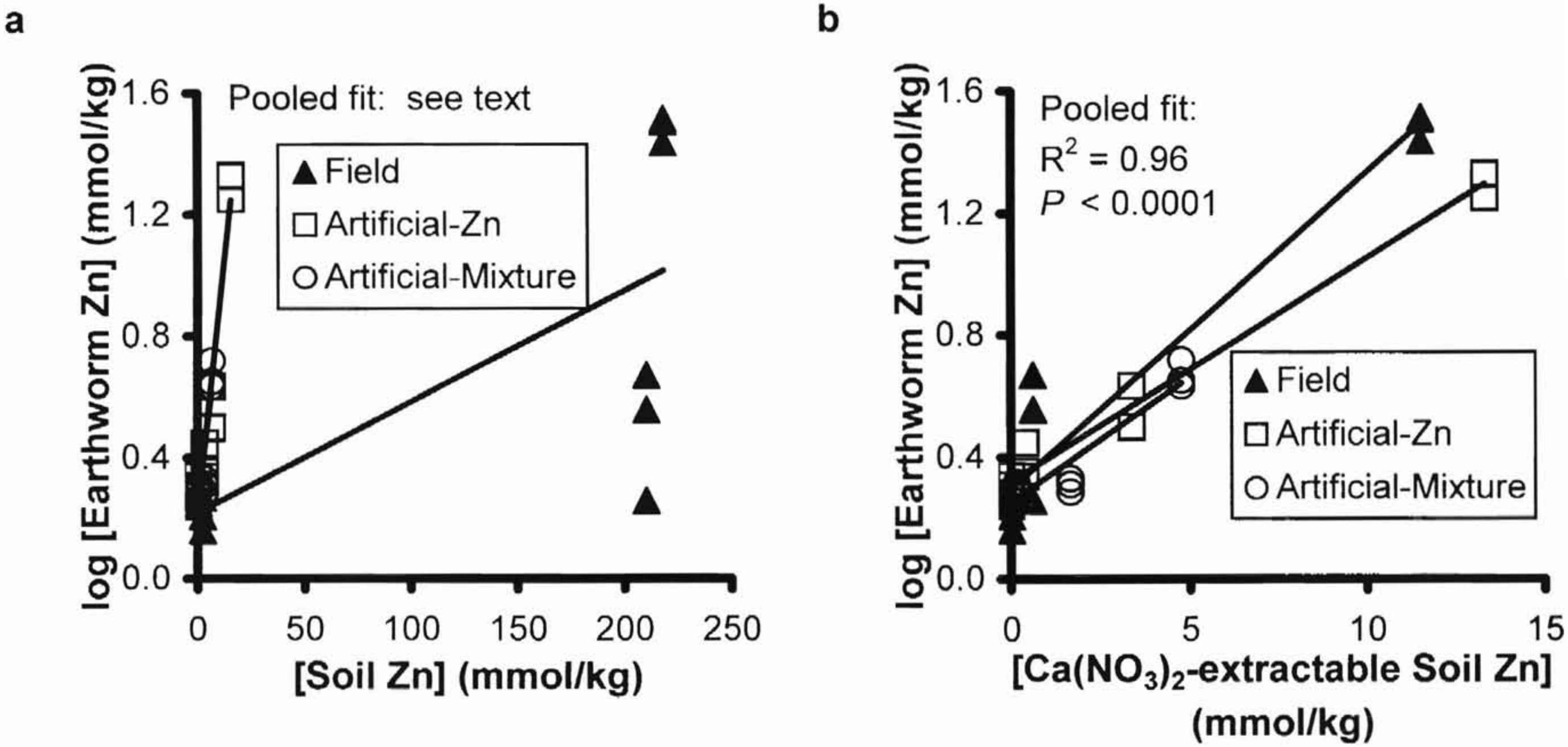

Figure 16. Total Zn concentrations (mean of 2-3 earthworm subsamples/marker, n = 3 per soil) of *Eisenia fetida* exposed to artificial soil spiked with Zn, artificial soil spiked with an equitoxic mixture of Cd, Pb, and Zn, or field soils amended with remediation treatments plotted versus total (a) or $Ca(NO_3)_2$-extractable (b) soil Zn concentrations.

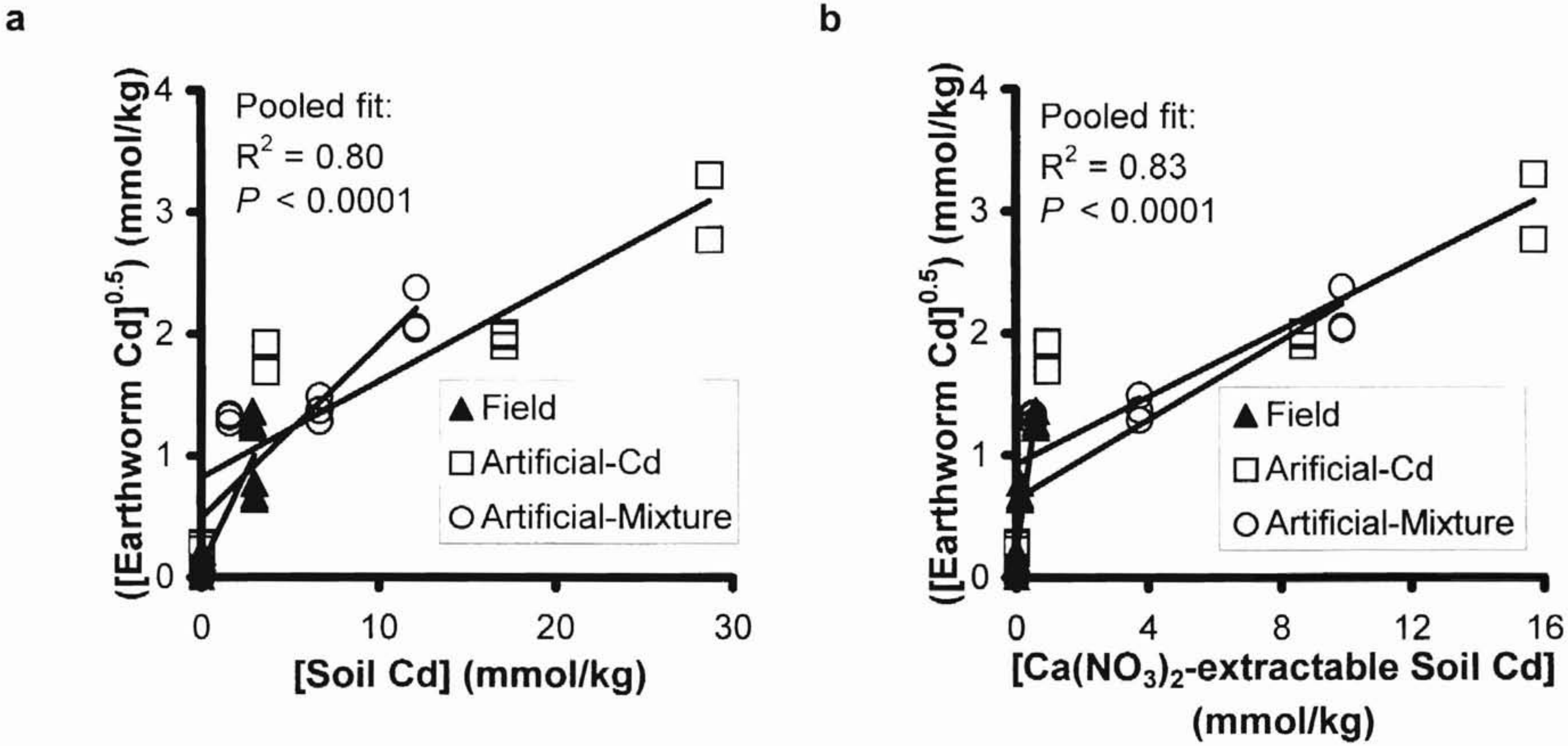

Figure 17. Total Cd concentrations (mean of 2-3 earthworm subsamples/marker, n = 3 per soil) of *Eisenia fetida* exposed to artificial soil spiked with Cd, artificial soil spiked with an equitoxic mixture of Cd, Pb, and Zn, or field soils amended with remediation treatments plotted versus total (a) or $Ca(NO_3)_2$-extractable (b) soil Cd concentrations.

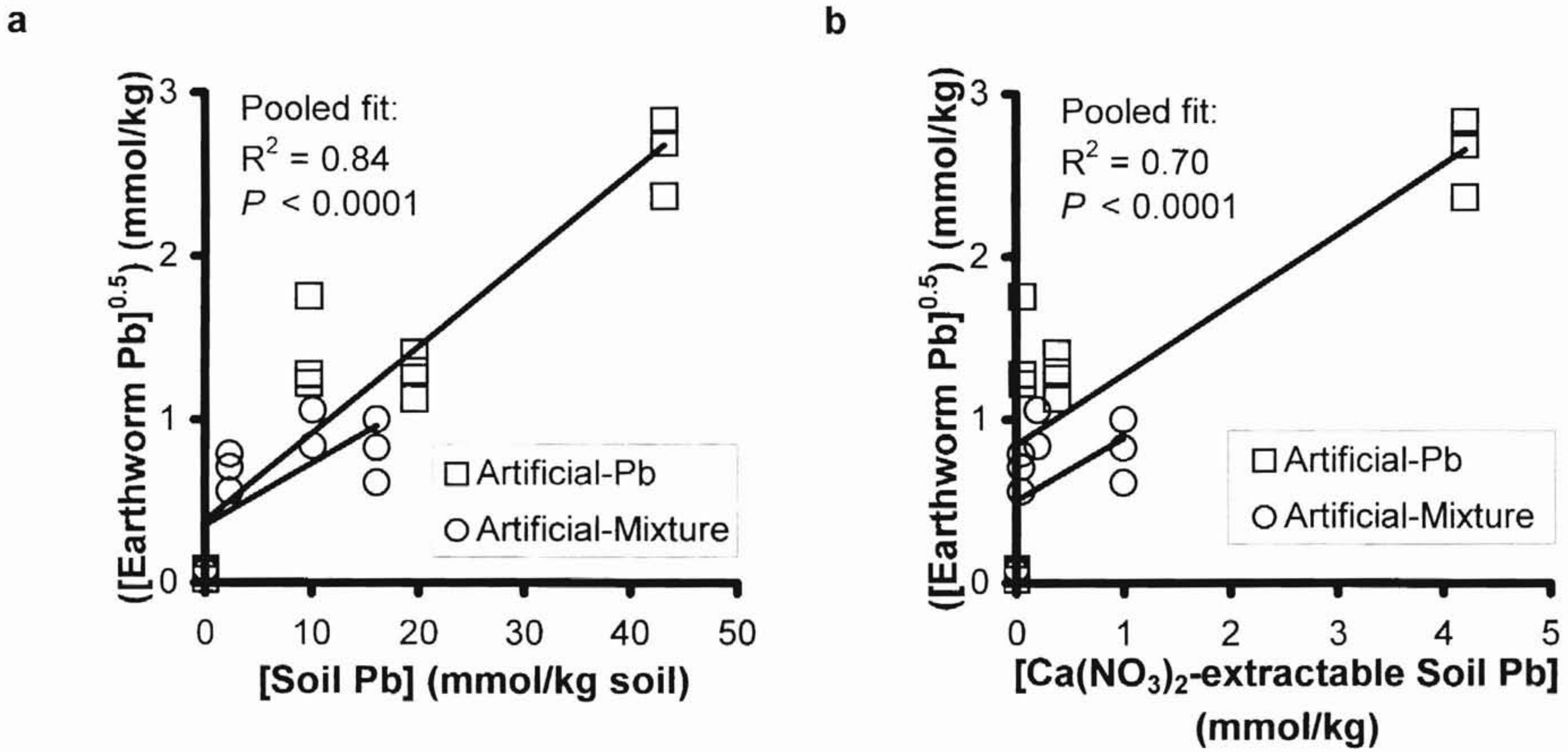

Figure 18. Total Pb concentrations (mean of 2-3 earthworm subsamples/marker, n = 3 per soil) of *Eisenia fetida* exposed to artificial soil spiked with Pb or an equitoxic mixture of Cd, Pb, and Zn plotted versus total (a) or $Ca(NO_3)_2$-extractable (b) soil Pb concentrations.

VITA

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Master of Science

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