BIOAVAILABILITY AND TOXICITY OF HEAVY METALS IN CONTAMINATED SOILS TO HUMAN AND ECOLOGICAL RECEPTORS

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INTRODUCTION

This document consists of four chapters, each reporting separate studies conducted during my doctorate program. All chapters are presented in formats suitable for publication in professional journals.

CHAPTER I

ECOTOXICOLOGICAL RISKS ASSOCIATED WITH LAND TREATMENT OF PETROCHEMICAL WASTE. I. RESIDUAL SOIL CONTAMINATION AND BIOACCUMULATION BY COTTON RATS (SIGMODON HISPIDUS)

ABSTRACT

Petrochemical waste contains both organic and inorganic contaminants that can pollute soil and may pose significant ecological risks to wildlife. Petrochemical waste typically is disposed of in land treatment units which are widespread throughout Oklahoma and the United States. Few studies have been conducted evaluating possible toxicity risks to terrestrial organisms residing on these units. In this study, the extent of soil contamination with fluoride (F), metals, and organic hydrocarbons, the bioaccumulation of F and metals in cotton rats (*Sigmodon hispidus*), the relationship between contaminants in soil and in tissues of cotton rats, and the level of potentially toxic polycyclic aromatic hydrocarbons (PAHs) in soil was determined on land treatment units. Over a two year period, cotton rats and soils were collected and analyzed from five land treatment and matched reference units. The number of land treatment units with soil metal contamination (in parentheses) included: Cr, Cu, Pb (5); Al, As, Ni, Sr, Zn (4); Ba (3); and Cd, V (2). The number of land treatment units with soil PAH contamination (in parentheses) were naphthalene, phenanthrene, benzo (g,h,i) perylene (3); acenaphthene, anthracene, pyrene, benzo (a) anthracene,

chrysene, benzo (b) fluoranthene, benzo (a) pyrene, indeno (1,2,3-cd) pyrene, dibenz (a,h) anthracene (2); and acenaphthylene, fluorene, fluoranthene, benzo (k) fluoranthene (1). Total PAH and total petroleum hydrocarbons (TPH) were elevated at all five land treatment units. Mean sum of benzo (a) pyrene (B(a)P) equivalents ($B(a)P_{equiv}$) were not affected on land treatment units as compared to reference units. Units 1 and 3 were significantly higher in levels of metals, total PAH, TPH and $B(a)P_{equiv}$ than the other units. Pb and F bioaccumulated in bone and Pb bioaccumulated in kidney of cotton rats. F in bone of 496 to 2212 mg/kg was 3- to 15-fold greater than mean F in bone of cotton rats from reference units. Elevated levels of Pb in bone of 4.6 to 24.8 mg/kg was 460- to 2500-fold greater than mean Pb in bone of cotton rats from reference units. Elevated levels of Pb in kidney of 0.31 to 1.08 mg/kg was 10- to 36-fold greater than mean Pb in kidney of cotton rats from reference units. Bone F was an accurate predictor of the severity of dental fluorosis. Strong relationships were found between bone F and HCl-extractable F and bone F and total F in soils of land treatment units. A strong relationship was discovered between Pb in bone and Pb in soil. Land treatment appears to have been effective as a remediation technology in reducing levels of individual PAHs and the sum of B(a)P equivalents to background levels on units 2, 4, and 5 but not on units 1 and 3. This study shows that certain contaminants such as Pb and F tend to bioaccumulate in cotton rats collected from land treatment units. Land treatment was ineffective in reducing levels of these contaminants to background levels that will not pose an increase in risk to terrestrial mammals. Therefore to avoid accumulation of these

contaminants in cotton rats and their possible deleterious effects, these contaminants should be measured and land application rates of petrochemical waste should be managed to avoid excessive loading into soil systems.

INTRODUCTION

The petrochemical refining industry utilizes a wide variety of chemicals in the processing of crude oil and produces a large quantity of hazardous and non-hazardous waste. In 1991, the petroleum industry produced approximately 123 billion kg of hazardous waste (Bass et al. 1995). Much of this waste is usually disposed of or stored on site in land treatment units or in storage pits. Land treatment is a waste management technology that involves controlled application of wastes onto or into soil for the purpose of biodegradation of organics, immobilization of inorganics, and avoiding the bioaccumulation of hazardous chemicals (Loehr and Malina 1986). Land treatment of waste on petrochemical units can pose risks of exposure to terrestrial vertebrates as many of these areas are heavily vegetated and support populations of small mammals and other vertebrates.

Land treatment of petrochemical wastes has been shown to reduce the concentrations of organic chemicals through biological degradation. However petrochemical waste usually contains various inorganics (i.e. metals and F) which do not biodegrade and which may pose significant ecological risks to wildlife. Excessive F exposure may cause weakening of bones and skeletal deformities in humans, domestic stock, and wildlife. Exposure to elevated F may result in dental and skeletal changes (fluorosis) in certain species of wildlife (Boulton et al. 1994; Kierdorf et al. 1995; Vikoren and Stuve 1996). Fluorosis has been reported in cotton rats (*Sigmodon hispidus*) collected from a F contaminated land treatment unit in Cyril, OK, USA (Paranjpe et al. 1994;

Schroder et al. 1999). Petroleum waste contains metals (Cr and V) and certain polycyclic aromatic hydrocarbons (PAHs) (benzo (a) pyrene, benzo (b and k) fluoranthene, chrysene, indeno (1, 2, 3,-c,d) pyrene, dibenz (a, h) anthracene, benzo (a) anthracene and benzo (g,h,i) perylene) which have been shown to produce immunotoxic effects (Silkworth et al. 1995; IPCS 1986; Schroder et al. 2000). Several of these chemicals are considered to be probable human carcinogens by the United States Environmental Protection Agency (U.S. EPA 1993a). PAHs occur in the environment as complex mixtures of many components, which have wide varying toxic potencies (Santodonato et al. 1981). Only one of the PAHs, benzo (a) pyrene (B(a)P), has been widely studied and characterized toxicologically. The toxicity equivalency factor (TEF) approach has been adopted by the United States Environmental Protection Agency as a basis for human risk assessment (U.S. EPA 1993b) of PAHs. The TEF approach involves the separation of PAHs into two subclasses consisting of the carcinogenic and noncarcinogenic PAHs. The noncarcinogenic PAHs are assigned a factor of zero; the carcinogenic ones are assigned a factor determined by bioassays, which compare their relative potencies to B(a)P. In this approach, concentrations of carcinogenic PAHs are multiplied by their appropriate TEF and summed as B(a)P equivalents for use in human risk assessments. Recently the World Health Organization (WHO 1997) has recommended TEF values for wildlife (fish, birds, and mammals) exposed to planar halogenated hydrocarbons such as dioxin for use in ecological risk assessments and it is possible that TEF values for wildlife exposed to PAH

mixtures will be developed in the near future. The World Health Organization (1997) concluded that there was insufficient data to discriminate between laboratory and wild mammalian species and decided that the TEFs for human risk assessment based on laboratory animals would be equally applicable to wild mammalian species. Therefore, the TEFs derived by the United States Environmental Protection Agency (1993b) for human risk assessment were used to calculate the sum of B(a)P equivalents in our study.

Small mammals residing on land treatment units may be exposed to contaminants through a number of pathways including ingestion of contaminated soil, water, and food. Mammals have been utilized as indicators of contamination with residue being analyzed in whole-body or specific organs. Uptake of contaminants and transfer between trophic levels in small mammals for elements such as Cd, Pb, and F have been shown to occur at contaminated sites (Hunter et al. 1978; Andrews et al. 1989a, 1989b). Small mammals have been successfully used to document exposure and toxicity of both F and metals (Johnson et al. 1978; Roberts et al. 1978; Walton 1986a, 1986b; Boulton et al. 1994; Schroder et al. 1999). Cotton rats are indigenous to Oklahoma and serve a critical as well as functional role in the terrestrial food chain. Cotton rats have proven beneficial as biomonitors for F, metals and organic chemicals (McMurry et al. 1991, 1994; Schroder et al. 1999, 2000; Rafferty et al. 2000; Kim et al. 2001). Land treatment units utilized for the disposal of petrochemical wastes are widespread throughout Oklahoma and the United States. However, few studies have been conducted on these units to evaluate possible toxicity risks to

terrestrial organisms residing in these units. The objectives of this study were to (1) evaluate the extent of soil contamination with F, metals, and organic hydrocarbons on land treatment units; (2) determine bioaccumulation of F and metals in cotton rats residing on these land treatment units; (3) determine the relationship between contaminants in soil and in tissues of cotton rats collected from land treatment units; and (4) to determine if land treatment results in elevated levels of potentially toxic PAHs in soil.

METHODS

Study Units

Five abandoned land treatment facilities that historically received petrochemical waste products generated by the oil refining industry were selected in Oklahoma. Location, climatological data, and a brief history of the units are shown in Table 1. Mean annual temperatures were about the same at all units but mean annual rainfall varied among units with precipitation at units 1 and 2 lower than the other units (Table 1). Over a two year period cotton rats and soils were collected and analyzed from these facilities and matched reference units. All land treatment facilities (Units 1-5) are privately owned (remaining anonymous) in Oklahoma and support resident populations of cotton rats. Adjacent reference units that showed no visible evidence of petrochemical contamination were identified within 5 km of each facility and chosen based on their vegetative and topographic similarity with their matched land treatment unit. Contaminated units and their matched reference units all consisted of disturbed terrestrial ecosystems with predominantly early seral stage plant species. The

most prominent plant species on these units were johnsongrass (*Sorghum halapense* L.), little bluestem (*Schizachyrium scoparium* Nash), big bluestem (*Andropogon gerardii* Vitman), brome (*Bromus* spp.), and bermuda grass (*Cynodon dactylon* L.). Trapping grids consisted of eight lines with eight traps per line and a total of 64 traps were utilized in the collection of animals.

Collection and Analysis of Soils

Surface soils (< 2cm) were collected from trapping grids on the land treatment facilities and the reference units. Land treatment facilities and their matched reference units were divided into six sub-units and a composite sample consisting of six subsamples was collected from each sub-unit. Soils were stored and transported in sealed acid washed glass jars. Soils were air-dried and sieved to pass a 2 mm screen prior to analysis.

Soil properties (pH, organic carbon, texture, and electrical conductivity) were measured from the collected samples. Soil pH was determined in a 1:2 soil: 0.01 M $CaCl_2$ suspension (Thomas 1996). Automated dry combustion was utilized to determine soil organic carbon (Nelson and Sommers 1996). Soil texture was determined by the hydrometer method (Gee and Bauder 1986). Electrical conductivity of a 1:5 soil:deionized water extract was measured (Rhoades 1996).

Metals (Al, As, Ba, Cd, Cr, Cu, Ni, Pb, Sr, Ti, V, Zn) in soil were digested by microwave according to U.S. EPA Method 3051 (USEPA 1994). Metals in acid digests were evaluated using inductively coupled plasma atomic emission spectroscopy (ICP-AES). Both total F content and potentially bioavailable F of

soils were measured. Because acid extractions of soils result in low recoveries of total F (Hall 1968; Cooke et al 1976; Venkateswarlu 1983; Andrews et al 1989b), fusion with NaOH was utilized to measure total F content of soil (McQuaker and Gurney 1977). F, in the fused soil sample, was determined using a combination F ion-selective electrode (Orion 960900, Beverly, MA, USA). A weak acid extraction (0.03 M HCl, pH 1.5) followed by potentiometric determination was used to measure the potentially bioavailable F (Walton 1987, Schroder et al. 1999, 2000).

PAHs (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo (a) anthracene, chrysene, benzo (b) fluoranthene, benzo (k) fluoranthene, benzo (a) pyrene, indeno (1,2,3-cd) pyrene, dibenz (a,h) anthracene, and benzo (g,h,i) perylene) were extracted using accelerated solvent extraction according to U.S. EPA Method 3545 (USEPA 1996a) (Dionex ASE Model 200, Dionex Corporation, Houston, TX, USA) and quantified by gas chromatography with mass spectroscopic detection (GC-MS, Hewlett-Packard 5890 Series II with 5971 MSD, Hewlett-Packard, San Fernando, CA, USA) according to U.S. EPA Method 8270 (USEPA 1996b). The following conditions were used for the extraction of PAHs and TPH: system pressure (1500 psi), oven temperature (100°C), sample size (10 g), oven heat up time (5 min), static time (5 min), solvent (1:1 mixture dichloromethane/acetone), number of cycles (2). The following GC-MS conditions were used for the analysis of PAHs: column 30 m, 0.25 mm ID, 0.25 μm df, Restek Rtx® -5 (Crossbond® 5% diphenyl/95% polysiloxane); 1 μL splitless

injection; oven temperature 40°C to 150°C at 15°C/min then 6°C/min to 300°C and hold for 10 min; 265°C injector temperature; 300°C detector temperature; helium carrier gas at linear velocity of 50cm/sec set at 300°C. Single ion monitoring (SIM) with mass spectroscopic detection was utilized to measure the low concentrations of PAHs in our study. Total petroleum hydrocarbons (TPH) were extracted using accelerated solvent extraction and analyzed by gas chromatography with flame ionization detection according to the Wisconsin method (Wisconsin Department of Natural Resources 1993). The following GC conditions were used during the analysis of TPH: column 30 m, 0.25 mm ID, 0.25 μm df; , Restek Rtx® -5 (Crossbond® 5% diphenyl/95% polysiloxane); 1 μL split injection; oven temperature 40°C to 150°C at 15°C/min then 6°C/min to 300°C and hold for 10 min; 265°C injector temperature; 300°C detector temperature; helium carrier gas at linear velocity of 50cm/sec set at 300°C.

Collection of Animals and Preparation of Tissue

A total of 12 adult cotton rats were collected from each grid for every land treatment unit and the matched reference unit during summer (August 1998, 1999) and winter (January - February 1999, 2000) using Sherman live traps baited with rolled oats (N = 240 per year). Following capture, the rats were housed for 48 hr. and sacrificed by exsanguination. Two humeri and two femurs from each rat were cleaned of excess tissue with a scalpel and scissors, freeze-dried, and weighed. The humeri were placed in petroleum ether for 96 h with daily changes to remove fat (Paranjpe et al. 1994) to prepare them for F analysis. One kidney per rat was removed, freeze-dried, and weighed for subsequent

metal analysis. Skulls were removed and fixed in formalin for later evaluation of incisors for evidence of dental fluorosis.

Analysis of Tissues

Each pair of femurs was acid digested by a method adapted from Andrews et al. (1989a). Each pair of femurs (200 mg) was refluxed with 5 ml of concentrated trace metal HNO_3 on a hotplate at 95°C for 1 hr. Digested material was diluted with deionized distilled water to 10 ml and metals (Ba, Cd, Cr, Cu, Ni, Pb, Sr, Ti, Zn) were analyzed by ICP-AES. Kidneys were digested in the same manner and analyzed by ICP-AES. For bone F analysis, each pair of humeri (100 mg) was ash dried in porcelain crucibles overnight at 550°C (Singer and Armstrong 1968). Ashed bone was ground with a mortar and pestle and subsequently dissolved in 0.25 M HCl. The digest was then neutralized with 0.125 M NaOH. Sample solution (5 ml) was combined with 5 ml of TISAB II buffer (Orion No. 940909) to adjust ionic strength and inhibit complexation of F by Fe and Al (Orion 1991). F was measured using an Orion F combination electrode. Calibration standards were prepared in a similar manner using certified 100 mg F/L standard (Orion No. 940907). Bone F content was expressed as mg F/kg on a freeze-dried basis.

Scoring Of Teeth for Dental Lesions

Scoring of incisors was performed to document gross morphological lesions commonly referred to as dental fluorosis, using a system previously described for mammals and summarized in Table 2 (Schupe et al. 1972; Boulton et al. 1994). All rats were assigned a random number to prevent bias and were

scored blinded by two different analysts for confirmation. Incisor scores were reported as an average of the two scores.

Quality Assurance and Quality Control

Blanks, spikes, and certified reference materials were digested/extracted and analyzed for quality assurance and quality control of metals, PAHs, and TPH in soil. Blanks, spikes, and certified reference materials were digested/extracted and analyzed for every six soil samples. Digested blanks contained below detection limit concentrations of all metals (Table 3). Mean recoveries of metal in certified reference soil (CRM020-050, RTC Corporation, Laramie, WY, USA) ranged from 91 to 110% with relative standard deviations ranging from 1.1 to 4.2% (Table 3). Spike recoveries for metals in soil ranged from 90 to 100%. Due to lack of an available certified reference material, blanks and spikes recoveries were used for quality assurance and quality control in the analysis of total and bioavailable F in soil. Laboratory quality assurance and quality control procedures showed that the fusion procedure recovered 95 to 105% of the F spikes added to soil and the HCl-extractable recoveries ranged from 90 to 95% of F spikes. Reagent blanks carried through both procedures contained below detection limits of F. Detection limits for the fusion procedure were 10 mg F/kg soil and the detection limits for the HCl-extractable procedure were 0.5 mg F/kg soil. Mean recoveries of PAHs in certified reference soil (CRM104-100, RTC Corporation, Laramie, WY, USA) ranged from 71 to 101% and the relative standard deviations ranged from 2.7 to 6.2 % (Table 3). Mean recovery of TPH in certified reference soil (CRM350-100, RTC Corporation, Laramie, WY, USA)

was 94% and the mean relative standard deviation was 6.3%. Spike recoveries for PAHs in soil ranged from 80 to 110% and spike recoveries for TPH in soil ranged from 90 to 100%. Extracted blanks consisting of sand that had been muffled furnaced overnight at 550°C contained below detection limit concentrations of all PAHs and TPH (Table 3).

Blanks, spikes, and standard reference material (National Institute of Standards and Testing bone meal SRM 1486) were analyzed for quality assurance and quality control of metals and F in tissue. Blanks, spikes, and certified reference materials were digested and analyzed for every ten tissue samples. Ashed and digested blanks contained below detection limit concentrations of metals and F. Mean recovery for F in bone was 97% with a relative standard deviation of 2.5% (Table 3). Recovery for Sr in bone averaged 92% with a relative standard deviation of 1.8%. The mean recovery for Zn in bone was 93% with a relative standard deviation of 1.2%. Spike recoveries of metals and F in the analyses of tissue ranged from 95 to 100%.

Statistical Analysis

Soil data were analyzed as a randomized complete block design using PROC GLM (SAS Institute 2001). Data were transformed using the natural logarithm function to adjust for heterogeneity of variance. The means of each contaminant of each land treatment unit were compared to the combined mean of all reference units for each contaminant using Duncan's multiple range test to determine organic and inorganic contamination of land treatment units.

Tissue data were analyzed using PROC MIXED (SAS Institute 2001) as a split block arrangement in a randomized block design with subsampling where units were considered blocks, treatments were the main factor, and season as the split-unit factor. A log transformation of data was used to control the heterogeneity of variance. Analysis of the simple effects of treatment (controlling for season) was performed using the SLICE option with the LSMEANS statement (SAS Institute 2001) when the treatment by season interaction was significant. All reference units were combined and Duncan's multiple range test was used to determine contamination of land treatment units. Pearson's linear correlation coefficients were calculated using PROC REG (SAS Institute 2001) to evaluate the relationship between mean metal and F content in tissue and soil for the study units.

RESULTS

Soil Properties

The soil pH of the units was near neutral (mean of 6.7) (Table 4) and typical of most Oklahoma soils which have an average pH of approximately 6.3 and may range in pH from 4.1 to 8.5 (Schroder 2001, unpublished data). All soils at the units had similar soil texture of loam. Soil electrical conductivity was not elevated and was less than 1.5 dS/m, which is the salinity level that might affect growth of salt sensitive plant species. Soil organic carbon was elevated as compared to typical Oklahoma soils and was likely due to residual petrochemicals. Oklahoma soils average 1.3% organic carbon and may range in organic carbon content from 0.3 to 2.6% (Schroder 2001, unpublished data).

Both the soil pH and the electrical conductivity of these units are conducive of good growth conditions and indeed the units in our study are heavily vegetated.

Extent of Soil Contamination

Metal concentrations in soil were significantly elevated on land treatment units as compared to reference unitsfor As, Cr, Ni, Pb and Zn (Table 5). Mean concentrations of all metals except Ti on reference units were similar to values reported for baseline soils of Oklahoma (Table 5). Titanium concentrations of baseline soils reported by Kabata-Pendias and Pendias (1984) were summarized from studies that used hydrofluoric acid (HF) in the digestion procedure. Most Ti in soil occurs as TiO_2 which can only be dissolved using HF. In this study, soil Ti levels from land treatment units and reference units were determined by U.S. EPA Method 3051, which doesn't utilize HF. Thus, Ti concentrations measured in our study are lower than soil Ti levels measured by HF digestion. The number of land treatment units (in parentheses) that had elevated levels of metal in soil as compared to the mean of all the reference units were Cr, Cu, Pb (5); Al, As, Ni, Sr, Zn (4); Ba (3); Cd, V (2); and Ti (0) (Table 6). Soils from three or more of the land treatment units were elevated in Al, As, Ba, Cr, Cu, Ni, Pb, Sr, and Zn. Aluminum in land treatment soil was significantly elevated and was approximately 1.5-fold greater than the overall mean for reference units. Similarly, land treatment contaminated soils were elevated in As (2- to 4-fold), Ba (approximately 1.5 fold), Cr (3- to 85-fold), Cu (2- to 140-fold), Ni (1.5 to 2-fold), Pb (2- to-150-fold), Sr (1.5 to 14-fold), and Zn (6- to 17-fold). In general, Units 1 and 3 had much higher levels of metal in soil than the other sites in our study.

Total F in soil and HCl-extractable F in soil were markedly elevated on all five of the land treatment units (Table 5). Total soil F of reference units was similar to total F in baseline soil, which ranges from 10 to 400 mg/kg (Table 5). The HCl-extractable F of reference site soils was also similar to HCl-extractable F in baseline soils. The total F was 6- to 34-fold greater on the land treatment units as compared to the overall mean of the reference units; HCl-extractable F was 50- to 210-fold greater (Table 6). In a prior investigation of petrochemical-contaminated units, Schroder et al. (2000) found elevated levels of both total F (range of 878 to 5257 mg/kg) and HCL-extractable F (range of 22 to 1026 mg/kg) in soil that received applications of oily sludges containing HF. In general, most land treatment units in our study had similar levels of both total F and HCl-extractable F as was reported by Schroder et al (2000).

PAH concentrations in soil were only significantly elevated on land treatment units as compared to reference units for naphthalene, acenaphthene, and benzo (g,h,i) perylene (Table 7). The majority of PAH concentrations in reference soils of our study were < 0.01 mg/kg which is the typical endogenous level for PAHs in soils as reported by Edwards (1983). Comparison using the Duncan's multiple range test indicates that there were differences between units in regard to the sixteen PAH concentrations. The number of land treatment units (in parentheses) that had elevated levels of PAHs in soil as compared to the mean of all the reference units were naphthalene, phenanthrene, benzo (g,h,i) perylene (3); acenaphthene, anthracene, pyrene, benzo (a) anthracene, chrysene, benzo (b) fluoranthene, benzo (a) pyrene, indeno (1,2,3-cd) pyrene,

dibenz (a,h) anthracene (2); and acenaphthylene, fluorene, fluoranthene, benzo (k) fluoranthene (1) (Table 8). TPH were elevated at all five land treatment units as compared to reference units (Table 7) and all five units exceeded the action level of 50 mg TPH/kg soil that has been established by Oklahoma (Table 8). Total PAH concentrations in soil were markedly elevated but the $B(a)P_{equiv}$ was not affected on land treatment units as compared to reference units (Table 7). Examination using the Duncan's multiple range test shows that Units 1 and 3 have much higher concentrations of total PAH and the sum of $B(a)P_{equiv}$ than the other units in our study (Table 8)

Tissue Content

Previous studies indicated that nine metals and F listed in Table 9 had a tendency to accumulate in tissue (Schroder et al. 2000). Therefore the investigation in this study was limited to the same nine metals and F. Metals that did not accumulate (Al, As, V) in tissue in previous studies are not reported in Table 7 (Schroder 2000). The mean content of Pb in bone was significantly elevated for cotton rats collected from land treatment units compared to reference units (Table 9). A significant treatment by season interaction did not exist for Pb content in bone or in kidney. The elevated concentrations of Pb in bone were approximately 460- to 2500-fold greater than the overall mean of the Pb in cotton rats collected from the reference units (Table 9). The elevated concentrations of Pb in kidney were approximately 10- to 36-fold greater than the overall mean of the Pb in cotton rats collected from the reference units (Table 10). The overall mean content of F in bone of cotton rats was markedly elevated

3- to 15-fold at land treatment units as compared to reference units (Table 9). A significant treatment by season interaction did not exist for F content in bone.

Dental Lesions

Approximately 62% of the cotton rats collected from land treatment units had dental lesions with approximately 43% of them having a score of ≥ 3. The majority (> 90%) of the cotton rats collected from the reference units did not have dental lesions. Severity of lesions varied among units and ranged from a score of zero (normal incisors) to a score of 5 (white chalky lower and upper incisors). F prevalence was not higher in winter than in summer. Regression analysis indicated there was a strong relationship ($r = 0.78$) between incisor score and F content in bone of cotton rats.

Bone and Soil Concentration Relationships

Regression analysis found a strong relationship between F content in bone and total F in soil ($r = 0.93$, Figure 1A) and F content in bone and HCl-extractable F in soil ($r = 0.73$, Figure 1B). A strong relationship was found between Pb content in bone of cotton rats and Pb in soil ($r = 0.96$). Significant relationships between other metal concentrations in soil (Ba, Cd, Cr, Cu, Ni, Sr, Ti, and Zn) and concentrations in tissue of cotton rats were not found.

DISCUSSION

Petrochemical waste disposal resulted in elevated levels of Al, As, Ba, Cr, Cu, Ni, Pb, Sr, Zn, and F in soil of the majority of land treatment units. The greatest degree (up to more than 50 times background) of soil metal contamination was found for Cr, Cu, Pb, and F and occurred on Units 1 and 3.

Loehr et al. (1993) investigated a land treatment unit where petrochemical waste had been applied for more than 30 years and found elevated Cr (280 mg/kg), Pb (130 mg/kg), Ni (110 mg/kg), and Zn (235 mg/kg) in soils. In general, the soils from Units 1 and 3 of our study had higher levels of soil Cr, Pb, and Zn but lower levels of Ni as reported by Loehr et al. (1993).

Total petroleum hydrocarbons and total PAH were elevated at all units with the degree of greatest contamination occurring at Units 1 and 3. Some PAHs (naphthalene, phenanthrene, and benzo (g,h,i) perylene) were also elevated in soils of the majority of land treatment units. The degree of soil contamination for these three PAHs was greater than 68 times background levels. Loehr et al (1993) found elevated levels of PAHs in the zone of incorporation of a land treatment unit where petrochemical waste had been applied for more than 30 years. However, the concentrations of PAHs reported by Loehr et al. (1993) in 15 cm core samples were 10- to 250-fold greater than the elevated concentrations of PAHs found on Units 1 and 3 of our study. The differences in PAH concentrations are probably due to different sampling depths because the type of petroleum waste applied to soils in this and other studies are similar. Our study sampled soils at depths of < 2 cm where enhanced microclimatic and biodegradation processes may be occurring in an attempt to better link soil concentrations to exposure of cotton rats. Other factors, such as climate, moisture, and the time since the last application of petrochemical waste may also account for the differences in concentrations between studies.

Wilson and Jones (1993) reviewed international clean up standards and reported that most countries use site-specific clean up standards while Canada has established a remediation criteria of 0.1 mg/kg for PAHs. This remediation criterion is the concentration that is considered generally protective of human and environmental health in soils and is used as a screening level to determine if further investigation is needed at a site. Units 1 and 3 have concentrations of PAHs that greatly exceed the Canadian remediation criteria and have much higher concentrations of PAHs than the other units in our study. Thus these units would require further investigation to determine the degree of risk posed to human and ecological health. Potential risk to humans from carcinogenic PAHs, measured by the sum of B(a)P equivalents, was on average slightly elevated across all land treatment units. Land treatment appears to have been effective as a remediation technology in reducing levels of individual PAHs and the sum of B(a)P equivalents to background levels on Units 2, 4, and 5 but not on Units 1 and 3. Units 1 and 3 but not Units 2, 4, and 5 may pose risk from elevated levels of carcinogenic PAHs in soil.

The soils from Units 1 and 3 contained higher levels of metals, PAHs, total PAH, and the sum of B(a)P equivalents than the other units in this study. There is limited historical data available for the units in our study but it possible that units 1 and 3 were managed differently than the other units of our study. The type of waste applied to each site was similar but it is possible the amount of petrochemical sludge applied to land was greater at Units 1 and 3 or that the waste applied to these units was more contaminated than at the other units.

Elevated levels of Pb and F were found in bone of cotton rats collected from some of the land treatment units. Total F in bone of cotton rats from reference units was similar to total F reported in other small mammalian studies conducted on uncontaminated units (Kay et al. 1975; Schroder et al. 1999). Levels of Pb in bone and kidney of cotton rats collected from reference units in our study were similar to reported values in bone and kidney of rodents collected from non-contaminated units which typically range from zero to < 3 mg/kg (Ma 1996). Venugopal and Luckey (1978) indicated chronic Pb exposure may result in renal dysfunction, reduced growth, and impairment of reproduction in mammals. Ingestion is the most common route of exposure for Pb and more than 90% of Pb tends to bioaccumulate in bone of small mammals (Talmage and Walton 1991). Several studies have shown that Pb tends to bioaccumulate in small mammals collected from highly contaminated Pb-zinc mining sites (Johnson et al. 1978; Roberts et al. 1978; Roberts and Johnson 1978; Andrews et al. 1989a). Our study shows that Pb accumulates in bone of cotton rats collected from land treatment units even though concentrations of Pb in soil at these units are much lower than the Pb-zinc mining sites. These results are very similar to those reported by Schroder et al. (2000) who found elevated levels of Pb in bone of cotton rats collected from petrochemical-contaminated sites. Cotton rats collected from Units 1 and 3 bioaccumulated Pb in bone and kidney to a greater extent than the cotton rats of the others units of our study and this is consistent with higher levels of Pb contamination in soils of these units. Although elevated levels of Cr, Cu, Ni, Sr, and Zn were found in soils of these units, these

metals did not bioaccumulate in tissues of cotton rats collected from land treatment units.

Dental lesions have been noted in several species of small mammals collected from sites with elevated levels of F in soil (Walton 1986 a, 1986 b, Cooke et al. 1996, Schroder et al. 1999, 2000). The greatest impact on cotton rats was associated with F contamination of land treatment units. Cotton rats collected from the land treatment units displayed dental lesions, a classic sign of fluorosis, consistent with elevated levels of F in all soils of the land treatment units. Fluoride contamination of soil resulted in severe degradation of teeth in cotton rats. Increased levels of F in bone have been associated with elevated levels of F in soil (Wright and Davison 1978; Andrews et al. 1982; 1989b). The results of this study are consistent with those of Schroder et al. (1999) who reported a mean of 1515 mg F/kg in bone of cotton rats collected from a landfarm that had received oily sludges containing hydrofluoric acid.

A strong relationship was found between F content in bone of cotton rats and HCl-extractable F and total F in soil. Regression analysis found a strong relationship between Pb content in bone and Pb in soil. However, the degree of contamination of bone with Pb was much smaller than F. Although elevated levels of other metals occurred in soils from the land treatment units, other metals did not bioaccumulate in bone of cotton rats and strong relationships were not found between metal content of bone and soil metal concentrations. The results of this study are similar to one conducted by Shore (1995) who examined published studies on small mammals and soil concentrations of Pb and F. He

reported strong relationships existed for Pb in bone of wood mice and field voles and Pb in soil.

Land treatment is a waste management technology where microbial communities in soil are used to degrade and detoxify oily petrochemical sludges. Up to 100 g oil waste/kg soil is commonly applied in land treatment operations. Land treatment is successful in degradation of most (>99%) of the organic compounds in the oily waste. However, small amounts of TPH and PAHs appear recalcitrant remaining in soil after 10-20 years. Land treatment was not effective in reducing levels of total PAHs and TPH to background levels on any of the units in our study. The potential risk to human and environmental health from small amounts of remaining TPH and PAHs is unclear. Limited information is available on risk posed from low level contamination of soil from TPH and PAH. Therefore, screening levels are usually based on background levels of these compounds. Most risk from these organic contaminants is associated with carcinogenic PAHs. Therefore, an index of potential toxicity associated with potential carcinogenic PAH, sum of B(a)P equivalents, should be more accurate than total contaminant concentrations at assessing risk from recalcitrant organic contaminants in soil. Land treatment effectively reduced both levels of individual PAHs and the sum of B(a)P equivalents to background levels on Units 2, 4, and 5. Organic contaminants should pose little risk at these units. However, individual PAHs and the sum of B(a)P equivalents were not reduced to background at Units 1 and 3. Land treatment increased risk of exposure to

carcinogenic PAHs at these units although the magnitude of the increase is unknown.

Soil contamination from inorganics (i.e. metals, F) in sludge associated with land treatment is much greater than organic compounds. Inorganic compounds do not biodegrade but can be immobilized by soil and made less available and pose less risk with time. Results from this and other studies clearly show that land application of petrochemical waste results in a large degree of soil contamination with Pb and F and subsequent bioaccumulation of these contaminants in cotton rats. Land treatment was ineffective in reducing these inorganic contaminants to levels that may not pose a significant risk to terrestrial mammals. Therefore to avoid accumulation of these contaminants in cotton rats and their possible deleterious effects, these contaminants should be measured and land application rates of petrochemical waste should be managed to avoid excessive loading of these contaminants into soil systems.

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Table 1. Location, climatological data, and history of land treatment units in Oklahoma.

Unit	Location	Mean annual rainfall (cm)	Mean annual temperature (°C)	History (Date of last petrochemical waste application)
1, 2	Southwest OK Stephens County	82.8	17.1	1983
3	Northcentral OK Kay County	132	15.4	Unknown
4, 5	Northeast OK Creek County	116	14.9	1981

Table 2. Scoring system for assessing severity of fluoride-induced lesions in incisors of cotton rats collected (1998-2000) from five land treatment units andaa matched reference units in Oklahoma.

Score	Incisor Characteristics
0	Normal: smooth, glossy deep yellow-orange
1	Slight striation or mottling in lower incisor
2	Definite mottling or striation (white chalky) in lower incisors
3	White chalky lower incisors; slight mottling in upper incisors
4	White chalky lower incisors; definite striation (or mottling) in upper incisor
5	White chalky lower and upper incisors

Table 3. Mean recovery, relative standard deviations, and detection limits for ten replicate analyses of metals, fluoride, PAHs, and TPH in standard reference materials(SRM).

SRM	Contaminant	Mean Recovery (%)	RSD (%)	Detection Limit
Soil CRM020-050	Al	99	1.2	0.50
	As	103	2.3	0.50
	Ba	99	2.3	0.05
	Cd	110	1.1	0.05
	Cr	107	3.8	0.05
	Cu	94	4.1	0.40
	Ni	100	2.4	0.10
	Pb	91	4.2	0.75
	Sr	107	1.9	0.35
	Zn	105	3.7	0.10
Soil CRM104-100	Naphthalene	81	3.5	0.004
	Acenaphthylene	84	3.8	0.004
	Acenaphthene	75	3.9	0.004
	Fluorene	71	3.1	0.004
	Phenanthrene	100	3.0	0.004
	Anthracene	81	3.6	0.004
	Fluoranthene	84	4.6	0.004
	Pyrene	93	4.4	0.004
	Benzo (a) anthracene	87	4.4	0.004
	Chrysene	79	6.2	0.004
	Benzo (b) fluoranthene	96	3.3	0.004
	Benzo (k) fluoranthene	96	4.5	0.004
	Benzo (a) pyrene	92	2.7	0.004
	Indeno (1,2, 3-cd) pyrene	98	4.0	0.004
	Dibenz (a,h) anthracene	85	4.4	0.004
	Benzo(g,h,i) perylene	101	3.1	0.004
Soil CRM350-100	TPH	94	6.3	10.0
Bone SRM 1486	F	97	2.5	25.0
	Sr	92	1.8	0.35
	Zn	93	1.2	0.10

Detection limits for soil are expressed as mg contaminant/kg soil and detection limits for bone are expressed as mg contaminant/kg bone.

Table 4. Soil properties of land treatment study units.

Unit	Soil pH	Soil OC[a]	Soil texture	Soil EC[b]
1	7.1	3.2	Sandy clay loam	0.17
2	6.0	1.9	Loam	0.14
3	7.3	4.9	Loam	0.39
4	6.5	2.7	Loam	0.12
5	6.4	3.3	Silt loam	0.01

[a] Organic carbon content in %.

[b] Electrical conductivity (dS/m).

Table 5. Comparison of the mean, median, and range of contaminant concentrations (inorganics) in soils collected (1998) from five land treatment units and matched reference study units in Oklahoma with baseline soil values.

	Mean Contaminant Concentration			Median Contaminant Concentration		Range of Contaminant Concentration		
Contaminant	Treatment units	Reference units	Baseline soils	Treatment units	Reference units	Treatment units	Reference units	Baseline soils
Al	23,900	17,500	71,000	25,600	15,600	8,260-38,400	9,040-33,800	10,000-300,000[a]
As	9.56*	3.44	7.20	9.29	3.38	1.92-19.6	1.96-5.34	0.10-97.0[b]
Ba	232	174	580	261	153	81.1-360	118-453	100-3000[b]
Cd	0.35	0.13	0.22	0.20	0.09	0.00-1.27	0.00-0.49	0.00-0.61[c]
Cr	429*	19.4	39.0	105	18.9	19.9-2,490	10.2-29.0	5.0-1,000[b]
Cu	219	6.83	10.5	19.0	6.06	6.62-1,290	3.55-13.2	2.7-23.9[c]
Ni	19.4*	11.0	21.0	20.1	10.1	5.62-43.6	6.16-19.8	6.1-41.7[c]
Pb	556*	10.2	16.5	60.6	8.90	14.8-2,450	5.16-29.9	5.1-27.2[c]
Sr	95.1	22.3	200	40.4	20.8	12.9-490	8.07-43.8	10.0-500[e]
Ti	230	245	2,770	246	183	81.1-332	136-559	684-4,080[c]
V	38.1	31.1	31.7	39.3	29.8	14.4-63.1	16.2-56.8	3.8-81.0[c]
Zn	330*	52.8	31.7	218	48.2	38.9-1160	29.6-102	22.3-127[c]
HCL F	591*	4.50	4.03	614	4.19	41.9-1,203	1.89-8.31	0.6-26.5[d]
Fusion F	3,490*	161	360	2710	140	374.7-10,100	86.7-345	10.0-400[e]

All values are in mg/kg soil.
[a]Lindsay 1979.
[b]Adriano 1986.
[c]Scott 1994.
[d]Schroder et al. 2000.
[e]Kabata-Pendias and Pendias 1984.
*significant from reference unit ($p < 0.05$)

Table 6. Mean concentrations of metals and fluoride in soils collected (1998) from five land treatment units and matched reference study units in Oklahoma.

Metal	Land Treatment Unit					Reference units
	1	2	3	4	5	
Al	29,700 a*	11,400 c	24,000 a*	24,000 a*	30,300 a*	17,500 b
As	12.0 a*	3.57 c	15.2 a*	7.55 b*	9.53 b*	3.44 c
Ba	289 a*	101 d	310 a*	210 bc	250 ab*	174 c
Cd	0.36 b*	0.09 cd	1.10 a*	0.19 c	0.02 d	0.13 cd
Cr	300 b*	31.0 e*	1650 a*	111 c*	53.1 d*	19.4 f
Cu	94.5 b*	11.4d*	955 a*	21.3 c*	12.6 d*	6.83 e
Ni	25.1 a*	7.63 d	30.2 a*	15.8 b*	18.4 b*	11.0 c
Pb	1490 a*	54.6 b*	1190 a*	29.8 c*	18.7 d*	10.2 e
Sr	77.7 b*	18.0 d	309 a*	38.4 c*	31.9c*	22.3 d
Ti	249 a	118 b	301 a	220 a	259 a	245 a
V	50.1 a*	17.4 c	32.7 b	39.0 ab	51.2 a*	31.1 b
Zn	253 b*	52.3 d	878 a*	309 b*	154 c*	52.8 d
HCl F	652 a*	946 a*	292 b*	843 a*	223 c*	4.50 d
Fusion F	5550 a*	2730 c*	3030 bc*	5170 ab*	981 d*	161 e

All values are in mg/kg soil. An asterisk indicates values are greater ($p < 0.05$) than the mean of reference units. Mean values with the same letter are not significantly different within a row.

Table 7. Comparison of the mean, median, and range of contaminant concentrations (organics) in soils collected (1998) from five land treatment units and matched reference study units in Oklahoma with baseline soil values.

	Mean Contaminant Concentration		Median Contaminant Concentration			Range of Contaminant Concentration	
Contaminant	Treatment units	Reference units	Treatment units	Reference units	TEF[a]	Treatment units	Reference units
Naphthalene	0.290*	0.003	0.195	0.005	0.0	0.019-1.120	0.000-0.009
Acenaphthylene	0.017	0.000	0.000	0.000	0.0	0.000-0.380	0.000-0.000
Acenaphthene	0.002*	0.000	0.000	0.000	0.0	0.000-0.010	0.000-0.000
Fluorene	0.009	0.000	0.000	0.000	0.0	0.000-0.128	0.000-0.000
Phenanthrene	0.514	0.024	0.138	0.002	0.0	0.009-2.36	0.000-0.130
Anthracene	0.354	0.000	0.044	0.000	0.0	0.006-3.21	0.000-0.000
Fluoranthene	0.070	0.001	0.009	0.000	0.0	0.000-0.472	0.000-0.007
Pyrene	0.390	0.002	0.062	0.000	0.0	0.008-2.46	0.000-0.017
Benzo (a) anthracene	0.309	0.003	0.044	0.000	0.1	0.008-1.98	0.000-0.17
Chrysene	0.685	0.003	0.081	0.000	0.001	0.012-5.59	0.000-0.032
Benzo (b) fluoranthene	0.308	0.007	0.031	0.008	0.1	0.013-2.50	0.000-0.018
Benzo (k) fluoranthene	0.023	0.000	0.008	0.000	0.01	0.000-0.302	0.000-0.004
Benzo (a) pyrene	0.325	0.006	0.050	0.007	1.0	0.000-2.23	0.000-0.021
Indeno (1,2, 3-cd) pyrene	0.386	0.004	0.046	0.006	0.1	0.016-3.25	0.000-0.010
Dibenz (a,h) anthracene	0.298	0.002	0.060	0.000	1.0	0.016-1.76	0.000-0.010
Benzo(g,h,i) perylene	3.029*	0.002	0.700	0.000	0.0	0.006-19.9	0.000-0.021
TPH	1,050*	28.7	810	24.5		127-5,910	0.000-78
Total PAH	6.99*	0.057	1.30	0.032		0.184-36.4	0.000-0.221
$B(a)P_{equiv}$	0.724	0.010	0.122	0.008		0.030-4.32	0.000-0.036

All values are in mg/kg soil.

[a]Toxicity Equivalency Factor (U.S. EPA 1993).

*significant from reference site ($p < 0.05$).

Table 8. Mean concentrations of polycyclic aromatic hydrocarbons in soils collected (1998) from five land treatment units and matched reference study units in Oklahoma.

	Land Treatment Unit					
Metal	1	2	3	4	5	Reference Units
Naphthalene	0.607 a*	0.033 c	0.505 a*	0.255 b*	0.053 c	0.003 c
Acenaphthylene	0.000 b	0.000 b	0.006 b	0.078 a*	0.002 b	0.000 b
Acenaphthene	0.000 c	0.001 bc	0.004 ab*	0.004 a*	0.002 bc	0.000 c
Fluorene	0.000 b	0.002 b	0.035 a*	0.008 b	0.002 b	0.000 b
Phenanthrene	0.596 b*	0.168 c*	1.63 a*	0.148 cd	0.003 d	0.024 d
Anthracene	0.278 b*	0.026 c	1.37 a*	0.079 c	0.015 c	0.000 c
Fluoranthene	0.026 b	0.009 b	0.301 a*	0.009 b	0.004 b	0.001 b
Pyrene	0.434b*	0.058 c	1.39 a*	0.058 c	0.014 c	0.002 c
Benzo (a) anthracene	0.271 b*	0.050 c	1.19 a*	0.024 c	0.013 c	0.003 c
Chrysene	0.468 b*	0.074 c	2.70 a*	0.045 c	0.025 c	0.003 c
Benzo (b) fluoranthene	0.239 b*	0.025 c	1.23 a*	0.032 c	0.017 c	0.007 c
Benzo (k) fluoranthene	0.000 b	0.013 b	0.090 a*	0.010 b	0.002 b	0.000 b
Benzo (a) pyrene	0.547 b*	0.046 c	1.00 a*	0.016 c	0.013 c	0.006 c
Indeno (1,2, 3-cd) pyrene	0.414 b*	0.028 c	1.40 a*	0.061c	0.026 c	0.004 c
Dibenz (a,h) anthracene	0.415 b*	0.032 c	0.907 a*	0.106 c	0.031 c	0.002 c
Benzo(g,h,i) perylene	3.81 b*	0.130 d	10.2 a*	0.806 c*	0.107 d	0.002 d
TPH	419 b*	760 ab*	2030 a*	1680 a*	370 b*	28.7 c
Total PAH	8.10 b*	0.696 d*	24.0 a*	1.79 c*	0.350 d*	0.057 e
$B(a)P_{equiv}$	1.06 b*	0.089 c	2.30 a*	0.134 c	0.049 c	0.010 c

All values are in mg/kg soil. An asterisk indicates values are greater ($p < 0.05$) than the mean of reference units.
Mean values with the same letter are not significantly different within a row.

Table 9. Mean concentration of metals and fluoride in bone of cotton rats collected (1998-2000) from five land treatment units and matched reference study units in Oklahoma..

Metal	Land Treatment Unit					Reference units
	1	2	3	4	5	
Ba	80.2 b	76.6 b	64.8 b	75.6 b	70.5 b	117 a
Cd	0.58 b	0.05 b	0.06 b	0.88 a*	0.08 b	0.12 b
Cr	2.81 ab	2.63 b	2.93 ab	2.71 b	3.07 a	2.74 ab
Cu	1.29 a	1.27 a	1.23 a	1.43 a	1.49 a	1.37 a
Ni	0.83 ab	0.61 ab	0.30 b	0.48 b	2.38 ab	3.58 a
Pb	24.8 a*	4.60 b*	12.1 a*	0.0 c	0.03 c	0.01 c
Sr	137 bc	125 c	147 ab	127 c	117 c	158 a
Ti	1.80 a	0.37 b	1.50 a	1.65 a	1.46 a	1.23 a
Zn	191 a*	157 c	176 ab	182 ab	170 bc	171 bc
F	2210 a*	1040 c*	1820 ab*	1390 b*	496 c*	144 d

All values are in mg/kg bone. An asterisk indicates values that are greater ($p < 0.05$) than the mean of the reference units. Mean values with the same letter are not significantly different within a row.

Table 10. Mean concentration of metals in kidney of cotton rats collected (1998-2000) from five land treatment units and matched reference study units in Oklahoma.

Metal	Land Treatment Unit					Reference units
	1	2	3	4	5	
Ba	18.3 c	34.9 a*	26.5 ab	21.9 bc	22.3 bc	23.9 bc
Cd	0.14 ab	0.06 b	0.24 a	0.12 b	0.09 b	0.30 a
Cr	0.11 a	0.19 a	0.18 a	0.15 a	0.11 a	0.27 a
Cu	3.76 a	3.76 a	3.50 a	3.47 a	3.40 a	3.60 a
Ni	0.21 b	0.40 ab	0.08 b	0.10 b	0.57 a	0.37 ab
Pb	1.08 a*	0.31 b	1.07 a*	0.08 c	0.00 c	0.03 c
Sr	0.47 a	0.39 a	0.54 a	0.41 a	0.50 a	0.47 a
Ti	0.16 b	0.22 a	0.21 a	0.16 b	0.22 a	0.19 ab
Zn	26.7 b	28.5 ab	31.7 a	26.2 b	28.2 ab	28.9 ab

All values are in mg/kg kidney. An asterisk indicates values that are greater ($p < 0.05$) than the mean of the reference units. Mean values with the same letter are not significantly different within a row.

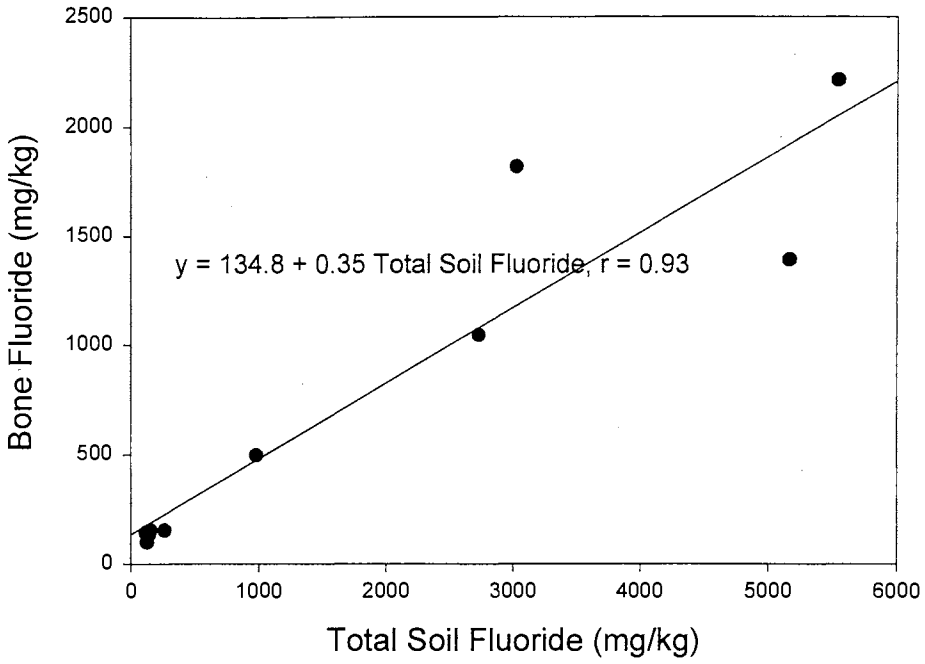

Figure 1A. Mean bone fluoride vs. mean total soil fluoride for soils and cotton rats collected (1998-2000) from five land treatment units and matched reference units in Oklahoma.

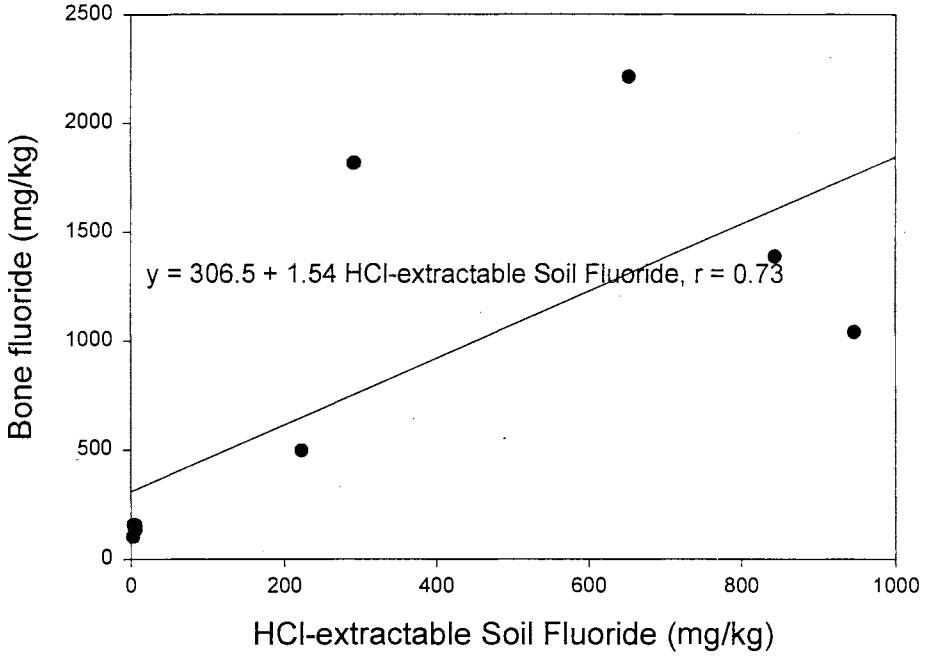

Figure 1B. Mean bone fluoride vs. mean HCl-extractable soil fluoride for soils and cotton rats collected 91998-2000) from five land treatmentunits and matched reference units in Oklahoma.

CHAPTER II

AN IN VITRO GASTROINTESTINAL METHOD TO ESTIMATE RELATIVE BIOAVAILABLE CADMIUM IN CONTAMINATED SOILS

Abstract

The capacity of an in vitro gastrointestinal (IVG) method to predict relative bioavailable Cd from soil ingestion was evaluated. Bioaccessible Cd, determined by the IVG method, was compared with relative bioavailable Cd measured from dosing trials using juvenile swine for 10 soils contaminated with Cd from 23.8 to 465 mg kg^{-1}. The effect of the food-dosing vehicle (e.g., dough) in the IVG method was evaluated. Bioaccessible Cd was measured in the gastric extraction and intestinal extraction steps of the IVG. Means for bioaccessible Cd, in parentheses, were gastric extraction step without dough (63.0%) > intestinal extraction step without dough (39.1%) > gastric extraction step with dough (38.2%) > intestinal extraction step with dough (12.9%). It is possible that phytic acid associated with the dough addition decreased bioaccessible Cd. In vivo relative bioavailable Cd ranged from 10.4 to 116% with a mean of 63.4%. Linear relationships between IVG gastric extraction step without dough ($r = 0.86$), IVG intestinal extraction step with dough ($r = 0.80$) and in vivo relative bioavailable Cd were found. Inexpensive in vitro methods may be useful in estimating the relative biovailability of Cd in soils from contaminated sites.

Introduction

Cadmium (Cd) is a naturally occurring metal found as a mineral combined with other elements such as oxygen (i.e., $CdCO_3$) or sulfur (CdS, $CdSO_4$). Cd typically ranges from 0.1 to 1.0 mg kg^{-1} in the earth's crust (1-3). Typical mean Cd for surface soils worldwide averages 0.53 mg kg^{-1} and ranges from 0.06 to 1.1 mg kg^{-1} (4). Cd levels in uncontaminated surface soils of the United States ranges from 0.005 mg kg^{-1} to 2.4 mg kg^{-1} with a geometric mean of 0.27 mg kg^{-1} (5). Cadmium is used for a variety of industrial and consumer materials, including nickel-cadmium batteries; stabilizers for polyvinyl chloride; pigments used in plastics, ceramics, and glasses; engineering coatings on steel and some non ferrous metals; and components of specialized alloys (2, 6-8). Cadmium contamination of soil may result from mining and smelting of (Zn) ores, atmospheric deposition from metallurgical industries, incineration of plastics and batteries, sewage sludge application to land, and burning of fossil fuels (9). Total releases of Cd to the environment due to anthropogenic activities is estimated to range from 2,000 to 6,500 MT yr^{-1} with major contributions from mining activities and burning of fossil fuels (3).

Cadmium is considered a human carcinogen by the International Agency for Research on Cancer (2) as well as a probable human carcinogen by the United States Environmental Protection Agency (10). Human exposure to Cd can occur through the consumption of contaminated foods or drinking water; the incidental ingestion of soil or dust; the inhalation of Cd-containing particles from ambient air; from the inhalation of vaporized Cd in cigarette smoke; or from

working in occupations involving exposure to Cd fumes and dust (1). Chronic exposure to Cd may result in obstructive pulmonary disease, emphysema, and kidney disease (3, 11). Cadmium is absorbed and retained by both terrestrial and aquatic plants, and, as a consequence, concentrated in the liver and kidney of animals that consume these plants. Extremely high dietary intake of Cd has been associated with osteomalacia, osteoporosis, and spontaneous fractures, which are conditions collectively termed "itai-itai" (ouch-ouch) and were originally documented in postmenopausal women living in the Cd-contaminated Fuchu area of Japan prior to and during World War II (12).

Cadmium pollution of soil has been reported in 433 of the 766 National Priorities List hazardous waste sites, and concentrations as high as 750 mg kg^{-1} have been reported in soils in the vicinity of Zn smelters (3, 13). Incidental ingestion of soil by children is an important pathway in the assessment of public health risks due to exposure of metal-contaminated soils. Most risk from Cd in ingested soil or waste materials is associated with the fraction of the ingested soil or waste material that is available for absorption from the gastrointestinal tract into the circulatory system. The amount of Cd absorbed through the gastrointestinal tract (bioavailable Cd) may be described in absolute or relative terms. Absolute bioavailability (ABA), also referred to as the oral absorption fraction, is equal to the absorbed dose/ingested dose as described by equation 1.

$$ABA = \frac{Absorbed\ Dose}{Ingested\ Dose} \quad (1)$$

Relative bioavailability (RBA) is the ratio of the ABA of Cd present in some test material (study soil) compared to the ABA of Cd in an appropriate reference material (equation 2).

$$RBA = \frac{ABA(study\ soil)}{ABA(reference\ material)} \quad (2)$$

Cadmium chloride ($CdCl_2$), a readily soluble form of Cd and thus easily absorbed from the gastrointestinal tract, is used as the reference material in the critical toxicity study reported in the Integrated Risk Information System (10). Relative bioavailability can be determined experimentally without specifically measuring absolute bioavailability. For example, the tissue concentration of Cd in animals dosed with study soil can be compared with tissue concentration of Cd in animals dosed with reference material. In this case, relative bioavailability is defined by equation 3.

$$RBA = \frac{Tissue\ Cd(study\ soil)}{Tissue\ Cd(reference\ material)} \quad (3)$$

Often, baseline risk assessments used for contaminated sites assume that the relative bioavailability of Cd is 100% (e.g. bioavailability of Cd in contaminated soil / media is the same as the bioavailability of $CdCl_2$ used in the IRIS critical toxicity study). However, due to the different geochemical and physical forms of Cd present in contaminated soils and waste, the relative bioavailability of Cd is likely to be less than 100% and may pose less risk to humans than highly soluble forms of ingested Cd. The soil matrix lowered the relative bioavailability of Cd to rats in a dosing study using Cd-spiked artificial soil

(14). The relative bioavailability of metal contaminants (e.g., Pb and As) in waste materials from hazardous waste sites has been assessed using in vivo animal dosing trials and used for risk assessment. In vivo dosing trials using animal models (juvenile swine, monkeys) are both lengthy and extremely expensive. Most dosing studies using contaminated soil have focused on Pb and As. Fewer studies have focused on measuring Cd relative bioavailability in contaminated soil using animal models (15).

Less expensive in vitro chemical extraction methods that simulate gastrointestinal biochemistry have been developed to estimate relative bioavailable Pb (16, 17) and As (18). The amount of contaminant dissolved in the gastrointestinal environment that is soluble and available for absorption is termed "bioaccessible." Several in vitro methods are sequential extractions with two distinct extraction steps: a gastric phase extraction that simulates the acidic biochemical stomach environment and a subsequent intestinal phase extraction that simulates the biochemical environment of the small intestine. The fraction of the contaminant dissolved by the in vitro procedure, the "bioaccessible" contaminant, has been used to estimate the relative bioavailability of the contaminant in soil (19). Hamel et al. (20) reported an in vitro method to estimate bioaccessible Cd in soils, but they did not relate their method to relative bioavailable Cd measured by an animal model. The in vitro physiologically based extraction test (PBET) of Ruby et al. (17), which does not use food in the extraction in order to mimic fasting conditions, has been shown to predict accurately relative bioavailable Pb in contaminated soil and media (19). The in

vitro gastrointestinal (IVG) method developed by Rodriguez et al. (18) is an accurate predictor of relative bioavailable As in contaminated soils and waste materials while utilizing food in the extraction procedure. To our knowledge, an in vitro method to estimate relative bioavailable Cd associated with soil ingestion has not been reported. Also, the effect of the presence or absence of food in in vitro extraction procedures on the capacity to estimate the relative bioavailability of Cd in contaminated soil has not been reported. The objective of this study was to determine the capacity of the IVG method of Rodriguez et al. (18), with and without food, to predict relative bioavailable Cd in contaminated soil as measured in vivo juvenile swine.

Methods and Materials

Contaminated Soils. Ten contaminated soils from seven different hazardous waste sites were evaluated using the IVG method of Rodriguez et al. (18). Air-dried soil was sieved through nylon mesh (< 250 μm) to obtain the soil fraction considered to adhere to fingers and likely to be ingested. Total metal content of soil was determined by acid digestion using U.S. EPA Method 3050 (21), and elemental analysis was conducted using a high resolution Thermo Jarrell Ash IRIS inductively coupled plasma atomic emission spectrophotometer (ICP-AES).

In Vivo Swine Dosing Study. In vivo relative bioavailable Cd in contaminated soil was determined by in vivo dosing trials using standard operating procedures developed by Dr. Stan Casteel of the University of Missouri-Colombia Veterinary Medical Diagnostic Laboratory and approved by U.S. Environmental Protection Agency (EPA) Region 8 for Pb contaminated soil

(22) with the exception that two dose groups per soil were used instead of three dose groups. Male swine (5-6 wk old) weighing 10-12 kg were dosed for 15 d with varying concentrations of Cd in substrates. Five swine were randomly assigned to treatment groups consisting of Cd-contaminated soil dosing groups, a negative control group (no substrate), and positive control groups that received oral $CdCl_2$. All swine were individually housed in stainless steel cages and daily fed a powdered grower's diet (referred to as dough in this paper) which approximated 5% of their body weight (Ziegler Bros., Inc., Gardner, PA). The diet was a commercially formulated to have a protein content of approximately 19% and contained less than 0.01 mg Cd/kg diet. After a 7-d acclimation period, the swine were dosed with contaminated soil that was placed in a 5-10 g doughball of moistened grower diet. The swine were dosed twice daily to mimic childhood cadmium ingestion which is likely to occur between meals while children are in a fasted or semi-fasted state. A dose of 6.25 mg soil per kg body weight per day was used with half of the first dose being delivered at 9:00 am after an overnight fast and the second half of the dose being delivered at 3:00 pm after a 4-h fast. All swine were fed 2 hr after dosing. The swine were euthanized, by electrical stunning and subsequent electrocution at the end of the dosing trail in accordance with procedures recommended by the American Veterinary Medical Association. All pigs within a treatment group were euthanized and necropsied on the same day over a 2 to 4 hour period of time. Kidneys were removed and frozen (–70°C) for subsequent metal analyses.

Tissue Analyses. Five grams of renal cortex kidney tissue (wet weight) were digested overnight at 90°C in 7.5 ml of concentrated trace metal HNO_3 and diluted to a final volume of 25.0 ml with deionized distilled water. Digested sample was filtered through a 0.45-µm membrane filter, and Cd was determined using ICP-AES. Blanks, spikes, and duplicate analyses were conducted every 20 samples to meet quality assurance and quality control (QA/QC) requirements. Relative Cd bioavailability was estimated using measured Cd concentration in kidney.

Calculation of In Vivo Relative Bioavailability. RBA was calculated using equation 3. Cadmium chloride was selected as the reference material in our study because it is a readily soluble form of Cd that is easily absorbed and used in IRIS. More specifically for each study substrate, the amount of Cd bioaccumulated in kidney (e.g., mg Cd kg^{-1} kidney) was plotted as a linear regression of Cd dosed (e.g., µg Cd kg^{-1} body weight day^{-1}) for both reference material and study substrate. The RBA was calculated by dividing the slope for the study substrate by the slope for the reference material.

In Vitro Gastrointestinal Method (IVG). Bioaccessible Cd was estimated in our study using the IVG method developed by Rodriguez et al. (18). The IVG method is a two-step sequential extraction; a gastric solution extraction followed by an intestinal solution extraction. An equivalent amount of the dosing vehicle (200 g of wet feed termed "dough") was added to the gastric solution to mimic the in vivo dosing of 100 mg soil to 5 g of dough. Gastric solution was 0.15 M NaCl and 1% porcine pepsin (Sigma Chemical Company, St. Louis, MO, cat. no.

P7000). The in vitro method was conducted using 1 L glass jars in a water bath at body temperature (37°C). Soil (4.0 g) was placed in 600 ml of gastric solution to which either 0 g (e.g., no dough) or 200 g of dough was added. The pH of the gastric solution was adjusted to pH 1.8 with trace metal grade HCl. Anaerobic conditions were maintained by constantly bubbling argon through the solution and pH was continuously monitored and adjusted to 1.8 throughout the 1-h procedure. Mixing (to simulate gastric mixing) was maintained during the procedure using individual paddle stirrers set at a speed of 100 rpm. After 1 h, 40 mL of gastric solution, removed for Cd analysis, was replaced with 40 mL of fresh gastric solution. Subsequently, the extraction solution was modified to simulate intestinal solution by adding saturated $NaHCO_3$ solution to adjust the pH to 5.5 followed by the addition of 2.10 g of porcine bile extract (Sigma Chemical Company, St. Louis, MO, cat. no. B8631) and 0.21 g of porcine pancreatin (cat. No. P1500). A small amount of anti-foam agent (decanol) was added to each reaction vessel. After 1 h, 40 mL of intestinal solution was collected for Cd analysis. Gastric and intestinal solution samples were centrifuged for 15 min at 10,000 rpm and filtered through 0.45-µm membrane filters immediately after their collection. The samples were acidified to pH of 2 using trace metal HCl, and Cd was determined using ICP-AES.

In Vitro Bioaccessibility. Bioaccessible Cd was calculated by dividing the Cd concentration measured in the in vitro gastric or intestinal solutions by the total soil Cd content (e.g., USEPA method 3050).

Statistical Analysis. Analysis of variance using PROC MIXED (23) was performed to evaluate the effects of extraction step (gastric or intestinal) and method (dough or no dough addition) on bioaccessible Cd. The data were analyzed as a split plot arrangement in a randomized complete block design. The combination of replicate and soil were used as blocks, method was the whole plot factor, and phase was the split plot factor. Simple effects of method given phase and phase given method were analyzed with a SLICE option in the LSMEANS statement. The relationship between mean in vitro bioaccessible Cd and mean in vivo relative bioavailable Cd was determined using PROC REG (23).

Results and Discussion

Soil Cd Concentrations. The Cd content of the contaminated soils ranged from 23.8 to 465 mg/kg (Table 1), which is well above the Cd content of 0.06 to 1.1 mg kg^{-1} reported for uncontaminated soils (4). The study soils were also contaminated with other heavy metals (e.g., Pb, Zn) and metalloids (As) (Table 1). The soils also contained significant amounts of elements known to affect Cd uptake and bioavailability, including Fe, Ca, and Zn.

Tissue Content and Soil Doses. Cadmium bioaccumulated in the renal cortex of juvenile swine dosed with contaminated soil. Kidney Cd ranged from 0.036 mg kg^{-1} for the control group to 2.41 mg kg^{-1} for the highest dose group (Table 2). Our results are similar to those of Schilderman et al. (14) which showed that rats bioaccumulated Cd in liver and kidney when dosed with a Cd-spiked artificial soil. The concentrations of Cd found in kidney of the control

group of our study are comparable to 0.019 mg kg^{-1}obtained in their study for control rats.

Doses in our study ranged from 0.59 µg Cd kg^{-1} body weight/day to 160 µg kg^{-1} body weight day^{-1}, and concentrations in the kidney of pigs increased in a dose-dependent manner within each soil (Table 2). Linear regression showed that Cd in the kidney of pigs increased as dose in soil increased ($p < 0.01$, $r = 0.92$; Figure 1). Our results are comparable to those of Lehman and Klassen (24) who administered $CdCl_2$ orally to rats and found that the retention of Cd after ingestion was dose-dependent and resulted from increased absorption of Cd at higher doses. Dose-dependent relationships have also been reported for mice, quail, dogs, and swine in other studies that used $CdCl_2$ (24-29).

In Vivo Relative Bioavailable Cd. Percentage relative bioavailable Cd estimated using the juvenile swine model ranged from 10.4 to 116% and averaged 63.4% for the soils evaluated in our study (Table 3). Relatively little literature has been generated regarding the oral bioavailability of Cd from contaminated soils. Schilderman et al. (14) exposed male rats to a dose of 650 µg Cd/day from a spiked artificial soil and reported the relative bioavailability of Cd as 46% based on kidney data. In another in vivo rat feeding study conducted on a soil sample from a zinc smelter in Bartlesville, OK; the relative bioavailability of Cd based on liver and kidney data was reported as 33% (30, 31).

In Vitro Bioaccessible Cd. Bioaccessible Cd measured by the gastric extraction step) using dough in the extraction ranged from 11.7 to 47.5% with an overall mean of 38.2% (Table 3). In vitro Cd measured by the intestinal

extraction step using dough in the procedure ranged from 4.05 to 19.5% and averaged 12.9% for soils, which was much lower extraction step Cd (Table 3). In part, the reduction of measured Cd between the gastric extraction step and the intestinal extraction step can be attributed to the reduced solubility of Cd in the higher solution pH of the intestinal extraction step vs. gastric extraction step (pH 5.5 vs. 1.8). Gastric extraction step Cd without using dough in the extraction ranged from 21.3 to 95.9% with an overall mean of 63.0% (Table 3). Within the intestinal extraction step, in vitro Cd without using dough in the extraction ranged from 15.0 to 55.0% with an overall mean of 39.1% (Table 3). Similar to results obtained with the IVG with dough method, Cd extracted for all 10 soils was intestinal extraction step < gastric extraction step. Mean gastric extraction step Cd both with and without dough were greater than mean intestinal extraction step Cd ($p < 0.001$, Table 3). Limited data are available concerning the estimation of the bioaccessibility of Cd in soils using in vitro extraction procedures. Hamel et al. (20) used an in vitro extraction procedure at a pH of 1.1 to evaluate the bioaccessibility of Cd and other contaminants in a National Institute of Standards and Testing standard reference material (NIST Soil SRM 2710). Their study investigated the effect of varying the liquid to solid ratio on the extractability of As, Cr, Ni, Cd, and Pb without using food in the extraction. Their results indicated that the solubility of Cd in the SRM 2710 was affected only slightly by changing the liquid-to-solid ratio, and they reported the bioaccessibility of Cd as 54.1% at a liquid to solid ratio of 100:1 and 61% at a liquid to solid ratio of 200:1. For comparison, in vitro bioaccessible Cd was measured in the same NIST SOIL

SRM 2710 both with and without dough. IVG gastric extraction step Cd (without dough) was 69% and was similar to that found by Hamel et al. (20), while intestinal extraction step Cd (without dough) was 38%. Measured gastric extraction step Cd (with dough) was 44% and intestinal extraction step Cd (with dough) was 20%.

Dough vs. No Dough. Comparison of the dough vs. no-dough methods showed that the mean Cd of 51.0% for the combined gastric and intestinal extraction steps without using dough in the extraction was greater than the mean Cd extracted of 25.6% for the combined gastric and intestinal extraction steps using dough in the extraction ($p < 0.001$). There were no significant interactions between method and extraction step (e.g., gastric vs. intestinal) ($p = 0.766$). In a review on human bioavailability. Ragan (32) reported that the solubility and absorption of Fe, Cd, and Pb may be lowered by dietary components such as oxalates, phosphates, and phytates. Lind et al. (33) fed mice diets containing 0.050 mg/kg Cd from wheat bran, sugar-beet fibre, carrots, or $CdCl_2$ mixed in a synthetic low-Cd feed and found that the group receiving the wheat-bran diet had significantly lower fractional Cd accumulation in the liver and kidneys as compared to the other groups in the experiment. The wheat-bran diet had significantly higher levels of phytates as compared to the other diets in their study, and they concluded that the decreased fractional absorption of Cd from the wheat-bran diet was due to the formation of insoluble Cd-phytate complexes. Turecki et al. (34) conducted an in vitro study using male rat intestines and showed that the absorption of Cd was significantly lowered in the presence of

phytic acid. The calcium-phytate complex has a strong affinity for both Pb and Cd (35). Chan et al. (29) fed mice a diet of field-grown, Cd-contaminated grain or grain that had been "amended" (i.e., spiked) with soluble cadmium nitrate and compared bioaccumulation of these diets with a gavage of soluble cadmium nitrate. Their results showed that for similar doses the administration of cadmium nitrate as a gavage resulted in higher accumulation of Cd in livers and kidneys of mice as compared to the spiked grain or when Cd was plant-incorporated into the grain. Results from the above studies show that the presence of food decreases the solubility and the absorption of Cd in the gastrointestinal system of mice. It is possible that Cd-phytate complexes or insoluble complexes involving phytic acid and Ca with Cd coprecipitating the complex were formed during the in vitro extraction of soils using dough, which resulted in lower bioaccessible Cd as compared to the extractions that did not use dough.

The dough material has a P content of 7580 mg kg^{-1}. A considerable amount of P was dissolved in the IVG methods. Results show that the inclusion of dough increased soluble P in the gastric extraction step from 53.9 to 1900 mg L^{-1} without soil. Similarly, inclusion of dough increased soluble P in the intestinal extraction step from 64.5 to 1740 mg L^{-1} without soil. Results show that the inclusion of dough increased soluble P in the gastric extraction step from 49.1 to 1810 mg L^{-1} with contaminated SRM 2710 soil. Similarly, inclusion of dough increased soluble P in the intestinal extraction step from 56.7 to 1570 mg L^{-1} with contaminated SRM 2710 soil. The equilibrium geochemical speciation model MINTEQA2 (ver. 4.0) was used to investigate the possibility that the addition of

dough to the IVG procedure resulted in precipitation of inorganic cadmium phosphates, thus lowering measured concentrations of in vitro Cd (36). In vitro concentrations of Ca, Cd, Fe, P, Zn, Na, Cl, and solution pH were used as model inputs. Total dissolved P was assumed to be present as orthophosphate ion, which would be consistent with the most likely scenario to form cadmium phosphate mineral precipitate. MINTEQA2 results predicted that the IVG gastric extraction and intestinal extraction step solutions, with and without dough, were unsaturated with respect to cadmium phosphate solid phases for contaminated soil. These results suggest formation of cadmium phosphate precipitates from dough addition could not be used to explain decreased in vitro Cd associated with dough addition.

Relationships Between Bioaccessible Cd and In Vivo Relative Bioavailable Cd. Linear regression indicated that the relationship was not significant ($p = 0.098$, $r = 0.55$) between the gastric extraction step Cd using dough in the extraction and in vivo relative bioavailable Cd (Figure 2A). Regression analysis showed that there was a strong linear relationship between the intestinal extraction step Cd using dough in the extraction and in vivo Cd ($p < 0.01$, $r = 0.77$) (Figure 2B). A strong linear relationship was found between the gastric extraction step Cd without using dough in the extraction and in vivo relative bioavailable Cd ($p < 0.01$, $r = 0.86$) (Figure 2C). However, a significant relationship between the intestinal extraction step and in vivo Cd was not found ($p = 0.111$, $r = 0.54$) (Figure 2D).

Gastrointestinal Interactions. Many interactions in the gastrointestinal systems of various animals affect Cd absorption. Gastrointestinal absorption of Cd is a complex and dynamic process involving dissolution, absorption, and interactions with other dietary components. Iron deficiencies in both humans and rats have been shown to increase Cd absorption in both humans and rats (37, 38). Additionally, dietary deficiencies of Ca, Zn, and protein enhanced absorption of Cd in humans and mice (3, 39-41). The ratios of total elemental content for Zn:Cd ranged from 25 to 1640 with a median value of 124; for Ca:Cd, ranged from 6 to 2950 with a median value of 372; and for Fe:Cd, ranged from 84 to 1630 with a median value of 447. The ratio of Zn:Cd extracted during the gastric extraction step with dough ranged from 33 to 1270 with a median value of 288, while the ratio of Fe:Cd extracted during the gastric extraction step with dough ranged from 6 to 7930 with a median value of 37. The extractable Zn:Cd ratio for the intestinal extraction step using dough ranged from 10 to 441 with a median value of 67, and the extractable Fe:Cd ratio (intestinal extraction step using dough) ranged from 3 to 4930 with a median of 8. It is possible large amounts of Ca, Fe, and/or Zn decreased Cd absorption in some soils more than others.

Gastrointestinal absorption of Cd and heavy metal contaminants is a dynamic process involving dissolution and absorption). Biological gastrointestinal digestive processes are quite complicated and difficult to simulate in vitro. In vitro gastrointestinal methods based solely on measuring heavy metal contaminant solubility do not account for active and passive

absorption processes and can only be accurate estimators of contaminant bioavailability if dissolution of the contaminant matrix is the rate-limiting step in this kinetic process (19, 42). Strong relationships from several studies between in vitro bioaccessible Pb or As and in vivo Pb or As suggest that dissolution of the contaminant matrix is the rate-limiting step for As and Pb in contaminated soils (19, 42). Bioaccessible Pb measured in the simulated gastric environment without dough (e.g., gastric extraction step) is correlated with in vivo Pb (17, 19, 43). Weaker relationships are found between bioaccessible Pb in the in vitro intestinal environment and in vivo Pb. Similarly, bioaccessible Cd was related to in vivo Cd in the in vitro gastric environment without dough addition (Figure 2). Strong relationships are found between bioaccessible As measured by IVG gastric extraction step and intestinal extraction step with dough and in vivo As (18) and bioaccessible As measured by IVG gastric extraction step and intestinal extraction step without dough and in vivo As where in vivo As was measured from juvenile swine dosing trials. The relationship between bioaccessible Cd and in vivo Cd (Figure 2) is more complex than As because it is affected by the presence of dough. The gastric extraction step of the IVG method of Rodriguez et al. (18) is related to in vivo Cd when dough is not present, but the intestinal extraction step of the IVG method is related to in vivo Cd when dough is present (Figure 2).

Precision of the IVG Method. The precision of the IVG method with and without dough was evaluated by conducting 12 replicated extractions of soil 1 (Table 4). The relative standard deviations for all IVG methods were < 10%,

indicating that the IVG method is precise. The inclusion of dough in the gastric extraction or intestinal extraction steps of the IVG method did not consistently affect precision.

Summary. In summary, only two of the phases (intestinal extraction step with dough and gastric extraction step without dough) of the IVG method were able to predict relative bioavailable Cd in contaminated soil as measured by in vivo swine dosing trials. The combination of the complex biochemistry and biological processes in the gastrointestinal system makes it difficult to measure bioavailable Cd by in vitro methods. However, the capacity of the IVG method to estimate relative bioavailable Cd shows some promise. Additional studies that compare in vitro results with in vivo bioavailable Cd should be conducted on more soils from a wide range of matrices (soil, slag, etc.). It is unlikely that an in vitro method can be developed to replace animal models in the estimation of in vivo bioavailability, but in vitro methods (i.e., the IVG method) may be useful as rapid screening tools in assessing relative bioavailability of Cd in soils from contaminated sites. Because in vitro methods are inexpensive, they can be used to analyze large numbers of soil samples and provide an estimate of the variability in bioavailable Cd at a single study site. The gastric extraction step of the IVG method without dough has the capacity to provide an estimate of the relative bioavailability of Cd, As, and Pb in contaminated soil.

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TABLE 1. Elemental Content of Select Metal Contaminants, Ca, and Fe in Study Soils

Soil	Cd (mg/kg)	As (mg/kg)	Pb (mg/kg)	Zn (g/kg)	Ca (g/kg)	Fe (g/kg)
1	465		1820			
2	43.0	240	8600	12.0	16.0	50.0
3	26.6	181	152	0.66	22.2	11.9
4	188	11.0	4050	50.0	81.8	18.0
5	139	16.0	6940	17.2	19.9	26.6
6	29.9	695	11500	48.9	88.1	16.9
7	23.8	310	3200	10.4	69.0	38.7
8	195	110	3230	6.50	1.16	25.9
9	319	134	2150	19.1	2.48	26.7
10	47.4	17.0	3870	4.11	17.3	23.0

TABLE 2. Cd Dose, Mean Kidney Cd (n = 5) and % Relative Standard Deviation for Juvenile Swine Exposed to Two Different Doses of Cd in Ten Soils

Soil	Cd Dose (µg/kg body weight/day)	Description of Dose	Mean Kidney Cd (mg/kg)	RSD (%)
Control	0	None	0.036	33.6
$CdCl_2$	20.0	Low	0.448	7.61
$CdCl_2$	80.0	High	2.27	24.8
1	40.0	Low	0.771	48.1
1	160	High	2.41	13.7
2	1.13	Low	0.033	57.3
2	3.38	High	0.061	73.5
3	2.94	Low	0.123	19.0
3	7.35	High	0.187	12.4
4	10.4	Low	0.088	71.3
4	31.3	High	0.481	17.5
5	4.51	Low	0.067	10.6
5	13.5	High	0.270	31.0
6	0.59	Low	0.014	20.2
6	1.76	High	0.038	30.0
7	1.67	Low	0.054	13.1
7	5.02	High	0.109	21.9
8	4.53	Low	0.126	32.9
8	13.6	High	0.375	12.8
9	11.1	Low	0.255	41.7
9	33.4	High	1.03	15.3
10	2.76	Low	0.05	28.4
10	8.27	High	0.212	26.2

RSD = relative standard deviation, the standard deviation divided by the mean expressed as a percentage. Mean kidney values are based on five replicates.

TABLE 3. Comparison of Soil Cd and In Vivo Relative Bioavailable Cd with Bioaccessible Cd Determined by the IVG Method With and Without Dough Additive

Soil	Soil Cd [a]	In Vivo Relative Bioavailable Cd [b]	Bioaccessible Cd			
			IVG with dough		IVG without dough	
			GE [c]	IE [d]	GE [c]	IE [d]
	mg/kg	%	%	%	%	%
1	465	55.4	34.0	11.2	54.8	43.2
2	43.0	29.9	11.7	4.05	21.3	15.0
3	26.6	73.4	37.9	9.81	75.6	42.9
4	188	53.6	28.7	11.2	53.2	33.5
5	139	63.3	46.8	17.9	69.0	55.0
6	29.9	10.4	40.4	6.69	42.1	25.6
7	23.8	56.8	42.9	16.2	75.0	49.2
8	195	94.2	46.1	16.1	75.2	38.1
9	319	116	46.1	19.5	95.9	40.8
10	47.4	80.6	47.5	16.6	68.1	47.9
Mean	148	63.4	38.2	12.9	63.0	39.1
Median	93.2	60.1	41.7	13.7	68.6	41.9
Minimum	23.8	10.4	11.7	4.05	21.3	15.0
Maximum	465	116	47.5	19.5	95.9	55.0

[a] SW 846, USEPA method 3050. [b] Determined from immature swine dosing trial.
[c] Gastric solution extraction step. [d] Intestinal solution extraction step.

TABLE 4. Precision of the IVG Methods for Determination of Bioaccessible Cd Determined by 12 Replicated Extractions of Soil 1

IVG Method	Mean (mg/kg)	Range (mg/kg)	RSD (%)
GE + Dough	159	154-162	2.08
IE + Dough	51.	46.0-56.7	7.80
GE, No Dough	255	233-273	6.61
IE, No Dough	201	192-207	2.78

GE = gastric solution extraction; IE = intestinal solution extraction; RSD = relative standard deviation, the standard deviation divided by the mean expressed as a percentage.

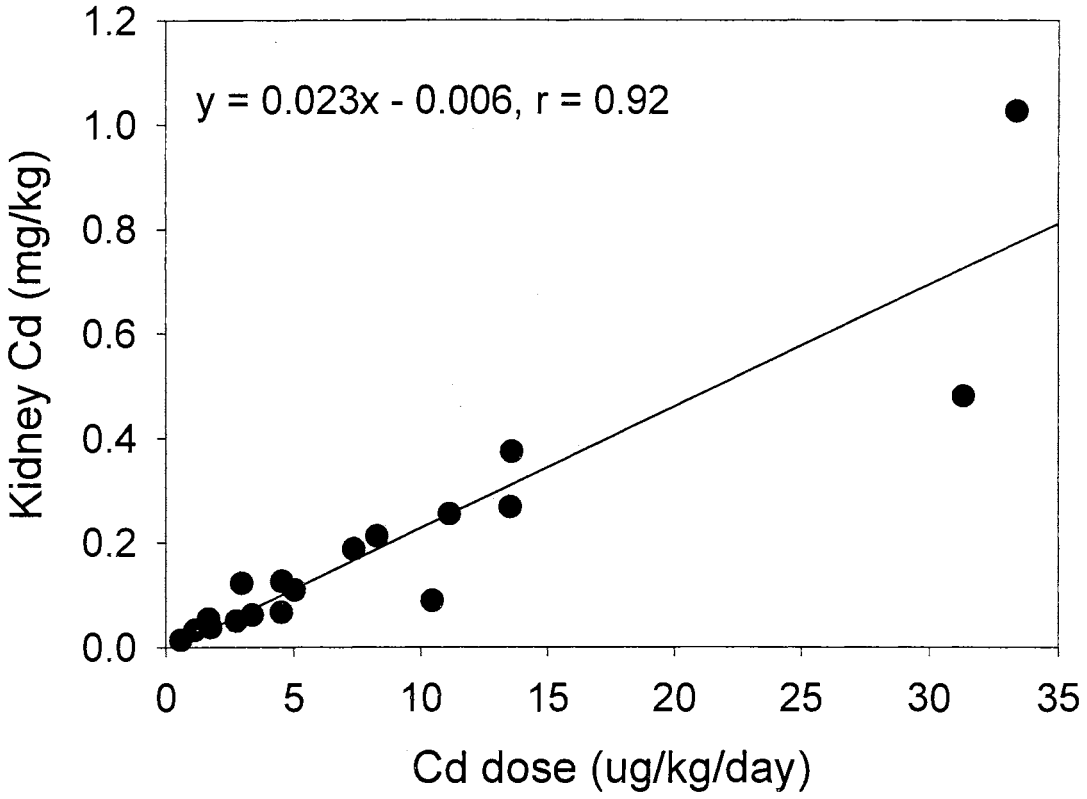

Figure 1. Concentration of Cd in kidney of juvenile swine as a function of increasing Cd dose in soil.

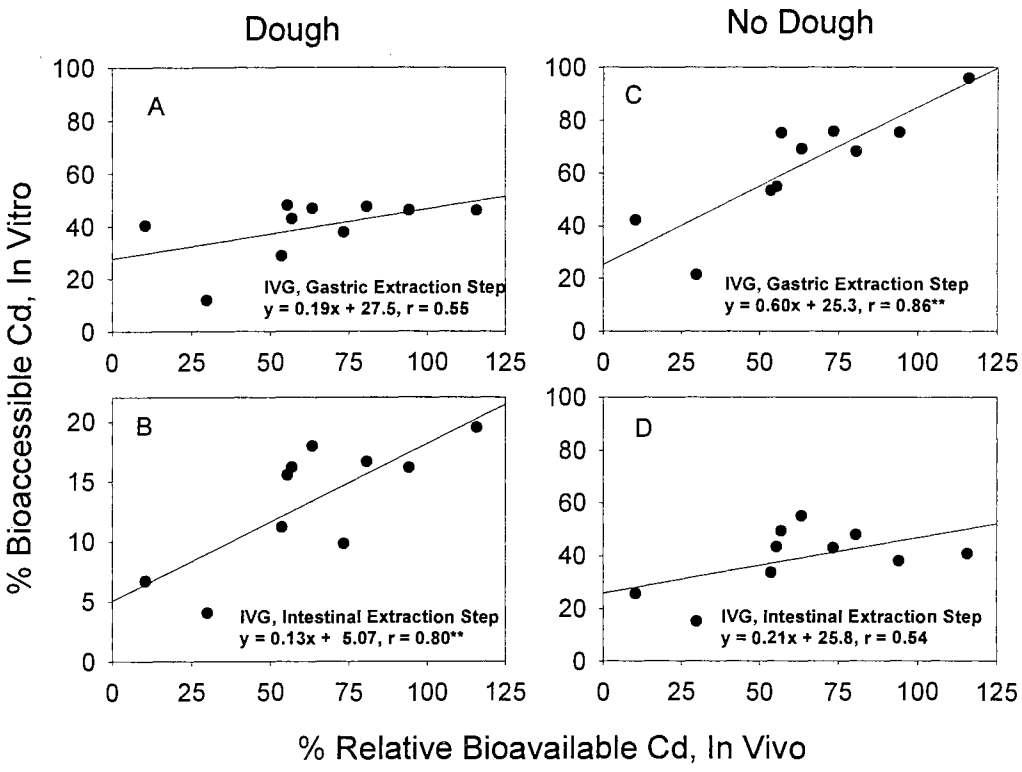

Figure 2. Linear regression of in vitro gastrointestinal (IVG) gastric or intestinal extraction steps with and without dough vs. relative bioavailable Cd. **P < 0.01.

CHAPTER III

VALIDATION OF THE IN VITRO GASTROINTESTINAL (IVG) METHOD TO ESTIMATE RELATIVE BIOAVAILABLE LEAD IN CONTAMINATED SOILS

ABSTRACT

The effect of the dosing vehicle (e.g., dough) on the ability of an in vitro gastrointestinal (IVG) method to predict relative bioavailable Pb associated with soil ingestion was evaluated. Bioaccessible Pb determined by the IVG method was compared with relative bioavailable Pb measured from dosing trials using juvenile swine for 18 contaminated soils ranging from 1,270 to 14,200 mg Pb kg^{1}. Bioaccessible Pb was measured in the IVG gastric extraction (GE) and intestinal extraction (IE) solutions. Mean bioaccessible Pb (in parentheses) were GE without dough (32.2%), GE with dough (23.0%), IE without dough (1.06%), and IE with dough (0.56%). It is possible that phytic acid associated with the dough addition decreased bioaccessible Pb. In vivo relative bioavailable Pb ranges for different swine tissues (in parentheses) were blood (1 to 87%), liver (0 to 110%), kidney (1 to 124%), and bone (0.04 to 94%). Strong linear relationships between IVG GE Pb with dough ($r > 0.76$, $P < 0.0002$), IVG IE Pb with dough ($r > 0.56$, $P < 0.015$), and IVG GE Pb without dough ($r > 0.81$, $P < 0.0001$) and in vivo bioavailable Pb as estimated with blood, kidney, liver, and bone were found. A weak but significant relationship was found between IVG IE Pb without dough (*r*

= 0.47, P = 0.049) and in vivo relative bioavailable Pb using bone data. Relationships between IVG IE Pb without dough and in vivo relative bioavailable Pb estimated using the other tissues were not significant ($P > 0.05$). Inexpensive in vitro methods may be useful in providing an estimate of the variability in relative bioavailable Pb at a single study site. The GE (no dough) can be used to estimate relative bioavailable Pb, As, and Cd in contaminated soil.

Lead (Pb) is a naturally occurring, bluish-gray metal usually found as a mineral combined with other elements, such as sulfur (i.e., PbS, $PbSO_4$) or oxygen ($PbCO_3$), and ranges from 10 to 30 mg kg^{-1} in the earth's crust (U.S. Department of Health and Human Services, 1999). Typical mean Pb for surface soils worldwide averages 32 mg kg^{-1} and ranges from 10 to 67 mg kg^{-1} (Kabata-Pendias and Pendias, 1992). Typical background Pb levels in surface soils of the United States range from 0.5 to 135 mg kg^{-1} with a median value of 11 mg kg^{-1} (Holmgren et al., 1993). Lead is used for a variety of industrial and consumer materials, including lead-acid batteries (63.0%), pigments and other compounds (12%), rolled and extruded products (7.7%), cable sheathing (4.5%), and gasoline additives (2.2%) (Adriano, 2001; U.S. Department of Health and Human Services, 1999). Lead contamination of soil may result from mining and smelting activities, sewage sludge usage in agriculture, contamination from vehicle exhausts, manufacturing processes involving Pb, and recycling and disposal of Pb-containing products (Adriano, 2001; Davies, 1990). Past uses of lead in the United States that have resulted in soil contamination include its addition to gasoline and its use in pesticides, batteries, firing ranges, and Pb-based paint chips (Adriano, 2001; Davies, 1990).

Lead is considered a possible human carcinogen by the International Agency for Research on Cancer (2002) as well as a probable human carcinogen by the United States Environmental Protection Agency (U.S. EPA, 1996b). Human exposure to Pb can occur through the consumption of contaminated foods or drinking water, incidental ingestion of soil or dust, inhalation of Pb-

containing particles from ambient air, ingestion of paint chips from Pb-painted surfaces, use of medications in the form of folk remedies, inhalation of automobile emissions, or from working in occupations involving exposure to Pb fumes and dust (Adriano, 2001; U.S. Department of Health and Human Services, 1999). Lead is a very toxic element, and exposure results in a variety of effects in humans. In both adults and children, the main target of lead toxicity is the central nervous system (U.S. Department of Health and Human Services, 1999). Acute exposure to high levels of Pb may result in gastrointestinal symptoms (cramping, colicky abdominal pain, nausea, and vomiting), brain damage, kidney damage, lowered sperm production, miscarriages, and possibly death. Chronic exposure to Pb may result in effects on the blood (anemia), central nervous system (CNS), blood pressure, kidneys, and Vitamin D metabolism (U.S. Department of Health and Human Services, 1999). Central nervous system effects on adults consist of subtle behavior changes, fatigue, and impaired concentration. Children are more susceptible to Pb exposure because they absorb and retain approximately 50% more in proportion to their body weight (Mushak et al., 1989). Exposure of children to Pb may result in impaired neurological development (both cognitive and behavioral) as evidenced by deficits in intelligence scores, speech and language processing, attention and classroom performance (da la Burde and Choate, 1972; Grant and Davis, 1989; Needleman et al., 1979, 1990; Rummo et al., 1979; Winneke, 1995).

Lead is ubiquitous in the environment primarily as a result of anthropogenic activities; and the U.S. Department of Health and Human Services

(1999) estimates that 89.4% of the total environmental release of Pb in 1996 was to soil. Pb ranks first on the priority list of hazardous substances found at Superfund sites (based on its frequency at sites, its toxicity, and its potential for human exposure) and has been identified in soils from 675 of the 1,026 National Priorities List (NPL) hazardous waste sites (Adriano 2001; U.S. Department of Health and Human Services, 1999). Concentrations as high as 60,000 mg kg^{-1} have been reported in soils adjacent to a smelter in Missouri (Palmer and Kucera, 1980). Additionally, soils adjacent to Pb-painted houses may contain > 10,000 mg kg^{-1} (U.S. EPA, 1986). The incidental ingestion of soil by children is an important pathway in the assessment of public health risks due to exposure of metal contaminated soils. Most risks from Pb in ingested soil or waste materials is associated with the fraction of the soil or waste material that is available for absorption from the gastrointestinal tract into the circulatory system. The amount of Pb absorbed through the gastrointestinal tract (bioavailable Pb) may be described in absolute or relative terms. Absolute bioavailability (ABA), also referred to as the oral absorption fraction, is equal to the absorbed dose/ingested dose as described by Eq. [1]:

$$ABA = \frac{Absorbed\ Dose}{Ingested\ Dose} \qquad [1]$$

Relative bioavailability (RBA) is the ratio of the ABA of Cd present in some test material (study soil) compared to the ABA of Cd in an appropriate reference material [Eq. 2]:

$$RBA = \frac{ABA(study\,soil)}{ABA(reference\,material)} \qquad [2]$$

Lead acetate, a readily soluble form of Pb and thus easily absorbed from the gastrointestinal tract, is used as the reference material in the critical toxicity study reported in the Integrated Risk Information System (IRIS; U.S. EPA, 1996b). Relative bioavailability can be determined experimentally without specifically measuring absolute bioavailability. For example, the tissue concentration of Pb in animals dosed with study soil can be compared with tissue concentration of Pb in animals dosed with reference material. In this case, relative bioavailability is defined by Eq. [3]:

$$RBA = \frac{Tissue\ Pb(study\ soil)}{Tissue\ Pb(reference\ material)} \qquad [3]$$

Often, baseline risk assessments used for contaminated sites assume that the relative bioavailability of Pb in soil is 60%, which is the default value used by the Integrated Exposure and Uptake Biokinetic (IEUBK) model for lead in children (U.S. EPA, 1994). However, because of the different geochemical and physical forms of Pb present in contaminated soils and waste, the relative bioavailability of Pb may be different than the default IEUBK value.. Therefore, a more accurate estimation of the relative bioavailability of metal contaminants

(e.g., Pb and As) in waste materials from hazardous waste sites has been assessed using in vivo animal dosing trials and used for risk assessment.

Less expensive in vitro chemical extraction methods that simulate gastrointestinal biochemistry have been developed to estimate relative bioavailable Pb (Ellickson et al., 2001; Hamel et al., 1998; Ruby et al., 1992, 1996), As (Rodriguez et al., 1999) and Cd (Schroder et al., in press). The amount of contaminant dissolved in the gastrointestinal environment and available for absorption is termed "bioaccessible" (Ruby et al., 1999). Most in vitro methods are sequential extractions with two distinct extraction steps: 1) a gastric phase extraction that simulates the acidic biochemical stomach environment, and 2) a subsequent intestinal phase extraction that simulates the biochemical environment of the small intestine. The fraction of the contaminant dissolved by the in vitro procedure, the "bioaccessible" contaminant, has been used to estimate the relative bioavailability of the contaminant in soil (Ruby, 1999). While many different in vitro methods have been developed to estimate bioacccessible Pb, few have related their results to relative bioavailable Pb as measured by an animal model. The in vitro physiologically based extraction test (PBET), which does not use food in the extraction in order to mimic fasting conditions, has been correlated with relative bioavailable Pb as estimated by two animal models (weanling rats and swine) (Medlin, 1997; Ruby et al., 1996, 1999). The in vitro gastrointestinal (IVG) method developed by Rodriguez et al. (1999) is an accurate predictor of relative bioavailable As in contaminated soils and waste materials as estimated by a juvenile swine model while utilizing food in the

extraction procedure. Recently, Schroder et al. (in press) showed that the IVG method was correlated with in vivo relative bioavailable Cd using a juvenile swine model. The objective of this study was to determine the ability of the IVG method of Rodriguez et al. (1999), with and without food, to predict relative bioavailable Pb in contaminated soil as measured by in vivo juvenile swine dosing trials.

MATERIALS AND METHODS

Contaminated Soils and Solid Media

Eighteen contaminated soils from eight different hazardous waste sites were evaluated using the in vitro gastrointestinal (IVG) method of Rodriguez et al. (1999). Air-dried soil was sieved through nylon mesh (< 250 μm) to obtain the soil fraction considered to adhere to fingers and likely to be ingested. Total metal content of soil was determined by acid digestion using U.S. EPA Method 3050 (1996a) and total elemental analysis was conducted using a high resolution Thermo Jarrell Ash IRIS inductively coupled plasma atomic emission spectrophotometer (ICP-AES).

In Vivo Swine Dosing Study

In vivo relative bioavailable Pb in contaminated soil was determined by in vivo dosing trials using standard operating procedures (Casteel, 1995). Male swine (5-6 wk old) and weighing 10-12 kg were dosed for 15 d with varying concentrations of Pb in substrates. Five swine were randomly assigned to treatment groups consisting of a dosing group, a negative control group (no substrate), and a positive control group that received oral lead acetate. All swine were individually housed in stainless steel cages and daily fed a powdered

grower's diet (referred to as dough in this paper), which approximated 5% of their body weight (Ziegler Bros., Inc., Gardner, PA). The diet was commercially formulated to have a protein content of approximately 19% and contained < 0.2 mg Pb kg^{-1} diet. After a 7-d acclimation period, the swine were dosed with contaminated soil that was placed in a 5-10 g doughball of moistened grower diet. The swine were dosed twice daily to mimic childhood Pb ingestion, which is likely to occur between meals while children are in a fasted or semi-fasted state. A dose of 6.25 mg soil per kg body weight per day was used with half of the first dose being delivered at 9:00 am after an overnight fast and the second half of the dose being delivered at 3:00 pm after a 4-h fast. All swine were fed 2 hr after dosing.

Tissue Analyses

Blood (1.0 ml) was mixed with 9.0 ml of a matrix modifier consisting of 0.2% v/v trace metal nitric acid, 0.5% v/v Triton X-100, and 0.2% w/v ammonium phosphate in deionized distilled water prior to analyses. Kidney or liver (1.0 g) were digested overnight at 90°C in 2.0 ml of concentrated trace metal HNO_3 and diluted to a final volume of 10.0 ml with deionized distilled water. Femurs were oven-dried overnight at 100°C and were ashed in a muffle furnace at 450°C for 48 h. Aliquots of ashed femurs (200 mg) were dissolved in 10.0 ml of a 1:1 mixture of trace metal nitric acid and deionized distilled water. All samples were filtered through 0.45 membrane filters prior to analyses by graphite furnace atomic absorption spectroscopy (GFASS). Blanks, spikes and duplicate analyses were conducted every 20 samples to meet quality assurance and

quality control (QA/QC) requirements. Relative Pb bioavailability was estimated using measured Pb concentrations in blood, liver, kidney, and bone.

Calculation of In Vivo Relative Bioavailability

RBA was calculated from Eq. [3]. Lead acetate was selected as reference material in our study because it is a readily soluble form of Pb that was used in critical toxicity studies as reported in IRIS. More specifically, for each study substrate, the amount of Pb bioaccumulated in tissue (e.g., μg Pb L^{-1} for blood and mg Pb kg^{-1} kidney, liver, or bone) was plotted as a function of Pb dosed (e.g., μg Pb kg^{-1} body weight day^{-1}) for both reference material and study substrate. The resulting best-fit straight lines (calculated by linear regression) for both the reference material and the study substrate were used to estimate the RBA. The RBA was calculated by dividing the slope for the study substrate by the slope for the reference material.

In Vitro Gastrointestinal Method (IVG)

Bioaccessible Pb was estimated in our study using the IVG method developed by Rodriguez et al. (1999). The IVG method is a two-step sequential extraction: a gastric solution extraction followed by an intestinal solution extraction. An equivalent amount of the dosing vehicle (200 g of wet feed termed "dough") was added to the gastric solution to mimic the in vivo dosing of 100 mg soil to 5 g of dough. Gastric solution was 0.15 M NaCl and 1% porcine pepsin (Sigma Chemical Company, St. Louis, MO, cat. no. P7000). The in vitro method was conducted using 1 L glass jars in a water bath at body temperature (37°C). Soil (4.0 g) was placed in 600 ml of gastric solution to which either 0 g (e.g., no

dough) or 200 g of dough was added. The pH of the gastric solution was adjusted to pH 1.8 with trace metal grade HCl. Anaerobic conditions were maintained by constantly bubbling argon through the solution; pH was continuously monitored and adjusted to 1.8 throughout the 1-h procedure. Mixing (to simulate gastric mixing) was maintained during the procedure using individual paddle stirrers set at a speed of 100 rpm. After 1 h, 40 mL of gastric solution, removed for Pb analysis, was replaced with 40 mL of fresh gastric solution. Subsequently, the extraction solution was modified to simulate intestinal solution by adding saturated $NaHCO_3$ solution to adjust the pH to 5.5 followed by the addition of 2.10 g of porcine bile extract (Sigma Chemical Company, St. Louis, MO, cat. no. B8631) and 0.21 g of porcine pancreatin (cat. No. P1500). A small amount of anti-foam agent (decanol) was added to each reaction vessel. After 1 h, 40 mL of intestinal solution was collected for Pb analysis. Gastric and intestinal solution samples were centrifuged for 15 min at 10,000 rpm and filtered through 0.45 µm membrane filters immediately after their collection. The samples were acidified to pH of 2 using trace metal HCl, and Pb was determined using ICP-AES.

In Vitro Bioaccessibility Calculations

Bioaccessible Pb was calculated by dividing the Pb concentration measured in the in vitro gastric or intestinal solutions by the total soil Pb content (e.g., U.S. EPA method 3050)

Statistical Analysis

Analysis of variance using PROC MIXED (SAS Institute, 2001) was performed to evaluate the effects of the extraction step (gastric or intestinal) and method (dough or no dough addition) on bioaccessible Pb. The data were analyzed as a split plot arrangement in a randomized complete block design. The combination of replicate and soil were used as blocks, method was the whole plot factor, and phase was the split plot factor. Simple effects of method given phase and phase given method were analyzed with a SLICE option in the LSMEANS statement. The relationship between mean in vitro bioaccessible Pb and mean in vivo relative bioavailable Pb in different tissues was determined using PROC REG (SAS Institute, 2001).

RESULTS AND DISCUSSION

Soil Pb Concentrations

The Pb content of the contaminated soils ranged from 1,270 to 14,200 mg kg^{-1} (Table 1), which is well above the Pb content of 10 to 67 mg/kg reported for uncontaminated soils (Kabata-Pendias and Pendias, 1992). The study soils were also contaminated with other heavy metals (e.g., Cd, Zn) and metalloids (As) (Table 1). The soils also contained significant amounts of elements known to affect Pb uptake and bioavailability including Fe, Ca, and Zn.

In Vivo Relative Bioavailable Pb

Ranges (in parentheses) for percent relative bioavailable Pb estimated using the young swine model varied by tissue and were blood (1 to 87%), liver (0 to 110%), kidney (1 to 124%), and bone (0.04 to 94%) for the soils evaluated in

our study (Table 2). In a review on the oral bioavailability of inorganics in soil, Ruby (1999) reported that the bioavailability of Pb in an ingested soil depends on the chemistry, particle size distribution, mechanism of dissolution, and geochemistry of the soil. Our results are similar to those of Ruby (1999) who reported that the relative bioavailability of Pb in contaminated soils ranged from 1 to 90% based on a combination of data from blood, bone, liver, and kidney using juvenile swine with blood data weighted more heavily.

In Vitro Bioaccessible Pb

The in vitro bioaccessible Pb measured by the IVG gastric solution extraction step (GE) using dough in the extraction ranged from 0.70 to 36.3% with an overall mean of 23.0% (Table 2). In vitro bioaccessible Pb measured by the IVG intestinal solution extraction step (IE) using dough in the procedure ranged from 0.02 to 1.16% and averaged 0.56% for the soils (Table 2). IVG GE Pb without using dough in the extraction ranged from 1.4 to 64.4% with an overall mean of 32.2% (Table 2). Within the IE, the in vitro Pb without using dough in the extraction ranged from 0.03 to 3.23% with an overall mean of 1.06% (Table 2). Much literature has been published concerning the estimation of bioaccessible Pb in soil using in vitro procedures (Davis et al., 1992; Ellickson et al., 2001; Hamel et al., 1998; Ruby et al., 1992, 1993, 1996). Previous work has reported a range of values for bioaccessible Pb in soils with most values < 100%. Davis et al. (1992) used an in vitro procedure to compare the solubility of lead acetate with the solubility of a soil from the Butte, MT, mining district and found that Pb in lead acetate was 70 and 5 times more available than an equivalent

mass of Pb found in the soil under simulated stomach and intestine conditions, respectively. Ruby et al. (1992, 1993) investigated mine-waste samples and found that bioaccessible Pb ranged from 0.5 to 6%. Hamel et al. (1998) used an in vitro extraction procedure composed of a gastric step at a pH of 1.1 to evaluate the bioaccessibility of Pb and other contaminants in a National Institute of Standards and Testing standard reference material (NIST Soil SRM 2710). Their study investigated the effect of varying the liquid to solid ratio on the extractability of As, Cr, Ni, Cd, and Pb without using food in the extraction. Their results indicate that the solubility of Pb in the SRM 2710 was affected only slightly by changing the liquid to solid ratio; they reported the bioaccessibility of Pb in SRM 2710 as 36% at a liquid to solid ratio of 100:1, 46% at a liquid to solid ratio of 200:1, and 35% at a liquid to solid ratio of 2000:1. The IVG extraction of Rodriguez et al. (1999) used a liquid:solid ratio of 150:1. In vitro bioaccessible Pb was measured in NIST SOIL SRM 2710 both with and without dough. IVG GE Pb (without dough) was 60%, while IVG IE Pb (without dough) was 4%. Measured IVG GE Pb (with dough) was 28%, and IVG IE Pb (with dough) was 0.4%. Ellickson et al. (2001) used a two step in vitro procedure (without food) composed of a saliva-gastric step (pH = 1.4) and an intestinal step (pH = 6.5) to evaluate the bioaccessibility of Pb and As in a National Institute of Standards and Testing standard reference material (NIST Soil SRM 2710). Using a liquid to solid ratio of approximately 3500:1, they reported the bioaccessibility of Pb in the saliva-gastric step as 76.1% and the bioaccessibility of Pb in the intestinal step as 10.7%. Ruby et al. (1996) used the PBET composed of a stomach step (pH =

2.5) and an intestinal step (pH = 7.0) at a liquid to solid ratio of 160:1 without food to estimate bioaccessible Pb in a set of seven soils. Percent bioaccessible Pb extracted by their stomach step ranged from 3.8 to 26%, while percent bioaccessible Pb for their intestinal step ranged from 0.6 to 29%.

Dough vs. No Dough

Pb extracted by the IVG GE was greater than Pb extracted by the IVG IE for the 18 individual soils for both dough and no dough methods (Table 2). Mean IVG GE Pb was also greater than mean IVG IE Pb for the soils using both methods ($p < 0.001$, Table 2). In part, the reduction of measured Pb between IVG GE and IVG IE can be attributed to the reduced solubility of Pb in the higher solution pH of the IE as compared to the GE (pH 5.5 vs. 1.8). During our study, mean Pb in the IE without dough decreased by approximately 97% as compared to mean GE Pb without dough. Our results are similar to those of Ruby et al. (1996) who showed that solubilized Pb decreased by 74% upon entering the small intestine step during the PBET due to adsorption and precipitation reactions removing Pb from solution as the pH increased.

Comparison of the dough vs. no-dough methods shows that the mean Pb of 16.6% for the combined GE and IE without using dough in the extraction was greater than the mean Pb extracted of 11.8% for the combined GE and IE using dough in the extraction ($p = 0.003$ (Table 2). Mean IVG GE Pb of 32.2% without dough in the extraction was significantly greater than mean IVG GE Pb of 23.0% using dough in the extraction ($p < 0.001$) (Table 2). There was a significant interaction between method and extraction phase (e.g., gastric vs. intestinal) ($p =$

0.011). Mean IVG IE Pb of 1.06% without dough in the extraction was not significantly greater than mean IVG IE Pb of 0.56% using dough in the extraction (p = 0.689, Table 2). Our results are similar to those of Ruby et al. (1993) who reported that the addition of rabbit chow to an in vitro procedure reduced the mass solubilized Pb during the stomach phase by approximately 10.8%.

In a review on human bioavailability. Ragan (1983) reported that the solubility and absorption of Fe, Cd, and Pb may be lowered by dietary components such as oxalates, phosphates, and phytates. The presence of food reduces absorption of ingested water-soluble Pb (e.g., lead chloride, lead nitrate, lead acetate) by humans primarily due to the presence of calcium and phosphate (Blake and Mann, 1983; Blake et al., 1983; Heard and Chamberlain, 1982; Rabinowitz et al., 1980). Madalonni et al. (1998) dosed human volunteers with contaminated soil from Bunker Hill, ID, and reported that the absorption of Pb was greatly affected by the presence of food in the gastrointestinal systems of test subjects. Their study reported the absorption of Pb in fasted test subjects as 26% and the absorption of Pb in fed test subjects as 2.5%. Phytic acid (myoinositol hexaphosphate) or its salt, phytate, is an important plant constituent accounting for up to 90% of total phosphorus in cereals, legumes, and oilseeds (Reddy et al., 1982). Phytic acid is capable of forming strong complexes with various metal cations under physiological conditions (Nolan et al., 1987). Wise (1981, 1983) conducted both acute (8 d) and chronic studies (6 mo) involving the addition of calcium phytate to Pb-contaminated diets fed to mice and reported that calcium phytate reduced blood Pb levels. Rose and Quarterman (1984) fed

rats a diet containing 200 mg Pb kg^{-1} supplemented with phytate (10 g kg^{-1}) or calcium (6 g kg^{-1}) and found that the addition of phytate or calcium separately reduced the accumulation of Pb in bone, blood, and liver. They also reported that the greatest reduction in tissue accumulation of Pb occurred when phytate and calcium were fed together. Bullock et al. (1995) investigated the effect of phytate on the in vitro solubility of Al, Ca, Hg, and Pb as a function of pH at 37°C. They varied the Pb to phytate ratio across the pH range of 3.0 to 7.0 and found that the solubility of Pb varied with both pH and the Pb to phytate molar ratio. Pb solubility in their study was greatly reduced by the formation of Pb-phytate precipitates. Maximum reduction in Pb solubilites occurred at a Pb:phytate ratio of approximately 3:1 with reductions ranging from 96% (pH = 3.0) to 88% (pH = 7.0). The calcium-phytate complex has a strong affinity for both Pb and Cd (Wise, 1983). Also, Wise and Gilburth (1981) reported that almost complete binding of both Cd and Pb occurred at Ca:phytate ratios that are common in stock diets of laboratory animals. It is possible that Pb-phytate complexes or insoluble complexes involving phytic acid and Ca with Pb coprecipitating the complex were formed during the in vitro extraction of soils using dough, which resulted in lower bioaccessible Pb as compared to the extractions that did not use dough.

The dough material has a phosphorus content of 7,580 mg kg^{-1}. A considerable amount of P was dissolved in the IVG methods. Results show that the inclusion of dough increased soluble P in the IVG GE solution from 53.9 to 1,900 mg L^{-1} without soil. Similarly, inclusion of dough increased soluble P in the

IVG IE from 64.5 to 1,740 mg L^{-1} without soil. Results show that the inclusion of dough increased soluble P in the IVG GE solution from 49.1 to 1,810 mg L^{-1} with contaminated SRM 2710 soil. Similarly, inclusion of dough increased soluble P in the IVG IE from 56.7 to 1,570 mg L^{-1} with contaminated SRM 2710 soil. The equilibrium geochemical speciation model MINTEQA2 (ver. 4.0) was used to investigate the possibility that the addition of dough to the IVG procedure resulted in precipitation of inorganic Pb phosphorus compounds, thus lowering measured concentrations of in vitro Pb (U.S. EPA, 1999). In vitro concentrations of Ca, Pb, Fe, P, Zn, Na, Cl, and solution pH were used as model inputs. Total dissolved P was assumed to be present as orthophosphate ion, which would be consistent with the most likely scenario to form Pb phosphate mineral precipitate. MINTEQA2 predicted that the IVG GE solutions, with and without dough, were oversaturated with respect to lead phosphate solid phases for the contaminated SRM 2710 soil. MINTEQA2 indicated that 24.6% of the total Pb could precipitate as pyromorphite without dough and that 79.6% of the total Pb could precipitate as pyromorphite with dough, which is consistent with decreased in vitro Pb associated with dough addition during the gastric step of the in vitro procedure. MINTEQA2 predicted that oversaturation occurred for the IVG IE solutions with and without dough. The model predicted that 99.98% of the total Pb could precipitate as pyromorphite without dough, while 99.96% of the total Pb could precipitate as pyromorphite with dough. However, MINTEQA2 failed to explain decreased in vitro Pb associated with dough addition (10x less for the SRM

2710) during the intestinal step and showed little difference between the dough and no dough situations.

Relationships Between Bioaccessible Pb and In Vivo Relative Bioavailable Pb

Concentrations of Pb in blood are the most widely used biomarkers of lead exposure (U.S. Department of Health and Human Services, 1999). However, approximately 94% of the total body burden of Pb is found in bones with Pb cycling between blood and bone (U.S. Department of Health and Human Services, 1999). The relationship between blood Pb and Pb exposure is nonlinear for gastrointestinal exposure at high exposure concentrations, and Pb in bone is considered a more appropriate biomarker of cumulative Pb exposure than Pb in blood (U.S. Department of Health and Human Services, 1999; U.S. EPA, 1986). Linear regression indicated there was a strong relationship between IVG GE Pb using dough in the extraction and in vivo relative bioavailable Pb estimated using blood data ($P < 0.0001$, $r = 0.93$) (Fig. 1A). Regression analysis showed there was a strong relationship ($P < 0.0001$, $r = 0.80$) between IVG IE Pb using dough in the extraction and in vivo relative bioavailable Pb estimated using blood data (Fig. 1B). A strong relationship was found between IVG GE Pb without using dough in the extraction and in vivo relative bioavailable Pb using blood data ($P < 0.0001$, $r = 0.89$) (Fig. 1C). However, a significant relationship between IVG IE Pb (no dough) and in vivo relative bioavailable Pb using blood data was not found ($P = 0.121$, $r = 0.38$) (Fig. 1D). Strong relationships also existed between IVG GE Pb using dough in the extraction and estimated in vivo

relative bioavailable Pb using other tissues (e.g., liver, kidney, and bone) with regression coefficients ranging from 0.76 to 0.85 (Table 3). Strong relationships were also found between IVG IE Pb using dough in the extraction and in vivo relative bioavailable Pb as estimated by the other tissues with regression coefficients ranging from 0.56 to 0.80 (Table 3). Significant relationships were also found between IVG GE (no dough) and in vivo relative bioavailable Pb using the other tissues, and regression coefficients ranged from 0.81 to 0.93 (Table 3). Conversely, within the IE without dough in the extraction, only the relationship between IVG Pb and in vivo relative bioavailable Pb using bone data was significant (P = 0.049, r = 0.47) (Table 3). Ruby et al. (1996) showed that the stomach phase of the PBET at pH values of 1.3 and 2.5 was highly correlated with in vivo relative bioavailable Pb as measured by a weanling rat model with blood as the target organ (r^2 = 0.93 for both pH values, n = 7). Their study reported a weaker relationship (r^2 = 0.76) between intestinal bioaccessible Pb and in vivo relative bioavailable Pb. In a review article on bioavailability of inorganics in soils, Ruby (1999) cited a study by Medlin (1997) and indicated that the stomach phase of the PBET was strongly correlated (r^2 = 0.79, n = 15) with a "weighted estimate" (i.e., blood weighted 3:1 over each tissues) of in vivo relative bioavailable Pb from a young swine model. However, correlations between the intestinal phase of the PBET and in vivo relative bioavailable Pb were not reported in the review. In general, the results of our study are very similar to those reported by Ruby et al. (1996) and Ruby (1999) in that the GE without

dough was highly correlated with in vivo relative bioavailable Pb as estimated with all tissues.

Many interactions in the gastrointestinal systems of various animals affect Pb absorption. Gastrointestinal absorption of Pb is a complex and dynamic process involving dissolution, absorption, and interactions with other dietary components. Iron deficiencies in children have been associated with elevated blood Pb concentrations, and several animal studies have shown that Fe deficiencies increase gastrointestinal absorption of Pb (Barton et al., 1978; Flanagan et al., 1979; Mahaffey and Annest, 1986; Morrison and Quarterman, 1987; Sullivan and Ruemmler, 1987). Dietary deficiencies of Ca, Zn, and P enhance absorption of Pb in humans, rats, mice, and pigs (Heard and Chamberlain, 1982; Hsu et al., 1975; Johnson and Tenuta, 1979; U.S. Department of Health and Human Services, 1999; Van Barneveld and Van Den Hamer, 1985; World Health Organization, 1996). The ratios of total elemental content for Zn:Pb ranged from 0.01 to 12 with a median value of 2, Ca:Pb ranged from 0.24 to 22 with a median value of 4, and Fe:Pb ranged from 0.89 to 308 with a median value of 8. It is possible large amounts of Ca, Fe, and/or Zn decreased Pb absorption in some soils more than others.

CONCLUSIONS

Three of the solution extraction steps (GE with dough, IE with dough, and GE without dough) of the IVG method were able to predict bioavailable Pb in contaminated soils as measured by in vivo pig dosing trials. The combination of the complex biochemistry and biological processes in the gastrointestinal system

make it difficult to measure bioavailable Pb by in vitro methods. However, the ability of the IVG method to estimate bioavailable Pb is promising. Additional studies that compare in vitro results with in vivo bioavailable Pb should be conducted on more soils from a wide range of matrices (soil, slag, etc.). It is unlikely that an in vitro method can be developed to replace animal models in the estimation of in vivo bioavailability; however, in vitro methods (i.e., the IVG method) may be useful as rapid screening tools in assessing bioavailability of Pb on contaminated sites. Because in vitro methods are inexpensive, they can be used to analyze large numbers of soil samples and provide an estimate of the variability in bioavailable Pb at a single study site. The GE step of the IVG method both with and without dough has the ability to provide an estimate of bioavailable As and Pb in contaminated soil. The GE (no dough) can be used to estimate relative bioavailable Pb, As, and Cd in contaminated soil.

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Table 1. Elemental content of select metal contaminants, Ca, and Fe in study soils.

Soil	Pb	As	Cd	Zn	Ca	Fe
	mg/kg	mg/kg	mg/kg	g/kg	g/kg	g/kg
1	1590	51	4.20	0.90	13.6	16.1
2	8600	240	43.0	1.20	16.0	50.0
3	11200	4.9	0.80	0.11	2.65	10.0
4	10800	25	33.7	10.0	45.8	40.2
5	4050	11	188	50.0	81.8	18.0
6	6940	16	139	17.2	19.9	26.6
7	7510	203	59.9	13.7	20.1	68.1
8	4320	110	38.5	2.65	3.93	27.5
9	10600	1050	12.8	67.3	117	207
10	1270	1290	4.00	0.44	8.29	391
11	7895	591	24.4	31.9	90.1	196
12	11500	695	29.9	48.9	88.1	169
13	3200	310	23.8	10.4	69.0	38.7
14	8350	5	4.00	1.88	11.8	8.89
15	3230	110	195	6.50	1.16	25.9
16	2150	134	319	19.1	2.48	26.7
17	14200	67	41.9	6.58	37.2	33.7
18	3870	17	47.4	4.11	17.3	23.0

Table 2. Comparison of soil Pb and in vivo relative bioavailable Pb with bioaccessible Pb determined by the IVG method with and without dough additive.

Soil	Soil Pb[‡]	In vivo relative bioavailable Pb[†]				Bioaccessible Pb			
						IVG with dough		IVG without dough	
		Blood	Liver	Kidney	Bone	GE[§]	IE[¶]	GE[§]	IE[¶]
	mg/kg	%	%	%	%	%	%	%	%
1	1590	33	33	21	21	19.7	0.54	21.1	2.79
2	8600	22	9	13	13	5.90	0.17	6.81	0.48
3	11200	1	0	1	1	0.70	0.02	1.40	0.32
4	10800	56	92	50	55	27.8	1.16	55.2	1.66
5	4050	78	110	77	70	31.6	1.06	64.4	0.49
6	6940	82	66	50	94	34.3	0.95	58.8	2.22
7	7510	71	92	91	62	26.4	0.47	41.0	1.93
8	4320	87	96	124	84	35.0	0.80	53.0	1.95
9	10600	20	11	10	18	8.24	0.04	7.50	0.09
10	1270	6	5	4	0.04	4.74	0.18	6.71	0.18
11	7895	20	8	8	9	13.8	0.06	6.85	0.03
12	11500	55	37	44	61	22.3	0.57	24.7	0.05
13	3200	67	87	102	63	31.6	0.32	51.9	0.07
14	8350	82	85	70	63	29.6	0.84	36.9	1.01
15	3230	74	50	42	47	31.1	0.59	32.2	0.75
16	2150	58	54	34	39	36.3	0.87	36.3	0.36
17	14200	56	86	68	72	23.3	0.66	37.7	1.43
18	3870	58	74	74	68	31.0	0.73	36.2	3.23
Mean	6740	51	55	49	47	23.0	0.56	32.2	1.06
Median	7230	57	60	47	58	27.1	0.58	36.3	0.62
Minimum	1270	1	0	1	0.04	0.70	0.02	1.40	0.03
Maximum	14200	87	110	124	94	36.3	1.16	64.4	3.23

[†]Determined from juvenile swine dosing trial.
[‡]SW 846, USEPA method 3050.
[§]Gastric solution extraction step.
[¶]Intestinal solution extraction step.

Table 3. Regression coefficients (r) and regression equations between percent bioaccessible Pb (in vitro) gastric and intestinal steps and percent relative bioavailable Pb (in vivo) as determined in different tissues of juvenile swine. Regression coefficients with asterisks are statistically significant ($P < 0.05$).

	Dough				No dough			
Tissue	GE[†]	Regression equation	IE[‡]	Regression equation	GE[+]	Regression equation	IE[‡]	Regression equation
Blood	0.93*	y = 0.39x + 2.97	0.80*	y = 0.01x + .0001	0.89*	y = 0.65x -1.44	0.38	y = 0.01x + 0.52
Liver	0.84*	y = 0.26x + 8.65	0.80*	y = 0.01x + 1.13	0.93*	y = 0.50x + 4.22	0.43	y = 0.01x + 0.41
Kidney	0.76*	y = 0.24x + 11.1	0.56*	y = 0.01x + 0.29	0.81*	y = 0.45x + 10.0	0.39	y = 0.01x + 0.53
Bone	0.85*	y = 0.33x + 7.40	0.78*	y = 0.01x + 0.12	0.89*	y = 0.61x + 3.53	0.47*	y = 0.02x + 0.30

[†]Gastric solution extraction step

[‡]Intestinal solution extraction step

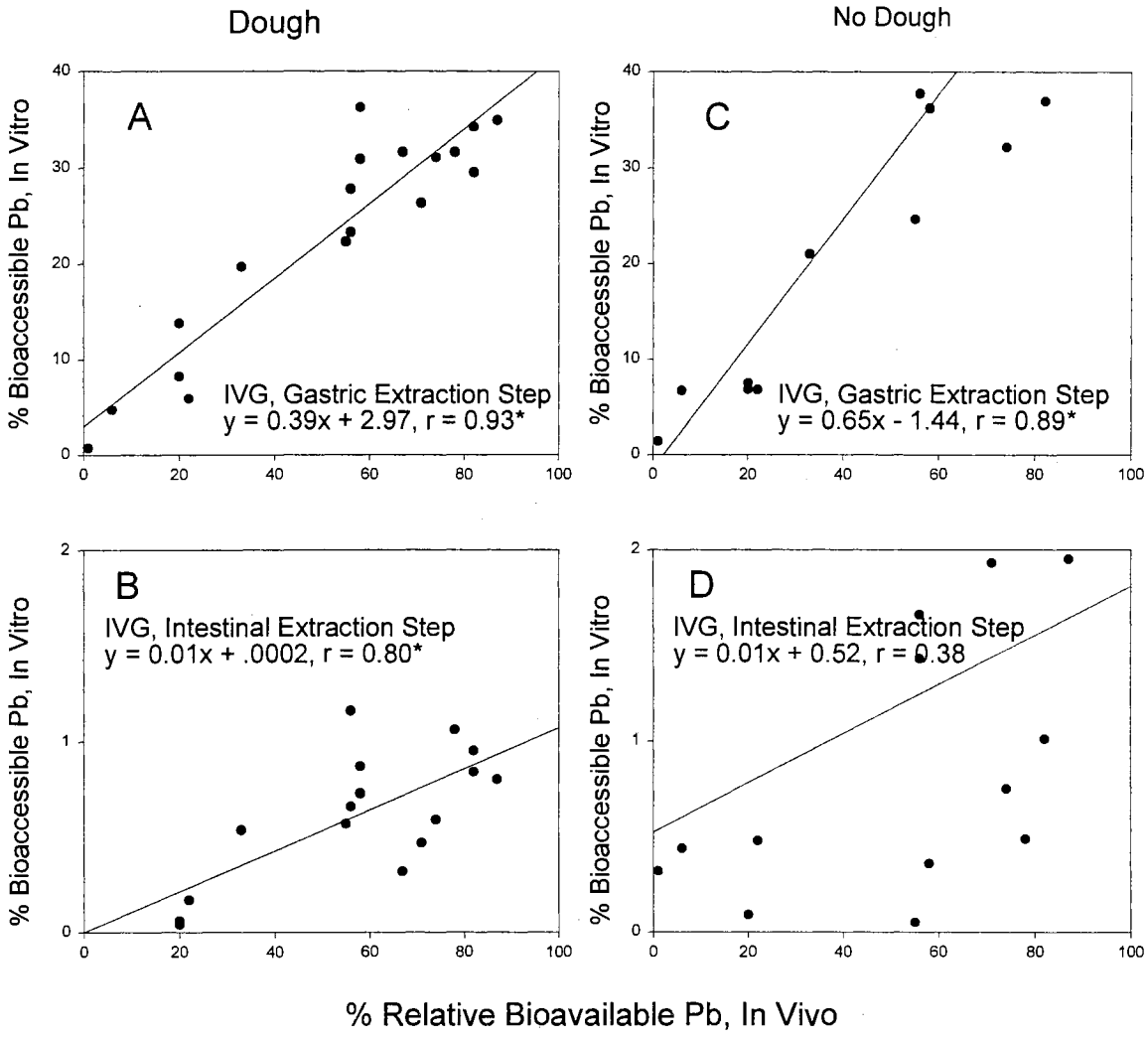

Figure 1. Linear regression of in vitro gastrointestinal (IVG) gastric or intestinal extraction steps with and without dough vs. relative bioavailable Pb, in vivo estiamted with bolld data. $^{*}P < 0.0001$

CHAPTER IV

BIOAVAILABILTY OF CADMIUM, LEAD AND ZINC TO LETTUCE AND EARTHWORMS IN SOILS OF DIFFERENT PROPERTIES.

ABSTRACT

Estimation of bioavailability is important in the characterization of contaminated sites and bioavailability should be considered in the risk assessment of these sites. Indirect methods like chemical extraction that do not use living organisms may be useful in estimating bioavailability of heavy metals and performing risk assessments. For chemical extraction methods to be useful, they must first be correlated with heavy metal bioavailability determined by bioassays. In this study the relationships between two chemical extraction methods (Potentially BioAvailable Sequential Extraction (PBASE) procedure and pore water extraction of soil) and heavy metal bioavailability determined by bioassays were evaluated in 22 field soils spiked with heavy metals. The PBASE procedure is a sequential extraction procedure using four different extractants: 0.5 M $Ca(NO_3)_2$ (E1), 1.0 M NaOAc (E2), 0.1 M Na_2EDTA (E3), and 4 M HNO_3 (E4). Heavy metal bioavailability was estimated with a lettuce (*Lactuca sativa var. Paris Island Cos*) bioassay and an earthworm (*Eisenia andrei*) bioassay. Significant linear relationships were found between E1 and lettuce metal content for the Cd50 ($p < 0.05$, $r = 0.41$), Cd300 ($p < 0.01$, $r = 0.62$), Pb ($p < 0.01$, $r = 0.74$) and the Zn ($p < 0.01$, $r = 0.71$) spiked soils. Strong linear relationships existed between E1 and earthworm mortality for the Pb ($p < 0.01$, $r = 0.86$) and

the Zn ($p < 0.01$, $r = 0.54$) spiked soils, however relationships between E1 and earthworm mortality were not significant ($p > 0.05$) for the Cd spiked soils. Significant relationships were found between E1 and metal content in earthworms for the Cd50 ($p < 0.01$, $r = 0.67$), Cd300 ($p < 0.01$, $r = 0.68$), Pb ($p < 0.01$, $r = 0.65$), and Zn ($p < 0.05$, $r = 0.44$) spiked soils. Regression analysis established significant relationships between soil pore water and lettuce metal content for th Cd50 ($p < 0.05$, $r = 0.43$), Cd300 ($p < 0.05$, $r = 0.48$) , Pb ($p < 0.01$, $r = 0.55$) and the Zn ($p < 0.01$, $r = 0.83$) spiked soils. Regression analysis revealed strong relationships ($p < 0.01$. $r > 0.62$) between soil pore water and earthworm mortality for the spiked soils. Significant relationships existed between soil pore water and earthworm metal concentrations for the Cd300 (p , 0.01, $r = 0.66$), Pb ($p < 0.01$, $r = 0.73$) and the Zn ($p < 0.05$, r 0.49) spiked soils. The E1 fraction of the PBASE procedure and soil pore water extractions can be employed to provide information on Cd, Pb and Zn phytoavailability as well as information on the toxicity and bioavailability of Cd, Pb and Zn to earthworms.

INTRODUCTION

Soils contaminated with heavy metals are a common problem throughout the United States and worldwide. Sources of heavy metal contamination include mining and smelting of Pb and Zn ores, atmospheric deposition from metallurgical industries, incineration of plastics and batteries, sewage sludge land application, burning of fossil fuels, pesticide use, contamination from vehicle exhausts, and manufacturing processes involving Pb and recycling and disposal of Pb-containing products (Davies 1990; Adriano 2001). Adverse environmental impacts due to soil contamination include risks to human health, phytotoxicity and ecotoxicity. Threats to humans and animals in relation to heavy metals may occur directly or indirectly. Direct threats are a result of inhalation or ingestion of contaminated soil; while indirect threats include contamination of groundwater and/or consumption of plants grown in contaminated soil. Risk of heavy metals is related to the bioavailability of these metals generally defined as "the amount or concentration of a chemical that can be absorbed by an organism thereby creating the potential for toxicity or the necessary concentration for survival" (Parametrix 1995). Estimation of bioavailability is important in the characterization of contaminated sites and bioavailability should be considered in the risk assessment of contaminated sites (Allen 2002).

Both direct and indirect methods exist for the estimation of heavy metal bioavailability. Direct methods involve the responses of organisms and/or the measurement of internal metal concentrations in an organism. Heavy metal bioavailability has been assessed by bioassays using plant or animal models.

Lettuce (*Latuca sativa* L..) is a cadmium (Cd) accumulator and has been used to assess the bioavailability of heavy metals in contaminated soils and food-chain risk to humans (Chaney and Ryan 1994; Brown et. al 1996; Brown et. al. 1998: Logan et. al. 1997; Basta and Gradwohl 2000). The earthworm toxicity test is a standardized toxicity test protocol (ASTM 1995) where several species of earthworms have been shown to be useful in the estimation of heavy metal bioavailability (Morgan and Morgan 1988; Van Gestel 1993; Jannsen et. al. 1997; Marinussen et al. 1997; Scaps et. al. 1997; Peijnenburg et. al. 1999; Lock and Jannsen 2001).

Indirect methods do not employ living organisms to estimate the bioavailability of heavy metals. Instead they involve the measurement of concentrations of chemicals in soils that are potentially available for uptake. In many cases total metal analyses is not a good predictor of heavy metal bioavailability to plants (McLaughlin 2002). Chemical extraction methods correlating well with bioassays of contaminants may be useful in estimating bioavailability of contaminants and performing risk analysis. Chemical fractionation methods involving sequential extraction can be used to determine the chemical forms of the contaminant ranging from water soluble to residual forms trapped in mineral lattices in soil (Chao 1984; Lake et al. 1984; Harrison 1987; Ure 1990). Although sequential extraction methods vary, heavy metal solubility and bioavailability decrease with each successive step of the sequential extraction method. Specific chemical pools have been identified and correlated with plant uptake of heavy metals (Ivenger et al. 1981, LeClaire et al. 1984, Xian

1989 a, b). Additionally, Aten and Gupta (1996) demonstrated that metals in soil extracted with several different weak salt solutions were highly correlated with heavy metal content in lettuce. Recently Basta and Gradwohl (2000) used a simplified sequential extraction procedure, Potentially BioAvailable Sequential Extraction (PBASE), to extract twelve soils contaminated from Pb and Zn mining and smelting activities. They illustrated that Cd and Zn extracted by $Ca(NO_3)_2$ was correlated with lettuce uptake of Cd and Zn. Additionally, it is believed that weakly bound metals extracted by weak salt solutions are available for uptake by earthworms (Jannsen et. al. 1997; Posthuma et. al. 1997; Peijnenburg et. al. 1999). Conder and Lanno (2000) demonstrated that earthworm toxicity was well related in a metal-spiked artificial soil with soluble metal extracted by 0.1 M $Ca(NO_3)_2$. Soft-bodied soil organisms (i.e. earthworms) are generally thought to be exposed to metals mainly by soil pore water with uptake tending to proceed via the soil solution (Peijnenburg 1997; Peijnenburg 2002). The equilibrium partitioning theory assumes a relationship exists between contaminant concentrations in pore water and tissue concentration in organisms and has proven useful for organic chemicals in earthworms (Oste et al. 2001). However, a few studies have examined the relationships between heavy metals in soil pore water and accumulation of heavy metals in earthworms (Jannsen et al.1997; Peijnenburg 1999; Oste et al. 2001). While the uptake of metals in plants is thought to occur via soil solution, few studies have examined the link between heavy metals in soil solution (i.e pore water metals) and plant metal uptake or toxicity (McLaughlin 2002). Research has been conducted showing that total

trace metal concentrations in pore waters of sediments are correlated with organism mortality (Swartz et. al. 1985; Di Toro et al. 1990). This work has not been extended to examine the relationship between pore water metal and earthworm mortality. The objectives of this study were to (1) evaluate the extent of heavy metal accumulation in earthworms and lettuce in soils with different properties; (2) determine the relationships between soil extracts (pore water and 0.5 M $Ca(NO_3)_2$) and lettuce metal content; and (3) determine the relationships between soil extracts (pore water and 0.5 M $Ca(NO_3)_2$) and metal content in earthworms.

MATERIALS AND METHODS

Selection of Soils

Approximately 40 soils were collected from the states of Oklahoma and Iowa to provide a wide range of soil properties including soil pH, organic carbon (OC) content, cation exchange capacity (CEC) and clay content. Oklahoma has a very diverse paleoclimatology and geology with soils that represent 8 of the 11 soil orders. Soils collected from central Iowa were Mollisols with a high organic C content. Chemical and physical properties were measured on the collected soils and twenty-two soils were selected from the larger set of 40 soils.

Selected soils showed a wide range in chemical properties including soil pH (4.0 to 8.0), organic carbon (0.3 to 3.0%), CEC (3.0 to 32.4 $cmol_c\ kg^{-1}$), and clay content (5.0 to 71.3%) (Table 1). Metal contaminants in selected soils were determined by acid digestion using microwave (CEM MDS 2100, CEM Corporation, Matthews, NC, USA) according to U.S. EPA Method 3051 for

confirmation of non-contamination prior to analysis of chemical and physical properties (U.S. EPA 1994). Duplicate analyses were conducted on all collected samples for the determination of baseline metal content. Heavy metal contaminant contents (Cd, Pb and Zn) of study soils were similar to uncontaminated background soil contents in literature (Table 2). Blanks, spikes and certified reference soil (CRM020-050, RTC Corporation, Laramie, WY, USA) were digested and analyzed as QA samples.

Preparation of Contaminated Soils by Contaminant Spiking

Soils were spiked with reagent grade $Cd(NO_3)_2$, $Pb(NO_3)_2$, or $Zn(NO_3)_2$ to obtain soil concentrations of 50 mg Cd/kg, 300 mg Cd/kg, 2000 mg Pb/kg, or 300 mg Zn/kg. Soils were spiked with only one metal (e.g. Pb spiked soil, Cd spiked soil, etc) to avoid competitive adsorption effects (Basta and Tabatabai, 1992a). One liter of spiking solution was prepared using reagent grade metal salt and deionized distilled water. Ten ml of the spiking solution was retained and analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES) to confirm the spiking concentration. Spiking solution (1 L) was added and mixed with 5.0 kg of soil in an aluminum pan. Additional deionized distilled water was added and thoroughly mixed with the soil to make a saturated paste. The soil was oven-dried at 105 °C for 24 h. After 24 h, the dried soil was removed from the oven and deionized distilled water was added to make a saturated paste followed by drying at 105 °C for 24 h. Excessive soil salinity (salt) may reduce the growth and yield of many crops and is commonly determined by measuring the electrical conductivity (EC) of a solution extracted from a water-saturated soil

paste. Therefore, soil (EC) of the spiked soils was determined after the second wetting and drying cycle as follows: Fifty grams of soil was shaken with 50 ml of deionized distilled water for 2 h. Soil EC was measured on filtered solution (0.45 μm Supor membrane filter; Fisher Scientific, Pittsburgh, PA). Soils with EC > 1.5 dS/m (i.e. a level that should not reduce yields of lettuce) were leached with the minimum amount of deionized distilled water to reduce EC < 1.5 dS/m (Soltanpour and Follett, CSU Fact Sheet No. 0.505). Soils that had EC < 1.5 dS/m were not leached. All soils underwent three wet-dry cycles to achieve adequate reaction with the soil matrix. Heavy metals added as salt to soil can result in "salt effect" where heavy metal availability is greater in spiked soil than untreated soil (Logan and Chaney 1983). The sequence minimized artifacts from spiking by reducing the "salt effect." Treated soils were crushed using a jar mill and corundum ball grinding media to pass a 2.0 mm sieve. Spiked soils were digested by microwave according to U.S. EPA Method 3051 to confirm the spiked concentration of each metal. Ranges of spiked metal concentrations, in parentheses, were Cd50 (40-54 mg Cd/kg soil), Cd300 (270-326 mg Cd/kg soil), Pb (1,697-2,023 mg Pb/kg soil) and Zn (292-445 mg/kg soil) (Table 3). Blanks, spikes and certified reference soil (CRM020-050, RTC Corporation, Laramie, WY, USA) were digested and analyzed for quality assurance in the determination of metal content in soil.

Chemical and Physical Properties

Soil pH was determined in 1:1 soil:water suspension (Thomas, 1996) as follows: 10.0 ml of deionized distilled water was combined with 10.0 g of air-dried

soil in a 50.0 ml plastic solo cup. The suspension was mixed for 15 min. The suspension was allowed to settle for 10 min and the pH was measured using a combination pH electrode. Duplicate soil pH analyses were conducted on each soil. Because metal salt additions result in acidification from metal hydrolysis (Basta and Tabatabai, 1992b), soil pH was measured on control (unspiked) and on metal-spiked soils.

Soil organic carbon content was determined by acid dichromate digestion according to Heanes (1984). Air-dried soil was ground to < 0.15 mm. Soil (0.5 g) was added to 5.0 ml of 0.5 M $K_2Cr_2O_7$ and 10.0 ml of concentrated H_2SO_4 in glass digestion tubes. Calibration standards of organic carbon were prepared from sucrose (Fisher Scientific, Pittsburgh, PA, USA) and treated similarly as to samples. Also, two reagent blanks were prepared for digestion and subsequent analysis. Samples, calibration standards and blanks were placed into a pre-heated digestion block set at 145 °C and digested for 30 min. The digested samples, calibration standards and blanks were diluted to 50.0 ml in a volumetric flask then filtered through 0.45 μm filters. Absorbance at 600 nm was measured on a spectrophotometer for samples, calibration standards and blanks. The amount of organic carbon in the sample was determined from a calibration curve generated using sucrose. Duplicate analyses were conducted on each soil in the determination of organic matter.

Cation exchange capacity of non-calcareous soil (soil pH < 7.0) was determined using a procedure adopted from Hendershot and Duquette (1986). Soil (0.5 g) was weighed into 50 ml centrifuge tubes. Twenty ml of 0.1 M $BaCl_2$

was added and the samples were shaken for 2.0 h. The extracted samples were centrifuged at 10,000 rpm for 10.0 min and filtered with 0.45 μm filters. Concentrations of cations were analyzed by ICP-AES and summation of cations (Al, Ca, Fe, K, Mg, Mn and Na) was used to represent the CEC of each soil.

Cation exchange capacity of calcareous soils (soil pH > 7.0) was determined according to the method of Polemio and Rhoades (1977) as follows. Soil (5.0 g) was weighed into 50 ml centrifuge tubes and saturated with 33.0 ml of a 60% ethanol solution of 4.0 N NaOAc-0.1 N NaCl. Equilibration was obtained by shaking the soil-solution mixtures for 5.0 min. Samples were centrifuged at 10,000 rpm for 10.0 min and the supernatant were discarded. The process was repeated three more times. Then the saturated sample was extracted with 33.0 ml of a 1.0 N $Mg(NO_3)_2$ solution by shaking for 5.0 min followed by centrifugation. Supernatant was retained in a 250 ml volumetric flask and the process was repeated three more times. The extracted supernatants were combined, diluted to a final volume of 250 ml and analyzed for Na using ICP-AES and for Cl using ion chromatography with the resulting concentrations were used to calculate CEC. Duplicate analyses were conducted on each soil in the determination of cation exchange capacity.

Soil texture was determined by the hydrometer method (Gee and Bauder, 1986). Each soil was pretreated to remove organic matter prior to particle size analysis as follows: Deionized distilled water (100 ml) was added to 100 g of soil in a 1 L glass beaker. The beakers were placed in a hot water bath set to 85 °C and 50 ml of H_2O_2 was added to each beaker. The addition of H_2O_2 resulted in

frothing from oxidation of soil organic matter. Samples were periodically stirred and heated until frothing subsided. They were re-treated with 50.0 ml H_2O_2 and heated until frothing subsided. The samples were treated until a total of 300 ml of H_2O_2 had been added. Samples were then oven-dried at 105 °C and ground to pass through a 2.0 mm sieve with a mortar and pestle. Soil (40.0 g) was weighed into a 200 ml container. One hundred ml of 5% sodium hexametaphosphate was added and each sample was shaken for 16 h. The suspension was then transferred into a 1 L glass graduated cylinder and deionized water was added to bring to 1.0 L final volume. Hydrometer readings were taken at 30 sec and 7 h 14 sec to determine the sand and clay content, respectively. Silt was determined by difference (100% - %sand -%clay). Hydrometer readings of blank solution were used to compensate for differences in temperature and solution viscosity. Duplicate analyses were conducted on each soil in the determination of soil texture.

Amorphous (i.e. reactive) Al and Fe oxides were determined on unspiked control soils using Tamm's reagent according to the procedure of Loeppert and Inskeep (1996). Soil (0.5 g) ground to < 0.15 mm was added to 30 ml of acidified ammonium oxalate solution (0.175 M ammonium oxalate plus 0.1 M oxalic acid, pH adjusted to 3.0) in 50 ml centrifuge tubes, shaken for 2.0 h, centrifuged at 10,000 rpm for 10.0 min, and analyzed for Al and Fe by ICP-AES. Duplicate analyses were conducted on each soil in the determination of aluminum and iron oxides.

Sequential Extraction Procedure

The solubility and potential bioavailability of Cd, Pb and Zn in the spiked soils were determined using a four step sequential extraction procedure developed by Basta and Gradwohl (2000) and termed the potential bioavailability sequential extraction or PBASE procedure. Soil (1.0 g) was placed in a 50 ml centrifuge tube and extracted with 20.0 ml of 0.5 *M* $Ca(NO_3)_2$ solution (E1 solution). The samples were shaken end-to-end on a reciprocal shaker for 16 h. The solution was then centrifuged at 10,000 rpm for 15 min and the supernatant decanted and filtered through a 0.45 μm membrane filter. The supernatant were acidified with 1.0 ml of trace metal concentrated HCl and stored at 4°C until analysis of metal by ICP-AES. In the second step of the PBASE, 20.0 ml of 1 *M* NaOAc (E2 solution) adjusted to pH 5.0 was added to the residue soil in the tube and shaken for 5 hr. After extraction, the supernatant was prepared for analysis as detailed above. In the third step of the PBASE, 20.0 ml of 1 *M* Na_2EDTA (E3 solution) adjusted to pH 7.0 was added to the residue in the tube and shaken for 6 h. The resulting supernatant was filtered but not acidified because acidification causes precipitation of EDTA salts. The EDTA extracts were stored at 4°C until analysis. In the final step of the PBASE, 20.0 ml of 4 *M* HNO_3 (E4 solution) was added to the residue in the tube and shaken for 16 h in a heated water bath (80 °C). The E4 extract was then filtered through a 0.45 membrane filter prior to metal analysis. Triplicate analyses were conducted for all soils in the study.

Pore Water Extraction

Approximately 40.0 g of soil was weighed into plastic solo cups. Deionized distilled water was added to each sample to make a saturated paste as described by Rhoades (1996) when measuring electrical conductivity. The soils were allowed to equilibrate for 48 h, transferred to 50 ml tubes and centrifuged at 10, 000 rpm for 15 min. The supernatant was filtered through a 0.45 μm membrane filter, acidified with trace metal HCl and retained for subsequent metal analyses.

Lettuce Bioassay

Lettuce (*Lactuca sativa var. Paris Island Cos*) was grown in 15-cm pots containing 750 g samples of control or spiked soil placed over a 2-cm layer of vermiculite in a completely randomized design with three replicates. Twenty seeds were planted per pot and the pots were kept in an environmental chamber for 40 days, during which they received 16 h light at 22°C ± 3°C and 8 h of darkness at 20°C ± 3°C per day. Seed germination was counted at 8 days. Lettuce was thinned to five plants per pot at 14 days. Lettuce was watered to "'field capacity" as needed and the pots were fertilized three times with a dilute nutrient solution (3.64 g L^{-1}) of commercial plant food (20-20-20). Lettuce was harvested at the soil surface and washed three times with deionized water. Dry matter production and metal content of lettuce was determined after harvest. The lettuce was dried at 75°C for 48 h then ground (< 2 mm) in a Wiley mill (Jones and Case 1990). Dry lettuce tissue (0.25 g) was predigested for 4 h in 10 ml of trace-metal nitric acid. Predigested samples were digested at 140 °C for 2

h. Digested samples were diluted to a final volume of 25.0 ml and analyzed for metals by ICP-AES. Blanks, spikes and certified reference materials (National Institute of Standards and Technology Spinach Leaves SRM 1570a for Cd, Zn and Commission of the European Communities Trace Elements in an Aquatic Plant, *Lagarosiphon major* BCR No 60 for Pb) were digested and analyzed for quality assurance in the determination of metal content in lettuce.

Earthworm Bioassays

Twenty-eight day earthworm bioassays were conducted using mature (clitellate) manure worms (*Eisenia andrei*) according to the American Society for Testing and Materials (ASTM) protocol (ASTM 1997). Testing was conducted in an environmental chamber set to 20°C ±1 °C with constant light and three replicates of each spiked metal. Each replicate contained 200 g of soil and 10 earthworms. Testing was also conducted on unspiked soils and ASTM artificial soil (69.5% silica sand, 20% kaolin clay, 10% 2-mm sieved sphagnum moss, and approximately 0.5% $CaCO_3$) to serve as controls during the experiment. Prior to testing, 200 g of each replicate was placed in glass jars with three small holes in the lid, moistened to between 1/3 bar and saturation and allowed to acclimate for 24 h in the environmental chamber. Twenty-four hours prior to the addition of earthworms to the test soils, earthworms weighing 0.2-0.4g were removed from in-house culture containers, rinsed with distilled water and placed on moist filter paper to depurate most of the bedding from their intestinal tract (van Gestel et al. 1993). At the start of the toxicity test, earthworms were removed from the filter paper, rinsed, blotted dry and separated into replicate groups of 10 earthworms.

Each replicate group was weighed and arbitrarily added to a randomly determined soil replicate. Earthworms were monitored daily for the first eight days and afterwards they were checked three times per week for the duration of the 28 day test. During each check, any dead earthworms were removed, rinsed thoroughly with distilled, deionized water, individually wrapped in aluminum foil and frozen at -20°C for storage. Earthworms were judged dead if they failed to respond to gentle stimulation with a blunt probe. Live earthworms present at the termination of the tests were rinsed, weighed and stored as described above.

Internal Concentrations of Earthwoms

Metal concentrations in earthworms were determined according to the method of Morgan et al. (1982). One worm from each replicate was unfrozen, dried for 24 h at 105°C in a 10 ml beaker and weighed. Individual worms were digested in 5.0 ml of concentrated trace metal nitric acid. Digests were evaporated to dryness, resolubilized at 60°C for 15 min in 3.0 ml of 0.5 M trace metal nitric acid and diluted to a final volume or 10 ml with 0.5 M trace metal HNO_3. Metal concentrations in earthworm tissue were measured by graphite furnace atomic absorption spectroscopy (GFAAS) and expressed as mg metal/kg dry weight earthworm. Blanks, spikes and certified reference material (lobster hepatopancreas, TORT-2, National Research Council, Canada) were digested and analyzed for quality assurance and quality control in the determination of metal in earthworms.

Quality Assurance and Quality Control

Blanks, spikes and certified/standard reference materials were digested and analyzed for quality assurance and quality control of metals in soil, plant tissue and earthworm tissue. Blanks, spikes and certified/standard reference materials were evaluated for every six samples of soil, plant tissue or earthworm tissue. Examples of certified/standard reference materials for different sample types include: Soil (CRM020-050, RTC Corporation, Laramie, WY, USA); Plant tissue (National Institute of Standards and Technology Spinach Leaves SRM 1570a for Cd, Zn and Commission of the European Communities Trace Elements in an Aquatic Plant, *Lagarosiphon major* BCR No 60 for Pb), and Earthworm Tissue (lobster hepatopancreas, TORT-2, National Research Council, Canada). Digested blanks contained below detection limit concentrations of Cd, Pb and Zn for all three types of samples (Table 4). Within the three types of samples spike recoveries of metals ranged from 95 to 100%. Mean recoveries of metal in certified reference soil (CRM020-050, RTC Corporation, Laramie, WY, USA) ranged from 98 to 99% with relative standard deviations ranging from 0.63 to 3.7% (Table 4). While mean recoveries of metals in certified plant materials ranged from 93 to 95% with relative standard deviations ranging from 2.6 to 2.7% (Table 4). Concurrently mean recoveries for metals in lobster hepatopancreas ranged from 96 to 102% with relative standard deviations ranging from 1.1 to 3.2% (Table 4).

.RESULTS AND DISCUSSION

Soil Extractions

The distribution of metal species within the four PBASE extraction fractions varied among metals (Figure 1). Comparison of the mean values for the 22 spiked soils show that 57% of soil Cd was in the E1 fraction and approximately 68% of soil Cd was extracted by the first two extracts of PBASE ($\sum$E1-E2) for the Cd50 soil. Slightly more soil Cd was extracted in the E1 fraction of the Cd300 soil (68%) with approximately 74% of the soil Cd being extracted by the first two extracts ($\sum$E1-E2). Approximately 36% of the soil Pb was extracted by the first two extracts ($\sum$E1-E2)) which is composed of 28% E1 extractable Pb and 8% E2 extractable Pb. Mean Zn extracted in the E1 fraction was 26% while 28% was extracted by the first two extracts of the PBASE. The bioavailability of metals is related to extractability, thus the relative bioavailability of the four fractions of the PBASE should be E1 > E2 > E3 > E4. The results of our study indicate that the metal solubility of spiked soils is Cd > Pb = Zn. The percentages of metal extracted in the E1 step of the sequential procedure are much higher than those reported by other researchers. Basta et al. (2000) used the same sequential extraction procedure to investigate twelve smelter-contaminated soils and reported that the E1 step extracted 25% of the total Cd, 0.2% of the total Pb, and 6% of the total Zn present in the soils. They reported the solubility of metals in smelter-contaminated soils as Cd > Zn > Pb. Sloan et al. (1997) studied agricultural soils that received applications of biosolids and found that metal extracted by 0.5 M $Ca(NO_3)_2$ ranged from 38 to 48% for Cd, 0.4

to 1.5% for Pb, and 10 to 17% for Zn. Conder et al. (2001) reported 0.1M $Ca(NO_3)_2$ extracted approximately 46.1% of the Cd, 0.3% of the Pb, and 6.9% of the Zn present in a smelter-contaminated soil. However our results are somewhat comparable to those of Conder and Lanno (2000) who spiked an artificial soil with five different concentrations of metals (Cd, Pb and Zn) and found that 0.1M $Ca(NO_3)_2$ extractable Cd ranged from 27 to 59%, extractable Pb ranged from 0.4 to 9.7% and extractable Zn ranged from 13 to 72%. Similarly, Geebelen et al. (2002) spiked an acid sandy soil with 2000 mg total Pb/kg and reported 0.1M $Ca(NO_3)_2$ extracted 24.6% of the total lead. Perhaps spiking of the soils resulted in increased amounts of metal in the soluble and exchangeable E1 fraction as compared to smelter-contaminated or biosolids amended soils.

Mean pore water concentrations of spiked metals, in parentheses, were Cd50 (2.39 mg/L), Cd300 (23.2 mg/L), Pb (24.4 mg/L), and Zn (28.5 mg/L) (Table 5). These concentrations are much greater than the concentrations reported by Kabata-Pendias and Pendias (1992) for Cd (0.006 mg/L), Pb (0.008 mg/L) and Zn (0.351 mg/L) in solutions of uncontaminated soils. Our results are similar to those reported by other spiking studies. Oste et al. (2001) spiked an uncontaminated sandy Dutch soil with cadmium nitrate to obtain a final soil concentration of 10 mg Cd/kg soil and reported total Cd in pore water as 1.47 mg/L. Mitchell et al. (1975) spiked sewage sludge with cadmium or zinc sulfate and added the spiked sludge to a calcareous and acidic soil to obtain final soil concentrations ranging from zero to 640 mg metal/kg soil. Their study reported pore water Cd in soils spiked with 40 mg Cd/kg as 3.9 mg Cd/L for calcareous

soil and 2.8 mg Cd/L for acidic soil while soils spiked with 320 mg/kg soil contained 6.8 mg Cd/L (calcareous soil) and 35 mg Cd/L (acidic soil). In the same study, water-soluble Zn concentrations for soils spiked with 320 mg Zn/kg soil were 4.0 mg Zn/L for the calcareous soil and 79 mg Zn/L for the acidic soil. The pore water concentrations in our spiked soils are orders of magnitude greater than those reported by Janssen et al. (1997a) who evaluated pore water concentrations in 20 Dutch field soils that contained Cd (range of 0.11 to 49.5 mg/kg), Pb (range of 70.4 to 847 mg/kg), and Zn (range of 5.23 to 3,110 mg/kg). Their reported medians for pore water metal concentrations, in parentheses, were Cd (0.002 mg/L), Pb (0.010 mg/L) and Zn (0.202 mg/L). Our results are comparable to those of Peijenburg et al. (2000) who spiked a field soil with metal salts and reported increased pore water concentrations of Cd, Pb and Zn in the spiked soils as compared to soils with approximately the same total metal content that received metal inputs from sources other than spiking.

Lettuce Germination, Yield and Metal Content

Lettuce germination rates in unspiked soils with germination ranged from 50.0% to 91.7% and averaged 77.5%. Lettuce also successfully germinated in all spiked soils with the exception of the Bernow B Zn spiked soil (Table 6). Germination for the Cd50 spiked soils ranged from 70.0 to 95.0% with an overall mean of 82.9%. Mean germination for the Cd300 spiked soils was 70.1% with germination ranging from 56.7 to 91.7%. Within the Pb spiked soils, germination ranged from 66.7 to 95.0% and averaged 83.6%. Germination results for the Pb spiked soils differ from those of Chang et al. (1997) who reported that

germination was reduced to approximately 68% in an artificial soil consisting of 100% silica sand spiked with lead nitrate to levels of 300 mg Pb/kg soil. In our study, only two of the Pb spiked soils (Mansic A and Summit A) displayed < 70% germination at a spiked concentration of 2000 mg Pb/kg soil. Perhaps the difference in germination between the studies is that the Pb was more available in the spiked sand than with our soils, which contain clay and organic matter having a higher capacity for adsorption of metals as compared to the silica sand. Germination for the Zn spiked soils (excluding the Bernow soil) ranged from 68.3 to 95.0% with an overall mean of 83.3%.

Mean yield of lettuce grown on unspiked soils ranged from 0.42 to 8.27 g with an overall mean of 5.66 g (Table 7). Yield of lettuce grown on spiked soils varied by soil and metal and was reduced in all spiked soils as compared to unspiked soils (Table 7). Lettuce yield for the Cd50 spiked soils ranged from 0.12 to 4.60 g with an overall mean of 1.87 g. Mean lettuce yield was reduced by approximately 67% for the Cd50 and 88% for the Cd300 spiked soils as compared to the mean yield for the unspiked soils. Mean yield of 0.69 g was more reduced on the Cd300 spiked soil as compared to the Cd50 spiked soils and ranged from 0.07 to 3.41 g (Table 7). The results for the Cd spiked soils are similar to those of other investigators. Moustakas et al. (2001) found lettuce yields were reduced (9%) when grown on a soil spiked with concentrations greater than 10 mg Cd/kg soil. In another study, Bingham et al. (1975) reported that soil Cd of 13 mg Cd/kg soil resulted in a 25% reduction in yield of lettuce grown on soils treated with cadmium-enriched sewage sludge. Mean yield for

the Pb spiked soils ranged from 0.13 to 6.45 g averaging 1.92 g (Table 6). Mean yield for the Pb spiked soils was reduced by approximately 66% as compared to the mean yield of the unspiked soils which is consistent with the yield reductions reported in other studies. John and Laerhoven (1972) reported that lettuce yield from soils spiked with 1000 mg Pb/kg soil was reduced by approximately 25% as compared to yields on unspiked soil. Yield for the Zn spiked soils (excluding the Bernow soil) ranged from 0.22 to 5.65 g with an overall mean of 2.84 g (Table 7). Mean yield of lettuce was reduced by approximately 50% as compared to mean yield for the unspiked soils. Mitchell et al. (1975) grew lettuce on a calcareous and an acidic soil that were amended with spiked sewage sludge to obtain soil concentrations of 320 mg Zn/kg soil and reported that lettuce yields were reduced by approximately 10% on the calcareous soil and approximately 40% on the acidic soil.

All three metals (Cd, Pb and Zn) accumulated in lettuce and metal content of lettuce varied by soil (Table 8). Cd content in lettuce grown on the Cd50 spiked soils ranged from 23.8 mg/kg to 221 mg/kg with an overall mean of 76.1 mg/kg (Table 8). Lettuce Cd for the Cd300 spiked soils ranged from 57.8 mg/kg to 403 mg/kg and averaged 156 mg/kg. The results for the Cd spiked soils are consistent with those of other researchers. Brown et al. (1998) spiked two soils (pH = 6.9, pH = 5.2) with cadmium chloride to a concentration of 5.7 mg total Cd/kg soil and evaluated Cd content in Paris Island Cos lettuce during four different growing seasons. Lettuce Cd for the spiked soil with a pH of 6.9 ranged from 19.4 to 37.2 mg/kg in their study while Cd in lettuce grown on the spiked soil

with a pH of 5.2 ranged from 22.6 to 51.0 mg/kg. Similarly, Moustakas et al. (2001) spiked a 50/50 mixture of peat and soil (pH = 7.1) with cadmium oxide to reach 50 mg Cd/kg soil and reported concentrations of approximately 50 mg/kg in Parris Island lettuce. Other studies involving spiking of sewage sludge with cadmium sulfate and its addition to soil to obtain final soil concentrations of 320 mg Cd/kg soil have resulted in lettuce Cd ranging from 413 to 780 mg Cd/kg lettuce (Mahler et al. 1978; Mitchell et al. 1978). Mean content of Cd for lettuce grown on unspiked soils was 0.23 mg/kg which is comparable to concentrations of 0.69 to 1.84 mg Cd/kg reported by Brown et al. (1998) for Paris Island Cos lettuce grown on control plots. Mean Pb content in lettuce grown on spiked soils ranged from 3.22 to 233 mg/kg averaging 63.9 mg/kg (Table 8). John and Laerhoven (1972) used lead nitrate to spike an acid soil (pH_{KCl} = 3.8) with 1000 mg Pb/Kg soil and reported Pb content in lettuce as 141 mg/kg. Nwosu et al. (1995) grew lettuce on a soil (pH = 6.2) spiked with a mixture of Cd and Pb and reported accumulations of 39.0 mg Pb/kg lettuce at a spiked mixture of 100 mg Cd/kg soil and 1000 mg Pb/kg soil. Mean Pb content in lettuce grown on unspiked soils was extremely low (i.e. < 0.5 mg/kg) similar to that reported by Kabata-Pendias and Pendias (1992) for lettuce grown on uncontaminated sites (range of 0.7 to 3.6 mg/kg). Mean Zn content in lettuce grown on spiked soils was 322 mg/kg with a range of 18.4 to 2,040 mg/kg (Table 8). Mitchell et al. (1975) analyzed lettuce grown on a calcareous and an acidic soil that were amended with spiked sewage sludge to obtain soil concentrations of 320 mg Zn/kg soil detailing that lettuce from the calcareous soils contained 380 mg Zn/kg

while lettuce from the acidic soils contained 1,585 mg Zn/kg. Zn content in lettuce grown on unspiked soils ranged from 11.9 mg/kg to 39.4 mg/kg with an overall mean of 21.1 mg/kg. Kabata-Pendias and Pendias (1992) reported that Zn concentrations in lettuce grown on uncontaminated areas ranged from 44 mg/kg to 73 mg/kg.

Earthworm Mortality and Internal Concentrations

The mortality of earthworms varied by soil type and metal species (Table 9). Mean earthworm mortality for the 22 unspiked soils never exceeded 10% and averaged 1.21%, which is indicative of a valid toxicity test. Mean earthworm mortality for the Cd50 soil ranged from zero to 93.3% with an overall mean of 7.27%. The Cd300 soils were more toxic to earthworms as compared to the Cd50 spiked soils with mortality ranging from zero to 100% and averaging 18.9% (Table 9). Our results differ from those of Spurgeon et al. (1994) who reported no significant mortality in *E. fetida* exposed for 28 days to a spike concentration of 300 mg Cd/kg soil in an artificial Organization for Economic Cooperation Development (OECD) soil. Within the Pb spiked soils, earthworm mortality ranged from zero to 100% with an overall mean of 39.1% (Table 9). Significant mortality (i.e. > 50%) occurred in several of the Pb soils at the spiked concentration of 2000 mg Pb/kg soil which is lower than levels reported by other researchers to cause significant mortality in earthworms. Davies et al. (2002) conducted the 28 day OECD draft earthworm reproduction test using an artificial soil and *E .fetida* as the test organism. Their study reported the LC_{50} for Pb as 5,395 mg/kg and the No Observed Effect Concentration (NOEC) as 3,000 mg/kg.

Earthworm mortality for the Zn spiked soils ranged from zero to 100% and averaged 14.5%. However, 100% mortality occurred in only one soil (Efaw) and mortality in the other soils was < 50% (Table 9). Zero mortality was observed for many of the soils in our study, consistent with the findings of Spurgeon and Hopkin (1996) who reported zero mortality in earthworms exposed for 14 days to the OECD artificial soil spiked with 350 mg Zn/kg. Conder and Lanno (2000) spiked an artificial soil, similar to the OECD soil with Cd, Pb or Zn salts and conducted toxicity tests with *Eisenia fetida.* They reported time-independent LC_{50}s for Cd, Pb, and Zn as 2,237; 5,822; and 631 total mg metal/kg soil, respectively. In our study, 100% mortality occurred in some soils at much lower total concentrations indicating that soil properties were affecting bioavailability of metals to earthworms.

Mean internal metal concentrations, in parentheses, for earthworms exposed to unspiked soils were Cd (7.30 mg/kg), Pb (0.60 mg/kg) and Zn (148 mg/kg) (Table 10). These findings are consistent with those of Janssen et al. (1997b) who found 5.17 mg Cd/kg, 2.07 mg Pb/kg and 196 mg Zn/kg in *Eisenia andrei* exposed to an uncontaminated OECD artificial soil for 21 days. Metals bioaccumulated in earthworms exposed to the spiked soils with mean internal concentrations in earthworms for the Cd50 spiked soils ranging from 71.0 to 1,190 mg/kg with an overall mean of 321 mg/kg (Table 10). More Cd accumulated in the tissues of earthworms exposed to the Cd300 spiked soils as compared to the Cd50 spiked soils. Internal concentrations in earthworms for the Cd300 soil averaged 529 mg/kg and ranged from 77.4 to 908 mg/kg (Table 10).

The results of the Cd spiked soils are similar to those of Lock and Jannsen (2001) who evaluated uptake and elimination of Cd in *E. fetida* by spiking the OECD soil with varying concentrations of Cd. They found that at the conclusion of their experiment (28 days) earthworms accumulated approximately 150 mg Cd/kg earthworm tissue at a spiked concentration of 56 mg Cd/kg soil and approximately 1000 mg Cd/kg earthworm tissue at a spiked concentration of 560 mg Cd/kg soil. Earthworm concentrations for the Pb spiked soils ranged from 28.7 to 1,030 mg/kg and averaged 306 mg/kg (Table 10). Scaps et al. (1997) found that *E. fetida* accumulated approximately 350 mg/kg tissue when exposed for 56 days to an artificial soil spiked with 2000 mg Pb/kg soil. Mean concentrations of Zn in earthworms exposed to spiked soils were not different from concentrations found in earthworm exposed to unspiked soils, thus Zn appeared to be regulated in earthworms exposed to spiked soils. Mean internal concentrations of Zn ranged from 38.2 to 188 mg/kg with an overall mean of. 136 mg/kg (Table 10). Several researchers have reported that Zn concentrations are regulated by different species of earthworms (Van Gestel et al. 1993; Peijunburg et al. 1999; Lock and Janssen 2001). Lock and Janssen (2001) reported that Zn was regulated by *E. fetida* to an internal concentration of approximately 100 to 120 mg/kg earthworm when exposed to concentrations of Zn in the OECD soil that ranged from 10 to 1000 mg Zn/kg soil.

Linear Regressions

Linear regressions between lettuce data and soil extractions are shown in Table 11. No significant relationships (i.e. $p > 0.05$) were found between

germination of lettuce in the Cd, Pb or Zn spiked soils and soil extractions. It appears that germination is not a sensitive enough indicator to be visibly affected by soil properties at the metal levels used in our study. Significant positive relationships were found between yield and PBASE E1 fraction for the Cd50 ($p < 0.05$, $r = 0.41$) and the Zn ($p < 0.01$, $r = 0.57$) spiked soils. Summations of E1 with other PBASE fractions did not improve the regressions found between yield and E1 for the Cd50 or the Zn spiked soils. Significant negative relationships were found between yield and the E3 PBASE fraction ($p < 0.05$, $r = -0.45$) and between yield and the E4 PBASE fraction ($p < 0.01$, $r = 0.51$) for the Cd50 spiked soils. A significant negative relationship was also found between yield and the E3 PBASE fraction for the Zn spiked soils ($p < 0.05$, $r = -0.42$). Significant relationships also existed between yield and pore water concentrations for the Cd50 spiked soils ($p < 0.05$, $r = 0.47$) and the Zn spiked soils ($p < 0.05$, $r = 0.46$). Strong relationships were found between lettuce uptake and metal extracted by PBASE E1 fraction for the Cd300 ($p < 0.01$, $r = 0.62$), Pb ($p < 0.01$, $r = 0.74$), and Zn ($p < 0.01$, $r = 0.71$) spiked soils (Figure 2). A weaker relationship was found between Cd in lettuce and the E1 fraction for the Cd50 spiked soils ($p < 0.05$, $r = 0.41$) (Figure 2). Summations of E1 with other PBASE fractions did not improve the regressions found between metal content of lettuce and E1. Our results are slightly different from those established by other researchers. Sloan et al. (1997) studied agricultural soils that received applications of biosolids and reported significant relationships between metal extracted by 0.5 M $Ca(NO_3)_2$ and lettuce Cd ($p < 0.01$, $r = 0.85$) and lettuce Zn ($p < 0.001$, $r = 0.87$) . However, they did

not find that a significant relationship existed between Pb extracted by 0.5 M $Ca(NO_3)_2$ and Pb in lettuce ($p > 0.05$, $r = 0.55$). Similarly, Basta et al. (2000) used the same sequential extraction procedure as our study to investigate twelve smelter-contaminated soils and reported that the PBASE E1 fraction was significantly correlated with lettuce Cd ($p < 0.001$, $r = 0.97$, and Zn ($p < 0.05$, $r = 0.68$) but was not significantly correlated with lettuce Pb ($p > 0.05$, $r = 0.54$). Perhaps spiking of the soils resulted in increased amounts of Pb in the soluble and exchangeable E1 as compared to their studies allowing significant relationships to be established. Strong relationships were found between metal content in lettuce and pore water metal for the Pb ($p < 0.01$, $r = 0.55$) and the Zn ($p < 0.01$, $r = 0.83$) spiked soils (Figure 3). Weaker relationships were found between lettuce metal and concentrations in pore water extracts for Cd50 ($p < 0.05$, $r = 0.43$) and Cd300 ($p < 0.05$, $r = 0.48$) spiked soils. These results are similar to those of Mitchell et al. (1975) who spiked sewage sludge with cadmium or zinc sulfate, added the spiked sludge to soils to obtain final soil concentrations ranging from zero to 640 mg metal/kg soil, and grew lettuce on a calcareous and an acidic soil. They reported water-soluble metal was significantly related to lettuce Cd ($p < 0.05$, $r = 0.91$) and lettuce Zn ($p < 0.05$, $r = 0.87$). Similarly, Peijnenburg et al. (2000) conducted a bioassay with lettuce using 17 different Dutch field soils that were collected at moderately contaminated sites or sites that were expected to contain background levels of metals and two artificially metal-contaminated soils. They reported a significant relationship using log-log transformed data between lettuce Cd ($p < 0.001$. $r = 0.79$) and pore water Cd.

Their study also found a significant relationship between lettuce Zn ($p < 0.001$, $r = 0.89$) and pore water Zn but no relationship was found for Pb due to low levels of Pb in their study soils and subsequently lack of accumulation of Pb in lettuce tissue.

Linear regressions between earthworm data and soil extractions are shown in Table 12. Strong relationships were found between earthworm mortality and the E1 fraction of the PBASE for the Pb ($p < 0.01$, $r = 0.86$) and the Zn ($p < 0.01$, $r = 0.54$) spiked soils (Figure 4). Summations of E1 with other PBASE fractions did not improve the regressions found between mortality in earthworms and E1. Conder and Lanno (2000) used a one-compartment first order kinetic model to estimate incipient lethal levels for *E. fetida* and showed that metal levels in 0.1 M $CaNO_3)_2$ extractions related well to Cd, Pb and Zn mortality in earthworms exposed to a spiked artificial soil. However, the E1 fraction (0.5 M $CaNO_3)_2$ was not a good predictor of earthworm mortality for the Cd spiked soils in our study (Figure 4). Strong relationships were found between earthworm mortality and pore water concentrations for the Cd50 ($p < 0.01$, $r = 0.62$), Cd300 ($p < 0.01$, $r = 0.76$), Pb ($p < 0.01$, $r = 0.84$), and Zn ($p < 0.01$, $r = 0.62$) spiked soils (Figure 5). Strong relationships were also found between metal content in earthworms and E1 extractable metal for the Cd50 ($p < 0.01$, $r = 0.67$), Cd300 ($p < 0.01$, $r = 0.68$), and the Pb ($p < 0.01$, $r = 0.65$) spiked soils while a weaker relationship ($p < 0.05$, $r = 0.44$) was found between earthworm Zn and pore water Zn for the Zn spiked soils (Figure 6). Our results are similar to those of Jannsen et al. (1997b) who investigated relationships between

concentrations of metals in *E. andrei* and soil extractions in 20 Dutch field soils. Their study found significant relationships between log-transformed metal extracted by a weak electrolyte (0.01 M $CaCl_2$) solution and log-transformed earthworm Cd ($r = 0.69$) and Pb ($r = 0.50$). They did not find a significant relationship between earthworm Zn and 0.01 M $CaCl_2$ extractable Zn during their study ($r = 0.17$). Strong relationships were found between metal content in earthworms and pore water metal for the Cd300 ($p < 0.01$, $r = 0.66$) and the Pb ($p < 0.01$, $r = 0.73$) spiked soils (Figure 7). A weaker but significant relationship was found between metal content in earthworms and concentrations in pore water extracts for Zn ($p < 0.05$, $r = 0.49$) spiked soils. However, a poor relationship was found between earthworm Cd and pore water Cd ($p > 0.05$, $r = 0.03$) for the Cd50 spiked soils. Jannsen et al. (1997b) reported significant relationships between log-transformed earthworm metal and log-transformed pore water metal for Cd ($r = 0.64$) and Pb ($r = 0.52$) but did not find a significant relationship between earthworm Zn and pore water Zn during their study ($r = -0.10$).

CONCLUSIONS

The bioavailability of metals in soils is related to extractability. The percentages of metal in soil extracted by the E1 step of the PBASE varied between metals and among soils with the mean solubility of metals in the spiked soils being Cd > Pb = Zn. The percentages of metal extracted in the E1 step of the sequential procedure are comparable to those found by others in metal spiking studies. Pore water concentrations of spiked metals varied between

metals and among soils but were consistent with yield reductions reported in other spiking studies.

Lettuce successfully germinated in both spiked and unspiked soils. There was little difference between germination in spiked and unspiked soils. It appears that germination in lettuce is not a sensitive indicator of metal toxicity for the spiked concentrations used in our study. Yield of lettuce grown was reduced on spiked soils as compared to unspiked soils. Yield reductions were similar to those reported in other studies. All three metals (Cd, Pb, Zn) bioaccumulated in lettuce and measured concentrations were equivalent to those found by other investigators.

Mortality of earthworms varied by soils and metal. Significant mortality occurred in many of the Cd and Pb spiked soils at much lower concentrations than those reported by other spiking studies that utilized artificial soils possibly due to the effect of soil properties on toxicity. Earthworm mortality was low in the Zn spiked soils, consistent with the findings of other researchers. Metals accumulated in tissue of earthworms were similar to concentrations reported in other spiking studies.

Significant relationships were not found between germination of lettuce in the spiked soils and soil extractions. Yield was not negatively correlated with the E1 step of the sequential extraction procedure. The E1 fraction of the sequential extraction procedure was an accurate predictor of phytoavailable Cd, Pb, and Zn in spiked soils. Other researchers did not report a significant relationship between Pb content in soils and Pb extracted by 0.5 M $Ca(NO_3)_2$. Perhaps

spiking of the soils resulted in increased amounts of soluble Pb as compared to other studies allowing significant relationships to be established. Additionally soil pore water was an accurate predictor of phytoavailable Cd, Pb, and Zn in spiked soils, which is consistent with other published studies.

Strong relationships were found between mortality in earthworms and the E1 fraction of the sequential procedure for the Pb and Zn soils even though the E1 step was not a good predictor of earthworm mortality for the Cd spiked soils. Yet significant relationships existed between metal content in earthworms and E1 extractions in soil for the Cd, Pb and Zn spiked soils. The findings also concluded that there were strong relationships between earthworm mortality and soil pore water concentrations for the Cd, Pb and Zn spiked soils. Although much literature has been published suggesting that pore water extractions are related to mortality of organisms, this work has not been shown specifically between mortality in earthworms and soil pore water. Strong relationships were discovered between earthworm metal content and soil pore water extractions for the Cd300, Pb and Zn spiked soils but not for the Cd50 spiked soils.

In summary, both the E1 fraction of the PBASE procedure and soil pore water extractions can be used to estimate phytoavailable Cd, Pb and Zn in spiked soils that display a wide range of properties. Additionally, our study shows that the E1 step can estimate the bioavailability of Cd, Pb and Zn to earthworms in soils with different properties potentially being useful in the prediction of earthworm mortality in Pb and Zn spiked soils. Pore water extractions can be used to accurately predict earthworm mortality in soils with different properties

that are spiked with Cd, Pb and Zn. Soil pore water concentrations may also be employed in the estimation of bioavailability of Cd, Pb and Zn to earthworms in soils with different properties.

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Table 1. Physical and chemical properties of study soils.

Soil	pH	OC (%)	CEC (cmol/kg)	Al (mg/kg)	Fe (mg/kg)	Soil Texture			
						Sand (%)	Silt (%)	Clay (%)	Class
Canisteo A	7.5	3.00	30.5	1150	795	27.4	33.8	38.8	clay loam
Dennis A	5.6	1.90	9.77	840	2880	40.3	35.9	23.8	loam
Dennis B	6.1	0.80	14.6	1160	1280	18.2	36.8	45.0	clay
Dougherty A	5.3	1.20	3.33	266	364	72.5	16.2	11.3	sandy loam
Efaw A	4.0	1.20	4.57	836	804	32.5	50.0	17.5	silt loam
Hanlon A	7.4	1.60	16.3	447	1530	60.0	22.5	17.5	sandy loam
Haskell A	5.1	1.20	4.85	608	1820	23.2	65.5	11.3	silt loam
Kirkland A	5.6	1.45	14.0	893	1580	16.9	51.8	31.3	silty clay loam
Luton A	7.1	2.00	32.4	701	2390	3.80	24.9	71.3	clay
Mansic A	7.8	1.50	16.5	597	222	32.5	37.5	30.0	clay loam
Mansic B	8.0	0.65	11.7	251	103	25.7	39.3	35.0	clay loam
Osage A	6.6	2.60	28.3	989	5090	11.9	32.4	55.7	clay
Osage B	6.8	2.00	27.5	1440	7900	3.80	34.9	61.3	clay
Perkins A	4.5	0.85	3.01	531	604	65.0	25.0	10.0	sandy loam
Pond Creek A	5.2	1.90	10.7	861	1450	15.0	56.2	28.8	silty clay loam
Pond Creek B	6.0	0.80	12.5	770	1150	17.5	50.0	32.5	silty clay loam
Pratt A	6.5	0.90	4.40	188	164	88.2	6.80	5.00	sand
Pratt B	6.4	0.50	3.40	163	152	86.3	7.45	6.25	loamy sand
Richfield B	7.7	1.10	22.4	710	395	12.5	46.2	41.3	silty clay
Summit A	7.2	2.40	29.4	1350	2190	10.0	44.3	45.7	clay
Summit B	7.0	1.25	27.6	982	983	6.9	36.3	56.8	clay
Mean	6.4	1.47	15.6	749	1612	31.9	35.9	32.2	
Median	6.5	1.25	14.0	770	1150	23.2	36.3	31.3	
Minimum	4.0	0.50	3.01	163	103	3.80	6.80	5.00	
Maximum	8.0	3.00	32.4	1440	7900	88.20	65.5	71.3	

Table 2. Comparison of mean contaminant concentrations of unspiked study soils with baseline soils. All values are expressed as mg contaminant/kg soil.

	Mean Contaminant Concentration (unspiked soils)			Baseline Soils		
Soil	Cd	Pb	Zn	Metal	Mean	Range
Canisteo A	< 0.50	6.61	55.4	Pb	18.0[a]	2.0-200[a]
Dennis A	< 0.50	7.39	43.6	Zn	54.0[a]	10.0-300[a]
Dennis B	< 0.50	13.7	56.8			
Dougherty A	< 0.50	7.69	24.5			
Efaw A	< 0.50	11.6	38.7			
Hanlon A	< 0.50	6.08	47.6			
Haskell A	< 0.50	7.34	26.5			
Kirkland A	< 0.50	9.07	41.1			
Luton A	< 0.50	12.3	150			
Mansic A	< 0.50	4.41	40.1			
Mansic B	< 0.50	< 2.50	34.8			
Osage A	< 0.50	14.4	145			
Osage B	< 0.50	14.3	134			
Perkins A	< 0.50	11.4	26.1			
Pond Creek A	< 0.50	10.0	48.0			
Pond Creek B	< 0.50	8.74	46.2			
Pratt A	< 0.50	2.88	28.2			
Pratt B	< 0.50	2.53	14.9			
Richfield B	< 0.50	12.6	64.3			
Summit A	< 0.50	12.7	56.9			
Summit B	< 0.50	7.6	58.1			

[a]Adriano 2001

Table 3. Mean contaminant concentrations of spiked soils. All values are expressed as mg contaminant/kg soil.

	Mean Contaminant Concentration (spiked soils)			
Soil	Cd50[a]	Cd300[b]	Pb[c]	Zn[d]
Canisteo A	42	306	1816	300
Dennis A	48	312	1924	353
Dennis B	46	315	1946	372
Dougherty A	46	300	1843	312
Efaw A	44	288	1842	296
Hanlon A	48	293	1813	344
Haskell A	53	315	1981	314
Kirkland A	48	303	1878	340
Luton A	51	326	1799	442
Mansic A	45	291	2023	360
Mansic B	47	309	1697	314
Osage A	50	290	1821	441
Osage B	48	306	1810	445
Perkins A	48	295	1812	303
Pond Creek A	46	285	1900	356
Pond Creek B	47	306	1895	333
Pratt A	54	308	2005	365
Pratt B	40	324	1846	292
Richfield B	44	278	1856	396
Summit A	50	314	1943	363
Summit B	54	309	1925	363
Mean	48	304	1875	353
Median	48	306	1856	353
Minimum	40	278	1697	292
Maximum	54	326	2023	445

[a]Nominal spiking concentration = 50 mg Cd/kg soil
[b]Nominal spiking concentration = 300 mg Cd/kg soil
[c]Nominal spiking concentration = 2000 mg Pb/kg soil
[d]Nominal spiking concentration = 300 mg Zn/kg soil

Table 4. Mean recovery, relative standard deviations, and detection limits for ten replicate analyses of metals in standard reference materials (SRM).

SRM	Contaminant	Mean Recovery %	RSD %	Detection Limit
Soil CRM020-050	Cd	99	0.63	0.50
	Pb	98	3.7	1.5
	Zn	99	2.5	0.50
Spinach Leaves SRM 1570a	Cd	94	2.6	0.50
	Zn	93	2.7	0.50
Aquatic Plant BCR No. 60	Pb	95	2.6	1.5
Lobster Hepatopancreas TORT-2	Cd	96	1.1	1.0
	Pb	98	1.2	0.04
	Zn	102	3.2	0.25

Detection limits are expressed as mg contaminant/kg soil, mg contaminant/kg plant tissue, or mg contaminant/kg earthworm tissue.

Table 5. Mean pore water concentrations of spiked soils. All values are expressed as mg contaminant/L solution.

	Mean Contaminant Concentration (spiked soils}			
Soil	Cd50	Cd300	Pb	Zn
Canisteo A	0.04	0.25	0.74	0.22
Dennis A	1.44	12.5	4.48	13.7
Dennis B	0.16	3.50	2.73	0.38
Dougherty A	4.70	53.3	31.9	107
Efaw A	9.19	76.4	56.1	73.5
Hanlon A	0.45	3.65	1.32	2.72
Haskell A	4.86	51.9	21.5	71.9
Kirkland A	1.29	16.0	3.16	16.1
Luton A	0.08	1.21	0.53	0.31
Mansic A	0.06	0.46	0.75	0.23
Mansic B	0.01	0.08	0.15	0.19
Osage A	0.16	1.21	1.70	2.05
Osage B	0.27	2.38	0.64	0.45
Perkins A	17.2	120	92.1	122
Pond Creek A	1.82	13.3	13.2	37.8
Pond Creek B	0.43	8.98	7.21	1.63
Pratt A	2.73	41.2	90.8	81.6
Pratt B	3.93	68.8	124	60.5
Richfield B	0.20	2.02	0.75	0.30
Summit A	0.09	0.74	0.46	0.18
Summit B	0.03	1.25	0.17	0.17
Mean	2.34	22.8	21.6	28.2
Median	0.43	3.65	2.73	2.05
Minimum	0.01	0.08	0.15	0.17
Maximum	17.2	120	124	122

Table 6. Mean germination (%) for three replicates of lettuce grown on unspiked and spiked soils.

		Spiked Soils			
Soil	UnspikedSoils	Cd50	Cd300	Pb	Zn
Canisteo A	78.3	78.3	66.7	75.0	68.3
Dennis A	72.5	86.7	58.3	90.0	73.3
Dennis B	85.0	78.3	73.3	83.3	75.0
Dougherty A	77.5	80.0	75.0	95.0	78.3
Efaw A	62.5	78.3	78.3	83.3	81.7
Hanlon A	83.3	86.7	62.5	80.0	86.7
Haskell A	55.0	86.7	68.3	90.0	78.3
Kirkland A	71.7	80.0	68.3	80.0	75.0
Luton A	76.7	83.3	91.7	83.3	88.3
Mansic A	80.0	78.3	80.0	66.7	90.0
Mansic B	86.7	70.0	61.7	86.7	81.7
Osage A	76.7	91.7	65.0	90.0	83.3
Osage B	91.7	83.3	68.3	88.3	90.0
Perkins A	50.0	85.0	66.7	82.5	95.0
Pond Creek A	83.3	91.7	75.0	85.0	80.0
Pond Creek B	71.7	80.0	56.7	83.3	76.7
Pratt A	81.7	95.0	60.0	83.3	91.7
Pratt B	86.7	80.0	66.7	92.5	88.3
Richfield B	81.7	81.7	76.7	83.3	85.0
Summit A	86.7	71.7	61.7	66.7	93.3
Summit B	88.3	93.3	78.3	90.0	83.3
Mean	77.5	82.9	69.5	83.7	83.0
Median	80.0	81.7	68.3	83.3	83.3
Minimum	50.0	70.0	56.7	66.7	68.3
Maximum	91.0	95.0	91.7	95.0	95.0

Table 7. Mean yield (g) for lettuce grown on unspiked and spiked soils.

Soil	Unspiked Soils	Spiked Soils			
		Cd50	Cd300	Pb	Zn
Canisteo A	7.00	0.89	0.14	3.32	1.26
Dennis A	6.87	4.60	1.20	1.26	2.00
Dennis B	6.43	0.20	0.11	0.16	0.22
Dougherty A	4.39	1.56	0.07	1.18	2.1
Efaw A	5.58	4.16	0.14	2.75	3.85
Hanlon A	8.05	3.05	0.11	3.18	3.00
Haskell A	6.10	1.45	0.11	4.01	5.21
Kirkland A	4.86	2.15	1.42	4.30	2.19
Luton A	5.07	0.78	1.12	1.14	1.06
Mansic A	4.41	0.25	0.20	1.07	1.20
Mansic B	3.59	0.12	0.25	0.13	2.08
Osage A	6.99	2.87	3.41	3.10	5.61
Osage B	5.44	3.07	3.01	4.08	3.28
Perkins A	5.45	3.08	0.13	1.66	3.76
Pond Creek A	8.01	4.55	2.23	6.45	5.65
Pond Creek B	6.71	1.88	0.08	0.96	3.90
Pratt A	6.03	2.02	0.08	0.23	5.16
Pratt B	5.37	1.01	0.11	0.17	3.69
Richfield B	5.21	1.91	0.20	0.94	1.37
Summit A	8.27	0.22	0.11	0.29	2.18
Summit B	4.35	0.73	0.20	1.63	0.87
Mean	5.91	1.93	0.69	2.00	2.84
Median	5.58	1.88	0.14	1.26	2.19
Minimum	3.59	0.12	0.07	0.13	0.22
Maximum	8.27	4.60	3.41	6.45	5.65

Table 8. Mean metal content in lettuce grown on unspiked and spiked soils. All values are expressed as mg metal/kg plant tissue.

	Unspiked Soils			Spiked Soils			
Soil	Cd	Pb	Zn	Cd50	Cd300	Pb	Zn
Canisteo A	0.17	0.00	11.9	54.7	124	8.72	25.4
Dennis A	0.89	0.00	24.9	60.4	242	88.7	333
Dennis B	0.31	0.00	16.2	49.0	122	160	110
Dougherty A	0.03	0.00	22.7	111	.	114	2040
Efaw A	0.15	0.00	16.6	82.9	237	61.3	739
Hanlon A	0.25	0.00	24.2	23.8	110	9.20	96.0
Haskell A	0.00	0.00	24.8	77.3	.	37.7	598
Kirkland A	0.14	0.00	16.8	52.3	.	50.4	190
Luton A	0.24	0.00	22.3	39.9	57.8	16.5	28.5
Mansic A	0.00	0.00	12.2	76.0	68.0	60.2	26.4
Mansic B	0.00	0.00	16.5	130	73.9	59.5	28.0
Osage A	0.28	0.00	33.2	28.1	64.2	3.22	69.7
Osage B	0.34	0.00	33.8	28.9	67.7	15.3	64.3
Perkins A	0.17	0.00	17.1	128	192	108	631
Pond Creek A	0.12	0.04	20.3	57.4	237	43.1	249
Pond Creek B	0.43	0.00	17.2	75.7	.	95.7	148
Pratt A	0.19	0.00	18.2	74.2	403	233	743
Pratt B	0.03	0.00	18.2	135	215	112	574
Richfield B	0.16	0.00	18.6	47.9	251	37.8	38.9
Summit A	0.21	0.00	23.6	76.6	68.6	30.2	25.6
Summit B	0.00	0.00	14.5	43.1	116	13.4	18.4
Mean	0.20	0.00	20.2	69.2	156	64.7	323
Median	0.17	0.00	18.2	60.4	122	50.4	110
Minimum	0.00	0.00	11.9	23.8	57.8	3.22	18.4
Maximum	0.89	0.04	33.8	135	403	233	2040

Table 9. Mean earthworm mortality (%) for unspiked and spiked soils.

		Spiked Soils			
Soil	UnspikedSoils	Cd50	Cd300	Pb	Zn
Canisteo A	0.00	0.00	0.00	0.00	0.00
Dennis A	0.00	0.00	0.00	46.7	0.00
Dennis B	0.00	0.00	3.33	0.00	0.00
Dougherty A	0.00	0.00	0.00	20.0	10.0
Efaw A	3.33	93.3	100	100	100
Hanlon A	0.00	0.00	0.00	53.3	6.67
Haskell A	0.00	6.67	3.33	60.0	43.3
Kirkland A	6.67	0	6.67	53.3	40.0
Luton A	3.33	0.00	0.00	0.00	0.00
Mansic A	0.00	0.00	0.00	0.00	0.00
Mansic B	6.67	16.7	13.3	20.0	13.3
Osage A	3.33	0.00	0.00	0.00	0.00
Osage B	0.00	0.00	6.67	0.00	0.00
Perkins A	0.00	33.3	100	100	100
Pond Creek A	0.00	6.67	30.0	56.7	6.67
Pond Creek B	3.33	0.00	3.33	46.7	0.00
Pratt A	0.00	0.00	0.00	100	0.00
Pratt B	0.00	0.00	46.7	100	0.00
Richfield B	0.00	0.00	0.00	3.33	0.00
Summit A	0.00	0.00	0.00	0.00	0.00
Summit B	0.00	3.33	3.33	0.00	0.00
Mean	1.27	7.62	15.1	36.2	15.2
Median	0.00	0.00	3.33	20.00	0.00
Minimum	0.00	0.00	0.00	0.00	0.00
Maximum	6.67	93.3	100	100	100

Table 10. Mean metal content in earthworms exposed to unspiked and spiked soils. All values are expressed as mg metal/kg earthworm.

	Unspiked Soils			Spiked Soils			
Soil	Cd	Pb	Zn	Cd50	Cd300	Pb	Zn
Canisteo A	4.44	0.30	141	173	441	76.4	129
Dennis A	8.77	0.40	164	322	598	176	151
Dennis B	9.83	0.20	140	251	450	172	142
Dougherty A	9.27	0.30	161	325	908	416	150
Efaw A	0.00	1.90	105	148	77.4	105	64.9
Hanlon A	8.66	0.20	149	198	466	183	155
Haskell A	13.7	0.60	162	333	765	366	143
Kirkland A	10.2	0.20	152	286	538	40.6	137
Luton A	9.49	0.10	162	135	339	28.7	128
Mansic A	7.91	0.20	189	150	319	106	153
Mansic B	9.74	0.40	141	85.8	331	195	135
Osage A	12.5	0.20	183	169	705	38.8	139
Osage B	5.59	0.20	156	196	383	38.3	139
Perkins A	1.73	6.00	132	71.3	366	1030	38.2
Pond Creek A	7.71	0.10	148	266	674	566	138
Pond Creek B	7.18	0.10	123	719	867	423	135
Pratt A	7.87	0.50	141	788	846	782	188
Pratt B	5.51	0.40	122	1190	642	701	161
Richfield B	7.33	0.10	140	241	434	261	139
Summit A	11.8	0.50	171	267	497	126	164
Summit B	1.49	0.10	132	232	444	83.1	130
Mean	7.65	0.62	148	312	528	282	136
Median	7.91	0.20	148	241	466	176	139
Minimum	0.00	0.10	105	71.3	77.4	28.7	38.2
Maximum	13.7	6.00	189	1190	908	1030	188

Table 11. Correlation coefficients (r) for paired relationships between metal extracted from spiked soils by PBASE or Pore Water and lettuce metal content, germination or yield.

	Lettuce Metal Content			
Soil	Cd50	Cd300	Pb	Zn
PBASE extracts				
E1	0.41[b]	0.62[a]	0.74[a]	0.71[a]
E2	-0.65[a]	-0.43[b]	0.49[b]	0.33
E3	-0.31	-0.42[b]	-0.70[a]	-0.58[a]
E4	-0.31	-0.44[b]	-0.44[b]	-0.68[a]
ΣE_{1-2}	0.33	0.40	0.74[a]	0.71[a]
ΣE_{1-3}	0.18	0.14	0.19	0.59[a]
ΣE_{1-4}	0.02	0.004	-0.12	-0.51[a]
Pore Water	0.43[b]	0.48[b]	0.55[a]	0.83[a]

Soil	Germination (%)			
PBASE extracts	Cd50	Cd300	Pb	Zn
E1	0.33	-0.09	0.32	0.01
E2	0.09	0.14	0.29	0.25
E3	-0.36	0.01	-0.32	0.26
E4	-0.30	0.30	-0.13	0.01
ΣE_{1-2}	0.39	-0.07	0.34	0.01
ΣE_{1-3}	0.25	-0.13	0.10	0.26
ΣE_{1-4}	0.19	-0.004	0.02	0.41
Pore Water	0.07	-0.01	0.23	0.34

Soil	Yield			
PBASE extracts	Cd50	Cd300	Pb	Zn
E1	0.41[b]	-0.03	-0.16	0.57[a]
E2	-0.19	-0.03	-0.03	0.26
E3	-0.52[a]	0.35	0.08	-0.42[b]
E4	-0.54[a]	0.05	-0.27	-0.20
ΣE_{1-2}	0.34	-0.36	-0.16	0.57[a]
ΣE_{1-3}	-0.06	-0.18	-0.32	0.51[a]
ΣE_{1-4}	-0.26	-0.25	-0.22	-0.28
Pore Water	0.47[b]	-0.28	-0.30	0.46[b]

[a] $p < 0.01$
[b] $p < 0.05$

Table 12. Correlation coefficients (r) for paired relationships between metal extracted from spiked soils by PBASE or Pore Water and earthworm mortality or earthworm concentration.

	Earthworm Mortality			
Soil Extract	Cd50	Cd300	Pb	Zn
PBASE extracts				
E1	0.08	0.36	0.86[a]	0.54[a]
E2	-0.25	-0.44	0.36	0.24
E3	-0.19	-0.37	-0.70[a]	-0.34
E4	-0.25	-0.39	-0.75[a]	-0.54[a]
ΣE_{1-2}	0.05	0.31	0.71[a]	0.54[a]
ΣE_{1-3}	-0.26	0.03	0.16	0.51[b]
ΣE_{1-4}	-0.36	-0.09	-0.30	-0.33
Pore Water	0.62[a]	0.76[a]	0.84[a]	0.62[a]
	Earthworm Concentration[c]			
Soil Extract	Cd50	Cd300	Pb	Zn
PBASE extracts				
E1	0.67[a]	0.68[a]	0.65[a]	0.44[b]
E2	-0.33	-0.52[b]	0.51[b]	0.52[b]
E3	-0.48[b]	-0.67[a]	-0.58[a]	-0.13
E4	-0.24	-0.67[a]	-0.52[b]	-0.47[b]
ΣE_{1-2}	0.68[a]	0.64[a]	0.68[a]	0.45[b]
ΣE_{1-3}	0.70[a]	0.24	-0.41	0.58[a]
ΣE_{1-4}	0.62[a]	0.09	-0.57[a]	-0.17
Pore Water	0.03	0.66[a]	0.73[a]	0.49[b]

[a] $p < 0.01$
[b] $p < 0.05$
[c]Soils with 100% mortality were not used in the regression analysis for earthworm concentration.

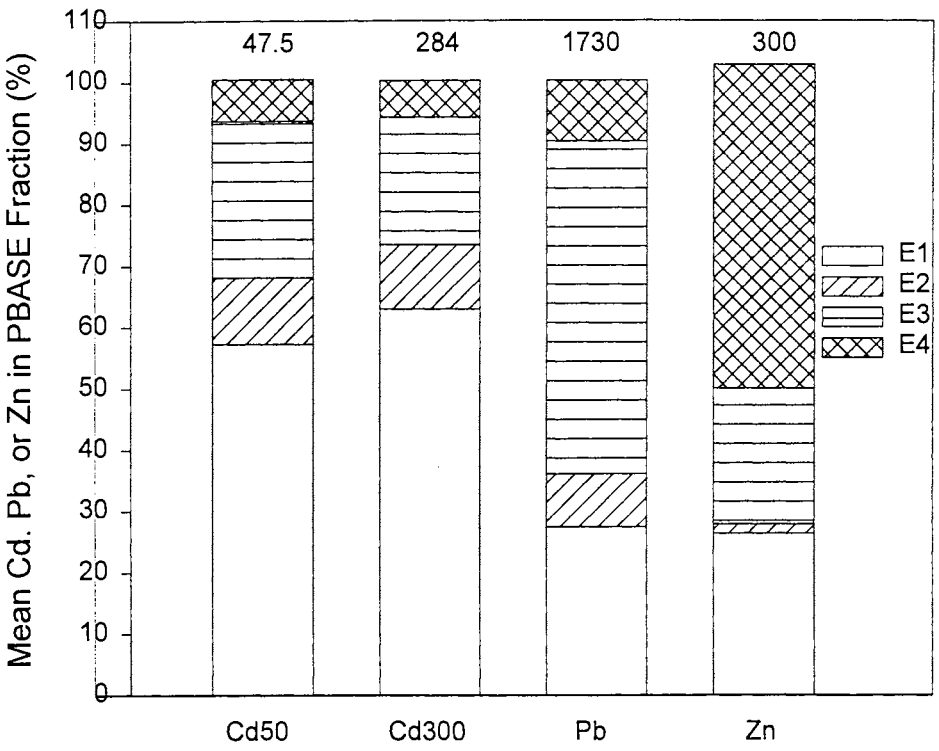

Figure 1. Mean distribution of Cd, Pb, and Zn in PBASE fractions E1-E4 expressed as as percentage of the sum of PBASE. Values at top of graph represent the mean sum of PBASE for 22 soils.

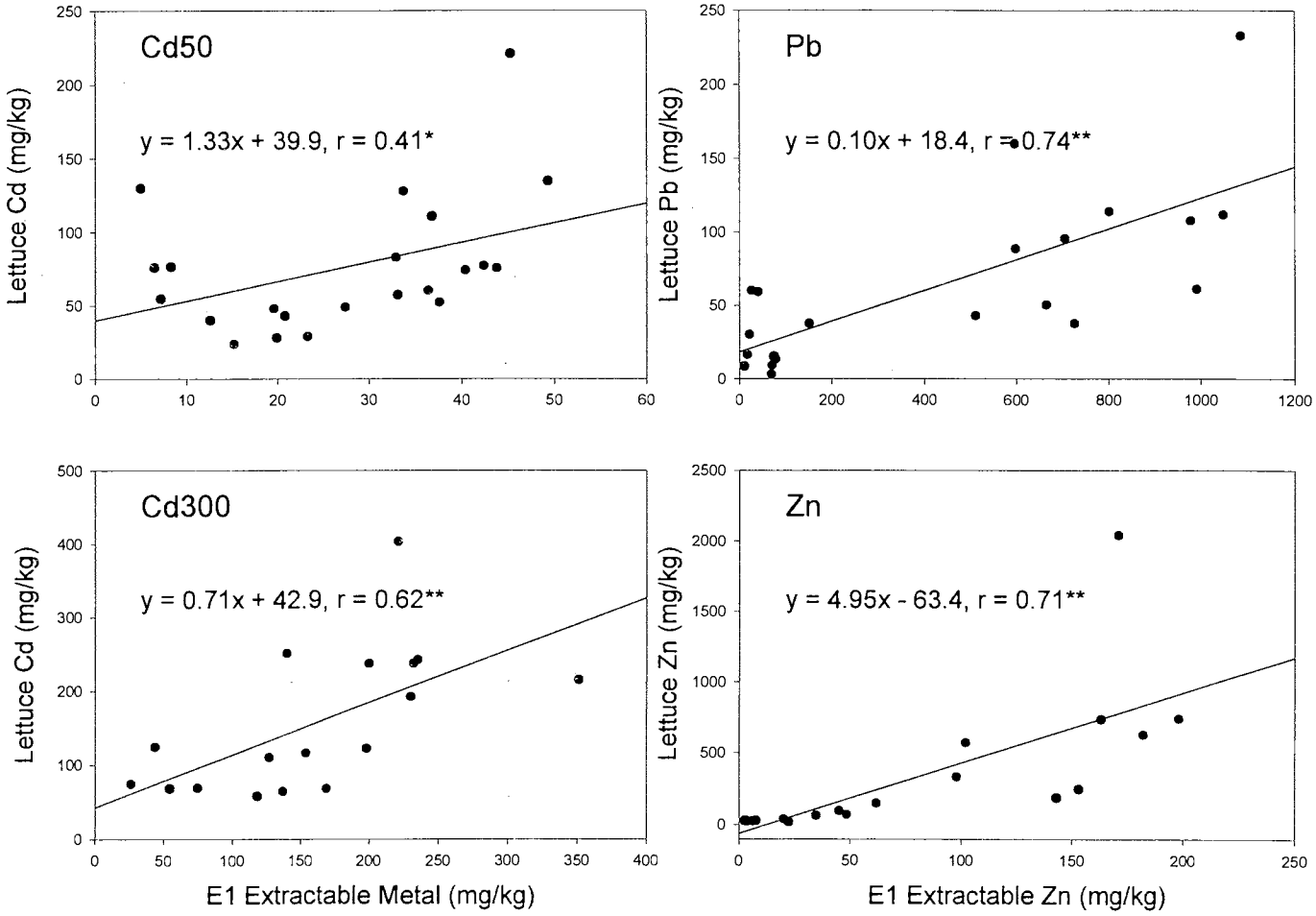

Figure 2. Linear regression of mean lettuce metal vs. mean E1 extractable metal.
* $p < 0.05$, ** $p < 0.01$

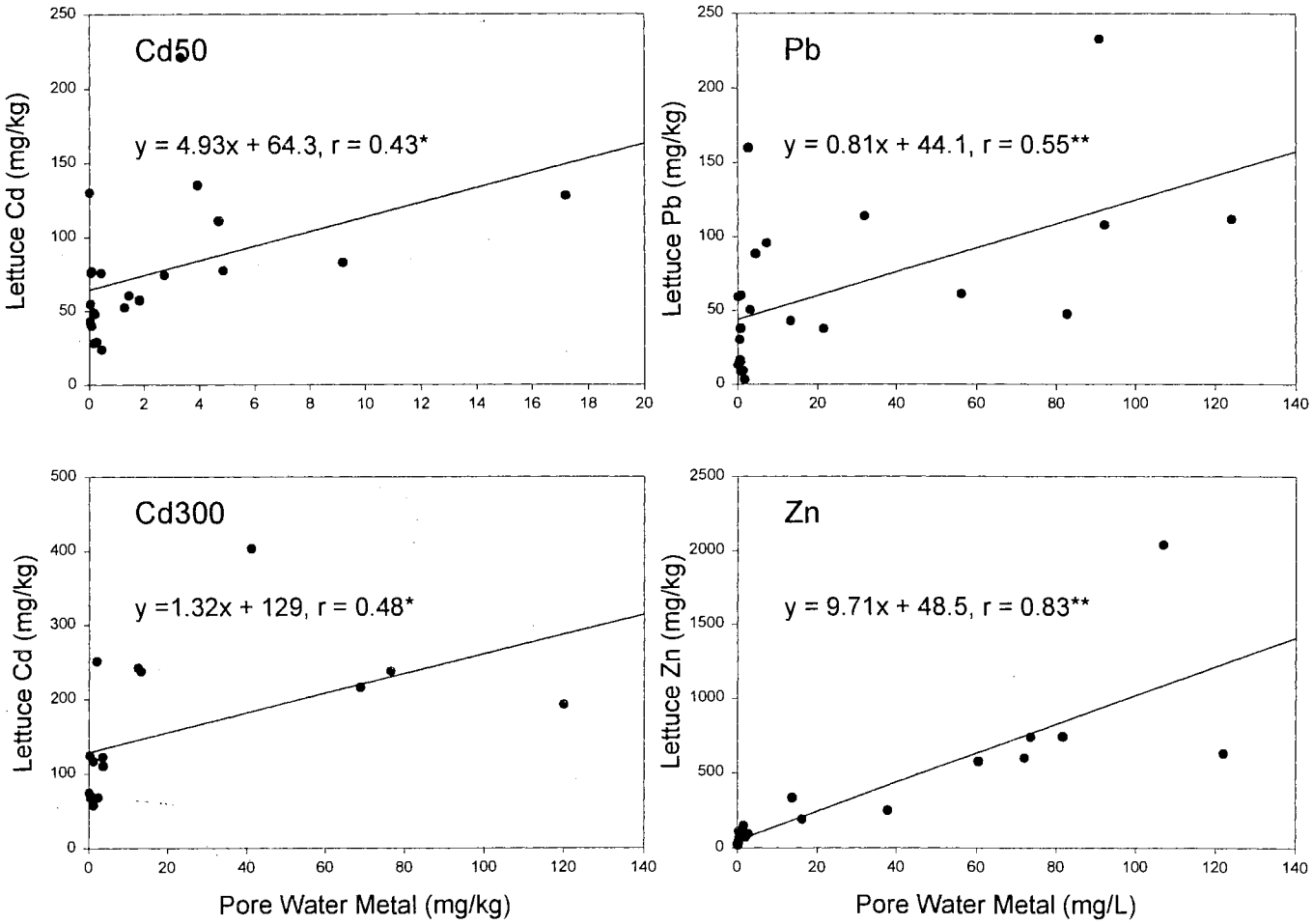

Figure 3. Linear regression of mean lettuce metal vs. mean pore water metal.
** $p < 0.01$, * $p < 0.05$.

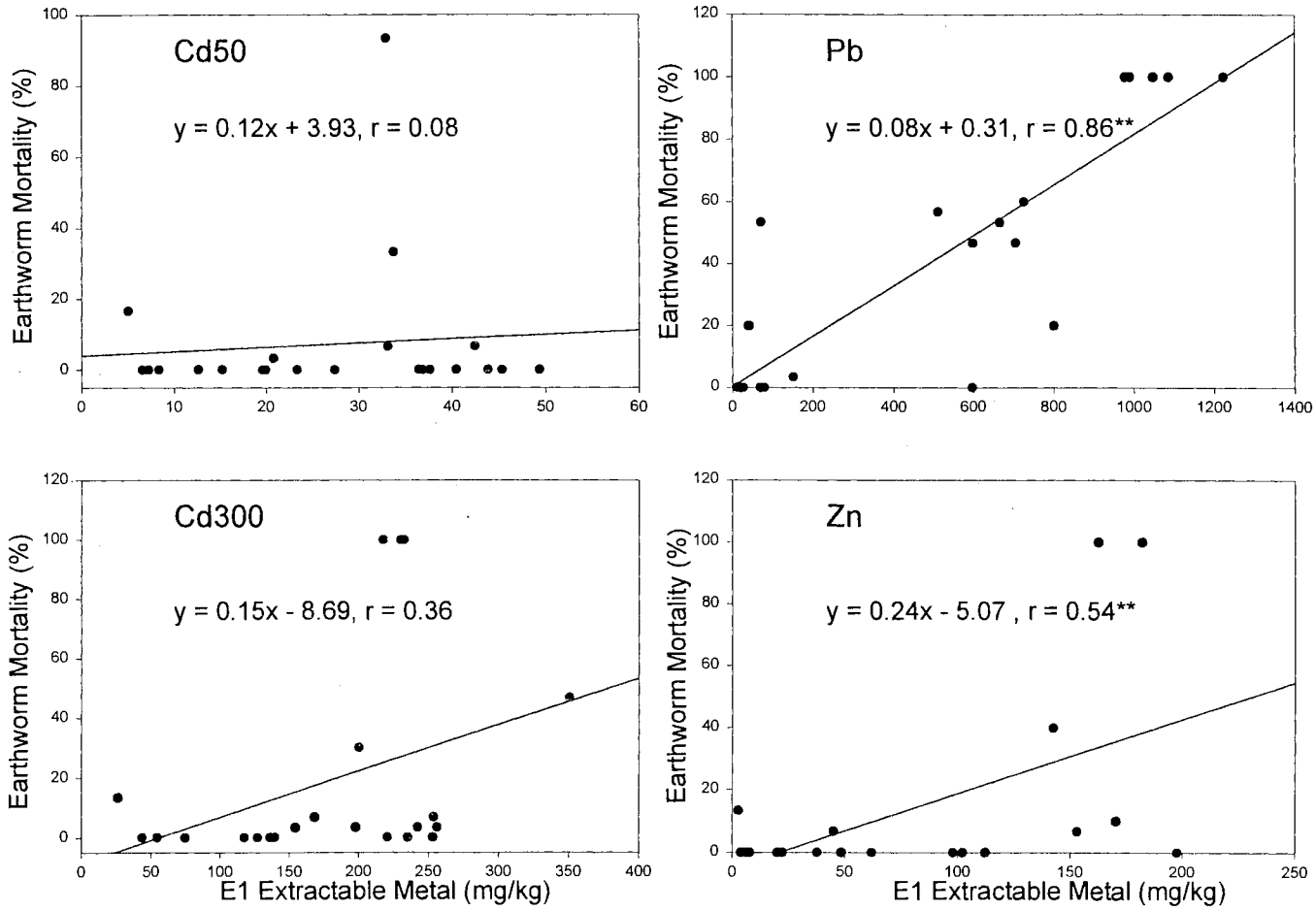

Figure 4. Linear regression of mean earthworm motality vs. mean E1 extractable metal. **p < 0.01.

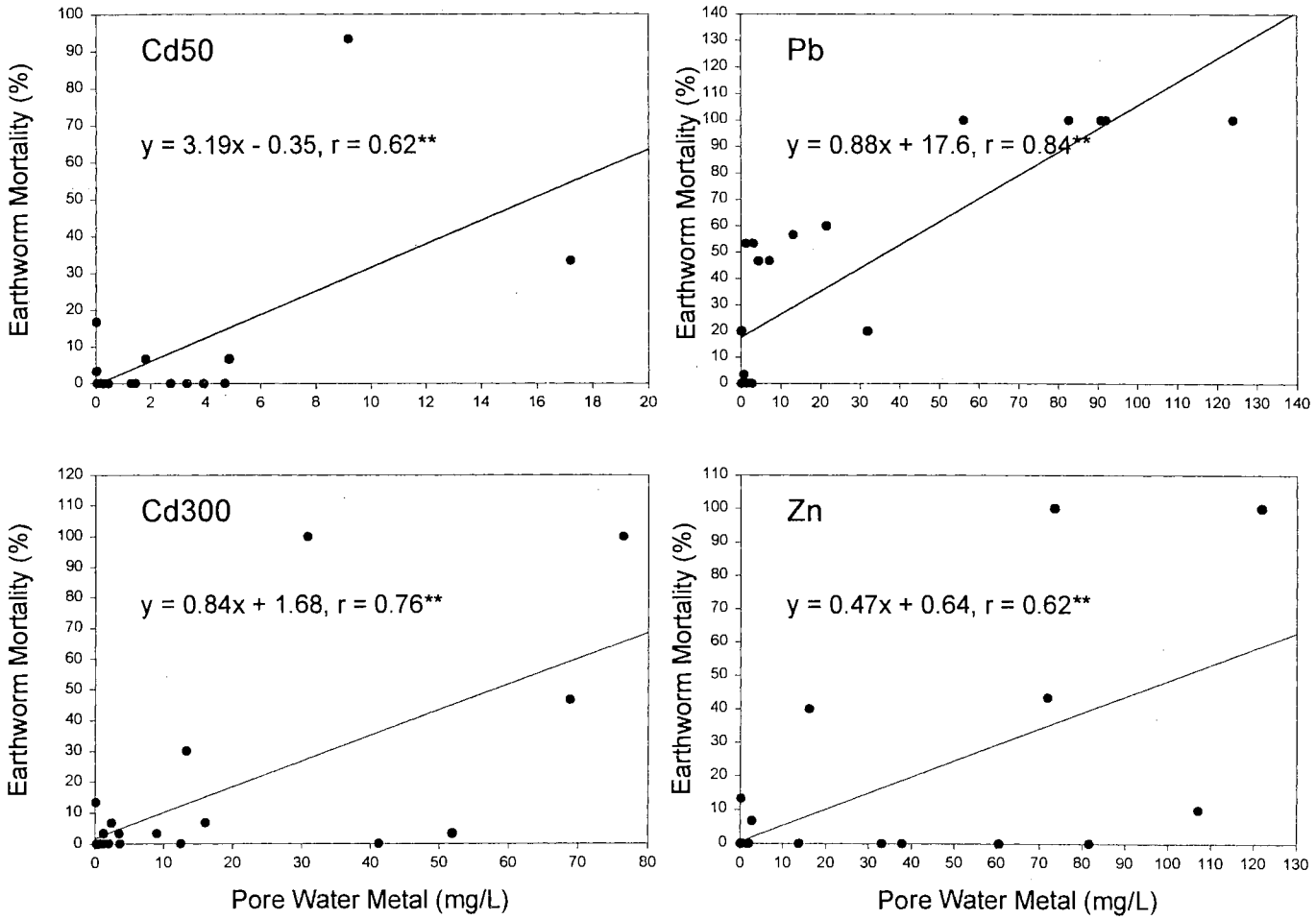

Figure 5. Linear regression of mean earthworm mortality vs. mean pore water extractable metal.
**p < 0.01

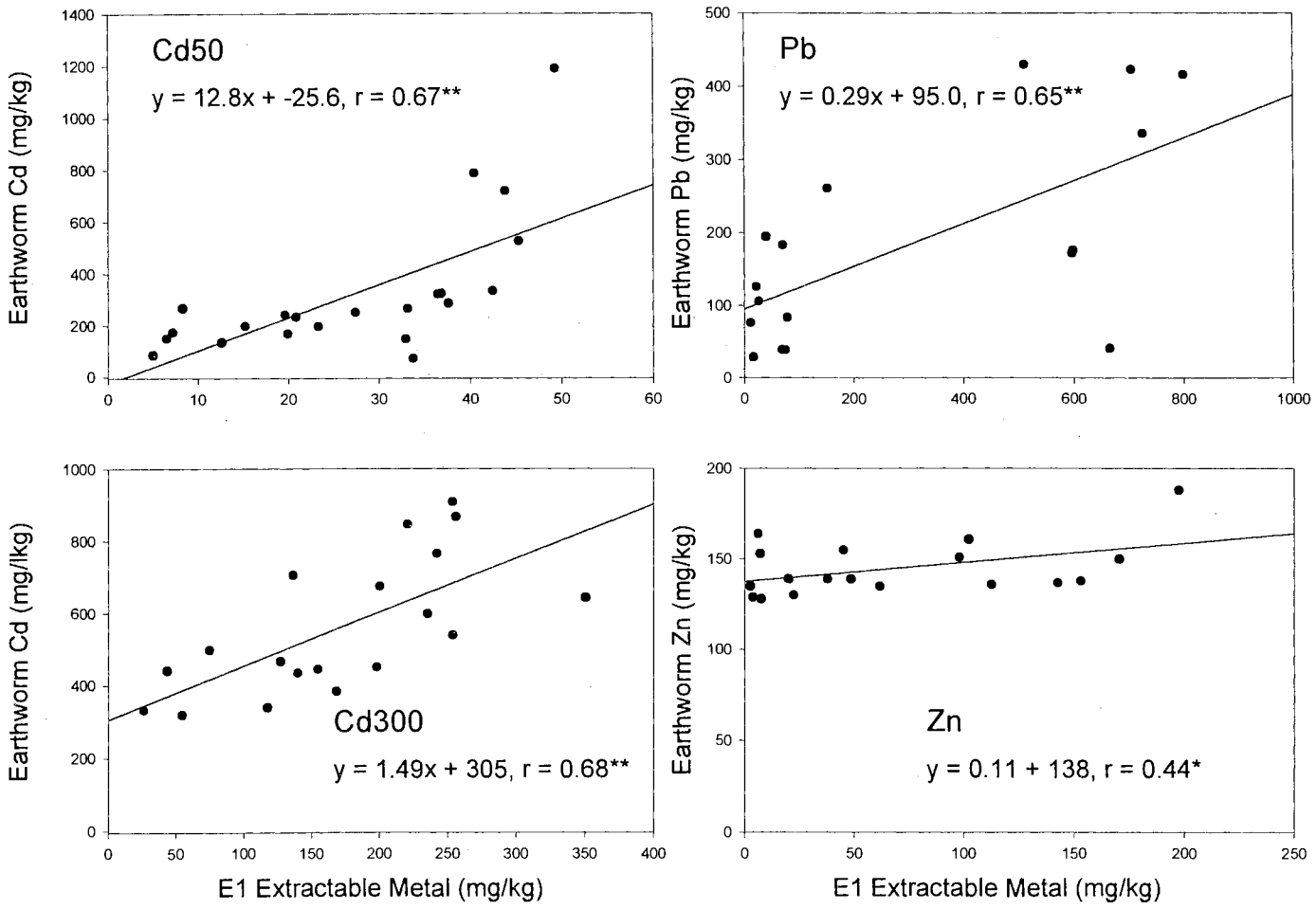

Figure 6. Linear regression of metal content in earthworms vs. E1 extractable metal.
*p < 0.05, ** p < 0.01

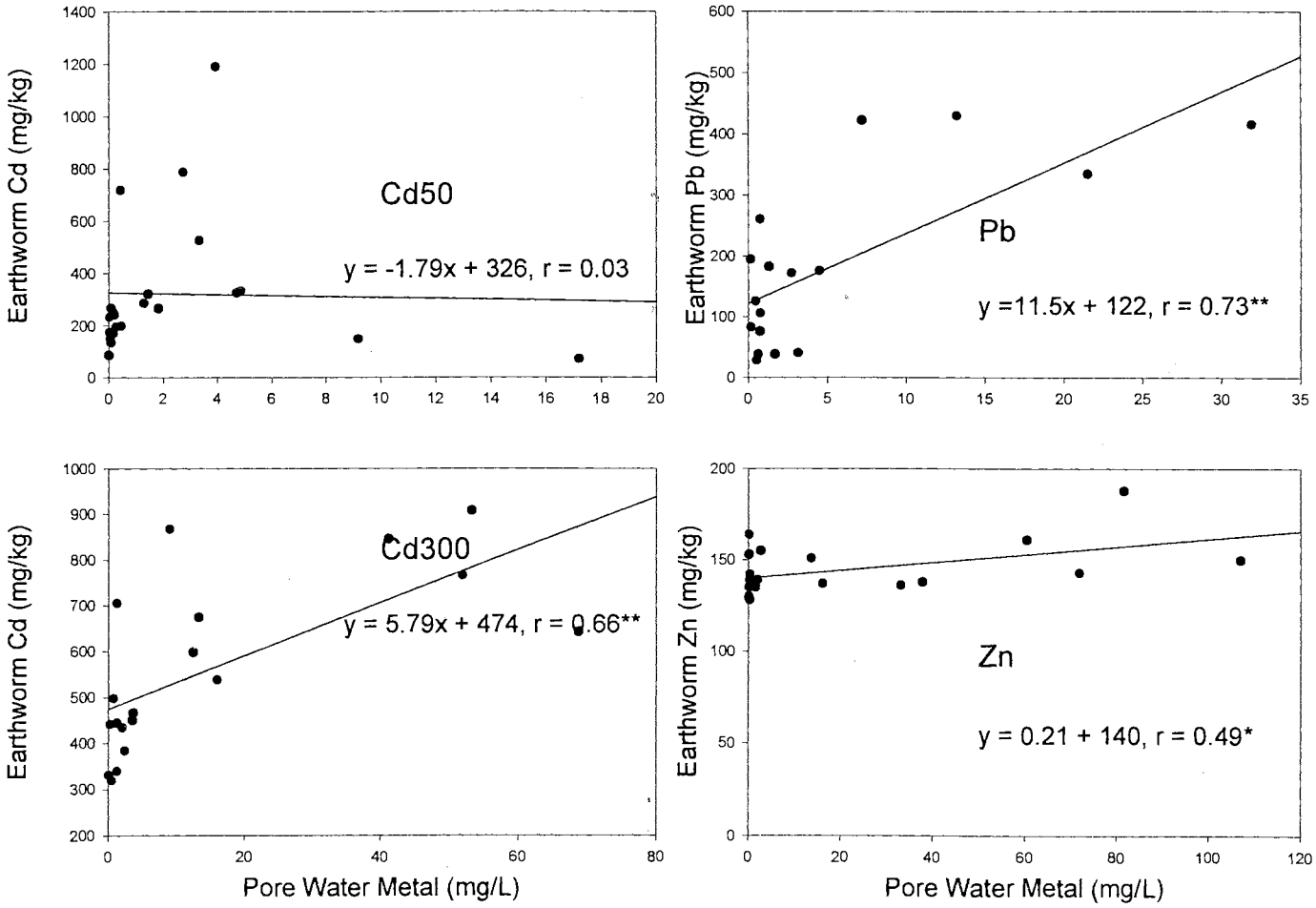

Figure 7. Linear regression of metal content in earthworms vs. pore water extractable metal. *p < 0.05, **p < 0.01.

VITA

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Doctor of Philosophy

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