Title
Bioavailability of Cadmium and Zin to Two Earthworm Species in High-metal Soils

Permalink
https://escholarship.org/uc/item/16v8k73r

Author
Liu, Ying

Publication Date
2012

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA
RIVERSIDE

Bioavailability of Cadmium and Zinc to Two Earthworm Species in High-metal Soils

A Dissertation submitted in partial satisfaction
of the requirement for the degree of

Doctor of Philosophy

in

Soil and Water Sciences

by

Ying Liu

June 2012

Dissertation Committee:
Dr. David R. Parker, Chairperson
Dr. Jay Gan
Dr. James Sickman

The Dissertation of Ying Liu is approved:

Committee Chairperson

University of California, Riverside

Acknowledgements

I gratefully acknowledge Dr. David R. Parker for his guidance and assistance in completing my Ph.D study. Dr. Parker is a great mentor with provocative ideas and patience, giving me critical feedback and support.

Sincerest thanks to Dr. Jay Gan and Dr. James Sickman for serving on the Dissertation and Qualify Exam Committee,

My deepest gratitude is to Woody Smith, as well as group members in Dr. Ling's Lab in Geology Department. They helped me a lot on instrument operation and sample analysis.

Many thanks to Guntram kiparski and Kelly for their assistance on revising my manuscript and giving good idea for my experiment.

Much appreciation to my husband, Naiyou Liu and to my parents who helped me a lot to take care my kids.

ABSTRACT OF THE DISSERTATON

Bioavailability of Cadmium and Zinc to Two Earthworm Species in High-metal Soils

by

Ying Liu

Doctor of Philosophy, Graduate Program in Soil and Water Sciences
University of California, Riverside, June 2012
Dr. David R. Parker, Chairperson

It was generally recognized that the bioavailability, other than total content of heavy metals in soil, is crucial to predict metals' toxic effect on environment. Stable Isotope Dilution technique (SID) equipped with inductively coupled plasma-mass spectrometry (ICP-MS) is a good way for measuring the potential bioavailable portion of metals in soils. In Chapter 2, we build method to accurately determined Cd (^{111}Cd/^{114}Cd) and the ratio of Zn (^{66}Zn/^{68}Zn) in soil suspension and then applied this to stable isotope dilution technique to successfully obtain the labile fraction of Cd and Zn in polluted soils.

In Chapter 3, it was found when compared to chemical extraction methods, the stable isotope dilution technique is the better way to assess bioavailability of Cd and Zn in soil to the earthworms *Eisenia fetida* and *Lumbricus terrestris*. Besides, the result showed on

average, the fraction of labile Cd (44.7%) was significantly higher than that of Zn (25.4%), indicating that Cd is more chemically reactive in soil, which may explain the larger BSAFs for Cd in earthworms. In addition, it was found the accumulation of heavy metals was not only element-specific, but also species-specific.

In Chapter 4, the uptake and elimination kinetics of Zn and Cd by *E. fetida* and *L. terrestris* were investigated then. The kinetic rates were calculated using a first-order, one-compartment, toxico-kinetic model. Pronounced differences were found on the earthworms' uptake and elimination kinetics between Zn and Cd ($p<0.01$).

Lastly in Chapter 5, it was found earthworms' activity increased the pH value and decreased organic carbon content of soils. In addition, the results showed that the Cd, Zn contents in the casts was significantly decreased, while the percentage of 0.01M $Ca(NO_3)_2$, 1M $CaCl_2$-extractable Cd and Zn, as well as the fraction of labile Cd and Zn in the casts were significantly increased, suggesting that the earthworms effectively improved heavy metals' lability.

TABLE OF CONTENTS

Page

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

LIST OF TABLES

LIST OF FIGURES

Chapter 1

General Introduction on bioavailability of Cd and Zn in polluted soils

1.1 Heavy metal contamination in soil

Soil is a precious and limited natural resource on earth. It provides habitat and nutrients for plants, soil organisms, as well as human beings. However, several human activities, such as mining, manufacturing, and the use of synthetic products (pesticides, paints, batteries, industrial waste), and land application of industrial or domestic sludges can result in metal contamination of urban and agricultural soils (Nriagu and Pacyna, 1988; Schalscha and Ahumada, 1998; McGrath et al., 2001).

Metals that have been identified in the polluted environment include Cu, Cd, Pb, Cr, Ni, Hg and Zn. These metals are mainly from endogenous sources and anthropogenic activities (Nriagu and Pacyna, 1988), which lead to excess metal levels in soils. Among these metals, zinc is an essential mineral of "exceptional biologic and public health importance" because the deficiency of Zn will lead to harmful effect to plants (Broadley et al., 2007), animals (Prasad, 2008), even human beings. It is "typically the second most abundant transition metal in organisms" after iron and it is the only metal which appears in all enzyme classes (Broadley et al., 2007). Although zinc is an essential requirement for good health, excess zinc can be harmful to organisms and human beings. The main pollution sources for Zn are electroplating industry, smelting and refining, mining and biosolids (Liu et al., 2005a). Soils contaminated with Zn through the mining of zinc-containing ores, refining, or where zinc-containing sludge is used as fertilizer, can contain up to several grams of zinc per kilogram of dry soil.

Cadmium occurs as a minor component in most zinc ores and therefore is often a byproduct of zinc production. Unlike Zn, Cd has no known useful role in higher organisms (Hogan, 2010), and can be viewed mainly as an environment hazard. Cadmium has many common industrial uses as it is a key component in battery production and is commonly used in electroplating (Scoullos et al., 2001). Also, repeat application of some phosphate fertilizers and sewage sludge can lead to high levels of Cd in soil (Alloway, 1995; Kabata-Pendias, 2001).

Soil contamination with heavy metals may cause changes in the composition of soil microbial community, adversely affecting soil characteristics (Giller et al., 1998; Kozdrój and van Elsas, 2001). High concentrations of Cd and Zn in soil can negatively affect crop growth, as these metals interfere with metabolic functions in plants, including physiological and biochemical processes, inhibition of photosynthesis, and respiration and degeneration of main cell organelles, even leading to death of plants (Garbisu and Alkorta, 2001; Schmidt, 2003; Schwartz et al., 2003). For human beings, directly exposure to high concentrations of these metals in environment can lead to acute gastrointestinal and respiratory damages and acute heart, brain and kidney damages (Kurek and Bollag, 2004), and may bring deficiency of other necessary elements (Fosmire, 1990).

1.2 Importance of study bioavailability of metals in soil

Metal contamination of soil is an important issue as these toxic elements could be transported in terrestrial ecosystem posing potential risk on food quality, soil health, and the environment (Gray et al., 2003). It is now generally recognized that the risk is not directly related to the total content of metals in soils. Another specific term "bioavailability" arises which reflects a growing awareness that soils and sediments bind chemicals to varying degrees, thus altering their availability to other environmental media (surface water, ground water, air) and to living organisms (microbes, plants, invertebrates, wildlife, and humans) (USEPA, 2000). Accordingly, it was proposed that "bioavailability" may represent the fraction of a chemical accessible to an organism for absorption (ASTM, 1999), may indicate the rate at which a substance is absorbed into a living system (Heuscher et al., 2004), or a measure of the potential to cause a toxic effect (Battelle and Exponent, 2000).

Often, environmental scientists consider bioavailability to represent the accessibility of a solid-bound chemical for assimilation and possible toxicity (Alexander, 2000), while toxicologists consider bioavailability as the fraction of chemical absorbed and able to reach systemic circulation in an organism. Currently, “bioavailability” is used in risk assessment most frequently as an adjustment or correction factor that accounts for the ability of a chemical to be absorbed by an organism. The extent to which chemicals are bioavailable has significant implications for the toxic effect on environment (Kelly et al., 2002). It is also recognized that the species of plant and animals, as well as properties of soils have influence on assessing availability of chemicals, i.e., it might be very different from one soil or one species to another when exposed to the same contaminated material (Dayton et al., 2006). The species-specific character of bioavailability will be investigated and described in our study.

1.3 Methods for studying bioavailability

Fractionation Methods

Numerous techniques and approaches have been developed to estimate the bioavailable pools of metals in contaminated soils. First, fractionation methods are widely used because the bioavailability of metals may be related to their partitioning into a number of discrete pools in the soils. Fractionation methods include the use of single (batch extraction) or more (sequential extraction) extractants to extract trace elements from different chemical pools in soil. The methods always allocate trace metals into operationally defined pools of putatively distinct biogeochemical forms (adsorbed, carbonate, organic, hydrous oxides, etc.) (Beckett, 1989; Tack and Verloo, 1995).

Single chemical extractions have been and are extensively used to assess metal availability in soils (Kelsey and Kottler, 1997; Kennedy et al., 1997; Cao et al., 2000; Li et al., 2001). The chemistry of metal interactions with soil components, such as clay minerals, organic matter, and/or soil solution, can be assessed by extracting soil with selective chemical solutions. These chemical extraction methods also can be utilized to assess metal mobility, retention, and availability to plants (Ure, 1996). There are three main types of chemicals utilized for extracting “available” soil metals: dilute solutions of neutral salts (e.g. $MgCl_2$, $CaCl_2$, $NaNO_3$, and NH_4NO_3), weak acids (especially acetic acid), and chelating agents such as DTPA and EDTA in the presence or absence of inorganic salts. However, the amount of metal extracted by stronger chemicals such as chelating agents or weak acids are more difficult to interpret because the regents can extract variable proportions of available and unavailable forms depending on soil properties, as shown for P by Kato et al. (1995). Furthermore, none of the above chemical approaces could give any direct information on the rate at which the metal is released from the solid phase to the soil solution (Sinaj et al., 2004).

Another widely used method for evaluating metal bioavailability is sequential extraction. In sequential extraction procedures (SEPs), increasingly "harsh" reagents are used in a stepwise procedure to extract increasingly refractory forms of the metal(s) of interest (Ahnstrom and Parker, 2001). Normally, soil trace elements can be partitioned into five or more operationally-defined fractions including: soluble/exchangeable; specifically-sorbed/carbonate; easily oxidizable; easily reducible; and residual (Beckett, 1989). It has been suggested that the first one, two, or occasionally three extracted fractions are taken to be the labile pool of the metal that is considered bioavailable and potentially mobile (Basta and Gradwohl, 2000).

However, chemical fractionation methods have some disadvantages and limitations because particularly harsh extractants can alter the chemistry of the soil system, which can lead to the redistribution of elements among the putative geochemical pools (Ahnstrom and Parker, 2001), or it can result in the release of non-labile metals (Gleyzes et al., 2002). Thus, although these fractionation methods can provide information regarding trace element speciation in soil, attention should be paid to the improvement of extractant specificity and to the prevention of possible redistribution.

Stable Isotope Dilution Methods

Isotope dilution technique (IDT) is a good substitute for measuring the potential bioavailable portion of metals in soils and has been employed in soil science for more than 50 years. IDT was initially used to characterize the phytoavailability or reactivity of some essential plant nutrients (e.g., P and K) in soils (Morel and Plenchette, 1994). More recently, IDT was applied to examine the labile pool of toxic trace elements in soils, such as Cd (Ahnstrom and Parker, 2001; Smolders et al., 1999; Young et al., 2000), As (Tye et al., 2002; Hamon et al, 2004), Pb (Degryse et al., 2007), Zn (Young et al., 2000), Ni (Echevarria et al., 1998) and Cu (Lombi et. al., 2003). The labile pool is the sum of isotopically exchangeable elements in the solution and solid phases and is considered to reflect the potentially bioavailable pool of metals in soil under equilibrium conditions (Degryse et al., 2007, Hamon et al., 2008). This can be achieved by adding an enriched isotope spike to a suspension of soil in water or neutral salt electrolyte such as $Ca(NO_3)_2$ to measure the extent of isotope dilution in the solution phase once equilibrated, which is known as the *E*-value (Ahnstrom and Parker, 2001; Hamon et al., 2008). The proportion of heavy metals in the labile fraction is of concern as this fraction may represent a hazard to the environment or pose a health risk.

Typically, radioactive isotopes of metals have been used to spike soil and calculate the metals' labile pool. Although the technique is relatively simple and inexpensive, it is not always feasible because of a short half-life for the radio-isotope, or because of potential environmental hazards (Hamon et al., 2008). To avoid the use of radio-isotopes, stable isotopes have been used to measure the labile pools (Degryse et al., 2007). More recently, rapid and sensitive analytical techniques for determination of isotopic ratios, such as inductively coupled plasma – mass spectrometry (ICP-MS), have prompted the use of enriched stable isotopes for labile pool studies (Gabler et al., 1999; Becker and Dietze, 2000; Ahnstrom and Parker, 2001). Even though the use of stable isotopes is analytically more demanding than the use of radio-isotopes, it provides a method to study elements for which appropriate radio-isotopes are not readily available. Stable isotopes also have the advantage of allowing in-field isotopic studies.

An isotope dilution technique using enriched stable isotopes is applied to determine the exchangeable or labile heavy-metal fractions in soils (Hamon et al., 2008). A spike of enriched stable isotopes is added to the suspension of a soil sample, and readily partitions between the solution- and exchangeable-phases in the same way as the other isotopes of the same element. The exchangeable portion is the portion of the element which rapidly exchanges between the soils and liquid phases under the extraction conditions and thus exchanges with the added spike. If equilibrium has been reached, the isotope ratios of the mixture of the spike solution and the exchangeable portion of the sample should be the same in both liquid and solid phases (Hamon et al., 2008). Thus, an aliquot of the liquid sample can be used to analyze the final isotope ratios. The exchangeable portion of the elements can be subsequently calculated using isotope dilution equations (Gabler et al., 1999). Using Cd as an example, enriched ^{111}Cd is added as a tracer to the soil, while ^{114}Cd can be used as reference isotope. After equilibration is reached, the labile Cd pool, or *E*-value (mg/kg), will be defined as the amount of Cd in the soil with the same isotopic signature as the solution phase derived from the ^{111}Cd to ^{114}Cd isotope ratio in solution (IR_{soil}) as:

$$IR_{soil} = \left(\frac{^{111}Cd}{^{114}Cd}\right)_{soil} = \frac{SA_{111}R + NA_{111}E}{SA_{114}R + NA_{114}E} \qquad (1)$$

where R is the total amount of Cd (in mg Cd/kg soil) added to the soil through the spike, SA_{111} and SA_{114} are the fractional abundance (on molar basis) of ^{111}Cd and ^{114}Cd in the spike, and NA_{111} and NA_{114} are the natural abundance (on molar basis) of ^{111}Cd and ^{114}Cd (Hamon et al., 2008). Equation (1) can be rearranged to obtain the E value:

$$E = \frac{R(SA_{111} - SA_{114}IR_{soil})}{NA_{111}IR_{soil} - NA_{114}} \qquad (2)$$

It should be noted that a certain period of time is needed for the system to reach equilibrium after adding enriched stable isotope. In theory the isotope would mix uniformly with the entire soil metal pool given infinite time (Tiller and Wassermann, 1972). Generally, researchers have chosen a few days (1-3 days) to reach equilibrium for E-value determinations (Hamon et al, 2008). The formula indicates that, with a stable isotope, the accuracy of the determined E-value depends on the accuracy of measuring isotope ratios in the spiked samples since the isotopic ratios in natural samples are known values. It should be recognized that several aspects of instrument performance can limit the precision and accuracy of isotope ratio measurements by ICP-MS. These include: signal stability, polyatomic mass spectral interferences, and mass discrimination (Nolan et al., 2004), which will be discussed in detail in Chapter 2.

1.4 Use of earthworms in bioavailability studies

Although much work has been done on measuring the phyto-available pool in soils by the isotopic dilution method (Hamon et al., 2008), there is the relative absence of similar studies using invertebrates in soil and some of these limited studies used radio isotopes other than enriched stable isotopes. Scott-Fordsmand et al. (2004) spiked soils with radioactive isotope ^{65}Zn for assessing the bioavailability of soil Zn to earthworms. Their results showed that the earthworms accessed 55-65% of the total Zn in the soils. The labile pool for the earthworms, *Eisenia andrei*, was similar to that for the plant *Lactuca sativa*, indicating that earthworms and plants generally access the same fraction of soil Zn. Another study showed that the bioavailable pool of Cd to a snail species was 58%, which was significantly higher than the *E* value (49.6%) (Scheifler et al., 2003). The result indicated that snails could also access some of the non-labile or non-bioavailable soil Cd pool. Although the difference is statistically significant, the conclusion may be not strong since such difference could come from uncertainties and random errors for *E* value determination (Hamon et al., 2008).

Earthworms are ubiquitous animals that inhabit soil. They are often selected for toxicokinetic studies because they have been found to accumulate higher levels of metals from polluted soils than most other soil organisms (Beyer et al., 1982), and they have a high tolerance capacity for heavy metals pollution (Díez-Ortiz et al., 2010). Metal bioaccumulation by these soil-dwelling organisms can provide a good ecological indicator of metal availability in soils (Suthara et al., 2008), and has been widely studied for some time (Morgan and Morgan, 1999; Kennette et al., 2002). Earthworms are crucial for soil ecosystem because they are actively involved in organic matter incorporation and decomposition, excavation of burrows, and production of casts (Lee, 1985; Edwards and Bohlen, 1996). Generally earthworm can be divided into three categories according to their morphological and behavioral characteristics (Sims and Gerard, 1999): litter dwelling (epigeic), horizontally burrowing mineral soil feeder (endogeic), and vertically burrowing surface feeder (anecic). The difference among these species in metal bioaccumulation partly reflects the differences in their food selectivity and niche separation (Morgan and Morgan, 1998), or physiological differences (Spurgeon and Hopkin, 1996). Different bioaccumulation patterns of heavy metals between earthworm species, such as endogeic and epegeic, have been observed (Hobbelen et al, 2006; Suthara et al., 2008). However, any differences in metal bioaccumulation by *E. fetida* (epigeic), a standardized earthworm for toxicity testing (ASTM, 1999), and *L. terrestris*, a representative anecic species, is still unknown.

Furthermore, although the accumulation of trace elements from soil to biota has been studied extensively for many species in various soils (Nahmani et al., 2007), less attention has been given to uptake and elimination kinetics (Spurgeon and Hopkin, 1996; Heikens et al., 2001; Hendriks and Heikens, 2001), which provide a more mechanistic basis for an element “bioavailability”. Limited research has indicated that uptake rates of trace metals in invertebrates are generally dependent on the metal species and concentrations, the soil characteristics, and the biological target (Spurgeon and Hopkin, 1996; Janssen et al., 1997; Peijnenburg et al., 1999). It has been reported that many soil organisms are able to sequester metals to various biological compartments following the uptake process (Rainbow, 2002). The sequestration mechanisms involved appear to differ in affinity for essential versus non-essential metals, and this might explain the pronounced differences observed in the uptake and elimination kinetics for essential (Zn, Cu) versus non-essential elements (Cd, Pb) (Vijver et al., 2005).

In order to estimate the metal uptake and elimination kinetics of an organism, the organism's internal metal concentration is measured as a function of time after exposure to a contaminated soil, and again after transfer to a noncontaminated control soil. Toxicokinetic models are then fit to the collected time-series data in order to obtain the uptake and elimination rate constants (Moriarty and Walker, 1987). A one-compartment, first-order kinetic model has often been used, and was shown to successfully obtain kinetic parameters and thus predict the body burden of a metal and toxicity (Kizilkaya, 2005).

1.5 Earthworm's influence on heavy metals availability

Earthworm species can survive in highly metal-contaminated soils, and can accumulate metals in their tissues (Morgan and Morgan, 1999; Lanno et al., 2004). It has been reported that, following treatment with earthworms, the distribution of metals in soil fractions was significantly changed if a large number of earthworms and a long period of exposure are provided (Devliegher and Verstraete, 1997; Cheng and Wong, 2002; Ma et al., 2002; Wen et al., 2004; Udovic and Lestan, 2007). Earthworms altered metals' fractionation mainly by their gut-associated processes to change the soil characteristics. It is known that earthworm can excrete ammonia into the intestine, and produce calcium carbonate in its calciferous glands and release it into the intestine, which may increase the pH of their casts (Lee, 1985). Also, dissolve organic carbon (DOC) and CEC was elevated by earthworm activity which may contribute to an increase in water-soluble metals (Wen et al., 2004; Liu et al, 2005b). It is found that plant nutrient availability and metal availability often elevated in the presence of earthworms (Springett and Gray 1997). Ma et al. (2002) used DTPA as an extractant to measure the bioavailable pool of Pb and Zn. After raised the two sub-species of earthworms in tested mine tailings, it was found the soil pH and available metals increased. Ireland (1975) reported a 50% increase in water-soluble Pb after culturing *Dendrobaena rubida* in a metal-contaminated soil. Devliegher and Verstraete (1997) found that gut-related processes in earthworms may increase both plant –nutrient and metal availability. Recently it was shown that earthworm activity might increase subsequent Cd bioavailability to snails (Coeurdassier et al., 2007).

However, there are also studies that suggested that earthworm can decrease heavy metals bioavailability in the soil. It was reported that bioavailable portion of Pb, assessed by DTPA extraction, was decreased by *Lumbricus terrestris* in the Pb/Zn mine spoils from UK which contained excessively high metal concentrations (Ma et al. 2000). These researchers thought the possible reason that metal availability was reduced with higher concentrations in the soil may be explained by the presence of dead and decaying earthworms. It is reported that earthworm activity was shown to decrease the amount of DTPA-extractable Pb^{2+} and Zn^{2+} in the cast, which implies the immobilization of metals (Maity et al., 2008). The increased soil pH by earthworm activities could possibly be the major reason for the decreased metal availability (Maity et al., 2008). Moreover, it is quite possible that inorganic ions and organic compounds released from dead or decaying earthworms could lead to the complexation of metals which could decrease the bioavailable metal to some extent (Ma et al., 2002). Furthermore, Zorn et al. (2005) found that different endogeic earthworm species have different effects on zinc distribution and availability in soils. While *A. chlorotica* is mixing the soil rather equally, *A. caliginosa* transports soil upward and lowers the zinc availability.

The discrepancies among these studies are probably not only due to the selection of earthworm species, but also because of selection of soils. A study by Cheng and Wong (2002), using the species *Pheretima sp.*, suggested that whether or not metals could be mobilized by earthworm activities was also dependent on soil type. They suggested that metals from various sources are bound in various pools within the soil, and vary in their availability to organisms. For example, geogenic metals are often tightly bound to or within soil particles, whereas anthropogenic metals are often bound in more exchangeable and labile pools (Scott-Fordsmand et al., 2004). Therefore, research is still needed to compare the possible difference of earthworm's effects on metals from various pollution sources, such as naturally metals enriched soils (geogenic), soils amended with sewage sludge, and those receiving smelter emission, which are all represented in Chapter 5. The possible difference between the two earthworm species, *Eisenia fetida* and *Lumbricus terrestris*, on metal bioavailability and soil characteristics are also evaluated in Chapter 5.

References

Ahnstrom, Z.A.S., Parker, D.R., 2001. Cadmium reactivity in metal-contaminated soils using a coupled stable isotope dilution-sequential extraction procedure. Environmental Science and Technology 35, 121-126.

Alexander, M. 2000. Aging, bioavailability, and overestimation of risk from environmental pollutants. Environmental Science and Technology 29, 2713-2717.

Alloway, B.J. 1995. Heavy Metals in Soils. London: Blackie Academic & Professional

ASTM, 1999. Standard guide for conducting laboratory soil toxicity or bioaccumulation tests with Lumbricid earthworm *Eisenia fetida*, standard E 1676-97. In: Annual Book of ASTM Standards, pp. 1056–1074.

Basta, N., Gradwohl, R.J. 2000. Estimation of Cd, Pb, and Zn bioavailability in smelter-contaminated soils by a sequential extraction procedure. Journal of Soil Contamination 2000, 9, 149-164.

Battelle, Exponent. 2000. Guide for Incorporating Bioavailability Adjustments into Human Health and Ecological Risk Assessments at U.S. Navy and Marine Corps Facilities. Part 1: Overview of Metals Bioavailability, Naval Facilities Engineering Service Center, Port Hueneme, CA.

Becker, J.S., Dietze, H.J. 2000. Inorganic mass spectrometric methods for trace, ultratrace, isotope, and surface analysis. Fresenius Journal of Analytical Chemistry 2000, 368, 23-30.

Beckett, P.H.T. 1989. The use of extractants in studies on trace metals in soils, sewage sludge and sludge-treated soils. Advances in Soil Science 9, 143-176.

Beyer, W.N., Chanery, R.L., Mulhern, B.M. 1982. Heavy metal concentrations in earthworms from soil amended with sewage sludge. Journal of Environmental Qaulity11, 381-385.

Broadley, M.R., White, P.J., Hammond, J.P., Zelko, I., Lux, A. 2007. Zinc in plants. New Phytologist. 173, 677–702.

Cao, X.D., Wang, X.R., Zhao, G.W. 2000. Assessment of the bioavailability of rare earth elements in soils by chemical fraction and multiple regression analysis. Chemosphere 40, 23-28.

Cheng, J.M., Wong, M.H. 2002. Effects of earthworms on Zn fractionation in soils.

Biology and Fertility of Soils 36, 72-78.

Coeurdassier, M., Scheifler, R., De, V.A., Crini, N., Saccomani, C., Salomon, D.M.L. 2007. Earthworms influence metal transfer from soil to snails. Applied Soil Ecology 35, 302-310.

Dayton, E. A., Basta, N. T., Payton, M. E., Bradham, K. D., Schroder, J. L., Lanno, R. P. 2006. Evaluating the contribution of soil properties to modifying lead phytoavailability and phytotoxicity. Environmental Toxicology and Chemistry 25, 719-725.

Degryse, F., Waegeneers, N., Smolders, E. 2007. Labile lead in polluted soils measured by stable isotope dilution. European Journal of Soil Science 58, 1-7.

Devliegher, W., Verstraete, W. 1997. The effects of *Lumbricus terrestris* on soil in relation to plant growth: Effects of nutrient enrichment processes (NEP) and gut-associated processes (GAP). Soil Biology and Biochemistry 29, 341-346.

Díez-Ortiz, M., Giska, I., Groot, M., Borgman, E.M., Van Gestel, C.A.M. 2010. Influence of soil properties on molybdenum uptake and elimination kinetics in the earthworm *Eisenia andrei*. Chemosphere 80, 1036–1043.

Echevarria, G., Morel, J. L., Fardeau, J. C., Leclerc-Cessac, E. 1998. Assessment of phytoavailability of nickel in soils. Journal of Environmental Quality 27, 1064-1070.

Edwards, C.A., Bohlen, P.J. 1996. Biology and Ecology of Earthworms. 3rd Edn., Chapman and Hall, London, ISBN: 0412561603, pp: 426

Fosmire, G. J. 1990. "Zinc toxicity". American Journal of Clinical Nutrition 51, 225–227.

Gabler, H. E., Bahr, A., Mieke, B. 1999. Determination of the interchangeable heavy-metal fraction in soils by isotope dilution mass spectrometry. Fresenius Journal of Analytical Chemistry 365, 409-414.

Garbisu, C., Alkorta, I. 2001. Phytoextraction: A cost effective plant-based technology for the removal of metals from the environment. Bioresouce Technology. 77, 229–236.

Giller K.E., Witter, E., McGrath, S.P. 1998. Toxicity of heavy metals to microorganism and microbial processes in agricultural soils: A review. Soil Biology and Biochemistry. 30, 1389–1414.

Gleyzes, C., Tellier, S., Astruc, M. 2002. Fractionation studies of trace elements in contaminated soils and sediments, a review of sequential extraction procedures. Trends in Analytical Chemistry 21, 451-467.

Gray, C.W., McLaren, R.G., Shiowatana, J. 2003. The determination of labile cadmium in some biosolids-amended soils by isotope dilution plasma mass spectrometry. Australian Journal of Soil Research 41, 589-597.

Hamon, R. E., Lombi, E., Fortunati, P., Nolan, A. L., McLaughlin, M. J. 2004. Coupling speciation and isotope dilution techniques to study arsenic mobilization in the environment. Environmental Sciences and Technology 38, 1794-1798.

Hamon, R. E., Parker D. R., Lombi, E. 2008. Advances in isotopic dilution techniques in trace element research: A review of methodologies, benefits and limitations. Advances in Agronomy 99, 289-343.

Heikens, A., Peijnenburg, W.J.G.M., Hendriks, A.J. 2001. Bioaccumulation of heavy metals in terrestrial invertebrates. Environmental Pollution 113, 385–393.

Hendriks, A.J., Heikens, A. 2001. Rate constants and equilibrium ratios for accumulation of inorganic substances related to species weight. Environmental Toxicology and Chemistry 20, 1421–1437.

Heuscher, S. A., Brandt, C. C., Jardine, P. M. 2004. SBAT: A Tool for Estimating Metal Bioaccessability in Soils, ORNL/TM-2004/49. Oak Ridge, TN, Oak Ridge National Laboratory.

Hobbelen, P.H.F., Koolhaas, J.E., van Gestel, C.A.M. 2006. Bioaccumulation of heavy metals in the earthworms Lumbricus rubellus and Aporrectodea caliginosa in relatin to total and available metal concentrations in field soils. Environmental Pollution 144, 639-646.

Hogan, C.M. 2010. Heavy metal. Encyclopedia of Earth. National Council for Science and the Environment.

Ireland, M.P. 1975. The effect of the earthworm *Dendrobaena rubida* on the solubility of lead, zinc, and calcium in heavy metal contaminated soil in Wales. Journal of Soil Science 6, 313-318.

Janssen, R.P.T., Peijnenburg, W.J.G.M., Posthuma, L., Van den Hoop, M.A.G.T., 1997. Equilibrium partitioning of heavy metals in Dutch field soils I. Relationship between metal partition coefficients and soil characteristics. Environmental Toxicology and Chemistry 16, 2470–2478.

Kabata-Pendias, A. 2001. Trace Elements in Soils and Plants. 3rd Ed. Boca Raton, Florida: CRC Press.

Kato, N., Zapata, F., Fardeau, J.C. 1995. The ability of chemical extraction methods to estimate plant-available soil P and a better understanding of P availability of fertilized andosols by using isotopic methods. Soil Science and Plant Nutrition 41, 781-789.

Kelley, M. E., Brauning, S. E., Schoof, R. A., Ruby, M. V. 2002. Assessing Oral Bioavailability of Metals in Soil. Columbus, OH, Battelle Press.

Kelsey, J.W., Kottler, B.D., Alexander, M. 1997. Selective chemical extractants to predict bioavailability of soil-aged pollutants. Environmental Sciences and Technology 31, 214–217.

Kennedy, V.H., Sanchez, A.L., Oughton, D.H. 1997. Use of single and sequential chemical extractants to assess radionuclide and heavy metal availability from soils for root uptake. Analyst 122, 89–100.

Kennette, D., Hendershot, W., Tomlin, A., Sauve, S. 2002. Uptake of trace metals by earthworm *Lumbricus terrestris* in urban contaminated soils. Applied Soil Ecology 19, 191 – 198.

Kizilkaya, R. 2005. The role of different organic wastes on zinc bioaccumulation by earthworm *Lumbricus terrestris* L. (Oligochaeta) in successive Zn added soil. Ecological Engineering 25, 322-331.

Kozdrój, J., van Elsas, J.D. 2001. Structural diversity of microbial communities in arable soils of a heavily industrialized area determined by PCR-DGGE finger printing and FAME profiling. Appl Soil Ecol. 17, 31–42.

Kurek, E., Bollag, J.M. 2004. Microbial immobilization of cadmium released from CdO in the soil. Biogeochemistry. 69, 227–239.

Lanno, R., Wells, J., Conder, J., Bradham, K., Basta, N. 2004. The bioavailability of chemicals in soil for earthworms. Ecotoxicology and Environment Safety 57, 39-47.

Lee, K.E., 1985. Earthworms: Their Ecology and relationship with soils and land use. Academic Press, London, UK. pp: 411

Li, F.L., Shan, X.Q., Zhang, S.Z. 2001. Evaluation of single extractants for assessing plant availability of rare earth elements in soil. Communications in Soil Science and Plant Analysis 32, 2577–2587.

Liu, X.M., Wu, Q.T., Banks, M.K. 2005(a). Effect of simultaneous establishment of *Sedum alfridii* and *Zea mays* on heavy metal accumulation in plants. International Journal of Phytoremediation. **7**, 43–53.

Liu, X., Hu, C., Zhang, S. 2005(b). Effects of earthworm activity on fertility and heavy metal bioavailability in sewage sludge. Environmental International 31, 874-879.

Lombi, E., Hamon, R. E., McGrath, S. P., McLaughlin, M. J. 2003. Lability of Cd, Cu, and Zn in polluted soils treated with lime, beringite, and red mud and identification of a non-labile colloidal fraction of metals using isotopic techniques. Environmental Sciences and Technology 37, 979-984.

Ma, Y., Dickinson, N.M., Wong, M.H. 2000. The effect of earthworm inoculation on metal bioavailability: potential use for phytoremediation of Pb/Zn mine spoils. International Conference on the remediation and management of degraded lands, Promaco Conventions Pty Ltd, Fremantle pp. 33-34.

Ma, Y., Dickinson, N.M., Wong, M.H. 2002. Toxicity of Pb/Zn mine tailings to the earthworm *Pheretima* and the effects of burrowing on metal availability. Biology and Fertility of Soils. 36, 79-86.

Maity, S., Padhy, P.K., Chaudhury, S. 2008. The role of earthworm *Lampito mauritii* (Kinberg) in amending lead and zinc treated soil. Bioresounce and Technology 99, 7291-7298.

McGrath S.P., Zhao F.J., Lombi, E. 2001. Plant and rhizosphere process involved in phytoremediation of metal-contaminated soils. Plant and Soil 232, 207–214.

Morel, C., Plenchette, C. 1994. Is the isotopically exchangeable phosphate of a loamy soil the plant-available P? Plant and Soil 158, 287–297.

Morgan, J.E., Morgan, A.J., 1998. The distribution and intracellular compartmentation of metals in the endogeic earthworm *Aporrectodea caliginosa* sampled from an unpolluted and a metal-contaminated. Environmental Pollution 99, 167-175.

Morgan, J.E., Morgan, A.J., 1999. The accumulation of metals (Cd, Cu, Pb, Zn and Ca) by two ecologically contrasting earthworm species (*Lumbricus rubellus* and *Aporrectodea caliginosa*): implications for ecotoxicological testing. Applied Soil Ecology 13, 9–20.

Moriarty, F., Walker, C.H. 1987. Bio-accumulation in food chains—A rational approach. Ecotoxicology and Environmental Safety 13, 208–215.

Nahmani, J., Hodson, M.E., Black, S. 2007. A review of studies performed to assess metal uptake by earthworms. Environmental Pollution 145, 402–424.

Nriagu, J.O., Pacyna, J.M. 1988. Quantitative assessment of worldwide contamination of air water and soils by trace metals. Nature. 333, 134–139.

Nolan, A.L., Ma, Y.B., Lombi, E. McLaughlin, M. J. 2004. Measurement of labile Cu in soil using stable isotope dilution and isotope ratio analysis by ICP-MS. Analytical and Bioanalytical Chemistry 380, 789-797.

Peijnenburg, W.J.G.M., Baerselman, R., De Groot, A.C., Jager, T., Posthuma, L., Van Veen, R.P.M. 1999. Relating environmental availability to bioavailability: soil-type-dependent metal accumulation in the oligochaete Eisenia andrei. Ecotoxicology and Environmental Safety 44, 294–310.

Prasad, A. S. 2008. Zinc in Human Health: Effect of Zinc on Immune Cells. Molecular Medicine. 14, 353–357.

Rainbow, P.S. 2002 Trace metal concentrations in aquatic invertebrates: why and so what? Environmental Pollution 120, 497–507.

Schalscha, E., Ahumada, I. 1998. Heavy metals in rivers and soils of central chile. Water Science and Technology 37, 251–255.

Scheifler, R., Schwartz, C., Echevarria, G., Vaufleury, A.D., Badot, P.M., Morel, J.L. 2003. “Nonavailable” Soil Cadmium is bioavailable to snails: evidence from isotopic dilution experiments. Environmental Science and Technology 37, 81-86.

Schmidt, U. 2003. Enhancing phytoremediation: The effect of chemical soil manipulation on mobility, plant accumulation, and leaching of heavy metals. Journal of Environmental Quality. 32, 1939–1954.

Schwartz, C., Echevarria, G., Morel, J.L. 2003. Phytoextraction of cadmium with *Thlaspi caerulescens* . Plant and Soil 249, 27–35.

Scott-Fordsmand, J.J., Stevens, D., McLaughlin, M. 2004. Do earthworms mobilize fixed zinc from ingested soil? Environmental Science and Technology 38, 3036-3039.

Scoullos, M. J., Vonkeman, G. H., Thornton, I., Makuch, Z. 2001. Mercury, Cadmium, Lead: Handbook for Sustainable Heavy Metals Policy and Regulation

Sims, R. W., Gerard, B. M. 1999. Earthworms: Notes for the Identification of British Species, Synopses of the British Fauna (New Series) No.31 (Revised). London: Linnean Society.

Sinaj, S., Dubois, A., Frossard, E. 2004. Soil isotopically exchangeable zinc: A comparison between *E* and *L* values. Plant and Soil 261, 17-28.

Smolders, E., Brans, K.m, Foldi, A., Merckx, R. 1999. Cadmium Fixation in Soils Measured by Isotopic Dilution. Soil Science Society of America Journal 63, 78-85.

Springett, J.A., Gray, R.A.J. 1997. Relationship between pasture plant roots and earthworm burrows. Soil Biology and Biochemistry 29, 621-625.

Spurgeon, D. J., Hopkin, S. P. 1996. Effects of variations of the organic matter content and pH of soils on the availability and toxicity of zinc to the earthworm *Eisenia fetida*. Pedobiologia 40, 80-96.

Suthara, S., Singhb, S., Dhawanc, Sachin. 2008. Earthworms as bioindicator of metals (Zn, Fe, Mn, Cu, Pb and Cd) in soils: Is metal bioaccumulation affected by their ecological category? Ecological Engineering 32, 99-107.

Tack, F.M.G., Verloo, M.G. 1995. Chemical speciation and fractionation in soil and sediment heavy metal analysis: a review. International Journal of Environmental Analytical Chemistry 59, 225-238.

Tiller, K.G., Wassermann, A. 1972. Isotopes and radiation in soil plant relationship, IRFA. Vienna.

Tye, A. M., Young, S. D., Crout, N. M. J., Zhang, H., Preston, S., Bailey, E. H., Davison, W., McGrath, S. P., Paton, G. I., Kilham, K. 2002. Predicting arsenic solubility in contaminated soils using isotopic dilution techniques. Environmental Sciences and Technology 36, 982-988.

Udovic, M., Lestan, D. 2007. The effect of *Eisenia fetida* on the redistribution of residual Pb, Zn and Cd in soil after remediation with EDTA leaching. Environmental Pollution 663–668.

Ure, A.M. 1996. Single extraction schemes for soil analysis and related applications. The Science of the Total Environment 178, 3-10.

USEPA. 2000. Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with the Amphipod Leptocherius phumulosus. U.S. Environmental Protection Agency, Office of Research and Development, Office of Water, Washington DC. EPA-600-R-99-064.

Vijver, M.G., Vink, J.P.M., Jager, T., Wolterbeek, H.T., van Straalen, N.M., van Gesterl, C.A.M. 2005. Biphasic elmination and uptake kinetics of Zn and Cd in the earthworm Lumbricus rubellus exposed to contaminated floodplain soil. Soil Biology and Biochemistry 37, 1843-1851.

Wen, B., Hu, X., Liu, Y., Wang, W., Feng, M., Shan, X. 2004. The role of earthworms (Eisenia fetida) in influencing bioavailability of heavy metals in soils. Biology and Fertility of Soil 40, 181–187.

Young, S. D., Tye, A., Carstensen, A., Resende, L., Crout, N. 2000. Methods for determining labile cadmium and zinc in soil. European Journal of Soil Science 51, 129-136.

Zorn, M.I., Van, G.C.A.M., Eijsackers, H. 2005. The effect of two endogeic earthworm species on zinc distribution and availability in artificial soil columns. Soil Biology and Biochemistry 37, 917-25.

Chapter 2

Determination of labile cadmium and zinc in soils by isotope dilution using with ICP-MS

Abstract

A stable isotope dilution technique combined with inductively coupled plasma-mass spectrometry (ICP-MS) was applied to determine the labile fractions of Cd and Zn in polluted soils. A key requirement in using this technique is to establish a reliable and accurate method for measuring isotope ratios of $^{111}Cd/^{114}Cd$ and $^{66}Zn/^{68}Zn$ in soil suspensions. First, it was found the ICP-MS detection remained in impulse mode and had the lowest relative standard deviation values when the Cd or Zn concentration is about 100 µg L^{-1} in solution. Next, it was found that when ICP-MS is used without specially gases, isotope ratio measurements of $^{111}Cd/^{114}Cd$ and $^{66}Zn/^{68}Zn$ are close to their natural abundance. After the standard protocol for measuring $^{111}Cd/^{114}Cd$ and $^{66}Zn/^{68}Zn$ in solution was established, the method was applied to determine the labile pool of Cd and Zn in 10 high-metal soils. The soils' labile fraction (*E*%) of Cd ranged from 31% to 58%, while labile Zn values were from 18% to 37%.

Keywords: ICP-MS, labile pool, isotope ratio, Cd and Zn

1. Introduction

Isotope dilution technique (IDT) is one approach to measure the potentially bioavailable pool of metals in soils, and has been employed in soil science for more than 50 years. IDT was initially used to characterize the phytoavailability or reactivity of some essential plant nutrients (e.g., P and K) in soils (Graham and Silva, 1979; Morel and Plenchette, 1994). More recently, IDT was applied to examine the labile pool of trace elements in soils, such as Cd (Smolders et al., 1999; Ahnstrom and Parker, 2001; Young et al., 2000), As (Tye et al., 2002; Hamon et al., 2004), Pb (Degryse et al., 2007), Zn (Tiller and Wassermann, 1972; Young et al., 2000) Ni (Echevarria et al., 1998) and Cu (Lombi et al., 2003). The labile pool is the sum of isotopically exchangeable elements in the solution and solid phases, and is considered to be the potentially bioavailable pool of metals in soil under equilibrium conditions (Degryse et al., 2007; Hamon et al., 2008). More recently, rapid and sensitive analytical techniques for determining isotopic ratios, such as inductively coupled plasma – mass spectrometry (ICP-MS), have made possible the use of enriched stable isotopes for isotope dilution studies possible (Gabler et al., 1999; Ahnstrom and Parker, 2001; Hamon et al., 2008). This can be achieved by adding an enriched isotope spike to a suspension of soil in water or neutral salt electrolyte such as $Ca(NO_3)_2$ to measure the extent of isotope dilution in the solution phase once equilibrated; thus is known as the *E*-value (Sinaj et al., 1997; Ahnstrom and Parker, 2001).

An isotope dilution technique involves spiking the soil (or soil suspension) with a small amount of an enriched isotope of the element of interest to determine the interchangeable or labile heavy-metal fractions in soils. The interchangeable portion of the elements can be subsequently calculated using isotope dilution equation (Gabler et al., 1999). Taking the *E* value of Cd as an example:

$$E = \frac{R(SA_{111} - SA_{114} IR_{soil})}{NA_{111} IR_{soil} - NA_{114}} \tag{1}$$

where R is the total amount of Cd (in mgCd/kg soil) added to the soil through the spike, SA_{111} and SA_{114} are the fractional abundance (molar basis) of ^{111}Cd and ^{114}Cd in the spike, and NA_{111} and NA_{114} are the natural abundance (molar basis) of ^{111}Cd and ^{114}Cd, and IR_{soil} is the isotope ratio of $^{111}\mathrm{Cd}/^{114}\mathrm{Cd}$ measured in soil suspension.

Since isotopic abundance in nature is known, the precise measurement of E value mainly depends on the accuracy of measuring isotope ratios in spiked samples (Janghorbani, 1984). Although ICP-MS is an accurate and reliable instrument to measure the concentration of elements in samples, it should be noted that several instrumental factors can compromise isotope ratio determination (Agilent, 2005). The most important factor is signal stability. The detector in ICP-MS has two modes to collect signal. One is analog mode, which is used to detect samples that contain high-concentrations of the elements. In samples that contain low concentrations of elements, the detector uses impulse mode, which is a more accurate mode and is better for isotope ratio (IR) determination (Agilent, 2005). However, low concentration of the element usually creates instability in detection which usually leads to high RSD (relative standard deviation) of repeated IR measurements (Agilent, 2005). In addition, selection of running mode also influences signal stability. ICP-MS has three running modes: In “No gas” mode, the only carrier gas is "argon" was used. In “Helium” and “Hydrogen” mode, helium or hydrogen is also added as collision gas (Agilent, 2005).

Another important instrumental factor is polyatomic mass spectral interference. In the extreme environment (extremely high temperature) in the ICP, some isotopes can combine with O, H, or OH to form polyatoms, which may have the same mass as the detected isotopes thus create interferences that interrupt isotopic ratio determination (McLaren et al., 1987). Thirdly, mass bias is another obstacle in accurately measuring IR because the detector of ICP-MS usually reacts more sensitively to heavier isotopes than to lighter isotopes (Agilent, 2005).

Several non-instrumental factors, such as pH, high levels of total dissolved solids, may result in the loss of accuracy and precision in ICP-MS (Beauchemin et al, 1987; Jarvis et al., 1992). However, these non-sepctral interferences are thought not to interfere with IR determination because the interferent acts on both isotopes equally (Jarvis et al., 1992).

The first aim of this study is to optimize the method for determine ^{111}Cd/^{114}Cd and ^{66}Zn/^{68}Zn in ICP-MS, which include: 1) find the best running concentration of metals, 2) selection the gas running mode in ICP, 3) investigate the possible mass spectral interferences, 4) correct mass bias. Secondly, soils spiked with enriched ^{111}Cd and ^{68}Zn and quantify the Cd and Zn labile pools (*E*-values) in polluted.

2. Materials and Methods

Enriched staple isotopes of ^{111}Cd, ^{114}Cd, ^{66}Zn and ^{68}Zn were purchased from Isoflex (San Francisco, CA). Information of the enriched isotopes listed as Table 2.1. An Agilent ICP-MS 7500ce was used for all isotope ratio determination. The isotope ratio analysis mode was selected. Before each run, the instrument was tuned and the standard instrumental operational parameters outlined in Table 2.2 were developed. All IR measurements were based on 5 replicate acquisition of 10 s each.

2.1 Selection of optimum concentration and running mode

To evaluate precision in $^{111}Cd/^{114}Cd$ and $^{66}Zn/^{68}Zn$ measurements, IR determination were conducted using a series of natural Cd (and Zn) standards (SPEX Industries, Edision, NJ) containing total Cadmium Cd_T (or Zn_T) concentrations ranging from10 to 200 µg L^{-1}. All standard solutions were prepared by diluting 1000 mg/L Cd or Zn stock solutions with 1% HNO_3 (trace-metal grade). At each concentration, isotope ratio measurements consisted of 10 replicate determination. RSD was calculated from the replicates.

Next, in order to choose running mode, standard solutions (containing Cd_T or Zn_T ranging from 10 to 200 µg L^{-1}) were run in the “No gas” and “Helium” modes separately. “Hydrogen” mode was not used here because it is not suitable for Zn determination (Agilent, 2005). The measured IR values were then compared with known IR values (0.446 for $^{111}Cd/^{114}Cd$, 0.336 for $^{66}Zn/^{68}Zn$) which were calculated from their natural abundance.

2.2 Isotope interference study

In this part, firstly the possible interference elements (isotopes) were identified by checking the element natural abundance table (Agilent, 2005). Secondly, the counts values of these isotopes and that of ^{111}Cd, ^{114}Cd, ^{66}Zn, ^{68}Zn in the same concentration (100 ug L^{-1}) were compared.

Possible interferences included that Mo and Zr, which could influence $^{111}Cd/^{114}Cd$ by forming $^{94}Zr^{16}OH$, $^{94}Mo^{16}OH$, $^{95}Mo^{16}O$ that would contribute to m/z 111, or forming $^{98}Mo^{16}O$ and $^{97}Mo^{16}OH$ tthat could contribute to m/z 114. 100 µg L^{-1} Zr, Mo and Cd standard solutions were prepared and were analyzed by ICP-MS. In these solutions, count values at m/z 111 and 114 were recorded separately (Table 2.3).

Similarly, it is assumed that Cr and V may impact $^{66}Zn/^{68}Zn$ ratios by forming $^{52}Cr^{16}O$ and ^{51}VOH to interfere with m/z 68. 100 µg L^{-1} Cr, V and Zn standards were made and were run in ICP-MS. These isotopes' count values at m/z 66 and 68 were recorded separately (Table 2.3).

2.3 Regression of measured IR vs. known IR to correct mass bias

To evaluate instrumental mass bias on $^{111}Cd/^{114}Cd$, enriched samples of ^{111}Cd and ^{114}Cd were obtained to make individual sub-stock solutions (Cd_T ≈100 µg L^{-1} for each) in 1% HNO_3. The precise concentrations of Cd_T in these solutions were measured by flame atomic absorption spectrometer (FAAS). Then a series of standard solution contains known IR ($^{111}Cd/^{114}Cd$) from 0.267 to 2.483 (including ^{Nat}Cd with $^{111}Cd/^{114}Cd$ at 0.446) were made by mixing the sub-stock solutions of enriched ^{111}Cd and ^{114}Cd. Isotope ratio measurements were then conducted for this series of IR standard solutions. Also, individual sub-stock solutions of ^{68}Zn and ^{Nat}Zn were prepared (Zn_T≈100 µg L^{-1} each) in 1% HNO_3. The precise concentration of Zn_T was also measured by FAAS. Then a series of standard solution contains known IR ($^{66}Zn/^{68}Zn$) from 0.336 to 1.488 were made by mixing the enriched ^{68}Zn and ^{Nat}Zn solutions. Isotope ratio measurements were then conducted. These measured IR values were plotted against known IR values to generate a standard curve which can be used to correct mass bias in determining $^{111}Cd/^{114}Cd$ and $^{66}Zn/^{68}Zn$.

Part 2.4 Soil Cd and Zn *E*-values determination

After optimization of isotope ratio determination procedures, the isotope dilution technique was applied to measure *E* value of Cd and Zn in polluted soils. Enriched ^{111}Cd and ^{68}Zn were used as tracer stable isotopes to spike soils; enriched ^{114}Cd and natural Zn were reference isotopes. The concentrations of possible interference elements (Mo, Zr, Cr, V) in the soils were measured at first, then *E* values were determined following these steps (Hamon et al., 2008):

1) Soil sample preparation: soil samples were air-dried and sieved to < 2 mm. 2.0 g portions of each soil samples (n=3) were weighed into 50 ml centrifuge tubes, and 20 ml of 0.01 M $Ca(NO_3)_2$ solution was added.

2) Spiking of soil suspension with enriched stable isotopes ^{111}Cd and ^{68}Zn: For each soil, it was spiked with enriched ^{111}Cd (96% enrichment) and ^{68}Zn (99% enrichment) separately. Spike size was adjusted to 1% of the 1 M $CaCl_2$-extractable concentration of Cd or Zn. The volume of the spiking solution was less than 0.2 ml.

3) Extraction of soil by low concentration neutral salt solution to reach equilibrium: The suspension was shaken end-over-end for 24h. After separation by centrifugation (3000 g, 20 min) and filtration with a 0.45-um membrane, a 5-ml sample was removed from each tube, acidified to pH 1 with 5 M HNO_3, and then diluted with 1% HNO_3 to Cd_T (or Zn_T) $\approx$100 µg L^{-1} separately.

4) Isotope ratio measurement in soil suspension: isotopic ratio measurement of

^{111}Cd/^{114}Cd and ^{66}Zn/^{68}Zn measurements were then conducted by ICP-MS. When running soil suspensions, IR standard solutions were run every 5 samples. Then the measured IR ratios were corrected by the IR standard curve to minimize mass bias. Finally *E* values of Cd and Zn were calculated by the equations:

$$E_{Cd} = \frac{R(SA_{111} - SA_{114} IR_{soil})}{NA_{111} IR_{soil} - NA_{114}}$$

$$E_{Zn} = \frac{R(SA_{66} - SA_{68} IR_{soil})}{NA_{66} IR_{soil} - NA_{68}}$$

3. Result and discussion

3.1 Optimization of sample running in ICP-MS

Optimal working concentrations: In a series of solutions with Cd_T and Zn_T ranging from 10 to 200 µg/L, RSD (relative standard deviation) of the measured isotope ratios (^{111}Cd/^{114}Cd and ^{66}Zn/^{68}Zn) was calculated using 10 repetitions. The RSD values decreased when Cd or Zn concentrations increased from 10 to 100 µg L^{-1} and remained constant thereafter (Figure 2.1). The ICP-MS detector remained in impulse mode until concentrations reached 100 µg L^{-1}. After that, the detector automatically switches to analog mode, which generally gives less accurate measurements (Agilent, 2005). The results indicate that the best concentration for IR measurements is 100 µg L^{-1} Cd_T and Zn_T when using ICP-MS. Thus, in all future experiments, the total concentrations of Cd and Zn in soil suspensions will be diluted to 100 µg L^{-1} before IR measurement in ICP-MS whenever possible.

Selection of gas mode for ICP-MS operation: Comparing IR measurements in "No gas" and "helium" mode, it was found under "No gas" mode, IR measurements for $^{111}Cd/^{114}Cd$ and $^{66}Zn/^{68}Zn$ were closer to their natural abundance (Figure 2.2). Generally, "Helium" or "Hydrogen" is added as collision gases to break up various polyatomic interferences that can occur in the "No gas" mode. However, "Hydrogen" mode is not suggested for Zn detection (Agilent, 2005) and this study showed that IR determination is not any better when using "Helium" mode, which suggested "No gas" mode should be selected in running soil suspensions.

Interference study: At concentrations of 100 μg L^{-1}, it was found that with V and Cr, the count values at m/z 66 were far lower than those for a comparable Zn solution, indicating that the potential interferences of element V and Cr on Zn are negligible (Table 2.3). At the same concentrations of Zr and Mo, the counts at m/z 111 and 114 were far lower than those for Cd, also indicating that the interferences on Cd could be ignored (Table 2.3).

Correcting mass bias: To examine mass bias for $^{111}Cd/^{114}Cd$ and $^{66}Zn/^{68}Zn$ determination, measurements were conducted in a series of IR standards prepared in 1% HNO_3. Then regression of mean measured IRs vs. known IRs was plotted. The measured IR values exhibited a strong linear relationship with the calculated IR values (Figure 2.3), over the entire range of IR values tested. It should be noted that daily variation in instrumental tuning may cause the fluctuating mass bias (Janghorbani and Ting, 1989), so the standard curve should be generated at every sample run.

3.2 Cd and Zn *E* value determination in soils

The Cr and V concentrations in soils were much lower than the Zn concentration. Also, Mo and Zr concentrations are very low compared to soil Cd concentrations (Table 2.4). In section 3.1, it was found the interference from the element (Cr, V, Mo and Zr) was negligible when they have the same concentration as Cd and Zn. In the soils, these elements' concentration are far lower than Cd and Zn which indicates there is no need to worry their possible interference on IR measurements in soil suspensions.

The *E*-values of Cd and Zn in soils were then determined followed the procedures described before. It was found as a proportion of total soil Cd, the fraction of labile Cd (*E*%) ranged between 31 and 58%, with an average of 45% in soils, which is much higher than the same fraction of Zn, which was ranged from 18 to 37% with a mean value of 25.4% ($p<0.001$) (Table 2.5).

References

Agilent Technologies, Inc. 2005. Agilent 7500 ICP-MS ChemStation (G1834B) Operator's Manual.

Ahnstrom, Z.A.S., Parker, D.R., 2001. Cadmium reactivity in metal-contaminated soils using a coupled stable isotope dilution-sequential extraction procedure. Environmental Science and Technology 35, 121-126.

Beauchemin, D., McLaren J.W., Berman S.S. 1987. Study of the effects of concomitant elements in inductively coupled plasma mass spectrometry. Spectrochimica Acta. 42B 467-490

Degryse, F., Waegeneers, N., Smolders, E. 2007. Labile lead in polluted soils measured by stable isotope dilution. European Journal of Soil Science 58, 1-7.

Echevarria, G., Morel, J. L., Fardeau, J. C., Leclerc-Cessac, E. 1998. Assessment of phytoavailability of nickel in soils. *Journal of Environmental Quality* 27, 1064-1070.

Gabler, H. E., Bahr, A., Mieke, B. 1999. Determination of the interchangeable heavy-metal fraction in soils by isotope dilution mass spectrometry. Fresenius Journal of Analytical Chemistry 365, 409-414.

Graham, E. R., Silva, C.G. 1979. Labile pools and distribution coefficients for soil calcium, magnesium, and potassium determined with exchange equilibria and radioisotopes. Soil Science 128, 17-22.

Hamon, R. E., Lombi, E., Fortunati, P., Nolan, A. L., McLaughlin, M. J. 2004. Coupling speciation and isotope dilution techniques to study arsenic mobilization in the environment. Environmental Science and Technology 38, 1794-1798.

Hamon, R. E., Parker, D. R., Lombi, E. 2008. Advances in isotopic dilution techniques in trace element research: A review of methodologies, benefits and limitations. Advances in Agronomy 99:289-343.

Janghorbain, M. 1984. Stable isotopes in nutrition and food science. Progress in Food and Nutrition Sciences 8, 303-332.

Janghorbani, M., Ting, B.T.G. 1989. Stable isotope tracer applications of ICP-MS. In application of ICP-MS. Date A.R. and Gray A.L. (eds.). 115-140. Blackie, London.
Jarvis, K.E., Gray A.L., Houk R.S. 1992. Interferences. In handbook of inductively coupled plasma mass spectrometry. Jarvis KE, Gray AL and Houk RS (eds). 125-152. Chapman and Hall, New York.

Lombi, E., Hamon, R. E., McGrath, S. P., McLaughlin, M. J. 2003. Lability of Cd, Cu, and Zn in polluted soils treated with lime, beringite, and red mud and identification of a non-labile colloidal fraction of metals using isotopic techniques. Environmental Science and Technology 37, 979-984.

McLaren, J.W., Beauchemin, D., Berman, S.S. 1987. Application of isotope dilution inductively coupled plasma mass spectrometry to analysis of marine sediments. Analytical Chemistry 59, 610-613.

Morel, C., Plenchette, C. 1994. Is the isotopically exchangeable phosphate of a loamy soil the plant-available P? Plant and Soil 158, 287–297.

Sinaj, S., Frossard, E., Fardeau, J.C. 1997. Isotopically exchangeable phosphate in size fractionated and unfractionated soils. Soil Science Society of America Journal 413-1417.

Smolders, E., Brans, K., Foldi, A., Merckx, R. 1999. Cadmium fixation in soils Measured by isotopic dilution. Soil Science Society of America Journal 63, 78-85.

Tiller, K.G., Wassermann, P. 1972. Radioisotopic techniques and zinc availability in soil. In "*Isotopes and radiation in soil-plant relationships including forestry*". pp. 517-529.

Tye, A. M., Young, S. D., Crout, N. M. J., Zhang, H., Preston, S., Bailey, E. H., Davison, W., McGrath, S. P., Paton, G. I., Kilham, K. 2002. Predicting arsenic solubility in contaminated soils using isotopic dilution techniques. Environmental Science and Technology 36, 982-988.

Young, S. D., Tye, A., Carstensen, A., Resende, L., Crout, N. 2000. Methods for determining labile cadmium and zinc in soil. European Journal of Soil Science 51, 129-136.

Tables and figures

Table 2.1 Relative isotopic abundances of Cd and Zn isotopes

Isotope	Natural Cd (%)	Enriched Cd-114 (%)	Enriched Cd-111 (%)	Isotope	Natural Zn (%)	Enriched Zn-68 (%)
^{106}Cd	1.25	<0.05	<0.01			
^{108}Cd	0.89	<0.05	<0.1	^{64}Zn	48.63	0.38
^{110}Cd	12.49	<0.05	0.9	^{66}Zn	27.90	0.33
^{111}Cd	12.80	<0.05	**96**	^{67}Zn	4.10	0.26
^{112}Cd	24.13	<0.05	2.7	^{68}Zn	18.75	**99**
^{113}Cd	12.22	5.6	0.3	^{70}Zn	0.62	0.03
^{114}Cd	28.73	**93.6**	<0.1			
^{116}Cd	7.49	0.8	0.01			

Table 2.2 Instrumental operating parameters in ICP-MS for isotope ratio analysis

Plasma Condition		Ion Lenses	
RF Power	1560 W	Extract 1	-1 V
RF Matching	1.66 V	Extract 2	-170 V
Sample Depth	8 mm	Cell Entrance	-30 V
Carrier Gas	0.95 L/min	QP Focus	5 V
Makeup Gas	0.15 L/min	Cell Exit	-30 V
Nebulizer Pump	0.1 rps		

Table 2.3 Counts values of possible interference isotopes (M_T=100 µg L^{-1}).

Element (C_T=100ppb)	66	68	111	114
Zn	$1.7*10^6$	$1.2*10^6$		
V	$8.7*10^3$	$6.2*10^3$		
Cr	$1.2*10^4$	$8.5*10^3$		
Cd			$1.3*10^6$	$2.9*10^6$
Zr			387	227
Mo			601	937
Blank	$3.3*10^2$	$2.4*10^2$	33	71

Table 2.4 Total concentration of possible interference elements in soils

Soil/element (mg kg^{-1})	Zn	Cr	V	Cd	Mo	Zr
P1	4600	88	133.1	28.75	6.6	2.7
P4	1165	85.6	127.4	18.23	11.3	1.34
P6	3079	80.1	134.7	62.42	14.1	0.21
P7	739	53.1	86.9	10.7	3.6	0.45
P9	19589	79.4	76.7	220.1	9.0	1.98
P12a	4088	62.5	95.6	71.23	22.2	2.3
P12b	506	51.2	106.4	15.76	13.2	0.55
Mill-01	129	12.3	12.5	18.9	5.3	0.76
T15	579	8.27	125	11.12	2.9	2.17
Romona	1393	67.9	54.2	31.17	9.8	4.2

Table 2.5 Labile Cd and Zn in soils

Soil	Zn		Cd	
	E	*E* %	*E*	*E* %
P1	826	18	8.99	31
P4	257	22	9.19	50
P6	1022	33	35.9	58
P7	182	25	5.16	48
P9	7234	37	99.3	45
P12a	985	24	40.5	57
P12b	125	25	6.14	39
Mill-01	*	*	8.83	47
T15	138	24	4.49	40
Romona	289	21	10.6	34

Figure 2.1 RSD (Relative Standard Deviation) of detection of (a) $^{111}Cd/^{114}Cd$ and (b) $^{66}Zn/^{68}Zn$ (n=10)

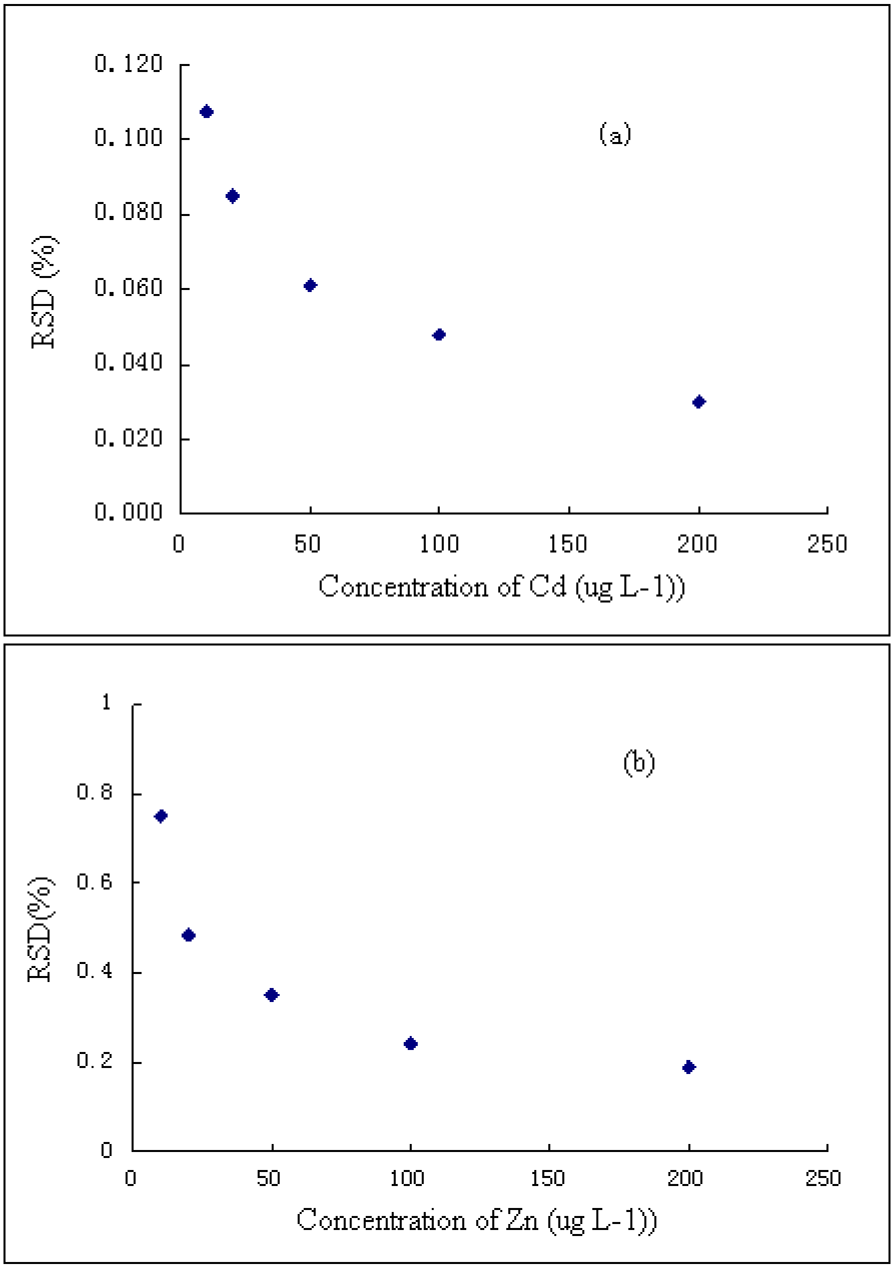


Figure 2.2 Determination of (a) $^{111}Cd/^{114}Cd$ and (b) $^{66}Zn/^{68}Zn$ in two running modes. (blank bar presents No gas mode; black bar presents Helium mode).

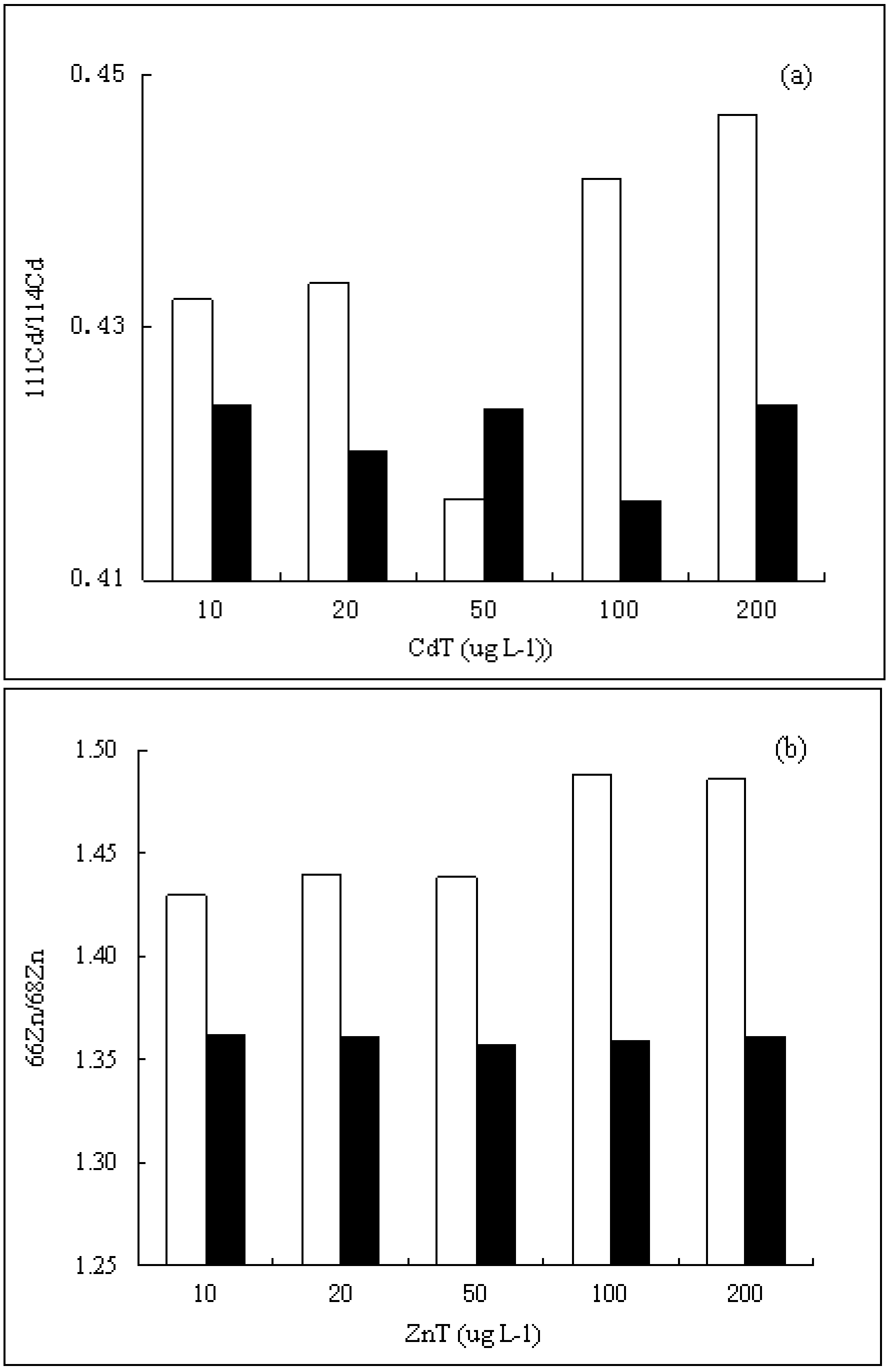
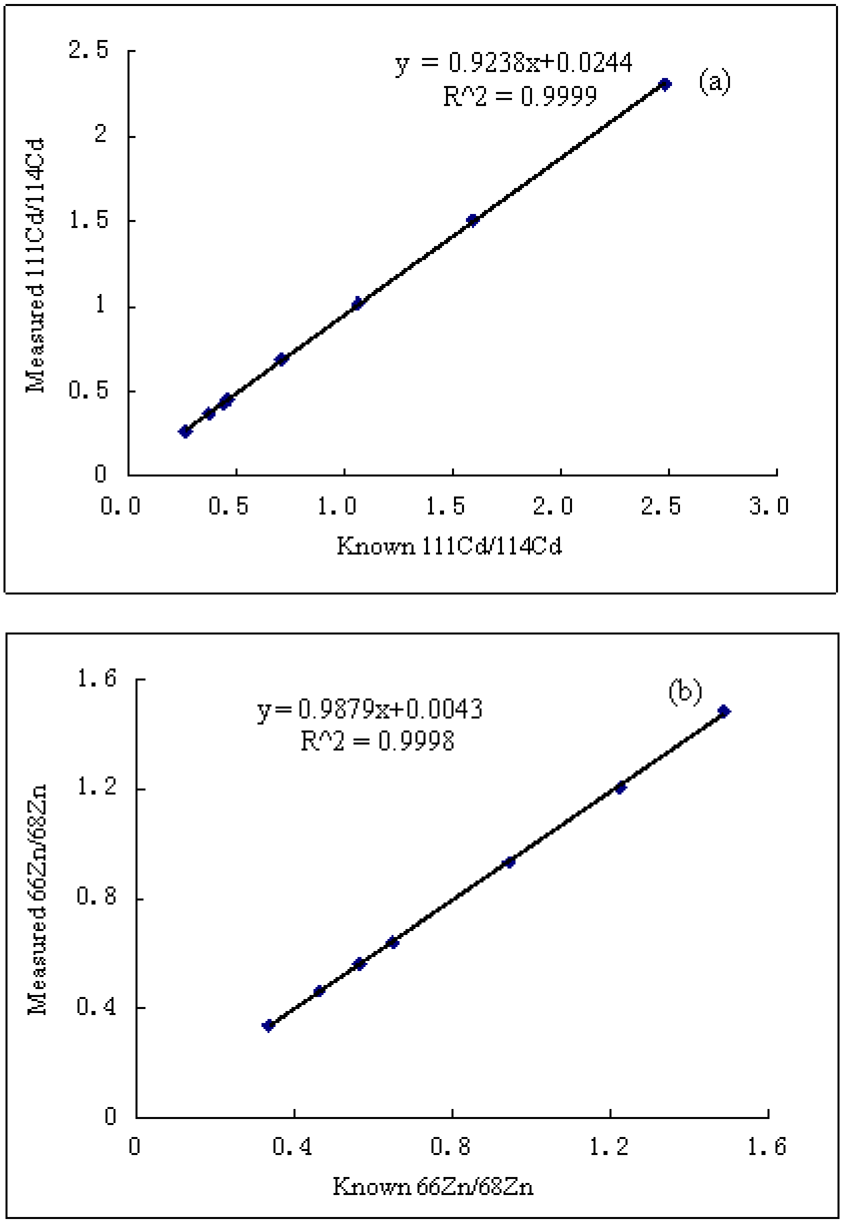


Figure 2.3 Regression of measured vs. Known $^{111}Cd/^{114}Cd$ and $^{66}Zn/^{68}Zn$ isotope ratios by ICP-MS for 1% HNO_3.



Chapter 3

Comparing chemical extractions and stable isotope dilution technique for assessing Cd and Zn bioavailability in contaminated soils

Abstract

The bioavailability of Cd and Zn to the earthworms *Eisenia fetida* and *Lumbricus terrestris* was investigated using stable isotope dilution (SID) technique and chemical extractions. Ten soils used for earthworm culturing represented various sources of metal contamination. The quantities of extractable metals followed the order: 0.05 M EDTA>1 M $CaCl_2$>0.005 M DTPA>0.05 M $Ca(NO_3)_2$>0.01 M $Ca(NO_3)_2$. Compared to extractions, the labile metal pools, estimated by SID, were highly correlated with the concentrations in earthworms ($p<0.01$), revealing that the SID method was the best way to predict Cd and Zn bioavailability. On average, the fraction of labile Cd (44.7%) was significantly higher than that of Zn (25.4%), indicating that Cd is more chemically reactive in soil, which may explain the larger Biota-Soil-Accumulation-Factors (BSAFs) for Cd in earthworms. In addition, the metal concentrations were higher in *E. fetida* than in *L. terrestris*. Thus, the accumulation of metals was element and earthworm species-specific.

Key words: BSAF, Trace metals; *E*-value; Earthworm

1. Introduction

Trace metal contamination of soil is an important issue as these toxic elements could be transported in terrestrial ecosystem posing potential risk on food quality, soil health, and the environment (Gray et al., 2003). Although the total content of metals in soil is used in many countries as an important criterion to assess pollution (Sheppard et al., 1992), it does not provide precise information on the bioavailability of the elements, nor does it predict the possibility of the elements' transferring up through the food chain. Thus, the possible risk associated with metal-contaminated soils can be unpredictable (Morel, 1997; Ahnstrom and Parker, 1999).

The bioavailability of heavy metals is related to their partitioning into a number of discrete pools in the soils. Quantifying these pools has, in the past, relied on a range of empirically derived single or sequential extractions to allocate trace metals into operationally defined pools of putatively distinct biogeochemical forms (adsorbed, carbonate, organic, hydrous oxides, etc.) (Tack and Verloo, 1995). There are three main types of chemicals utilized for extracting "available" soil metals: dilute solutions of neutral salts (e.g. $MgCl_2$, $CaCl_2$), weak acids, and chelating agents such as DTPA and EDTA (Young et al., 2000). Although chemical extractions provide valuable information on predicting bioavailability of heavy metals, these extraction methods have some limitations for wide application as the regents can extract variable proportions of available and unavailable forms depending on soil properties (Kato et al., 1995), lead to the redistribution of elements among the putative geochemical pools (Ahnstrom and Parker, 2001), or can result in release non-labile metals during extraction (Gleyzes et al., 2002).

Isotope dilution technique (IDT) is a good substitute for measuring the potential bioavailable portion of metals in soils (Hamon et al., 2008). The heavy metal labile pool estimated from IDT is the sum of isotopically exchangeable elements in the solution and solid phases and is considered to reflect the potentially bioavailable pool of metals in soil under equilibrium conditions, often is referred to as the *E*-value (Ayoub et al, 2003, Degryse et al., 2007). Several (or even most) of these studies used radio isotopes, such as, ^{109}Cd (Young et al., 2000; Lombi et al., 2003), ^{65}Zn (Young, et al., 2000; Lombi et al., 2003; Scott-Fordsmand et al., 2004), ^{73}As (Tye et al., 2002; Hamon et al., 2004), ^{64}Cu (Lombi et al., 2003), ^{83}Ni (Echevarria et al., 1998). However, using of radio isotopes is not always feasible because of a short half-life for some radio-isotopes, or potential environment hazards. To avoid the use of radio-isotope, rapid and sensitive analytical techniques (e.g. inductively coupled plasma-mass spectrometry (ICP-MS)) for determining stable isotope ratios have prompted the use of enriched stable isotopes for assessing isotopically labile metal pools in soil (Gabler, 1999; Becker, 2000; Ahnstrom and Parker, 2001; Degryse et al., 2007).

Although much work has been done on measuring the phyto-available pool in soils by the isotopic dilution method (Hamon et al., 2008), there is a relative absence of similar studies using invertebrates in soil, including earthworms (Scott-Fordsmand et al., 2004). Earthworms are ubiquitous animals inhabiting the soil, and have been found to accumulate much higher levels of metals from polluted soils than most other soil organisms (Beyer et al., 1982). Metal bioaccumulation by these soil-dwelling organisms can provide an ecological indicator of metal availability in soils (Suthara et al., 2008) and has been widely studied for some time (Morgan and Morgan, 1999; Kennette et al., 2002). In general, earthworms can be divided into three categories according to their morphological and behavioral characteristics (Sims and Gerard, 1999): litter dwelling (epigeic), horizontally burrowing mineral soil feeders (endogeic) and vertically burrowing surface feeders (anecic). The difference among species in metal bioaccumulation partly reflects the differences in their food selectivity and niche separation (Morgan and Morgan, 1992), or physiological differences (Spurgeon and Hopkin, 1999). Different bioaccumulation patterns of heavy metals between earthworm species, such as endogeic and epegeic, have been studied (Hobbelen et al, 2006; Suthara et al., 2008); however, the difference of heavy metal bioaccumulation by *E. fetida* (epigeic), a standardized earthworm for toxicity testing (ASTM, 1999), and *L. terrestris*, the representative of anecic species, is still unknown.

In this paper, two earthworm species *E. fetida* and *L. terrestris* were chosen and cultured in ten soils with three sources of Cd contamination: Zn smelter emissions, long-term sewage sludge applications, and naturally Cd-rich parent material. The first aim of this study was to compare the novel SID technique with traditional chemical extraction methods in order to determine the best way to predict Cd, Zn bioavailability to earthworms. Secondly, bioaccumulation between the essential element (Zn) and nonessential element (Cd) by earthworms was compared. Then, differences in Cd and Zn bioaccumulation between the two ecologically different earthworm species were investigated.

2. Materials and methods

2.1 Soil sample collection and preparation

Ten soils were collected: The 7 Palmerton soils constituting a gradient in metal pollution were collected from an abandoned Zn smelter emission area in Palmerton, PA. The Romona and T15 soils were collected from two fields in California, both of which were contaminated with Cd from applications of anaerobically digested sewage sludge. The Soil designated Mill01 was collected from an area near Pt. Dume, Los Angeles County, and was derived from Cd-rich shale and thus be viewed as a naturally polluted soil. The sample collection area was 1 m^2 from the soil surface layer with the exception of P12b from subsurface. At each area, three soil samples were collected and combined together, sealed in a plastic bag and transferred to the lab. Samples were air-dried at ambient temperature, ground and sieved (<2mm). Soil samples were subsequently sealed in clean, sterile plastic bags for further analysis.

Accurately weighed crushed soils (<0.2mm) were placed in a C/N/S combustion furnace for total N analysis. Organic C was determined by the Walkley-Black procedure (Nelson and Sommers, 1982). Soil texture was measured by hydrometer (Bouyoucous) method (Anderson and Ingram, 1993). Soil pH was measured in a de-ionized water-soil suspension using a ratio of 1:1. Soil characteristics are listed in Table 3.1.

2.2 Extractable Cd and Zn in soils

Each soil was independently extracted by six chemical solutions (0.01M $Ca(NO_3)_2$, 0.05M $Ca(NO_3)_2$, 1M $CaCl_2$, 0.05M EDTA, 0.005 M DTPA, 0.01M $CaCl_2$

with TEA). The detailed operations and nominal "target" fractions in soils were reported previously (Gray et al., 2003; Fang et al., 2007) and are summarized in Table 3.2. For all extraction procedures, after shaking, the suspensions were centrifuged and filtered through a 0.45μm cellulose acetate membrane filter prior to heavy metal determination. All extractions, including blanks, were carried out in triplicate. The Cd and Zn concentrations were measured by ICP-MS.

2.3 Earthworm culturing

Earthworms used for the experiment included *Eisenia fetida* (*E. fetida*) and *L. terrestris* (*L. terrestris*), and were purchased from Timeline Fishery Company (IL, USA). Earthworms were hosted in clean soil and fed with peat (contained in shipping package from the supplier) for two weeks at 20℃ prior to experiment. Mature earthworms were selected (100-300 mg fresh weight of *E. fetida*, 2.0-3.0 g fresh weight of *L. terrestris*) and transferred to the 10 studied soils. Each soil had three replicates with weights of 500 g, and each contained three *L. terrestris* and six *E. fetida* together. Soil moisture was maintained at 60% of field capacity. Soils and earthworms were maintained in a large controlled-environment chamber at a constant temperature of 20℃. Light/dark cycle was set as 16h/8h to monitor the natural environment; humidity set at 65%. Soil moisture was maintained 60% field capacity by adding DI water every two days. Worms were not fed during culturing to allow them to fully assimilate nutrients and metals from soil alone. After 30 days, earthworms were carefully removed from soils, washed, and transferred to moist filter paper at 20℃ for 48 h. The filter paper was changed daily to allow complete evacuation of the gut contents. Earthworms were then placed in the freezer at -20℃ and subsequently freeze dried for a period of 48h to obtain a constant dry weight.

2.4 Determination of total concentration of Cd, Zn in soil and earthworms

For determination of the total concentration of Cd and Zn, dried soil samples and earthworms were ground and passed through a 0.2 mm plastic sieve. Samples were then digested by using a microwave oven digestion system (CEM MARS 5) following the EPA 3501-HP500 method (USEPA, 2007). All digested soil samples, including the NIST standard soil 2710 (from Montana Soil contains highly elevated trace element concentrations) and blanks were carried out in triplicates for quality control. NIST standard soil 2781 (from domestic sludge with high organic matter content) was used for quality control of earthworm analysis. The recovery was 100% ± 5% (n=3). The concentrations of Cd and Zn in the final solutions were determined by ICP-MS. ^{115}In was added as an internal standard to monitor matrix effects and signal drift while analyzing samples.

2.5 *E*-value determination

Labile Cd and Zn in soils were determined using a stable isotope dilution technique with ICP-MS in isotope ratio analysis mode. Enriched stable isotopes ^{111}Cd and ^{68}Zn were added to a water suspension of each soil and the labile metal pool (*E*-value) was calculated from the diluted isotope ratio in the suspension (Hamon et al., 2008). The size of the isotope spike was adjusted to 1% 1 M $CaCl_2$ -extractable Cd and Zn concentration for each soil. Detailed procedures have been described previously (Gray et al., 2003; Ayoub et al., 2003; Hamon et al., 2008) and showed in Chapter 2. The *E*-value for Cd calculation was from:

$$E = \frac{R(SA_{111} - SA_{114} IR_{soil})}{NA_{111} IR_{soil} - NA_{114}}$$

Where R is the total amount of Cd (in mg Cd kg^{-1} soil) added to the soil through spiking, SA_{111} and SA_{114} are the fractional abundance (molar basis) of ^{111}Cd and ^{114}Cd in the spike, and NA_{111} and NA_{114} are the natural abundance (molar basis) of ^{111}Cd and ^{114}Cd. IR_{soil} is the isotope ratio of ^{111}Cd/^{114}Cd measured in soil suspension. Analogous calculation was done using ^{66}Zn and ^{68}Zn to calculate the Zn *E*-value.

2.6 Statistical analysis

Statistical analysis was conducted with the Minitab 15 software package (Minitab Inc., 2010). Paired t-test analysis was used to test significant differences between the fraction of extractable Cd and Zn in each extraction, as well as labile Cd and Zn in soils. Pearson's correlation test was used to identify the significantly correlated relationship between extractable or labile Cd and Zn pools in soils with the metal total concentrations of earthworms. A linear regression model was used to determine the amount of variance in the metal concentrations, containing the significantly correlated variables as predictors. A two-way ANOVA was performed to determine whether significant differences existed in metal accumulation between the two species of earthworm.

3. Results

3.1 Soil characteristics

The basic soil characteristic, along with the total Cd and Zn concentrations are shown in Table 3.1. The Palmerton soils, except for P12b from the subsurface, had much greater Zn contents than the soils obtained from the other two sources. Statistic analysis found that there was a significant correlation between total Cd and Zn in the ten tested soils ($r=0.86$, $p<0.05$), indicating co-occurence of Cd and Zn in most of these soils.

3.2 Extractable and labile Cd, Zn in soils

On average, the amount of extractable Cd and Zn by the extractants followed the order: 0.05 M EDTA > 1M $CaCl_2$ >0.005 M DTPA > 0.05 M $Ca(NO_3)_2$ > 0.01 M $Ca(NO_3)_2$ (Table 3.3), which was consistent with the chemicals' extraction strength. Comparing the extractable Cd with the extractable Zn in each fraction, it was found the proportion of 1M $CaCl_2$-extractable Cd was significantly higher than that of Zn ($p<0.001$). The same results were observed for the other four extractants (Table 3.3). A correlation study on extractable heavy metals with soil properties was carried out showing in proportion, that 0.05 M $Ca(NO_3)_2$ and 0.01 M $Ca(NO_3)_2$ –extractable Cd and Zn have significantly negative correlation with soil pH (Cd: $r= -0.97$ or -0.95 $p<0.001$; Zn: $r= -0.91$ or -0.92, $p<0.01$).

As a proportion of total soil Cd, the fraction of labile Cd (*E*%) ranged between 31 and 58%, with an average of 45%. This was much higher than the labile fraction of Zn, which ranged from 18 to 37% with a mean value of 25% ($p<0.001$) (Table 3.3). The correlation study found that the *E*-values of Cd and Zn were independent of soil properties. Comparing *E*-values with chemical extractions, it was found that the amount of labile Cd is highly correlated with, and quite close to, the 1M $CaCl_2$-extractable Cd (r^2=0.99, p<0.001). The comparison study also found that 0.05M EDTA extracted on average 14% more Cd and 12% more Zn than soil labile Cd and Zn (Table 3.3), while other extractants extracted less Cd and Zn than the labile pools as determined by SID. It was found except for the DTPA extraction, extractable Cd, as well as labile Cd were highly correlated with soil total Cd concentration (Table 3.4). With regard to Zn, only DTPA extractable Zn and labile Zn were highly correlated with soil total Zn concentration (Table 3.5)

3.3 Heavy metal concentrations in two earthworms

During earthworm culturing, there was no mortality found in soils except that in P9, in which all of the worms were dead after four day. The two-way ANOVA analysis showed that overall *E. fetida* accumulated significantly more Cd and Zn than *L. terrestris* from soils (Table 3.6).

The Biota-Soil Accumulation Factor (BSAF) of the metals in the earthworms was calculated by the following formula: BSAF = metal concentation in earthworm/total metal concentration in soil (Cortet et al., 1999). Except in P12a, BSAF values of Cd of the two worms were higher than 1 (Figure 3.2). For Zn accumulation, the BSAF values of the two worms were generally lower than 1 except in Mill-01, which contained lowest total Zn in soil (Figure 3.2). The two-way ANOVA test showed the BSAF values of Cd were significantly higher than that of Zn for both earthworm species (Table 3.7).

3.4 Relationship between soil properties and bioavailable heavy metals

Stepwise linear regression was performed here to model the relationship between accumulated heavy metals and soil properties, including Cd, Zn total concentrations in soil, soil pH, clay content, and TOC. Results showed Cd, Zn total concentration is the most important factor in the metal accumulation by the earthworms ($r^2 > 0.60$, $p < 0.05$), while added soil properties (pH, organic carbon, clay content) did not enhance the models' predictability, except adding TOC value to the regression equation was shown to increase the r values from to 0.72 to 0.89 for Cd accumulation by earthworm *L. terrestris*.

3.5 Evaluation of heavy metals bioavailability using various chemical methods

Pearson’s correlation test was used to find the relationship between the Cd and Zn concentrations in earthworms and the extractable or labile pools in soils. Zinc concentration in *L. terrestris* and *E. fetida* were found to be significantly positively correlated to the labile Zn ($p < 0.01$), and the DTPA extraction ($p < 0.01$) and soil total Zn concentration ($p < 0.01$) (Table 3.8). Cadmium concentrations in *L. terrestris* and *E. fetida* were significantly positively correlated with labile Cd ($p < 0.001$ and $p < 0.01$, respectively) and with 1M $CaCl_2$–extractable Cd ($p < 0.001$ and $p < 0.01$, respectively) (Table 3.8). Cadmium concentration in *L. terrestris* also positively correlated with soil total Cd concentration ($p < 0.01$) (Table 3.8). Moreover, it was found that the two earthworms’ Cd and Zn concentrations had no significant relationship with the 0.05 M $Ca(NO_3)_2$ or the 0.01 M $Ca(NO_3)_2$ ($p > 0.05$) extractable fractions (Table 3.8). Although 0.01 M $CaCl_2$ + 0.1 M triethanolamine (buffered at pH 7.2) was considered to simulate earthworm gut environment and showed linearly related to the Cd concentrations in the earthworm tissues (Oste et al., 2001), it didn't show such strong correlation in this study (Table 3.8).

By logarithmically transforming the metal concentrations in the earthworms and in extractions, a linear regression model was used to determine the significant variables in explaining the variance of metal concentrations in *L. terrestris* and *E. fetida*. (Table 3.9) In the case of Zn, most of the variance in the two earthworms was explained by the labile Zn pool, and total Zn concentration in soil (Table 3.9). With Cd, the variance in *E. fetida* was best explained by the labile Cd pool (78%), while most of the variance in the *L. terrestis* concentration was explained by either the labile Cd pool (91%) or the 1M $CaCl_2$-extractable Cd (89%). Furthermore, when adding soil organic matter content as the second variable in equations containing *E* values, it was found that the predictability (r^2) of the equations was effectively improved (Table 3.9).

4. Discussion

4.1 Chemical extractable Cd and Zn in soils

In our study, the amount of extractable Cd and Zn followed the order: 0.05 M EDTA > 1M $CaCl_2$> 0.005 M DTPA > 0.05 M $Ca(NO_3)_2$ > 0.01 M $Ca(NO_3)_2$ in the ten tested soils. This order was in accordance with Gray et al (2003), where it was found that the magnitude of the Cd fractions decreased in the order: EDTA>$CaCl_2$> $Ca(NO_3)_{2.}$ This order was mainly ascribed to the extractants' extraction strength. EDTA is a strong chelating reagent of metals, which has been reported to extract both labile and non-labile metals, including exchangeable and weakly organically bound fractions (Bermond et al., 1998). We found that EDTA extracted more Cd and Zn than their labile pools by SID, and others have reported similar results (Nakhone and Young, 1993; Gray et al, 2003). Also as a chelating reagent, DTPA can release the soluble, exchangeable, adsorbed and organically bound metals, and possibly some of the metals fixed by oxides (Lindsay and Norvell, 1978). However, its chelating strength is far lower than EDTA in this study because of lower concentration of DTPA utilized here. The 0.05 M $Ca(NO_3)_2$ and 0.01 M $Ca(NO_3)_2$ extractions are generally considered good indicators of soluble metals in soil solution (Young et al., 2000). In our study, 0.05 M $Ca(NO_3)_2$ and 0.01 M $Ca(NO_3)_2$ extracted the smallest portion of soil Cd and Zn compared to other extractants due to their weak extraction capacity, which indicated the soils only contain a small portion of readily soluble Cd and Zn after long-term pollution.

4.2 Labile portion of Cd and Zn in soils

Our study showed that there was proportionally more extractable and labile Cd in the soils than with Zn, indicating that Cd is more chemically reactive than Zn in soil. Alloway (1990) reached a similar conclusion, and found that Cd is more available to plants than many other metals. Morera et al. (2001) reported that Cd tends to be more mobile in soil than Zn because Cd has a lower affinity for soil collides than does Zn, which can be explained by the value of the first hydrolysis constant of the cations: Cd (pK = 10.1) < Zn (pK = 9.0) (Basta and Tabatabai, 1992). We found that the 1M $CaCl_2$ extraction could provide a good estimation of the labile Cd pool in soils, which was consistent with previous studies (Young et al., 2000; Gray et al., 2003). However, there was no such relationship shown between 1M $CaCl_2$ extractable Zn and labile Zn, which may be explained by the fact that Cl^- is a moderately strong complexing agent for Cd, while it has less affinity for Zn^{2+}, making it an inefficient complexing agent for absorbed Zn (Gray et al., 1998 and 2003).

No significant linear relationship was found between *E* values and other soil properties in our study. It has been reported before that the Cd *E*-value was not influenced by soil pH, TOC and clay content (Gray et al., 2003; Kawasaki and Yada, 2008). Gray et al. (2003) suggested that the proportion of labile Cd in soils varies greatly depending on its origin and the physiochemical properties of the soils.

4.3 Chemical methods to assess bioavailability

Pearson's correlation test was used to find the linear relationships between accumulated metals in the earthworms and extractable or labile metal pools. A good correlation indicates that a certain extractable fraction in the soils is available to plants or organisms (Wang et al., 2004). Based on this approach, our study showed that the good chemical methods to predict bioavailability of Cd in soil were the *E*-value estimated from SID and the 1M $CaCl_2$ extraction, as well as soil total Cd concentration (Table 3.8). With regard to Zn, the best chemical methods were *E*-value and the DTPA extraction and total Zn concentration in soil (Table 3.8). Dai et al. (2004) also reported that the DTPA extraction could predict bioavailability of Zn to earthworms, but that is not a good choice for Cd. A possible explanation of the failure of chemical extractions could be related to the lack of specificity, which can dissolve an arbitrary fraction of the soil metals and do not provide an accurate measure of the bioavailable metal pool (Hutchinson et al., 2000).

Adding a small isotope spike soil for *E*-value determination do not alter metal speciation, and a number of studies used this method to assess bioavailability of metals in soil. However, most of them focused on phytoavailability (Gray et al., 2001; Ayoub et al., 2003; Hamon et al., 2004, 2008) and found that *E* values were numerically close to the *L* value, a biological estimate of the labile pool (Smolders et al., 1999), than the chemical extractions (Ayoub et al., 2003). Only a few studies have used isotopic dilution to investigate zooavailability. Scheifler et al (2003) found snail could access "nonavailable" Cd when exposed in a polluted soil spiked with ^{109}Cd. Scott-Fordsmand et al. (2004) assessed the bioavailability of soil Zn to earthworms by spiking soil with ^{65}Zn, and suggested that the *E*-value can accurately reflect the bioavailable pool of Zn in soil.

Generally, there are two main routes for metal uptake by earthworm. The first one is via dermal exposure for absorbing soluble elements in soil solution; the other way is absorbing metals associated soil solids during gut transit and digestion (Weltje, 1998). The result in this study showed that both of the two earthworms' body metal concentration were not correlated with 0.05 M and 0.01 M $Ca(NO_3)_2$ extraction (Table 3.8), which suggested that dermal absorbing of soluble metals was not the primary mode of Cd and Zn uptake by earthworms. The strong correlation between body concentration and the labile metal pools, as well as soil total metal concentration (Table 3.8) suggested that the intestinal pathway is more important.

Regression models were developed based on the metal solid-liquid partitioning in soil (Table 3.9). The predictors selected for the modeling were from the highly correlated variables from the correlation study. The pH in the gut of earthworms is constant and close to 7.0 (Oste et al. 2001). Thus the solution concentration in gut can be expressed as:

$$c(gut) = k \times E \tag{2}$$

At a given pH, high organic matter (OM) or oxide (ox) content can be quantified. The metal's concentration in gut can be showed as follow:

$$c(gut) = k' \times E \times (OM \text{ or } ox)^{k3} \tag{3}$$

So, theoretically,

$$\log Cworm = k1 + k2 \times \log E + k3 \times \log(OM \text{ or } ox). \tag{4}$$

In this study, it was showed the predictability (r^2) of the model could be improved by including both *E* value and organic carbon content (Table 3.9), which again indicates the gut uptake indeed prevails. In addition, the r^2 value for models containing *E* are higher than the equations containing other variables, which indicates *E* value is the best predictor to assess metals' availability to earthworms.

Researchers have also shown the important role of the gut uptake route other than dermal contact with soil solution (Morgan et al., 2004), and found that 66% of the Cd body concentration of *P. minuta* can be explained by using soluble-exchangeable, organic- and oxide-bound Cd concentrations fractions in the tested four soils (Nursita et al., 2009). Oste et al. (2001) found that uptake of Cd by earthworms is independent of soil pH, and concluded that intestinal uptake prevailed. However, other studies have presented the opposite view that dermal uptake is predominantly via the soil solution, thus the soluble metals best describe the bioaccumulation of heavy metals in earthworms (Vijver et al., 2003; Hobbelen et al., 2006). The differences between these studies may be due to differences in earthworm species, in soil properties, and/or in culturing conditions.

4.4 Differences of metal accumulation by the two earthworms

BSAF values represent earthworms' accumulation of metals (Cd, Zn) in soils, thus it indicates bioavailability of Cd and Zn to earthworms. Although Cd^{2+} and Zn^{2+} are both divalent metal ions, their bioavailability was significantly different to the earthworms. The BSAFs of Cd in the two earthworm species were much higher than their BSAFs of Zn in most soils (Figure 3.2), which indicated that Cd was more bioavailable than Zn to the earthworms. Dai et al. (2004) reported the same observation, determining that the BSAFs of four metals were ranked as Cd>Zn>Cu>Pb in earthworms. The accumulation of heavy metals in earthworms is the result of the balance between uptake and excretion processes. Taking the uptake process into account first, the fraction of labile Cd and extractable Cd in soils were much higher than Zn in the same pool, which showed that Cd was more chemically reactive in soils and therefore, more available to earthworms than Zn, leading to greater accumulation of Cd^{2+} by the earthworms. It has also been suggested that the different accumulation patterns in the earthworm species were ascribed to the differences in essential metal (Zn) versus non-essential element (Cd) intake and physiological utilization (Kizilkaya, 2005). Considering the earthworm's ability to eliminate excess metals, Spurgeon and Hopkin (1999) showed that for essential metals (e.g. Zn), a fast initial uptake was followed by equilibrium after a few days of exposure, highlighting a physiological control and a possible excretion of these elements. For nonessential elements, such as Cd, the excretion was slow or absent (Dai et al., 2004). Thus, on the one hand, there was more bioavailable Cd present in the soil, but the other, the slower excretions rates of Cd by earthworm was found which could explain the higher

BSAF of Cd in the earthworms.

Earthworms are well known to be selective consumers (Edwards and Bohlen, 1996) and are divided into three categories. Researchers investigated different accumulation capacity of metals between endogiec and epigeic species (Hobbelen et al., 2006; Suthara et al., 2008) and it was found that epigeic earthworm *L. rubellus* had generally lower concentrations of Cd and Zn than did *A. caliginosa*, an endogiec earthworm (Morgan and Morgan, 1999; Dai et al., 2004). Dai et al. (2004) confirmed such observation and found that an endogiec species *A. caliginosa* had higher BSAFs of Cd, Pb and Zn, while there was no difference in the BSAF for Cu and Fe between the two species. Our study used two species of earthworm belonging to the anecic and epigeic groups. It was found that *E. fetida*, an epigeic species accumulated more metals than *L. terristris*, an anecic species in most soils. The possible reasons for the observation include: 1) more soluble heavy metals could be absorbed by *E. fetida* due to its larger specific dermal surface area than *L. territri*s 2) as a smaller sized earthworm, *E. fetida*'s narrow gut could make sufficient contact possible between soil solid particles thus benefits greater gut uptake. 3) it is possible that *E. fetida*'s has a lower gut pH to favor Cd assimilation.

5. Conclusions

Our study demonstrated that there were significant correlations between metal (Zn, Cd) concentrations in two earthworms (*L. territri*s, *E. fetida*) and the elements' labile fractions *(E*-value*)* in 10 contaminated soils. This implied that heavy metal labile pools

(*E*-value), derived from a stable isotope dilution technique, could be considered the most accurate chemical method to assess bioavailability of Cd, Zn in soils. Moreover, the correlation results suggested that dermal absorption is not the major mode for Cd and Zn uptake by the earthworms. Instead, absorbing available Cd, Zn from soil solids during gut transit may play the more important role. Secondly, both of the earthworm species accumulated more Cd than Zn from the soils. Possible reasons include the fact that Cd is more reactive in soil, as indicated by the greater proportion that was extractable or isotopically labile. Moreover, it has been suggested that earthworms could homeostatically adjust their accumulation of the essential element (Zn), while they cannot control their accumulation of the nonessential element Cd. Finally, it was shown that *E. fetida* accumulated more Cd and Zn than *L. terrestris*, showing that heavy metal uptake was not only element-specific, but also species-specific.

References

Ahnstrom, Z.A.S., Parker, D.R., 1999. Development and assessment of a sequential extraction procedure for the fractionation of soil cadmium. Soil Science Society of America Journal 63, 1650 – 1658.

Ahnstrom, Z.A.S., Parker, D.R., 2001. Cadmium reactivity in metal-contaminated soils using a coupled stable isotope dilution-sequential extraction procedure. Environmental Science and Technology 35, 121-126.

Alloway, B.J., 1990. Cadmium. In: Alloway, B.J., (Ed.), Heavy metals in soils. Blackie, Glasgow, pp. 100-124.

Anderson, J.M., Ingram, J.S. 1993. Tropical Soil Biology and Fertility: A Handbook of Methods. 2nd ed. CAB International, Wallingford, UK. pp. 93-94.

ASTM, 1999. Standard guide for conducting laboratory soil toxicity or bioaccumulation tests with Lumbricid earthworm Eisenia fetida, standard E 1676-97. In: Annual Book of ASTM Standards, pp. 1056 – 1074.

Ayoub, A.S., McGaw, B. A., Shand, C. A., Midwood, A.J., 2003. Phytoavailability of Cd and Zn in soil estimated by stable isotope exchange and chemical extraction. Plant and Soil 252, 91-300.

Basta, N. T., Tabatabai, M. A., 1992. Effect of cropping systems on adsorption of metals by soils. II Effect of pH. Soil Science 153, 195-204.

Becker, J.S., Dietze, H.J. 2000. Inorganic mass spectrometric methods for trace, ultratrace, isotope, and surface analysis. Fresenius Journal of Analytical Chemistry 2000, 368, 23-30.

Bermond, A., Yousfi, I., Ghestem, J.P., 1998. Kinetic approach to the chemical speciation of trace metals in soils. Analyst 123, 785 – 789.

Beyer, W.N., Chanery, R.L., Mulhern, B.M. 1982. Heavy metal concentrations in earthworms from soil amended with sewage sludge. Journal of Environmental Quality 11, 381-385.

Cortet, J., Vauflery, A.G.D., Balaguer, N.P., Gomot, L., Texier, Ch., Cluzeau, D. 1999. The use of invertebrate soils fauna in monitoring pollutant effects. European Journal of Soil Biology 35, 115-134.

Dai, J., Becquer, T., Rouiller, J.H., Reversat, G., Bernhard-Reversat, F., Nahmani, J., Lavelle, P. 2004. Heavy metal accumulation by two earthworm species and its relationship to total and DTPA-extractable metals in soils. Soil Biology and Biochemistry 36, 91-98.

Degryse, F., Waegeneers, N., Smolders, E. 2007. Labile lead in polluted soils measured by stable isotope dilution. European Journal of Soil Science 58, 1-7.

Echevarria, G.., Morel, J. L., Fardeau, J. C., Leclerc-Cessac, E. 1998. Assessment of phytoavailability of nickel in soils. Journal of Environmental Quality 27, 1064-1070.

Edwards, C.A., Bohlen, P.J. 1996. Biology and Ecology of earthworms, third ed., Chapman & Hall, London.

Fang, J., Wen, B., Shan, X.Q., Wang, H.H., Lin, J.M., Zhang, S.Z. 2007. Evaluation of bioavailability of light rare earth elements to wheat (Triticum aestivum L.) under field conditions. Geoderma 141, 53-59.

Gabler, H.E., Bahr, A., Mieke, B. 1999. Determination of the interchangeable heavy metal fraction in soils by isotope dilution mass spectrometry. Fresenius Journal of Analytical Chemistry 365, 409-414.

Gleyzes, C., Tellier, S., Astruc, M. 2002. Fractionation studies of trace elements in contaminated soils and sediments, a review of sequential extraction procedures. Trends in Analytical Chemistry 21, 451-467.

Gray, C.W., McLaren, R.G., Roberts, A.H.C., Condron, L.M. 1998. Sorption and desorption of cadmium from some New Zealand soils: effect of pH and contact time. Australian Journal of Soil Research 36, 199-216.

Gray, C.W., Mclaren, R.G., Roberts, A.H.C. 2001. Cadmium phytoavailability in a New Zealand pastoral soil. In 'Workshop on Chemical bioavailaility in the terrestrial environment Abstracts'. Adelaide, Australia, pp. 85-86.

Gray, C.W., McLaren, R.G., Shiowatana, J. 2003. The determination of labile cadmium in some biosolids-amended soils by isotope dilution plasma mass spectrometry. Australian Journal of Soil Research 41, 589-597.

Hamon, R.E., Lombi, E., Fortunati, P., Nolan, A.L., McLaughlin, M.J., 2004. Coupling Speciation and Isotope Dilution Techniques to study Arsenic mobilization in the Environment. Environmental Science and Technology 38, 1794-1798.

Hamon, R. E., Parker, D. R., Lombi, E. 2008. Advances in isotopic dilution techniques in trace element research: A review of methodologies, benefits and limitations. Advances in

Agronomy 99:289-343.

Hobbelen, P.H.F., Koolhaas, J.E., van Gestel, C.A.M., 2006. Bioaccumulation of heavy metals in the earthworms Lumbricus rubellus and Aporrectodea caliginosa in relatin to total and available metal concentrations in field soils. Environmental Pollution 144, 639-646.

Hutchinson, J. J., Young, S. D., McGrath, S.P., West, H. M., Black, C. R., Baker, A.J.M., 2000. Determining uptake of “non-labile” cadmium by Thlaspi caerulescens using isotopic dilution technique. New Phytologist 146, 453-460.

Kato, N., Zapata, F., Fardeau, J.C. 1995. The ability of chemical-extraction methods to estimate plant-available soil P and a better understanding of P availability of fertilized andosols by using isotopic methods. Soil Science and Plant Nutrition 41, 781-789.

Kawasaki, A., Yada, S., 2008. Determination of labile cadmium in agricultural soils by isotope dilution plasma mass spectrometry and a coprecipitation separation technique, Journal of Nuclear Science and Technology Supplement 5, 138-142.

Kennette, D., Hendershot, W., Tomlin, A., Sauve, S. 2002. Uptake of trace metals by earthworm *Lumbricus terrestris* in urban contaminated soils. Applied Soil Ecology 19,191–198.

Kizilkaya, R. 2005. The role of different organic wastes on zinc bioaccumulation by earthworm Lumbricus terrestris L. (Oligochaeta) in successive Zn added soil. Ecological Engineering 25, 322-331

Lindsay, W.L., Norvell, W.A. 1978. Equilibrium relationships of Zn^{2+}, Fe^{2+}, Ca^{2+} and H^{+} with EDTA and DTPA in soils. Proceedings - the Soil Science Society of America 33, 62-68.

Lombi, E., Hamon, R. E., McGrath, S. P., McLaughlin, M. J. 2003. Lability of Cd, Cu, and Zn in polluted soils treated with lime, beringite, and red mud and identification of a non-labile colloidal fraction of metals using isotopic techniques. Environmental Science and Technology 37, 979-984.

Morel, J.L. 1997. Bioavailability of trace elements to terrestrial plants. In: Tarradellas, J., Bitton, G., Rossel, D. (Eds.), Soil Ecotoxicology. Lewis, Boca Raton, pp. 141-178.

Morera, M.T., Echeverria, J.C., Mazkiaran, C., Garrido, J.J. 2001. Isotherms and sequential extraction procedures for evaluating sorption and distribution of heavy metals in soils. Environmental Pollution 113, 135-144.

Morgan, J.E., Morgan, A.J. 1992. Heavy metal concentrations in the tissues, ingesta and faeces of ecophysiologically different earthworm species. Soil Biology and Biochemistry 12, 1691 – 1697.

Morgan, J.E., Morgan, A.J. 1999. The accumulation of metals (Cd, Cu, Pb, Zn and Ca) by two ecologically contrasting earthworm species (Lumbricus rubellus and Aporrectodea caliginosa): implications for ecotoxicological testing. Applied Soil Ecology 13, 9-20.

Morgan, A.J., Stürzenbaum, S.R., Winters, C., Grime, G.W., Aziz, N.A.A., Kille, P. 2004. Differential metallothionein expression in earthworm (Lumbricus rubellus) tissues. Ecotoxicology and Environmental Safety 57, 11 – 19.

Nakhone, L.N., Young, S.D. 1993. The significance of (radio-) labile cadmium pools in soil. Environmental Pollution 82, 73-77.

Nelson, D.W., Sommers, L.E. 1982. Total carbon, organic carbon, and organic matter. In: Methods of Soil Analysis. 2nd Ed. ASA Monogr. 9(2). Amer. Soc. Agron. Madison, WI, pp. 539-579.

Nursita, A. I., Singh, B., Lees, E. 2009. Cadmium bioaccumulation in Proisotoma minuta in relation to bioavailability in soils. Ecotoxicology and Environmental Safety 72, 1767-1773.

Oste, L.A., Dolfing, J., Ma, W., Lexmond, T.M. 2001. Cadmium uptake by earthworms as related to the availability in the soil and the intestine. Environmental Toxicology and Chemistry. 20, 1785-1791.

Scheifler, R., Schwartz, C., Echevarria, G., Vaufleury, A.D., Badot, P.M., Morel, J.L. 2003. "Nonavailable" soil cadmium is bioavailable to snails: Evidence from isotopic dilution experiments. Environmental Science and Technology 37, 81-86.

Scott-Fordsmand, J.J., Stevens, D., Mclaughlin, M. 2004. Does earthworms mobilize fixed zinc from ingested soil? Environmental Science and Technology 38, 3036-3039.

Sheppard, S.C., Gaudet, C., Sheppard, M.I., Cureton, P.M., Wong, M.P., 1992. The development of assessment and remediation guidelines for contaminated soils, a review of the science. Canadian Journal of Soil Science 72, 359-394.

Sims, R.W., Gerard, B.M. 1999. Earthworms. Notes for the identification of British species. Field Studies Council, Shrewsbury, pp. 169.

Smolders, E., Brans, K., Foldi, A., Merckx, R. 1999. Cadmium Fixation in Soils Measured by Isotopic Dilution. Soil Science Society of America Journal 63, 78-85.

Spurgeon, D.J., Hopkin, S.P., 1999. Comparisons of metal accumulation and excretion kinetics in earthworms (Eisenia fetida) exposed to contaminated field and laboratory soils. Applied Soil Ecology 11, 227-243.

Suthara, S., Singhb, S., Dhawanc, Sachin. 2008. Earthworms as bioindicator of metals (Zn, Fe, Mn, Cu, Pb and Cd) in soils: Is metal bioaccumulation affected by their ecological category? Ecological Engineering 32, 99-107.

Tack, F.M.G., Verloo, M.G. 1995. Chemical speciation and fractionation in soil and sediment heavy metal analysis: a review. International Journal of Environmental Analytical Chemistry 59, 225-238.

Tye, A. M., Young, S. D., Crout, N. M. J., Zhang, H., Preston, S., Bailey, E. H., Davison, W., McGrath, S. P., Paton, G. I., Kilham, K. 2002. Predicting arsenic solubility in contaminated soils using isotopic dilution techniques. Environmental Science and. Technology 36, 982-988.

US EPA. 2007. Method 3051: Microwave assisted acid digestion of sediments, sludges, soils and oils. Revision 1. Washington, DC, USA.

Vijver, M.G., Vink, J.P.M., Miermans, C.J.H., Van Gestel, C.A.M. 2003. Oral sealing using glue: a new method to distinguish between intestinal and dermal uptake of metals in earthworm. Soil Biology and Biochemistry 35, 125-132.

Wang, X.P., Shan, X.Q., Zhang, S.Z.,Wen, B. 2004. A model for evaluation of the phytoavailability of trace elements to vegetables under the field conditions. Chemosphere 55, 811－822.

Wear, J.I., Evans, C.E. 1968. Relationship f zinc uptake by corn and sorghum to soil zinc measured by three extractants. Soil Science Society of America Proceedings, 32, 543-546.

Weltje, L. 1998. Mixture toxicity and tissue interactions of Cd, Cu, Pb and Zn in earthworms (Oligochaeta) in laboratory and field soils: a critical evaluation of data. Chemosphere 36, 2643-2660.

Young, S. D., Tye, A., Carstensen, A., Resende, L., Crout, N. 2000. Methods for determining labile cadmium and zinc in soil. European Journal of Soil Science 51, 129-136.

Tables and Figures

Table 3.1 Soil properties

Soil	Classification	Total N (%)	TOC (%)	Clay (%)	pH	Total Zn (mg kg^{-1})	Total Cd (mg kg^{-1})	*E*-Zn (mg kg^{-1})	*E*-Cd (mg kg^{-1})
P1	Loam	0.33	5.04	14.5	5.98	4600	28.8	826	8.99
P4	Loam	0.48	5.56	26.7	5.86	1165	18.2	257	9.19
P6	Clay loam	0.19	2.39	33.9	5.60	3079	62.4	1022	35.9
P7	Loam	0.17	2.62	23.9	5.67	739	10.7	182	5.16
P9	Loam	0.36	7.34	10.7	6.03	19589	220	7234	99.3
P12a	Silt loam	0.7	13.1	13.3	3.78	4088	71.2	985	40.5
P12b	Loam	0.07	2.04	27.3	3.96	506	15.8	125	6.14
Mill-01	Clay	0.27	1.73	40.7	5.34	129	18.9	*	8.83
T15	Sandy loam	0.18	2.58	8.48	7.06	579	11.1	138	4.49
Romona	Sandy loam	0.62	8.08	2.88	6.10	1393	31.2	289	10.6
Range min		0.07	1.73	2.88	3.78	129	10.7	125	4.49
max		0.62	13.1	40.7	7.06	19589	220	7234	99.3

Table 3.2
Extraction Procedures and target fractions

Extractant	Procedure	Fraction	Reference
1M $CaCl_2$	4.00 g soil was mixted with 20 ml 1mol l^{-1} $CaCl_2$ in a 50 ml plastic centrifuge tube, and shake for 24 h	Exchangeable	Gray et al. (2003)
0.05M $Ca(NO_3)_2$	2.00 g soil was extracted with 20 ml 0.05 M $Ca(NO_3)_2$ in a 50 ml plastic centrifuge tube, and shake for 24 h	Soluble and easily exchangeable pool	Gray et al. (2003)
0.01M $Ca(NO_3)_2$	2.00 g soil was extracted with 20 ml 0.01 M $Ca(NO_3)_2$ in a 50 ml plastic centrifuge tube, and shake for 24 h	Soluble, mimic to soil solution	Gray et al. (2003)
0.05 M EDTA	2.00 g soil was mixed with 20 ml 0.05 mol l^{-1} EDTA and its pH was adjusted with ammonia solution to 7.0 in a 50 ml plastic centrifuge tube, and the suspension was shaken for 1 h	Organically bound and metals occluded in oxides and secondary clay minerals in part	Wear and Evans (1968)
0.005M DTPA	10.0 g soil was mixed with 20 ml 0.005 mol l^{-1} DTPA+0.01 mol l^{-1} $CaCl_2$ + 0.01 mol l^{-1} TEA, pH of suspension was adjusted to pH 7.3 in a 50 ml plastic centrifuge tube, and shaken for 2 h	Iron and manganese oxides bound	Lindsay and Norvell (1978)
0.01 M $CaCl_2$ + TEA	2.00 g soil was extracted with 20 ml 0.01 M $Ca(NO_3)_2$ + 0.1 M TEA (pH adjusted to 7.2) in a 50 ml plastic centrifuge tube, and shake for 24 h	Simulated to earthworm gut environment	Oste et al. (2001)

Table 3.3 Comparison of fraction of extractable and labile Cd with Zn

Element		Fraction of extractable Cd and Zn (%)					
		1M $CaCl_2$	0.05 M $Ca(NO_3)_2$	0.01 M $Ca(NO_3)_2$	0.05 M EDTA	0.005 M DTPA	Stable isotope
Cd	Range	38-71	2.5-51	0.8-38	47-76	3.8-58	31-58
	Mean	49.8	17.1	9.5	59.10	23.8	44.7
Zn	Range	0.2-53	0.33-22.5	0.14-20	5.1-66	1.7-30	18-37
	Mean	18.5	6.8	4.8	38.2	11.8	25.4
Paired T-test (Cd>Zn)	p	<0.001	0.010	0.05	0.005	0.048	<0.001

Table 3.4 Correlation of extractable Cd and total Cd concentration in soils

Cd	0.05M $Ca(NO_3)_2$	0.01M $Ca(NO_3)_2$	0.05 M EDTA	0.005 M DTPA	0.01 M $CaCl_2$ + TEA	Stable isotope	Total
1 M $CaCl_2$	0.86^{**}	0.82^{**}	0.98^{**}	0.002	0.87^{**}	0.99^{**}	0.99^{**}
0.05 M $Ca(NO_3)_2$		0.99^{**}	0.88^{**}	0.16	0.99^{**}	0.89^{**}	0.85^{**}
0.01 M $Ca(NO_3)_2$			0.85^{**}	0.18	0.98^{**}	0.85^{**}	0.82^{**}
0.05 M EDTA				0.09	0.90^{**}	0.96^{**}	0.98^{**}
0.005 M DTPA					0.12	0.03	-0.008
0.01 M $CaCl_2$+ TEA						0.90^{**}	0.87^{**}
Stable Isotope							0.98^{**}

Table 3.5 Correlation of extractable Zn and total Zn concentration in soils

Zn	0.05M $Ca(NO_3)_2$	0.01 M $Ca(NO_3)_2$	0.05 M EDTA	0.005 M DTPA	0.01 M $CaCl_2$ + TEA	Stable isotope	Total
1 M $CaCl_2$	0.76^{*}	0.71^{*}	0.67^{*}	0.87^{**}	0.53	0.83^{*}	0.56
0.05 M $Ca(NO_3)_2$		0.99^{**}	0.74^{*}	0.57	0.94^{**}	0.74^{*}	0.58
0.01 M $Ca(NO_3)_2$			0.71^{*}	0.50	0.96^{**}	0.70^{*}	0.55
0.05 M EDTA				0.66	0.69^{*}	0.74^{*}	0.66
0.005 M DTPA					0.38	0.88^{**}	0.73^{*}
0.01 M $CaCl_2$ + TEA						0.63	0.58
Stable Isotope							0.93^{**}

Table 3.6 ANOVA test for comparing accumulation of metals between the two earthworms.

	Source	DF	SS	MS	F	P
C_{Cd} versus Earthworm, Soil	earthworm	1	1127.7	1127.69	43.54	0.000
	Soil	8	24287.3	3035.91	117.23	0.000
	Interaction	8	656.4	82.05	3.17	0.008
	Error	36	932.3	25.90		
	Total	53	27003.7			
	R-Sq = 96.55%	R-Sq(adj) = 94.92%				
C_{Zn} versus Earthworm, Soil	Source	DF	SS	MS	F	P
	earthworm	1	589693	589693	26.49	0.000
	Soil	8	5843197	730400	32.81	0.000
	Interaction	8	251298	31412	1.41	0.225
	Error	36	801386	22261		
	Total	53	7485575			
	R-Sq = 89.29%	R-Sq(adj) = 84.24%				

Table 3.7 Two-way ANOVA test for comparing BSAF values between Cd and Zn in each earthworm.

	Source	DF	SS	MS	F	P
$BSAF_{E.fetida}$ versus Element, Soil	Element	1	19.8138	19.8138	736.37	0.000
	Soil	8	12.0357	1.5045	55.91	0.000
	Interaction	8	4.7357	0.5920	22.00	0.000
	Error	36	0.9687	0.0269		
	Total	53	37.5539			
	R-Sq = 97.42%	R-Sq(adj) = 96.20%				
$BSAF_{L.terrestris}$ versus Element, Soil	Source	DF	SS	MS	F	P
	Element	1	4.1279	4.12787	191.06	0.000
	Soil	8	6.5234	0.81542	37.74	0.000
	Interaction	8	2.7807	0.34759	16.09	0.000
	Error	36	0.7778	0.02161		
	Total	53	14.2097			
	R-Sq = 94.53%	R-Sq(adj) = 91.94%				

Table 3.8 Correlation analysis between Cd/Zn concentration in earthworms and the available Cd/Zn pools in soils

	Earthworm	Pearson's correlation coefficient							
		Total Digestion	1M $CaCl_2$	0.05 M $Ca(NO_3)_2$	0.01 M $Ca(NO_3)_2$	0.05 M EDTA	0.005 M DTPA	0.01 M $CaCl_2$ with TEA	Stable isotope
Cd	*L. terristris*	0.85*	0.90**	0.67^{ns}	0.58^{ns}	0.79	-0.21^{ns}	0.63^{ns}	0.87**
	E. fetida	0.78	0.83*	0.63^{ns}	0.58^{ns}	0.72	-0.3^{ns}	0.63^{ns}	0.84*
Zn	*L. terristris*	0.85*	0.76	0.5^{ns}	0.44^{ns}	0.76	0.89**	0.34^{ns}	0.90*
	E. fetida	0.87*	0.69	0.52^{ns}	0.47^{ns}	0.82*	0.84*	0.43^{ns}	0.89*

*: $p<0.01$; **: $p<0.001$; ns: $p>0.05$

Table 3.9 modeling of Cd, Zn concentrations in earthworms and their available pools in soils

Metal	Earthworm	Model obtained	R^2	*p*
Cd	*L. terrestris*	log Cworm= 0.82 + 0.64×log *E*	0.91	0.001
		log Cworm= 0.86 + 0.73×log *E* - 0.20×log*OM*	0.96	0.001
		log Cworm= 0.53 + 0.69×log *Total*	0.80	0.001
		log Cworm= 0.74 + 0.71×log $CaCl_2$	0.89	0.001
	E. fetida	log Cworm= 1.05 + 0.54×log *E*	0.78	0.001
		log Cworm= 1.08 + 0.62×log *E*- 0.18×log*OM*	0.84	0.004
		log Cworm= 0.84 + 0.55×log *Total*	0.63	0.01
		log Cworm= 1.01 + 0.57×log $CaCl_2$	0.70	0.005
Zn	*L. terrestris*	log Cworm= 1.79 + 0.44×log *E*	0.87	0.001
		log Cworm= 1.78 + 0.46×log *E* - 0.03×log*OM*	0.87	0.006
		log Cworm= 1.53 + 0.44×log *Total*	0.84	0.001
		log Cworm= 1.80 + 0.50×log *DTPA*	0.57	0.03
	E. fetida	log Cworm= 1.58 + 0.48×log *E*	0.86	0.001
		log Cworm= 1.62 + 0.43×log *E* - 0.13×log*OM*	0.89	0.005
		log Cworm= 1.26 + 0.48×log *Total*	0.85	0.001
		log Cworm= 1.54 + 0.55×log *DTPA*	0.60	0.024

Figure 3.1 Concentration of metals (mg kg^{-1}) in earthworms cultured in the soils. filled column: *L. terrestris*, open column: *E. fetida*; bars represent standard deviation with n=3.

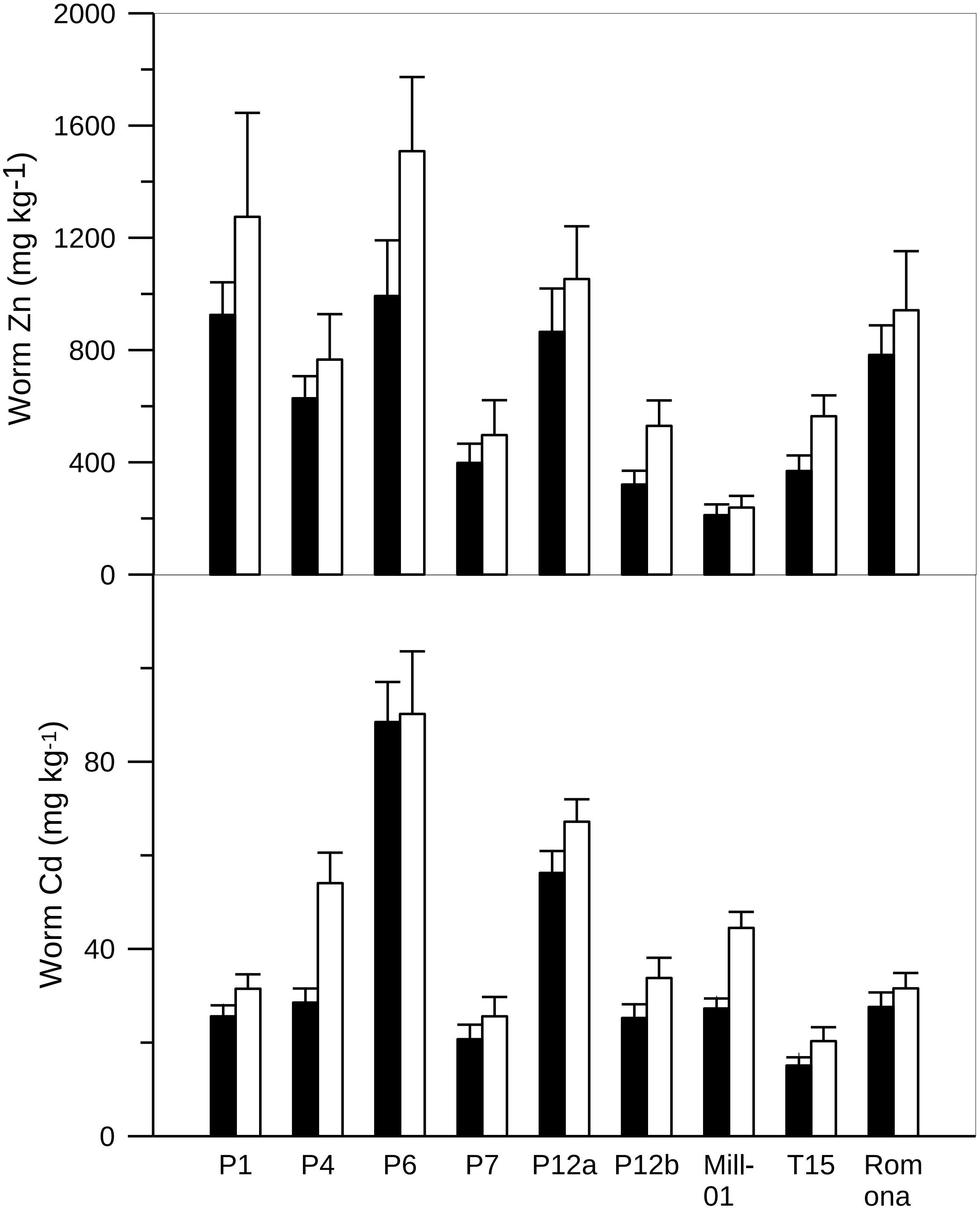
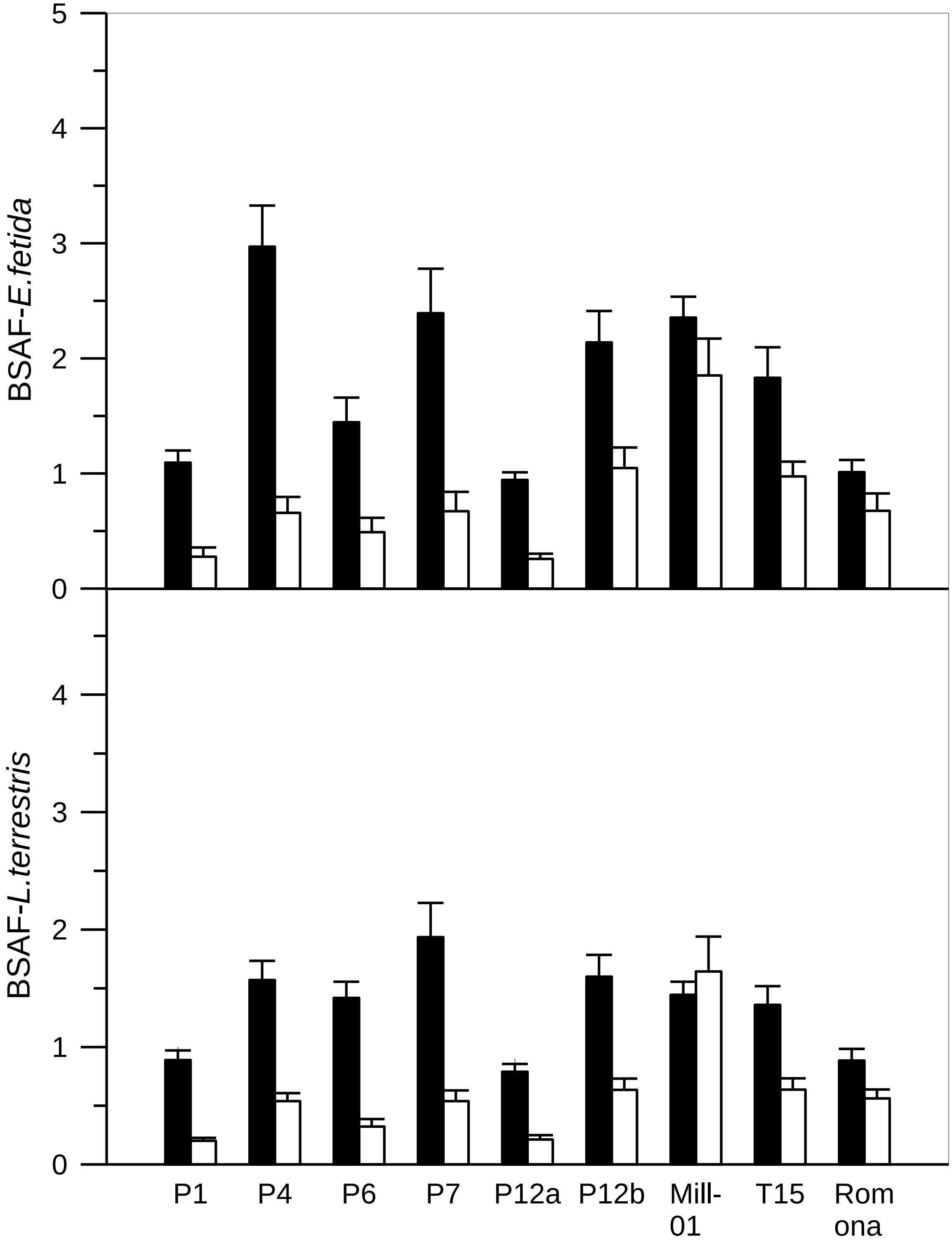


Figure 3.2. BSAF (Metal concentration in earthworm/metal concentration in soils) of metals. filled column: Cd, open column: Zn; bars represent standard deviation with n=3.



Chapter 4

Metal uptake and elimination kinetics in two earthworm species exposed to soils with cadmium and zinc

Abstract

The uptake and elimination kinetics of zinc (Zn) and cadmium (Cd) were investigated in two physiological contrasting earthworm species (*Eisenia fetida* and *Lumbrius terrestris*) reared in three field-contaminated soils. Earthworms were sampled from the soils at 1, 2, 4, 7, 10, 15, 20, 25, 30 days as an uptake experiment. Then, pre-loaded earthworms were transferred to clean soil and incubated for another 30 days to allow for metal elimination. Uptake and elimination rates were calculated using a first-order, one-compartment, toxico-kinetic model. Pronounced differences were found on the earthworms' uptake and elimination kinetics between Zn and Cd ($p<0.01$). For the essential element Zn, rapid initial uptake rates ranged from 40 to 140 mg Zn kg^{-1} worm day^{-1} for *L. terrestris,* and from 55 to 202 mg Zn kg^{-1} worm day^{-1} for *E. fetida*. Steady-state body burdens were observed after only 4-7 days of exposure and fast elimination rates were observed. Compared to Zn, slow and nearly linear uptake was observed in Cd uptake, the rates of 1.2 to 1.6 mg Cd kg^{-1} worm day^{-1} were observed with *L. terrestris,* and higher values of 1.3 to 2.6 mg Cd kg^{-1} worm day^{-1} for *E. fetida*. An equilibrium plateau was not reached in the first 25 days, and very slow elimination was observed. In addition, it was found that more than 90% of the accumulated Zn could be eliminated by earthworms after transfer to the clean soil, while less than 70% of internal Cd was eliminated. The highest accumulation rates of heavy metals were for worms exposed to the most contaminated soil P4, while for elimination rates there were no significant differences among the three soils ($p>0.05$). The results showed the kinetic rates are both metal- and earthworm species-specific, which would need to be considered when predicting steady-state tissue metal concentrations when using kinetic models.

Key words: uptake, elimination kinetic, accumulation, Cd, Zn, earthworm

1. Introduction

It is generally recognized that heavy metals' bioavailability, rather than its total concentration in soil, is the most critical measurement when assessing its potential for ecotoxicological effects and environmental risk. The bioavailability of these contaminants in the environment is a complex issue and it has been suggested that bioavailability should be viewed more as a dynamic process (Peijnenburg et al., 1997). That is, the rate of uptake, not the internal concentration in exposed organisms, is a better measure of bioavailability, potential metal toxicity, (Rainbow, 2002; van Straalen et al., 2005), and overall ecotoxicological effects (Walker et al., 1996).

Although the accumulation of trace elements from soil to biota has been studied extensively for many species in various soils (Nahmani et al., 2007), less attention has been given to uptake and elimination kinetics (Spurgeon and Hopkin, 1999; Heikens et al., 2001; Hendriks and Heikens, 2001). Limited research has indicated that the uptake rates of trace metals in invertebrates are generally dependent on the metal species and concentrations, the soil characteristics, and the biological target (Janssen et al., 1997; Peijnenburg et al., 1999; Spurgeon and Hopkin, 1999). It has been reported that many soil organisms are able to sequester metals to various biological compartments following uptake (Rainbow, 2002). The involved sequestration appear to differ in affinity for essential versus non-essential metals, and this might explain the pronounced differences observed in the uptake and elimination kinetics for essential (Zn, Cu) versus non-essential elements (Cd, Pb) (Vijver et al., 2005). However, more studies are needed to confirm the conclusion because only one earthworm species *L. rubellus* was used in that study.

In order to estimate the metal uptake and elimination kinetics in an organism, the organism's internal metal concentration is measured as a function of time after exposure to a contaminated soil and again after transfer to a non-contaminated control soil. Toxicokinetic models are then fit to the collected time-series data in order to get the uptake and elimination rate (Moriarty and Walker, 1987). A one-compartment, first-order kinetic model has often been used, and was shown to successfully obtain steady-state tissue metal concentrations and thus predict possible toxic effects (van Straalen et al., 2005).

Earthworms are usually selected for toxicokinetic studies because they are ubiquitous animals in soil which have a high tolerance capacity for metal pollution (Díez-Ortiz et al., 2010), and typically have a high capacity to take up metals (Nahmani et al., 2007). Earthworms are crucial for soil ecosystems because they are actively involved in organic matter incorporation and decomposition, excavation of burrows, and production of casts (Lee, 1985; Edwards and Bohlen, 1996). Generally earthworm can be divided into three categories according to their morphological and behavioral characteristics (Sims and Gerard, 1999): litter dwelling (epigeic), horizontally burrowing mineral-soil feeders (endogeic) and vertically burrowing surface feeders (anecic). Research found that bioaccumulation of heavy metals by earthworms is metal-specific (Marinussen et al., 1997), as well as earthworm species-specific (Morgan and Morgan, 1999; Hobbelen et al., 2006; Suthara et al., 2008). However, the difference in metal uptake and elimination kinetics between earthworm species, such as *E. fetida* (epigeic), a standardized earthworm for toxicity testing (ASTM, 1999), and *L. terrestris*, the representative of anecic species, is still unknown.

In this paper, our objective is to study the uptake and elimination kinetics of Cd and Zn for two physiologically distinct species of earthworms: *E. fetida* and *L. terrestris* exposed to three field-contaminated soils for 30 days of metal uptake, then another 30 days for elimination. It may provide supplementary information on metals' availability to soil invertebrates. The data were then fitted to a one-compartment, first-order model for calculating toxicokinetic parameters (uptake and elimination rates). The kinetic rates between the two worms, between the two metals and among the three soils were compared.

2 Materials and Methods

2.1 Soil sample collection and preparation

Three field soils were used here to culture earthworms. Two Palmerton soils (identified as P4 and P7) were collected from a Zn-smelter emission area in Palmerton, PA. The third soil (identified as Mill01) was from Pt. Dume, Los Angeles County, CA, which is derived from Cd-rich shale and considered a naturally enriched Cd source. The sampled area were about 1 m^2 each. At each area, three soil samples were collected from the upper layer (15cm) of the field soil and thoroughly mixed together. The soil samples were sealed separately and transferred to the lab. After thorough air-drying at ambient temperature, each soil was ground and sieved to <2mm.

After sieving, a subsample of each soil was taken for soil characteristics. Accurately weighed crushed soils (<0.2mm) were placed in a C/N/S combustion furnace (Carlo Erba NA 1500) for total N analysis. Organic C was determined by the Walkley-Black procedure (Nelson and Sommers, 1982). Soil texture was measured by the hydrometer (Bouyoucous) Method (Anderson and Ingram, 1993). Soil pH was measured in de-ionized water-soil suspension at a ratio of 1:1. The chemical and physical properties for each soil are listed in Table 4.1.

2.2 Earthworm Culturing

Earthworms used for the experiment were *Eisenia fetida* and *Lumbrius terrestris*, which were purchased from Timeline Fishery Company (IL, USA). Earthworms were initially kept in an uncontaminated soil (designated as T1) collected from California and fed with ped (contained in the shipping package from the supplier) for two weeks at 20℃ prior to the beginning of the uptake experiment. The mature earthworms (100-300 mg fresh weight of *E. fetida*, 1.5-2.5 g fresh weight of *L. terrestris*) were then transferred to the three metal-rich soils. Each of them had three replicates with 3000 g of soil hosting thirty of *L. terrestris* and eighty of *E. fetida*, which were kept in a large controlled-environment chamber (temperature = 20℃; 16h/8h light/dark cycle, and relative humidity =65%). Sufficient distilled water was added to each soil container to maintain the soil moisture content at approximately 60% of field capacity, and left to stabilize for a week at 20 ℃ under 65% relative air humidity before worms were introduced.

2.3 Exposure experiment for uptake and elimination

The uptake and elimination kinetics of the two metals were assessed in time series studies. The two species of earthworms were exposed to the three soils (P4, P7, Mill01) for 30 days for uptake and then another 30 days in clean soil T1 for elimination. During this procedure, 0.5 g (dry weight) peat per *L. terrestris* and 0.15g per *E. fetida* per week was added to each replicate pot. This quantity of food is sufficient to maintain earthworm weight (Cluzeau and Fayolle, 1989), and it has been reported that additions of uncontaminated food does not significantly affect metal accumulation rates (Spurgeon and Hopkin, 1996; van Gestel, 1997).

During the uptake experiment, earthworms were subsampled from the three soils at 1, 2, 4, 7, 10, 15, 20, 25, 30 days after introduction. Earthworms in soil T1 were without exposure, and were collected to obtain background concentrations of heavy metals in earthworms. For each time interval, six of *E. fetida* and two of *L. terrestris* were collected from each triplicate of each soil, and were starved on moist filter paper for 48h to cause them depurate. The filter paper was changed twice during the depuration period. After starvation, the worms were killed by placing them in a freezer at -20 °C. Before chemical analysis, earthworms were freeze-dried for 48h to achieve constant dry weight.

For the elimination process, earthworms were firstly exposed to the three field soils for 30 days to allow them to uptake metals (hereafter, referred to as 'pre-loaded' worms). Then pre-loaded worms were transferred into the clean soil T1 for the elimination phase of the experiment. Six of *E. fetida* and two of *L. terrestris* were separated from each test soil at 0, 1, 2, 4, 7, 10, 15, 20, 25, 30 days after the start of the excretion phase. The worms were processed in an identical manner as that for the uptake experiments.

2.4 Measurement of Cd, Zn concentration in soils and earthworms

In order to determine the total concentrations of Cd and Zn in samples, air dried soil samples or earthworms were ground and passed through a 0.2 mm (No. 100) mesh plastic sieve. Then samples were total digested by using microwave oven digestion system (CEM MARS 5) followed EPA 3501-HP500 method (USEPA, 2007). Quality assurance/quality control was conducted by including NIST standard soils 2710 (from Montana Soil contains highly elevated trace element concentrations) and 2781 (from domestic sludge with high organic matter content) and blanks in triplicate for every digestion batch. The concentrations of Cd, Zn in the final digestion solutions were determined by ICP-MS. Dilutions were made when necessary. ^{115}In was added as an internal standard to monitor matrix effects and signal drifting while running samples.

2.5 Accumulation and elimination kinetics modeling and statistical analysis

To analyze the change of earthworm body concentrations with time, and allow uptake and elimination rate constants to be calculated, a one-compartment, first-order kinetic model was fitted to the data (Van Straalen et al., 2005).

$$\mathrm{Cworm}\ (t) = C_{\mathrm{worm},i(0)} + a/k_e * (1-e^{-k_e t}) \tag{1}$$

for uptake phase, and

$$\mathrm{Cworm}\ (t) = C_{\mathrm{worm},i}(e^{-k_e' t}) \tag{2}$$

for elimination phase.

Where: Cworm(t) = metal concentration in the earthworm (mg kg^{-1} dry weight) at time t (days), $C_{i(0)}$ is the initial body concentration (mg kg^{-1} dry weight), which is measured in the animals before exposure, a is the rate of uptake (mg metal kg^{-1}.animal day^{-1}), k_e is the elimination rate constant (day^{-1}) during the uptake phase, while k_e' is the elimination rate during elimination phase. The uptake rate constant k_u (day^{-1}) is calculated as follows: the rate of uptake a divide by total metal concentration in soil.

The model fitting of the uptake and elimination parameters was estimated by least square regression in Origin7.5 (Originlab Corporation, 2007) statistical software package. Other statistical analysis was performed by using ANOVA in MINITAB 16 (MINITAB Inc, 2010). Three-way ANOVA was used to compare accumulation and elimination rates between the two metals, between the two earthworm species, as well as the difference among the three soils.

3. Results and Discussion

3.1 Accumulation kinetics of Cd and Zn

The soil characteristic analysis shows the variety of soil properties of tested soils (Table 4.1). The soil Cd, Zn concentrations were also showed in Table 4.1.

All earthworms were healthy throughout the exposure and no mortality occurred.

Initial metal concentrations in the earthworms prior to exposure to the contaminated soil, i.e. $Ci_{(0)}$, are given in Table 4.2. Metal concentrations remained constant over the exposure period when cultured in the uncontaminated Soil T1 (data not shown). Uptake and elimination rates of earthworm *L. terrestris* and *E. fetida* were fitted with the one-compartment model (Table 4.3 and 4.4).

With regard to uptake, differences were observed between the essential element Zn and the non-essential element Cd. Cd internal concentrations in the two species of earthworms increased nearly linearly even close to exponential over the exposure period and the concentrations did not reach equilibrium within first 25 days when exposed to the three contaminated soils (Fig 4.1). However, for the essential element Zn, rapid uptake was observed and the internal concentrations of the two earthworms reached steady-state very quickly (i.e., within 4 to 7 days) after introducing them to the soils (Fig 4.2).

In order to get the rate of uptake and elimination kinetics, the one-compartment model was applied to fit the time series data of earthworm body concentration (Table 4.3 and 4.4). It was found that Zn uptake by the two earthworms was very rapid with uptake rates ranging from 40 to 202 mg Zn kg^{-1} worm day^{-1} (Table 4.3 and 4.4). Uptake rates of Cd, with 1.2 to 2.6 mg Cd kg^{-1} worm day^{-1} for the two earthworm species, were far lower than that of Zn, which indicated that Cd was relatively slowly taken up by the earthworms in the contaminated soils (Table 4.3 and 4.4). ANOVA tests showed the uptake rates of Cd, were significantly lower than that of Zn in corresponding soils (Table 4.5). By using the one compartment model, other researchers reported similar findings and stated that uptake rates of essential elements were significantly higher than that of non-essential elements. For instance, uptake rates of Zn by *E. fetida* were relatively rapid (Marinussen et al., 1997; Peijnenburg et al., 1999) while rates for non-essential element Co (Crossley et al., 1995), Cd, Pb (Spurgeon and Hopkin, 1999) were slow or zero. However, when comparing the uptake rate of constant k_u (uptake rate a divided by total metal concentration in soil), it was found except in soil Mill01, there is no significant difference exist between the two metals, which indicates the big difference on uptake rates (a) between the two metals was largely ascribe to the metal levels in soil.

3.2 Elimination kinetics of Cd and Zn

The study of elimination kinetics further demonstrated the differences of kinetics between non-essential (Cd) and essential (Zn) metals. During accumulation phase of the experiment, elimination is occurring concurrently with uptake heavy metals. The elimination rates k_e of Cd were very small, and ranged from 0.025 to 0.036 day in the three soils, which leads to nearly linear accumulation of Cd in earthworms during uptake phase. During the elimination phase, a marked decrease in Cd concentration was observed when the earthworms exposed to the clean soil T1. This elimination was more rapid with rates (k_e') at 0.04 to 0.07 day^{-1} (Table 4.3 and 4.4), and body concentrations approached steady state within 10 days. For each earthworm species, the estimates of the elimination rates across the three soils were reasonably close to each other (Table 4.3, 4.4 and 4.5). The elimination processes was insufficient to allow the earthworm body burdens to return to their initial levels after 30 days culturing in uncontaminated soil. Indeed, only 67% to 70% of the bioaccumulated Cd was eliminated by the earthworm *L. terrestris* and only 63% to 67% by *E. fetida*. Consequently, the remaining Cd in both earthworm species was much higher than the earthworms' initial body concentration before exposure. Moreover, when the k_e and k_e' value are compared, it is apparent that the elimination rates were higher during the elimination phase (k_e') than during uptake (k_e) phase (Table 4.3 and 4.4) which may indicate the elimination is somewhat faster when earthworms exposed to uncontaminated soil.

In contrast to Cd, the essential element Zn had significantly larger elimination rates in corresponding soils (Table 4.3, 4.4 and 4.5). During uptake phase, the k_e values of Zn were approximately ten-fold higher than those of Cd (Table 4.3 and 4.4). When pre-loaded earthworms were transferred to the clean soil T1, Zn concentrations decreased very quickly with rates of 0.30 to 0.33 day^{-1} in *L. terrestris* and 0.62 to 0.66 day^{-1} in *E. fetida*, and reached steady-state after just 3 days after transfer (Figure 4.2). Subsequently, the level of Zn stabilized and showed no further fluctuations during the elimination phase of the experiment. Indeed, after transferring to the clean soil T1, more than 90% of accumulated Zn was eliminated by the both earthworms.

A two-distinct compartment theory has been proposed to explain the difference on uptake and elimination kinetics between essential and non-essential elements. It was stated that two distinct compartments could be identified in the earthworm (Vijver et al., 2005). The compartments have different affinities for essential element Zn and non-essential element Cd which cause the two elements to follow different uptake and excretion route due to their difference in physiological functions in the earthworm. It was reported that Zn was accumulated very quickly and mainly distributed in a loosely-bound compartment (C1) because Zn is an essential element actively involved in many metabolic process (Brandao-Neto et al., 1995), and therefore likely exhibits high turnover rates. As an non-essential element, Cd was mainly distributed in an inert storage compartment (C2) (Morgan and Morgan, 1998; Sturzenbaum et al., 2001) in which accumulated Cd is harder to eliminate, possibly leading to the lower uptake, and especially a lower elimination flux in the earthworms.

It was also suggested that the different elimination kinetics between the two metals might be well explained by differing detoxification. It has been proposed that soil-dwelling animals cannot regulate bioaccumulation of non-essential metals because the elements are detoxified by effectively storing metals in non-toxic forms (inorganic matrices or binding to organic ligands) rather than through excretion (Rainbow, 2002; Cain et al., 2004; Sturzenbaum et al., 2004). It was reported that a significant proportion (>70%) of Cd accumulated by the earthworm *Lumbricus terrestris* (Dallinger et al., 1997) or snail *H. Pomatia* (Gimbert et al., 2006) was sequestered by cysteine-rich metal-binding proteins, such as metallothionein (MT), and MT-2 (isoform to metallothionein) (Dallinger et al., 1997; Sheppard et al., 1997; Spurgeon and Hopkin, 1999, Morgan et al., 2004; Sturzenbaum et al., 2004) and very low elimination rates have been observed in these studies. Species relying on sequestration as a detoxification mechanism may excessively accumulate metals with increasing exposure concentrations without suffering toxic effects, unless the capacity of the detoxification mechanism is exceeded (Lock and Janssen, 2001; Rainbow, 2002). Unlike Cd, the detoxification mechanism of Zn may be mainly by excretion instead of storage (Spurgeon and Hopkin, 1999). As an essential element, Zn was mainly stored in loosely bound compartment C1 and regulation of zinc has been observed in several earthworm species, *Eisenia fetida* (Spurgeon and Hopkin, 1999; Lock and Janssen, 2001), *Eisenia andrei* (Peijnenburg et al., 1999) and *L. rubellus* (Ireland, 1979). It was reported that after transferring the earthworms to clean soil, C1 emptied rapidly and thoroughly (Spurgeon and Hopkin, 1999; Peijnenburg et al., 1999; Lock and Janssen, 2001), which is consistent with the result in this study in that only very small amount of accumulated Zn remained after the elimination phase.

3.3 Factors affecting kinetics

In our study, the order of uptake rates *a* of Zn in the three soils is P4>P7>Mill01 (Table 4.3 and 4.4) for both earthworms, which follows the same order of the soil total Zn concentration, as well as extractable and labile Zn content. Also, the order of uptake rate Cd for the two earthworms have the same order of soil total Cd concentration, as well as extractable and labile Cd (P4=Mill01>P7) (Table 4.3 and 4.4). This may be explained by the fact that the uptake process is well fitted by the first-order kinetic model ($r>0.8$, $p<0.05$). First-order conformity is expected when the accumulation rate is simply a function of an intrinsic rate coefficient and the size of bioavailable metal pool. Other studies have reported the same phenomenon, and found that the higher uptake rates always occurred when organisms were exposed to the most contaminated soils (Spurgeon and Hopkin, 1999; Gimbert, 2006).

Other studies have proposed that metal uptake and elimination rates are organism species-specific. It was found that the species, behavior and the physiology of the organism impact on uptake rates of trace elements (Spurgeon and Hopkin, 1996; Langdon et al., 2005). In our study, two physiologically different earthworm species, one epegic and one anecic, were selected. Uptake of Zn by *E. fetida* was rapid with uptake rates *a* from 55 to 202 mg Zn kg^{-1} worm day^{-1} (Table 4.4). ANOVA test showed the rates were significantly higher than that of *L. terrestris* with uptake rates ranged between 40 and 140 mg Zn kg^{-1} worm day^{-1} ($p<0.001$)(Table 4.5) . Similarly, Spurgeon and Hopkin (1999) reported rapid zinc uptake and elimination in *E. fetida*, while slower uptake in *L. rubellus*. They proposed that this was probably because *E. fetida* is less sensitive to contaminants than other species, which may leads to its higher uptake rates and accumulation of more contaminants. Such difference in uptake rates not only exists among earthworm species, but also among various soil organisms. For instance, researchers have compared uptake rates between earthworm and snails (Spurgeon and Hopkin, 1999) and showed that the accumulation rate of metals for earthworm is much lower than that for snails when they were exposed to a Cd-spiked soil.

Comparing elimination rates between the two earthworms, it was found there is no such huge difference between them in each soil ($p>0.01$, table 4.5). The higher values of uptake rates in *E. fetida* and the similar values of elimination rates in the two worms adequately explaine the higher internal concentration of metals in *E. fetida*. Unlike the discrepancy in uptake rates among the soils, it was observed the elimination rates (k_e and k_e') of both Cd and Zn were similar in all three soils. This finding is in accordance with a study of Mo bioavailability, which found that although external soil metal concentration was shown to influence uptake rates, in most cases they have a negligible effect on metal excretion rates, which indicates elimination rate is intrinsic properties of earthworm which are not impacted by soil metal concentrations or soil characteristics (Díez-Ortiz et al., 2010).

In this study, other than total content of metals in soils, we could not investigate soil properties' impact on uptake rates of the metals by earthworms because only a limited number of soils could be used. Indeed, the influence of soil properties on metal bioavailability, transfer and toxicity for soil invertebrates has been well established by others (Weltje, 1998; Lock and Janssen, 2001). These studies recognized the particular importance of pH, organic matter, aging, and metal interactions in the reduction of metal bioavailability in soils. The most significant correlation between uptake parameters and soil characteristics was found using the total heavy metal soil concentration (Mo). Organic carbon content was the main soil factor explaining the variation between uptake rates and bioaccumulation factors. Also, it was reported CEC and pH can play an important role, too (Díez-Ortiz et al., 2010).

References

Anderson, J.M., and Ingram, J.S. 1993. Tropical Soil Biology and Fertility: A Handbook of Methods. 2nd ed. CAB International, Wallingford, UK. pp. 93-94.

ASTM. 1999. Standard guide for conducting laboratory soil toxicity or bioaccumulation tests with Lumbricid earthworm Eisenia fetida, standard E 1676-97. In: Annual Book of ASTM Standards, pp. 1056-1074.

Brandao-Neto, J., Stefan, V., Mendonca, B.B., Bloise, W., Castro, A.V.B. 1995. Nutrition Research 15 (3), 335-358.

Cain, D.J., Luoma, S.N., Wallace, W.G. 2004. Linking metal bioaccumulation of aquatic insects to their distribution patterns in a mining-impacted river. Environmental Toxicology and Chemistry 23,1463-1473.

Cluzeau, D., Fayolle, L. 1989. Croissance et feÂconditeÂ comparees de Dendrobaena rubida tenuis (Eisen, 1874), Eisenia andrei (BoucheÂ, 1972) et Lumbricus rubellus (Hoffmeister, 1843) (*Oligochaeta, Lumbricidae*) en eÂlevage controÃleÂ. Rev. Ecol. Biol. Sol 26, 111-121.

Crossley, D.A., Jr., E. R., Blood, P.F., Hendrix, T.R. 1995. Seastedt. Turnover of cobalt-60 by earthworms (*Eisenia fetida*) (*Lumbricidae, Oligochaeta*). Applied Soil Ecology 2, 71-75.

Dallinger R., Berger B., Hunziker P.E., Kägi J.H.R. 1997. Metallothionein in snail Cd and Cu metabolism. Nature 388, 237-238.

Díez-Ortiz, M., Giska, I., Groot, M., Borgman, E.M., Van Gestel, C.A.M. 2010. Influence of soil properties on molybdenum uptake and elimination kinetics in the earthworm *Eisenia andrei*. Chemosphere 80, 1036-1043.

Edwards, C.A., Bohlen, P.J. 1996. Biology and Ecology of Earthworms. 3rd Edn., Chapman and Hall, London, ISBN: 0412561603, pp: 426

Fang, J., Wen, B., Shan, X.Q., Wang, H.H., Lin, J.M., Zhang, S.Z. 2007. Evaluation of bioavailability of light rare earth elements to wheat (Triticum aestivum L.) under field conditions. Geoderma 141, 53-59.

Gimbert, F., Vaufleury, A., Douay, F., Renaud, S., Coeurdassier, M., Badot, P.M. 2006. Modelling chronic exposure to contaminated soil: A toxicokinetic approach with the terrestrial snail Helix aspersa. Environment International. Environment International 32, 866-875.

Gray, C.W., McLaren, R.G., Shiowatana, J. 2003. The determination of labile cadmium in some biosolids-amended soils by isotope dilution plasma mass spectrometry. Australian Journal of Soil Research 41, 589-597.

Heikens, A., Peijnenburg, W.J.G.M., Hendriks, A.J. 2001. Bioaccumulation of heavy metals in terrestrial invertebrates. Environmental Pollution 113, 385-393.

Hendriks, A.J., Heikens, A. 2001. Rate constants and equilibrium ratios for accumulation of inorganic substances related to species weight. Environmental Toxicology and Chemistry 20, 1421-1437.

Hobbelen, P.H.F., Koolhaas, J.E., van Gestel, C.A.M. 2006. Bioaccumulation of heavy metals in the earthworms Lumbricus rubellus and Aporrectodea caliginosa in relatin to total and available metal concentrations in field soils. Environmental Pollution 144, 639-646.

Ireland, M.P. 1979. Metal accumulation by the earthworms Lumbricus rubellus. Dendrobaena vetena and Eiseniella tetraedra living in heavy metal polluted sites. Environmental Pollution 19, 201-206.

Janssen, R.P.T., Peijnenburg, W.J.G.M., Posthuma, L., Van den Hoop, M.A.G.T. 1997. Equilibrium partitioning of heavy metals in Dutch field soils I. Relationship between metal partition coefficients and soil characteristics. Environmental Toxicology and Chemistry 16, 2470-2478.

Langdon, C.J., Hodson, M.E., Arnold, R.E., Black, S. 2005. Survival, Pb-uptake and behaviour of three species of earthworm in Pb treated soils determined using an OECD-style toxicity test and a soil avoidance test. Environmental Pollution 138, 368-375.

Lee, K.E., 1985. Earthworms: Their Ecology and relationship with soils and land use. Academic Press, London, UK. pp: 411

Lock, K., Janssen, C.R. 2001. Zinc and cadmium body burdens in terrestrial oligochaetes: use and significance in environmental risk assessment. Environmental Toxicology and Chemistry 20, pp. 2067-2072

Marinussen, M.P.J.C., Van der Zee, S.E.A.T.M., De Haan, F.A.M., Bouwman, L.H., Hefting, M. 1997. Heavy metal (copper, lead, and zinc) accumulation and excretion by the earthworm, *Dendrobaena veneta*. Journal of Environmental Quality 26, 278-284.

Morgan, J.E., Morgan, A.J. 1998. The distribution and intracellular compartmentation of metals in the endogeic earthworm *Aporrectodea caliginosa* sampled from an unpolluted and a metal-contaminated. Environmental Pollution. 99, 167-175.

Morgan, J.E., Morgan, A.J. 1999. The accumulation of metals (Cd, Cu, Pb, Zn and Ca) by two ecologically contrasting earthworm species (Lumbricus rubellus and Aporrectodea caliginosa): implications for ecotoxicological testing. Applied Soil Ecology 13, 9-20.

Morgan, A.J., Stürzenbaum, S.R., Winters, C., Grime, G.W., Aziz, N.A.A., Kille, P. 2004. Differential metallothionein expression in earthworm (Lumbricus rubellus) tissues. Ecotoxicology and Environmental Safety 57, 11 – 19.

Moriarty, F. Walker, C.H. 1987. Bio-accumulation in food chains—A rational approach. Ecotoxicology and Environmental Safety 13, 208-215.

Nahmani, J., Hodson, M.E., Black, S. 2007. A review of studies performed to assess metal uptake by earthworms. Environmental Pollution 145, 402-424.

Nelson, D.W., Sommers, L.E. 1982. Total carbon, organic carbon, and organic matter. In: Methods of Soil Analysis. 2nd Ed. ASA Monogr. 9(2). Amer. Soc. Agron. Madison, WI, pp. 539-579.

Peijnenburg, W.J.G.M., Posthuma, L., Eijsackers, H.J.P., Allen, H.E. 1997. A conceptual framework for implementation of bioavailability of metals for environmental management purposes. Ecotoxicology and Environmental Safety 37,163-172.

Peijnenburg, W.J.G.M., Baerselman, R., De Groot, A.C., Jager, T., Posthuma, L., Van Veen, R.P.M. 1999. Relating environmental availability to bioavailability: soil-type-dependent metal accumulation in the oligochaete Eisenia andrei. Ecotoxicology and Environmental Safety 44, 294-310.

Rainbow P.S. 2002 Trace metal concentrations in aquatic invertebrates: why and so what? Environmental Pollution120, 497-507.

Sheppard, S.C., Evendsen, W.G., Cornwell, T.C. 1997. Depuration and uptake kinetics of I, Cs, Mn, Zn and Cd by the earthworm (Lumbricus terrestris) in radiotracer-spiked litter. Environmental Toxicology and Chemistry 16, 2106-2112.

Sims, R. W, Gerard, B. M. 1999. Earthworms: Notes for the Identification of British Species, Synopses of the British Fauna (New Series) No.31 (Revised). London: Linnean Society.

Spurgeon, D. J., Hopkin, S. P. 1996. Effects of variations in the organic matter content and pH of soils on the availability and toxicity of zinc to the earthworm *Eisenia fetida*, Pedobiologia 40, 80-96.

Spurgeon, D.J., Hopkin, S.P. 1999. Comparisons of metal accumulation and excretion kinetics in earthworms (*Eisenia fetida*) exposed tocontaminated field and laboratory soils. Applied Soil Ecology 11, 227-243.

Sturzenbaum, S.R., Winters, C., Galay, M., Morgan, A.J., Kille, P. 2001. Metal ion trafficking in earthworms. The Journal of Biology Chemistry 36, 34013-34018.

Sturzenbaum, S.R., Georgie, O., Morgan, A.J., Kille, P. 2004. Cadmium detoxification in earthworms: from genes to cells. Environmental Science and Technology 38, 6283-6289.

Suthara, S., Singhb, S., Dhawanc, Sachin. 2008. Earthworms as bioindicator of metals (Zn, Fe, Mn, Cu, Pb and Cd) in soils: Is metal bioaccumulation affected by their ecological category? Ecological Engineering 32, 99-107.

USEPA. 2007. Method 3051: Microwave assisted acid digestion of sediments, sludges, soils and oils. Revision 1. Washington, DC, USA.

van Gestel, C. A. M. 1997. Scientific basis for extrapolating results from soil ecotoxicity tests to field conditions and the use of bioassays, In: van Straalen, N.M., Lùkke H., (Eds.), Ecological Risk Assessment of Contaminants in Soil, Lewis Publishers, Chapman and Hall, London, pp. 25-50.

van Straalen, N.M., Donker, M.H., Vijver, M.G., van Gestel, C.A.M. 2005. Bioavailability of contaminants estimated from uptake rates into soil invertebrates. Environmental Pollution 136, 409-417.

Vijver, M.G., Vink, J.P.M., Jager, T., Wolterbeek, H.T., van Straalen, N.M., van Gesterl, C.A.M. 2005. Biphasic elmination and uptake kinetics of Zn and Cd in the earthworm Lumbricus rubellus exposed to contaminated floodplain soil. Soil Biology & Biochemistry 37, 1843-1851.

Walker, C. H., Hopkin, S. P., Sibly, R. M., Peakall, D. B. 1996. Principles of Ecotoxicology, Taylor and Francis, London.

Weltje, L. 1998. Mixture toxicity and tissue interactions of Cd, Cu, Pb and Zn in earthworms (*Oligochaeta*) in laboratory and field soil: a critical evaluation of data. Chemosphere 36, 2643-2660.

Tables and Figures

Table 4.1 Soil properties

Soil	Classification	Total N (%)	TOC (%)	Clay (%)	pH	Zn (mg kg^{-1})	Cd (mg kg^{-1})
P4	Loam	0.48	5.6	26.7	5.86	1165	18.2
P7	Loam	0.17	2.6	23.9	5.67	739	10.7
Mill-01	Clay	0.27	1.7	407	5.34	129	18.9

Table 4.2 Cd, Zn initial and final concentration in earthworms after accumulation

	Zn(mg.kg^{-1})				Cd (mg.kg^{-1})			
Earthworm	C_0	Ct			C_0	Ct		
		P4	P7	Mill01		P4	P7	Mill01
L. terrestris	58	515	270	158	0.86	28.6	21.2	27.2
E. fetida	77	639	403	178	1.35	51.9	23.7	45.8

Table 4.3 Fitted kinetic parameters by one-compartment modeling in earthworm *L. terrestris.*

Soil	Zn						Cd					
	a	k_u	k_e	k_e'	r_u^2	r_e^2	a	k_u	k_e	k_e'	r_u^2	r_e^2
P4	139.6 ±22	0.12 ±0.02	0.27 ±0.04	0.33 ±0.02	0.78	0.75	1.64 ±0.14	0.09 ±0.008	0.032 ±0.004	0.062 ±0.01	0.68	0.75
P7	71.8 ±8.9	0.10 ±0.01	0.27 ±0.04	0.30 ±0.05	0.82	0.85	1.22 ±0.15	0.11 ±0.014	0.028 ±0.004	0.073 ±0.01	0.62	0.79
Mill01	39.7 ±4.2	0.31 ±0.03	0.25 ±0.05	0.31 ±0.04	0.76	0.78	1.63 ±0.12	0.09 ±0.006	0.036 ±0.007	0.056 ±0.01	0.71	0.78

Table 4.4 Fitted kinetic parameters by one-compartment modeling in earthworm *E. fetida.*

Soil	Zn						Cd					
	a	k_u	k_e	k_e'	r_u^2	r_e^2	a	k_u	k_e	k_e'	r_u^2	r_e^2
P4	201.8 ±31	0.17 ±0.02	0.31 ±0.04	0.66 ±0.08	0.75	0.73	2.61 ±0.23	0.14 ±0.01	0.025 ±0.004	0.049 ± 0.01	0.63	0.73
P7	126.9 ±19	0.17 ±0.02	0.31 ±0.03	0.64 ±0.09	0.72	0.73	1.29 ±0.14	0.12 ± 0.01	0.029 ±0.005	0.041 ± 0.01	0.62	0.75
Mill01	54.9 ±7.9	0.43 ±0.05	0.32 ±0.05	0.62 ±0.08	0.71	0.74	2.48 ±0.29	0.13 ±0.02	0.029 ±0.007	0.048 ± 0.01	0.69	0.71

Table 4.5 Three-way ANOVA test of the uptake rate (a), elimination rate (k_e) between earthworms, elements, as well as among soils.

a versus	Source	DF	Seq SS	Adj SS	Adj MS	F	P
Soil,	Soil	2	23087	23087	11544	11.29	0.000
Earthworm,	Earthworm	1	4515	4515	4515	4.42	0.044
Element	Element	1	97291	97291	97291	95.19	0.000
	Error	31	31684	31684	1022		
	Total	35	156577				
	S = 31.9695 R-Sq = 79.76% R-Sq(adj) = 77.15%						
ke versus	Source	DF	Seq SS	Adj SS	Adj MS	F	P
Soil,	Soil	2	0.00000	0.00000	0.00000	0.00	0.999
Earthworm,	Earthworm	1	0.00469	0.00469	0.00469	5.22	0.029
Element	Element	1	0.60140	0.60140	0.60140	668.89	0.000
	Error	31	0.02787	0.02787	0.00090		
	Total	35	0.63397				
	S = 0.0299851 R-Sq = 95.60% R-Sq(adj) = 95.04%						

Figures 4.1 Uptake and excretion kinetics of Cd in two earthworm species (black square: *E. fetida*; grey diamond: *L. terrestris*) (Error bars represents standard deviation with n=3)

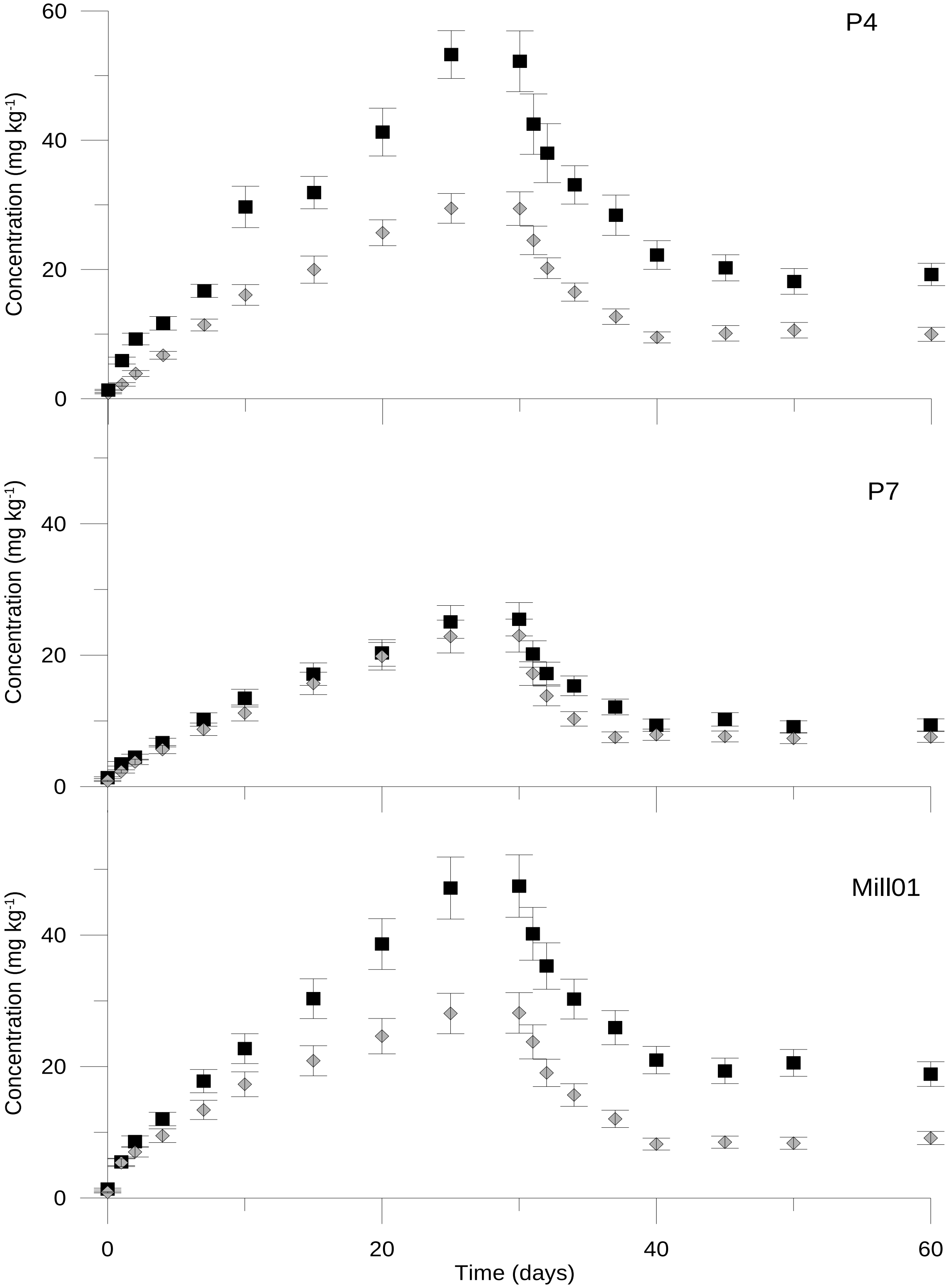
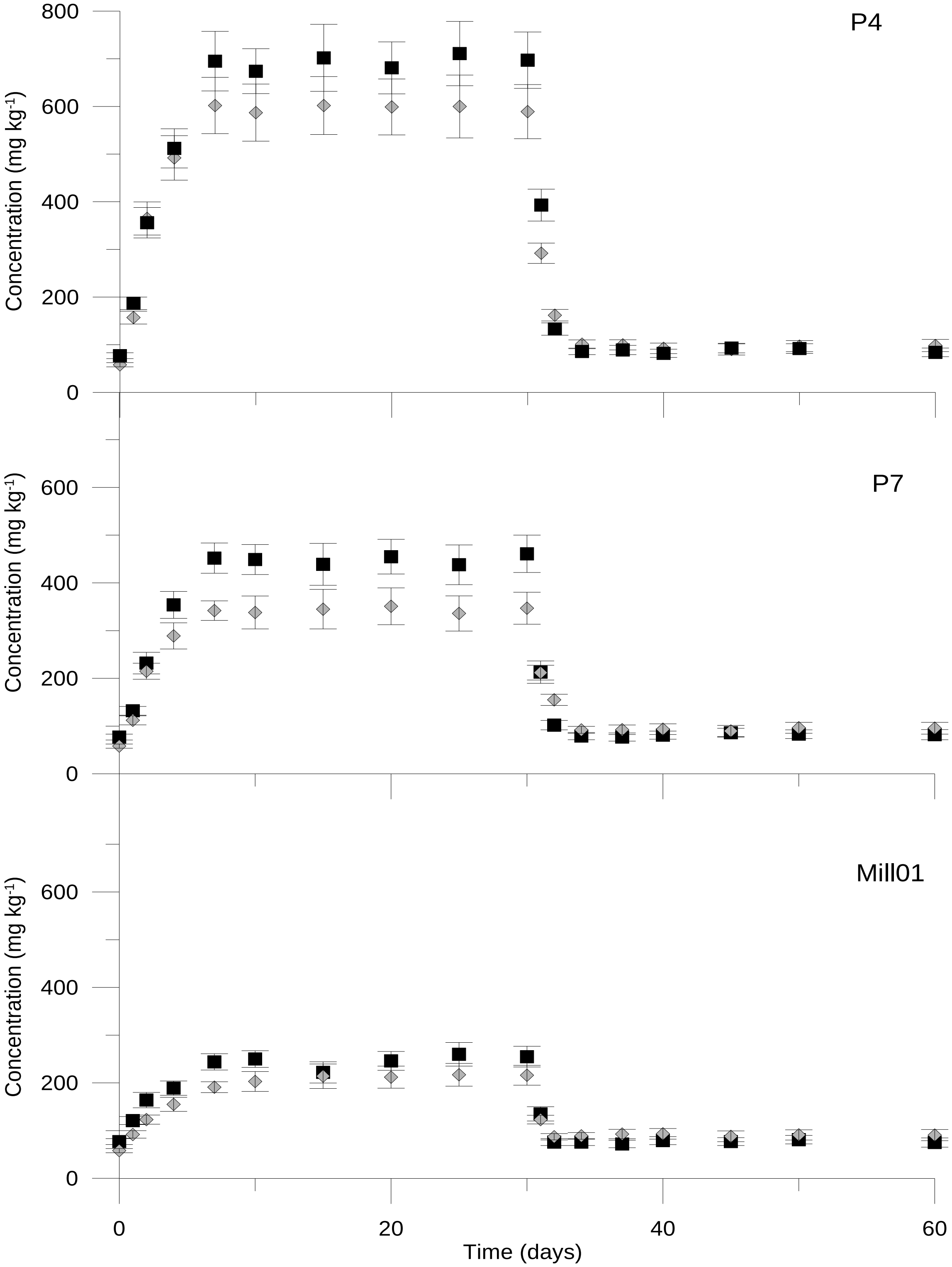


Figure 4.2 Uptake and excretion kinetics of Zn in two earthworms (black square: *E. fetida*; grey diamond: *L. terrestris*) (Error bars represents standard deviation with n=3)



Chapter 5

Effects of earthworms on bioavailability of trace metals in casts

Abstract

The effects of earthworm activity on soil properties (pH, total organic carbon), as well as their influence on total, labile and extractable pool of metal were investigated in this study. The soils used were obtained from sites affected by different pollution sources: a Zn smelter emission polluted area, a site where sewage sludge has been applied, and a naturally Cd-rich soil. By analyzing and comparing the soil properties with those of the corresponding casts produced by earthworms, it was shown that the two species of earthworms (*Lumbricus terrestris*, *Eisenia fetida*) increased the pH values in all the soils after 60 days of culturing in the soil. The earthworms' casts exhibited decreased organic carbon content. In addition, Cd, Zn contents in the casts were decreased relative to the bulk soil. Moreover, the percentages of 0.01M $Ca(NO_3)_2$- and 1M $CaCl_2$-extractable Cd and Zn, as well as the fraction of labile Cd and Zn determined by isotope dilution in the soils, were all increased by earthworm activity, suggesting that passage through the earthworm gut effectively mobilized both metals.

Key words: Earthworms, Extractability, Bioavailablity, Polluted soils

1 Introduction

Trace metals are present in soils as natural components or as a result of human activities, such as industrial, mining and agricultural activities. These activities have, in some cases, introduced excess amounts of metals, such as Zn, Cd, Hg, Cu and Pb into soils, leading to potential risks to both human and ecosystem health (Schalscha and Ahumada, 1998; McGrath et al., 2001).

Earthworms are considered the dominant macrofauna in most soils (Lee, 1985). It has been shown that after long-term habitation, earthworms can decrease soil metals by accumulating them in their body (Ma et al., 2003). Also, earthworms can alter bioavailable metals in soil, and enhance soil fertility by burrowing activity and gut process (Ma et al., 2003). Several earthworm species have been reported to survive in highly metal contaminated soils, and can even accumulate metals in their tissues (Morgan et al., 1998; Lanno et al., 2004). Studies have also showed that following treatment with earthworms, the distribution of metals in soil fractions was significantly changed (Devliegher and Verstraete, 1997; Cheng and Wong, 2002; Ma et al., 2002; Wen et al., 2004; Udovic and Lestan, 2007); specifically the portion of water-soluble and extractable heavy metals were increased.

The changes in metal speciation were ascribe to digestion processes in the earthworms' gut, along with burrowing activity that altered the soil characteristics, including DOC, pH and CEC (Diez-Ortiz et al., 2010). However, disagreement still exists on earthworm's ability to accumulate heavy metals, to change heavy metal fractionations, as well as their effect on soil properties. This could be due to selection of different earthworm species. Generally earthworms are grouped in three species according to their morphological and behavioral characteristics (Sims and Gerard, 1999): litter dwelling (epigeic), horizontally burrowing mineral soil feeders (endogeic), and vertically burrowing surface feeders (anecic). Few studies have compared the difference among the species type in terms of their ability to change soil properties, or metal fractionation. Another explanation for the discrepancy among the studies may be to the selection of soils. It is generally known that metals from various sources bound in different pools within the soil and varying in their bioavailability. For example, geogenic metals are often tightly bound to or within soil solid particles, whereas anthropogenic metals are often bound in more exchangeable and labile pools (Scott-Fordsmand et al., 2004). Thus, research is still needed to compare the possible difference of earthworm' effects on metal speciation when soils were collected from various sources.

The objective of this study was to verify whether earthworms could alter the total and bioavailable metals in naturally enriched, smelter emission polluted, and sewage sludge-amended soils. Representatives of epigeic (*Eisenia fetida*) and anecic (*Lumbrius terrestris*) earthworm species have been used in this study. More specifically, we investigated: (i) the tolerance of two species of earthworms to metals and the accumulation of metals in their tissues; (ii) earthworm effects on soil properties (iii) changes in metal fractionation, including water soluble, exchangeable, labile and total content of metals in earthworm casts.

2 Materials and Methods

2.1 Soil and soil analysis

The three soils used in this study represented three distinct sources of trace metal pollution. Palmerton soil P6 was heavily polluted by heavy metals collected from a Zn smelter emission area in Palmerton, PA. The area received Zn smelter emission for more than 100 years, but emissions ceased 30 years ago. Romona soil, collected from the Moreno Field Station of the University of California in Moreno Valley, CA, was contaminated with heavy metals through constant applications of anaerobically digested sewage sludge. The soil designated Mill01 was collected from Pt. Dume, Los Angeles County, CA, and was derived from Cd-rich shale and will be treated as a naturally contaminated Cd source. The sampled area were about 1 m^2 each. At each area, three soil samples were collected from the upper layer (15cm) of the field soil and thoroughly mixed together, then transferred to the lab. Samples were subsequently air-dried at ambient temperature, ground and sieved (<2mm), for further analysis.

Organic C was determined using the Walkley-Black procedure (Nelson and Sommers, 1982), soil texture was measured by hydrometer (Bouyoucous) method (Anderson and Ingram, 1993), and soil pH was measured in a de-ionized water-soil suspension using a ratio of 1:1. Soil and casts characteristics are given in Table 5.1.

2.2 Earthworm culturing and casts collection

Clean plastic pots (height 50 cm, diameter 12.5 cm) were filled with 500 g of air-dried soil in triplicate. Soils were wet with deionized water to approximately 60% of field capacity, and left to stabilize for a week at 20 °C under 65% relative air humidity.

Earthworms used for the experiment were *Eisenia fetida* (*E. fetida*) and *Lumbricus terrestris* (*L. terrestris*), purchased from Timeline Fishery Company (IL, USA). They were hosted in clean soil and fed with peat (contained in shipping package from the supplier) for two weeks at 20 °C prior to performing any experiment. Mature earthworms (two of *L. terrestris* and six of *E. fetida*.) were selected (100-300 mg fresh weight of *E. fetida*, 1.5-2.5 g fresh weight of *L. terrestris*) and transferred to each triplicate pot for every soil sample. Soil moisture was maintained at 60% of field capacity by adding DI water every two days. The pots were maintained in a large environment controlled chamber at a constant temperature at 20℃. Light/dark cycle was set as 16h/8h and humidity was kept at 65%. During the procedure, 0.5 g (dry weight) peat per *L. terrestris* and 0.15 g per *E. fetida* per week was added to each pot. Casts were deposited on top of the soil, making them easily identifiable through visual observation. Casts were manually collected daily from soil surface. After 60 days, earthworms were carefully removed from soils. The casts were pooled from the 3 replicate pots into a single sample for each worm-soil combination.

2.3 Extractable metals and *E*-value determination

Each soil and corresponding cast were extracted independently via chemical extraction, utilizing 0.01 M $Ca(NO_3)_2$, 1M $CaCl_2$ solutions to obtain water-soluble and exchangeable fractions of Cd, Zn. The detailed operations and nominal target fractions in soils were previously reported (Lindsay and Norvell, 1978; Gray et al., 2003). After shaking each extraction, suspensions were centrifuged and filtered through a 0.45 μm cellulose acetate membrane filter prior to heavy metal determination. All extractions, including blanks, were carried out in triplicate. Cd and Zn contents in samples were measured by ICP-MS.

The labile portion of Cd and Zn in soils was investigated using a stable isotopic dilution technique with ICP-MS for measuring isotope ratio of $^{111}Cd/^{114}Cd$ and $^{66}Zn/^{68}Zn$.

Enriched stable isotopes ^{111}Cd and ^{68}Zn were added to a water suspension of each soil and the labile metal pool (*E*-value) was calculated from the changed diluted isotope ratio and followed the *E*-value calculation equation. Detailed procedures have been described in Chapter 2.

2.4 Metal measurement in samples

For determination of total Cd and Zn concentrations, dried soil samples and corresponding casts were ground and passed through a 0.2 mm plastic sieve. Samples were subsequently digested using a microwave oven digestion system (CEM MARS 5) following the EPA 3051-HP500 method (USEPA, 2007). Quality assurance/quality control was conducted by including NIST standard soils 2710 (from Montana Soil contains highly elevated trace element concentrations) and 2781 (from domestic sludge with high organic matter content) and blanks in triplicate for every digestion batch. The total Cd and Zn concentrations were determined by ICP-MS. ^{115}In was added as an internal standard to monitor matrix effects and signal drift while analyzing samples.

3 Results and Discussion

3.1 Earthworm's effect on soil pH

The pH values of the three soils listed in Table 5.1, indicated the soils were slightly acidic. The pH values of the casts from the two worms were greater than the corresponding hosting soil, especially for soil P6, in which the pH increased by 0.92 and 0.67 units in casts from earthworm *L. terrestris* and *E. fetida*, respectively. The pH value in the casts collected from soil Mill01 was only slightly elevated by about 0.2 units. In general the pH change in the casts from *L.terrestris* was larger than that from *E. fetida*.

Soil pH is a significant factor that affects the mobility and bioavailability of heavy metals in soil. As a soil "ecological engineer", the earthworm has been considered to have a significant impact on soil properties (Ma et al., 2002; Wen et al., 2004; Udovic and Lestan, 2007). However, disagreement still exists on the effect of earthworm on soil pH. Many studies have shown that earthworms can increase soil pH probably due to N-rich excreta produced by the earthworms, including alkaline urine (Salmon, 2001), or to earthworm's calciferous glands (Lee, 1985; Edwards and Bohlen, 1996). In our study, it is the first time that two physiological different earthworms (epegic and anecic) have been compared for their effects on soil pH. The result confirmed the general view that earthworms tend to raise soil pH. It was also observed that the anecic species, *L. terrestris*, had a greater effect in increasing soil pH, compared to the epegic species, *E. fetida*. In contrast, it was reported that earthworms (*Pheretima sp.)* have ability to decrease soil pH (Cheng and Wong, 2002). Edwards and Bohlen (1996) stated that the earthworm have ability to increase or decrease soil pH in order to keep soil pH in a neutral range. The discrepancy among these studies is probably due to the selection of different species of earthworm and different type of soils. More soils, with wider range of pH, should be used in future studies.

3.2 Earthworm's effect on soil total organic matter

As shown in table 5.1, the Mill01 soil had the lowest total organic carbon content at 1.7%, while soil Romona had the highest value, over 8%, most likely due to the repeated amendment with sewage sludge. Comparing the TOC content in the soils to the corresponding casts, it was found that earthworm activity slightly decreased the TOC in all three soils. The order of the change follows: Romona>P6>Mill01. In detail, TOC of casts were decreased from 0.12 to 0.65 units by *L. Terrestris*, and from 0.18 to 0.78 unit by *E. fetida*, suggesting that the earthworm, *E. fetida*, has stronger capacity to alter soil total organic matter content.

It has been well documented that earthworm activity improves soil properties through physical, chemical, biological and biochemical mechanisms, such as enhancing the fragmentation and humidification of coarse particulate organic matter into finer fractions and influencing the distribution of organic matter in soil (Martin, 1991). This was confirmed by Ponder et al., 2000, who found that the dissolved organic carbon in earthworm-treated soil was much higher than that in non-treated soil, indicating that earthworms and existing microbes decomposed soil organic matter and transferred it to the dissolved phase (Edwards and Bohlen, 1996). It has also been shown that microbial activity, and nutrient and carbon mineralization were increased in earthworm casts over a period of a few days (Edwards and Bohlen, 1996). Under laboratory conditions, as in our study, higher earthworm density in the soil may lead to higher metabolic rates of the microflora community thus leading to a higher rate of carbon mineralization, explaining the decreased organic carbon content in the casts. Our results also indicated that the earthworm, *E. fetida*, has a larger capacity to decrease soil organic carbon content, due in part to the fact that it is an epegiec species, which tend to feed on organic rich debris and have higher rates of carbon mineralization.

3.3 Earthworm's effect on total and extractable metals in soils

Total Zn content in soils ranged from 3079 mg kg^{-1} in P6 to 129 mg kg^{-1} in Mill01. In the Romona and P6 soils, total Zn content decreased by 14% and 18%, respectively in the corresponding casts from *L terrestris*, and decreased 19% and 23% respectively in casts from *E. fetid*a. (Figure 5.1). However, Zn content in Mill01 soil was only slightly lowered (<5%) in the two worms' casts. The total Cd concentration in the soils ranged from 18.9 mg.kg^{-1} in Mill01 to 62.4 mg.kg^{-1} in P6. The change in Cd content in the three soils was also follows: P6>Romona>>Mill01, with the Cd content lowered from 16.5% to 3.7% by *L. terrestris*, and lowered from 22.6% to 5.3% by *E. fetida*. Earthworms could lower metal concentrations in casts due to possible two reasons. First, the worm retained a significant mass of Cd, Zn in its body when soil passing by. Second, somehow the Cd, Zn was diluted in the casts via addition of exogenous material (e.g. The added food). If the biomass of earthworm and that of casts were recorded, we could quantify the mass balance of heavy metal concentrations among earthworm, soil, and corresponding casts. However, this was not done here since it is difficult to quantitatively collect all the casts, especially casts produced sub-surface. Further work is needed to use such mass balancing in order to assure the mechanism of apparent metal decrease in the casts. Moreover, the results showed that casts from *E. fetida* had lower Cd and Zn concentration, which may coincide with our previous findings that *E. fetida* could accumulate more metals from the soils than *L. terrestris.* Further observation found that the higher the initial concentration of metals, the greater the decrease in the casts. It has been well documented that earthworms can tolerate high concentrations of heavy metals in soil (Díez-Ortiz et al., 2010). High concentrations of heavy metals, if below lethal levels, have been shown to promote earthworm uptake and allow for this detoxification process to occur, thus reducing the heavy metal concentration in casts (Maity et al., 2008). Besides, it was observed that the order of change in metal concentration follows the same order of metal *E* value (P6>Romona>Mill01 for both two metals) (data was shown in Chapter 2 and 3), which indicates the labile metal pool is easily accessible for soil organisms and could be easily uptake by them.

3.4 Earthworm's effect on fractionation of heavy metals

Soils and corresponding casts were extracted by 0.01 M $Ca(NO_3)_2$ in order to obtain the water-soluble metal. P6 soil had the highest fraction of water soluble Zn at 8% and over 12% of water soluble Cd (Figure 5.2). The water soluble Cd and Zn in Romona, especially in Mill01 soil was relatively small, less than 1%. The earthworms strongly enhanced water solubility of Zn in P6 and Romona soils. The values increased by factor of 1.38 and 1.54, respectively, in the casts of *L. terrestris* , and increased by 1.67- to 1.92-fold, respectively, in the casts of *E. fetida*. The fraction of water soluble Cd in the casts produced in P6 and Romona soils were also much higher than that in the original soils, however, the fraction of water soluble Cd and Zn in the cast produced in Mill01 were largely unchanged (Figure 5.2).

Soils and casts were subsequently extracted with 1M $CaCl_2$ to obtain the exchangeable heavy metal fraction. The fraction of exchangeable Zn showed a large variation between the three soils. Mill01 contains only 2% of 1M $CaCl_2$-extractable Zn and a similar value was found in the casts. The two worm species increased the fraction of exchangeable Zn in Romona soil from 12% to nearly 20% in the casts. P6 soil had the highest level of exchangeable Zn which was shown to be over 50% of the total Zn content in soil. This fraction increased to over 60% during passage through the gut of the worms (Figure 5.3). Although from different sources, the fractions of 1M $CaCl_2$-extractable Cd in the three soils were relatively the same, ranging from 44% to 53%, which were enhanced by factor of 1.18-1.23 and 1.11-1.28, respectively, in the casts of *L. terrestris* and *E. fetida*.

Labile Zn (*E*-value) in the P6 and Romona soils was determined by stable isotope dilution. The detailed procedure for this technique was described in Chapter 3. Labile Zn in Mill01 soil was not obtainable due to the fact that the value was below detection. In the P6 soil, the original fraction of labile Zn was determined to be 33% and was increased by earthworm activity to nearly 50% in the casts. The %*E* of Zn was 20% in Romona soil, and was increased by a factor of 1.2 and 1.3, respectively in the casts obtained from *L. terrestris* and *E. fetida* (Figure 5.4). The %*E* of Cd in P6 and Romona soils was increased by the earthworm activity, while it remained unchanged in the casts produced in Mill01 soil (Figure 5.4).

The bioavailable metal to these organisms may come from a variety of pools in the soil, including pore water, cation exchange sites, specific adsorption sites, and perhaps part of metal-bearing minerals (Sizmur and Hodson, 2009). Our study found an increase in the fraction of water-soluble, exchangeable, and labile heavy metal pools in the earthworm casts produced in both P6 and Romona soils. These results indicate that the chemical partitioning of heavy metals within the soil was affected by *E. fetida*, leading to a higher concentration of metals in the non-residual fractions of the soil. It was previously shown that metal availability increased due to earthworm (*Ensenia fetida*, *Aporrectodea tuberculate*, *Lumbricus terrestris*, *Lumbricus rubellus*, *Pheretima sp*) activity in both contaminated (Rada et al., 1996; Ma et al., 2002; Ma et al., 2003; Coeurdassier et al., 2007, Udovic and Lestan, 2007) and uncontaminated (Stephens et al., 1994, Wen et al., 2004) soils. Moreover, when exposed together with earthworms, snails had higher concentrations of Cd, Cu and Zn than when they were exposed alone, which probably due to the increase in available metal pools, especially the water-soluble metals in casts (Coeurdassier et al., 2007). However, it was also shown the impact of the earthworms on concentration and metal speciation was both soil- and metal- specific (Coeurdassier et al., 2007; Sizmur et al., 2011). In our study, such difference between the two metals was not observed, but the change in metal fractionation was soil specific.

Soils used in this study were either naturally metal-rich soil or soils artificially amended with metals. Generally speaking, metals that are added as an artificial amendment, such as downwind from a metal smelting complex (e.g. P6 soil), or sewage sludge application (e.g. Romona soil) are likely to be more chemically reactive than metals from naturally contaminated ones (e.g. Mill01 soil) (Spurgeon and Hopkin, 1995), which well explained our findings that the fraction of water soluble metals in P6 and Romona soils were much higher than that in Mill01 soil.

In addition, it was found although the soils were collected from the different pollution sources, the fractions of extractable, and labile Cd are similar across the soils. However, these fractions were little changed in the Mill01, where Cd source is generic.

The lower content of organic carbon in Mill01 is another factor that could explain the trends in Fig 5.2-5.4. The increase in exchangeable and labile fractions of metals by earthworms has been shown to be accomplished by mobilizing the organic matter- and sulfide- bound fractions (Wen et al., 2004). Edwards and Bohlen (1996) concluded that the ingested soil undergoes chemical and microbial changes during passage through the worm's gut: part of the organic matter is digested, and the pH and microbial activity of the gut increases. Consequently, the possibility of metals binding to organic matter increases in ingested material leading to a greater concentration of extractable and labile metals in the casts (Kizikaya, 2005).

It is known that an increase in soil pH leads to less heavy metal concentration in soil solution because it increases the enhancement of the affinity of soils for heavy metals due to increased pH-dependent surface-charge density on colloids (Cao et al., 2001; Shan et al., 2002). However, we observed the opposite phenomenon in our experiments. In our study, the pH values in the casts were elevated relative to the original soil, but the fractions of water soluble Cd and Zn also increased. This concentration may be due to metal-complexity metallophores produced by the earthworms. In addition, it was reported that the soil DOC increased during passage through the earthworm (Wen et al., 2004). Jones (1998) suggested trace elements in solution may bound to DOC, thereby increased their desorption to the water phase. Besides, the exogenous food added during earthworm cultural process is a more likely source of labile carbon that could result in elevated DOC in the gut and casts. Comparing the two earthworm species, it was found that *E. fetida* had a higher capacity to decrease total content of heavy metals and to increase the fraction of available heavy metals in soils. As a smaller and debris-dwelling species, *E. fetida* has a preference to ingest organic matter and its narrower gut may promote complete decomposition of organic matter which leads to more heavy metals bound to DOC accumulated by the earthworm.

It has been shown that essential metals are regulated and excreted, while non-essential metals are probably detoxified through binding with organic ligands or sequestration within inorganic matrices (Spurgeon and Hopkin, 1999). The difference between these two mechanisms may cause differences in the effects of earthworm activity on individual metals in the soil. Ireland (1975) found that earthworms affected the availability of Pb and Zn in different ways, with Pb being made more available when Zn was made less available. Sizmur and Hodson (2008) reported that water extractable Pb increased, but the Zn decreased, after *E. veneta* inoculation to soils from metalliferous mining sites. Devliegher and Verstraete (1997) reported a decrease in ammonium acetate-EDTA extractable Ni contrasting with an increase in a range of essential and non-essential metals after *L. terrestris* inoculation. Despite these examples, the vast majority of studies, including ours, have shown that the effect of earthworms on metal availability not to be metal-specific. It can be concluded that the individual metals are not as important as the earthworm species or the soil properties when determining the impact of earthworms on metal availability and lability.

References

Anderson, J.M., and Ingram, J.S. 1993. Tropical Soil Biology and Fertility: A Handbook of Methods. 2nd ed. CAB International, Wallingford, UK. pp. 93-94.

Cao, X., Chen, Y., Wang, X., Deng, X. 2001. Effects of redox potential and pH value on the release of rare earth elements from soil. Chemosphere 44, 655-661.

Cheng, J., Wong, M.H. 2002. Effects of earthworms on Zn fractionation in soils. Biology and Fertility of Soils 36, 72-78.

Coeurdassier, M., Scheifler, R., de Vaufleury, A., Crini, N., Saccomani, C., Du Mont, L.S., Badot, P.M. 2007. Earthworms influence metal transfer from soil to snails. Applied Soil Ecology 35, 302-310.

Díez-Ortiz, M., Giska, I., Groot, M., Borgman, E.M., Van Gestel, C.A.M. 2010. Influence of soil properties on molybdenum uptake and elimination kinetics in the earthworm Eisenia andrei. Chemosphere 80, 1036-1043.

Devliegher, W.; Verstraete, W. 1997. The effects of *Lumbricus terrestris* on soil in relation to plant growth: Effects of nutrient enrichment processes (NEP) and gut-associated processes (GAP). Soil Biology and Biochemistry 29, 341-346.

Edwards, C.A., Bohlen, P.J. 1996. Biology and Ecology of Earthworms. 3rd Edn., Chapman and Hall, London, ISBN: 0412561603, pp: 426.

Gray, C.W., McLaren, R.G., Shiowatana, J. 2003. The determination of labile cadmium in some biosolids-amended soils by isotope dilution plasma mass spectrometry. Australian Journal of Soil Research 41, 589-597.

Ireland, M.P. 1975. The effect of the earthworm *Dendrobaena rubida* and the solubility of lead, zinc, and calcium in heavy metal contaminated soil in wales. Journal of Soil Science. 26, 313-318.

Jones, D.L. 1998. Organic acids in rhizosphere-a critical review. Plant and Soil. 205, 25-44.

Kizikaya, R. 2005. The role of different organic wastes on zinc bioaccumulation by earthworm *Lumbricus terrestris L. (Oligochaeta)* in successive Zn added soil. Ecological Engineering 25, 322-331.

Lanno, R., Wells, J., Conder, J., Bradham, K., Basta, N. 2004. The bioavailability of chemicals in soil for earthworms, Ecotoxicology and Environment Safety 57, 39-47.

Lee, K.E. 1985. Earthworms: their ecology and relationships with soils and land use. Academic, Sydney.

Lindsay, W.L., Norvell, W.A. 1978. Equilibrium relationships of Zn^{2+}, Fe^{2+}, Ca^{2+} and H^{+} with EDTA and DTPA in soils. Proceedings - the Soil Science Society of America 33, 62-68.

Ma, Y., Dickinson N.M., Wong M.H. 2002. Toxicity of Pb/Zn mine tailings to the earthworm *Pheretima* and the effects of burrowing on metal bioavailability. Biology and Fertility of Soils 36, 79-86.

Ma, Y., Dickinson, N.M., Wong, M.H. 2003. Interactons effects of earthworms trees, soil nutrition and metal mobility in amended Pb/Zn mine tailings from Guangdong, China. Soil Biology and Biochemistry 35, 1369-1379.

Maity, S., Padhy, P.K., Chaudhury, S. 2008. The role of earthworm Lampito mauritii (Kinberg) in amending lead and zinc treated soil. Bioresource Technology 99(15), 7291-7298.

Martin, A. 1991. Short and long term effects of the endogenic earthworm Millsonia anomalia (*Megascolecidae, Oligochaeta*) of tropical savannas, on soil organic matter. Biology and Fertility of Soils 11, 234-238.

McGrath, S.P., Zhao, F.J., Lombi, E. 2001. Plant and rhizosphere process involved in phytoremediation of metal-contaminated soils. Plant and Soil 232(1/2), 207-214.

Morgan, J.E., Morgan, A.J. 1998. The distribution and intracellular compartmentation of metals in the endogeic earthworm *Aporrectodea caliginosa* sampled from an unpolluted and a metal-contaminated. Environmental Pollution. 99, 167-175.

Nelson, D.W., Sommers, L.E. 1982. Total carbon, organic carbon, and organic matter. In: Methods of Soil Analysis. 2nd Ed. ASA Monogr. 9(2). Amer. Soc. Agron. Madison, WI, pp. 539-579.

Ponder, F.Jr., Li F., Jordan, D., Berry, E.C. 2000. Assessing the impact of *Diplocardia omata* on physical and chemical properties of compacted forest soil in microcosms. Biology and Fertility of Soils 32, 166-172.

Rada, A., El Gharmali, A., Elmeray, M., Morel, J.L. 1996. Bioavailability of cadmium and copper in two soils from the sewage farm of Marrakech city (Morocco): effect of earthworms. Agricoltura Mediterranea 126, 364-368.

Shan, X.Q., Lian, J., Wen, B. 2002. Effect of organic acids on adsorption and desorption of rare earth elements. Chemosphere 47, 701-710.

Salmon, S. 2001. Earthworm excreta (mucus and urine) affect the distribution of springtails in forest soils. Biology and Fertility of Soils 34, 304-310.

Schalscha E, Ahumada I. 1998. Heavy metals in rivers and soils of central chile. Water Science and Technology 37(8), 251-255.

Scott-Fordsmand, J.J., Stevens, D., McLaughlin, M. 2004. Do earthworms mobilize fixed zinc from ingested soil? Environmental Science and Technology 38(11), 3036-3039.

Sims, R.W. and Gerard, B.M. 1999. Earthworms: Notes for the Identification of British Species, Synopses of the British Fauna (New Series) No.31 (Revised). London: Linnean Society.

Sizmur, T., Hodson, M.E. 2008. The impact of Eisenia veneta on As, Cu, Pb and Zn uptake by rygrass (Loliium perenne L.). Mineralogical Magazine 72(1), 495-499.

Sizmur, T., Hodson, M.E. 2009. Do earthworms impact metal mobility and availability in soil? A review. Environmental Pollution 157, 1981-1989.

Sizmur, T., Palumbo-Roe, B., Watts, M.J., Hodson, M.E. 2011. Impact of the earthworm Lumbricus terrestris on As, Cu, Pb and Zn mobility and speciation in contaminated soils. Environmental Pollution 159, 742-748.

Spurgeon, D.J., Hopkin, S.P. 1995. Extrapolation of the laboratory-based OECD earthworms (*Eisenia fetida*) exposed to contaminated field and laboratory soils. Applied Soil Ecology 11, 227-243.

Spurgeon, D.J., Hopkin, S.P. 1999. Tolerance to zinc in populations of the earthworm Lumbricus rubellus from uncontaminated and metal-contaminated ecosystems. Archives of Environmental Contamination and Toxicology, 37, 332-337.

Stephens, P.M. Davoren, C.W., Double, B.M., Ryder, M.H. 1994. Ability of the earthworms *Aporrectodea rasea* and *Aporrectodea trapezoides* to increase plant growth and the foliar concentration of elements in wheat (*Triticum aestivum cv. spear*) in a sandy loam soil. Biology and Fertility of Soils 18, 150-154.

USEPA. 2007. Method 3051: Microwave assisted acid digestion of sediments, sludges, soils and oils. Revision 1. Washington, DC, USA.

Udovic, M., Lestan, D. 2007. The effect of *Eisenia fetida* on the redistribution of residual

Pb, Zn and Cd in soil after remediation with EDTA leaching. Environmental Pollution 148, 663-668.

Wen, B., Hu, X., Liu, Y., Wang, X., Hu, F. Shan, X. 2004. The role of earthworms (*Eisenia fetida*) in influencing bioavailability of heavy metals in soils. Biology and Fertility of Soils 40, 181-187.

Table and Figures

Table 5.1 Soil and casts properties

	pH			Organic Carbon (%)		
Sample	Soil	Casts from *L. terrestris*	Casts from *E. fetida*	Soil	Casts from *L. terrestris*	Casts from *E. fetida*
P6	4.80 ±0.04	5.76 ±0.10	5.51 ±0.08	2.39 ±0.03	2.27 ±0.02	2.21 ±0.05
Romona	5.30 ±0.06	5.86 ±0.07	5.62 ±0.09	8.58 ±0.08	7.93 ±0.10	7.80 ±0.08
Mill01	5.21 ±0.06	5.46 ±0.08	5.41 ±0.07	1.73 ±0.03	1.56 ±0.04	1.48 ±0.02

The values listed in the table are mean value ± standard deviation for three triplicate analysis.

Figure 5.1 Total content of Cd, Zn in soils and corresponding casts. (a) Zn (b) Cd. (blank: soil; grey: casts from *L. terrestris*; black: casts from *E. fetida*). Error bars represents standard deviationfor three triplicate analysis.

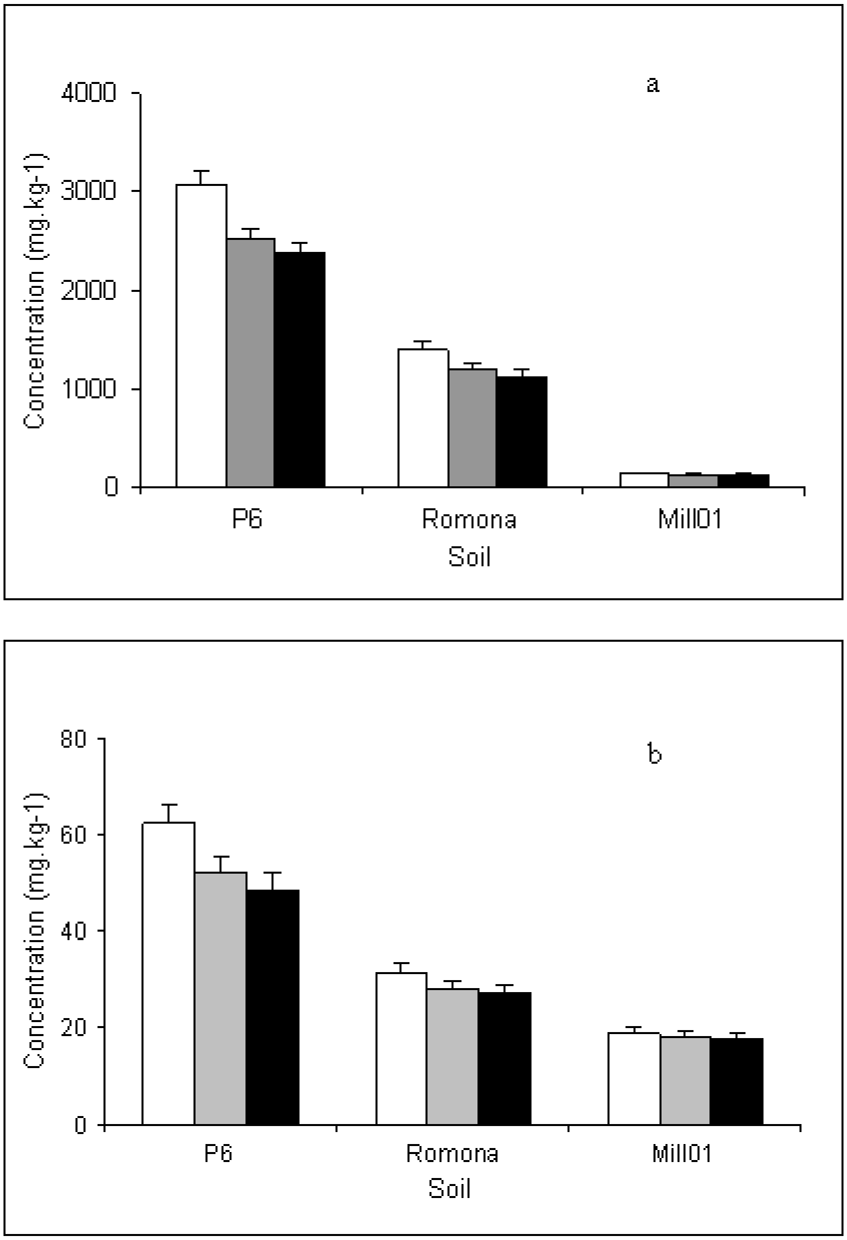


Figure 5.2 0.01 M $Ca(NO_3)_2$-extractable heavy metals in soils and corresponding casts. (a) Zn (b) Cd. (blank: soil; grey: casts from *L. terrestris*; black: casts from *E. fetida*). Error bars represents standard deviation for three triplicate analysis.

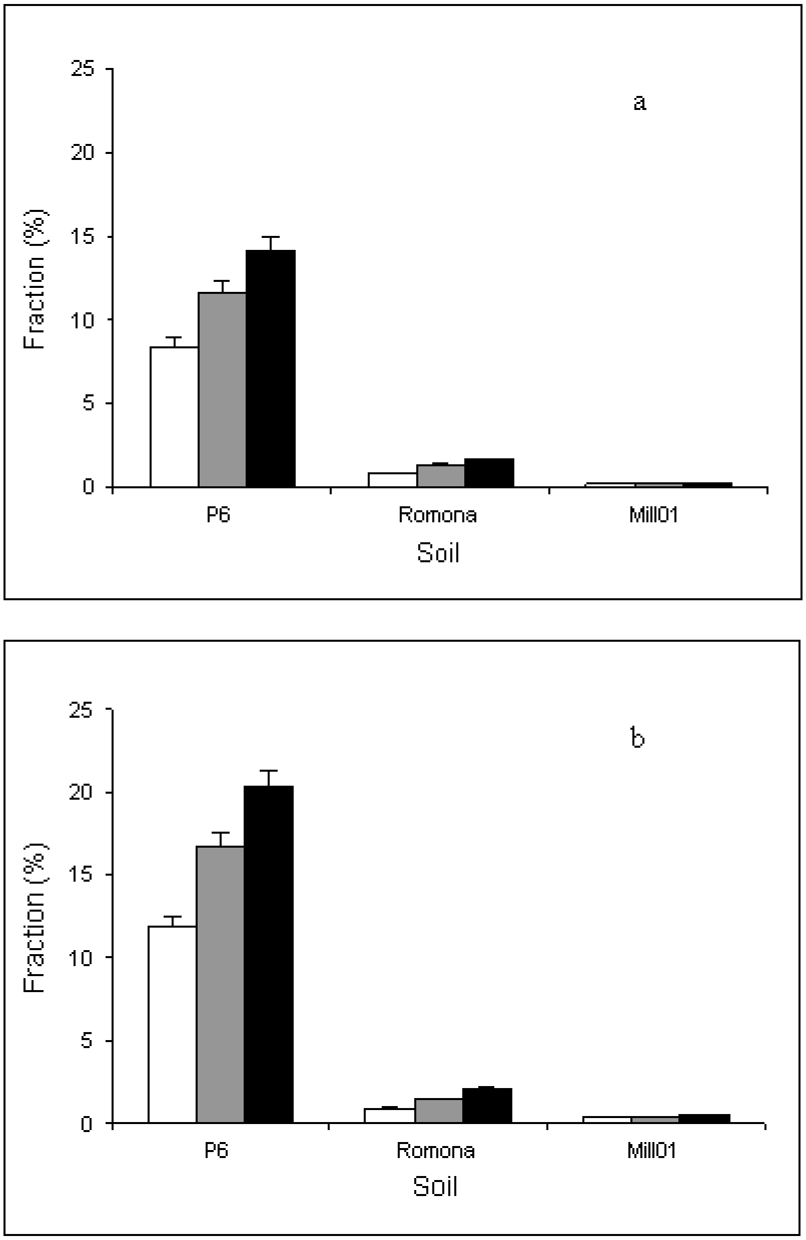


Figure 5.3 1M $CaCl_2$-extractable heavy metals in soils and corresponding casts. (a) Zn (b) Cd. (blank: soil; grey: casts from *L. terrestris*; black: casts from *E. fetida*). Error bars represents standard deviation for three triplicate analysis.

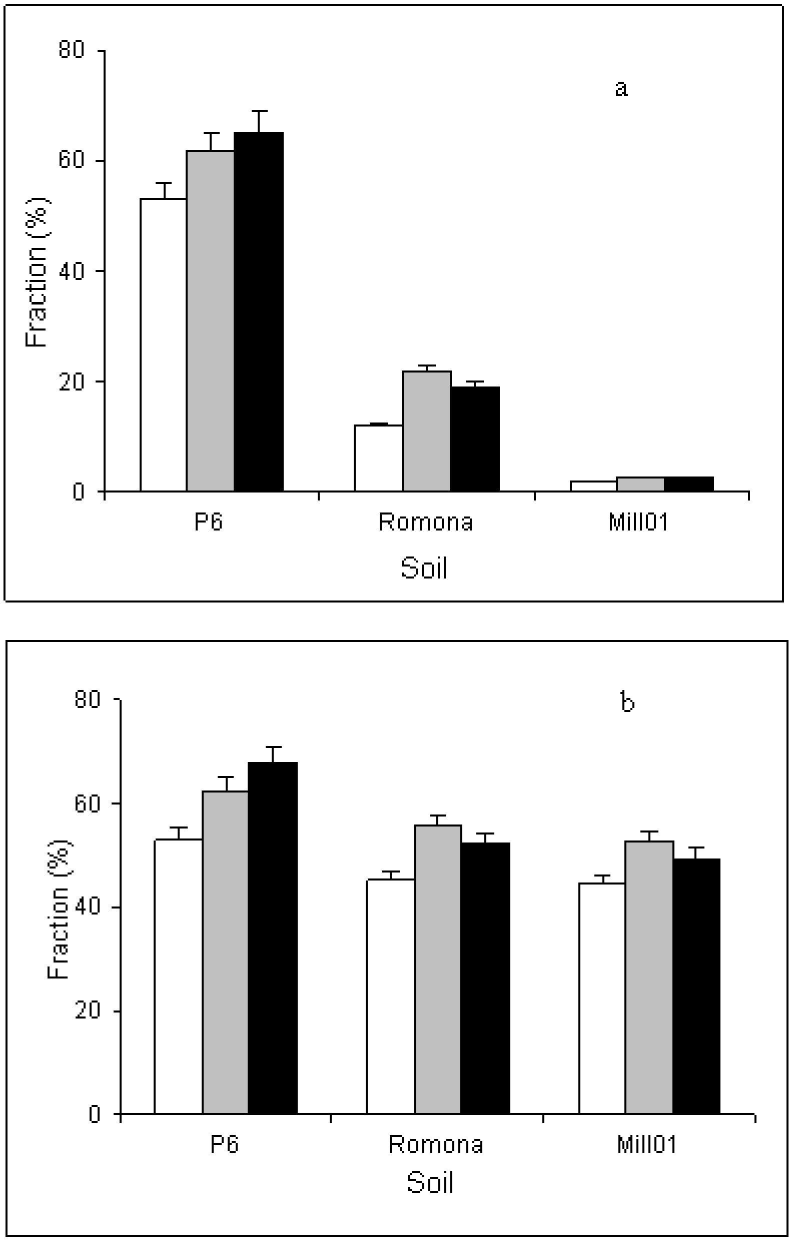


Figure 5.4 Fraction of labile heavy metals in soils and corresponding casts. (a) Zn (b) Cd. (blank: soil; grey: casts from *L. terrestris*; black: casts from *E. fetida*). Error bars represents standard deviationfor three triplicate analysis.