The Impact of Long Term Biosolid Application on Soil Health

By

Abdul Wahab Mossa

(BSc Agricultural Sciences; MSc Soil Science)

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

May 2017

Abstract

The disposal of biosolids poses a major environmental and economic problem. Agricultural use is generally regarded as the best means of disposal. Although the impact on soil ecosystems remains uncertain. Biosolids can improve soil properties by supplying nutrients and increasing organic matter content but there is also a potentially negative impact arising from the introduction of heavy metal contaminants into soils. It is widely acknowledged that the bioavailable fraction, rather than total metal content, is indicative of plant metal uptake and toxicity. The bioavailable metal fraction in turn is dependent on soil properties. Therefore, the overall aim of this work was to determine the bio-geochemical factors that control the dynamics of trace element bioavailability in soils that have been subject to the disposal of sewage sludge for over 100 years. Three main investigations were undertaken. In order to determine the current metal composition of the site and identifying the geochemical factors that control the dynamics of metals bioavailability, thirty -eight fields, from a dedicated sewage sludge disposal site for over 100 years, were sampled for both soil (bulk and rhizosphere) and plant. Special attention was devoted to determining soil properties that govern metal partitioning between different metal pools (i.e. total, isotopically exchangeable, $Ca(NO_3)_2$-extractable and free ion activity).

In order to identify the best estimate of plant uptake and toxic response, a pot experiment was carried out to compare the effects of Zn on plant growth in soils recently spiked with Zn and soils historically amended with biosolids

to identify soil properties that best predict metal uptake and subsequent phytotoxicity.

The effect of biosolids on soil microorganisms was assessed. Terminal restriction fragment length polymorphism, a fingerprint molecular technique, in combination with multivariate data analysis were used to relate soil microbial diversity and community structure to metal accumulation and bioavailability.

High levels of contamination, exceeding the current limits for the use of biosolids in agriculture, were observed in the studied soils reflecting extensive long-term biosolid application. Enrichment factors in relation to background levels in the area were greater than 5 and followed the trend Cd>Cu>Zn>Pb>Ni. Copper and Cd exhibited extremely high enrichment levels, up to 106 and 151 respectively.

Except for Pb, the isotopically exchangeable pool of the studied metals (*E*-value) was mainly controlled by the total metal content in soil, accounting for more than 90% of the variation in *E*-values. Lead lability was primarily controlled by the total P, LOI and Fe oxides. Metal labilities expressed as % of total metal content were <40% for the five studied elements following decreasing order of Cu > Cd > Zn ≈ Ni > Pb.

Apart from Pb, all the bioavailability estimates (total, E-values, $Ca(NO_3)_2$-extractable and free ion activity) correlated strongly with metal concentration in plant, accounting for more than 70% of the variation in plant concentrations. $Ca(NO_3)_2$-extractable provided the best estimate out of the four measures of bioavailability, accounting for 87, 77, 87 and 83% of the variation in plant concentration of Ni, Cu, Zn and Cd respectively.

The results of the pot trial showed that 67-90% of the added Zn remained isotopically exchangeable after 3 months of Zn addition, suggesting that rapid adsorption processes take place, followed by a slow aging process that cannot be detected over the period of the experiment (3 months). The speciation of soil solution showed that Zn was present mainly (80% on average) as free ion indicating the low affinity of this metal to complexation by dissolved organic matter. An antagonistic relationship was observed between Zn and Cd suggesting that greater Zn availability suppressed Cd uptake by plant. Although Zn addition increased Cd concentration in the soil solution, Cd transfer factor was simultaneously inversely correlated with Zn concentration in soil solution.

The free ion activity model (FIAM), based on the biotic ligand model (BLM), accounted for 94% of the variation Zn concentration in plant. Cadmium appeared to play an important role in competing with Zn for uptake. A simple regression model utilising soil total Zn, soil organic matter and soil pH accounted for 88% of the variation in plant uptake. This indicates the possibility of using soil properties that are measured routinely as input for prediction of plant uptake. The results of the Zn phytotoxicity test indicated that the intensity of the exposure (i.e. free ion activity) was the key quantity in the context of predicting plant toxic response, describing 80% of the variation in the response of barley growth to Zn toxicity. Only labile Zn from the quantity based extraction was able to describe the toxic response explaining only 46% of the variation.

The study of the effect of biosolids on soil microorganisms showed that soil total Zn concentration could be adopted as a good indicator of the overall (historical) biosolids loading. A biosolids loading, equivalent to 700 – 1000

mg kg^{-1} Zn appeared to be optimal for maximum bacterial and fungal diversity. This markedly exceeds the maximum soil Zn concentration of 300 mg kg^{-1}permitted under the current UK Sludge (use in agriculture) Regulations. Redundancy analysis (RDA) suggested that the soil microbial communities had been altered in response to the accumulation of trace metals, especially Zn, Cd, and Cu.

Based on the findings of this thesis, it can be concluded that (i) the estimation of metal speciation, both in the solution and solid phase is a key factor in determining the bioavailability and thus, has greater chemical and biological significance than soil total metal content; (ii) the maximum beneficial effect of biosolids on soil microbial diversity occurred at a metal (Zn) concentration well in excess of current regulations governing application of biosolids to agricultural land. This indicates that soil microbial diversity is unlikely to be determining factor for regulatory limits for biosolids disposal to agricultural lands.

ACKNOWLEDGEMENT

I gratefully acknowledge the financial support from the Islamic Development Bank.

My sincere gratitude and appreciation for Professor Neil Crout and Dr. Scott Young for their supervision and patient support throughout the study. I would also like to express my gratitude for Professor Mathew Dickinson and Dr. Helen West for their guidance and contribution with Chapter 5.

I am thankful to the administrative and technical team at the Agricultural and Environmental Sciences division, Emma Hooley, John Corrie, Darren Hepworth, Saul Vazquez, Laura Holt and James Verran, for their support and help.

Finally, I would like to thank Severn Trent Water Ltd. for their co-operation and access to the study site.

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1. General introduction and literature review

Ten million tonnes of dry sewage sludge, a by-product of wastewater treatments, are generated per annum in EU-27 countries alone (Heimersson et al., 2017). This is expected to increase to 13 million tonnes by 2020 (Verlicchi and Zambello, 2015). The management of this sludge involves substantial cost and effort (Babel and del Mundo Dacera, 2006). According to Fang et al. (2017), treatment and disposal of each tonne of sludge cost $10-31 USD. Therefore, developing an appropriate disposal method, that is economically sound and environmentally safe, is imperative. Currently, the most common disposal methods include incineration, landfilling, dumping at water (sea/ocean) and land application (Sánchez-Monedero et al., 2004a; Verlicchi and Zambello, 2015). Land application has been adopted as a common practice worldwide for a number of reasons: (I) sewage sludge contains a substantial amount of plant nutrients (especially N and P) and organic matter (Hale et al., 2012) which makes it a suitable option as a fertilizer and soil amendment; (II) prohibition of dumping into oceans and (III) the high cost involved in incineration and landfilling. For example, the cost of recycling sewage to agricultural land is approximately 30-50% less than that of landfilling and incineration (Antille et al., 2017)

Once sewage sludge has been treated (i.e. digested, composted and dried) it is often refer to as "biosolids" (Verlicchi and Zambello, 2015).

1.1. Use of biosolids as a soil amendment

The high organic matter content and concentrations of macro and micronutrients in biosolids will result in modification of most soil physical, chemical, and biological properties.

1.1.1. Impact on soil physical properties

Biosolids, due to their high content of organic matter, are considered an effective material for improving soil physical properties (Tsadilas et al., 2005; Weber et al., 2007). Reducing bulk density, increasing porosity, improving structural stability and aggregate stability are among the potential benefits arising from of application of biosolids (Annabi et al., 2011). Generally, these changes increase water retention capacity and, in the long-term, enhance water transmission properties and resistance to soil erosion. Angin and Yaghanoglu, (2011) found that sewage sludge application at rates of 0, 40, 80, and 120 t ha^{-1} significantly decreased bulk density and increased both soil porosity and permeability coefficient significantly. However, the effectiveness of the application of sewage sludge decreased during the experimental period, which was attributed to the mineralization of sewage-borne organic matter with time. Likewise, the results of Samaras et al. (2008) showed that applying sewage sludge at rates of 10, 30 and 50 t dry solids $ha^{-1}year^{-1}$ decreased soil bulk density and increased field water holding capacity with increasing rate of application.

1.1.2. Impact on soil chemical properties

The application of biosolids enriches soil with organic carbon, affects soil pH and, due to the high content of trace elements, its application may result in

trace elements accumulating in the soil. AlWabel et al. (1998) investigated the effects of biosolids application at different rates (0, 25, 50, 75, 100 t ha^{-1}) on soil pH and heavy metal concentration. Their results indicated that there was a positive correlation between the application rate and total soluble salts, whereas a negative correlation was found between the application rate and pH values. They also found that heavy metal concentration and mobility increased with increasing application rate, but this was restricted to the upper layer (0-30 cm). The results of Bergkvist et al. (2003) showed a reduction in pH in the 0-20 cm layer in biosolids-amended soil, which was attributed to the mineralization of added organic compounds rich in N and S in addition to the accumulation of the stable humic compounds. They also found that soil organic matter was increased by 60% in the topsoil layer mainly due to the application of biosolids. Similarly, the results of Samaras et al. (2008) showed that repeated application of biosolids at 10, 30 and 50 t dry solids ha^{-1} $year^{-1}$ increased organic matter, decreased pH and increased total N and extractable P. The reduction of pH was partly attributed to low pH of applied sludge in addition to nitrification of ammonium contained in the added sludge. Their results also indicated an increase in soil available P and nitrate-N beyond crop requirements which might result in surface and ground water contamination at high rate of biosolid application. In addition to the nutrient enrichment effects of biosolids application, biosolids contain high levels of persistent organic and inorganic pollutants (Fang et al., 2017; Hale et al., 2012; Kim et al., 2017). Consequently, biosolids application may result in significant increases in the concentration of these pollutants in soil. Wang et al. (2008) found that, besides greater soil nutrient content, heavy metal

concentrations also increased in soil with increasing biosolids application rates at 0, 15, 30, 60, 120 and 150 t ha^{-1}. They reported 86, 46, 28 and 25% concentration increase for Cd, Pb, Cu and Zn in biosolids-treated soils compared to a control soil. However, only Cd concentration was higher than permissible levels for environmental quality standards for agricultural soil, while the concentrations of Pb, Cu and Zn were within the Chinese environmental quality standards for soil. Roig et al. (2012) also reported that levels of potentially toxic metals were significantly higher in sludge-amended plots than in control plots. Nevertheless, the concentrations were well below the regulatory limits set by international guidelines. Creamer et al. (2008) found that high application rates of biosolids resulted in high Zn and Cu concentration in soil exceeding current UK statutory limits for safe use in agriculture. The results of Carbonell et al. (2009) revealed that moderate doses of biosolids (30–60 t ha^{-1}) applied to agricultural soil did not produce an increase in heavy metal content, but high doses (120 t ha^{-1}) caused a significant increase in Cd, Cu, Zn and Hg.

1.1.3. Impact on soil microbial activity

Soil microorganisms play a fundamental role in soil quality as they are responsible for many ecological processes, most notably decomposition of organic matter and nutrient recycling (Banerjee et al., 1997). Therefore, evaluating the impact of biosolids on soil microorganisms is vital. Increased organic matter in soil through biosolid application has the potential to stimulate soil microbial activity as it provides a new source of energy (Marschner et al., 2003). However, the high content of heavy metals in biosolids may have negative effects on soil microbial activity (Singh and Agrawal, 2008a). Poulsen et al. (2013) observed no detrimental impact on

soil microbial diversity and activity after substantial biosolid additions (i.e. equivalent to normal annual application rates over 50-60 yr of biosolids application). On the contrary, their results indicated beneficial short to medium-term effects on the microbial community compared to a control soil to which no addition of organic substrates has been made. The results of Sastre et al. (1996) also suggested that sewage-sludge applications at two rates (50 and 100 t ha^{-1}) increased microbial activity in soil. Sánchez-Monedero et al. (2004) studied the effect of different degrees of biosolids stabilization, achieved through composting, on soil microbial biomass. They found that the soil microbial biomass increased in all biosolids treatment and the increase was related to the degree of stabilization of the biosolids. Higher degrees of stabilization produced lower microbial biomass development during the incubation period. Gomes et al. (2010) reported that although the incorporation of biosolids into soil is an acceptable agricultural practice from a nutrient cycling point of view, the application of highly metal-contaminated sludge may affect the soil microbial communities. Their results suggested particularly that the amendment of soil with sludge contaminated by Cd and Zn might have both short-term and long-term effects on soil bacterial groups.

Another challenge to the use of biosolids in agriculture is the potential of the spread of anti-biotic resistant genes in soil (Bondarczuk et al., 2016). Threedeach et al. (2012) found that 80-87% of E-coli isolated from semi-aerobic and anaerobic treated municipal waste were resistant to antibiotics. Rahube et al. (2014) studied the effect of treated and untreated biosolids on pathogenic bacteria and antibiotic-resistant genes in soil. Their results showed that 49% of coliform bacteria were resistant to the antibiotic

Cefoxitin in control soils, whereas 86% were resistant in sewage-treated soils. They also found that all the targeted antibiotic-resistant genes were detectable, in both soil and crops, in sewage-treated soils but not in the control soils.

1.2. The concept of metal bioavailability in soils

The accumulation of inorganic contaminants in the soil ecosystem, particularly potentially toxic elements, is of great concern due to potential threats to human health (food chain), crop production (phytotoxicity) and soil health. Furthermore, it is a general consensus that those risks are not directly related to the total metal content which is only indicative of the risk arising from contamination in soil (Kim et al., 2015a). Accordingly, regulatory frameworks and policies are moving toward adopting the "bioavailable fraction", rather than the total content, in risk assessments. Therefore, assessing the bioavailability of metals is crucial in determining the environmental impact of inorganic contaminants in soils.

According to Caussy et al. (2003) bioavailability is a dynamic process comprising two distinct phases: (I) dissolution and release from soil matrix "external bioavailability" and (II) absorption causing toxicological effects in organisms " internal bioavailability". The total metal concentration in soil comprises various fractions that are distributed between the soil solid and solution phases. Generally, these fractions can be grouped into four compartments (Chaignon et al., 2003; Hooda, 2010):

(I) The solution phase contains free metal ions and organically and inorganically complexed species

(II) Adsorbed metal ions on the soil particulate phases (clays, organic matter and oxides)

(III) Metal associated with soil secondary minerals (e.g. phosphates, sulphides, phosphates)

(IV) Metal ions occluded in the lattice of soil primary minerals.

Generally, only the fraction in the first compartment is considered bioavailable, while the second compartment is responsible for buffering the soil solution concentration in response to depletion (e.g. plant uptake or leaching). These two compartments together constitute the soil 'labile' metal pool (Hooda, 2010). The fraction that is associated with primary and secondary minerals may be regarded as inert and does not react to changes in metal concentrations in the soil solution. Therefore, while total soil metal concentration provides the maximum pool of metal in the soil, other soil characteristics are likely to have a greater importance in determining metal bioavailability.

1.2.1. Soil properties influencing metal bioavailability

In general, metal bioavailability is governed by sorption and desorption processes in soil (Zeng et al., 2011). Soil characteristics such as pH value, organic matter, clay and mineral oxide contents and redox status have been demonstrated to be the main factors influencing sorption/desorption of metals in soil (Boshoff et al., 2014; Zeng et al., 2011).

Soil pH directly, and indirectly, influences almost all soil biogeochemical processes that govern bioavailability in soil. These processes include precipitation, sorption, solubility and soil microbial activity (Carrillo-González et al., 2006; Naidu, 2008). Metal solubility and consequently

bioavailability generally increase with decreasing pH, which can be attributed to increased proton competition for sorption (Rieuwerts et al., 2006; Shaheen et al., 2013) and decreasing pH-dependent negative charge on the sorption complex (Naidu, 2008). Zeng et al. (2011) observed a negative correlation between soil pH and plant concentration of Cr, Cu, Mn and Zn. Rieuwerts et al. (2006) observed that a decrease in soil pH from 6 to 4 corresponded to approximately a 10-fold increase in $CaCl_2$-extractabilty of Zn, Cd and Pb. Twining et al. (2004) reported a strong dependency of Zn partition coefficient (K_d) on soil pH, increasing by more than an order of magnitude from 40 to 3000 ml g^{-1} over the pH range 4.4 – 7.3.

Soil organic matter content (SOM) is another factor influencing metal bioavailability. SOM is a major contributor to soil cation exchange capacity (CEC) through its surface negative charge resulting from the dissociation of carboxylic and phenolic acid groups (Hodson, et al., 2011), which will strongly affect the ability of soil to retain cationic metals. In addition, organic compounds such as humic and fulvic acids act as chelating agents forming complexes with more than one bond leading to the formation of a very stable ring structures (Hodson, et al., 2011) and thereby reducing metal bioavailability.

It is important however to consider the effect of the relationship between soil pH and SOM on metal bioavailability. For instance, even though dissolved metal concentration decreases at high soil pH, the solubility of organic matter increases. Hence, depending on the metal affinity for dissolved organic matter (DOM), metal bioavailability may increase as pH rises due to formation of metal complexes with DOM (Sauvé et al., 2000). Hooda and Alloway, (1998) studied sorption behaviour of Cd and Pb in

semi-arid (Indian) soils and English soils. They found that the English soils retained Cd and Pb more strongly than Indian soils, despite having lower pH value (4.7-7.6); this was attributed to higher organic matter content in English soils.

Clays and mineral oxides provide more sites for adsorption, thus increased soil content of clay and mineral oxides will result in reduced metal bioavailability. Soil redox conditions will also have an impact on metal bioavailability, because under reducing conditions Fe and Mn oxides become unstable and dissolve leading to the release of any sorbed metal into the soil solution (Hodson, et al., 2011)

Soil microorganisms also play an important role in the solubilisation, mobilization, and leaching of trace elements in soils (Qureshi et al., 2003). Leita et al. (1999) reported that the concentrations of DTPA-extractable Cd and Ni were strongly correlated with microbial biomass and activity, suggesting that the mobilization of both metals were mediated by microbial biomass. The results of Qureshi et al. (2003) indicated that microbial acidification enabled leaching of trace elements from the application of sludge where the condition for microbial growth were favourable and where no matrix was present for re-adsorption of the released metals. Olivie-Lauquet et al. (2001) found greater concentrations of trace elements in soil solution during summer months than in winter months, coupled with greater dissolved organic carbon contents. They suggested that microorganisms were responsible for higher concentrations of trace elements and dissolved organic carbon.

1.2.2. Methods for determining metal bioavailability in soils

The total content of metals in soil is still commonly used in risk assessments and guidelines (Prasad et al., 2005). This is measured by dissolution with strong acids (HF, $HClO_4$ and HNO_3). This content comprises both geogenic and anthropogenic inputs and hence is a poor indicator of bioavailability. Therefore, a more relevant to bioavailability measure, "pseudototal" using aqua regia digest (HNO_3/HCl), is often used. This pseudototal does not include the fraction that is locked within silicate lattices and therefore is a measure of the maximum amount that could potentially be mobilised over a long period of time (Rao et al., 2008).

Several extraction approaches have been developed to measure the bioavailable pool of metals in soil. These approaches can be classified as either single extractions, in which a single extractant is used to dissolve an operationally defined form of metal in soil, and sequential extractions, where a series of increasingly aggressive extractants are used to target various, operationally defined, fractions that are associated with soil solid phases (Hooda, 2010). The most widely used sequential extraction procedures are those suggested by Tessier et al. (1979); in which five fractions are identified: (I) exchangeable; (II) bound to carbonates; (III) reproducible, bound to Fe and Mn oxides; (IV) oxidisable, bound to organic matter and (V) residual, bound to primary mineral structure (i.e. insoluble)

Dilute salt extractants e.g., $Ca(NO_3)_2$ and $CaCl_2$ with an ionic strength close to that of the soil solution have been extensively used as a single extraction to mimic soil solution concentrations (Datta and Young, 2005; Fien Degryse et al., 2009; Hamels et al., 2014; Marzouk et al., 2013a). The use of neutral

salt solutions as extractants is based on the hypothesis that readily soluble metals are weakly adsorbed on soil surfaces; and can be easily replaced by other cations such as Ca^{2+} (Kim et al., 2015a; Menzies et al., 2007). The ionic strength of 0.01 M is the most commonly used. However, other concentrations have been used; for example Schröder et al. (2005) concluded that 0.0025 M $CaCl_2$ rather than 0.01 M $CaCl_2$ was in better agreement with the ionic strength of pore water in floodplain soils, implying that 0.01 M would overestimate metal concentration in soil solution. Hamels et al. (2014) used 0.001 M $CaCl_2$ to investigate the phytotoxicity of Zn and Cu in field-contaminated soils.

Soil solution is the main exposure route for soil biota, including plants (Hooda, 2010). As a result, determination of metal concentration in soil solution has gained recognition as a mean for assessing bioavailability. Several analytical techniques (e.g. ion-selective electrodes, ion-exchange resins and Donnan membrane systems) and geochemical computational tools (e.g. WHAM, GEOCHEM and ECOSAT) have been used to determine metal concentration in soil solution (Hooda, 2010). A key limitation of such techniques is that they provide only a snapshot of the system at the sampling time and do not account for the dynamic nature of the relationship between the solid and solution phases in soil (Hooda, 2010). The main feature of this relationship is that a replenishment from the soil labile metal pool, associated with solid phase, may be induced in response to metal depletion in soil solution. Therefore, metal bioavailability in soil is related to metal flux toward plant roots or the cell membrane of soil organisms, which in turn depends on both soil solution concentration and the rate of change of the lability in response to changes in the soil solution concentration

(Hooda, 2010; Peijnenburg et al., 2007). This dynamic process can be mimicked using diffusive gradients in thin films (DGT) (Zhang et al., 2001) which have been widely applied to measure metal bioavailability in soil (Hamels et al., 2014; Nolan et al., 2005). The DGT device consists of a chelating resin gel layer covered by a filter through which ions can diffuse. DGT devices simulate plant roots when deployed to soil by depleting the soil solution through metal accumulation on the resin layer, thus stimulating resupply from the labile pool on the soil solid phase. The diffusional flux toward the DGT device equates to what is called "effective concentration" (Zhang et al., 2001). This concentration is dependent on soil solution concentration (intensity) and labile pool and the rate of the resupply (capacity).

For strongly adsorbed metals where neutral salt extractants are too weak to extract these potentially available pools, chelating agents such ethylene-diamine-tetra-acetic acid (EDTA) (0.1–1 M) and diethylene-triaminepenta-acetic acid (DPTA) (0.005 M), have been used (Fang et al., 2007; Kim et al., 2015a; Menzies et al., 2007). Degryse et al. (2009) suggested that EDTA extracts are the most appropriate choice to approximate the labile metal pool, when used at concentrations ranging from 50 to 100 mmol EDTA per kg soil. They speculated that smaller concentrations would not completely extract all the labile metal whereas larger concentrations would probably mobilise non-labile metal. Dilute mineral acids (e.g.0.43 HNO_3) have also been proposed for extracting the labile metal pool (F. Degryse et al., 2009; Tipping et al., 2003).

Isotopic dilution (ID) is another approach to estimate labile metal in soil. The isotopically exchangeable pool reflects the fraction of metals that is in

equilibrium with the soil solution metal concentration (F. Degryse et al., 2009). The main assumption of this technique is that when an isotope is introduced into a soil suspension, it will redistribute itself between solid and solution phases in the same ratio as other isotopes of the same element, within a operationally defined time frame (F. Degryse et al., 2009; Hamon et al., 2008). The technique was originally used with radioactive isotopes. However, because of the potential risk of exposure to radiation (e.g. ^{210}Pb) and short/relatively long half-lives, the use of enriched stable isotopes has become more common recently, particularly after sensitive analytical instruments for determination of isotope ratios, such as inductively coupled plasma mass spectrometry (ICP-MS), have become more accessible (Hamon et al., 2008; Nolan et al., 2004a). The stable isotopic dilution procedure involves adding an enriched isotope spike of element of interest to a suspension of soil in a neutral salt solution such as $Ca(NO_3)_2$. After isotopic equilibration is achieved, dilution of the spike isotope in soil solution is used to calculate the labile pool, known as the *E*-value (Hamon et al., 2008). The *E*-value calculation is based on three isotopic ratios: the natural abundance ratio, the ratio of the isotopes in the spike solution and the measured ratio in spiked soil suspensions (Eq. 1.1).

$$E = \left(\frac{M_{soil}}{W}\right)\left(\frac{C_{spike}V_{spike}}{M_{spike}}\right)\frac{(^{Iso1}IA_{Spike} - {}^{Iso2}IA_{Spike}R_{SS})}{(^{Iso2}IA_{Soil}R_{SS} - {}^{Iso1}IA_{Soil})} \qquad 1.1$$

Where M_{soil} and M_{spike} are the average atomic mass metal in soils and spike solutions respectively, W is the weight of the soil (kg), C_{spike} is the gravimetric concentration of the metal in the spike solution, V_{spike} is the volume of spike added (L), IA is the isotopic abundance, R_{ss} is the ratio of isotopic abundances for the two isotopes in the spiked soil solution.

Three key considerations should be taken into account when using the ID assay: the amount of the added isotopic tracer (spiking level), equilibration time and suspension matrix.

The quantity of the added isotope tracer should meet two criteria: (I) it should be sufficient to induce a quantifiable shift in the isotopic ratio of the spiked soil in comparison to the natural ratio of the control soil; (II) it ideally should not perturb the equilibration of the system, i.e. there should be no access to any non-labile metal pool through processes such as precipitation. Nolan et al. (2004a) used different concentrations of ^{65}Cu for measuring labile Cu. They concluded that an addition of ^{65}Cu equivalent to 5% of the *E*-value produces reliable results; they suggested an amount equivalent to 1% of Cu when the *E*-value is unknown. Some authors have used proxies for *E*-value to estimate the spike levels, for instance Young et al. (2000a) used 1M $CaCl_2$ as a proxy in case of Cd. The abundance of the spiked isotope is also a factor in determining the spike level, with the lower the abundance the lower the amount of the spike needed (Hamon et al., 2008). For example reliable results could be achieved using only 0.1% of ^{111}Cd (Gray et al., 2003) because ^{111}Cd has a natural abundance of only 12.8% compared to 30.8% for ^{65}Cu.

The introduced isotopic tracer is expected to be in a dynamic equilibrium with soil constituents (Hamon et al., 2008). Therefore, an operationally defined time is usually chosen for equilibration when determining *E*-values. Generally, it is believed that 1-3 days is long enough for the reaction to be complete and very little change in *E*-value will occur beyond that equilibration time (Hamon et al., 2008; Oliver et al., 2006; Young et al., 2006, 2000a).

The key consideration when choosing the suspension matrix is solubilising a measurable amount of the element of interest without bringing non-labile metal into solution. Deionized water and dilute electrolyte solutions, such as 0.01 M $CaCl_2$ (Smolders et al., 1999) and 0.01 M $Ca(CO_3)_2$ (Hamels et al., 2014; Marzouk et al., 2013a), have been used. A possible advantage in using a dilute salt solution is increasing the concentration of the analyte in the suspension due to some degree of the displacement from soil adsorption sites. This is particularly important when the concentration of the analytes in the solution are close to the detection limits (Hamon et al., 2008). Atkinson et al. (2011a) found that 0.01 M $Ca(NO_3)_2$ did not solubilise enough Pb to reliably measure *E*-values in soils amended with biosolids. They suggested 5×10^{-4} M EDTA as a viable suspending electrolyte for determining Pb *E*-value in biosolids contaminated soils.

Extraction based methods can be divided into two categories: (i) intensity-based methods estimate the concentration in the soil solution (total or free metal ions), (ii) quantity-based methods measure the metal fraction held in or on the soil solid phase; the fraction that constitutes the metal reservoir which resupplies soil solution following depletion (Fien Degryse et al., 2009; Hamels et al., 2014; Hough et al., 2005; Kim et al., 2015a). Neutral salt extraction and DGT best reflect intensity based methods, whereas the *E*-value provides the best approach of all the quantity based methods (Hamels et al., 2014; Kim et al., 2015a).

Mechanistic assemblage models have also been developed to compute free metal ion concentrations in soil solution (speciation models). Assemblage models consist of sub-models each describing metal binding to different soil constituents. WHAM/model VI (Tipping, 1998) or the NICA-Donnan model

(Kinniburgh et al., 1999) are currently the most advanced models for metal speciation in soils. Several studies have reported the applicability of these models to metal speciation in the soil solution (Ge et al., 2005; J. E. Groenenberg et al., 2010; Pampura et al., 2006). Both models have also been used to predict metal partitioning between soil solid and solution phases. Marzouk et al. (2013a) used WHAM to predict total concentrations of Zn, Cd and Pb in soil solution. They reported that WHAM accurately predicted soluble concentrations of Zn and Cd, especially when using isotopically exchangeable (E-value) instead of total metal content as the input variable. Bonten et al. (2008) also showed that solution concentrations of Cd, Cu and Zn can be predicted well for a large set of soils with a large variation in soil properties.

1.3. Plant uptake

A range of models has been used to predict plant uptake. These models vary in their complexity, from simple 'equilibrium' approaches where plant concentration is related to specific soil parameters to more mechanistic models where, in addition to soil parameters, certain plant parameters such as root growth are taken into account (McLaughlin, et al., 2011).

One very commonly used approach is the transfer factor (TF), expressed simply as the ratio of total metal concentration in plants or organisms to the concentration in soil. But although the TF approach is simple and easy to apply, it has some limitations: (I) it relies on soil total metal concentration, which may not correspond to the bioavailable fraction (Adamo et al., 2014); (II) it implies a constant linear relationship between the concentration in plant and soil. These assumptions do not reflect what

is generally observed. For instance Adamo et al. (2014) and Wang et al. (2004) reported that TF values decrease with increasing soil total metal concentration. This decreasing trend implies excluding mechanisms operating in plant roots (Adamo et al., 2014; Moodley et al., 2012; Wang et al., 2004). Another possible explanation is that increased total metal concentration will have toxic effects thereby impairing the ability of the organisms for uptake (Wang et al., 2004).

Regression approaches are often used to relate measured plant concentration to the concentration in soil using various extractions (McLaughlin, et al., 2011). Freundlich type equations where

$$\log(M_{plant}) = a + b\log(M_{soil})$$

are often used in this type of approach. Since it is well established that metal uptake is dependent not only on total metal content, but also on other soil properties such as pH and organic matter, extended Freundlich-type equations have also been used (Adams et al., 2004; Ding et al., 2013, 2014; Liang et al., 2013; Liu et al., 2015) to predict plant uptake. Such equations take the following form:

$$\log(M_{plant}) = a + b\log(M_{soil}) + c\,(pH) + d\,log(SOM)$$

where M_{plant} is metal concentration in plant, M_{soil} is the concentration in soil, SOM is soil organic matter, and *a*, *b*, *c* and *d* are constants. Liang et al. (2013) have shown that prediction of Cd variation in spinach was improved from 89%, using only total Cd, to 94% with inclusion of pH and organic carbon.

The free ion activity model (FIAM) is another approach for prediction of plant uptake. The FIAM was developed based on the assumption that metal uptake, and subsequent toxicity, is related to the free ion metal in the soil solution (He et al., 2014; Hough et al., 2005; Kalis et al., 2006; Sauvé et al., 1996). However, this assumption has been challenged by the competition effect of other cations and protons on metal uptake (Degryse et al., 2006; Hough et al., 2005; Kalis et al., 2006; McLaughlin, et al., 2011). For example, Li et al. (2009) reported that pH-adjusted free Cd^{2+} better predicted body Cd concentration than free Cd^{2+} alone in the earthworm *Eisenia fetida.* Hough et al. (2005) also showed that incorporation of protons as competition ions in the FIAM improved the prediction of Cd and Zn uptake in perennial ryegrass (*Lolium perenne* L). Another limitation of the FIAM is that it does not account for other forms of dissolved metal (i.e. complexed) that may be taken up by soil organisms (Degryse et al., 2006). These limitations have led to the development of the biotic ligand model (BLM). There are two main assumptions of BLM theory (Ardestani et al., 2014a; Spurgeon, 2010): (I) free metal ions are the chemical species which are taken up through soil pore water as the main route for uptake; (II) the rate of the uptake is dependent on the concentration of the free metal ion and the composition of soil pore water, particularly the concentration of major cations (mainly H^+, Ca^{2+}, Mg^{2+}) that may compete for binding and uptake. Originally the BLM was developed to predict the toxic effects of metal bound to the gills of fish (Di Toro et al., 2001; He et al., 2014). However, the BLM has also been used to describe Cu and Ni toxicity to barley (Thakali et al., 2006a), Cu and Ni toxicity to invertebrates and microbes in soil (Thakali et al., 2006c), Cu toxicity to

lettuce (Le et al., 2012a) and the effect of Co on root growth of barley (Lock et al., 2007a).

1.4. Current study

This study was conducted at Stoke Bardolph, a sewage treatment works and farm, which is located adjacent to the River Trent east of Nottingham city and operated by Severn Trent Ltd, a major UK water company. The farm has been used for recycling sewage waste since 1880. Successive geochemical surveys of the c. 60 fields that comprise the farm have shown a wide range of metal loadings ranging from background levels to high concentrations of metal contamination (e.g. several thousand mg kg^{-1} Zn). Thus, many of the fields have topsoil that violate the Sludge Regulations and therefore the farm cannot be used to grow crops for direct human consumption. However, traditionally the farm was used to grow a mix of wheat, maize and grass for animal feed, partly for the farm's dairy herd and partly for sale. In 2010, the farm management discontinued dairy farming and invested in an energy crop anaerobic digestion plant (ADP). Therefore, most of the farm is now sown with 'fodder maize' to provide feedstock for the ADP. The maize is first ensiled and the silage is used as ADP feedstock to produce methane, which is then burned to generate electricity on site. The waste produced at the end of the process provides fertiliser material, which is re-applied to the land.

Generally, soil health is defined as the ability of soil to function as vital living system, in accordance with its potential, to maintain biological productivity, maintain the quality of air and water environment, and sustain plant, animal and human health (Doran and Safley, 1997; Doran and Zeiss, 2000).

Therefore, the aim of this work is to characterize arable soils that, historically, have been used for recycling sewage waste. In particular, the study aims to assess the benefits to soil health arising from increased humus content and soil fertility against the potential hazards presented by the suite of trace metal loadings. Implications for both plant and microbial health are assessed in this context.

To accomplish these aims, three main strands of investigation were undertaken:

- Thirty-eight fields were sampled for both soil and plants to:
    - Examine soils in terms of metal accumulation and associated soil characteristics (e.g. humus content, phosphate concentration).
    - Identify soil properties that play a role in governing metal activity, solubility and availability.
    - Investigate the influence of major soil properties on metal partition between soil solid and solution phase.
    - Investigate the relationships between plant uptake and different pools of metal in soil.
- A detailed study involving adding Zn salts to 8 soils with different contamination levels to investigate plant uptake and plant toxic response in relation to various measureable fractions of Zn: total, isotopically exchangeable, EDTA-extractable, $Ca(NO_3)_2$-extractable and the free ion activity in solution.
- A study of the microbial diversity and community structure in relation to metal accumulation and fractionation through biosolids application. This was achieved by using a molecular fingerprinting technique, terminal restriction fragment length polymorphism (T-RFLP).

2. General material and methods

2.1 Soil characterization

2.1.1 Soil pH

Soil pH was measured in a water suspension of air-dried soil (< 4 mm) and Milli-Q water (1:2.5 m/v) after shaking for 30 minutes in an end-over-end shaker. Soil solution (0.01 M $Ca(NO_3)_2$ suspension) pH was also measured. The pH meter was calibrated using buffers at pH 4.01 (potassium hydrogen phthalate) and pH 7.00 (K/Na phosphate).

2.1.2 Organic matter content

Soil organic matter was estimated from loss on ignition (LOI). Approximately 5 g of oven-dried soil (< 4 mm) was ignited in a muffle furnace at 550 °C for 4 hours. The loss of weight was expressed as a percentage of soil organic matter.

2.1.3 Soil solution analysis

Estimates of dissolved trace and major elements were obtained using a 0.01 M $Ca(NO_3)_2$ extraction. Approximately 3 g of soil were weighed into centrifuge tubes and end-over-end shaken for 3 days with 30 mL 0.01 M $Ca(NO_3)_2$ to achieve equilibrium. The soil suspensions were then centrifuged (2200 g) and filtered through 0.22µm syringe filters before analysis. An aliquot of the filtered supernatant solution was analysed for dissolved organic and inorganic carbon using a Shimadzu TOC –Vcp analyser. Another aliquot was acidified to 2% HNO_3 prior to analysis of major and trace elements by ICP-MS (2.1.6).

2.1.4 Soil total metal content

Total soil elemental concentrations were determined following acid digestion. Approximately 200 mg of finely ground soil were digested with 2 mL of concentrated HNO_3 (70% TAG) and 1 mL of $HClO_4$ (70% TAG) at 80°C for 8 hr and then at 100°C for a further 2 hr. An aliquot (2.5 mL) of HF (40 %) was then added and the samples were heated to 120°C for 8 hr. A further 2.5 mL of HNO_3 and 2.5 mL MilliQ water were then added to the dry residue and the vessels heated at 50 °C for 30 min. After the digestion was complete the final volume was made up to 50 mL using Milli-Q water (18.2 MΩ cm). Samples were diluted 1-in-10 with Milli-Q water prior to multi-element analysis by ICP-MS (Section 2.1.6).

2.1.5 Metal lability: *E*-value determination

The concentrations of isotopically exchangeable Ni, Cu, Zn, Cd and Pb were determined using isotopic dilution. Four replicates of each soil were suspended in 0.01 M $Ca(NO_3)_2$ (3 g : 30 mL) and pre-equilibrated using an end-over-end shaker for 2 days. An aliquot (0.4 mL) of multi element isotopic spike solution enriched with $^{62}Ni^{2+}$, $^{65}Cu^{2+}$, $^{70}Zn^{2+}$, $^{108}Cd^{2+}$ and $^{204}Pb^{2+}$, was added to two of the suspensions and the other two were left as controls. The suspensions were shaken for a further 3 days to attain isotopic equilibrium (Marzouk et al., 2013b), centrifuged (2200 g) for 15 minutes and syringe-filtered (<0.22 µm).

The isotopic ratios ($^{62}Ni/^{60}Ni$, $^{65}Cu/^{63}Cu$, $^{70}Zn/^{66}Zn$, $^{108}Cd/^{111}Cd$, $^{204}Pb/^{206}Pb$, $^{204}Pb/^{207}Pb$, and $^{204}Pb/^{208}Pb$), in the filtered supernatant, were determined by a quadrupole Thermo-Fisher Scientific X-Series^{II} ICP-MS (Thermo Fisher Scientific Inc., Waltham, MA, USA). To reduce polyatomic interferences, the

instrument was operated in 'collision cell with kinetic energy discrimination (KED) mode'. To minimize plasma flicker noise, multiple runs (c. 10×100 sweeps) and short dwell times were employed (Mao et al., 2014; Marzouk et al., 2013a) as follows: ^{60}Ni (2.5 ms), ^{62}Ni (10 ms), ^{63}Cu (5.0 ms), ^{65}Cu (10 ms), ^{66}Zn (2.5 ms), ^{70}Zn (10 ms), ^{107}Ag (5.0 ms), ^{108}Cd (10 ms), ^{111}Cd (2.5 ms), ^{202}Hg (10 ms), ^{20}4Pb (10 ms), ^{206}Pb (2.5 ms), ^{207}Pb (2.5 ms) and ^{208}Pb (2.5 ms). To minimise mass bias, a correction factor was estimated at a regular interval throughout the analytical session (every 15 samples). The correction factor was defined as the ratio between the true isotopic ratio (expected) and the isotopic ratio in standard reference material NIST SRM-981 (lead wire).

The isotopic abundance (IA) of the spike isotope was calculated from the isotopic ratios listed above, and the concentrations of 'isotopically exchangeable' Ni, Cu, Zn, Cd, and Pb (*E*-value, mg kg^{-1}) were calculated from the Equation 2.1.

$$E = \left(\frac{M_{soil}}{W}\right)\left(\frac{C_{spike}V_{spike}}{M_{spike}}\right)\frac{(^{Iso1}IA_{Spike} - {}^{Iso2}IA_{Sike}R_{SS})}{(\,{}^{Iso2}IA_{Soil}R_{SS} - {}^{Iso1}IA_{Soil})} \qquad (2.1)$$

Where M_{soil} and M_{spike} are the average atomic masses of Ni, Cu, Zn, Cd and Pb in soils and spike solutions respectively, W is the weight of the soil (kg), C_{spike} is the gravimetric concentration of the metal in the spike solution, V_{spike} is the volume of spike added (L), IA is the isotopic abundance and R_{ss} is the ratio of isotopic abundances for the two isotopes in the spiked soil solution.

2.1.6 ICP-MS settings for multi-element analysis

The concentrations of major and trace elements extracted from both soil and plants were determined by ICP-MS (Thermo-Fisher Scientific X-Series^{II}; Thermo Fisher Scientific Inc., Waltham, MA, USA). The instrument was run employing collision-cell technology with kinetic energy discrimination (CCT-KED) to remove polyatomic interferences; the collision cell gas was 7% H_2 in He. Samples were introduced using an autosampler (Cetac ASX-520 with 4 x 60-place sample racks) via a concentric glass venturi nebuliser (Thermo-Fisher Scientific; 1 mL min^{-1}). Internal standards comprising of Ge (20 µg L^{-1}), Rh (20 µg L^{-1}), Sc (100 µg L^{-1}), and Ir (10 µg L^{-1}) in 2% HNO_3 (TAG) were introduced to the sample stream through a T-piece. Two sets of external calibration standards were used for trace elements (0–100 µg L^{-1}) and major elements (0-30 mg L^{-1}). Plasmalab software was employed to process the samples. When necessary, the software utilized internal cross-calibration between pulse-counting and analogue modes.

3. Characterization of soils subject to long-term biosolids application

3.1 Introduction

The application of waste biosolids to soil is the preferred disposal practice because of its cost effectiveness compared with alternative methods. For example, the cost of disposing biosolids to agricultural lands is approximately 30-50% lower than landfilling or incineration (Antille et al., 2017). Application of biosolids to land supplies additional nutrients and organic matter which improves soil physical, chemical and biological properties (Samaras et al., 2008; Sánchez-Monedero et al., 2004a; Wang et al., 2008). On the other hand, biosolids contain high levels of potentially toxic elements (e.g. Zn, Pb, Cd, Cu, Ni, As and Hg) that may pose a risk to humans and/or ecosystems. (Roig et al., 2012; Singh and Agrawal, 2008a; Wang et al., 2008).

The potential impact of the biosolids-born metals on soil depends more on the soluble and bioavailable fractions than on the total content (Islam et al., 2013). Metal solubility in turn is dependent on soil properties, of which soil organic matter and pH are the most important factors (Antoniadis et al., 2008).

Statistical and mechanistic models have been used to predict metal concentration in the soil solution from soil properties. Statistical models can be either a simple linear relationship between soil properties and the partitioning coefficient K_d (Sauvé et al., 2000) or a Freundlich type equation (Tye et al., 2003). Mechanistic models usually comprise several sub-models

that describe metal sorption on a set of independent adsorption surfaces in soil (Lofts and Tipping, 1998)

In this chapter, a survey was conducted to characterise the geochemical status of soils subject to long-term biosolids application. Soil and plant samples were collected from 31 agricultural fields that have been historically used for recycling sewage waste. The main aim was to quantify the current trace metal composition of the site and determine their solubility and availability to plants. An attempt was made to assess how these characteristics are affected by soil properties. The availability of Ni, Cu, Zn, Cd and Pb was assessed by plant metal content and soil extractability with acid digestion, isotopic dilution and 0.01 M $Ca(NO_3)$.

3.2 Materials and methods

3.2.1 Sample collection.

Soil and plant samples (n = 38) were collected on 7th and 8th of August 2013 from 31 fields on the Stoke Bardolph Estate, Nottinghamshire. Four maize plants were cut 3-4 cm above ground level from within one square meter. Four bulk soil samples within rows were taken from the same square meter area from the upper 20 cm and mixed into one composite sample.

3.2.2 Sample preparation

Leaves were removed from the maize stems and both leaves and stems were washed with tap water followed by deionised water, and oven dried at 60 °C for four days. After drying both stems and leaves were ground and combined into one composite sample using a cutting mill (Retsch, Model SM 100). Soil samples were sieved to <4 mm and air-dried. A subsample (c. 20 g) was agate ball milled (Retsch, Model PM 400) prior to acid digestion.

3.2.3 Plant material digestion and analysis

A portion of finely ground plant material (c. 200 mg) was weighed into digestion vessels and 6 mL of concentrated *Primar* grade HNO_3 was added. The samples were digested using a Multiwave PRO Anton Paar microwave reaction system, in which they were heated at 140°C for 20 minutes and then cooled to 55°C for 15 minutes. Once the digestion was complete, the samples were made up to a final volume of 20 mL with Milli Q water. Replicate digestions of Standard Reference Material NIST 1573a *Tomato Leaves* (National Institute of Standards and Technology) and 3 reagent blank digestions were all undertaken in a similar manner to check accuracy and precision of the digestion and analyses. Full dissolution was achieved in all cases. The recoveries for the certified reference material were in the 78-120% range (median =102). The acid digests were diluted 1-in-10 prior to analysis by ICP-MS (section 2.1.6).

3.2.4 Soil analyses

3.2.4.1 Soil characterization

Soil pH in Milli-Q water (1:2.5 m/v) and 0.01 M $Ca(NO_3)_2$ (1:10 m/v) suspensions were measured (see section 2.1.1). Soil organic matter was estimated from loss on ignition (% LOI) as described in section 2.1.2.

3.2.4.2 Available phosphorus

Available (Olsen) P in soil was estimated using the bicarbonate extraction method described by Olsen et al. (1954). Approximately 2.0 g of soil was weighed into 50 mL centrifuge tubes with approximately 0.3 g of low phosphate charcoal and 30 mL of 0.5 M sodium bicarbonate were added. Samples were end-over-end shaken for 30 minutes before centrifuging at

2500 g for 15 minutes. An aliquot of the extracted supernatant was added to a 50 mL flask to which 2 mL 3 M H_2SO_4 was added to neutralise the $NaHCO_3$, followed by 4 mL of acid molybdate reagent and 4 mL of ascorbic acid as a reducing agent. The final volume was made up to 50 mL using Milli-Q water. The colour was allowed to develop for 20 minutes before transferring an aliquot to 1 cm^3 cuvettes for reading absorbance at 880 nm wavelength by means of a spectrophotometer.

3.2.4.3 Mineral oxides

Estimates of total Al, Fe and Mn oxides were determined using the Dithionite–Citrate–Bicarbonate (DCB) extraction method. The predominant process of this method is reduction using sodium dithionite (0.07 M). Reduced Al, Mn, and Fe are maintained in solution by complexation with citric acid in the system. Sodium bicarbonate (1 M) is used to buffer the system at pH ≈ 7.3 to prevent decomposition of dithionite to hydrogen sulphite and complex Fe^{2+} ions. Twenty mL of reagent was added to 0.25 g of finely ground soil in centrifuge tubes. Samples tubes were shaken upright at 20 °C with lids left slightly open to allow gas escape. The concentrations of Al, Mn, Fe were determined by ICP-MS (Section 2.1.7) after centrifuging and syringe filtration (<0.22 µm).

3.2.4.4 Total soil elemental concentration

Total concentrations were determined in acid digests (section 2.1.5). Samples were diluted 1-in-10 with Milli-Q water prior to multi-element analysis by ICP-MS (Section 2.1.7). Data were corrected using 10 blank digestions. The Standard Reference Material, SRM 2711a *Montana soil*, supplied by the National Institute of Standards and Technology (NIST), was

used to assess the accuracy of the digestion and analysis. Elemental recovery ranged from 75% to 113% (median = 89.2%).

3.2.5 Metal soluble concentrations

Dissolved trace and major elements were measured by ICP-MS (Section 2.1.6) in the solution phase of a 0.01 M $Ca(NO_3)_2$ suspension as described in Section 2.1.3. Dissolved organic and inorganic carbon were also measured in the solution phase of a 0.01 M $Ca(NO_3)_2$ suspension using a Shimadzu TOC –Vcp analyser (section 2.1.4).

3.2.6 E-value determinations

The concentrations of isotopically exchangeable Ni, Cu, Zn, Cd and Pb were measured as described in section 2.1.6. A stock solution of enriched stable isotopes with certified isotopic abundances (using metal foils or oxides purchased from ISOFLEX USA) was prepared to include ^{62}Ni (603 mg L^{-1}), ^{65}Cu (841 mg L^{-1}), ^{70}Zn (142 mg L^{-1}), ^{108}Cd (123 mg L^{-1}) and ^{204}Pb (442 mg L^{-1}). The stock solution was preserved in ≈ 5% HNO_3. The isotopic abundances (IA) of the certified material were as follow ^{62}Ni (IA ≥ 98.2%), ^{65}Cu (IA ≥ 98.9%), ^{70}Zn (IA ≥ 92.8%), ^{108}Cd (IA ≥ 69.7%) and ^{204}Pb (IA ≥ 98.8%). Multi-elemental 'spike solutions' were freshly prepared from the stock solution with the following concentrations: ^{62}Ni (12.1 mg L^{-1}), ^{65}Cu (33.6 mg L^{-1}), ^{70}Zn (5.68 mg L^{-1}), ^{108}Cd (2.5 mg L^{-1}) and ^{204}Pb (53 mg L^{-1}). The enrichment of spike solution concentration was determined after conducting a preliminary experiment to test a range of spike levels on two soils with contrasting (low and high) metal concentrations to ensure analytically reliable shifts in the isotopic ratios between spiked and unspiked samples. The concentrations of isotopically exchangeable Ni, Cu, Zn, Cd,

and Pb, *E*-values, were calculated using equation 2.1. The same experimental procedure was repeated for all soils using 10^{-5} M Na_2EDTA as the suspending solution instead of 0.01 M $Ca(NO_3)_2$. This was in response to the extremely low solubility of Pb in the Stoke Bardolph soils (Atkinson et al., 2011b), which resulted in the 0.01 M $Ca(NO_3)_2$ solubilising an insufficient proportion of the labile pool for a robust measurement of Pb isotope ratios. Thus, Pb data contain only *E*-values obtained using EDTA as the background electrolyte.

3.2.7 Geochemical modelling using WHAM

The geochemical model, WHAM VII (Tipping, 1994), was used to speciate Ni, Cu, Zn, Cd and Pb in the solution phase of the 0.01 $Ca(NO_3)_2$ soil suspension that was used to determine E-values. Inputs to the model included cation and anion concentrations and pH in the $Ca(NO_3)_2$ suspension. The dissolved organic carbon (DOC) was converted to fulvic acid concentration by assuming (i) a carbon content of 50% and (ii) that fulvic acid constituted 65% of DOC (Buekers et al., 2008a; Lofts et al., 2008; Marzouk et al., 2013a). Partial pressure of CO_2 (pCO_2) was set to 0.004 atm and the temperature was set to 25 °C. The speciation model was also used to predict the concentrations of dissolved metal in the soil solution (0.01 M $Ca(NO_3)_2$ suspension). Additional inputs for these calculations included: (I) either total (acid digests) or reactive (*E*-values) of Ni, Cu, Zn, Cd and Pb, (II) particulate phases included humic acid, suspended particulate matter (converted from 3g solid in 30 mL of electrolyte) and mineral oxides (DCB extraction). Humic particulate phase was estimated from soil organic matter (%LOI) by assuming 50% is active humic acid.

3.3 Results and discussion

3.3.1 General soil properties

The general characteristics of soils used in the study are given in Table 3.1. Soil pH in 0.01 M $Ca(NO_3)_2$ showed near-neutral values with very little variation across the soil dataset (6.0-6.9). Soil organic matter contents, expressed as Loss on ignition values, covered a broad range between 3-23%. As expected in biosolids-amended soils, total P concentrations positively correlated with LOI ($r=0.98$ $p<0.001$) and also varied widely, ranging from 945 to 13700 mg kg^{-1}. These observations reflect different biosolids application rates throughout the sampling area and thus a range of responses of soil to variable biosolids inputs can be anticipated. Available P (Olsen) accounted for 1.97-9.4% of the total P in soils. There was a declining trend in the proportion of available P with increasing total P (i.e. increasing biosolids application rate) (Fig 3.1). This is likely due to some P occurring in insoluble forms. Total oxides in soils did not vary greatly, ranging between 0.1–0.9% Al, 1.1–3.6% Fe and 0.02–0.3% Mn in their oxide forms.

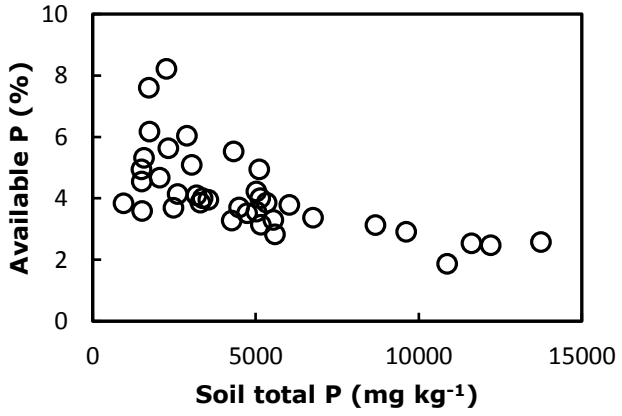


Figure 3.1. Relationship between soil total P and the available proportion of P

Table 3.1. General properties of studied soils

	Min	Max	Median	Mean	St. Deviation
pH	6.43	7.68	6.98	7.00	0.35
LOI (%)	3.52	23.4	9.24	10.2	4.96
	(mg kg^{-1})				
P	945	13700	4300	4750	3230
Mg	3160	21500	5780	6980	426
K	4940	30900	13800	14400	5530
Ca	3830	41800	9490	12700	9290
Al	19300	53600	30600	33500	8230
V	33.9	109	48.4	53.4	17.0
Cr	43.2	1670	327	433	440
Mn	205	1170	546	573	241
Fe	17600	44600	24800	26600	6570
Co	6.72	16.5	11.4	11.4	2.47
Ni	24.4	415	90.4	114	107
Cu	25.3	766	123	198	211
Zn	122	2050	407	565	532
As	8.59	45.6	17.7	18.7	8.21
Se	0.32	2.53	1.01	1.07	0.56
Rb	40.5	96.1	51.9	55.8	13.3
Sr	46.6	136	69.7	74.1	22.9
Mo	1.02	4.04	2.27	2.33	0.70
Ag	0.25	28.23	5.23	6.57	6.45
Cd	0.43	48.6	5.19	9.9	12.7
Sn	5.02	160	34.0	46.5	45.8
Sb	1.65	98.1	13.6	18.7	21.8
Cs	1.89	9.43	2.94	3.55	1.68
Ba	323	1520	568	648	332
Pb	67.7	688	195	245	194
Th	5.55	11.1	7.74	7.99	1.38
U	1.39	3.42	2.63	2.61	0.39

3.3.2 Total metal concentrations in soils

Soil total metal concentrations strongly correlated with soil organic matter (LOI) ($r=0.93–0.98$, $p<0.001$, Table 3.2), suggesting the same source of contamination. Zn was the most abundant metal, spanning over almost 2 orders of magnitude (122–2047 mg kg^{-1}), followed by Cu (25–766 mg kg^{-1}), Pb (66–689 mg kg^{-1}), Ni (24–415 mg kg^{-1}) and Cd (0.4-49 mg kg^{-1}).

Table 3.2. Pearson correlation matrix for soil total metal concentration, soil organic matter (LOI) and soil pH. Bold numbers are not significant at p>0.05.

pH											
-0.58	*LOI*										
-0.65	0.96	*Cr*									
0.13	0.39	**0.26**	*Co*								
-0.63	0.95	0.98	**0.31**	*Ni*							
-0.62	0.97	0.99	**0.29**	0.98	*Cu*						
-0.60	0.97	0.98	0.34	0.98	0.99	*Zn*					
-0.49	0.85	0.85	**0.31**	0.76	0.83	0.84	*As*				
-0.66	0.96	0.97	**0.31**	0.95	0.96	0.96	0.87	*Se*			
-0.69	0.86	0.85	0.33	0.85	0.85	0.86	0.73	0.89	*Mo*		
-0.54	0.78	0.83	**0.23**	0.82	0.83	0.83	0.71	0.81	0.76		
-0.62	0.92	0.96	**0.26**	0.99	0.96	0.96	0.69	0.92	0.84	*Cd*	
-0.50	0.94	0.94	0.40	0.90	0.95	0.96	0.91	0.93	0.79	0.87	*Ba*
-0.60	0.95	0.96	**0.32**	0.93	0.97	0.97	0.89	0.96	0.83	0.90	0.96	*Pb*

The large variation in metal content indicates variable biosolids application rates throughout the sampling area and the time period that each field has been used for biosolids disposal.

Table 3.3. Maximum permissible concentrations of metals in sludge amended soils, permitted by the UK Sludge (Use In Agriculture) Regulations. 1989, No 1263. http://www.legislation.gov.uk/uksi/1989/1263/made

	Soil limits (mg kg^{-1}) according to soil pH			
	pH 5-5.5	pH 5.5-6.0	pH 6.0-7.0	pH > 7.0
Zn	200	250	300	450
Cu	80	100	135	200
Ni	50	60	75	110
	For pH 5.0 and above			
Cd	3			
Pb	300			
Cr	400			
Mo	4			
Se	3			
As	50			

Soil total metal concentrations for most fields exceeded the maximum concentrations of heavy metals in sludge-amended soils permitted by the UK Sludge (Use In Agriculture) Regulations reported in Table 3.3. In

particular, Zn concentrations appeared to be remarkably higher (almost 10-fold) than the regulatory limits (Table 3.3).

Figure 3.2 shows ratios between Zn and several metals in soil as a function of soil organic matter (%LOI), as a proxy of biosolids application in this study. Rubidium was selected as a fingerprint of presence of clays and therefore as an indication of native soil. The positive correlation between Zn:Rb ratio and organic matter indicates that biosolids application caused (i) increased Zn contents in soil and (ii) a dilution effect of *native* soil through increased organic matter content, thus shifting Zn:Rb to greater values. By contrast, Zn to Cu, Cd and Cr ratios were found to decline with increasing biosolids application rate. This indicates that the composition of the biosolids changes over time. Zinc to Ni and Pb ratios did not correlate with the biosolids application rate, also supporting the hypothesis that biosolid composition is not consistent over time and the concentration of biosolid-borne metals in soil are subject to variability. Considering the complexity of the factors (e.g. biosolids composition, leaching, plant uptake) that most likely control the ratios between different metals, it is difficult to infer the change of metal composition of soils that have been subject to biosolids application for over 100 years.

3.3.2.1 Enrichment factors

In order to contextualise the contamination level in the studied soils, enrichment factors (EF) were determined using Eq. 3.1 (Bing et al., 2016; Izquierdo et al., 2013a):

$$EF = \frac{(\frac{Me}{Al})sample}{(\frac{Me}{Al})background} \qquad (3.1)$$

Where $(Me/Al)_{sample}$ is the ratio of the concentration of an element (*Me*) related to *Al* concentration in the sample normalised to the ratio in the regional background $(Me/Al)_{background}$.

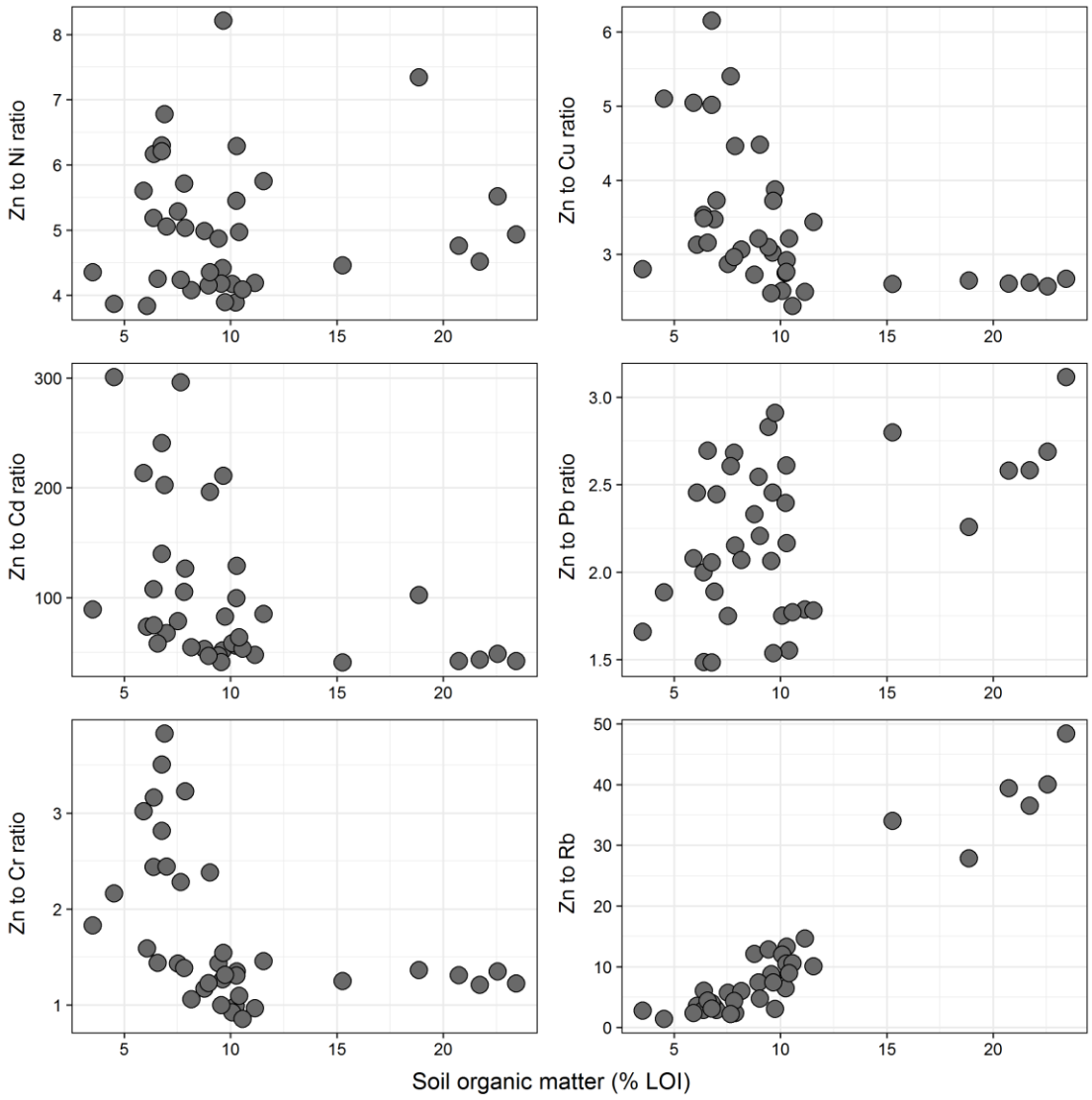


Figure 3.2. Ratios of total metal concentrations between Zn and different metal as a function of organic matter

Background levels used for comparison were the 0.75 percentile of topsoils derived from the Triassic Mercia Mudstone of the UK (Izquierdo et al., 2013a). Figures 3.3 and 3.4 show the enrichment factor plotted against soil organic matter and a box plot of the enrichment factor for Ni, Cu, Zn, Cd, and Pb.

Sutherland (2000) identified five contamination categories based on the enrichment factor: EF<2 depletion to minimal enrichment, EF=2-5: moderate enrichment, EF=5-20: significant enrichment, EF=20-40: very high enrichment, EF>40 extremely high enrichment. In this study, all elements had a mean EF greater than 5 reflecting a significant contamination (Figure 3.4). The mean EFs followed a decreasing order of: Cd>Cu>Zn>Pb>Ni.

Nickel was the least enriched element; nearly 50% of the soils revealed EF<5. The majority of EFs for Zn and Pb were between 5-20. For Cd and Cu, approx. 34% of the samples had an EF greater than 20 for Cu and Cd indicating very high to extremely high enrichment.

3.3.2.2 Cluster analysis

Figure 3.5 shows a dendrogram obtained from hierarchical clustering analysis (Ward's linkage; Minitab 17) of soil total metal concentrations. Two distinct clusters are apparent. The first cluster, comprising Fe, V, Mg, Al, Rb, K, Mn and Co, reflects the presence of main soil components such as aluminosilicates and oxides. The association between Co and Mn is expected due to the isomorphic substitution of Co with Mn in Mn oxides (Ma and Hooda, 2010).

The second group clustered away from lithogenic elements (e.g., Al and K), suggesting non-crustal sources, consists of three sub-clusters (Ca, Sr), (Cu, Zn, Cr, Cd, Ni) and (Pb, Ba, Se). Strontium and Ca clustered together indicating the substitution Ca by Sr in the structure of carbonate minerals (Towett et al., 2015) and may suggest a similar origin such as liming. The second and third sub-group also clustered away from lithogenic elements (e.g., Al and K) suggesting non-crustal sources. Metals that are most likely

to be associated with biosolid application, such as Cu, Zn, Cr, Cd, Ni, cluster together in one group (Fig. 3.5).

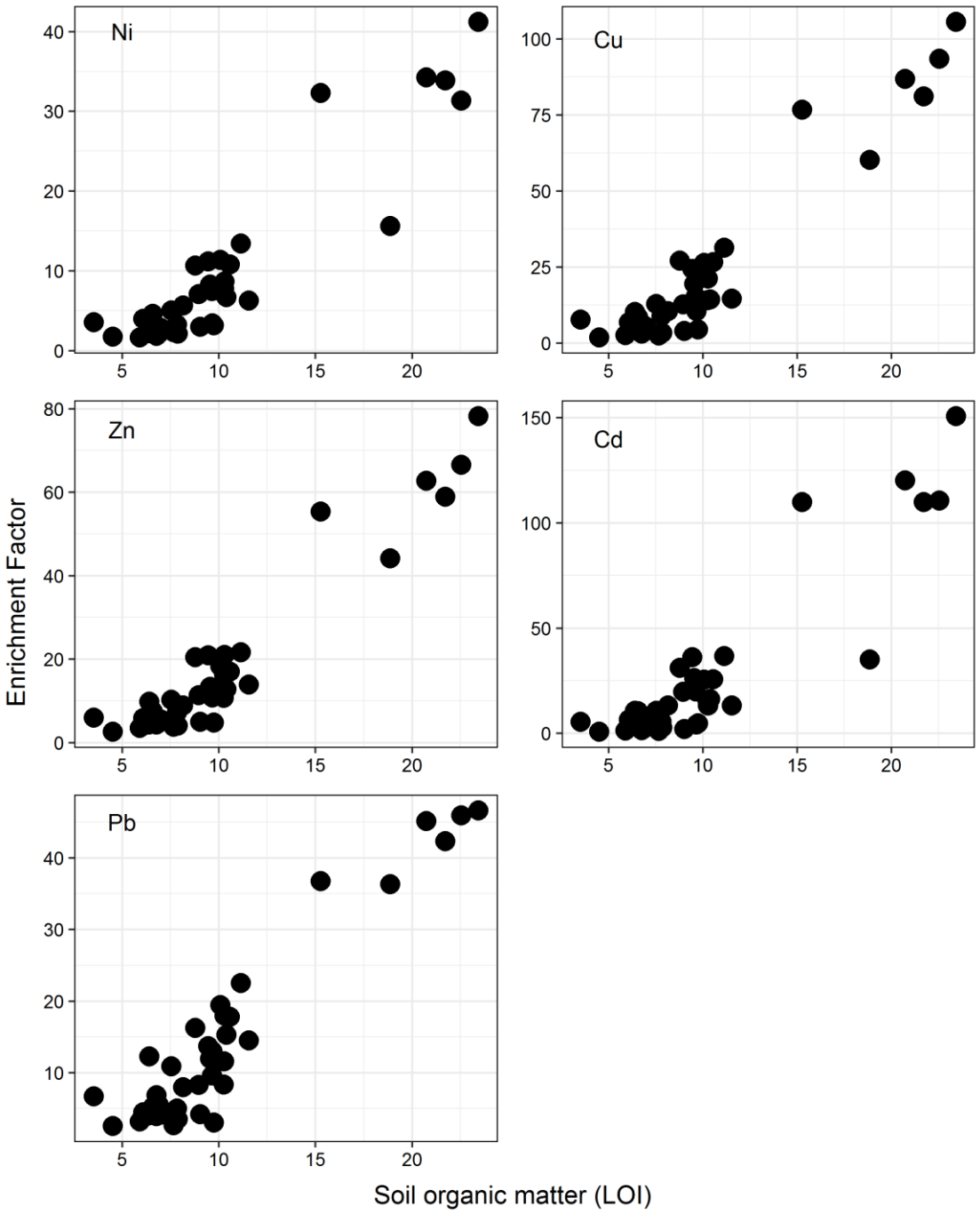


Figure 3.3. Relationships between metal enrichment factors and soil organic matter

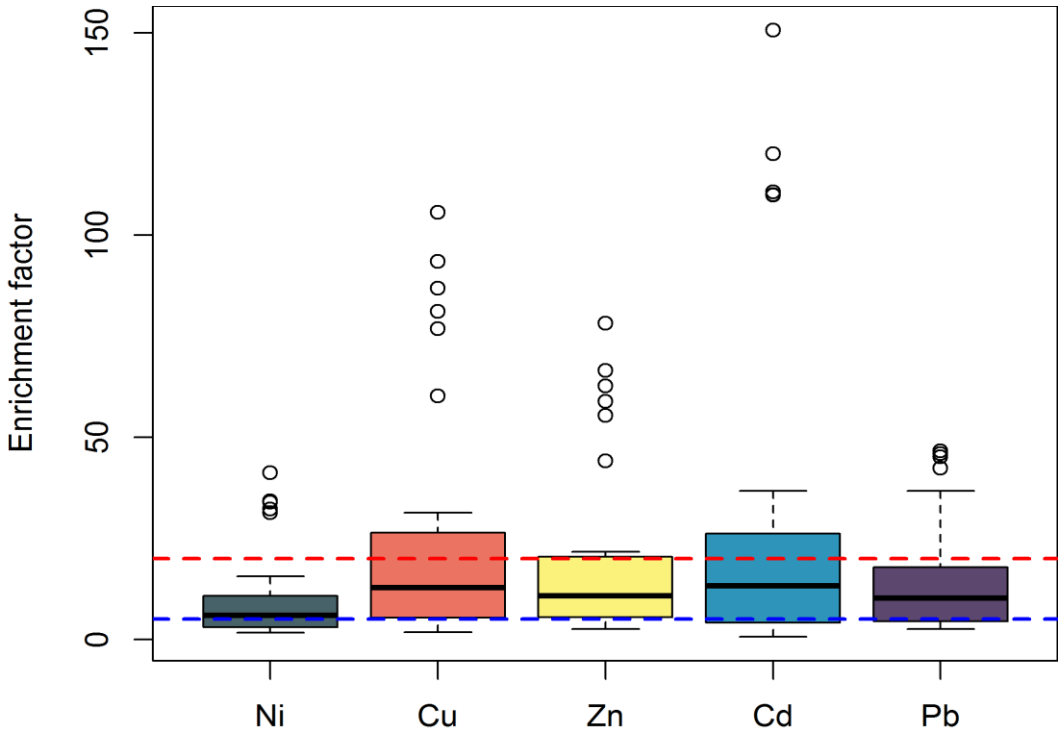


Figure 3.4. Boxplots of enrichment factors in the studied soils. The bold black lines inside the box represent the median; the boxes mark the 25th and 75th percentiles; the horizontal line outside the box (whisker) mark the minimum and maximum observations; individual points above the whiskers are the outliers. Blue and red horizontal dashed lines represent EF of 5 and 20 respectively.

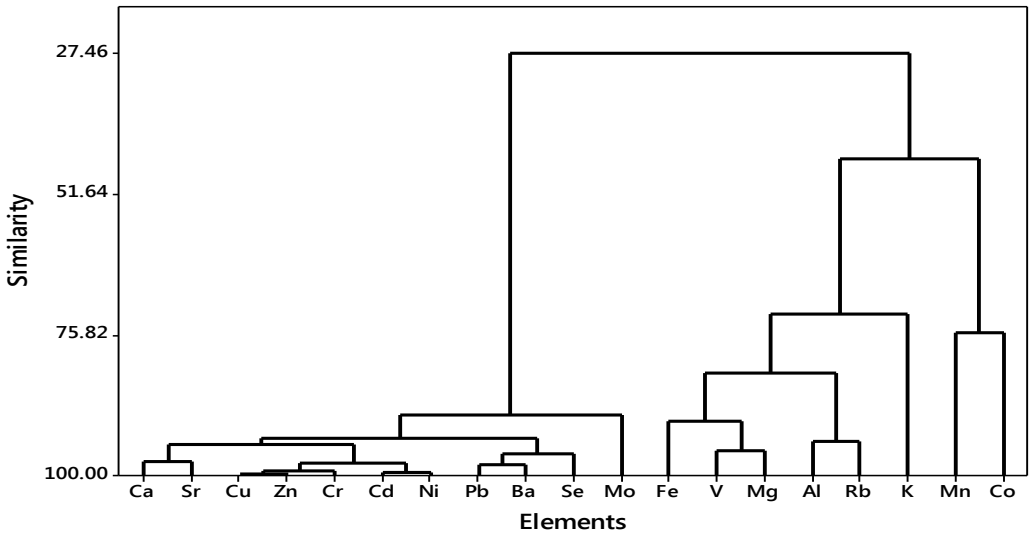


Figure 3.5. Hierarchical cluster analysis (dendogram; Ward's linkage) of soil's elemental analysis

3.3.3 Soil metal lability

E-values of the studied metals (Table 3.4) covered a wide range spanning over 2 orders of magnitude. Soil total metal content for Ni, Cu, Zn and Cd accounted for more than 98% of the variation in *E*-values (Fig. 3.6); while the *E*-values for Pb poorly correlated with Pb total content in soil (Fig 3.6).

Table 3.4. *E*-values and lability (*E*-value relative to soil total metal content)

	E-values (mg kg^{-1})				Lability (%)			
	Mean	Median	Min	Max	Mean	Median	Min	Max
Ni	32.1	17.3	1.1	176	19.9	18.8	3.3	42.4
Cu	76.5	45.3	5.8	304	36.0	37.0	22.9	44.2
Zn	149	81.1	12.0	679	21.5	22.1	7.6	33.2
Cd	2.5	1.5	0.1	11.3	28.0	28.3	19.7	37.3
Pb	10.9	9.4	1.3	36.4	5.3	4.8	1.5	12.6

The range of *E*-values expressed as a proportion (%) of total soil metal content are shown in Table 3.4. Copper was the most labile metal and the lability decreased in the order Cu > Cd > Zn ≈ Ni > Pb. This order is in accordance with the findings of Garforth et al. (2016) on the same soils. However, they found Ni to be the most labile metal, whereas Ni in the present study was less labile than Cu and Cd. It is worth noting that %*E*-values of Ni in the current study exhibited a large variation (from 3.3 up to 42.4 %). Lead was the least labile metal with average %*E*-value of 5.3 (Table 3.4). This relatively low lability of Pb is probably because of the high content of the studied soil of P (up to 13700 md kg^{-1}) which promotes precipitation of Pb as insoluble phosphate compounds. (Atkinson et al., 2011b; Garforth et al., 2016; McBride, 2016).

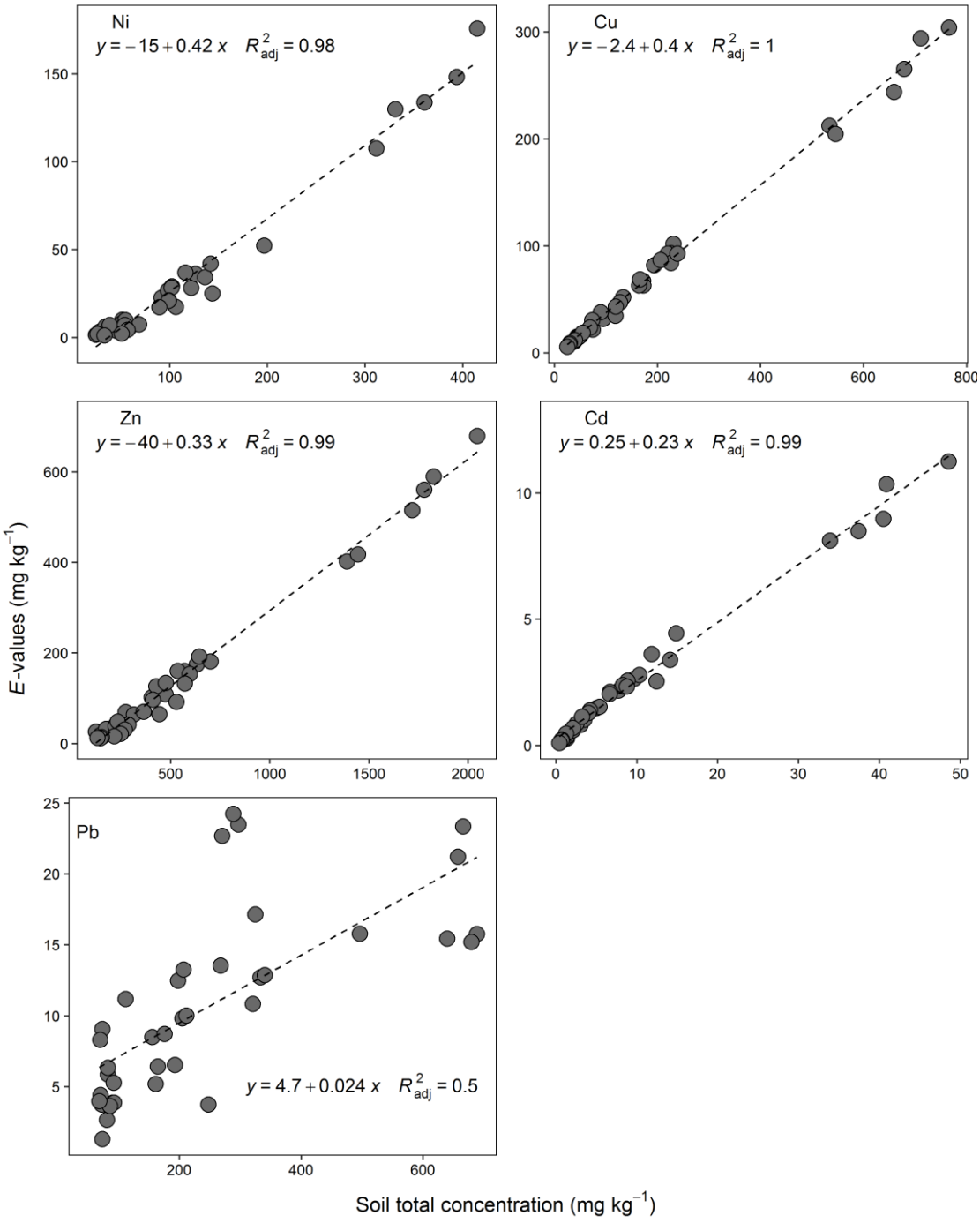


Figure 3.6. *E*-values as a function of total metal content in soil

Soil organic matter content, pH and total metal content are the main soil variables that influence metal lability (Marzouk et al., 2013a). Increased negative charge on soil adsorption surfaces is expected with increasing pH, thereby stronger adsorption is expected (Crout et al., 2006). Soil organic

matter is a crucial soil component that controls metal behaviour in soil. Dissolved organic matter can retain metals in the soil solution and the soil humus can chelate metals thus reducing metal lability (Clemente et al., 2005).

Multiple linear regression was used to predict metal lability (%*E*-values) from soil pH, %LOI and soil total metal content (M_{soil}) (Eq. 3.2).

$$\%E - value = \mathrm{a} + \mathrm{b}(\mathrm{pH}) + \mathrm{c}(M_{soil}) + d(\%LOI) \qquad 3.2$$

The results of the multiple regressions are shown in Figure 3.7 and Table 3.5. Predicted values of Ni an Zn were in reasonable agreement with the measured values, while the model failed to predict %*E*-value of Cu, Cd and Pb explaining only 30, 25 and 32%, respectively, of the variation (Fig 3.7 and Table 3.5). The low performance of the model indicated that other soil properties influence metal lability in these cases.

One feature of the studied soils is their exceptionally high phosphate content (up to 13700 mg kg^{-1}) which is known to reduce metal mobility (Datta and Young, 2005). This was particularly important for Cd and Pb where there was a negative correlation between soil total P content and %*E*-values of Cd and Pb (r = -0.45; *P* = 0.005 and r = -0.47; *P* = 0.003 for Cd and Zn respectively). It is well known that both Cd (Azzi et al., 2017; Hong et al., 2010) and Pb (Atkinson et al., 2011b; McBride, 2016) can be retained in soil as insoluble P compounds. Soil mineral oxides are also known to be important sorbents for metals and are expected to influence metal lability (Marzouk et al., 2013a). For instance, manganese oxide is known to be a strong binding phase for Pb (Bonten et al., 2008; Cancès et al., 2003).

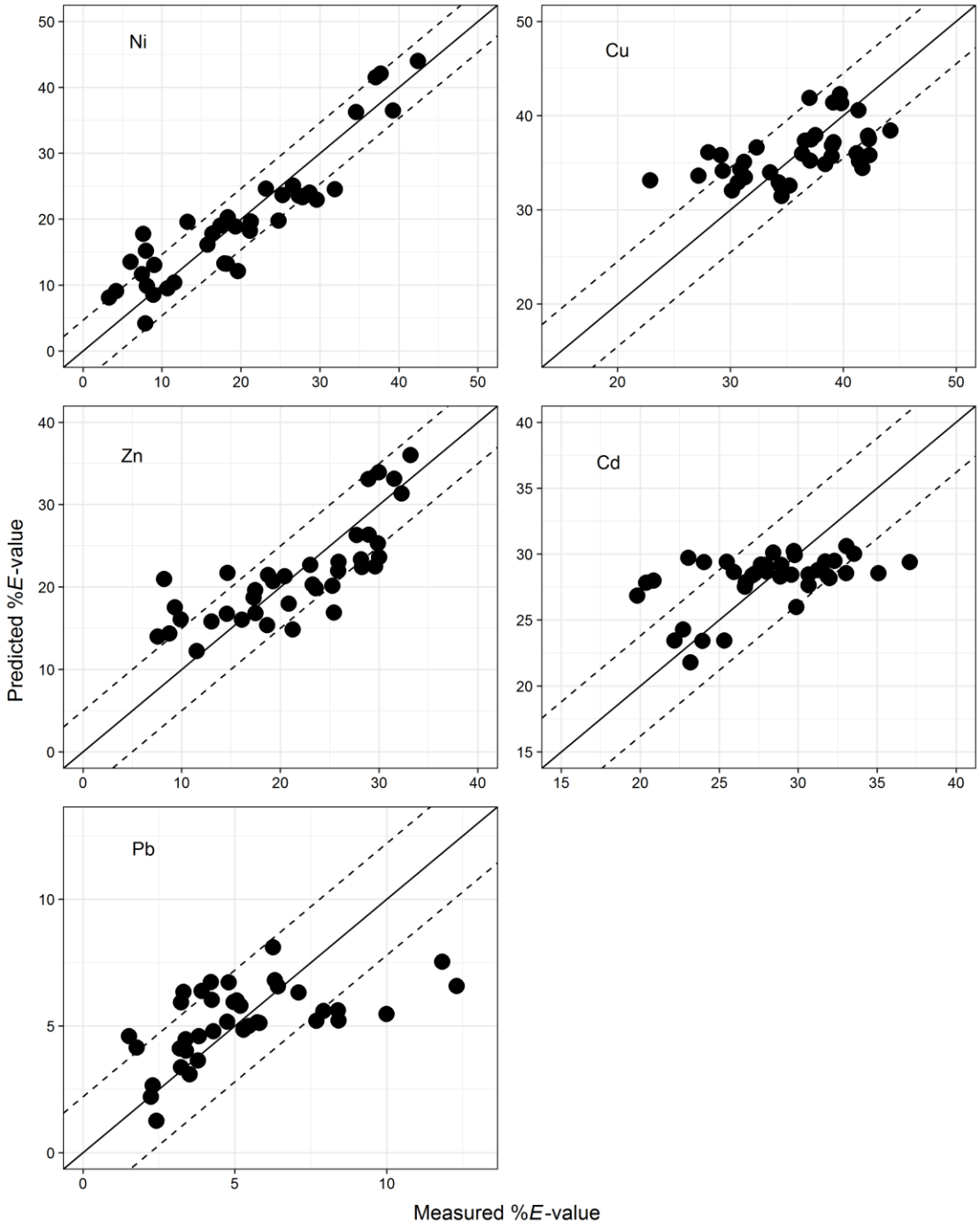


Figure 3.7. Predicted vs measured metal lability (%*E*-value). Predictions were derived from Eq. 3.2. The solid line represents a 1:1 relation. Dashed lines represent ± RSD of the model.

The inclusion of soil total P within the regression equation for Cd and total P and Mn oxide within the equation for Pb increased the explained variation

in %*E*-values for Cd and Pb to 37% and 73% respectively. No such improvement was observed for Cu.

Table 3.5. Coefficients of the multiple regression (Eq. 3.2) used for predicting metal lability (%*E*-value). Superscripted 'N.S' denote that the coefficient was not significant at the level $P<0.05$.

Metal lability	Intercept (a)	pH (b)	M_{soil} (c)	LOI (d)	R^2	RSD
(%)			(mg kg^{-1})	(%)		%*E*-value
Ni	107.6	-15.4	0.05	$0.5^{N.S}$	0.83	4.65
Cu	70.0	$-5.40^{N.S}$	$0.01.^{N.S}$	$-0.24^{N.S}$	0.30	4.52
Zn	81.5	-9.53	0.02	$-0.79^{N.S}$	0.60	4.99
Cd	43.3	$-2.51^{N.S}$	-0.30	$0.36^{N.S}$	0.25	3.81
Pb	19.2	-3.59	$-0.01^{N.S}$	$0.20^{N.S}$	0.32	2.20

3.3.4 Metal solubility

Table 3.6 shows the concentration of metals in 0.01 M $Ca(NO_3)_2$ suspension. Nickel, Zn, and Pb were found to be poorly soluble showing $Ca(NO_3)_2$ extractable proportions <1% of the total metal content. By contrast, Cu and Cd displayed a greater solubility with the extractable proportions varying across the soil dataset (0.1–23%; 5% on average and 0.6-66%; 10% on average for Cu and Cd respectively).

Table 3.6. Metal concentration (µg L^{-1}) in solution phase (0.01 M $Ca(NO_3)_2$)

	Ni	Cu	Zn	Cd	Pb
Minimum	1.38	2.68	2.01	0.07	0.02
Maximum	688	204	393	11.2	1.36
Median	54.6	37.9	43.7	1.88	0.09
Mean	141	54.3	96.7	3.02	0.20

3.3.4.1 Partitioning coefficient K_d

Partitioning coefficients were calculated for each element as the ratio between the adsorbed proportions of isotopically exchangeable metal (M_E–M_{soln}) and the free ion activity (M^{2+}) in the soil solution. Values ranged over

2 orders of magnitude. Nickel, Zn and Cd positively correlated with pH reflecting increased adsorption at higher pH, while no distinctive trend was observed for Cu and Pb (Fig. 3.8).

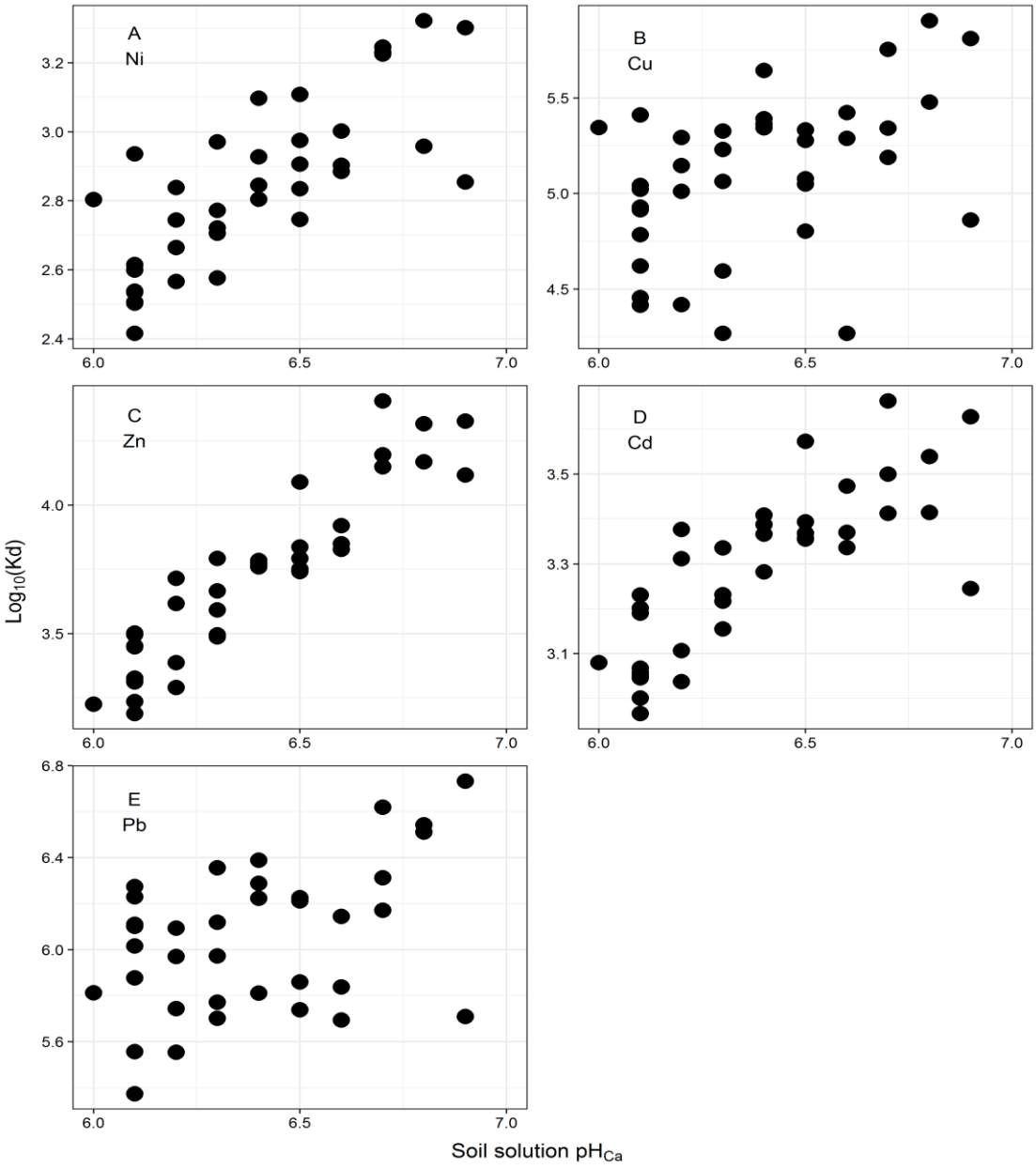


Figure 3.8. Partitioning coefficient ($Log_{10}(K_d)$) as a function of soil solution pH (0.01 M ($Ca(NO_3)$)).

To identify the most influential soil properties controlling metal partitioning between the soil solid and solution phases, stepwise regression was used

to relate $Log_{10}(K_d)$ with log-transformed soil characteristics. The results of the regression models are reported in Figure 3.9 and Table 3.7. The regression models had different structures, no single variable was systematically present in all models, suggesting that the models are probably not applicable to another data set.

Table 3.7. Coefficients of multiple regression models used to predict partitioning coefficient K_d. Only soil variables that were significant at the level 5% were included the model.

	intercept	pH	SOM	$M_{E\text{-}value}$	DOC	Al_{Ox}	Fe_{Ox}	Mn_{Ox}	R^2
Ni	-0.20	0.46	1.30	-0.34	-0.50	-	-	0.14	0.94
Cu	-0.30	0.70	2.26	-0.78	-	-	-	-	0.38
Zn	-3.43	1.16	-	-	-	-	-	0.24	0.88
Cd	-0.46	0.58	0.74	-	-0.34	-	-	0.26	0.87
Pb	4.45	-	-	-		-1.27	1.5-4	-0.54	0.36

The regression model was successful in predicting the partitioning coefficient for Ni, Zn and Cd explaining most of the variation (Table 3.7), while only 37% of the variation in K_d values for Cu and Pb was accounted for suggesting that other soil factors control the partitioning of these metals. As expected soil pH had positive coefficients in all the regression model indicating the dual role of pH in controlling metal solubility through increasing proton competition for binding sites and decreasing pH-dependent surface charges with decreasing pH. (Sauvé et al., 2003; Shaheen et al., 2013). Soil organic matter (%LOI) was another important factor influencing K_d values by providing greater binding surface for metal adsorption favouring partitioning to the solid phase. Despite the high affinity for Cu and Pb for soil organic matter, %LOI was only significant in the case of Cu, whereas only mineral oxides were significant in predicting K_d for Pb (Table 3.7). DOM, when significant, inversely correlated with the

partitioning coefficient Kd suggesting increased metal solubility with increased DOM.

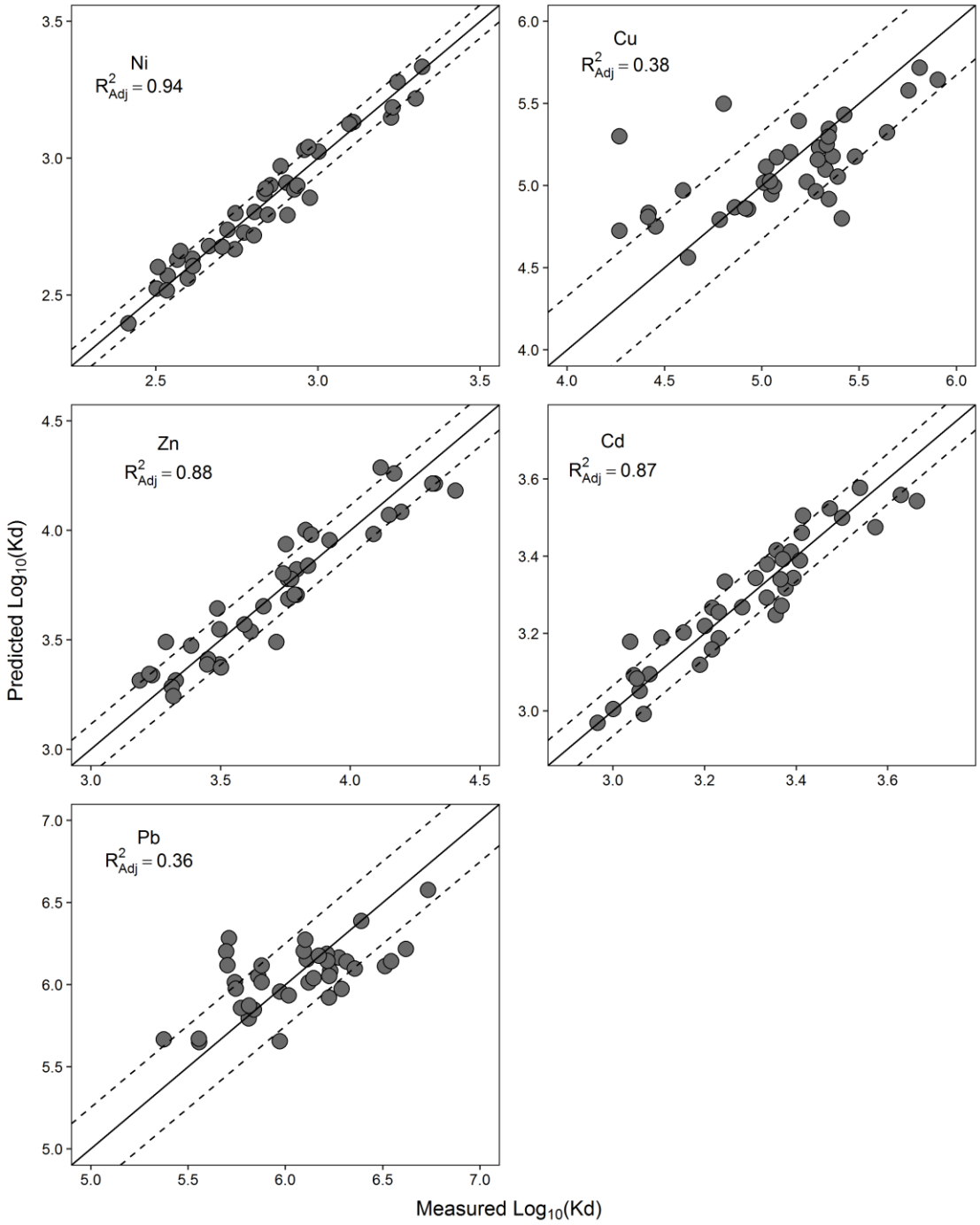


Figure 3.9. Predicted and measured partitioning coefficient ($log_{10}(K_d)$). Solid lines represent 1:1 relation; dashed lines represent ± residual standard error of the multiple regression model

3.3.4.2 Soil solution speciation using assemblage models.

Geochemical assemblage models, a combination of different models simulating ion binding to several reactive soil components (organic matter, oxides, clays), are widely used to predict metal speciation in soil (Ahmed et al., 2014; Ponthieu et al., 2016; Rennert et al., 2017; Schneider et al., 2016a). Dissolved organic matter DOM is considered to be the most important complexing ligand for ion binding in the soil solution (Cornu et al., 2016; Jan E. Groenenberg et al., 2010). Due to the lack of the characterization of DOM in most cases, certain assumptions are made regarding the composition and the reactive fraction of DOM. In general 50% of the DOM is assumed to be C, and that 65% of the DOM is active fulvic acid and 35% is inert (Buekers et al., 2008a; Lin et al., 2015; Marzouk et al., 2013c; Ren et al., 2015).

To highlight the importance of DOM, the WHAM model was used to speciate the soil solution assuming four different reactive fractions of the DOM (i.e. assuming 50, 65, 80 and 100% DOM is potentially reactive). Figure 3.10 shows estimated free ion activity of Ni, Cu, Zn, Cd, and Pb using different fractions of the DOM as reactive. Consistent predictions of free ion activities for Ni, Zn, Cd were obtained with the 4 modelling scenarios. This suggests that these metals exhibit low affinity for organic matter colloids. This in agreement with Zhang et al. (2015) who found that assuming 0% active fraction of DOC had little effect on predicting dissolved Ni using WHAM, indicating that DOC could be neglected in the prediction. Ivezić et al. (2012) also reported that only 9% and 7% of Cd and Zn, respectively, were

associated with FA in the soil solution. By contrast, Cu and Pb free ion activities were strongly dependent on the proportion of the DOM assumed to be reactive (Fig 3.10), reflecting the high affinity of these two elements for DOM (Ponthieu et al., 2016; Schneider et al., 2016a).This in agreement with the observation of Ivezić et al. (2012) who reported that 66% of Cu and Pb were bound to FA in the soil solution. These observations underline the potential impact of DOM composition on the accuracy of the model predictions particularly for metal with high affinity for DOM such as Cu and Pb.

The free ion activity in the soil solution assuming 65% of the DOM as active FA will be used throughout the rest of this chapter (Table 3.8).

Table 3.8. Fractions of dissolved metals that present as free ion and DOM-bound in soil solution (0.01 M $Ca(NO_3)$ suspension) calculated by WHAM.

	Me^{2+} (%)			Me-DOM (%)		
	Mean	Min	Max	Mean	Min	Max
Ni	98.8	97.9	99.7	1.24	0.3	2.18
Cu	41.8	28.9	62.4	58.1	37.6	72.7
Zn	94.0	89.4	98.5	6.2	1.56	10.9
Cd	91.2	86.7	96.9	9.13	3.1	15.3
Pb	59.3	34.4	91.2	40.9	8.8	65.6

3.3.4.3 Predicting metal solubility in soil using WHAM

WHAM model was used to predict metal solubility (concentration in the 0.01 M $Ca(NO_3)_2$ suspension). Input variables for the model included either soil total (M_{soil}) or labile (M_E) metal content, in conjunction with measured major and minor cations, DOC, pH in soil suspension, Fe/Al/Mn oxides content and LOI. The accuracy of the prediction was evaluated by means of root mean square error (RSME) and mean error (ME) between predicted and observed values (Table 3.9).

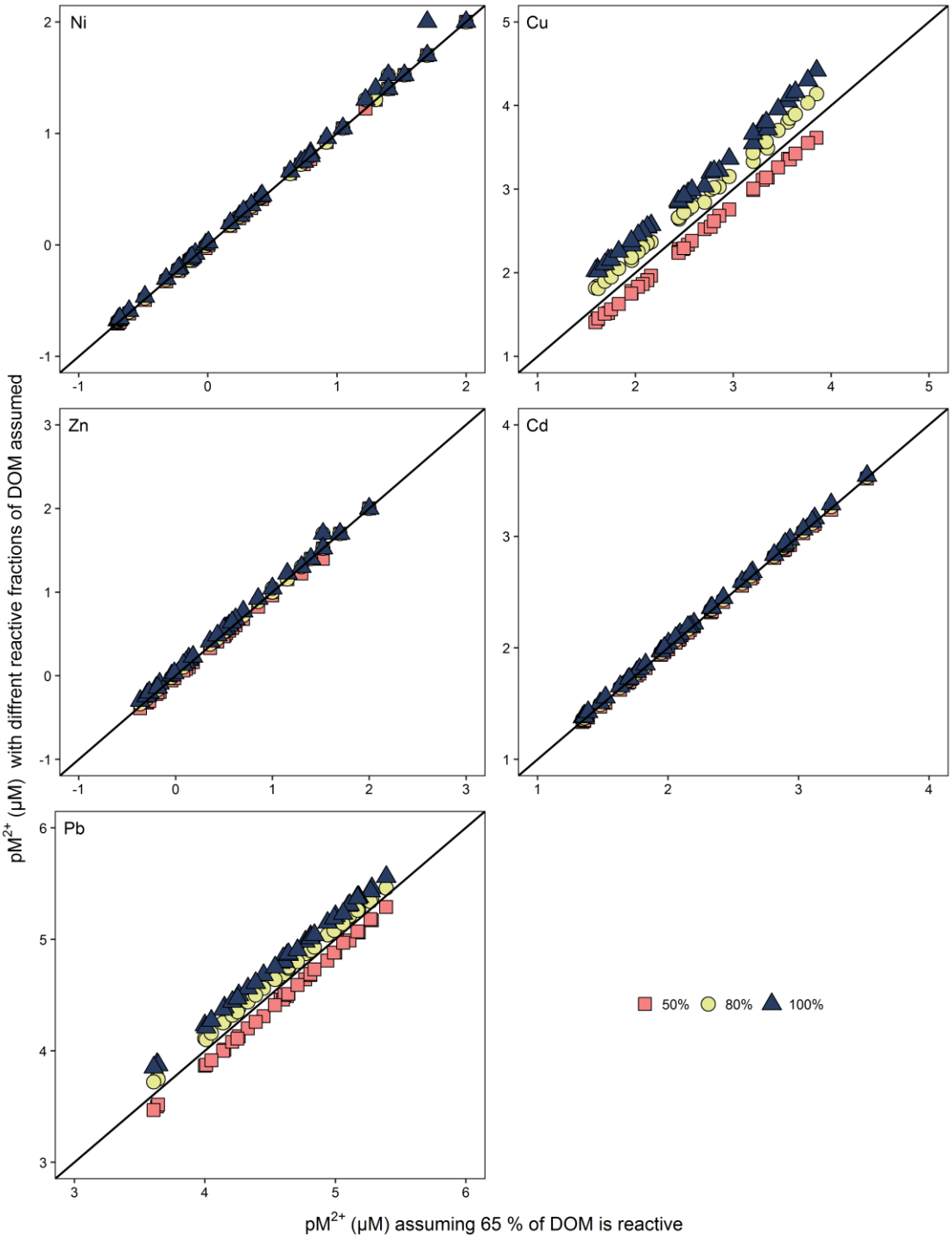


Figure 3.10. Comparison between free metal ion activity (pM^{2+}) calculated by WHAM assuming different active fractions of DOM. Solid lines represent 1:1 relation.

The results (Fig.3.11 and Table 3.9) demonstrate that using total metal concentrations as input in WHAM largely overestimated the solubility of Cd, Ni and Zn. Using "reactive metal concentration" (M_E) substantially improved the prediction of soluble metal concentration, particularly for Ni and Zn.

Table 3.9. Root mean square (RMSE) and mean error (ME) between predicted (using WHAM) and measured concentration in soil solution (0.01 Ca(NO3)2 suspension).

	RMSE		ME	
	M_{soil}	$M_{E\text{-value}}$	M_{soil}	$M_{E\text{-value}}$
Ni	1.40	0.34	-1.33	-0.31
Cu	0.28	0.28	-0.28	0.26
Zn	1.62	0.74	-1.57	-0.69
Cd	3.13	2.37	-3.13	-2.35
Pb	0.50	0.31	0.29	-0.02

Errors in the estimates of dissolved Cu were consistent whether they were predicted using soil total Cu_{soil} or labile Cu_E (RMSE=0.28). Total Cu overestimated the solubility whilst it was underestimated when Cu_E was used as input in WHAM. Copper has a strong affinity for soil organic matter. Therefore, the proportion of soil organic matter assumed reactive may have substantial effects on model predictions for Cu. Decreasing the proportion of SOM (%LOI) assumed to be HA from 50% to 40% resulted in a considerable improvement in the predictions of soluble Cu (RSME = 0.1 and ME = 0.02). This is in line with the observations of Bonten et al. (2008) who also reported that 40% of SOM assumed as HA provided the best estimate of Cu solubility. However, simultaneous prediction for the 5 studied metals considering 40% of SOM as HA reduced overall surface area available for binding which resulted in overestimation of Ni, Zn and Cd soluble concentrations.

Cadmium concentration in the soil solution was systematically overestimated in both cases (Fig. 3.10), although slightly improved estimates were obtained when Cd_E was used as input. The poor predictions obtained for Cd may be because of: (i) low default binding constant for Cd to SOM will results in solubility overestimation (Bonten et al., 2008); (ii) overestimation of Cd_E values due to the presence of non-labile colloidal complexed Cd (Lombi et al., 2003).

Several authors have reported poor predictions of Pb solubility using WHAM (Bonten et al., 2008; Marzouk et al., 2013a; Rennert et al., 2017). In contrast, good predictions were obtained for Pb soluble concentration in the this study, using both the total and labile pools (Fig.3.11). Using Pb_E as inputs yielded in a better prediction particularly in reducing the average displacement (Table 3.9).

Figure 3.12 Shows the ratio between predicted and measured soluble concentrations of Ni, Cu, Zn and Cd (using M_E as inputs in WHAM) as a function of soil organic matter. For Cd, Ni and Zn there was a declining trend in the predicted/measured ratio with increasing organic matter in soil. Metal solubility in soils with organic matter levels <10% was largely overestimated, suggesting that the active fraction of organic matter available for metal binding is greater than assumed (50%). By contrast, better predictions were obtained with increasing organic matter contents suggesting that the 50% default assumption may be valid. This indicates that the reactive fraction (i.e. available for metal binding) of soil organic matter decreases as soil organic matter increases (Shi et al., 2013a, 2013b; Tipping et al., 2003). These observations emphasise that soil organic matter should not be considered as a chemically homogenoeus soil component.

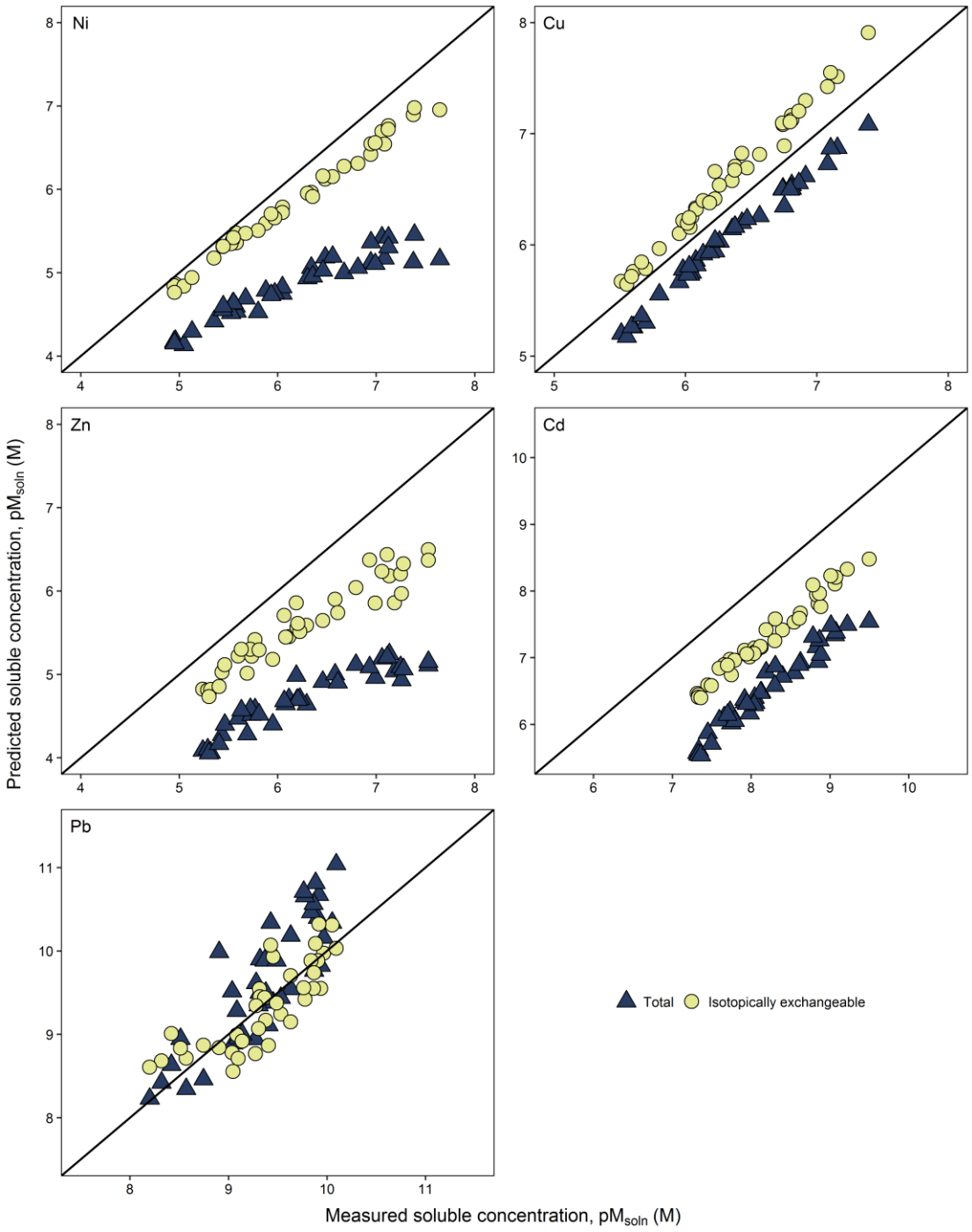


Figure 3.11. Predicted and measured soluble metal concentration (0.01 M $Ca(NO_3)_2$ suspension). Solids lined represent 1:1 relation

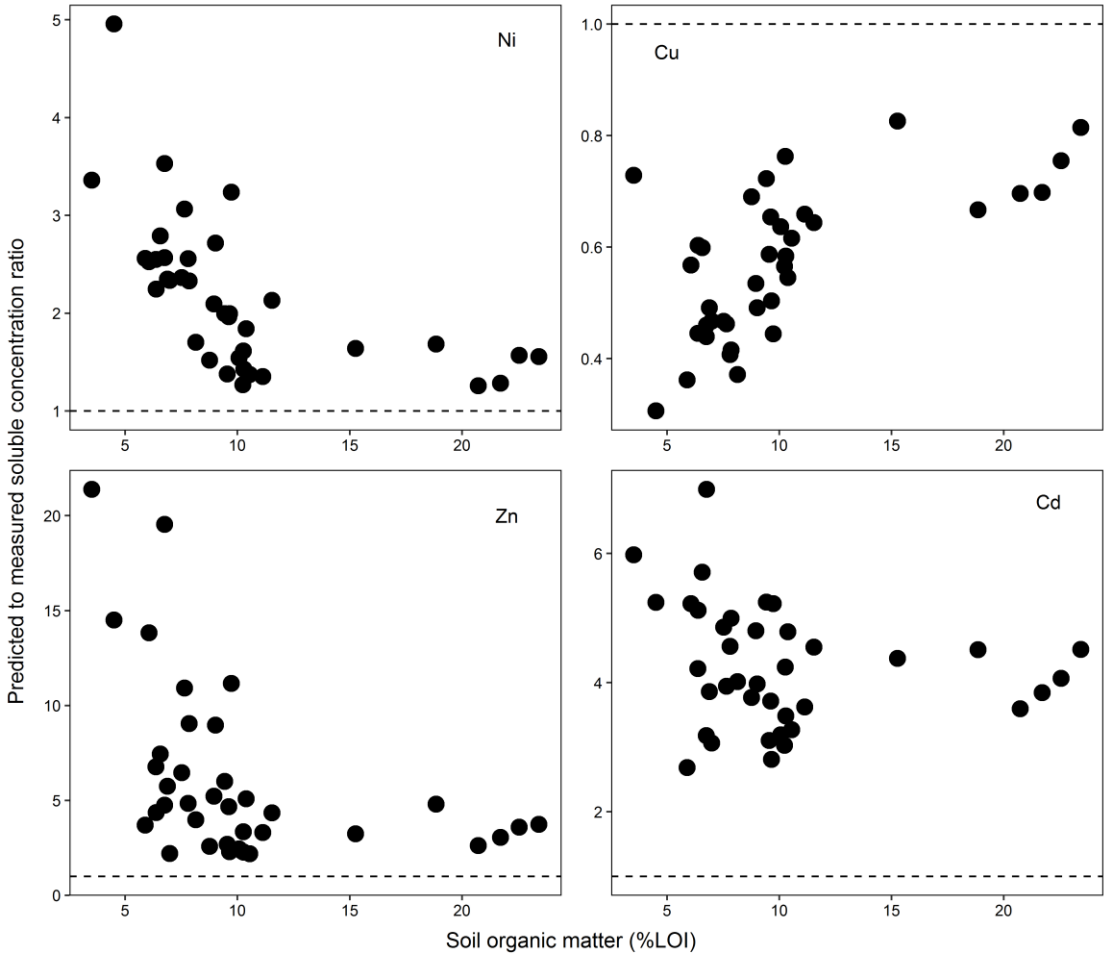


Figure 3.12. The ratio between predicted and observed soluble metal as a function of soil organic matter (%LOI)

3.3.5 Plant uptake

The relationships between metal concentration in the plant shoot and the different measurements of metal availability in the soil are shown in Figures 3.13 to 3.17. All relations were significant ($p<0.01$) for the five studied elements.

The four measures of metal bioavailability gave good predictions of metal concentration in plants, explaining more than 70% of the variation in Ni, Cu, Zn and Cd concentrations in the plant shoot (Fig 3.13 to 3.16).

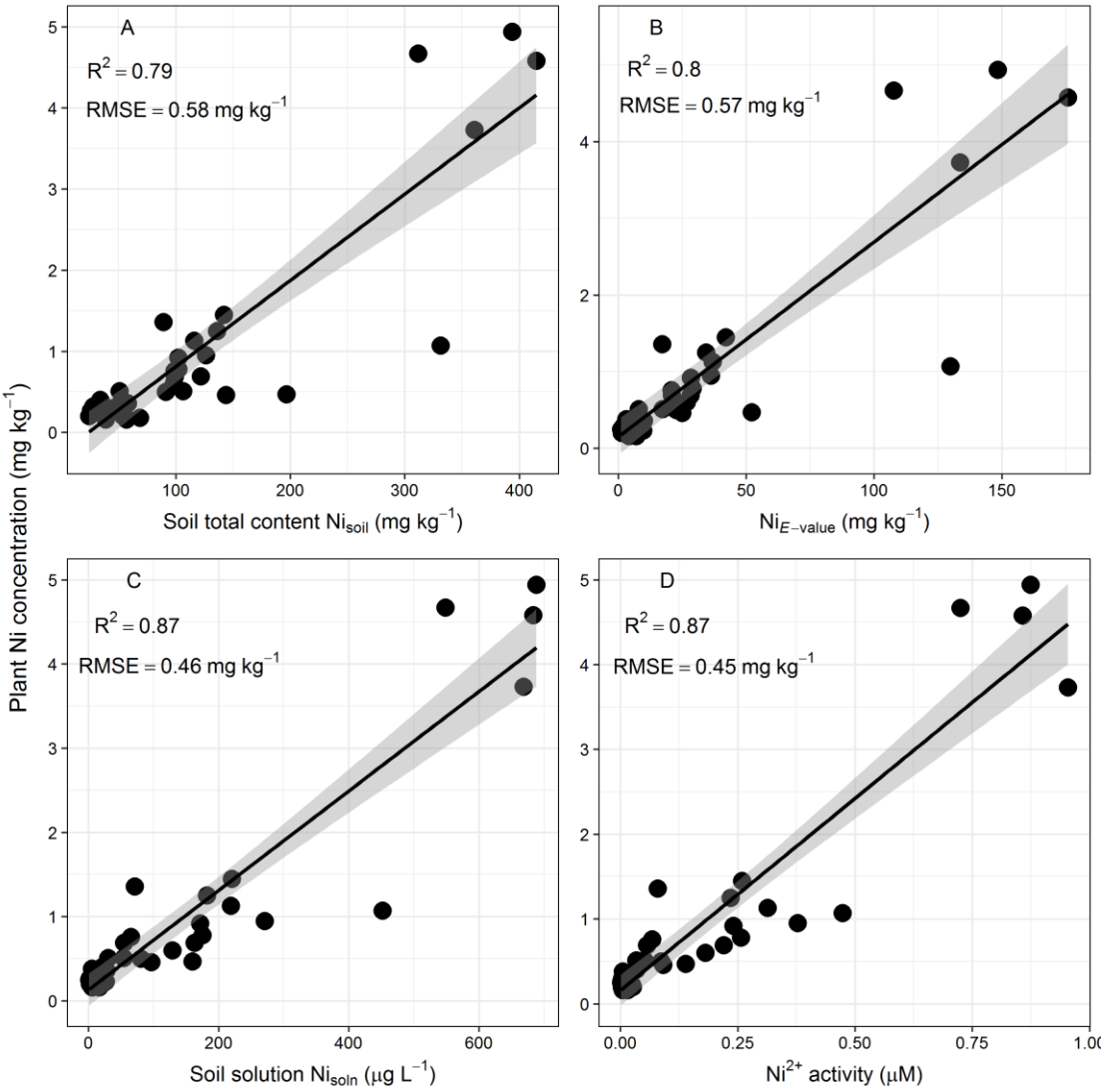


Figure 3.13. Relationships between Ni concentration in plant and Ni concentration in soil obtained using (A) total Ni_{soil}, (B) labile Ni_E, (C) $Ca(NO_3)_2$ extractable Ni_{soln}, (D) Ni^{2+} activity. The solid lines represent linear regression fit. Grey shaded areas represent 95% confidence interval.

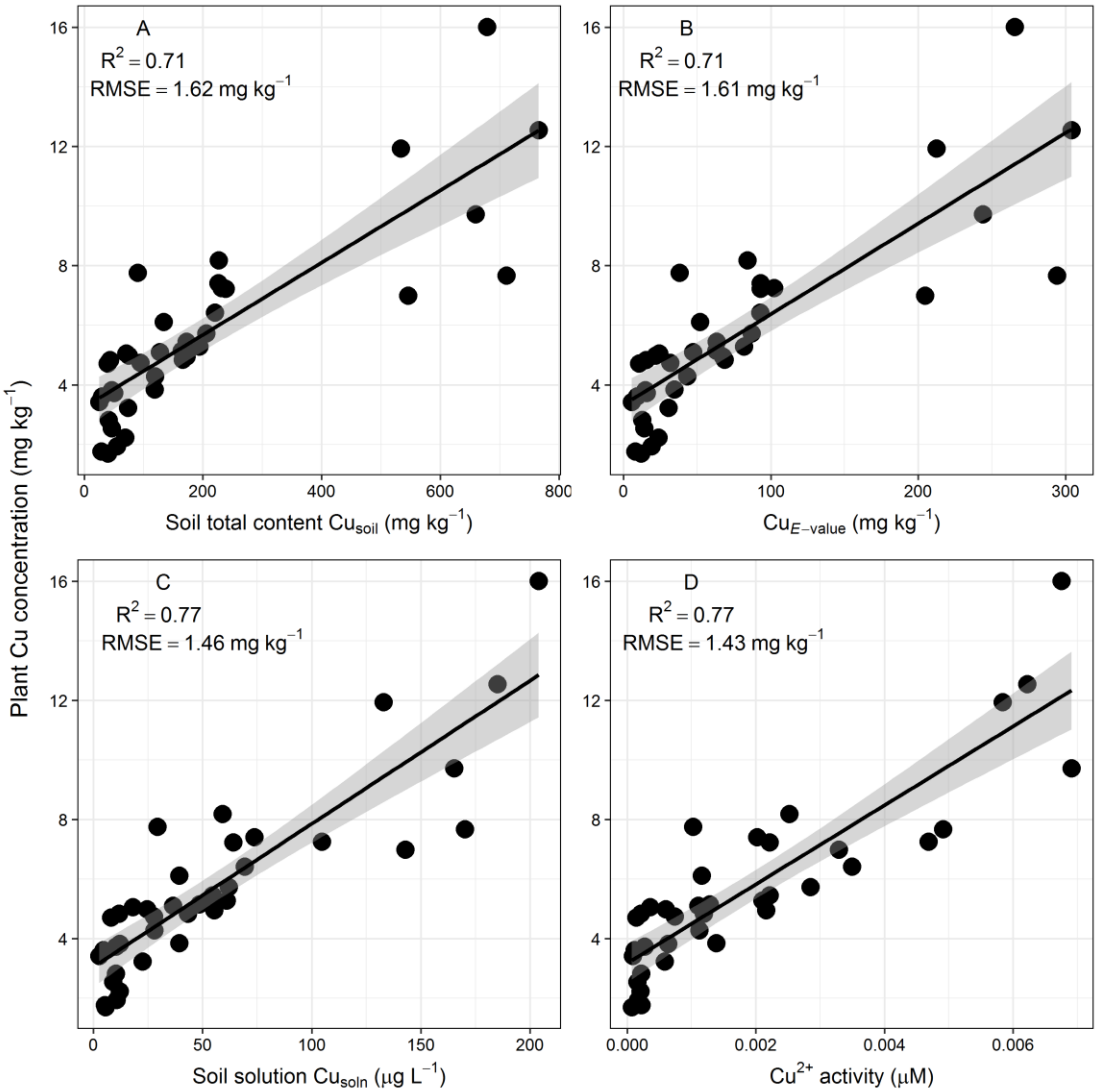


Figure 3.14. Relationships between Cu concentration in plant and Cu concentration in soil obtained using (A) total Cu_{soil}, (B) labile Cu_E, (C) $Ca(NO_3)_2$ extractable Cu_{soln}, (D) Cu^{2+} activity. The solid lines represent linear regression fit. Grey shaded areas represent 95% confidence interval.

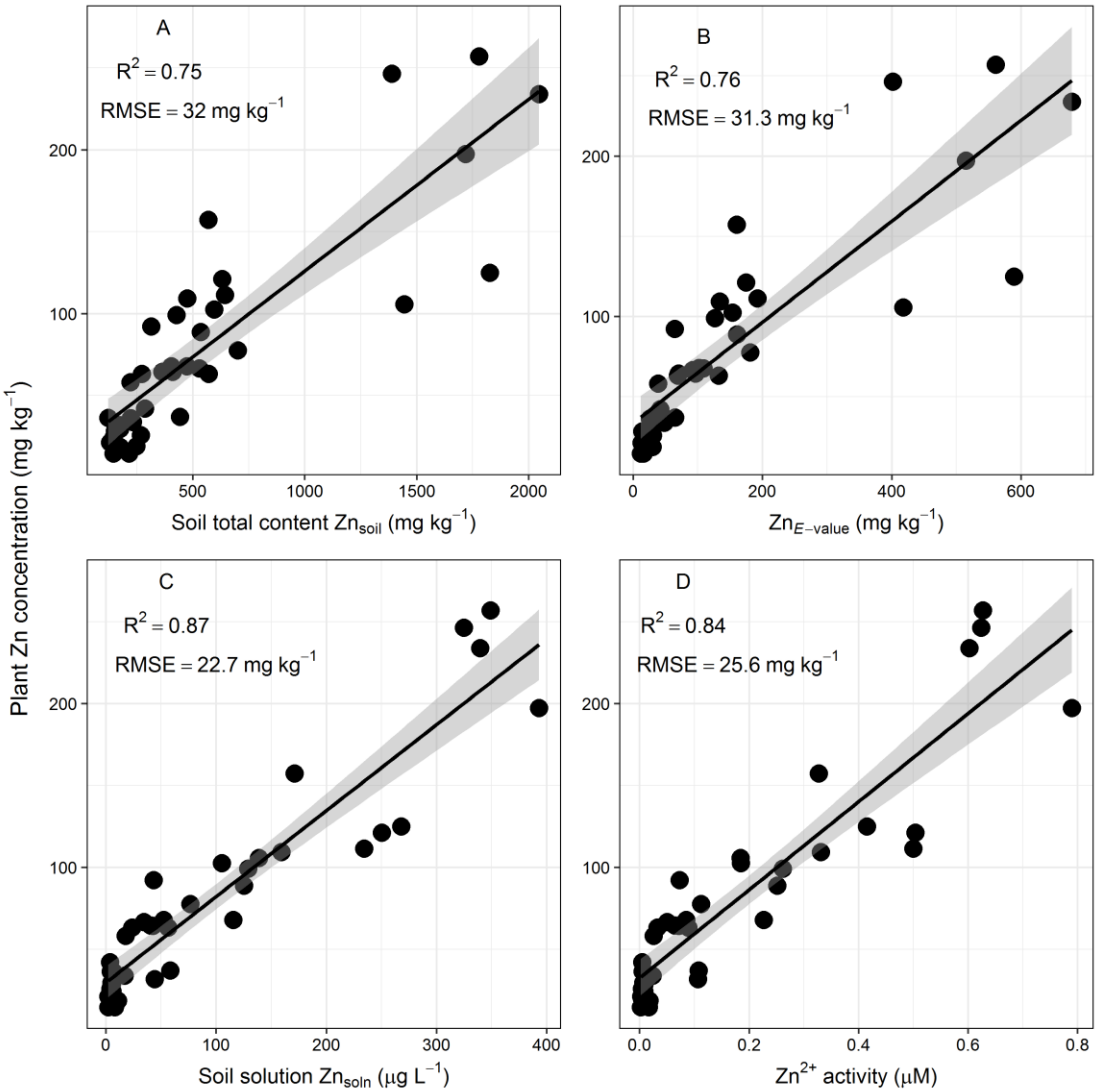


Figure 3.15. Relationships between Zn concentration in plant and Zn concentration in soil obtained using (A) total Zn_{soil}, (B) labile Zn_E, (C) $Ca(NO_3)_2$ extractable Zn_{soln}, (D) Zn^{2+} activity. The solid lines represent linear regression fit. Grey shaded areas represent 95% confidence interval.

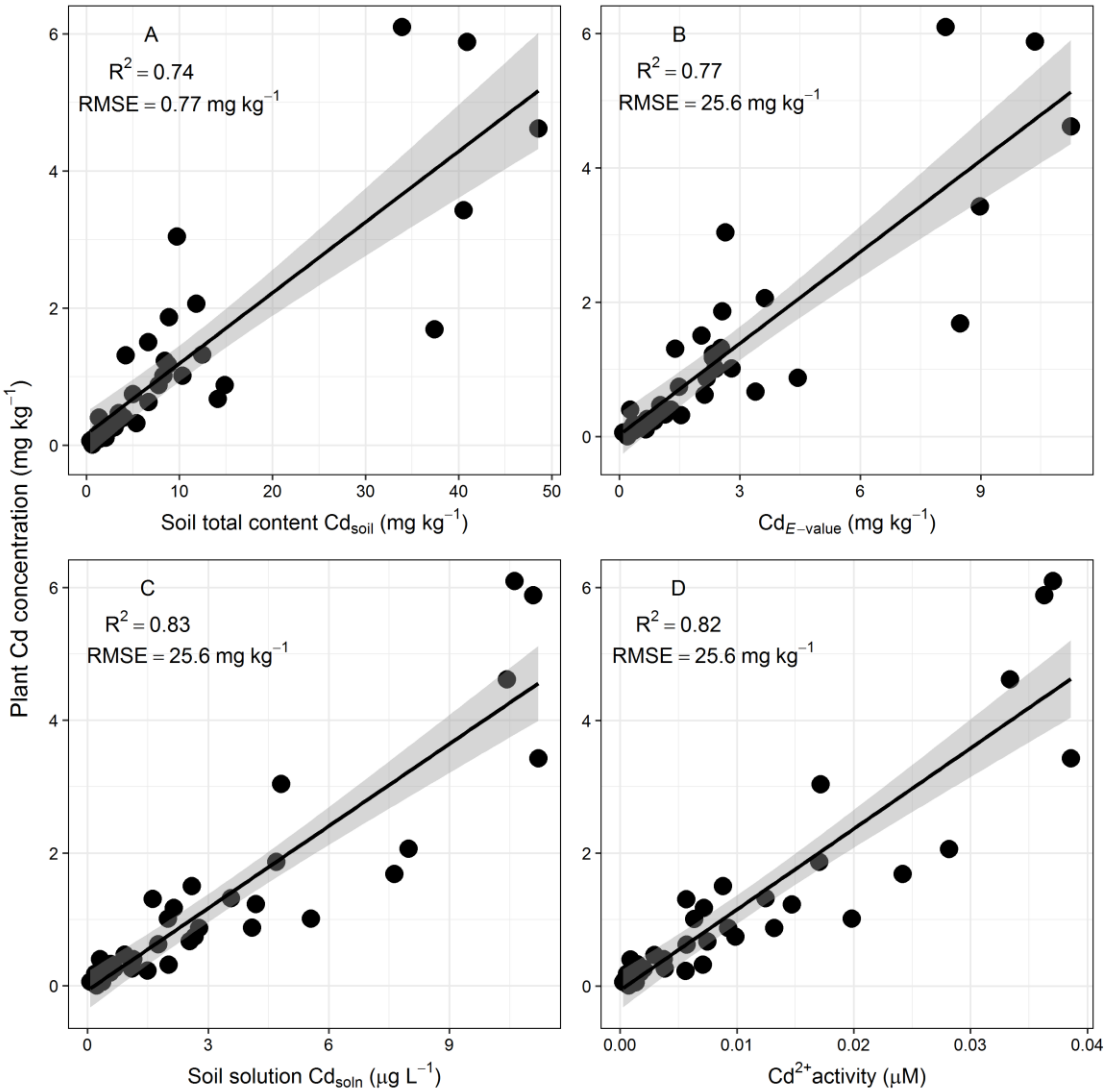


Figure 3.16. Relationships between Cd concentration in plant and Cd concentration in soil obtained using (A) total Cd_{soil}, (B) labile Cd_E, (C) $Ca(NO_3)_2$ extractable Cd_{soln}, (D) Cd^{2+} activity. The solid lines represent linear regression fit. Grey shaded areas represent 95% confidence interval.

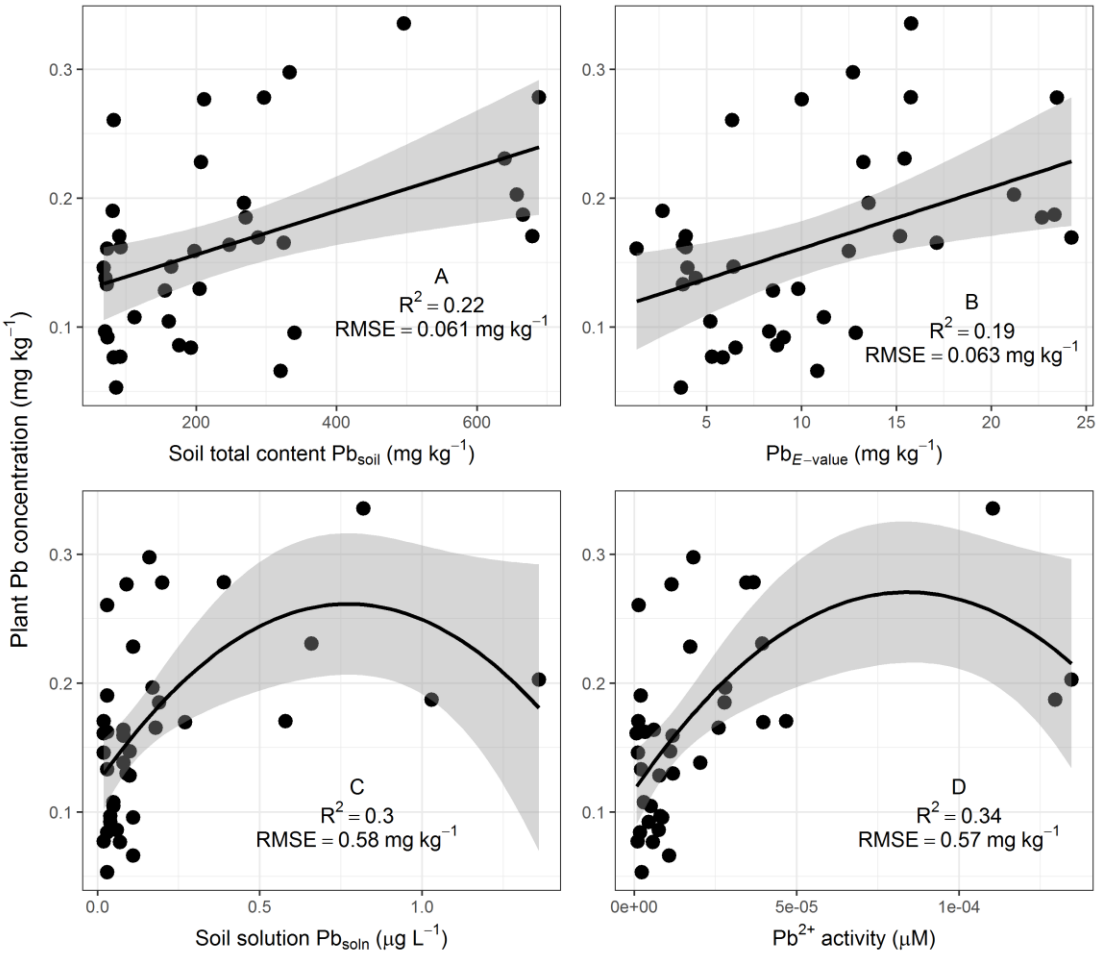


Figure 3.17. Relationships between Pb concentration in plant and Pb concentration in soil obtained using (A) total Pb_{soil}, (B) labile Pb_E, (C) $Ca(NO_3)_2$ extractable Pb_{soln}, (D) Pb^{2+} activity. The solid lines represent linear regression fit. Grey shaded areas represent 95% confidence interval.

In the case of Pb, even though the four measurements resulted in significant relationships with Pb concentration in plant (Fig. 3.17), these were not able to explain more than 34% of the variation in plant Pb concentration (using free Pb^{2+} activity as the explanatory variable (Fig. 3.17D)). The relationship between Pb concentration in plant shoots and Pb concentration in the soil solution (Pb_{soln} and Pb^{2+}) was better described by a quadratic rather than a linear relationship. The poor and curved relationship between Pb concentration in the soil solution and the concentration in the plant shoots

suggests that other soil and plant factors influence this relationship. For instance, the ability of plant roots to restrict Pb uptake and/or reduce translocation from roots to the areal parts of plant (Nolan et al., 2005; Nordløkken et al., 2015). Similarly, the low Pb solubility in the studied soils relative to other cations present at much higher levels would also result in limiting uptake of Pb due to the competitive uptake of cations with higher concentration (Black et al., 2011).

From a predictive perspective, soil solution concentrations, M^{2+} activity and M_{soln}, were the best measure for plant concentration (Fig 3.13-3.17). This emphasises that soil dissolved metals are more relevant fractions for metal bioavailability and subsequent toxicity than soil total metal content (Black et al., 2011; Zhang et al., 2015).

In general, soil total metal content provides a poor indication of plant uptake (McBride et al., 2009). In the present study, biosolids are probably the single source of metal contamination. Thus, it is reasonable to assume that metals would mainly be held in organically bound forms and hence would be potentially bioavailable (Menzies et al., 2007). This combined with the narrow pH range (6.0 - 6.9) of the studied soils and the covariance of soil organic matter with soil total metal content (Table 3.2) might explain the small differences between the measures of soil *capacity* (i.e. metal reservoir; M_{soil}, M_E) and *intensity* (M_{soln}, M^{2+}) in predicting metal concentration in the plant shoots.

3.4 Conclusions

In this chapter, the geochemical status of soils from 31 fields that have been subject to long-term biosolids application was reported. Metal

accumulation, metal partitioning between different fractions in relation to different soil properties and plant uptake were investigated.

High levels of contamination were observed in the studied soils. However, metal lability (%*E*-values) was less than 40% for the five studied elements. This emphasises that plant uptake, risk assessment and toxicological guidelines should be evaluated using the reactive fraction rather than the total metal content in soil. This is particularly important for soil subject to long-term biosolids application where metals are introduced into the soil system with high levels of organic matter, consequently increasing soil adsorptive properties and metal retention (Frost and Ketchum., 2000; Mamindy-Pajany et al., 2014).

Empirical and mechanistic models showed similar performance in predicting metal solubility. The strength of empirical models lies in their simplicity and low demand for inputs. The drawbacks of these models are (i) their limited applicability to the soils they were derived from; (ii) over simplification (i.e. complex role of soil variables, such as pH, are reduced to a one single coefficient in the regression models) (Groenenberg, 2011). DOM was inversely correlated with the partitioning coefficient K_d suggesting increased metal solubility with increased DOM. Therefore, potential environmental risks caused by metal leaching and toxicity associated with the use of biosolids in agricultural soils should be considered, because DOM derived from biosolids might significantly increase metal solubility through the formation of dissolved M-DOM complexes in soil (Wong et al., 2007).

Mechanistic models on the other hand offer a more holistic and scientifically sound approach in describing metal interactions with reactive soil

constituents, both in the solid and the solution phases, which determine metal partitioning and speciation in the soil. When soil total metal content (M_{soil}) was used as an input in WHAM, metal solubility was largely overestimated, indicating that soil capacity to buffer the soil solution was overestimated. Predictions of metal solubility were improved when the isotopically exchangeable metals (M_E) were used as inputs. This highlights the importance of defining the reactive fraction of metals in soil that is in equilibrium with the soil solution within the relevant timeframe.

Free metal ion in soil solution is believed to be the major bioavailable form and therefore potentially the most toxic species for soil biota (Ardestani et al., 2014b; Djae et al., 2016). The results of metal speciation in the soil solution using WHAM indicated that Cd, Ni and Zn were mainly present as free ionic species in the soil solution, whilst approximately 50% of dissolved Cu and Pb was associated with DOC complexes. Therefore, inaccurate assumptions regarding the proportion of active dissolved organic matter may result in unreliable predictions when modelling Cu and Pb speciation in the soil solution.

Soil extraction with 0.01 M $Ca(NO_3)_2$ proved to be simple, inexpensive and highly effective in predicting Ni, Cu, Zn and Cd uptake by plants. In contrast, none of the bioavailability estimates used in this study appeared to be indicative of Pb concentrations in the plant shoot suggesting plant-controlled mechanisms (e.g. translocation from roots to shoots) are involved in Pb uptake by plants.

4. Zinc and Cd dynamic in contaminated soil amended with Zn

4.1. Introduction

Heavy metals in soils originate from parent materials and anthropogenic sources including mining, industries, agrochemicals, sewage sludge and industrial waste (Cambrollé et al., 2013; Jiao et al., 2015). Their concentration can reach elevated levels in soil. This may pose a serious environmental problem due to the toxicity, persistency and bioaccumulation ability of all trace metals (Cambrollé et al., 2013; Prasad et al., 2005).

Zinc and Cd have similar properties and are often associated with each other in the environment (Chaney, 2010; Gharaibeh et al., 2016). Therefore, both synergistic and antagonistic relations between them are expected (Balen et al., 2011; Gharaibeh et al., 2016; Reiser et al., 2014). Zinc is an essential micronutrient for plant growth and physiological functions (Cherif et al., 2011). However, an excess of Zn can result in adverse effects on plants such as leaf chlorosis, nutrient imbalances and inhibition of photosynthesis at various stages, resulting in impairment of plant growth (Cambrollé et al., 2013; Sidhu, 2016). Elevated levels of Zn in the soil environment can arise from different sources, including mining and smelting activities, processing of Zn ore bodies, and Zn plating industries (Cruz et al., 2014; Kader et al., 2016; Zhang et al., 2012). Cadmium, by contrast, is not essential for plant growth and is a highly toxic metal known to interfere with several plant metabolic processes (Balen et al., 2011; Cherif et al., 2011). Elevated levels of Cd in soil may occur through several anthropogenic activities such as

land application of biosolids, mining activities and the use of phosphate fertilizers (Hooda, 2010; Reiser et al., 2014).

Generally, elemental toxicity depends on the fraction of the element that can actually be absorbed by an organism (Kim et al., 2015b). As a result, the concept of bioavailability is an important consideration in toxicology and agricultural science. This importance is illustrated by the fact that the pollutants in soil sometimes appear to cause less harm to the biological receptors than is suggested by the total content (Scheckel et al., 2009). Research has clearly indicated the decisive role of metal *speciation* in assessing metal bioavailability in the environment (McNear. et al., 2007; Scheckel et al., 2009). Therefore, a simple measure of the total soil metal content is not adequate to develop regulations governing the fate and effects of metals in the environment. Studies have shown that there are a number of environmental conditions related to the strength of metal adsorption (e.g., pH, competing ions, organic matter) that play an important role in the exposure of organisms to metals in soil. Again, this renders reliance on total metal concentration inappropriate for risk assessment and may result in poor estimates of potential risks (Kim et al., 2015b; Meers et al., 2007).

A great deal of research has been conducted to find a suitable soil-based assay for estimating the bioavailable metal pool. Broadly, two approaches have been used: quantity-based and intensity-based soil tests. 'Quantity approaches' measure the pool of metal held onto the soil, which is potentially available (i.e. able replenish the soil solution). EDTA-extractable metal and isotopically exchangeable metal (the *E*-value) are well known methods which attempt to quantify metal availability (Hamels et al., 2014).

By contrast, the 'intensity approach' measures the concentration or activity of metal ions in the soil solution. Determination of soil metal concentration using weak and neutral salt extractants, (Gray et al., 2004; Izquierdo et al., 2015; McBride et al., 2009; Menzies et al., 2007) and measurement or modelling of metal ion activity are recognized attempts to represent metal intensity (Hamels et al., 2014; Hough et al., 2005).

One of the frequently used approaches to assess metal bioavailability in soil is the Free Ion Activity Model (FIAM). The FIAM hypothesizes that the free metal ion in the soil solution is the most readily available for plant uptake (Hooda, 2010). Therefore the biological response (accumulation and/or toxicity) is a function of the free ion activity in the soil solution (Nolan et al., 2003). However, the variation in the biological response cannot be explained by the free ion activity alone. For example, the FIAM does not account for competition with other metals and protons for the binding sites. In addition, the FIAM fails to take into account the other forms of dissolved metal that may be taken up by soil organisms (Degryse et al., 2006; Wang et al., 2009). The Biotic Ligand Model (BLM) is an extension of the FIAM which integrates competition from protons and other metal ions for receptors in biotic ligands (e.g. root surfaces), and has been applied to the terrestrial ecosystem (Le et al., 2012b; Lock et al., 2007b; Thakali et al., 2006b, 2006d).

In the current work, it was hypothesized that when competition from other cations and protons are considered, free ion activity can provide a good prediction of plant metal uptake and toxic response. It was further hypothesized that the prediction will be universal across the soils tested. Therefore, the aim of this study was to assess the ability of different

extraction methods to predict metal uptake and identify soil properties that best predict the toxic effect of Zn. To achieve this aim, a range of Zn concentrations was added to 6 soils with different Zn contents, and the effect on barley growth and metal uptake was observed. Our specific objectives were to (i) determine different estimates of Zn bioavailability (total, EDTA-extractable, isotopically exchangeable, dissolved, metal free ion activity); (ii) evaluate plant uptake of metal by implementing different form of the FIAM based on the BLM; (iii) evaluate the plant's toxic response against different soil extraction methods.

4.2. Materials and methods

4.2.1. Soil collection

Soil samples were collected from a sewage treatment works in the East Midlands (UK). The site is located adjacent to the River Trent, close to the city of Nottingham (UK) and has been dedicated to sewage sludge disposal since 1880. Topsoils (0–15 cm) from 8 fields covering a wide range of zinc concentration (139 ~ 2000 mg kg^{-1} Zn) were sampled. Each sample consisted of approximately 6 kg of soil collected from each field. The soils were air-dried, homogenised, and sieved to < 4 mm.

4.2.2. Experimental setup

4.2.2.1. *Soil spiking*

Replicates of six soils were spiked with soluble Zn to produce a contamination gradient of six Zn concentrations (control + five Zn doses). The concentrations applied were intended to cover the range of anticipated *E*-values in the sampled fields (initially assuming that ≈ 90% of added Zn

would be isotopically exchangeable). The maximum concentration added to each soil varied from 1036 to 1523 mg kg^{-1} Zn. The required concentrations were achieved by adding variable volumes of a stock solution containing 15000 mg L^{-1} Zn, which was prepared using zinc sulphate. In addition to the spike solution, an additional volume of deionized water was added to achieve moist, but aerobic, soils capable of free gas exchange. The spiking solution was sprayed over the soils while they were being mixed using a stainless-steel food mixer. To ensure thorough incorporation, the mixing was continued for a further 5–10 minutes after the whole amount of the solution was dispensed. Deionized water, equivalent to the volume of solution added to the 5 spiked soils, was added to each control soil. The final water content of the incubated soils was an arbitrary choice based simply on an observation of the integrity of the soil's aggregate structure (Table 4.1). To counterbalance any potential acidifying effect arising from adding Zn, KOH was added at a rate equivalent to the metal salt addition, assuming a proton⇌metal stoichiometry of 2 (Tye et al., 2003). Soils were then placed in 2 L plastic containers (20×14×8 cm) with holes in their lids, to allow gas exchange, and incubated for 4 weeks at 10 °C.

4.2.2.2. *Pot experiment*

After the incubation period, each soil (n = 38; 6 soils amended with 5 levels of Zn + 2 un-amended soils) was split into 4 replicates of 250 g and transferred to pots (total of 176 pots). Each pot was initially planted with six pre-germinated barley seeds, which were thinned later to four. A nitrogen addition of 200 kg ha^{-1} as KNO_3 was made to all the pots with the first watering event following planting. For the duration of the experiment, watering was done only when soils became noticeably dry on the surface.

After 28 days of growth chlorophyll content was measured with a SPAD-502 meter (Konica-Minolta, Japan) on three randomly selected leaves of each plant. The photosynthetic rate was also measured on two consecutive days from one randomly selected leaf from each replication using a LI-COR instrument (LI-6400XT Portabl, Lincoln, NE). After 28 days of growth, all above-ground plant tissues (leaves and stems) were harvested. Plant tissues were washed with tap water and deionised water, to remove any soil particles, and oven dried at 50 °C for four days.

4.2.3. Soil analysis

Soil pH was measured in a suspension of air-dried soil (< 4 mm) and Milli-Q water (1:2.5 m/v) (section 2.1.1). Soil organic matter content was estimated from loss on ignition (LOI) (section 2.1.2).

Total soil elemental concentration was determined by ICP-MS following the acid digestion of approximately 200 mg of finely ground soil (See section 2.1.4). Data were corrected using blank digestions and a certified reference material, NIST-2711 Montana soil supplied by the National Institute of Standards and Technology (NIST), was used to assess the accuracy of the analysis.

An extraction with 0.01 M $Ca(NO_3)_2$ was used to mimic the soil solution. To this end, a portion of 2 g soil was weighed into tubes and 30 mL of 0.0 1M $Ca(NO_3)_2$ was added (section 2.1.3). Dissolved organic and inorganic carbon concentrations were measured in the solution phase of calcium nitrate suspensions using a Shimadzu TOC –Vcp analyzer. The pH values of the soil suspensions were also determined (section 2.1.1).

The concentrations of isotopically exchangeable Zn and Cd were measured in calcium nitrate suspensions as described in section 2.1.5. Briefly, two replicates of each soil were suspended in 0.01 M $Ca(NO_3)_2$ and pre-equilibrated for 2 days, then an aliquot (0.4 mL) of multi element isotopic spike solution, prepared from stock solution enriched with ^{70}Zn (250 mg L^{-1}) and ^{108}Cd (123 mg L^{-1}) was added to the suspensions. Depending on total metal content of each soil, four different spike levels were used.

Spike levels were designed to equate to ≈ 1% of the total metal content. The suspensions were shaken for a further 3 days to attain isotopic equilibrium. Samples were then centrifuged (2200 g) and syringe-filtered (<0.22 µm) and the isotopic ratios ($^{70}Zn/^{66}Zn$ and $^{108}Cd/^{111}Cd$) were measured in the filtered supernatant by ICP-MS. The "isotopically exchangeable" Zn and Cd (*E*-value, mg kg^{-1}) were calculated from equation 2.1 (section 2.1.5).

E-value was measured (i) after 28 days of soil amendment with Zn (before transferring amended soils into pots for plant growth), (ii) 3 times during the growth period and (iii) after harvesting and removal of plant residues.

In order to assess the robustness of the isotopic dilution technique, two of the Zn-amended soils, were spiked with different amounts of ^{70}Zn and ^{108}Cd equivalent to (0.1, 0.3, 0.5, 1, 2, 3, 4, 5 % of measured *E*-value) to estimate the uncertainty associated with *E*-value measurement.

Estimates of potentially bioavailable metal were also obtained by means of a 0.05 M EDTA extraction. This was performed by shaking approximately 2 g soil with 0.05 M NH_4-EDTA at a liquid to solid ratio of 20:2 mL g^{-1} for 1 h

and then analysed by ICP-MS (section 2.1.6) following centrifuging and filtering through 0.45 µm filter.

4.2.4. Partitioning coefficient (K_d value) estimation

The tendency of a metal to distribute between soil solid and liquid phases can be simply quantified using a distribution coefficient (K_d) (Sauvé et al., 2000). Values of K_d provide an insight to metal retention and solubility (Janik et al., 2015) and are generally expressed as the ratio of soil total metal content to soil solution concentration. However, K_d can be defined in different ways which may provide more accurate predictions for risk assessment models. For example, K_d can be expressed as the ratio between exchangeable metal and metal in the soil solution (Gooddy et al., 1995), the isotopically exchangeable pool in relation to the concentration in soil pore water (Izquierdo et al., 2013b), or the ratio between sorbed and dissolved metal (Carlon et al., 2004). In the present study, K_d was calculated as the ratio between the isotopically exchangeable adsorbed and the free ion activity in the soil solution and therefore was denoted as $K_{d\text{-}FIA}$ as follow:

$$\boldsymbol{K_{d-FIA}} = \frac{\mathrm{E} - \mathrm{D}}{\mathrm{FIA}} \qquad (4.1)$$

Where $K_{d\text{-}FIA}$ is the distribution coefficient (L kg^{-1}), *E* is the isotopically exchangeable metal concentration (mole kg^{-1}), D is the dissolved metal concentration (mole kg^{-1}) in the $Ca(NO_3)_2$ extraction, and FIA is the free divalent ion activity (M) in the soil solution.

4.2.5. Chemical speciation calculations

Zinc and Cd speciation were modelled using the geochemical speciation model WHAM 7 (Tipping, 1994). Input to the model included pH and the measured cation and anion concentrations in the $Ca(NO_3)_2$ suspension used to measure E-values. Soluble organic matter was assumed to be fulvic acid. The measured concentration of dissolved organic carbon in 0.01 M $Ca(NO_3)_2$ was converted to fulvic acid concentration by assuming that dissolved organic matter contains 50% C and that 65% of this organic matter consists of active fulvic acids (Buekers et al., 2008b; Marzouk et al., 2013d).

4.2.6. Plant analysis

The whole dried plant tissues were ground and weighed into hot-plate vials for acid digestion with 10 mL of HNO_3 and 5 mL of H_2O_2 for 2 h at 95 °C. Digested samples were then analysed using ICP-MS (section 2.1.6)

4.2.7. Modelling plant uptake

A FIAM based on the BLM principles was implemented to predict metal concentration in plants as a function of free ion activity. The free ion activity was either measured (with speciation using WHAM) or predicted, using a simple pH-dependent Freundlich equation (Hough et al., 2005; Meena et al., 2016; Tye et al., 2003) (Eq. 4.2):

$$(M^{2+}) = \frac{M_L}{K_m(H^+)^{-n}} \qquad (4.2)$$

where K_m and n are the empirical constants of the Freundlich equation, (M^{2+}) is the free ion activity in the soil solution, M_L is the labile (isotopically exchangeable) metal pool assumed to be exclusively adsorbed on humus. Isotopically exchangeable metal was used as an estimate of the labile pool

and expressed as mol kg^{-1} of SOC. This form of FIAM assumes sorption of metal from soil solution on hypothetical sorption sites (biotic ligands) while considering the competition between cations and protons for sorption sites. The total ligand sites on roots can be written as (Eq. 4.3):

$$BL_t = BL^- + HBL + \sum MBL \qquad (4.3)$$

Where BL^-, HBL, and MBL refer to free, metal bonded, and protonated root sites. The absorption reactions for both metals ions and protons were described as follows:

$$M^{2+} + BL^- = BLM; \quad K_M = \frac{BLM}{(M^{2+})BL} \quad (4.4)$$

$$H^+ + BL^- = BLH; \quad K_H = \frac{BLH}{(H^+)BL} \quad (4.5)$$

Where K_M and K_H are reaction constants. Combining equations (4.3-4.5), with the assumption that the metal uptake is proportional to the metal bound to the sorption sites on plant roots:

$$Zn_{plant} = \frac{K_t BL_t K_{Zn}(Zn^{2+})}{1 + K_{Zn}(Zn^{2+}) + K_M(M^{2+}) + K_H(H^+)} \quad (4.6)$$

Where K_t is the proportionality constant, which express the assumption that plant metal concentration is proportional to the concentration of metal adsorbed on root sites. Combining equation 4.2 and 4.6 and assuming competition with other cations and H^+, Zn uptake may be described as follows (Eq. 4.7):

$$Zn_{plant} = \frac{K_t BL_t K_{Zn}\left(\frac{Zn_L}{K_{Zns}(H^+)^{-n}}\right)}{1 + K_{Zn}\left(\frac{Zn_L}{K_{Zns(H^+)^{-n}}}\right) + K_M(M^{2+}) + K_H(H^+)} \quad (4.7)$$

Parameters in Equations 4.2, 4.6 and equation 4.7 were derived by minimizing root mean square residual (RMSD) between the predicted and

measured values of plant concentration using the Solver function in Microsoft Excel. Four modelling scenarios were tested:

Model A: measured (WHAM) free ion activities (mol L^{-1}) in the soil solution were used and competition from other cations and protons was considered (Eq.4.6).

Model B: predicted (Eq. 4.2) free ion activities (mol L^{-1}) in the soil solution were used and competition from other cations and protons was considered (Eq.4.6).

Model C: predicted free ion activity (mol L^{-1}) of Zn was optimised based on Zn_{plant} (Eq. 4.7) and competition from other cations and protons was considered.

Model D: 0.01 M ($Ca(NO_3)_2$)-extractable metal concentrations (mol L^{-1}) were used in Eq 4.6.

4.2.8. Zinc toxicity

Biomass of the 28-day growth, photosynthetic measurements, and chlorophyll content were used to establish the effective EC_{50} values (effective concentration of Zn added to soil that reduces the plant response to 50 % of control values). Values of EC_{50} were established by fitting the dose–response data to a log-logistic curve according to Eq. (4.8).

$$Y = \frac{100}{1 + e^{b(X-M)}} \qquad (4.8)$$

Where y is a measurement endpoint (shoot mass, chlorophyll content and photosynthetic rate), x is the logarithm (Log_{10}) of different measurements of Zn availability in soil. The fitted parameter M is the logarithm of the effective concentration (EC_{50}) and b is a slope parameter. To ensure a

robust fit, dose-response data were fitted to the log-logistic curve using SPSS (Version 22, NY, IBM), *drc package in R* (Ritz et al., 2015) and SOLVER function in Excel.

4.3. RESULTS AND DISCUSSION

4.3.1. General soil characteristics

The general characteristics of soils used in the study are given in Table 4.1. Most studied soils were near neutral, with soil pH in the range 6.1–7.1 except for soil S1 which was found to be slightly alkaline (soil pH = 7.8). Organic carbon contents (OC) ranged between 2.0–9.9%. The concentration of Zn varied over one order of magnitude, ranging from 140 to 1768 mg kg^{-1}. For Cd, almost a 30-fold variation was observed, with concentrations between 1.5 and 43 mg kg^{-1}. The broad range of variation reflects different biosolid application rates throughout the site.

Table 4.1. Main characteristics of the experimental soils

Soil ID	pH	LOI	Total Zn	Total Cd	IC	OC	N	Moisture content of incubated soils
		%	mg kg^{-1}	mg kg^{-1}	%	%	%	% dry weight
S1	7.8	4.4	139	1.5	0.4	2.0	0.19	10
S2	6.9	8.6	415	10.7	0.1	4.2	0.36	11
S3	7.1	13.7	623	11.4	0.2	7.2	0.47	18.2
S4	6.1	13.8	1020	15.1	0.2	6.6	0.60	11.4
S5	6.7	17.3	1740	33.7	0.5	9.4	0.87	12.7
S6	6.6	19.7	1990	37.1	0.5	9.9	0.92	14.5
S7	6.6	17.1	1770	37.4	0.3	9.3	0.78	12.7
S8	6.5	18.7	1850	42.8	0.4	9.6	0.91	15

4.3.2. EDTA-extractable Zn

The EDTA-extractable Zn concentrations varied widely ranging between 46 and 2560 mg kg^{-1} and showed a strong relationship with the total Zn

content (r=0.97 p<0.0001). Figure 4.1A shows the relationships between the total soil Zn concentration (native Zn plus added) and the available Zn, expressed as EDTA-extractable Zn.

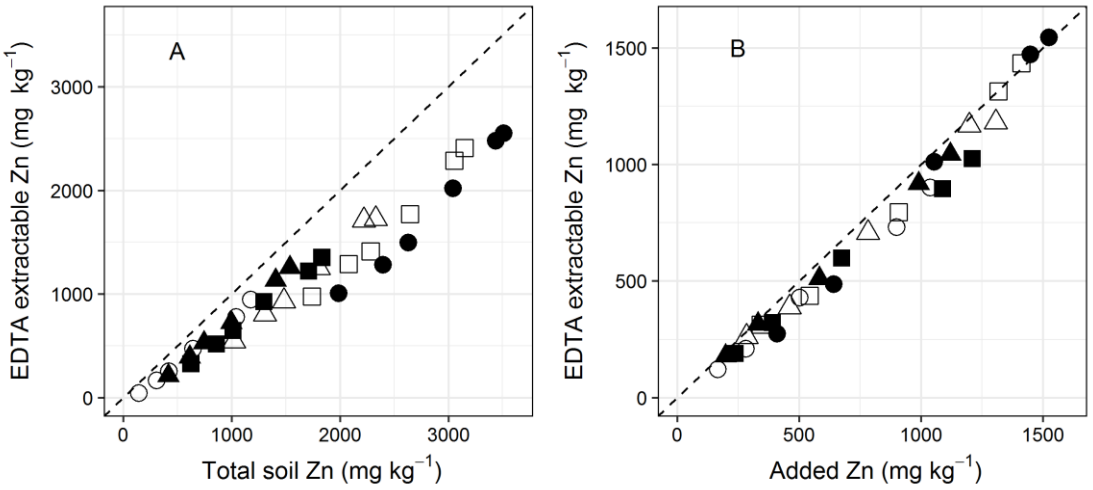


Figure 4.1. Relationships between total soil Zn and EDTA-extractable Zn (A), and EDTA-extractability of added Zn after subtraction of the native Zn concentration (B). (○) soil1, (▲) soil 2, (■) soil 3, (Δ) soil 4, (□) soil 5, (●) soil 6. Dashed lines represent a 1:1 relation

It was found that whilst 32–60% of the native Zn was EDTA-extractable, 75−100% of the added Zn remained EDTA-extractable, providing evidence that added Zn remained in more labile forms over the timescale of the experiment. Figure 4.1B shows that, when plotting added Zn against EDTA-extractable excluding the native EDTA-extractable Zn in soil, data from all soils fell on a single line. This suggests that the studied soils had a similar response (in terms of 0.05M EDTA extractability) to the same Zn addition regardless of their different properties e.g. organic C content and pH.

4.3.3. Zinc lability in soils

4.3.3.1. *Effect of isotope spike level on* E-*value determination*

Figure 4.2 shows the effect of variable amounts of isotopic tracer, ^{70}Zn and ^{108}Cd, on *E*-values determination. In general, different levels of isotopic enrichment did not have a major impact on the *E*-values which remained consistent across the different spike levels studied, particularly for Zn.

Isotopic tracer additions as small as 0.1% of the total labile metal were found to cause an analytically measurable shift in the isotopic ratios and provide a reasonable estimate of the *E*-value. However low tracer amounts (<0.5%) appeared to slightly underestimate the *E*-values in comparison to the use of greater tracer additions. In addition, the greater variations between replicates suggest that these *E*-values are subject to a greater uncertainty, especially in the case of Cd (Figure 4.2). This is likely due to a combination of factors including a less precise determination of isotope enrichment in spiked suspension, and increased error associated with handling small volumes of the isotope solution.

Expressed in terms of variation in %*E*-value relative to the total pool of metal in soil, % lability for Zn in both soils showed <10% variation across the different spike levels tested (Fig 4.3). However, for Cd % lability revealed a greater variation. Differences up to 20% in %*E*-value were reported between the lowest (10% lability) and highest spike level assayed (30% lability) for both soils. This suggests that optimisation of spike level can substantially improve the accuracy in %*E* determinations in some cases. It is not clear why Cd %*E* is more sensitive to spike levels than Zn but this could be associated with overall lower total and labile concentrations.

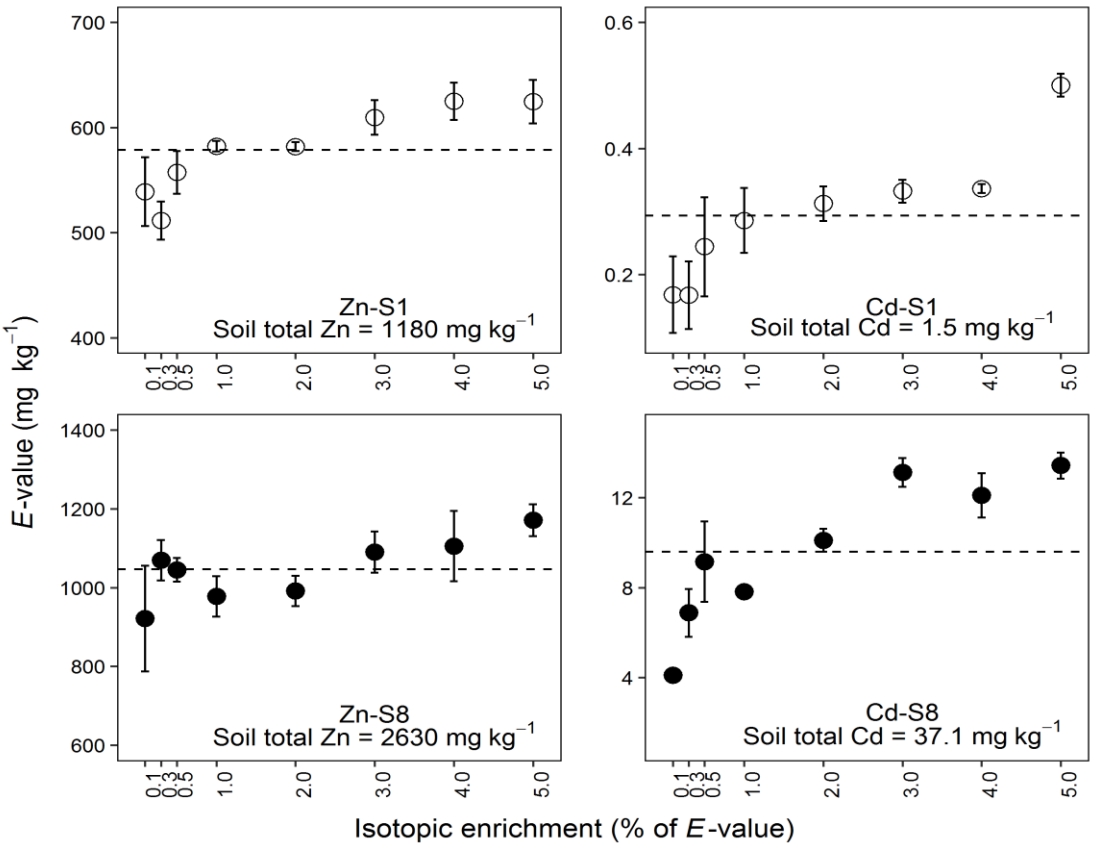


Figure 4.2. Zinc and Cd *E*-values for soils S1 (○) and S8 (●) as a function of the added isotopic tracer. The dashed horizontal line represents the mean of *E*-values (n=8).

Adding the isotopic tracer in an amount more than 4% of the total labile metal appeared to overestimate *E*-value determination. In the case of Cd, there was a 72 and 40% deviation from the average measured *E*-value for S1 and S8 respectively, while in the case of Zn 8 and 12% deviation for S1 and S8 respectively. This suggests that the soil equilibrium might have been disrupted and/or there was an acidification effect caused by over-spiking the isotopic tracer. In general, the lowest standard deviations were obtained when using an amount of the spike equivalent to 1–5% of *E*-value (Fig.4.3). These findings suggest that there is a stable range of isotopic enrichment where the error bars suggest no variation with spike level. This is broadly

in agreement with (Nolan et al., 2004b) who reported less than 5% uncertainty in *E*-value determination when the isotope were added in an amount equivalent to 5% of the *E*-value.

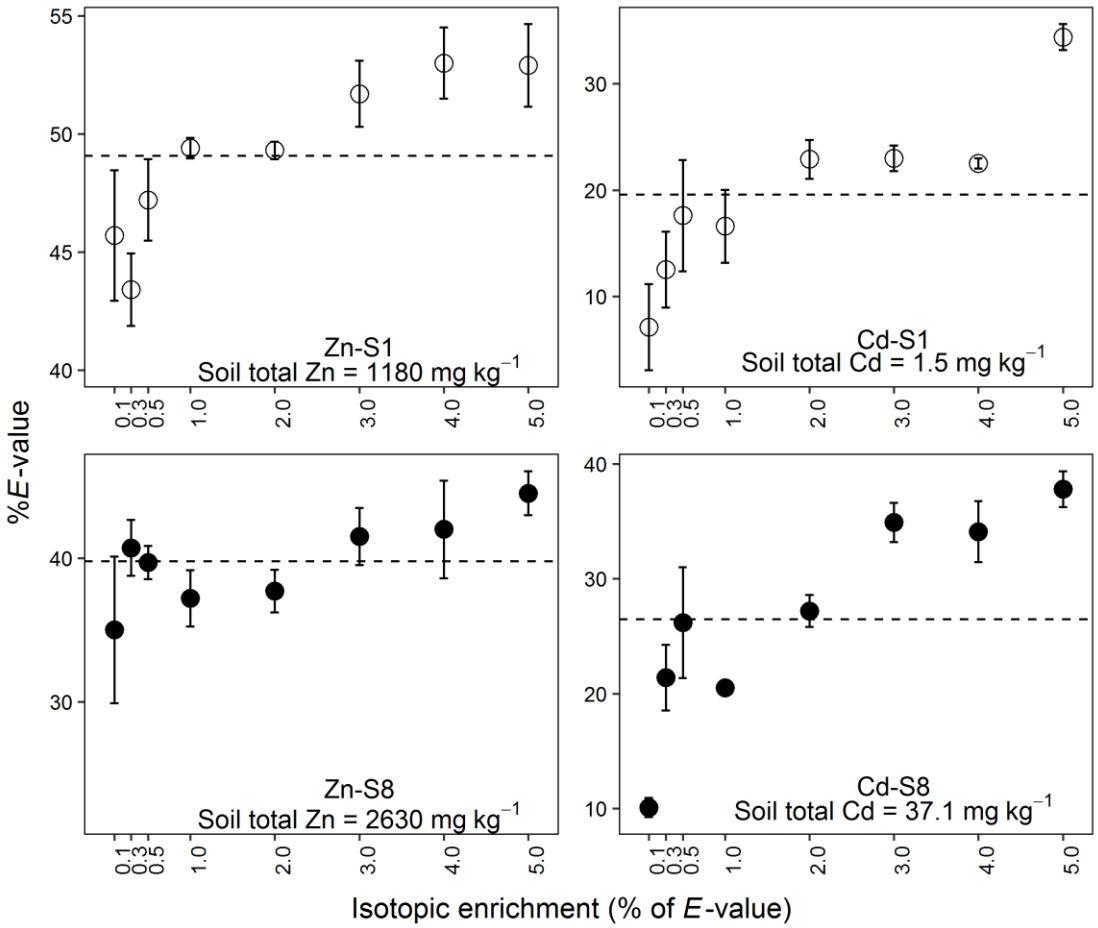


Figure 4.3. Zinc and Cd *E*-values for S1 (○) and S8 (●) as a function of the added isotopic tracer. The dashed horizontal line represents the mean of %*E*-values (n=8). Error bars represent %CV between three replicates.

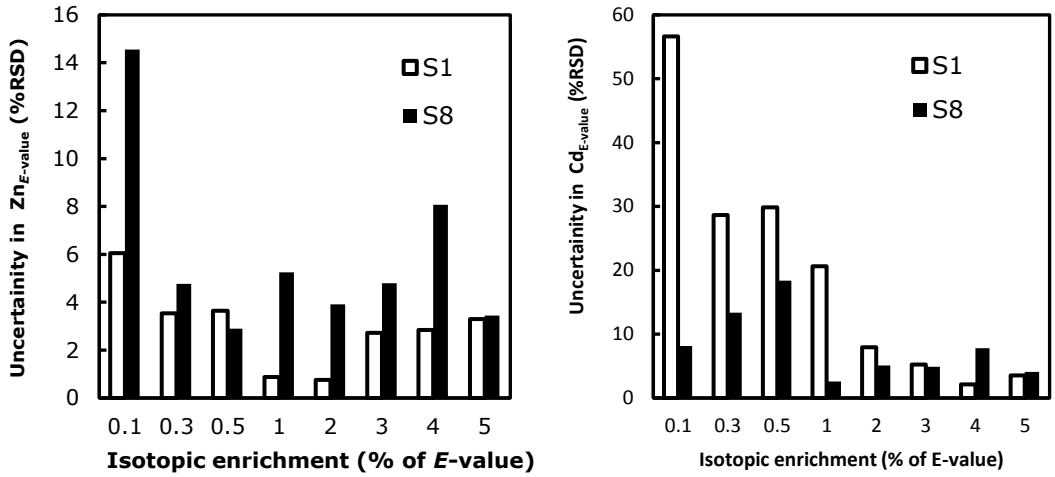


Figure 4.4. Uncertainty in determining *E*-value for Cd and Zn as a function of isotopic tracer addition

4.3.3.2. Zinc E-*values (Zn_E)*

Based on the findings of the spiking level optimisation experiment, a spike level equivalent to 1% of total labile metal was used to measure subsequent Zn_E. Values of Zn_E for soils ranged from 51 to 1630 mg kg^{-1}. Expressed as % of the total soil content, $\%Zn_E$ in control soils was approximately 30%. This is in line with values reported in the literature for polluted and unpolluted soils, which are typically in the range 10–37% (Degryse et al., 2004; Gäbler et al., 2007; Izquierdo et al., 2013b; Young et al., 2000b). The addition of Zn to soils resulted in increased values of $\%Zn_E$ to 40–60% of total Zn in soil.

As was found for EDTA extractions, *E*-values were strongly correlated with the total Zn concentration in soil ($r = 0.93$, $p<0.0001$) (Fig. 4.5A). However, there was a lower slope for the relationship compared to EDTA, with some differentiation between spiked soils.

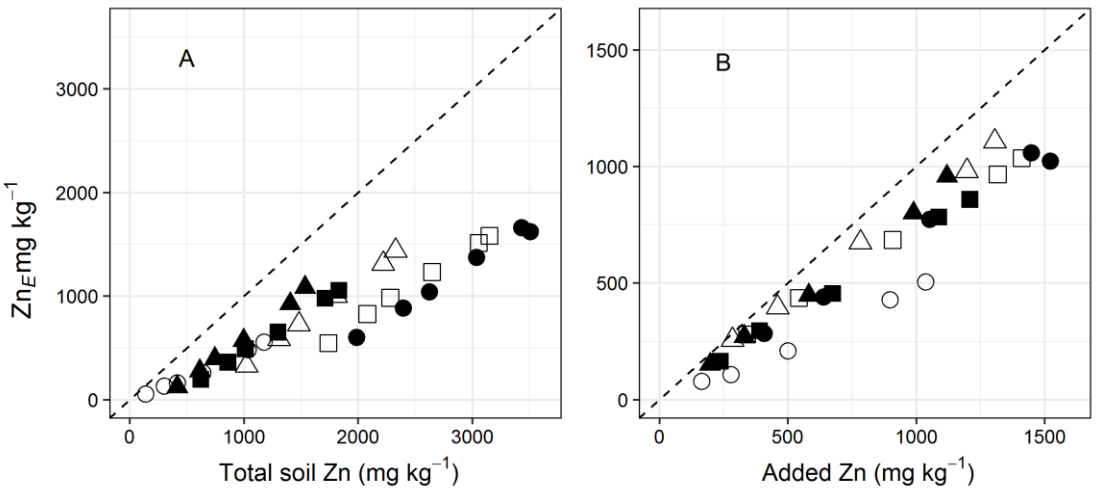


Figure 4.5. Relationships between total soil Zn and isotopically exchangeable Zn (A) and added Zn with isotopically exchangeable Zn after subtraction of the native Zn concentration (B). (○) soil1, (▲) soil 2, (■) soil 3, (Δ) soil 4, (□) soil 5, (●) soil 6.

As seen for the EDTA-extractable Zn, the comparison after subtracting the native Zn (Fig. 4.5B) revealed that all soils followed a single line suggesting that Zn lability is similar for a given Zn addition level regardless of the soil considered. The exception was Soil 1 (open circles, Fig.4.5B) which showed a distinctive trend with a lower slope indicating lower *E*-values compared to other studied soils. This could be explained by the higher pH (pH_{Ca} = 7.6) of this soil (Table 4.1) (Fig.4.6) enhancing Zn adsorption and thus resulting in 50% fixation of added Zn. Excluding Soil 1, 67–90% of the added Zn remained isotopically exchangeable. These observations indicate that initial rapid adsorption processes took place at different rates and some of the added Zn rapidly became non-isotopically exchangeable in the soil, although it largely remained extractable by 0.05 M EDTA (Fig. 4.1). This situation is probably followed by a slow aging process (Oorts et al., 2007), which would progress to some degree during the span of the experiment (3 months).

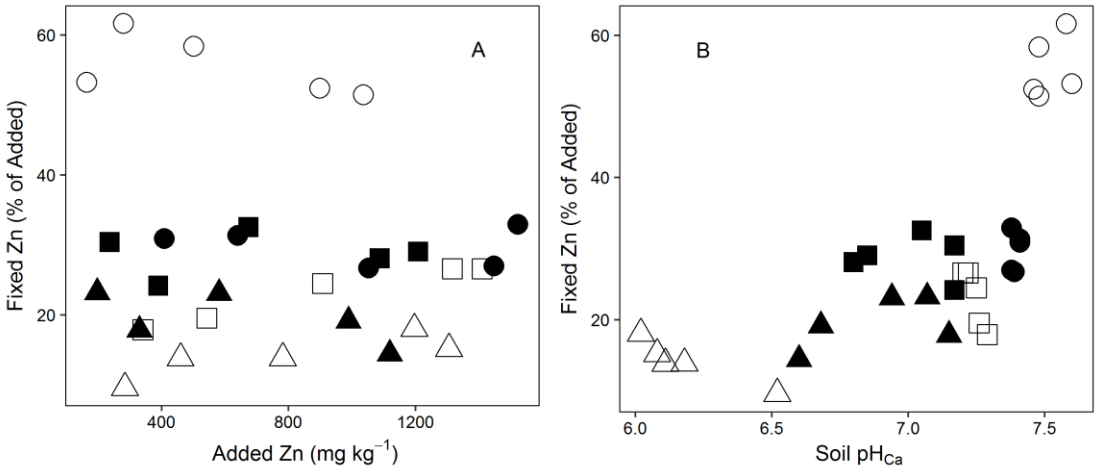


Figure 4.6. Fixed Zn (%) as a function of added Zn (A) and pH in 0.01M $Ca(NO_3)_2$ suspension (B). (○) soil 1, (▲) soil 2, (■) soil 3, (Δ) soil 4, (□) soil 5, (●) soil 6.

To investigate any effect of plant growth on Zn lability, values of Zn_E were determined before planting and after harvesting. Two potential processes are likely to affect Zn lability during plant growth: (i) rhizosphere processes associated with plant growth which may affect soil chemistry and (ii) short term (2 months) aging reactions enhancing the transfer of freshly added Zn from the soluble pool into less mobile pools in soil. The comparison depicted in Fig. 4.7 revealed little change in the Zn_E values throughout the growth period, with most soils falling along the 1:1 line. This suggests that neither of the processes mentioned above were sufficiently active or intense to effectively change Zn lability within the time span of this study. Based on these observations, it can be assumed that the barley plants were exposed to the same pool of labile Zn in soil (i.e. Zn_E) throughout their growth and therefore any variations in plant uptake or toxicity for a given soil cannot be attributed to the variation in Zn lability during the growing period.

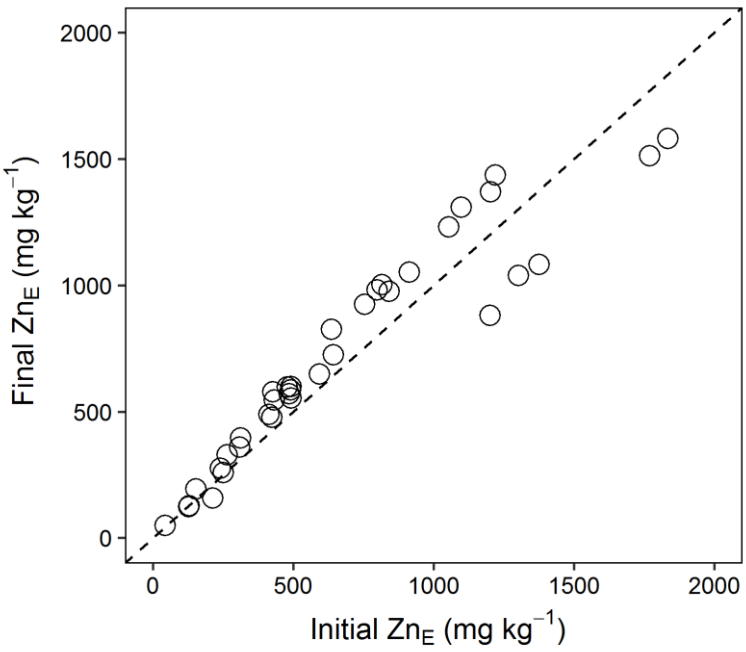


Figure 4.7. Comparison between Zn_E before planting (after 1 month incubation) and after harvesting. The dashed line represents a 1:1 relation.

4.3.4. Comparison between Zn_E and EDTA-extractable Zn

The concentrations of EDTA-extractactable Zn were compared to Zn_E values to determine whether 0.05 M EDTA can provide a simple 'non-isotopic' estimate of the labile pool of Zn in soil. There was a strong linear relationship ($r = 0.98$, $p<0.0001$) between the EDTA-extractable and Zn_E values (Fig. 4.8). However, the regression slope of 0.69 indicates that EDTA mobilised non-isotopically exchangeable Zn, which indicates that, in the current study, the use of 0.05M EDTA extraction cannot be used as a simple analogue for Zn_E.

Gäbler et al. (2007) and Izquierdo et al. (2016) reported a good agreement between the two assays using 0.025 M EDTA and 0.05 M respectively for uncontaminated soils, concluding that both methods are likely to access a similar pool of Zn and consequently EDTA extraction can be effectively used

as a simple estimate of the geochemically reactive pool of Zn in soil. This appears to be valid for uncontaminated soils, where metal concentrations are low, but for contaminated soils, 0.05 M EDTA has been reported to access the non-labile reservoir of Cd and Zn (Degryse et al., 2004; Izquierdo et al., 2013b; Nakhone and Young, 1993; Young et al., 2000b), thus resulting in overestimated *E*-values.

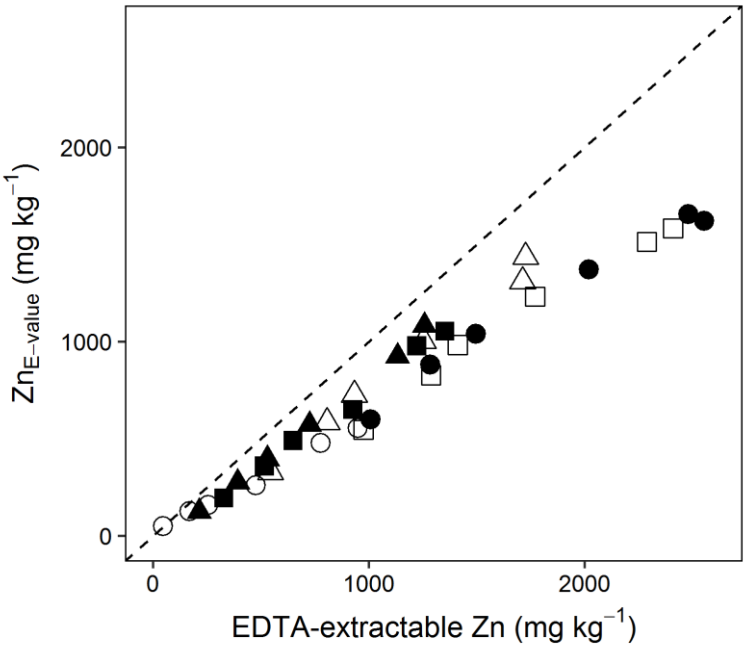


Figure 4.8. Relationship between EDTA-extractable Zn and Zn_E. (○) soil 1, (▲) soil 2, (■) soil 3, (Δ) soil 4, (□) soil 5, (●) soil 6. The dashed line represents a 1:1 relation.

4.3.5. Zinc solubility

Soil suspension in 0.01 M $Ca(NO_3)_2$ has been used to provide samples for the estimation of soil pore water Zn concentrations (Degryse et al., 2011; Marzouk et al., 2013). Dissolved Zn concentration in soil suspension ranged from 0.02 to 0.56 mg L^{-1} in the control soils. These concentrations are very similar to those reported for soil pore waters from the River Trent floodplain

in the vicinity of the studied sampling site (0.02–0.78 mg L^{-1}) (Izquierdo et al., 2013). Zinc concentration in the soil solution (i.e. $Ca(NO_3)_2$-extractable) increased linearly as a function of added Zn in all amended soils (Fig 4.9A), suggesting that a constant proportion of the added metal was extractable and potentially available for plant uptake. The results also exhibited an influence of the soil on the portion extractable by $Ca(NO_3)_2$. For instance, greater Zn extractability was found for S2 and S4. This indicate that the amended soils possessed different binding ability for the added Zn.

Speciation of soil solution using the WHAM 7 geochemical assemblage model revealed that solution Zn was largely (68–90%; average 80%) present as Zn^{2+}. Bicarbonate-complexed Zn typically accounted for 9% of Zn_{soln} except for soil 1, which showed up to 19% bicarbonate complexed Zn_{soln}. Fulvic acid bound Zn ranged between 4–20 % with an average of 10%. Free Zn^{2+} activity, as calculated by WHAM, in soil suspension ranged from 0.05 to 3.4 µM in control soils which increased to 6.5 to 82 µM at the highest Zn additions.

Figure 4.9A and 4.9B show that, in contrast to quantity based methods (i.e. EDTA extraction, isotopic dilution), intensity-based approaches were independent of soil total metal content. There was a generally increasing trend in free ion activity and soil solution concentration with increasing Zn concentration. However, a poor correlation was found due to each soil responding differently to Zn addition. For example, similar Zn additions of ≈1100 mg kg^{-1} resulted in Zn_{soln} of 1, 4 and 12 mg L^{-1} in soils S1, S3 and S4 respectively. The variable response to similar Zn additions appeared to

be primarily controlled by soil pH and organic matter content. Differences in soil pH may have accounted for variable Zn adsorption, with lower pH values enhancing Zn partition to the soil solution (Fig 4.9C). Acidification effects (i.e. resulted from Zn addition) may also increase solubility. An attempt was made to offset acidification effects caused by Zn addition, nevertheless a decrease in solution pH of up to 0.6 pH units was found in soils S1-S4. Soils with lower organic matter contents, e.g. S4, were found to be more sensitive to acidification whilst no decline in the pH was noted for soils S5 and S6, which are enriched in organic matter in relation to other soils. This could be due to the buffering capacity of soil organic matter. The difference in soil organic matter content in the studied soils may also play a role in the variable response to Zn addition, as greater organic matter contents, such as those in S5 and S6, provide a larger surface area available for metal binding - thus decreasing Zn_{soln}.

The partitioning coefficient was calculated as the ratio between the isotopically exchangeable adsorbed and the free ion activity in the soil solution (Eq. 4.1). Zinc $K_{d\text{-}FIA}$ values ($Zn_{kd\text{-}FIA}$) varied between soils. Log($K_{d\text{-}FIA}$) values were between 2.4–4.2 L kg^{-1}. These values are in the same range as previous studies (Luo et al., 2006; Sauvé et al., 2000) and in good agreement with those from alluvial soils in the area i.e. log ($K_{d\text{-}FIA}$) = 2.0 – 4.2 L kg^{-1} reported as Zn_E/Zn_{soln} (Izquierdo et al., 2013).

Figure 4.9C shows the relationships between soil solution pH and log-transformed $K_{d\text{-}FIA}$ values. Values of $Zn_{kd\text{-}FIA}$ showed a generally increasing trend with increasing pH; this is likely in response to increased strength of adsorption, which indicates that pH plays a critical role in controlling Zn solubility and thus Zn partitioning. However, the slightly scattered dataset

in Fig 4.9C suggests that other soil properties may also affect metal partitioning in soil.

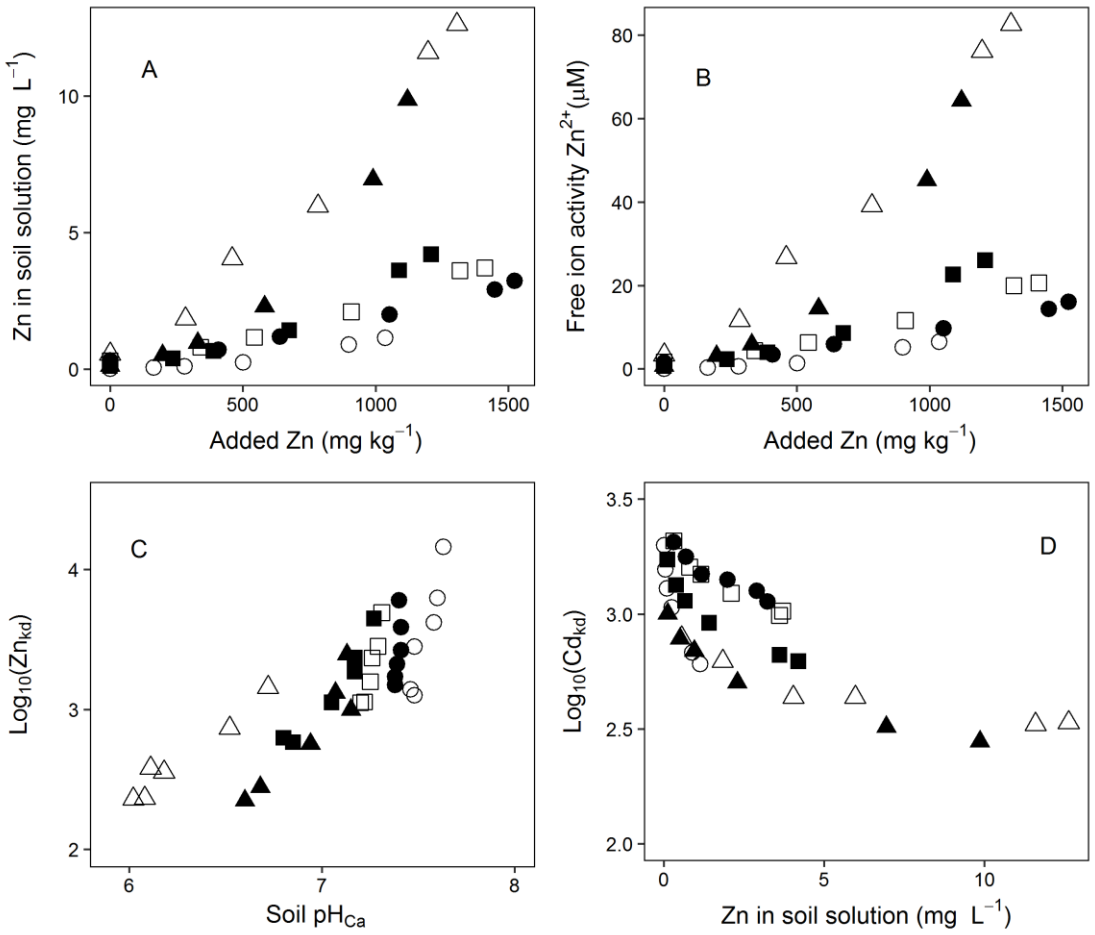


Figure 4.9. Relationship between Zn addition and solution Zn (A) and Zn FIA (B); Zn partition coefficient ($K_{d\text{-}FIA}$) as a function of soil suspension pH (C) and relationship between solution Zn and Cd partition coefficient ($K_{d\text{-}FIA}$) (D). (○) soil1, (▲) soil 2, (■) soil 3, (Δ) soil 4, (□) soil 5, (●) soil 6.

Zinc and Cd usually occur together as contaminants from anthropogenic sources in agricultural soils (McBride et al., 2017; Shute and Macfie, 2006). Evidence for competition effects between Zn and Cd was found in the studied soils. Figure 4.9D shows that Cd_{kd} values decreased with increasing Zn_{soln} concentrations. This suggests that greater Zn additions cause stronger competition with Cd for the limited available sorption sites, thus

displacing Cd into the solution phase and thereby decreasing Cd_{Kd} values. Given the same Zn solution concentration, the competition effect is more obvious in soils with low organic matter content, i.e. S1 and S2, (log Cd_{Kd} < 3) than in organic-rich soils with a greater number of sorption sites (log Cd_{Kd} > 3). Previous studies have also reported competitive interaction between Zn and Cd for sorption processes (Ming et al., 2016; Shaheen et al., 2015).

Previous works (Datta and Young, 2005; Tye et al., 2003) have demonstrated that free ion activity can be predicted as a function of labile metal content and pH using pH-dependent Freundlich equations (Eq. 4.2).

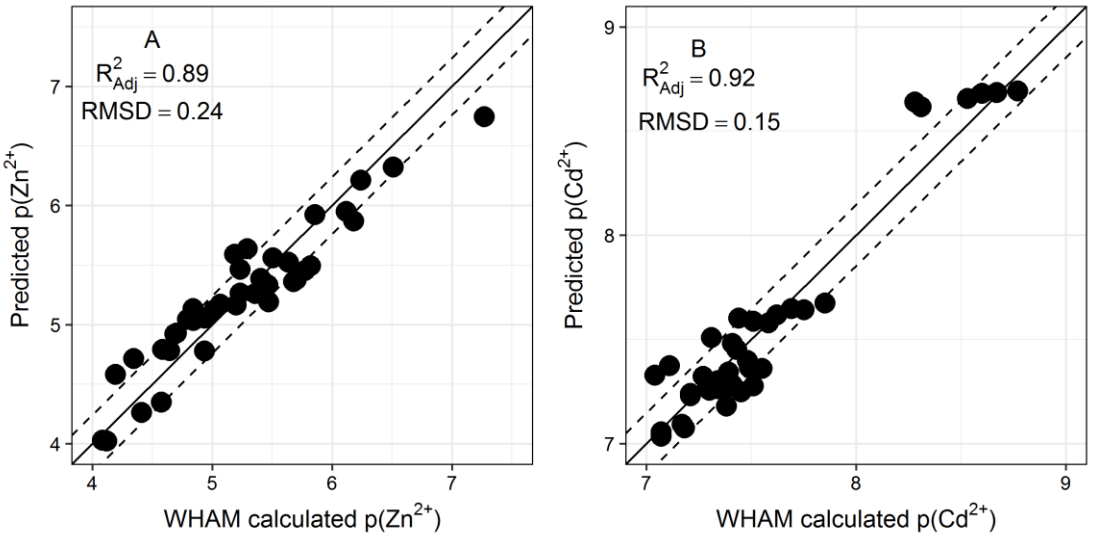


Figure 4.10. Predicted and measured values of free metal ion activity (pM^{2+}). Predicted values were derived from a parameterized version of Equation 4.2 using measured Zn_E and Cd_E; free ion activities were calculated from the speciation of the soil solution using WHAM. (○) soil 1, (▲) soil 2, (■) soil 3, (Δ) soil 4, (□) soil 5, (●) soil 6. The diagonal solid lines represent a 1:1 relation, the dashed lines represent ± residual standard error.

Figure 4.10 shows the relationships between metal ion activity (M^{2+}) predicted from a simple pH-dependent adsorption model using soil characteristics and calculated from speciation of soil suspensions using

WHAM. Between 89 and 92% of the variation observed in soil solution activity of Zn^{2+} and Cd^{2+}, respectively, were explained by parameterizing equation 4.2. This suggests that (M^{2+}) can be effectively predicted from accessible soil characteristics with high accuracy.

4.3.6. Plant uptake

4.3.6.1. *Zinc accumulation in plants*

Zinc addition to soils resulted in significantly increased uptake of Zn in barley (Zn_{plant}). The range of Zn concentration in plants over the control soils was 28 – 116 mg kg^{-1}, whereas the range at the highest Zn addition was 421 – 1220 mg kg^{-1}. The concentration of metal in plants increased with increasing levels of added Zn to soil but the response varied as a function of the soil considered. It is worth noting that, concomitant with a progressive increase in Zn_{plant}, a general decline in the concentration of Cd in plants (Cd_{plant}) was observed. This reflects Zn/Cd competition effects and will be discussed in more detail in the following sections.

4.3.6.2. *Relationships between estimates of Zn availability and Zn_{plant}*

In an attempt to predict plant metal uptake from soil properties, the relationships between Zn_{plant} and the different measures of available Zn in soil were investigated. Figure 4.11 shows the relationships between soil Zn concentration obtained by different extractions and Zn concentrations in the harvested barley plants. There is a general increasing trend in Zn_{plant} with increasing total Zn concentration in soil, but the observed correlations were weak and none of the quantity-based estimates examined i.e. total pool, EDTA extractable pool or isotopically exchangeable pool provided a good prediction of plant uptake under the current experimental conditions.

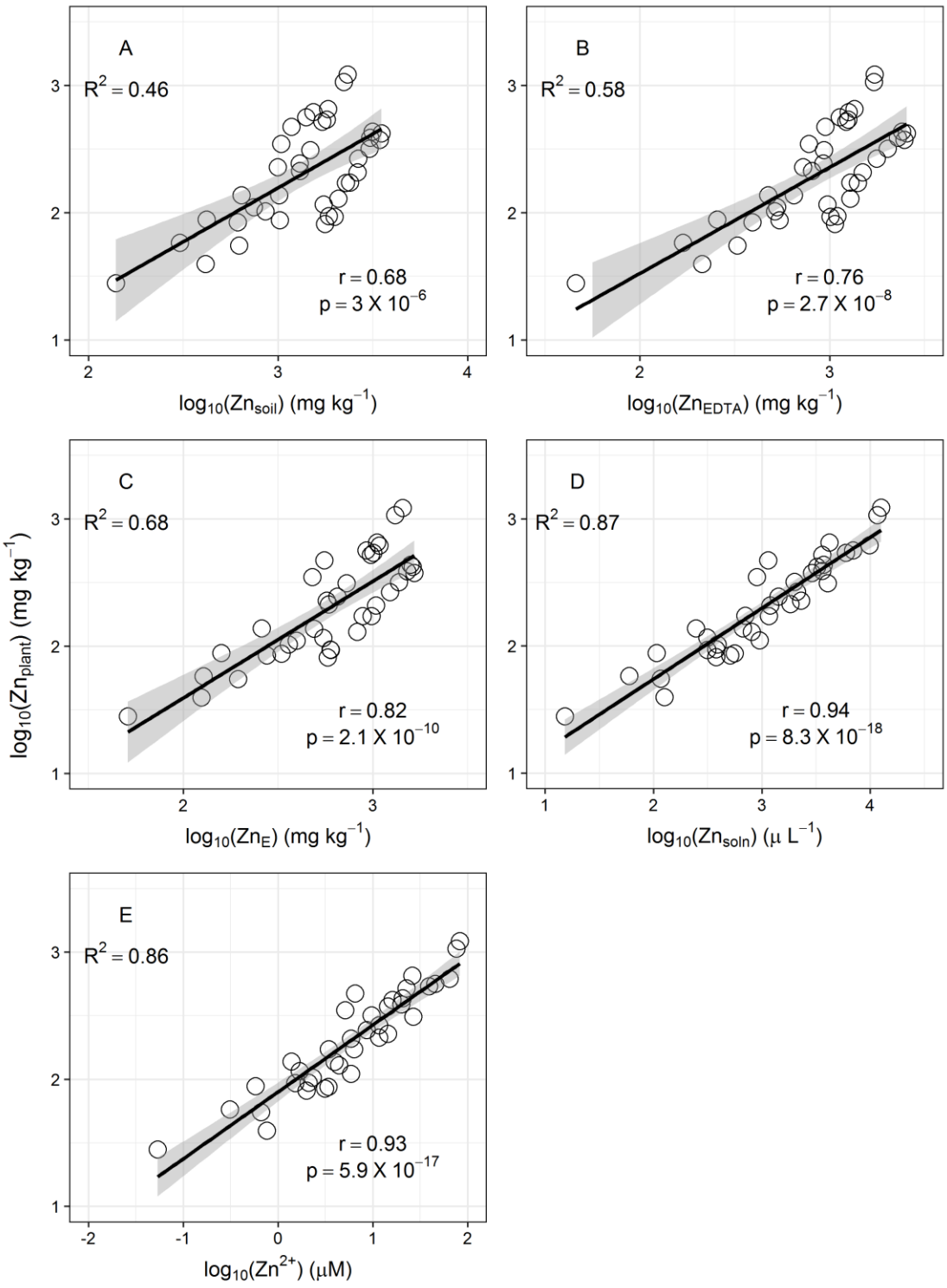


Figure 4.11. The concentration of Zn in plant [Zn_{plant}] as a function of (A) total soil concentration [Zn_{soil}], (B) concentration of labile Zn in soil [Zn_E], (C) EDTA-extractable Zn in soil [Zn_{EDTA}], (D) Zn in the soil solution (0.01 M $Ca(NO_3)_2$) [Zn_{soln}], and (E) free ion activity of Zn in the soil solution (Zn^{2+}). Shaded areas are 95% confidence intervals of the linear models.

Nevertheless, out of these 3 approaches, there was evidence that measures of 'reactive' Zn provide better indices of plant-availability than total soil Zn content. Specifically, Zn_E showed the strongest relationship accounting for 68% variability in plant uptake.

By contrast, intensity-based measurements, solution Zn and free ion activity, showed much stronger linear relationships and explained 87 and 86% of the variability in plant Zn concentration (Fig 4.11D+E). In addition, there was no clear advantage of Zn^{2+} activity over Zn_{soln} in predicting plant uptake. This suggests that a simple batch soil extraction may provide reliable prediction of plant uptake, at least specifically for the conditions of this experiment.

One index commonly used to express trace metal affinity is the transfer factor (TF), expressed as the ratio between total metal concentration in soil and the concentration in biota, as in (Eq 4.10).

$$TF = \frac{Zn_{plant}}{Zn_{total}} \qquad 4.10$$

One of the limitations of the TF is its reliance on total metal content, which may not correspond to the bioavailable fraction (Adamo et al., 2014). Therefore, values of free ion activity (Zn^{2+} and Cd^{2+}) were used to calculate TF.

The metal transfer factor index implies a linear relationship between metal concentration in plant and soil. However, that is not reflected in the decreasing trend of Zn_{TF} with increasing soil total Zn (Fig 4.12A). This indicates that plant root affinity for Zn declines with Zn concentration in the soil solution, suggesting plant control over transport of metal from soil to

plant (e.g. excluding mechanisms) (Adamo et al., 2014; Moodley et al., 2012). Moreover, Zn addition to the soil will increase other cationic metals' solubility through substitution on the adsorption sites (e.g. Cd; Fig 4.12E), which may increase competition for plant uptake. Another possible explanation is that increased total metal concentration will have toxic effects thereby impairing the ability of the organisms for uptake (Wang et al., 2004). Figure 4.12B shows greater plant affinity for Zn^{2+} with increasing pH. However, metal solubility decreases at high soil pH, which may explain the limited effect of pH on plant uptake (Fig. 4.12C). An antagonistic interaction in soil between Zn and Cd has been reported in the literatures (Murtaza et al., 2017; Sikka and Nayyar, 2012). In the current study, a dual relationship is expected. On one hand, Zn addition will increase Cd solubility through competition between Zn and Cd for adsorption sites on soil constituents (Fig. 4.12E). On the other hand, Zn addition will increase Zn in soil solution thereby suppressing Cd uptake (Fig. 4.12D).

Figure 4.12F shows that in spite of the complexity of the underlying mechanisms that control both Zn and Cd uptake, there was a remarkably strong relation between Zn to Cd ratio in the soil solution and the ratio in plant. This consistent relationship may be explained by two possible factors: (i) As Zn is added to soil, it solubilizes more Cd, but the ratio of (Zn/Cd) in solution should increase as Zn solubilizes strongly bound Cd from the soil; (ii) as Zn in solution increases it adsorbs to progressively weaker root sites which may offset the increasing (Zn/Cd) ratio in solution.

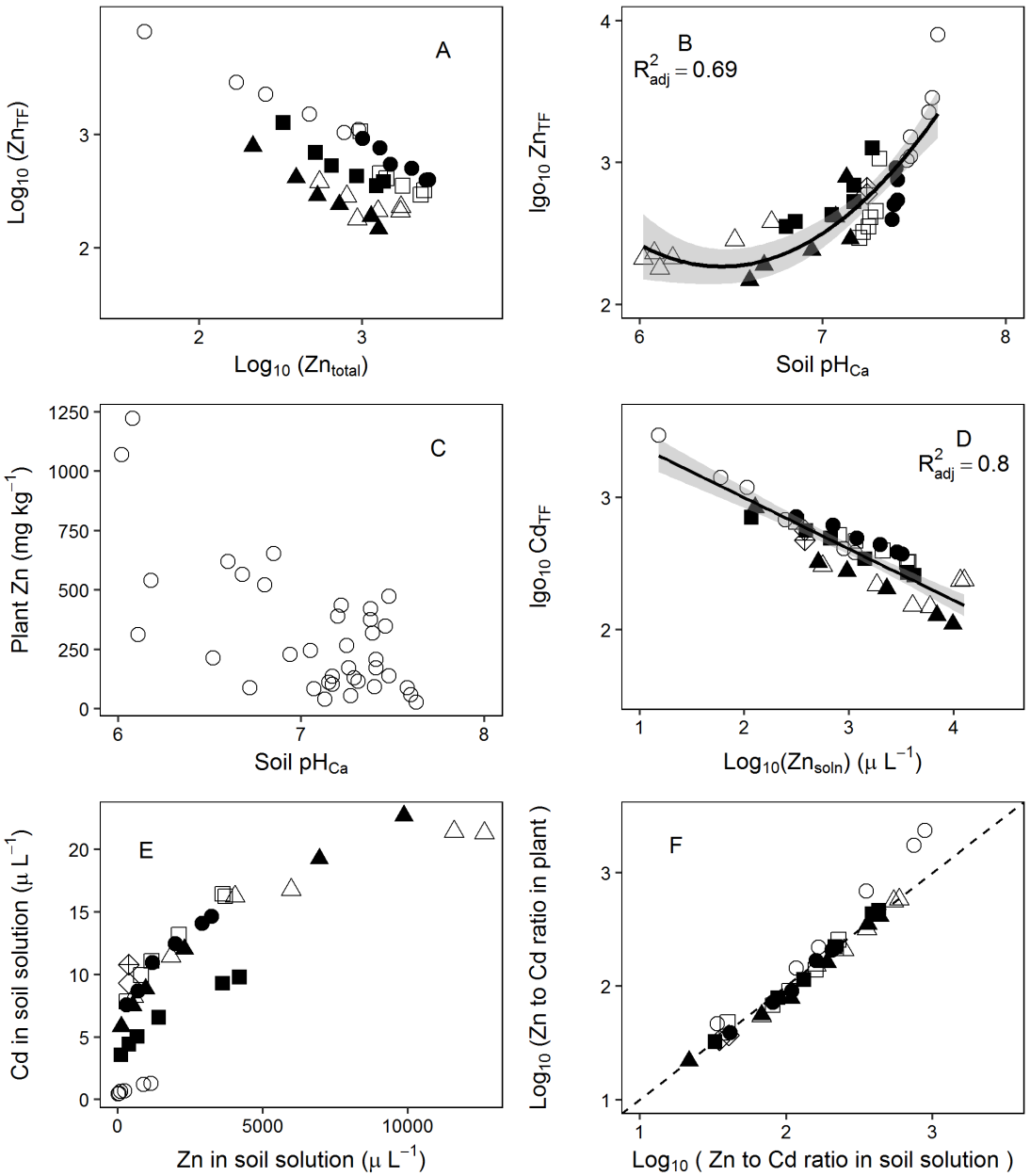


Figure 4.12. Zinc transfer factors as a function of soil total Zn (A); Zinc transfer factors as a function of soil pH (0.01 M $Ca(NO_3)_2$ suspension) (B); plant Zn concentration as a function of soil pH (C); Cd transfer factors as a function of Zn concentration in soil solution (0.01 M $Ca(NO_3)_2$ suspension) (D); relationship between Zn and Cd in soil solution (E); relationship between Zn to Cd ratio in soil solution and plant (F). Shaded areas in B and D indicate 95% confidence intervals around the polynomial and linear models respectively. The dashed line in F represents a 1:1 relation.

4.3.6.3. *Predicting plant uptake*

As stated above, weak relationships between Zn_{plant} and soil quantity-based measures were observed. By contrast, intensity-based estimates revealed stronger relationship with Zn_{plant}. Furthermore, it is increasingly recognized that models such as FIAM can provide a firmer basis for predictions of trace metal uptake into plants (Hough et al., 2005). Therefore, Zn_{plant} was related to Zn concentration in soil based on FIAM and BLM principles (Fig 4.13 and Table 4.2).

Results reported in Table 4.2 demonstrate the shortcomings of the FIAM (i.e. competition from other cations and protons is not considered) in predicting Zn_{plant} predictions. This limitations of the FIAM can be explained by three potential mechanisms:

First, there is evidence that competition from other cations and protons can modify metal uptake and toxicity. For example, Hough et al. (2005) reported that inclusion of protons as competitor in the FIAM improved the prediction of Cd and Zn uptake in perennial ryegrass (*Lolium perenne* L). Ardestani et al. (2015) confirmed proton competition with the free metal ions to bind to the BL sites of different plant species and soil invertebrates. Thakali et al. (2006d) also concluded that incorporation of protons in their BLM-based model significantly improved the fits of the logistic dose–response to barley root elongation and tomato shoot yield responses to Ni and Cu toxicity.

Second, other forms of dissolved metal (i.e. complexed) may be taken up by soil organisms. For instance, Krishnamurti et al. (1997) showed that organically bound Cd species significantly contributed to the bioavailability

of Cd. Similarly, Weggler et al. (2004) demonstrated increased Cd uptake by wheat with increasing chloride concentration in soil solution, which they attributed to potential uptake of cadmium–chloride complexes. López et al. (2005) also reported enhanced Cd uptake by alfalfa following the addition of EDTA suggesting non-ionic species uptake of Cd (i.e. EDTA-Cd complexes).

Finally, the FIAM hypothesises that the free ion activity at plant membrane is buffered from other labile species, but this assumption may not be valid where the rate of uptake is fast and where metal speciation is altered in soil, for example due the chemical and biological processes in the rhizosphere such as pH change, plant root exudates and soil microbial activity (Hooda, 2010; Kim et al., 2010).

The predictions of Zn_{plant} were significantly improved when the implementation of the FIAM assumed competition from both Cd and proton (Table 4.2). This confirms the relevance of competition effects in assessing plant uptake.

In Model B and C predicted free ion activities (Eq. 4.7), rather than measured (WHAM) were used in predicting Zn_{plant}. Because Zn_E is a relatively robust measurement that probably applies in the rhizosphere and bulk soil, it may provide better predictions of free ion activity than using Zn_{soln} values measured in a simple 1:10 soil:solution suspension.

Although using measured free ion activity (Model A) seems to be superior to using predicted Zn^{2+} in describing plant uptake (Fig. 4.13 and Table 4.2). However, measured Zn^{2+} (i.e. calculated using WHAM) assumes the same constant value of Zn^{2+} activity in both bulk soil solution and the rhizosphere.

This assumption is in doubt when concentration gradients around the root uptake zone are formed (i.e. the free ion at the root surface is smaller than in the bulk solution) (Maxted et al., 2007). In that case, the free ion activity will be largely controlled by soil buffering capacity from other labile species (Hooda, 2010), and the uptake will be limited by the diffusional flux toward the roots (Fien Degryse et al., 2009).

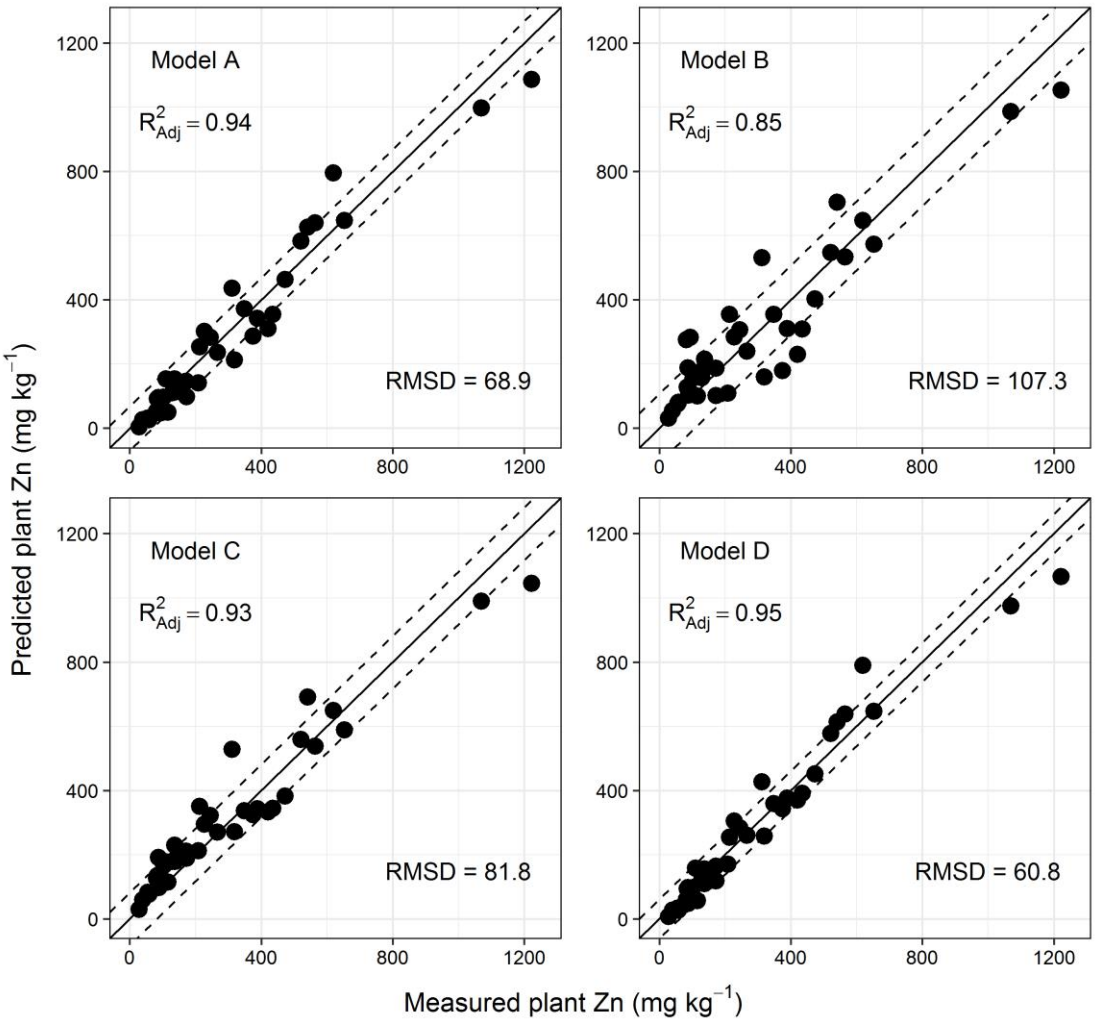


Figure 4.13. Predicted vs measured Zn concentration in plant using different modelling scenarios. The diagonal solid line represents a 1:1 relation and the dashed lines represent ± RMSD.

In addition, metal speciation in the rhizosphere will undoubtedly be different from the bulk soil due to several plant-driven mechanisms such as decrease of redox potential and acidification (Kim et al., 2010; Li et al., 2011; Zhang et al., 2014). The potential differences in the free ion activity between the rhizosphere and the bulk soil is suggested by the improvement in predicting plant uptake using Model C (i.e. optimizing predicted FIA based on plant uptake) compared to Model B (i.e. optimizing predicted FIA based on WHAM-measured FIA) (Fig 4.13 and Table 4.2).

Model D (using Zn_{soln} (in 0.01 M $Ca(NO_3)_2$) in equation 4.6) resulted in the best prediction of Zn plant uptake, accounting for 95 % of the variation in Zn_{plant} and RMSD = 56.4 mg kg^{-1} (Fig. 4.13). This is probably because a large proportion of soil solution Zn was present as free ion species (> 69% as determined by WHAM). In addition, there is evidence that free ions are not the only species absorbed by plant roots (Fien Degryse et al., 2009; Hough et al., 2005)

Table 4.2. Coefficients of determinations and RMSD for the prediction of Zn_{plant} (mg kg^{-1}) using different modelling scenarios

	Absorbing ions			
	Zn^{2+}		Zn^{2+}, Cd^{2+}, H^+	
	R^2	RMSD (mg kg^{-1})	R^2	RMSD (mg kg^{-1})
Model A	0.85	113.1	0.94	68.9
Model B	0.68	158.2	0.86	107.3
Model C	0.84	112.5	0.93	81.8
Model D	0.86	107	0.95	60.8

It has been well established that soil properties, such as pH and organic matter, affect plant uptake of metals. Therefore, incorporating these properties in extended Freundlich-type functions have often been used to predict metal transfer from soil to plant (Ding et al., 2013; Liang et al.,

2013; Liu et al., 2015). Equation 4.9 shows a log-transformed form of an extended Freundlich model.

$$Log_{10}(Zn_{plant}) = 1.51Log_{10}(Zn_{soil}) - 1.46Log_{10}(SOM) - 0.40pH_{ca} \quad 4.9$$

Figure 4.14 shows that the fit of this simple empirical regression was within the range of the various implementations of the mechanistic models (Fig. 4.13). However, a limitation of empirical regressions is their applicability to conditions (i.e. pH, M_{soil} and SOM) beyond those used in deriving such models. For instance, Hough et al. (2005) compared the performance of mechanistic models (FIAM) and a simple regression model in prediction of Cd uptake, when applied to conditions beyond those that were used to parameterize the two approaches. They demonstrated that regression models predicted a continuous increase in Cd_{plan} in response to pH decrease and Cd_{soil} increase, whereas a FIAM, in both cases, showed a limited increase in Cd_{plant} due to the proton competition for root absorption and the capacity term BL_t in Eq. 4.6 (absorption sites on the root surfaces).

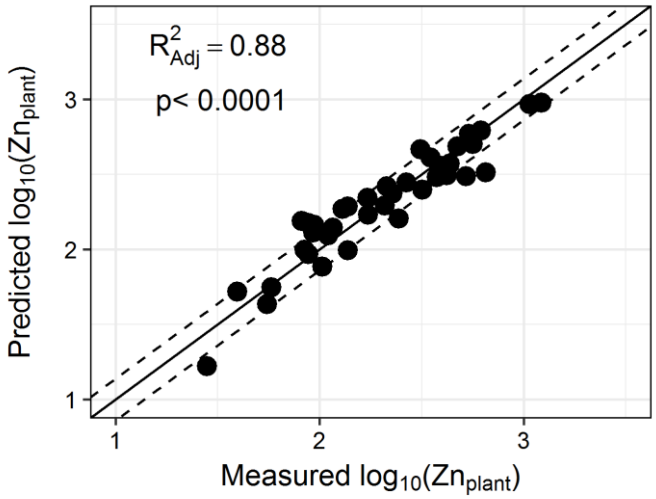


Figure 4.14. Measured and predicted $\log_{10}(Zn_{plant})$. The solid line is a 1:1 relation, and the dashed lines are ±residual standard error of the regression model (Eq. 4.9).

4.3.7. Zinc phytotoxicity

Zinc additions had an obvious visual impact on plant growth. Increased Zn addition showed severe yellowing of leaves which caused a reduction in yield indicating Zn toxicity, even though it is difficult to confidently infer the exact cause of the yellowing. However, it is probable that impairment of nutrient transfer occurred, particularly those nutrients that play a key role in chlorophyll synthesis (Adriaensen et al., 2006). Other possible mechanisms of the chlorosis caused by elevated levels of Zn is deficiency of Fe and its interference with Ca metabolism (Sidhu, 2016).

Barley growth, photosynthetic rate, and chlorophyll content exhibited a significant response to Zn additions in all Zn-amended soils. Compared to the control soils, the inhibitory effects on barley, at the maximum concentration of added Zn, were up to 53, 45, and 28% for yield, photosynthetic rate and chlorophyll content respectively.

In an attempt to predict Zn toxicity threshold, different estimates of bioavailability were used in the log-logistic model (Eq. 4.8). Figures 4.15, 4.16, 4.17 and Table 4.2 show that the biological response curve shape and the predicted toxicity threshold (EC_{50}) values varied between the different estimates of Zn bioavailability.

For relative plant growth, expressed as a percentage of the control, (Zn^{2+}) and solution concentration Zn_{soln} (intensity measures) described the relative growth very well, account for 82% and 81% of the variation in the relative plant growth, respectively (Fig. 4.15). Estimated EC_{50} were 85 and 198 µM for Zn^{2+} and Zn_{soln}, respectively. These values are in agreement with the values of Kader et al. (2015) who found that soil pore-water and Zn^{2+}

successfully predicted Zn toxicity to cucumber explaining 78% and 80% of the variation in the relative growth, they found that the EC_{50} was 79.2 µM for Zn^{2+}which is similar to the result of the current study (85 µM).

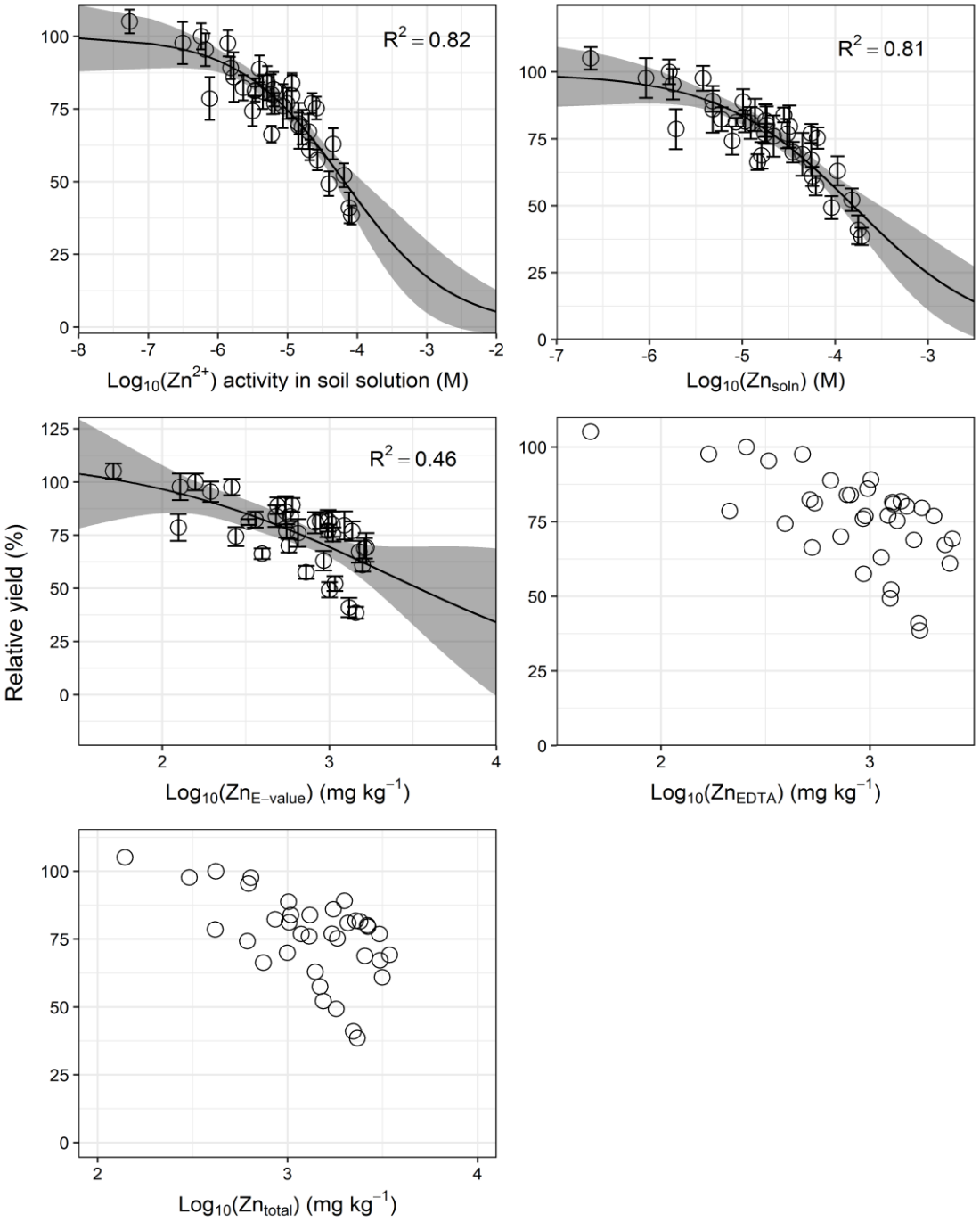


Figure 4.15. Relationships between barley relative growth (% of controls) and Zn concentration: free ion activity Zn^{2+}, in soil solution Zn_{soln} (0.01 M $Ca(NO_3)_2$) suspension), labile in soil Zn_E, soil EDTA-extractable Zn_{EDTA}, and soil total Zn_{total}. Shaded areas indicated 95% confidence interval around the curve.

In contrast to the intensity measures, estimates of the available metal reservoirs (quantity measures) could not describe the biological response to Zn toxicity (Fig 4.15). Only labile Zn_E could be fitted into the log-logistic model, accounting for 46% of the variation in the relative growth (Fig. 4.15).

Table 4.3. Phytotoxicity threshold (EC_{50}) expressed as different estimated of Zn bioavailability.

Relative plant endpoint (%)		Zn_{total} (mg kg^{-1})	Zn_{EDTA} (mg kg^{-1})	Zn_E (mg kg^{-1})	Zn_{soln} (µM)	Zn^{2+} (µM)
Plant growth	EC_{10}	No fit	No fit	35.9	14.0	1.4
	EC_{50}	No fit	No fit	3283	198	85.0
Chlorophyll content	EC_{10}	No fit	No fit	4732	316	148
	EC_{50}	No fit	No fit	4786	627	306
Photosynthetic rate	EC_{10}	No fit	No fit	No fit	1659	105
	EC_{50}	No fit	No fit	No fit	1660	881

Relative to the controls, the highest Zn addition resulted in a 10-26% reduction in the chlorophyll content. As was found for the biomass response, a log–logistic dose–response model successfully explained the toxic response. Values of EC_{50} for the chlorophyll content were 306 and 627 µM when the dose was expressed as free ion activity (Zn^{2+}) and solution concentration (Zn_{soln}) respectively (Table 2). Again, only Labile metal (Zn_E), out of the three measures of soil metal quantity, could (rather poorly) describe the relative photosynthetic rate (Fig 4.16 and Table 2.4) with less than 10% of the variation explained and an EC_{50} value of 4786 mg kg^{-1}.

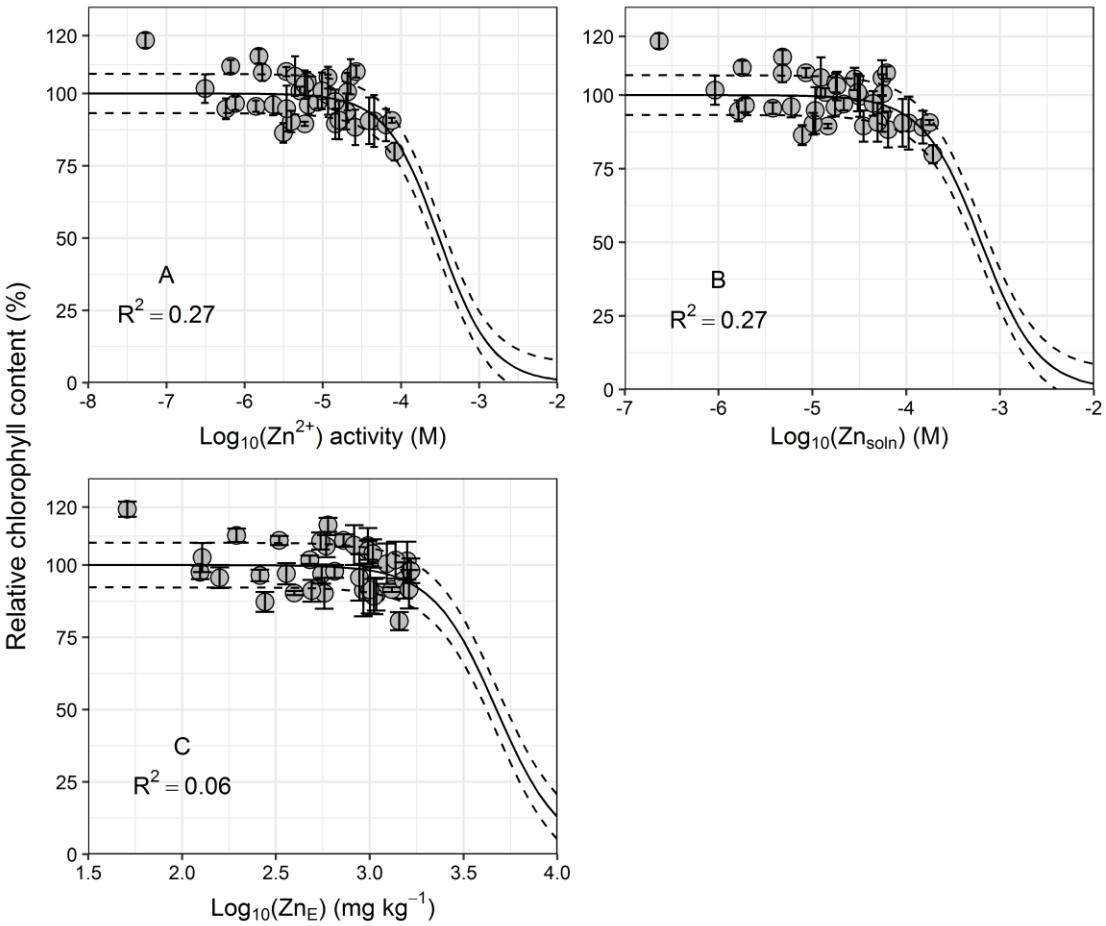


Figure 4.16. Relationships between barley relative chlorophyll content (% of controls) and Zn concentration: (A) free ion activity Zn^{2+}; (B) soil solution concentration Zn_{soln} (0.01 M $Ca(NO_3)_2$) suspension); (C) labile metal in soil Zn_E. Dashed lines indicate ± RMSD.

Compared to fresh biomass and chlorophyll content, the Photosynthetic rate was less sensitive to Zn addition. The intensity based measurements could be fitted to the log-logistic response model, but with rather weak relationships (Fig. 4.17). Values of EC_{50} for the photosynthetic rate were 881 and 1660 µM when the dose was expressed as free ion activity (Zn^{2+}) and solution concentration (Zn_{soln}) respectively. The three quantity-based measurements failed to provide a coherent prediction of the toxic response.

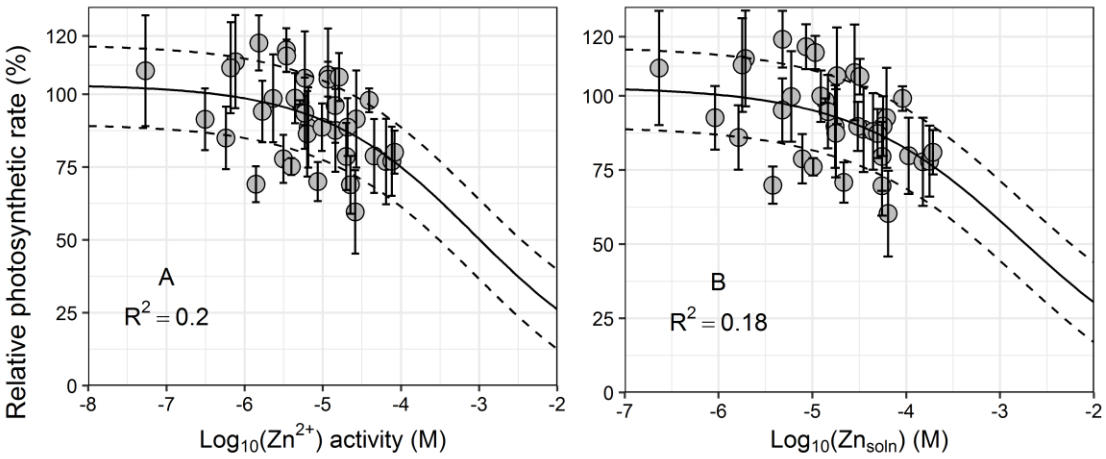


Figure 4.17. Relationships between barley relative photosynthetic (% of controls) and Zn concentration: (A) free ion activity Zn^{2+}; (B) soil solution concentration Zn_{soln} (0.01 M $Ca(NO_3)_2$) suspension). Dashed lines indicate ± RMSD.

It is worth noting that the reduction in plant growth could not be attributed to the toxic effect of Zn on chlorophyll content and photosynthetic rate as the reduction in the chlorophyll content and the photosynthetic rate, as response to Zn toxicity, was mild (Fig 4.16 and 4.17) compared to the reduction in the plant growth (Fig 4.15). This may be because of the tendency of Zn to accumulate in roots, which can result in retarded root growth and thereby limit plant uptake of water and nutrients (Beyer et al., 2013). This can be seen in Figure 4.18, where the ratio between fresh and dry weight decreased with increasing Zn concentration in soil.

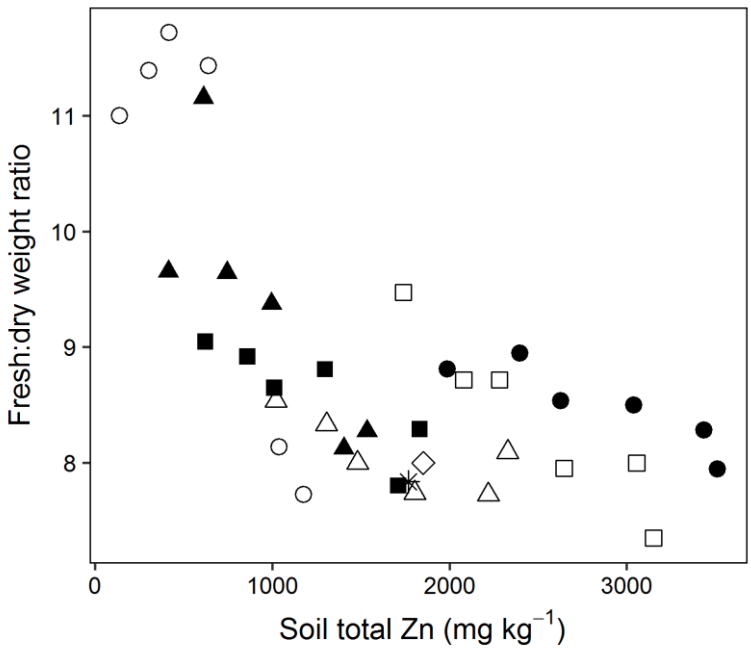


Figure 4.18. The ratio between the fresh and dry weight of plants as a function of soil total Zn concentration. (○) soil1, (▲) soil 2, (■) soil 3, (Δ) soil 4, (□) soil 5, (●) soil 6, (*) soil 7, (◇) soil 8.

The results of the phytotoxicity test indicate that the *intensity* of the Zn exposure was a better predictor of toxicity, and only when considering the variation in the chemically reactive proportion of the metal (i.e. labile metal), could quantity-based method describe plant toxic response. This is consistent with the findings of Hamels et al. (2014) who also found that 0.001 M $CaCl_2$ extraction and the DGT method (intensity-based estimates) were the most robust indices of Zn inhibition of barley shoot growth.

Discrepancies in metal toxicity between freshly spiked and long-term contaminated soils have been reported in the literature (Oorts et al., 2006; Smolders et al., 2009, 2004). However, the finding of the current study suggest that metal in the soil solution, either in ionic form or complexed, is the best predictor for plant uptake and subsequent toxic response (Hooda,

2010; Liu et al., 2015). The differences in the Zn solubility of individual soils, in response to Zn addition (i.e. spiked vs field contaminated), are probably a function of metal distribution between the solid (capacity) and solution (intensity) phases. The ratio between these two terms, capacity and intensity, may be highly variable between soils, depending on soil properties such as SOM, pH and clay content, thus both factors may contribute to metal bioavailability. Figure 4.19 shows different ratios between different measures of metal intensity and quantity as a function of different soil properties. As can be seen from Figure 4.19A and 4.19B, complex formation with dissolved organic matter and bicarbonate may be the main causes of the difference in the free ion activity concentrations. The enhanced sorption of metal at high pH values will decrease the solution concentration and thus reduce metal bioavailability (Fig 4.19D). Soil organic matter is a major source of pH-dependent negative charge in soil (Liu et al., 2015) which enhances soil's ability to retain cationic metals and reduces solubility. It must be emphasized, however, that SOM may have contrasting roles in controlling metal bioavailability depending on whether it is dissolved or solid.

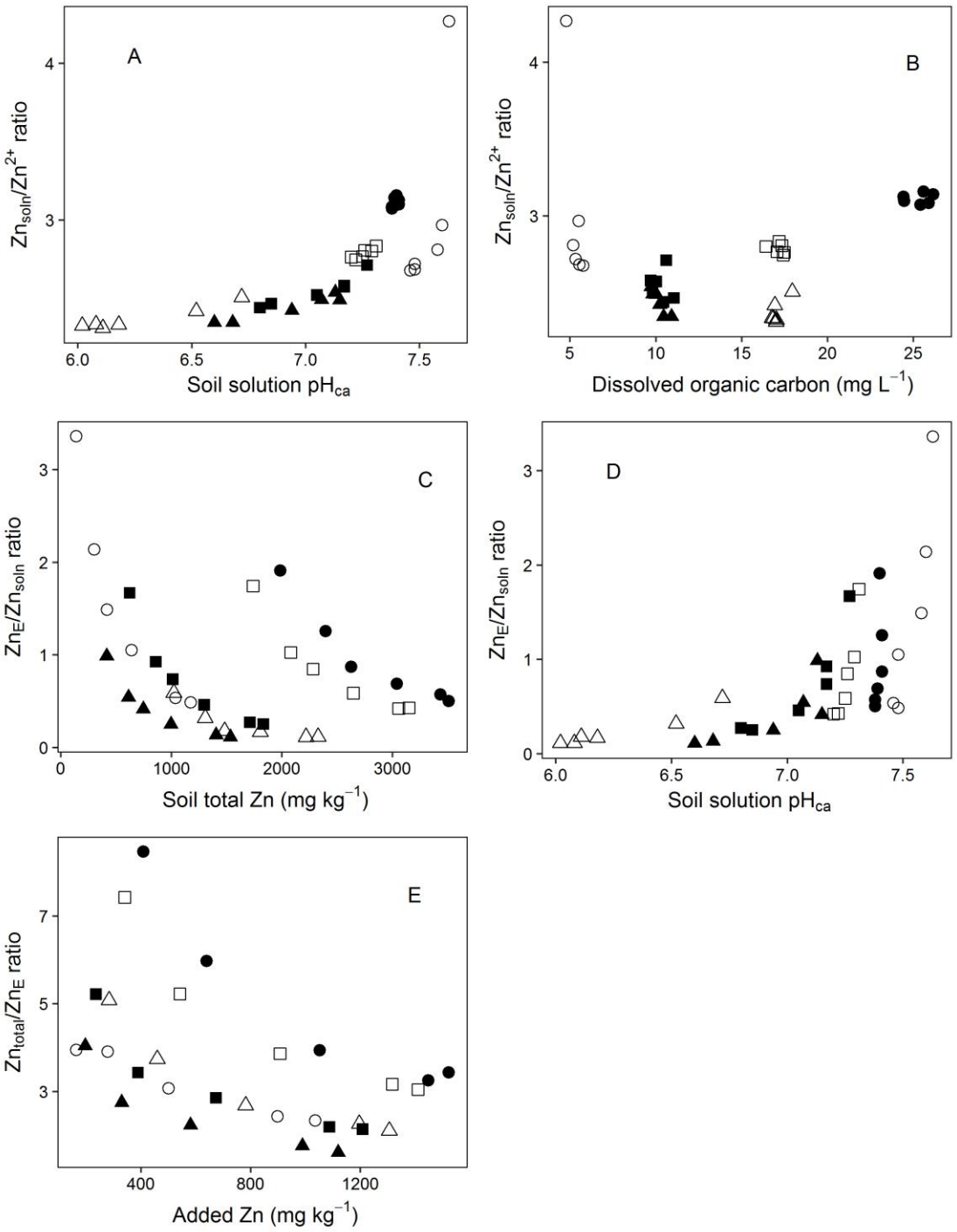


Figure 4.19. The ratio between Zn_{soln} and Zn^{2+} as function of: (A) soil solution pH (0.01 M $Ca(NO_3)_2$); (B) dissolved organic matter; the ratio between labile Zn_E and Zn_{soln} as a function of: (C) total soil Zn; (D) soil pH; the ratio between soil total and Labile Zn

4.4. Conclusions

Six historically contaminated soils were spiked with soluble Zn to investigate a range of proxies for metal bioavailability and, ultimately, determine the assay that can potentially give the best explanatory variable for plant uptake and toxicity.

Zinc lability in the studied soils was approximately 30% which is in line with values reported in the literature. The addition of Zn to soils resulted in a soil dependent increase in $\%Zn_E$ to variable degrees as a function of added Zn. Approximately 50-90% of the added Zn remained isotopically exchangeable suggesting that initial rapid adsorption processes were soil dependent (largely pH-controlled). Some of the added Zn rapidly became non-isotopically exchangeable in the soil, although it largely remained EDTA-extractable. Whilst it is reasonable to assume that this was followed by slower aging processes progressing to some degree during the span of the experiment (2 months), there is little evidence to support this as no significant change in the E-values throughout the growth period was seen. This suggests that aging reactions are not sufficiently active to substantially transfer freshly added soluble Zn into less mobile pools in soil over the time period of this experiment. Constant *E*-values also indicate that rhizosphere processes associated with plant growth did not drastically change the soil chemical conditions. Zinc solubility was largely independent of the added Zn and was primarily controlled by differences in soil properties (e.g. pH and SOM).

Quantity-based estimates of Zn availability i.e. total, EDTA-extractable or isotopically exchangeable pools were poorly correlated to plant uptake and

therefore are of limited use in predicting Zn uptake by plants. By contrast, intensity-based measurements i.e. solution Zn and free ion activity provided a more reliable estimate. This suggests that a reasonable estimate of Zn_{plant} may be obtained by performing a simple batch soil extraction with water or dilute neutral salt.

Evidence of an antagonistic relationship between Zn and Cd was observed for competition for both sorption on soil constituents (Cd_{soln} increased with increased Zn_{soln}) and for plant uptake (Decreased Cd_{TF} with increased Zn_{soln}).

The FIAM alone could not predict plant uptake but a reasonably good prediction was obtained using the BLM. When competition from Cd was assumed 94% of the variation was accounted for. Incorporating predicted (soil pH and E-values) rather than measured Zn^{2+} in FIAM also yielded an acceptable prediction of plant uptake which indicates the possibility of using routinely measured soil properties as a substitute for M^{2+} which is not readily measured on a routine basis.

The plant's toxic response was described remarkably well by the log-logistic dose-response curve when using the intensity of the exposure in a clear contrast to the capacity of the exposure. Therefore, since the ratio of capacity to intensity varies as a function of soil properties, both factors are important in determining metal bioavailability and subsequent toxicity.

5 The response of soil microbial diversity and abundance to long-term application of biosolids*

5.1 Introduction

Municipal wastewater treatment results in large amounts of biosolids that require disposal and this is a major worldwide environmental concern. A number of disposal methods are used, including application to agricultural soils, dispose to landfill, and incineration (Sánchez-Monedero et al., 2004b; Singh and Agrawal, 2008b). Land application is suggested to be the best practicable environmental option and the most cost-effective method for the disposal of biosolids (Singh and Agrawal, 2008b). This is because the practice supplies valuable nutrients and improves soil physical and biological properties by providing organic matter to soils (Coors et al., 2016; Singh and Agrawal, 2008b). However, there are potentially damaging effects from long-term disposal of biosolids to soil including increased concentrations of potentially toxic metals and organic compounds (Sánchez-Monedero et al., 2004b; Singh and Agrawal, 2008b), nitrate and phosphorus contamination of water (Joshua et al., 1998), and the introduction of pathogens to arable soils (Hong et al., 2009). It has been demonstrated that excessive application of biosolids to soil can cause stress to soil microorganisms. Macdonald et al. (2011) reported changes in the soil microbial community in response to 11 years exposure to sewage sludge enriched in Zn and Cu. Charlton et al. (2016) reported a 7-12%

* The content of this chapter was published in Environmental Pollution Journal:
Mossa, A.-W., Dickinson, M.J., West, H.M., Young, S.D., Crout, N.M.J., 2017.The response of soil microbial diversity and abundance to long-term application of biosolids. Environ. Pollut. 224, 16–25. doi:10.1016/j.envpol.2017.02.056

decrease in soil microbial biomass carbon in soils which had received sewage sludge contaminated with Zn and Cu over a period of 8 years. Understanding the effect of biosolids on soil microorganisms is important due to the critical role of microorganisms in biogeochemical processes and ultimately in ecosystem functions, such as decomposition of organic matter and nutrient cycling (Nannipieri et al., 2003).

The impact of biosolids application on soil microbiota has been explored previously (Chen et al., 2016; El Azhari et al., 2012; Kelly et al., 1999; Macdonald et al., 2011). However the time scale of most previous work was relatively short and therefore the resilience of microbiota to long-term exposure to extended successive biosolids application over time has not been assessed. In the relevant literature, there is little quantitative research that relates heavy metal contamination and biosolids loading to soil microbial diversity and community composition. This is because previous observations reported in the literature have been made at sites where the heavy metal concentrations were close to, or below, the regulatory limits and where the duration of disposal was short (ca. 10 years). Therefore, the study site presents an excellent opportunity to investigate the impact of heavy metal loading in soils subject to biosolids applications over >100 yr and where the metal concentrations are above the regulatory limits for normal arable land which does not have 'dedicated site' status (e.g. several thousand mg kg^{-1} Zn).

Several molecular techniques are available to characterise the soil microbial community and these can be used to monitor any potential changes in response to environmental stress. Techniques include ribosomal intergenic spacer analysis (RISA), denaturing and temperature gradient gel

electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) (Leckie, 2005). These techniques generally involve producing a fingerprint of the microbial communities based on amplifying genes, using polymerase chain reaction (PCR), extracted from the community. One of the most frequently used profiling techniques is T-RFLP, which depends on the variation of restriction sites within the sequences of different organisms (Schütte et al., 2008).

This chapter was driven by the hypothesis that prolonged application of biosolids to the soil will induce an increase in activity and diversity of soil microbial populations arising from increased humus content. It was further hypothesized that biosolids, due to heavy metal loadings, will induce shifts in soil microbial community structure. To address these hypotheses, the study aimed to (i) investigate the effect of biosolids application on several soil chemical properties (pH, organic matter, and labile and soluble metal concentrations), (ii) assess the long-term impact of biosolids on soil microbial diversity, (iii) investigate soil bacterial and fungal community structure assessed by the T-RFLP fingerprinting technique. The overall aim was to assess the balance of positive and negative effects on soil health arising from biosolids application and determine whether an optimum loading was apparent at the study site.

5.2 Materials and methods

5.2.1 Study site

The study was conducted on a dedicated sewage sludge disposal site, located adjacent to the River Trent, east of Nottingham, UK. The site comprises approximately 60 fields, some of which have been used for

disposal of sewage sludge since 1880. The topsoils show a wide range of metal content ranging from background levels to high levels of metal contamination. It is important to note that the study location is a working site run by a water company for practical sewage disposal purposes. There are no records of biosolid disposal across the site, or control fields. The value of the site lies in its size and the range and duration of biosolids disposal (for >100 year). It is the resulting variation in soil organic matter, phosphate and metal concentrations that provides the experimental basis for testing the hypotheses.

5.2.2 Sample collection

Soil samples were collected from 17 fields sown with maize. Four bulk soil samples within rows were taken from a 1 m^2 area from the upper 20 cm and combined into one composite sample. Four plant roots from maize were uprooted and the loosely adhered soil shaken off the roots in order to conserve the remaining adhering soil as 'rhizosphere soil'. Soil samples from bulk sampling and the rhizosphere (n=34) were sieved to <4 mm and then split into two sub-samples for analysis of microbial diversity, using T-RFLP assays, and soil chemical properties. The subsamples for molecular analysis were initially stored at 4 °C for 24 hours before storing at -20 °C; soil for chemical analysis was air dried and a subsample (c. 20 g) was agate ball milled (Retsch, Model PM 400) prior to acid digestion.

5.2.3 Soil chemical analysis

5.2.3.1 Soil characteristics

Total soil elemental concentration was determined using ICP-MS (Thermo-Fisher Scientific X-Series^{II}; Thermo Fisher Scientific Inc., Waltham, MA,

USA), following acid digestion (section 2.1.4). Soil organic matter was estimated from loss on ignition (LOI) (section 2.1.2). Soil pH was measured in a suspension (1:2.5 m/v) of air dried soil (< 4 mm) and Milli-Q water after shaking for 30 minutes on an end-over-end shaker. Dissolved organic and inorganic carbon were measured, using a Shimadzu TOC –Vcp analyser, in the solution phase of soil suspensions in 0.01 M $Ca(NO_3)_2$ (3 g: 30 mL) (2.1.3). The concentrations of isotopically exchangeable Ni, Cu, Zn, Cd and Pb were determined as described in section 3.2.6.

5.2.3.2 Soil solution speciation

Nickel, Cu, Zn, Cd and Pb speciation were resolved using the geochemical model, WHAM VII (Tipping, 1994). Inputs to the model included cation and anion concentrations and pH value in the $Ca(NO_3)_2$ extract (section 2.1.3). The dissolved organic carbon (DOC) was converted to fulvic acid concentration by assuming (i) a carbon content of 50% and (ii) that fulvic acid constituted 65% of DOC (Buekers et al., 2008c; Marzouk et al., 2013a)

5.2.4 Microbial community structure

T-RFLP was used to investigate soil microbial community structure. Briefly, DNA was extracted from 0.25 g of each soil sample using a Power Soil DNA extraction kit (Mo Bio, Carlsbad, CA, USA) as instructed by the manufacture. Extracted DNA was amplified targeting the bacterial 23S ribosomal subunit gene and fungal ITS-2 region. For bacterial DNA, (5'-GCG ATT TCY GAA YGG GGR AAC CC-3') primer and the reverse primer (5' TTC GCC TTT CCC TCA CGG TAC T-3'), labelled with the blue WellRED dye D4 (Sigma–Proligo, Gillingham, UK), were used (Martin et al., 2012). The fungal DNA was amplified using 5.8S for (5'-GCA TCG ATG AAG AAC GCA

GC-3') and FITSrev (5'- ATA TGC TTA AGT TCA GCG GGT-3'), labelled with the green WellRED dyeD3 (Sigma–Proligo, Gillingham, UK) (Martin et al., 2012). Prior to fragment analysis, PCR products were digested with restriction enzymes in 20 µL reactions made of 10 µL PCR product, 2 µL10x RE buffer (New England BioLabs, Hitchin, Hertfordshire, UK), 7 µl de-ionised water, and 1 µL of restriction enzyme. For bacteria, T-RFLP double digest (0.5 µL) was carried out using both MseI and HindIII enzymes (New England BioLabs, Hitchin, Hertfordshire, UK). For fungal T-RFLP only HaeIII enzyme (New England BioLabs, Hitchin, Hertfordshire, UK) was used. Digests were incubated at 37°C for 2 hours to complete the digestion followed by deactivation of enzymes by heating to 80 ºC for 20 min. Digestion products were verified on 1% of agarose gel containing ethidium bromide in 1X TBE buffer. Gels were run for 1 hr at 90V and then viewed under UV light. Both bacterial and fungal digests were then mixed in a 2:1 ratio prior to fragments analysis to accommodate differences in signal strength, because different dyes were used for each. One µL aliquots of the digest mix were loaded into a 96 well plate with each well containing 38.5 µL of GenomeLab sample loading solution and 0.5 µL internal 600 base pairs (bp) standard ladder (Beckman Coulter Inc.) to allow fragments between 60-640 bp to be considered. The samples were loaded with mineral oil to prevent oxidation of the sample and then separated by electrophoresis using a CEQ 8000 DNA analysis system (Beckman Coulter Inc, HighWycombe, UK).

5.2.5 Data analysis

Numerical data were obtained from transforming T-RFLP using CEQ 8000 (Beckman Coulter Inc, High Wycombe, UK). Only T-RFs larger than 60 bp were included in the analysis to avoid any dimmer signals. Data were

further analysed using the T-REX platform (Culman et al., 2009) for noise reduction (Abdo et al., 2006) and fragments with a band width of 1.25 bp or less were binned (Martin et al., 2012). The relative abundance of individual T-RFs was calculated as the percentage of total peak height in a given T-RFLP profile. Only those T-RFs with a relative abundance of greater than 1% were considered (Azziz et al., 2016). These refining techniques increase the independent integrity of the T-RFLP profiles and enable comparisons to be made across soil samples. Nevertheless, individual TRFs will include more than one species.

The *Vegan* package (Oksanen et al., 2016) in R (R Core Team, 2015) was used to conduct all statistical analyses. The compositions of T-RFLP profiles were visualized with non-metrical multidimensional scaling (NMDS), using vegan's *metaMDS()* function, based on Bray–Curtis dissimilarity matrix of Hellinger transformed T-RFLP profiles (Legendre and Gallagher, 2001). Relationships between soil properties and soil microbial communities were further analysed with Redundancy Analysis (RDA); total, isotopically exchangeable, dissolved and the free ion activities of Ni, Cu, Zn, Cu, and Pb in addition to the total elemental concentrations were included to calculate the explained variance in RDA. The most appropriate statistical model for RDA was determined using forward selection as implemented in vegan's *ordistep ()* and *anova*() function. Only variables with $P<0.05$ were included in the selected model; to avoid any collinearity, the variance inflation factor (VIF) was calculated and only variables with VIF < 10 were included in the model (Borcard et al., 2011).

T-RF richness refers to the number of peaks, whilst Simpson's Diversity Index was calculated as implemented in R Vegan's *diversity()* function

(Oksanen et al., 2016) using equation 5.1, which accounts for T-RF abundance in addition to number.

$$D = 1 - \sum_{i=1}^{S} \left(\frac{n_i}{N}\right)^2 \qquad (5.1)$$

Where n_i is the abundance of an individual TRF, N is the total abundance of all TRFs, s is the total number of TRFs.

5.3 Results and discussion

5.3.1 Soil characteristics

The general characteristics of soils used in the study are given in Table 5.1. Loss on Ignition (%LOI), ranged from 3.5% to 23 %. Soil pH showed near neutral values with very little variation across the 17 fields (soil pH= 6.9–7.6). Total N ranged between 0.4 – 1.2 %.

The soil metal concentrations covered a wide range, in some cases spanning over one order of magnitude (Table 5.1). This is the product of variable biosolids application rates over the operational time period of the site. A Pearson correlation matrix (Table 5.2) showed very strong relationships between individual trace metals in addition to strong correlations between the expected indicators of biosolid application such as soil organic matter (LOI), total P content, and metal loading. It should be noted that (i) the site has been exclusively used for biosolids disposal, (ii) the original soils would have similar background metal concentrations, and (iii) the site consists of free draining soil used for arable agriculture. Therefore, the observed LOI reflects different levels of biosolids application, rather than individual field soil properties. Based on the above observations and, in the absence of past historical records of disposal, it is reasonable to assume that any of the

metals could be used as a proxy measure of biosolids application. In this work Zn was adopted as an indicator for biosolids application because of the highly significant correlation with other biosolids tracers i.e. soil organic matter and phosphate content (Table 5.2). Furthermore, very similar results were obtained when different metals e.g. Cd or Cu were used instead of Zn (data not shown).

Table 5.1. Descriptive statistics of the main properties of soils sampled

		Mean	Minimum	Median	Maximum
pH		6.9	6.4	6.8	7.6
LOI (%)		12.5	3.5	10.3	23.4
N (%)		0.7	0.4	0.5	1.2
P (mg kg^{-1})		6330	1510	5130	13700
Cr (mg kg^{-1})		656	67	483	1670
Ni (mg kg^{-1})		171	28	122	415
Cu (mg kg^{-1})		311	44	172	766
Zn (mg kg^{-1})		861	122	570	2050
Cd (mg kg^{-1})		16.8	1.4	8.4	48.6
Pb (mg kg^{-1})		353	70	270	688

With the exception of Pb, isotopically exchangeable metal concentrations (Cu, Zn, Cd, Ni) strongly correlated with their respective total concentration in soil ($r>0.98$, $P<0.001$). Copper was the most labile metal (31 - 44 %), whereas Pb had limited lability (1.5 – 8.4 %) (Fig. 5.1) as also determined for this site by Atkinson et al. (2011c) and Mao et al. (2014). The isotopically exchangeable metals of studied elements did not show any obvious pH dependence although this is probably due to the restricted range of pH values. The comparison between bulk soil and rhizosphere soil did not show any rhizosphere effect on the lability of Ni, Cu, Zn, and Cd. However, Pb lability was significantly higher ($P<0.001$) in the rhizosphere compared to the bulk soil (Fig. 5.1).

Table 5.2. Pearson correlation matrix for soil total metal concentration, soil organic matter (%LOI) and soil total carbon content

LOI								
0.98	P							
0.98	0.98	Cr						
0.95	0.97	0.98	Ni					
0.98	0.98	0.99	0.98	Cu				
0.98	0.98	0.99	0.98	0.998	Zn			
0.92	0.96	0.96	0.99	0.96	0.96	Cd		
0.97	0.95	0.96	0.92	0.97	0.97	0.89	Pb	
0.94	0.90	0.90	0.87	0.92	0.92	0.86	0.88	Total C

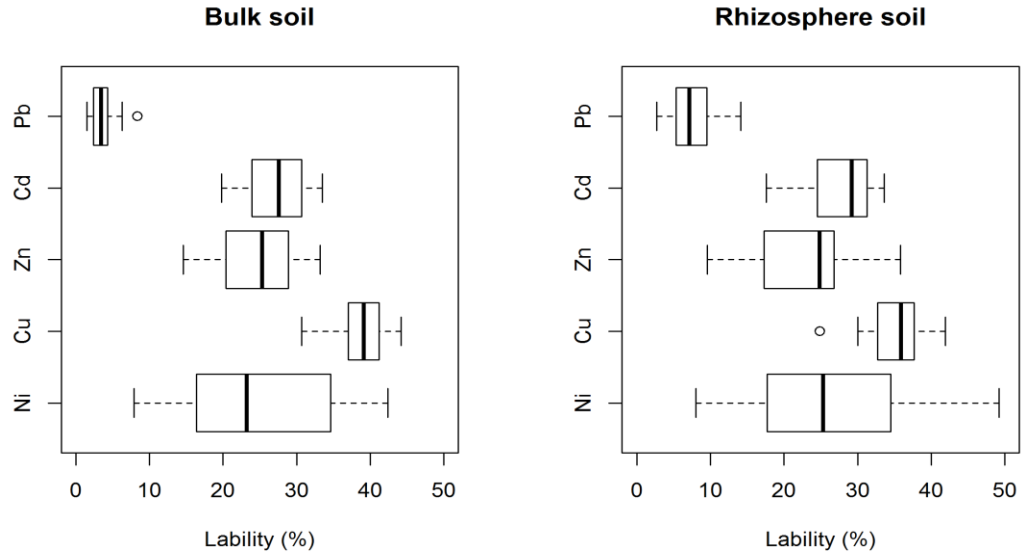


Figure 5.1. Variation in metal lability (E-value) across the sampling sites expressed as a percentage of the total soil metal concentration

5.3.2 Soil microbial diversity

Figure 5.2 shows the relationship between soil Zn concentration, and the richness and diversity index of bacterial and fungal T-RFs, which revealed a range of trends. It is important to acknowledge that, while this data span a broad range of Zn concentrations, the middle range is less well represented. Bacterial diversity increased slightly with increasing soil Zn

and peaked around 500-700 mg kg^{-1} before declining at higher Zn concentrations. As shown in Fig. 5.2, a significant polynomial model was found for bacteria suggesting an optimal biosolids application for bacterial diversity and richness. Thus moderate biosolids applications may increase the diversity of the soil ecosystem i.e. additional organic matter and nutrient input supports the growth of microbial populations and increases diversity. However, for soils with greater than 1000 mg kg^{-1} Zn, diversity steadily decreased with increasing Zn concentration. This suggests that, above a certain level of biosolids accumulation, any beneficial effects of the additional organic matter inputs are offset by the deleterious impact of high metal contamination on soil microorganisms. The toxic response appears to be the dominant factor at Zn >1000 mg kg^{-1}. These observations are in qualitative agreement with those of El Azhari et al. (2012) who reported that while 10 t ha^{-1} sewage sludge application resulted in higher bacterial diversity than that observed in control and farmyard manure treatments, at a higher dose, 100 t ha^{-1}, the lowest diversity was observed and any beneficial effect of sewage sludge was lost.

Bacteria were affected by the presence of biosolids to different degrees compared to fungi. Bacterial populations had a limited response to biosolids application rates as the diversity index across the studied soils did not vary widely and typically remained in the range 0.80-0.90. This may indicate that bacterial communities are resistant over these ranges of metal concentration. In contrast, fungi appeared to be more sensitive and whilst low application rates promoted an increase in diversity as described above, high application rates (>1000 mg kg^{-1}) caused a major decrease in diversity index from 0.8 to 0.4. These findings are consistent with the observations

reported by Garcia-Sánchez et al. (2015) and Pennanen et al. (1996), although other authors have reported that fungi are more tolerant to heavy metal stress than bacteria (Bååth et al., 1998; Kandeler et al., 2000; Stefanowicz et al., 2008). Fungal diversity decreased linearly with increasing biosolids application (Fig. 5.2) in contrast to the curved response of bacteria which appeared to pass through a maximum value.

5.3.3 Effects of biosolids on soil microbial abundance

Figure 5.3 visualizes the differences in soil microbial communities. A Bury-Curtis dissimilarity matrix was calculated from T-RFLP profiles, which was then coordinated into two dimensions with nMDS. Each point in the figure represents a field (n=17); fields that ordinated close to one another are similar in terms of microbial abundance. The differences in soil microbial abundance are evident across the studied fields across the sewage disposal site. In bulk soil, the bacterial communities showed greater similarity in the fields with high concentrations of Zn, clustered at the right of the nMDS biplot, in clear contrast to the fields with low Zn concentration (Fig. 5.3).

In common with bacteria, differences in fungal community structure were evident in the nMDS biplots. Although the trend was less obvious than that observed for bacteria, the fields enriched in metals tended to cluster together. Moreover, the clustering of the fields with high metal loading was more apparent in the rhizosphere than the bulk soil (Fig. 5.3). This pattern suggests an effect of the rhizosphere on soil fungal abundance.

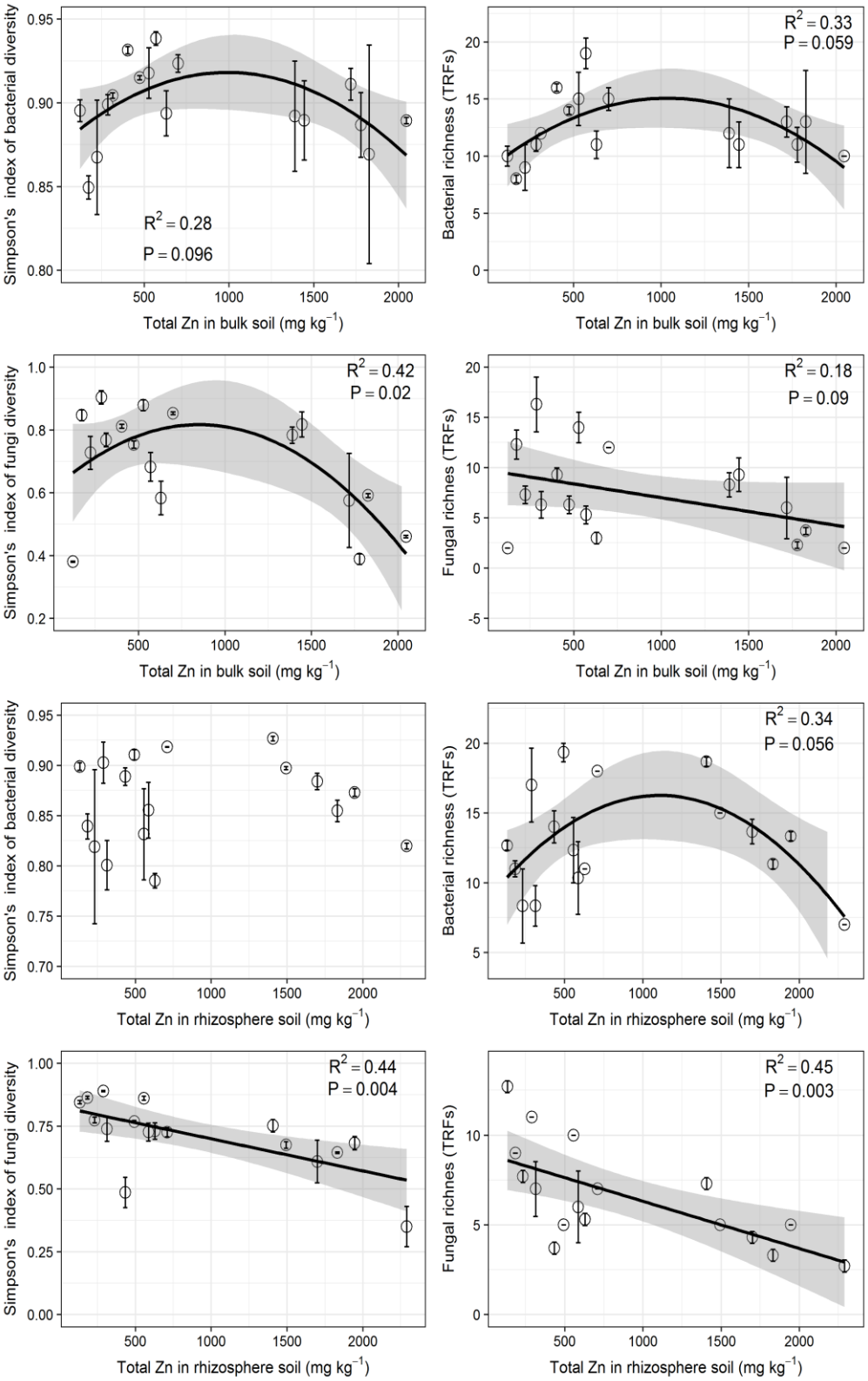


Figure 5.2. The relationship between total Zn concentration and soil microbial diversity, estimated using the Simpson index (left), and richness (right), defined as the number of T-RFs in each soil profile. The solid line is the regression model fit. The grey shaded areas represent 95% confidence intervals

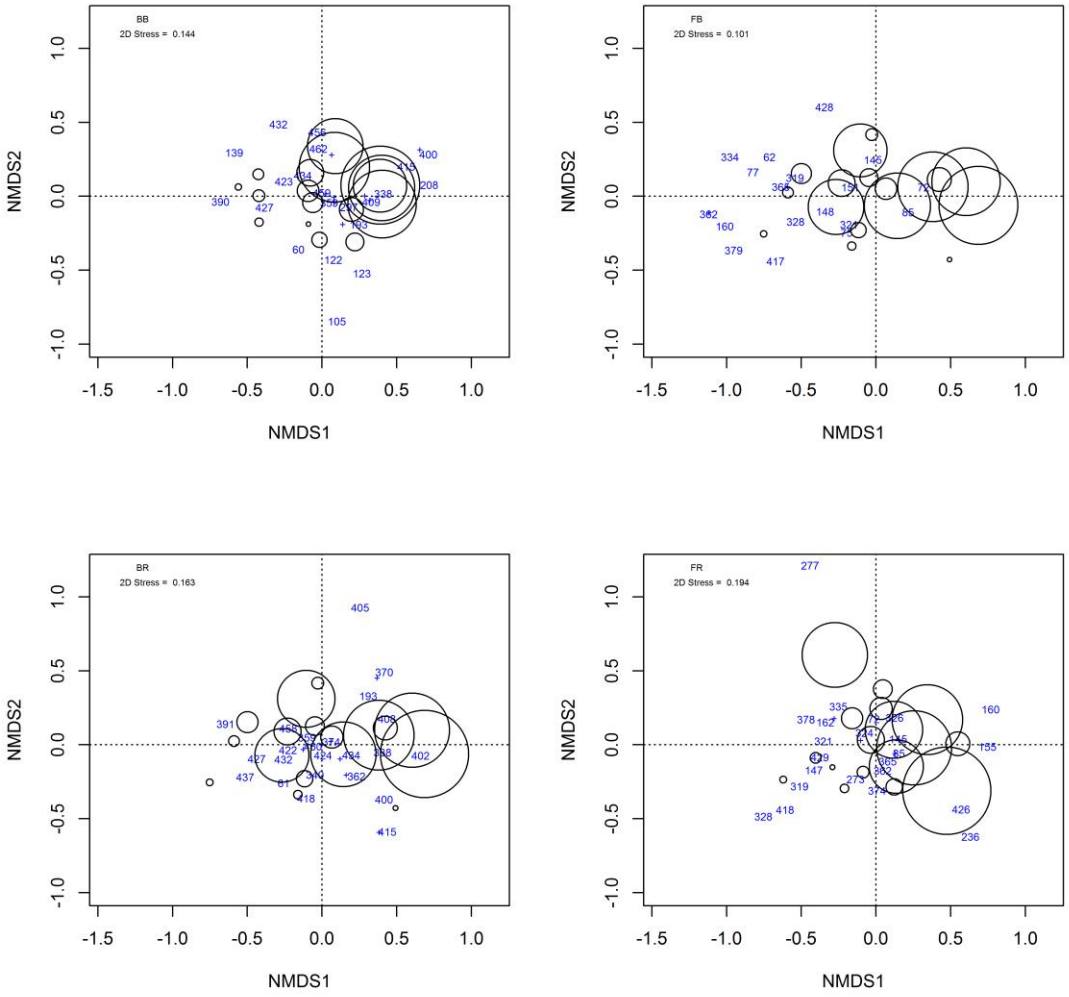


Figure 5.3. nMDS ordination biplot showing bacterial (left panels) and fungal (right panels) T-RFLP community composition in both bulk and rhizosphere soil (BB: bacteria in bulk soil, FB: fungi in bulk soil, BR: bacteria in rhizosphere soil, FR: fungi in rhizosphere soil). Circles represent the studied fields, the size of the circles reflects total soil Zn concentration (mg kg^{-1}). 2D stress represents the level of confidence in the ordination.

Overall, these results show a clear impact of historical biosolids application on soil microbial community structure. This agrees with the findings of Macdonald et al. (2011) who reported a shift in the structure of soil microbial communities in response to sludge-derived metal. Tipayno et al. (2012) also found a change in bacterial communities in metal contaminated sites.

5.3.4 Effect of soil variables on soil microbial abundance

Redundancy analysis (RDA) was used to identify the main soil variables that explained the variance in soil microbial structure generated from T-RFLP. In the case of the five metals assayed for E-values and free ion activities, it was observed that isotopically exchangeable, dissolved and free ion activity concentration explained more variation in T-RFLP profiles and hence microbial community structure, than the total soil metal concentration. This supports the notion that total soil metal concentration is a poor estimate of bioavailability and subsequent toxicity of metals in soil (Scheckel et al., 2009). RDA showed that LOI, C:N ratio, total As and Ag, significantly affected the structure of the soil microbial community. In bulk soil, RDA explained 40% ($P<0.001$) of the variation in both bacterial and fungal T-RFs respectively, while in rhizosphere soil, the corresponding figures were 30 and 23.9 % ($P<0.001$) respectively. The RDA showed that isotopically exchangeable Zn, dissolved Cu, and free ion activity Cd^{2+} were significant in explaining the distribution of bacterial T-RFs, while the free ion activity Zn^{2+}, dissolved Cu, and C:N ratio were significant in explaining fungal community composition (Fig. 5.4). Bacterial T-RFs 400, 402, and 415 correlated positively with Cd free ion activity and these T-RFs were only present in fields with high metal loadings (> 1500 mg kg^{-1} Zn). In contrast, bacterial T-RFs 390, 427, and 423 negatively correlated with Cd free ion activity and these T-RFs were found in fields with low metal loadings (<570 mg kg^{-1} Zn). Most of the fungal T-RFs correlated negatively with LOI and hence metal loading (Fig. 5.4).

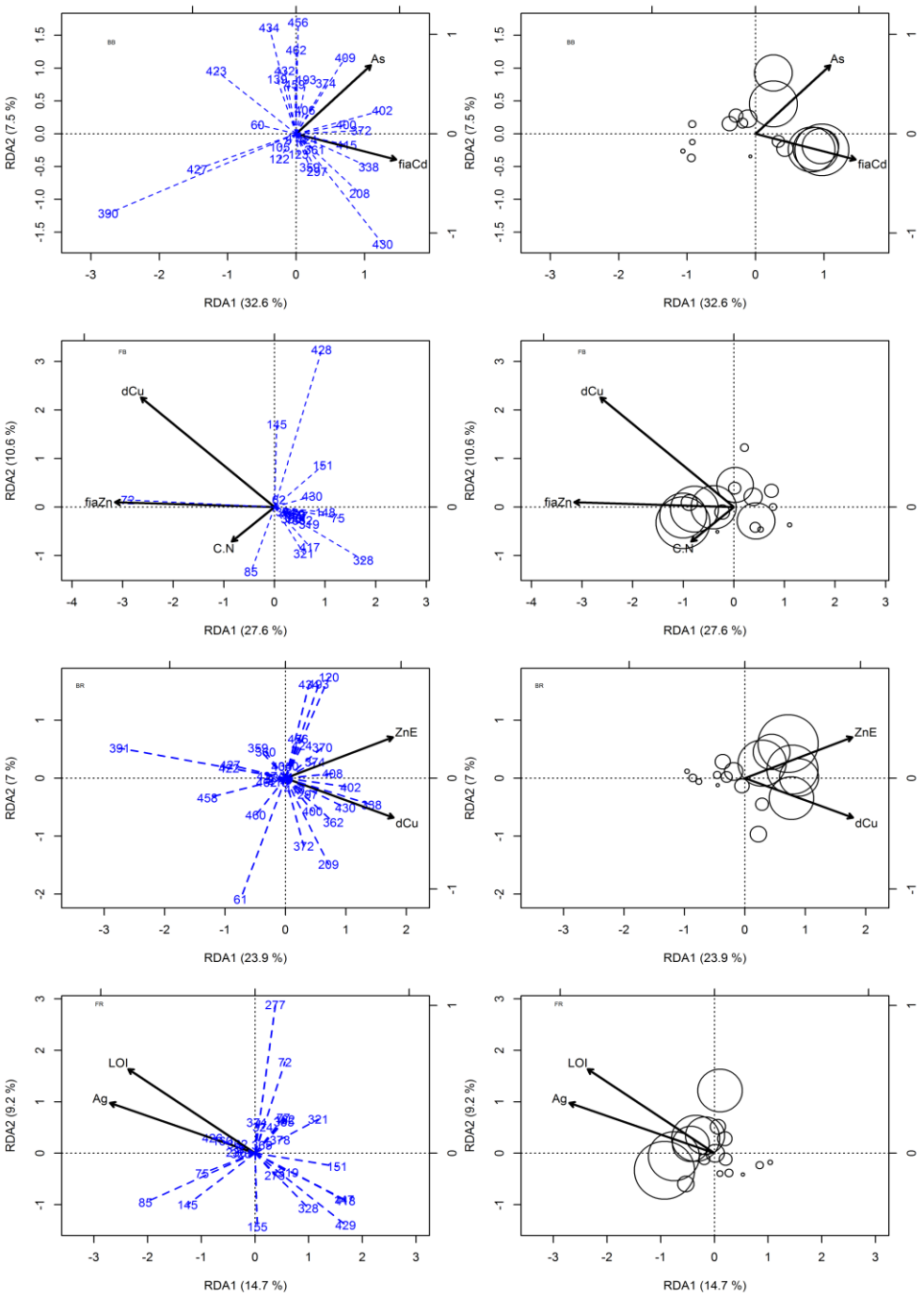


Figure 5.4. Ordination plot of the RDA results generated from T-RFLP profiles with constrained soil properties chosen by forward selection. T-RFs are shown in dashed blue lines and soil variables are shown in black arrows. (BB: bacteria in bulk soil, FB: fungi in FB: fungi in bulk soil, BR: bacteria in rhizosphere soil, FR: fungi in rhizosphere soil, fia: free ion activity, d: dissolved, E: isotopically exchangeable, LOI: loss on ignition). Circles represent the studied fields, the size of the circles reflects total soil Zn concentration (mg kg^{-1}). The plots are scaled so that the angles between T-RFs and environmental variables reflect the correlations. For clarity, the triplot of RDA was split into species plots (left) and site plots (right)

Trends between the abundance of selected T-RFs and soil Zn concentration were shown by bacterial T-RFs 427 and 390 which were reduced or completely absent in fields with high Zn content (Fig. 5.5). In contrast, bacterial T-RFs 402 and 415 were dominant in these fields. This, combined with the results of RDA, suggests that the long-term application of biosolids induces a change in soil microbial community structure towards dominance of particular species and reduction or even the complete absence of sensitive species. This is in agreement with (Kelly et al., 1999) who found a 20-fold increase in Zn-resistant bacteria in response to biosolids application. Chen et al. (2016) also found a substantial increase in the abundance of antibiotic resistant bacteria as a result of long-term application of sewage sludge.

The results indicate that a total sludge loading equivalent to accumulation in the soil of 180-220, 350-450, 700-1000, 20-30, 350-450 mg kg^{-1} of total Ni, Cu, Zn, Cd, Pb is optimal for (maximum) soil microbial diversity at the study site. This contrasts markedly with the maximum soil Zn concentration permitted under the current UK Sludge (use in agriculture) Regulations' of 135, and 300 mg kg^{-1} of Cu and Zn respectively (Charlton et al., 2016).

Regulations for biosolids disposal take into account a range of potential harmful effects, including phytoxicity, metal transfer to the human/animal food supply, and possible impacts on the soil invertebrate community etc. This study, at a site subject to long-term biosolids disposal (>100 years) suggests that microbial diversity is unlikely to be the limiting factor for

biosolids disposal rates. In addition, the relationship between accumulated soil metal and sludge loading will depend explicitly on the sludge composition and presumably on soil conditions governing decomposition rate. Thus, the relative importance of each causal influence must be considered before microbial diversity could be incorporated into policy making.

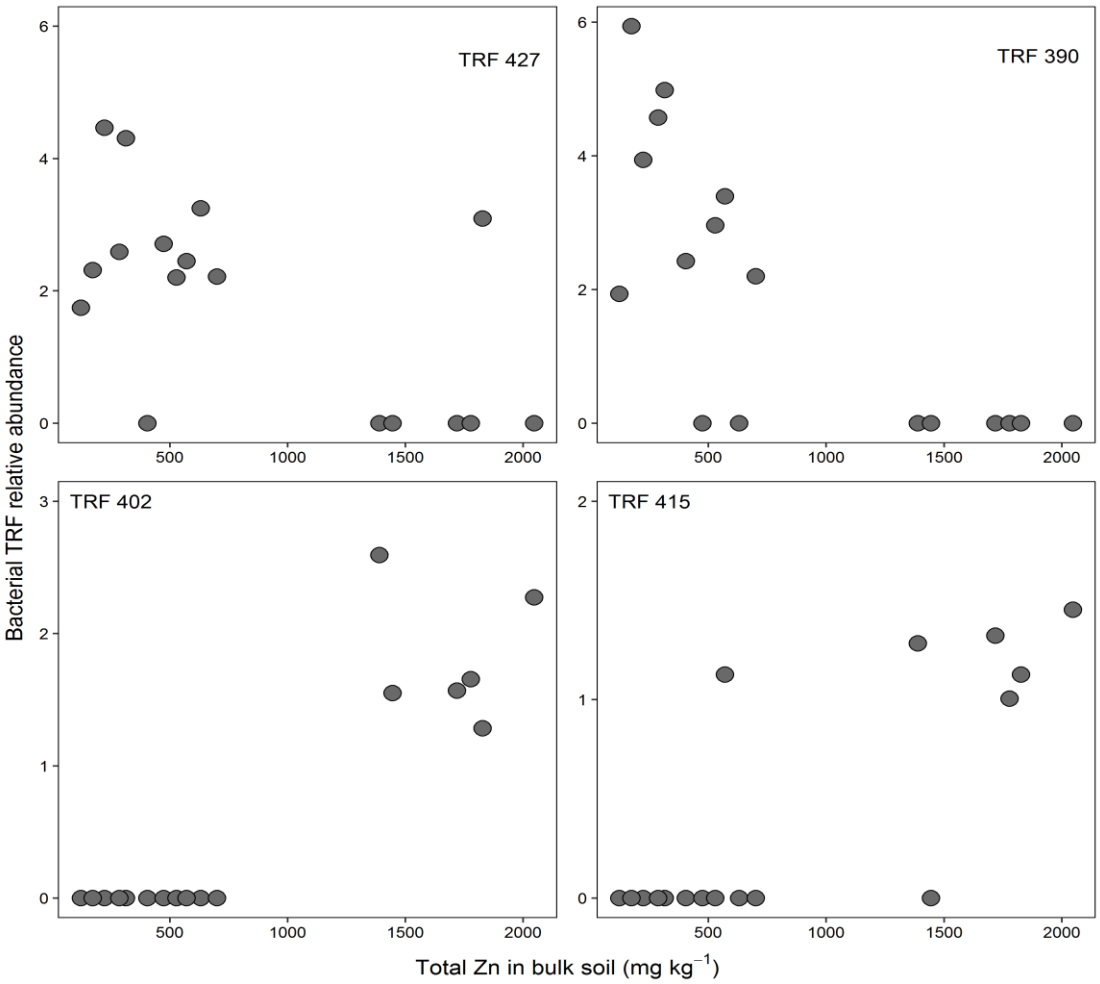


Figure 5.5. Trend in selected individual T-RF numbers with soil Zn concentration.

5.4 Conclusion

While low application rates of biosolids can increase soil microbial diversity, this trend is reversed at higher rates of application. This is probably due to increased concentration of heavy metals at higher application rates. Moreover in the current study, there was a change in the structure of soil microorganism communities with increasing application of biosolids. The trade-off between positive and negative effects may depend on the specific characteristics of a site and the biosolids applied. However, compared to current statutory standards, it appears that microbial diversity is unlikely to be the limiting factor for regulatory limits for biosolids disposal rates. In the current study, the maximum beneficial effect on microbial diversity occurred at a metal (Zn) concentration well in access of current regulations governing application of biosolids to agricultural land which address issues of phytotoxicity and transfer to the foodchain.

6. General conclusions and future work

The current study was conducted to examine the geochemical and biological status of soils that have been subject to long-term application of biosolids. The study was divided into three main strands: (i) quantifying the current metal composition of the study site and determining the geochemical factors that control metal solubility and bioavailability (Chapter 3); (ii) identifying the soil measurement that best predicts metal uptake and subsequent toxicity; and determining soil properties that affect metal dynamics and availability (Chapter 4); (iii) examining the effects of biosolids on soil microbial diversity and community structure. This work tested the hypothesis that biosolids will have beneficial effects, arising from organic matter inputs, or will have a potential detrimental effect due to accumulation of heavy metals (Chapter 5).

6.1 Main findings

Soil organic matter, pH, DOC and mineral oxides were the key soil properties controlling solid-solution partitioning of metals. Different ranges of *E*-values were found for Ni, Cu, Zn, Cd and Pb; relative lability followed the sequence Cu > Cd > Zn ≈ Ni > Pb. Soil total metal content was the single most important variable determining isotopically exchangeable metal (*E*-values), explaining more than 98% of the variation for Ni, Cu, Zn and Cd. In contrast, Pb lability was poorly correlated with soil total Pb and mainly controlled by soil organic matter, oxides and total phosphorus.

Speciation modelling of Ni, Zn and Cd appeared to be not sensitive to the reactive proportion of DOM. In contrast, the reactive fraction of the DOM played a major role in the speciation of Cu and Pb in the soil solution. The

free ion activity of Cu and Pb, due to their high affinity for DOM, varied substantially depending on the fraction of DOM assumed reactive. This emphasises the importance of characterizing the composition of the DOM by measuring the respective proportions of HA and FA and determining the active fraction of DOM (i.e. the fraction that can complex with metals) rather than relying on simple assumptions regarding these two parameters.

Using *E*-values as inputs in WHAM proved to be more accurate in predicting the concentration of dissolved metal than total metal concentration, whilst the prediction based on total metal content was overestimated. This illustrates that total metal content overestimates soil ability to buffer the concentration in the soil solution.

The 0.01 M $Ca(NO_3)_2$ extraction provided the best indication for plant uptake when compared to *E*-values, EDTA and total metal content (i.e. quantity based methods). This indicated that neutral salt extractants have the potential as a universal estimate of metal bioavailability in soil.

The plant's toxic response was described remarkably well (R^2 = 0.81) by the log-logistic dose-response curve using the intensity based exposure measures (FIA and dissolved concentration). This was in marked contrast to the quantity based exposure measures (total, EDTA, *E*-values), where even when the variation in the reactive proportion was considered (i.e. *E*-values), plant toxic response could be poorly described (R^2 = 46%). This emphasises the importance of determining the reactive contents of metals in soil as a tool in identifying potential risk for the environment and human health, as opposed to determining total metal content.

The free ion activity appeared to be the key metal species in the context of bioavailability and toxicity. However, competition from other metals for adsorption on both soil constituents and biological membranes should be considered if a reasonable prediction of plant uptake and toxicity is to be made.

The results of the study of the impact of biosolids on soil microorganisms demonstrated that low application rates of biosolids can increase soil microbial diversity while, due to increased concentration of heavy metals, higher rates of application may decrease microbial diversity. Moreover, a change in the structure of soil microorganism communities was observed in response to increasing the application of biosolids. The maximum beneficial effect of biosolids on soil microbial diversity occurred at a metal (Zn) concentration well in excess of current regulations governing application of biosolids to agricultural land. This indicates that soil microbial diversity is unlikely to be a determining factor for regulatory limits for biosolids disposal to agricultural lands.

6.2 Future work

The findings presented in this study suggest further investigation in the following areas:

- Further investigation is needed to better characterize the composition of the DOM (i.e. the proportion of humic acid, fluvic acids and non-humic substances) and to determine the reactive fraction of DOM. This will lead to a better understanding of the impact of DOM on metal speciation in the soil solution. This is particularly important when using geochemical models to quantify metal speciation, which requires

significant assumptions regarding the composition the reactive proportion of DOM. Such assumptions may lead to very different results depending on the quantity (reactive fraction) and the nature (whether FA or HA) assumed, especially for metals with a high affinity for DOM such as Cu and Pb. Furthermore, it is generally assumed that the humic substances of DOM are the most reactive fraction of DOM that control metal mobility in soil solution. However, it has been reported that non-humic substances have a strong affinity for metal ions and may constitute the major part of the DOM (Buzier et al., 2006; Schneider et al., 2016b). Therefore, further investigation into role of non-humic substances in soil solution is needed.

- The results of the pot trial indicate that the biotic ligand model is a promising approach for predicting plant uptake and toxic response. Further investigation is needed to better understand the competitive effects of cations and anions on metal uptake. This will require application of the model to mixtures of potentially toxic metals.
- The findings of this thesis suggest a significant impact of long-term biosolids application on soil microbial diversity and community structure. Further investigation is required to identify soil microbial species that are tolerant and sensitive to biosolids application, and to determine the potential implications on the vital role of soil microorganisms in the functioning of ecosystem.
- Modern regulation has greatly reduced conventional metal contamination of biosolids. However, new challenges are emerging, most notably increased global production of nanoparticles (NPs) in the global market has resulted in their increased release into domestic and

industrial waste streams and accumulation in sludge from waste water treatment plants. NPs thereby enter the soil environment via the application of biosolids to land (Keller et al., 2013; Rodrigues et al., 2016; Whitley et al., 2013).

Results from recent studies indicate that metals introduced into soil as NPs do not behave similarly to the metal ions (Whitley et al., 2013). Thus, bioavailability of NPs cannot be fully explained using the commonly applied soil–solution partitioning processes that are commonly used to assess metal ions in soil (Rodrigues et al., 2016). There is an urgent need for assessment of the presence, nature and environmental impact of NPs which requires a comprehensive understanding of their environmental behaviour. Therefore, identifying the key processes that govern the dynamic of NPs in soil and developing a set of novel extraction methods and advanced analytical techniques, for example using (i) 'single particle inductively coupled plasma mass spectrometry' (SP-ICP-MS) coupled with ultra-centrifugation and (ii) size-exclusion chromatography coupled with ICP-MS, for fractionation and speciation of NPs in soils subject to biosolid amendment.

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