ZINC UPTAKE AND PHYTO-TOXICITY: COMPARING INTENSITY- AND CAPACITY-BASED DRIVERS

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ABSTRACT

Metal bioavailability and phytotoxicity may be exaggerated when derived from studies based on amending soils with soluble metal salts. It is therefore important to evaluate soil tests for their consistency in estimating plant uptake and phytotoxicity in both field-contaminated and freshly-spiked soils. This study aimed to compare the effects of Zinc (Zn) on plant growth in soils (i) recently spiked with soluble Zn and (ii) historically amended with biosolids. The objective was to reconcile methods for determining bioavailability in both cases by testing a range of 'quantity-based' and 'intensity-based' assays. Soils with a range of Zn concentrations, from an arable farm used for biosolids disposal for over a century, were further amended with Zn added in solution, and were incubated for one month prior to planting with barley seeds in a glasshouse pot trial. The majority (67-90%) of the *added* Zn remained isotopically exchangeable after 60 days. Zinc in the solution phase of a soil suspension was present mainly as free Zn^{2+} ions. Cadmium bioaccumulation factors were inversely proportional to Zn concentration in the soil solution confirming that greater Zn availability suppressed Cd uptake by plants. Measurements of soil Zn '*quantities'* (total, EDTA-extractable and isotopically exchangeable) and '*intensity'* (solution concentration and free ion activity) were correlated with Zn uptake and toxicity by barley plants. Correlations using Zn *intensity* were much stronger than those using *quantity*-based measurements. The free Zn^{2+} ion activity appears to be a consistent driver for plant uptake and phytotoxic response for both metal-spiked soils and historically contaminated soils. Surprisingly, soil Zn accumulation of up to 100 times the current regulations for normal arable land only produced a mild toxic response suggesting that constituents in biosolids (e.g. organic matter and phosphates) strongly restrict metal bioavailability.

Keywords: Bioavailability; free ion activity model; phyto-toxicity; Zinc

1. INTRODUCTION

Potentially toxic elements (PTEs) are naturally present in soils or occur as a result of anthropogenic activities, such as mining, and contamination from agrochemicals, sewage sludge and industrial waste (Cambrollé et al., 2013; Jiao et al., 2015). Concentrations of bio-accessible PTEs in soil can be sufficiently large to have dangerous implications for components of the biosphere, hydrosphere and lithosphere (Concas et al., 2015).

In general, multi-metal contamination is more common than contamination from a single element (Qiu et al., 2016). This is particularly relevant in the case of sewage sludge disposal to soils where inevitably there is enrichment of several contaminants, such as Zn, Cd, Cu, Pb and Ni. Consequently, elemental interactions may influence metal dynamics in soil-plant systems in ways that are difficult to predict. For example, increased competition for adsorption sites in soil will increase metal solubility (Qiu et al., 2016) but, simultaneously, this will also supress uptake of individual elements by plants through competition for sorption sites on roots (Komjarova and Blust, 2009).

Zinc is an essential micronutrient for plants (Cherif et al., 2011), however, an excess of Zn can result in adverse effects on plants such as photosynthesis inhibition, leaf chlorosis, and nutrient imbalances at different stages (Cambrollé et al., 2013; Sidhu, 2016). 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). Zinc and Cd have similar properties and co-occur in primary sources (e.g. sphalerite ore) and so are often associated with each other in soils (Chaney, 2010; Gharaibeh et al., 2016). Zinc and Cd also compete with each other for plant uptake and for translocation from roots to shoots (Zare et al., 2018). Both synergistic and antagonistic relations are reported in the literature (Gharaibeh et al., 2016; Reiser et al., 2014), therefore Zn toxicity should not be studied without consideration of Cd in soil.

Identifying the bioavailable fraction of PTEs is a prerequisite for predicting plant uptake and potentially phytotoxic response. It is well established that the *total concentration* of PTEs in soil provides a relatively poor means of predicting toxic response because this depends on the fraction of the element that can actually be absorbed by an organism (Kim et al., 2015). Thus, assessing bioavailability is a vital consideration in environmental toxicology and agronomy. In particular the importance of metal *fractionation* and *speciation* in determining metal bioavailability in the environment has been shown (Scheckel et al., 2009). Nevertheless, it is the *total* soil metal content that is normally used to develop regulations governing disposal of metal-enriched wastes to soil with little consideration given to contaminant interactions (McBride, 2003; Oliver et al., 2004).

Generally, two approaches have been adopted to assess metal bioavailability in soil; these may be classed as ‘quantity-based’ and ‘intensity-based’ soil tests (Q and I) (Hamels et al., 2014). Quantity-based approaches measure the pool of reactive metal that is able to replenish the soil solution in response to depletion. EDTA-extractable metal and isotopically exchangeable metal (the *E*-value) are well known quantity-based assays of the reactive metal fraction (Hamels et al., 2014). ‘Intensity’ approaches measure the concentration or activity of metal ions in the soil solution or in dilute neutral salt extractions of soil intended to simulate the ionic environment of soil pore water (Hamels et al., 2014; Hough et al., 2005; McBride and Cai, 2015). The Free Ion Activity Model (FIAM) is an intensity-based model based on the hypothesis that free metal ions in solution are the immediately available species for uptake and determine the biological response of organisms (Hooda, 2010). However, the basic FIAM does not consider metal interaction with ligands in the soil solution and on roots, nor possible uptake of soluble complex metal species (Degryse et al., 2006; Wang et al., 2009). The Biotic Ligand Model (BLM) is an extension of the FIAM which incorporates competition from protons and other metal ions for receptor sites on root surfaces when applied to the terrestrial ecosystem (Le et al., 2012; Thakali et al., 2006).

The aim of this study was to assess a range of quantity- and intensity-based measurements of soil Zn status to predict metal uptake and identify the soil properties that provide the best prediction of phytotoxicity. To achieve this aim, soils historically amended with biosolids were further enriched with several levels of soluble Zn and the effects on barley growth and metal uptake were observed. Our specific objectives were to: (i) test contrasting estimates of Zn bioavailability (total, EDTA-extractable, isotopically exchangeable, dissolved, metal free ion activity) as drivers for Zn uptake and plant toxic response; (ii) assess the role of Zn in suppressing uptake of Cd in soils amended with biosolids; (iii) evaluate different forms of the FIAM – BLM as predictive models for uptake. The EDTA-extractable and isotopically exchangeable metal fractions were chosen to reflect quantity-based soil tests. EDTA was chosen because it is possibly the most extensively used in the literature for estimating trace metal availability. The isotopic dilution method was used because it is a robust mechanistically-based method for assessing the potentially available or 'reactive' fraction in soil; and because the EDTA generally tends to overestimate the reactive fraction in soil. The 0.01 M $Ca(NO_3)_2$ extraction of soil was chosen to reflect intensity-based methods. This soil test is a proxy for porewater metal concentrations or readily available metals and it is routinely used.

2. MATERIALS AND METHODS

2.1. Soil collection

Eight soil samples were collected from a sewage treatment facility, operated as an arable farm, in the East Midlands (52.58°N, 1.03°W), U.K. The site is located adjacent to the River Trent, close to the city of Nottingham and has been dedicated to sewage sludge disposal since 1880. Topsoils (0−15 cm) from 8 fields covering a wide range of zinc concentrations were sampled.

Each sample consisted of approximately 6 kg of soil collected as an aggregated sample from each specified field. The soils were air-dried, homogenised, and sieved to < 4 mm.

2.2. Experimental setup

2.2.1. Soil amendment.

Of the eight soils sampled, six were amended with 6 levels of Zn including a zero Zn addition. The remaining two soils were used without any Zn amendment – this gives a total of 38 soil-treatment combinations. A table detailing Zn addition levels can be seen in the supplementary material (Table S1). The concentrations of Zn applied (Zn_{Added}; mg kg^{-1}) were intended to enrich the existing bioavailable soil Zn up to double the level of isotopically exchangeable Zn (Zn_E) in the sampled fields. To accomplish this, it was assumed that initially ≈ 90% of added Zn would remain isotopically exchangeable, based on previous studies by Crout et al., (2006). Thus different levels of soluble Zn were applied to each soil, depending on the native Zn_E value of each soil. The intended purpose was to produce a comparable range of both available native and added Zn to enable discrimination between their effects on plant uptake and eventual phytotoxicity. The highest concentration added to each of the six test soils varied from 1037 to 1523 mg kg^{-1} Zn. Zinc was added to the soils as variable volumes of a $ZnSO_4$ stock solution containing 15 g L^{-1} Zn. The spiking solution was added to the soils while they were being mixed using a stainless-steel food mixer for 5–10 min. To counterbalance acidity arising from Zn adsorption, KOH solution was also added at a rate equivalent to the metal salt addition, assuming a proton⇌metal exchange stoichiometry of 2 (Tye et al., 2003). The final water content of the incubated soils was made up with Milli-Q water (18.2 MΩ cm) to achieve a friable moist soil capable of free gas exchange; no attempt was made to achieve a fixed moisture potential, or content, across the range of soils. Soils were then placed in 2 L plastic containers (20×14×8 cm) with holes in their lids and incubated for 4 weeks at 10°C to allow the added Zn to react with the soil.

2.2.2. Pot experiment

After the incubation period, each soil (n = 38; 6 soils amended with 6 levels of Zn + 2 un-amended soils) was split into 4 replicate samples of 250 g and transferred into pots (n = 152 pots). Each pot was initially planted with six pre-germinated barley seeds, which were thinned later to four plants. Seeds were germinated on moist paper in a Petri dish in the dark at room temperature. The pots were randomly placed in a glasshouse with a day temperature of 22 °C and night temperature of 20 °C. The photoperiod was 16 h with a mixture of natural and supplementary light; the latter was set to switch on when the outdoor light level falls below 25000 lux. Addition of N equivalent to 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, treated soils were maintained at approximately 60% water holding capacity by adding distilled water to reach the original weight of the pots. 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. Light saturated rates of net carbon assimilation (A_{sat}) were also measured on two consecutive days on one randomly selected fully expanded leaf from each replicate pot using a portable infrared gas analysis system (LI-COR 6400-XT, LI-COR, Nebraska, USA). Leaf temperature during the measurement was maintained at 26 °C. The area of the leaf cuvette was measured for individual plant, the photosynthetic photon flux density (PPFD) was set to 500 µmol photons m^{-2} s^{-1}, the CO_2 partial pressure was set to 400 ppm, and the relative humidity was maintained at 55%. The measurement were conducted between 9:00 am – 4:00 pm for two days. After taking chlorophyll and photosynthesis measurements, the above-ground biomass (leaves and stems) was harvested. Plant tissues were washed with tap water, then deionised water, and oven-dried at 50 °C for four days.

2.3. Plant analysis.

Dried ground plant tissues were acid-digested on a block digester with 10 mL of HNO_3 and 5 mL of H_2O_2 for 2 h at 95 °C and analysed using ICP-MS (Thermo-Fisher Scientific X-Series^{II}; Thermo Fisher Scientific Inc., Waltham, MA, USA). A standard reference material, NIST 1573a Tomato Leaves (National Institute of Standards and Technology), and 4 operational blanks, were included to assess the accuracy and precision of the digestion and analysis. The elemental recoveries for NIST 1573a Tomato Leaves were 99.5% Zn and 91.2% for Cd. Biological Accumulation Coefficients for Zn (Zn_{BAC}) and Cd (Cd_{BAC}) were calculated as the ratio of Zn_{Plant} to (Zn^{2+}) and Cd_{Plant} to (Cd^{2+}) respectively (Eq. 1)

$$Zn_{BAC} = \frac{Zn_{Plant}}{\{Zn^{2+}\}}\ ;\ Cd_{BAC} = \frac{Cd_{Plant}}{\{Cd^{2+}\}} \qquad (1)$$

2.4. Soil analysis

2.4.1. General characterization

Soil pH was measured in a suspension of air-dried soil (< 4 mm) and Milli-Q water (1:2.5 m/v). Total carbon content in soil was measured using Shimadzue TOC-Vcp analyser. Soil organic matter content was also estimated from loss on ignition (LOI).

2.4.2. Total elemental concentration

Total soil elemental concentrations were determined by ICP-MS following acid digestion in a block digester (Model A3, Analysco Ltd, Chipping Norton, UK). Approximately 200 mg of finely ground soil were digested with 2 mL of HNO_3 (70% trace element grade) and 1 mL of $HClO_4$ (70% analytical grade) at 80 °C for 8 hrs followed by 2 h of heating at 100 °C. This was followed by addition of 2.5 mL of HF, (40% trace element grade) and heating at 120 °C for 8 hrs. A further 2.5 mL of HNO_3 and 2.5 mL of Milli-Q water were then added to the dried residue and the vessels were 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. A certified reference material, NIST-2711 Montana soil (National Institute of Standards and Technology) and 10 operational blank digestions were used to determine the accuracy of the analysis and the limit of detection (LOD) for quality control. The elemental recoveries for NIST-2711 Montana soil were 109% Zn and 129% for Cd.

2.4.3. Isotopic dilution assay

Concentrations of 'isotopically exchangeable' Zn and Cd (Zn_E and Cd_E) were measured in calcium nitrate suspensions. Two replicate samples of each soil were suspended in 0.01 M $Ca(NO_3)_2$ (2 g : 30 mL) and pre-equilibrated on an end-over-end shaker for 2 days, then an aliquot (0.4 mL) of an isotopic spike solution with known isotopic abundance (IA), prepared from a stock solution enriched with ^{70}Zn (250 mg L^{-1}; IA = 95.47%) and ^{108}Cd (123 mg L^{-1}; IA = 69.74%) was added to the suspensions. Isotope spike levels were calculated as equivalent to ≈ 1% of the estimated labile metal following an assessment of the effect of isotope spike level on the robustness of *E*-value determination (details are given in the supplementary material). The suspensions were shaken for a further three 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. Isotopically exchangeable Zn and Cd (M_E, mg kg^{-1}) were calculated from equation 2.

$$M_E = \left(\frac{M_{soil}}{W}\right)\left(\frac{C_{spike}V_{spike}}{M_{spike}}\right)\frac{({}^{Iso1}IA_{Spike} - {}^{Iso2}IA_{Sike}R_{SS})}{({}^{Iso2}IA_{Soil}R_{SS} - {}^{Iso1}IA_{Soil})} \qquad (2)$$

Where M_{soil} and M_{spike} are the average atomic masses of the 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 of a particular isotope in the spike or soil and R_{ss} is the ratio of isotopic abundances for the two isotopes in the spiked soil suspension (^{70}Zn to ^{66}Zn and ^{108}Cd to ^{111}Cd). In the case of Zn_E, Iso1 and Iso2 refer to ^{66}Zn and ^{70}Zn; for Cd_E, Iso1 and Iso2 refer to ^{111}Cd and ^{108}Cd respectively. Values of Zn_E and Cd_E were measured (i) after 28 days of soil amendment with Zn (before transferring amended soils into pots for plant growth), (ii) three times during the growth period and (iii) after harvesting and removal of plant residues. Soil moisture content was determined at each time and M_E (mg kg^{-1}; dw basis) was corrected accordingly.

2.4.4. *Chemical extractions*

Estimates of EDTA-extractable metal (M_{EDTA}) were obtained by extraction of 2.0 g soil in 20 mL 0.05 M EDTA, shaking for 1 h end-over-end, and analysis by ICP-MS following centrifuging and filtration (0.45 µm syringe filter). Soluble metal concentrations (M_{Soln}) were determined in the 0.01 M $Ca(NO_3)_2$ suspensions used for E-value determination. Dissolved organic and inorganic carbon concentrations (DOC and DIC) and pH were also measured in the suspension using a Shimadzu TOC –Vcp analyzer. The free ion activities (M^{2+}) of Zn^{2+} and Cd^{2+} were calculated using the geochemical speciation model WHAM (VII) (Tipping, 1994). Input to the model included pH and measured cation and anion concentrations in the $Ca(NO_3)_2$ suspension. Fulvic acid concentration was also included as an input variable to WHAM (VII); this was estimated by assuming that DOC contains 50% C and that 65% of DOC consists of active fulvic acid (Buekers et al., 2008; Marzouk et al., 2013).

2.5. Modelling plant uptake of metals.

The concentration of Zn in plant tissue (Zn_{Plant}, mg kg^{-1}) was described as an asymptotic function of Zn^{2+} ion activity (Zn^{2+}; µM) incorporating competition from other divalent metal ions (M^{2+}) and protons (Eq. 3)

$$Zn_{Plant} = \frac{K_{Zn1}(Zn^{2+})}{1 + K_{Zn2}(Zn^{2+}) + K_M(M^{2+}) + K_H(H^+)} \quad (3)$$

Parameters in Equation 3 (K_{Zn1}, K_{Zn2}, K_M, K_H) were derived by minimizing the root mean square deviation (RMSD) between the predicted and measured values of plant concentration using the *'nls2'* package in R (Grothendieck, 2013).

2.6. Zinc phytotoxicity

Biomass of the 28-day growth, photosynthetic measurements, and chlorophyll content were used to establish EC_{50} values, i.e. the 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).

$$Y = \frac{100}{1 + e^{b(X-M)}} \qquad (4)$$

Where Y is a response variable (yield, 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 EC_{50} and b is a slope parameter. Dose-response data were fitted to the log-logistic curve using the *'nls2'* package in *R* (Grothendieck, 2013).

3. RESULTS AND DISCUSSION

3.1. General soil characteristics

The general characteristics of soils used in the study (prior to addition of Zn) are shown in Table 1. Soil pH was in the range 6.1–7.1, except for soil S1 which was more alkaline (soil pH = 7.8). Soil total organic carbon content (OC) ranged between 2.0–9.9%. The total concentration of Zn varied over one order of magnitude, from 140 to 1990 mg kg^{-1}; for Cd, there was almost a 30-fold variation, 1.5 - 43 mg kg^{-1}. The broad range of characteristics in Table 1 reflects different rates, or durations, of biosolid application to individual fields throughout the site.

3.2. Zinc lability in soil

Following plant harvest, Zn_{EDTA} concentrations ranged from 46 to 2560 mg kg^{-1} and showed a strong relationship with total soil Zn concentration (Zn_{Total}), $r = 0.97$ $p<0.0001$ (Fig 1A). Only 32–60% of the native Zn (control soils) was EDTA-extractable, while 75−100% of the *added*

Zn (assessed as the difference between Zn_{EDTA} in amended soil and corresponding controls) remained EDTA-extractable, indicating its greater availability for the duration of the experiment (Figure 1B).

Values of Zn_E for soils ranged from 51 to 1630 mg kg^{-1}. Expressed as a percentage (%) of the total soil content, $\%Zn_E$ in *control* soils was, on average, 32.1% (SD = 2.07%). This is in line with values reported in the literature for polluted and unpolluted soils, which are typically in the range 10–40% (Degryse et al., 2004; Gäbler et al., 2007; Izquierdo et al., 2013). However, the addition of Zn to soils resulted in increased values of $\%Zn_E$ to 40–60% of Zn_{Total}. Values of Zn_E were also strongly correlated with Zn_{Total} ($r = 0.93$, $p<0.0001$) (Fig. 1C). However, there was a lower slope than that found for EDTA extraction (Fig 1A), with greater differentiation between amended soils. Similarly, when subtracting the native Zn_E (controls soils) (Fig. 1D) it was clear that the added Zn (Zn_{Added}) remained largely labile. The exception was Soil 1 (Fig.1D) which showed a distinctive trend with a lower slope indicating lower Zn_E compared to the other studied soils. This could be explained by the higher pH (pH = 7.6) of this soil (Table 1) enhancing Zn adsorption and thus resulting in approximately 50% fixation of Zn_{Added}. 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 remained extractable by 0.05 M EDTA (Fig. 1A and B). This initial adsorption is probably followed by a slow aging process (Oorts et al., 2007), which would be expected to progress to some degree during the span of the experiment (60 days). However, Zn_E was determined throughout the growth period (4 times) and revealed no change in Zn lability, indicating that any variation in Zn uptake or toxicity cannot be attributable to a change in Zn lability during the growth period.

3.3. Zinc solubility and speciation

The concentration of soluble Zn (Zn_{Soln}) increased linearly as a function of Zn_{Added} for *individual* amended soils, suggesting that a constant proportion of the Zn_{Added} was potentially available for plant uptake. However, the extractable proportion varied between soils, indicating an influence of soil properties, primarily soil pH and organic matter content. Lower pH values increased Zn solubility; correlation coefficients between soil pH and Zn_{Soln} or the free ion activity (Zn^{2+}) were -0.77 and -0.79 respectively. Soils with greater organic matter contents, such as S5 and S6, had lower values of Zn_{Soln}.

Chemical speciation of the solution phase of the soil suspensions, using WHAM (VII), showed that free ionic Zn^{2+} was the predominant species present (68–90%; average 80% of Zn_{Soln}). Values of (Zn^{2+}) ranged from 0.05 to 3.4 µM in control soils and up to 6.5 - 82 µM across the greatest Zn additions to the six soils. Bicarbonate-complexed Zn ($ZnHCO_3^+$), on average, accounted for 9% (SD = 4.69 %) of Zn_{Soln} except for soil 1, which had up to 19% HCO_3^--complexed Zn_{Soln}. Fulvic acid bound Zn ranged between 4.1–19.0 % with an average of 9.8% of Zn_{Soln} (SD = 3.8%).

3.4. Plant uptake of Zn

Zinc addition to soils increased the concentration of Zn in the barley (Zn_{plant}). For the control soils, the range of Zn_{plant} was 28 – 116 mg kg^{-1}, whereas for the highest level of Zn_{Added} it was 421 – 1220 mg kg^{-1} for the six soils tested. Figure 2 shows the relationships between different estimates of Zn bioavailability in soil and Zn_{plant}. There was a general increase in Zn_{plant} with all estimates of available Zn in soil although the quantity-based indices (Zn_{Total}, Zn_{EDTA} and Zn_E) provided only weak correlations with Zn_{plant}. Nonetheless, it was evident that measures of 'reactive' Zn pools (Zn_{EDTA}, Zn_E) provided better indices of plant-availability than Zn_{Total}; Zn_E showed the strongest relationship, accounting for 67% of the variability in Zn_{plant}. Intensity-based measurements, Zn_{Soln} and (Zn^{2+}), provided much better predictions of Zn_{plant}, explaining 87% and 86%, respectively, of the variability (Figs 2D and 2E). Thus there was no apparent advantage in speciating Zn_{Soln} to derive (Zn^{2+}), suggesting that a simple soil extraction with 0.01 M $Ca(NO_3)_2$ may provide a reliable prediction of plant uptake. However, the strength of this relationship may reflect a strong underlying covariance between concentrations of Zn and soil organic matter at the study site.

Figure 2 clearly emphasises the importance of assessing intensity-based indices of trace metal availability in any assessment of potential harm to the environment or human health (Adamo et al., 2014; Bravo et al., 2017; Rodrigues et al., 2010). Therefore, Biological Accumulation Coefficients (BAC) for Zn and Cd were calculated based on the free metal ion activity in soil, rather than total concentration in soil (Eq. 1). Values of Zn_{BAC} decreased with Zn_{Soln}, indicating that plant root affinity for Zn declines with the greater supply provided by increased Zn concentration in the soil solution. This is consistent with physiological control over trace metal uptake (Adamo et al., 2014; Moodley et al., 2012), but may also arise from increased solubility of other metals (e.g. Cd; Fig 3A) providing competition for plant uptake (Murtaza et al., 2017). Factors causing increased Zn solubility (lower pH or increased Zn loading) are also likely to increase the solubility of other metals through competition for adsorption sites from Zn^{2+} and H^+ ions. Antagonistic interactions in soil between Zn and Cd are well known (Murtaza et al., 2017; Sikka and Nayyar, 2012). However, competition effects apply to both soil and plant adsorption sites: Zn addition will increase Cd solubility (Fig. 3A) but will also cause increased competition for plant uptake (Fig. 3B). Despite this complexity, Fig. 3C shows a remarkably strong relationship between the ratios Zn_{Soln}:Cd_{Soln} and Zn_{Plant}:Cd_{Plant}. This consistent relationship may be explained by factors relating to the heterogeneity of both soil and root adsorption sites. Thus, (i) as Zn_{Added} increases it solubilizes more Cd but the ratio Zn_{Soln}/Cd_{Soln} should increase as Zn^{2+} ions compete with increasingly strongly bound soil Cd; (ii) as Zn_{Soln}

increases it is progressively absorbed by lower-affinity root sites which will tend to offset the effect of the increasing ratio Zn_{Soln}/Cd_{Soln}.

3.5. Modelling plant uptake

Table 2 shows the results of two approaches to modelling plant uptake of Zn. In Models 1 and 2, values of Zn_{Plant} were related to (i) (Zn^{2+}) only; (ii) (Zn^{2+}) with competition from other cations and H^+ ions. Model 1 assumed that the driver for uptake was (Zn^{2+}) and the relative values of K_{Zn1} and K_{Zn2} (Table 2) indicate that the relationship between plant uptake and Zn did not reach an asymptote. Including proton competition (Model 2), produced no improvement in the prediction of Zn_{Plant}. This may appear to be in contrast to previous studies. For example, Hough et al., (2005) reported that inclusion of H^+ ions as a competitor in a FIAM improved the prediction of Cd and Zn uptake by perennial ryegrass (*Lolium perenne* L); Ardestani et al., (2015) confirmed proton competition with free metal ion binding onto BL sites of different plant species and soil invertebrates; Thakali et al., (2006) showed that including H^+ ions improved models describing the response to Ni and Cu toxicity of barley root elongation and tomato shoot yield. The lack of response of the current model to proton competition may be explained not only by the strong correlation between H^+ and Zn^{2+} ion activity (r = 0.80) in the solution phase, but also because of the contrasting effects of pH: at lower pH there will be greater Zn solubility but at the same time there will be greater competition from H^+ for root uptake. It is possible that these contradictory effects are largely cancelled out in the restricted pH range (6.1 – 7.8) of the studied soils. Including competition from Cd, produced a significant improvement in predicting Zn_{Plant}. However the coefficient representing root affinity for Zn (K_{Zn2}) was negative and insignificant, implying that root affinity for Zn increased with Zn_{Soln} and that Cd played a decisive role in governing Zn uptake. However, values of Cd_{Soln} were very low compared to Zn_{Soln}, so that restriction of root site occupancy and mass transfer of Zn, by Cd, seems unlikely. The apparent effect of Cd is probably an artefact of the high correlation between Zn_{Soln} and Cd_{Soln} (r = 0.82) which arises because of the single source of soil contamination (biosolids).

Replacing (M^{2+}) with M_{Soln} in Eq. 3 provided a minor improvement in prediction of Zn_{Plant}. This small difference partly reflects the observation that c. 89% of Zn_{Soln} was present as Zn^{2+} ions (as calculated by WHAM VII) but may also indicate that complexed metal species, in addition to free divalent ions, may be taken up by plant roots. For instance, Weggler et al., (2004) demonstrated uptake of Cd chloride complexes by wheat; López et al., (2005) reported enhanced Cd uptake by alfalfa following the addition of EDTA suggesting uptake of non-ionic Cd species (i.e. EDTA-Cd complexes). Dissociation of organically complexed Zn on the root surface may also contribute to plant uptake; Krishnamurti et al., (1997) showd that soluble organic-Cd species can contribute to the bioavailability of Cd.

3.6. Zinc phytotoxicity

Zinc addition typically induced (visual) leaf chlorosis in plants receiving the two greatest Zn treatments suggesting a toxic response affecting chlorophyll synthesis (Adriaensen et al., 2006). Iron or manganese deficiency and interference with Ca metabolism are other possible mechanisms causing Zn-induced chlorosis (Sidhu, 2016). Barley growth, photosynthetic rate, and chlorophyll content exhibited a significant response to Zn additions in all Zn-amended soils. This is congruent with the findings of Sun et al., (2014) who reported that increased Zn uptake led to increased toxic symptoms in wheat and maize plants. Toxic responses included leaf chlorosis and a significant decrease in shoot and root biomass. They highlighted a threshold of 400 mg kg^{-1} and 800 mg kg^{-1} of Zn concentration in soil, for maize and wheat respectively, beyond which plants exhibited toxic responses. They suggested nutrient (eg. Mn, Fe) deficiency as possible reasons for Zn toxicity. Compared to the control soils, the maximum inhibitory effects on barley, at the maximum level of Zn_{Added} (1310 mg kg^{-1} to soil 4) were

53%, and 28% for yield and chlorophyll content respectively, and it was 45% for photosynthetic rate at the maximum level of Zn_{Added} (1210 mg kg^{-1} to soil 3).

Different estimates of bioavailability were used in the log-logistic model (Eq. 4) to predict Zn toxicity thresholds. For relative plant growth, expressed as a percentage of the control, (Zn^{2+}) and Zn_{Soln} (intensity measures) accounted for 79% and 78% of the variation in the toxic response of the relative plant growth, respectively (Figs. 4A and 4B). Estimated EC_{50} values for biomass yield were 85.5 and 204 µM for (Zn^{2+}) and Zn_{soln}, respectively. These values are in agreement with those 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 relative growth. In particular, they found that the EC_{50} was 79.2 µM for (Zn^{2+}) which is very similar to that of the current study (85.5 µM). When the dose was expressed as a function of one of the labile 'quantity' measurements, only Zn_E resulted in significant parameters of the log-logistic model, but explained only 38% of the variation in the yield; the use of Zn_{EDTA} resulted in insignificant (0.05 level) parameters and using Zn_{Total} failed completely as model parameters could not be resolved (Fig. 4 and Table 3).

Relative to the control soils, the greatest Zn additions resulted in a 10-28% reduction in the chlorophyll content. The toxic response described by the log–logistic dose–response model only provided a viable fit when the intensity variables, ((Zn^{2+}) and Zn_{Soln}) were used to represent the exposure (Fig. 5 and Table 3). Values of EC_{50} for the chlorophyll content were 725 and 1105 µM when exposure was expressed as (Zn^{2+}) and Zn_{Soln} respectively. Compared to the biomass and chlorophyll content, the photosynthetic rate was less sensitive to Zn toxicity. Only intensity-based variables resolved a solution to the log-logistic response model, but explained only 39% of the variation in the photosynthetic response to Zn toxicity (Fig. 5, Table 3). Values of EC_{50} for the photosynthetic rate were 259 and 567 µM when the exposure to Zn was expressed as (Zn^{2+}) and Zn_{soln} respectively.

Reductions in the chlorophyll content and the photosynthetic rate, as a response to Zn toxicity, were quite small (Fig. 5) compared to the reduction in the biomass (Fig. 4). This is consistent with analogous findings by Dias et al., (2013), who examined the effect of Cd toxicity on lettuce. They found that even though the net photosynthetic rate was not affected by Cd concentration up to 1 µM, there was a significant decrease in plant biomass, and only at a very high concentration of the exposure, 50 µM, there was impairment of photosynthetic rate. These authors attributed the biomass reduction to induced genotoxicity and delayed cell division. Therefore, it is unlikely that the reduction in plant growth could be attributed solely to the toxic effect of Zn on chlorophyll content and photosynthetic rate. A possible reason for the differences observed may simply be that whereas biomass reflects conditions experienced during the growth period of the plant, photosynthesis rate was measured just once, shortly before harvest.

Previous studies (Smolders et al., 2015; Stevens et al., 2003) have shown that metal toxicity is confounded by pH decrease associated with adding metal salts to soil. For instance, Smolders et al., (2015) observed a decrease in pH of up to two units in soil spiked with Pb in solution. In the current study an average decrease of only 0.3 pH units (maximum of 0.5 pH unit at high rates of metal addition) was observed, probably because of the addition of KOH with added Zn and the buffer capacity of the soils provided by the organic matter originating from biosolids application.

3.7. Implications for biosolids regulations

The results of the phyto-toxicity test indicate that differences in the pattern of toxic response to Zn due to soil type and soil-metal contact time are minor when the exposure is expressed as Zn_{Soln} or (Zn^{2+}). This is consistent with the findings of Hamels et al., (2014) who also found that 0.01 M $CaCl_2$ extraction and the DGT method (intensity-based estimates) were the most robust indices of Zn inhibition of barley shoot growth. To put these results in the context of biosolids use in agriculture regulations, Table 4 shows a comparison between Zn^{2+} free ion activity in individual control soils and the Predicted No Effect Concentration (PNEC). The Table shows that the current limit of 200 mg kg^{-1} of Zn in soil (The Sludge Use in Agriculture Regulations, 1989) is broadly in line with the point at which there is a negligible toxic response in barley plants grown in the control soils. However, at greater values of Zn_{Total} in the control soils barley showed only a very limited yield response even though Zn_{Total} extended to an order of magnitude above the current regulations for 'non-dedicated' arable sites. This may reflect the co-occurrence in biosolids of potentially toxic metals and constituents that limit metal bioavailability, such as organic matter and phosphate. These findings appear to validate the 'protection' theory (Frost and Ketchum, 2000; McBride, 1995; Stietiya and Wang, 2011), which hypothesizes that the hazard from heavy metals introduced with biosolids into soils is limited by the high adsorptive capacity of organic sludge constituents.

4. CONCLUSIONS

Our results demonstrate that while soil characteristics are important in determining metal solubility, it is the *intensity*, rather than the *quantity*, of metal in soil that best predicts metal uptake and subsequent phytotoxic response. Therefore, assessing the ecological consequences of pollutants should be based on the intensity of the exposure. Moreover, it can be concluded from the observed pattern of toxic response by barley plants that the current U.K. (E.U) regulation governing the use of biosolids in agriculture are appropriate, and conservative, in terms of phytotoxicity.

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Free metal ion activity is the driver for plant uptake and phytotoxicity

Greater Zn availability suppressed Cd uptake by plants

Ratios of Zn:Cd in barley strongly reflected those in the soil solution

Only mild phytotoxicity was found in biosolids-amended soils with up to 2000 mg Zn/kg

E.U. rules on biosolid use in agriculture seem appropriate in terms of phytotoxicity

Table captions

Table 1. Main characteristics (pH, organic matter composition and trace metals) of the soils (mean of three replicates ± standard errors)

Table 2. Coefficients of determinations and root mean squared deviation (RMSD) for the prediction of Zn_{Plant} (mg kg^{-1}) using the asymptotic function (Equation 3). NS denotes not significant.

Table 3. Phytotoxicity 'effective concentration' values (EC_{50}) for different estimates of Zn bioavailability. L.CL and U.CL are the 95% lower and upper confidence limits; NS signifies a non-significant fit of the model; 'No fit' indicates that the parameter could not be resolved.

Table 4. Comparison between Zn^{2+} activity (Zn^{2+}) at the Predicted No-Effect Concentration (PNEC, 0.51 μM) and Zn^{2+} activity in control soils; Zn_{Tot} is the total soil Zn concentration.

Table 5. Main characteristics (pH, organic matter composition and trace metals) of the soils (mean of three replicates ± standard errors)

Soil sample	pH	LOI	TIC	TOC	N	Ni	Cu	Zn	Cd	Pb
		%				mg/kg				
S1	7.84 ± 0.009	4.44 ± 0.086	0.440 ± 0.018	2.03 ± 0.15	0.19 ± 0.106	31.7 ± 1.42	44.7 ± 2.20	139 ± 5.75	1.49 ± 0.042	71.1 ± 2.70
S2	6.89 ± 0.006	8.55 ± 0.160	0.150 ± 0.054	4.24 ± 0.076	0.361 ± 0.076	110 ± 3.50	174 ± 5.41	415 ± 18.8	10.7 ± 0.234	199 ± 4.25
S3	7.15 ± 0.307	13.7 ± 0.359	0.230 ± 0.034	7.18 ± 0.055	0.474 ± 0.055	135 ± 3.84	249 ± 4.76	623 ± 14.3	11.4 ± 0.15	251 ± 2.99
S4	6.13 ± 0.020	13.8 ± 0.145	0.163 ± 0.036	6.57 ± 0.016	0.60 ± 0.016	201 ± 2.63	408 ± 4.86	1020 ± 14.8	15.1 ± 0.15	413 ± 6.31
S5	6.66 ± 0.003	17.3 ± 0.230	0.462 ± 0.034	9.43 ± 0.14	0.87 ± 0.14	339 ± 7.53	672 ± 14.7	1740 ± 35.3	33.7 ± 0.646	717 ± 5.29
S6	6.56± 0.030	19.7 ± 0.346	0.463 ± 0.155	9.94 ± 0.457	0.92 ± 0.46	335 ± 4.57	664 ± 10.6	1850± 30.4	42.8 ± 0.678	588 ± 11.3
S7	6.57 ± 0.009	17.1 ± 0.392	0.315 ± 0.042	9.34 ± 0.088	0.78 ± 0.088	364 ± 4.43	742 ± 10.1	1990 ± 24.1	37.1 ± 0.399	678 ± 6.31
S8	6.54 ± 0.009	18.7± 0.083	0.385 ± 0.006	9.58 ± 0.127	0.906 ± 0.13	335 ± 6.96	655 ± 14.8	1770 ± 35.4	37.4 ± 0.336	647.9 ± 4.74

Table 6. Coefficients of determinations and root mean squared deviation (RMSD) for the prediction of Zn_{Plant} (mg kg^{-1}) using the asymptotic function (Equation 3). NS denotes not significant.

Coefficients	Model 1 (M^{2+})	Model 2 (M^{2+})
K_{Zn1}	29.3	29.2
K_{Zn2}	0.018	0.018
K_H	-	- 0.00736 ^{NS}
R^2	0.85	0.85
RMSD (mg kg^{-1})	113	115

Table 7. Phyto-toxicity ‘effective concentration’ values (EC_{50}) for different estimates of Zn bioavailability. L.CL and U.CL are the 95% lower and upper confidence limits; NS signifies a non-significant fit of the model; ‘No fit’ indicates that the parameter could not be resolved.

Relative Endpoint (%)	Dose	EC_{50}	L.CL	U.CL
Plant growth	Zn^{2+} (µM)	85.5	54.3	134.4
	Zn_{Soln} (µM)	204.3	136.1	306.9
	Zn_{E} (mg kg^{-1})	2495.2	439.7	14159
	Zn_{EDTA} (mg kg^{-1})	1100^{NS}	0.0038	3×10^{8}
	Zn_{Total} (mg kg^{-1})	No fit	-	-
Chlorophyll content	Zn^{2+} (µM)	725	111	4719
	Zn_{Soln} (µM)	1105	235	5195
	Zn_{E} (mg kg^{-1})	No fit	-	-
	Zn_{EDTA} (mg kg^{-1})	No fit	-	-
	Zn_{Total} (mg kg^{-1})	No fit	-	-
Photosynthetic rate	Zn^{2+} (µM)	259	57	1179
	Zn_{Soln} (µM)	567	126	2546
	Zn_{E} (mg kg^{-1})	No fit	-	-
	Zn_{EDTA} (mg kg^{-1})	No fit	-	-
	Zn_{Total} (mg kg^{-1})	No fit	-	-

Table 8. Comparison between Zn^{2+} activity ((Zn^{2+})) at the Predicted No-Effect Concentration (PNEC, 0.51 μM) and Zn^{2+} activity in control soils; Zn_{Tot} is the total soil Zn concentration.

	Zn_{Tot} (mg kg^{-1})	(Zn^{2+}) μM
PNEC	-	0.51
Soil 1	139	0.05
Soil 2	415	0.76
Soil 3	623	0.66
Soil 4	1022	3.41
Soil 5	1739	1.69
Soil 6	1987	1.52
Soil 7	1768	1.99
Soil 8	1852	2.10

Figure captions

Figure 1. Variation in (A) EDTA-extractable Zn (Zn_{EDTA}) and (C) isotopically exchangeable Zn (Zn_E) with total soil Zn (Zn_{Total}); variation in (B) Zn_{EDTA} and (D) Zn_E, with added Zn (Zn_{Added}) following subtraction of the native Zn concentrations. Dashed lines represent a 1:1 relation.

Figure 2. Concentration of Zn in plants [Zn_{Plant}] as a function of (A) soil total content [Zn_{Total}], (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+}), on a log-log scale. Shaded areas are 95% confidence intervals of the linear models.

Figure 3. Cadmium concentration in soil solution (Cd_{Soln}) as a function of Zn_{Soln} (A); Cd bioaccumulation factor (Cd_{BAC}) as a function of Zn_{Soln} (B); relationship between Zn to Cd ratio in the soil solution and plant (C). Shaded areas in B indicate 95% confidence intervals around the linear regression model. The dashed line in C represents a 1:1 relation.

Figure 4. Relationships between relative barley yield (% of controls) and five estimates of available Zn: (A) (Zn^{2+}), (B) Zn_{Soln}, (C) Zn_E, (D) Zn_{EDTA}, and (E) Zn_{Total}. Shaded areas indicate ± RMSD.

Figure 5. Toxic response of photosynthetic rate of barley (% of controls) to: (A) (Zn^{2+}) activity (B) Zn_{Soln}. Toxic response of chlorophyll content of barley to: (C) (Zn^{2+}) activity (D) Zn_{Soln}. Shaded areas indicate ± RMSD.

Figure 6. Predicted toxic response in control soils as a function of total Zn concentration in soil. Vertical dashed line indicate the maximum permissible limits (MPL) in the soil because of biosolids application. Error bars represent standard errors of the mean.

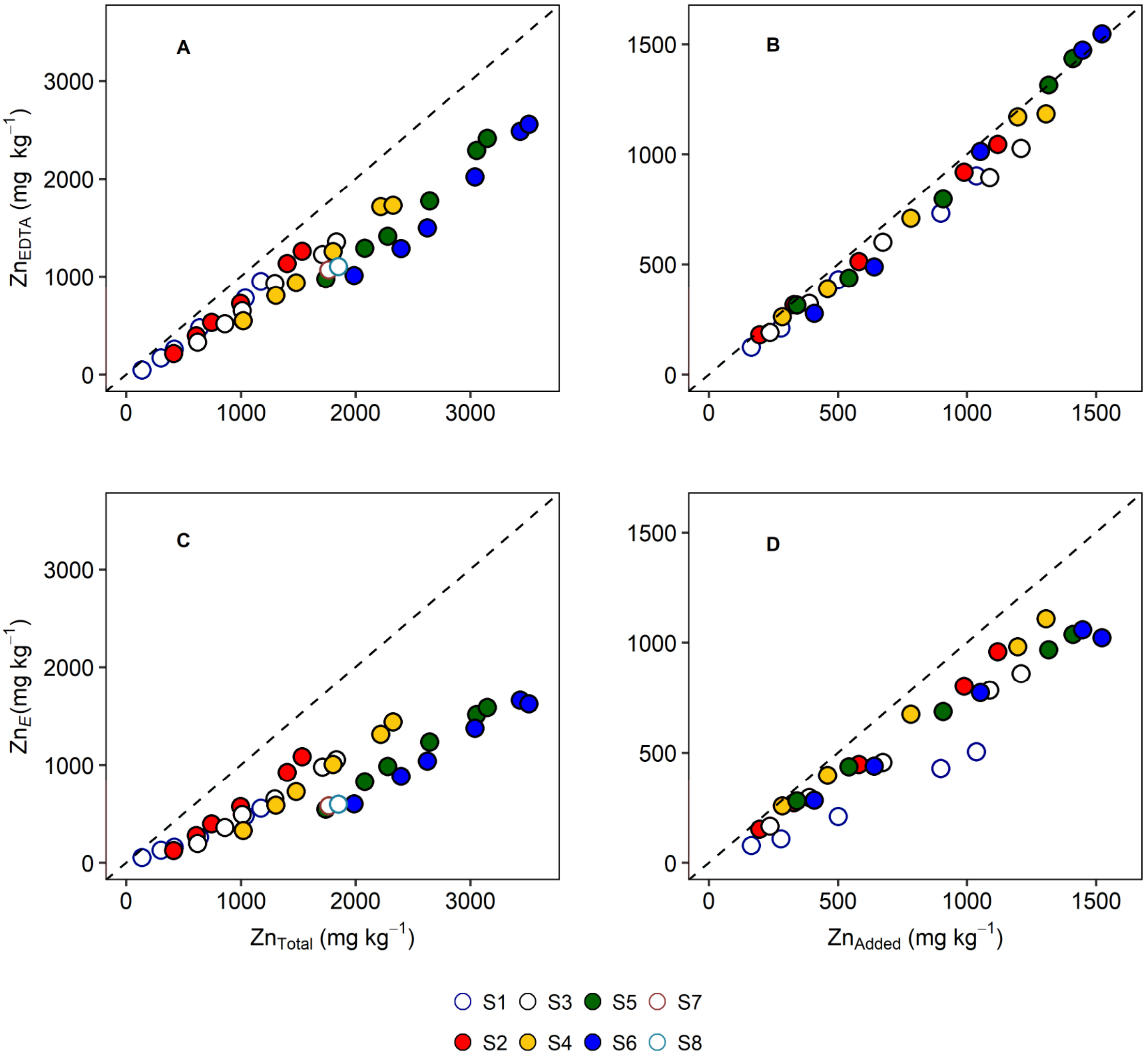

Figure 1. Variation in (A) EDTA-extractable Zn (ZnEDTA) and (C) isotopically exchangeable Zn (ZnE) with total soil Zn (ZnTotal); variation in (B) ZnEDTA and (D) ZnE, with added Zn (ZnAdded) following subtraction of the native Zn concentrations. Dashed lines represent a 1:1 relation.

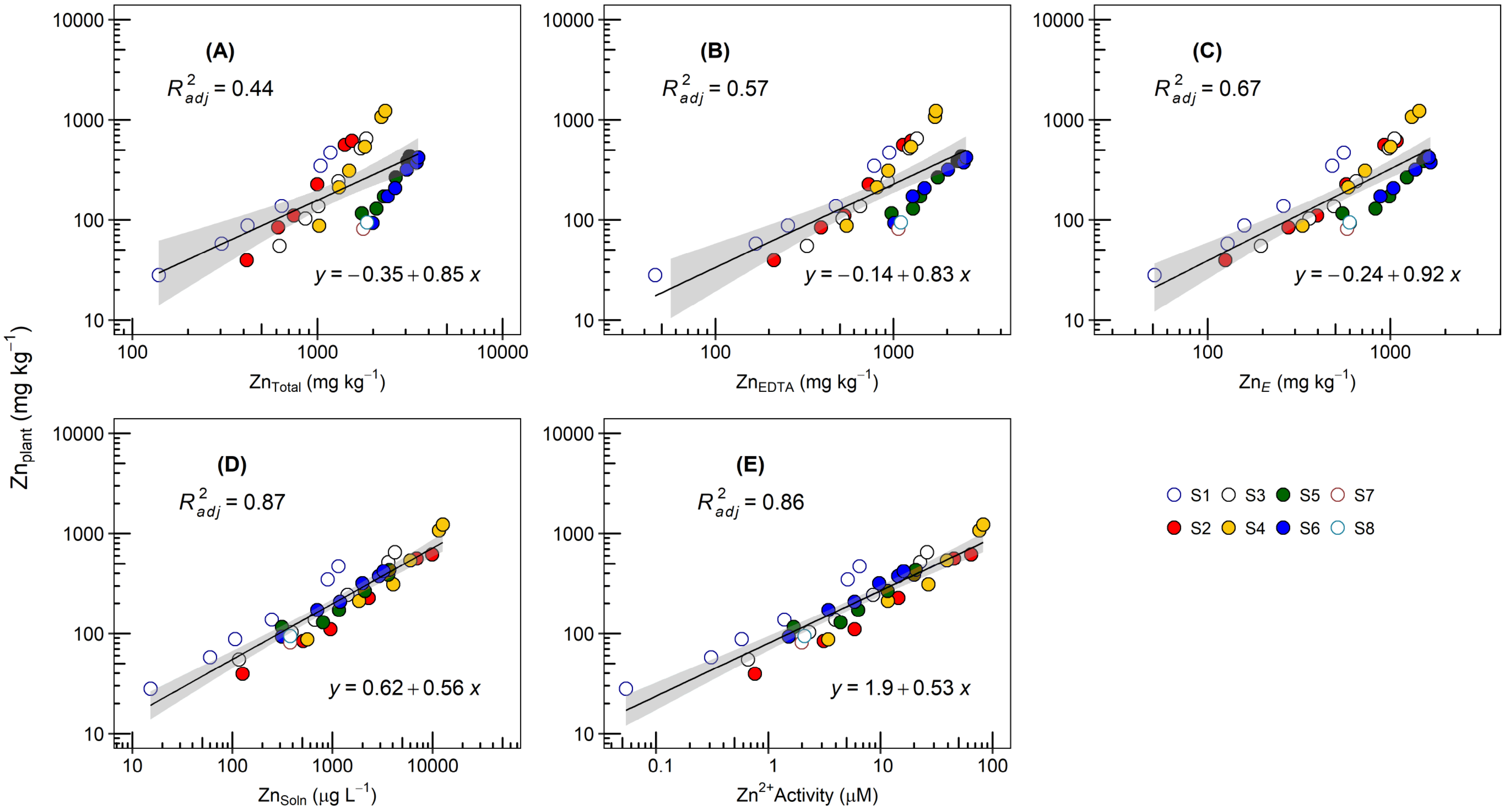

Figure 2. Concentration of Zn in plants [ZnPlant] as a function of (A) soil total content [ZnTotal], (B) concentration of labile Zn in soil [ZnE], (C) EDTA-extractable Zn in soil [ZnEDTA], (D) Zn in the soil solution (0.01 M Ca(NO3)2) [ZnSoln], and (E) free ion activity of Zn in the soil solution (Zn^{2+}), on a log-log scale. Shaded areas are 95% confidence intervals of the linear models.

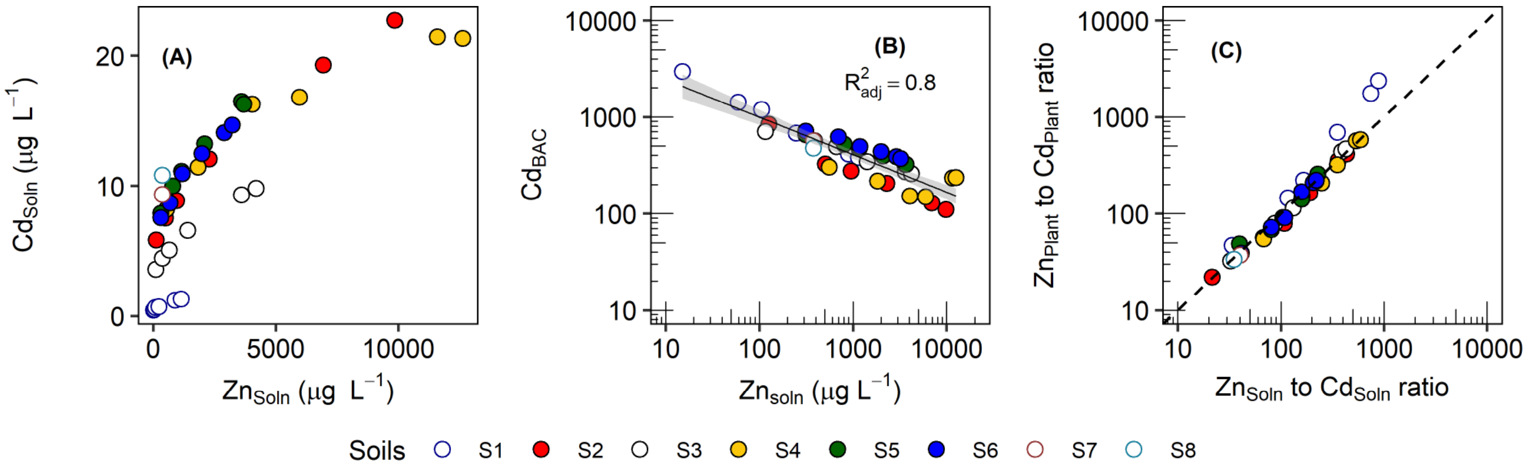

Figure 3. Cadmium concentration in soil solution (CdSoln) as a function of ZnSoln (A); Cd bioaccumulation factor (CdBAC) as a function of ZnSoln (B); relationship between Zn to Cd ratio in the soil solution and plant (C). Shaded areas in B indicate 95% confidence intervals around the linear regression model. The dashed line in C represents a 1:1 relation.

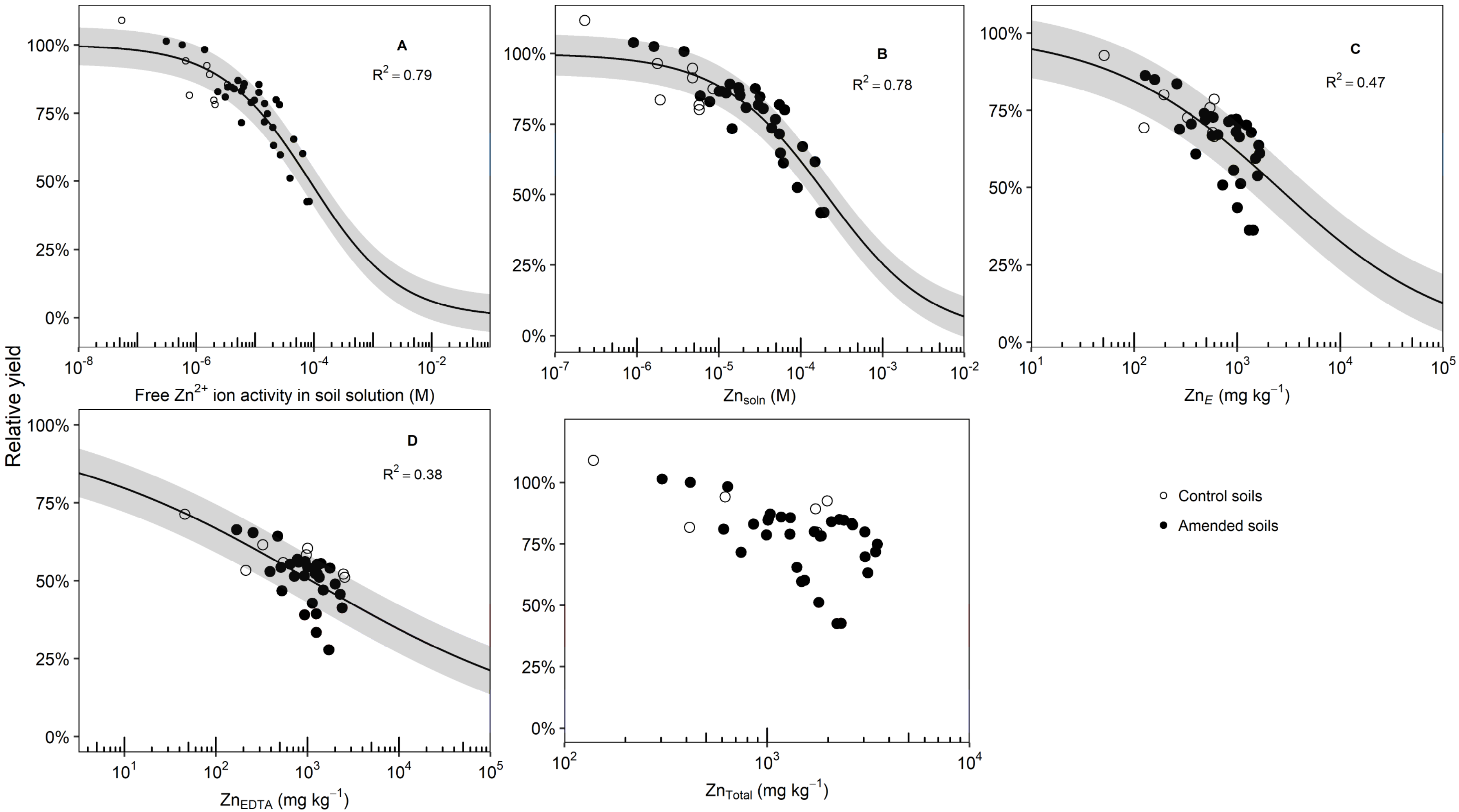

Figure 4. Relationships between relative barley yield (% of controls) and five estimates of available Zn: (A) (Zn2+), (B) ZnSoln, (C) ZnE, (D) ZnEDTA, and (E) ZnTotal. Shaded areas indicate ± RMSD.

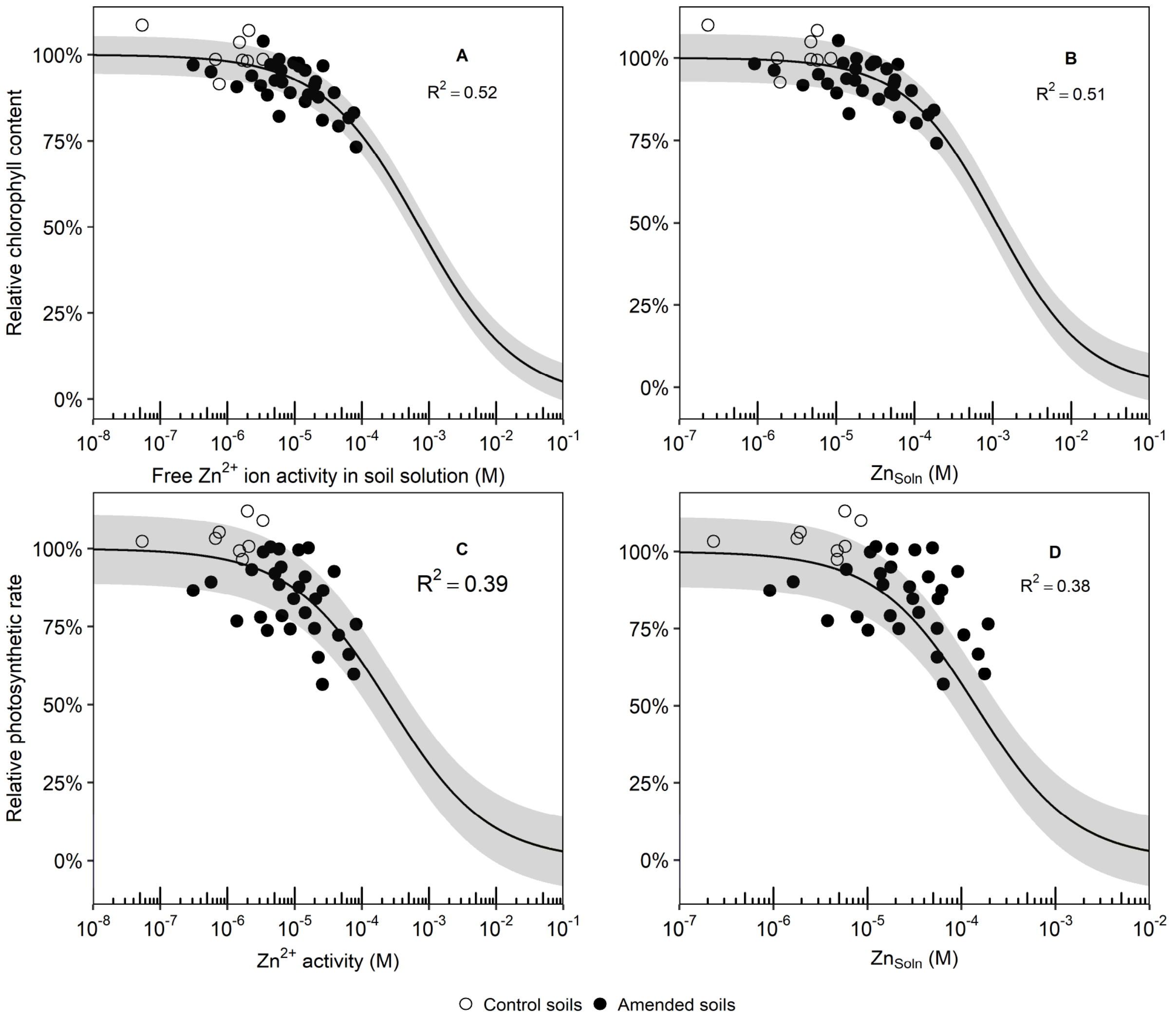

Figure 5. Toxic response of photosynthetic rate of barley (% of controls) to: (A) (Zn2+) activity (B) ZnSoln. Toxic response of chlorophyll content of barley to: (C) (Zn2+) activity (D) ZnSoln. Shaded areas indicate ± RMSD.

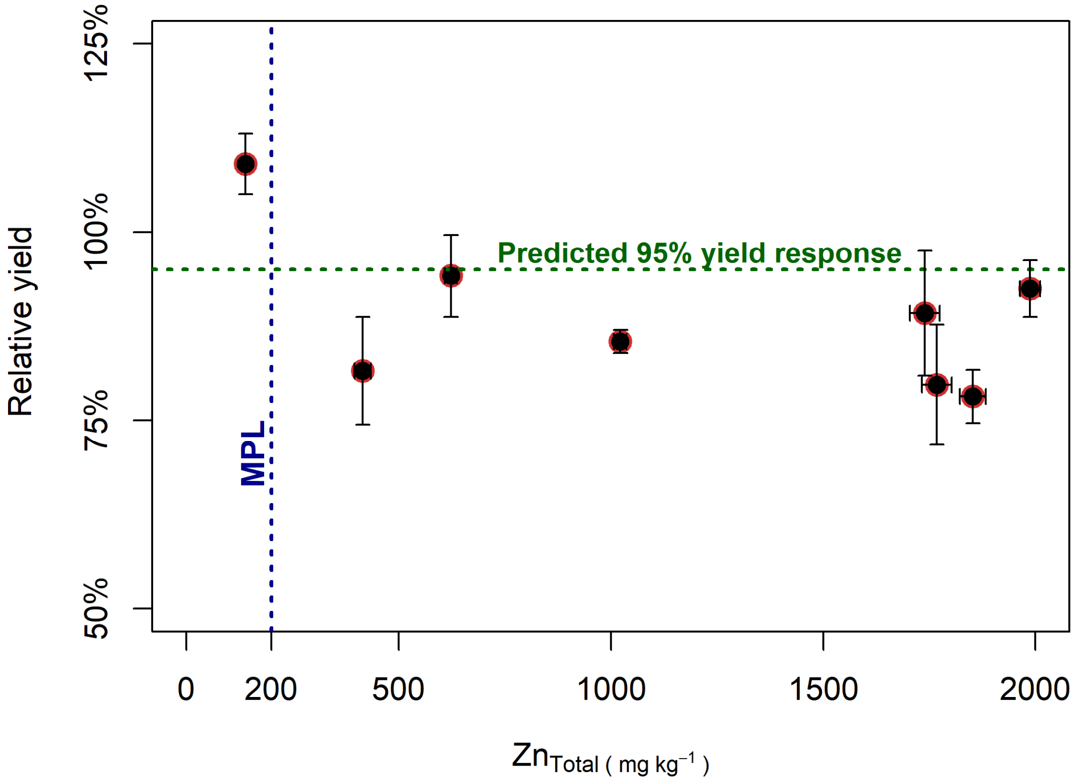

Figure 6. Predicted toxic response in control soils as a function of total Zn concentration in soil. Vertical dashed line indicate the maximum permissible limits (MPL) in the soil because of biosolids application. Error bars represent standard errors of the mean.

ZINC UPTAKE AND PHYTOXICITY: COMPARING INTENSITY AND CAPACITY BASED DRIVERS

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Table S1. Zinc levels added to individual soils S denotes soil; L denotes Zn addition level)

	Background	Added Zn	Total
	(mg kg-1)	(mg kg-1)	(mg kg-1)
S1L0	139	-	139.4
S1L1	139	164	303.8
S1L2	139	280	419.3
S1L3	139	501	640.7
S1L4	139	899	1038.7
S1L5	139	1037	1176.2
S2L0	415	-	415.3
S2L1	415	197	612.6
S2L2	415	330	745.5
S2L3	415	582	996.8
S2L4	415	989	1404.6
S2L5	415	1120	1535.0
S3L0	623	-	623.2
S3L1	623	237	860.0
S3L2	623	390	1012.9
S3L3	623	675	1297.8
S3L4	623	1088	1711.4
S3L5	623	1209	1832.5
S4L0	1022	-	1022.2
S4L1	1022	284	1306.4
S4L2	1022	460	1482.0
S4L3	1022	782	1804.7
S4L4	1022	1197	2219.2
S4L5	1022	1306	2328.3
S5L10	1739	-	1739.4
S5L11	1739	341	2080.4
S5L12	1739	543	2282.0
S5L13	1739	908	2647.1
S5L14	1739	1317	3056.1
S5L15	1739	1411	3150.0
S6L0	1987	-	1987.1
S6L1	1987	409	2396.3
S6L2	1987	640	2627.4
S6L3	1987	1053	3040.0
S6L4	1987	1448	3435.5
S6L5	1987	1523	3510.5
S7L0	1768	-	1767.6
S8L0	1852	-	1852.5

Effect of isotope spike level on E-value determination

Figure S1 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 caused an analytically measurable shift in the isotopic ratios and provided 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. Moreover, the greater variations between replicates suggest that these *E*-values are subject to a greater uncertainty, especially in the case of Cd (Figure S1). 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, % Zn_E in both soils showed <10% variation across different spike levels tested (Fig S2). Whereas for %Cd_E revealed a greater variation. Differences up to 20% in %Cd_E were found 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.

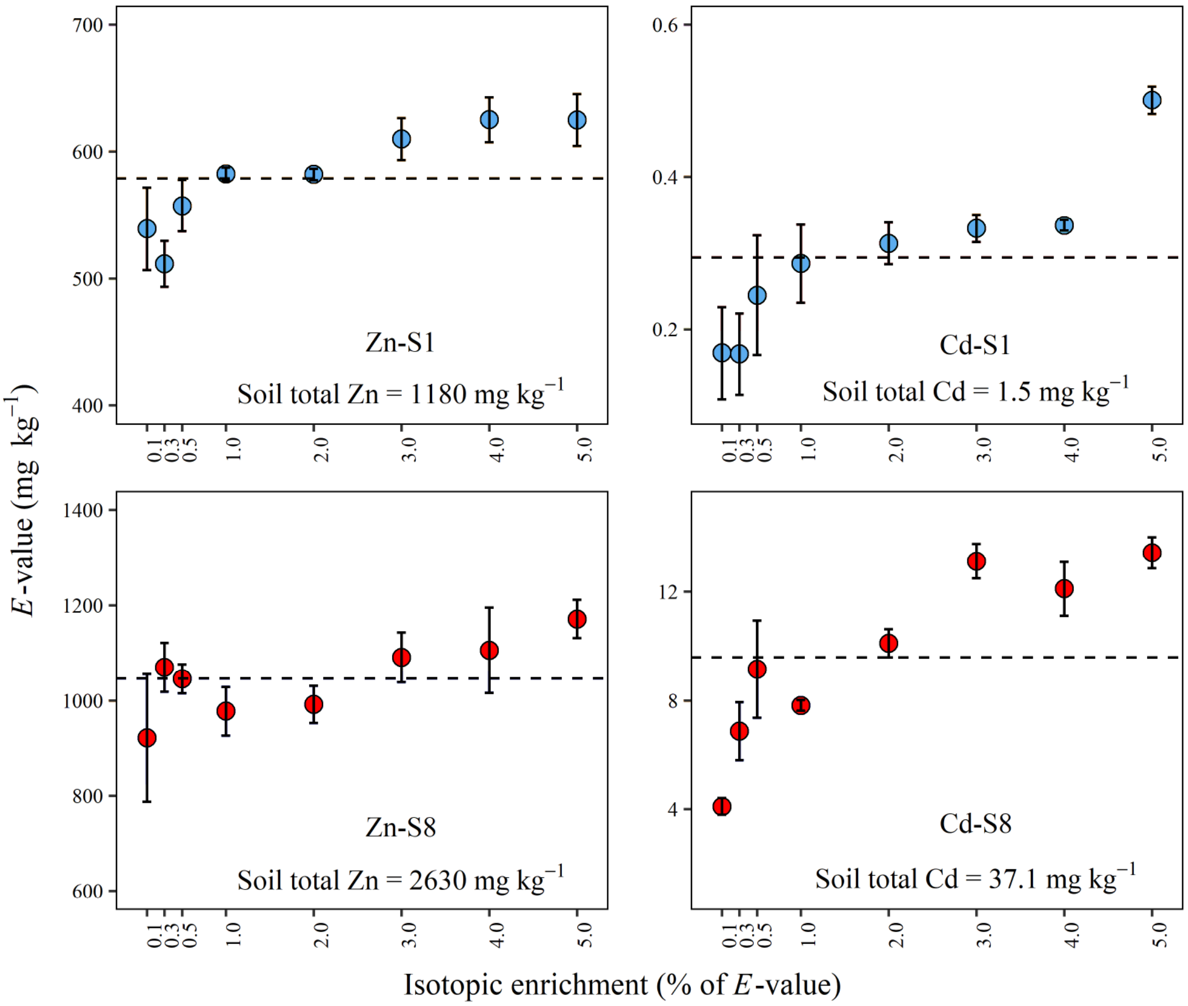

Figure S1. Zinc and Cd *E*-values for two soils (S1 and S8) as a function of the added isotopic tracer. The dashed horizontal line represents the mean of *E*-values (n=8).

Adding the isotopic tracer in an amount more than 4% of the total labile metal overestimated *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.S2). 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., 2004) 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.

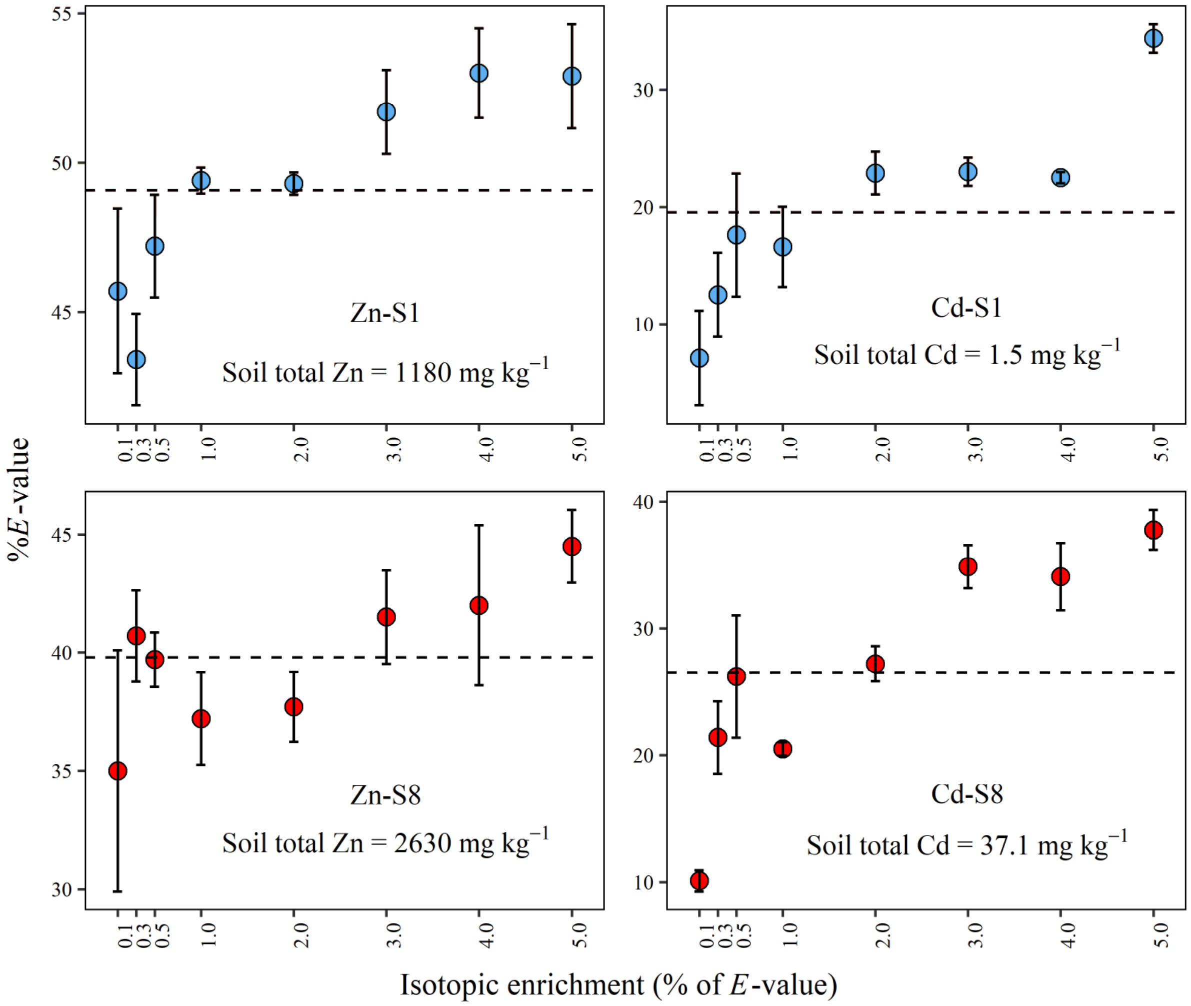

Figure S2. Zinc and Cd % *E*-values for two soils (S1 and S8) as a function of the added isotopic tracer. The dashed horizontal line represents the mean of % *E*-values (n=8). Error bars represent % CV between three replicates.

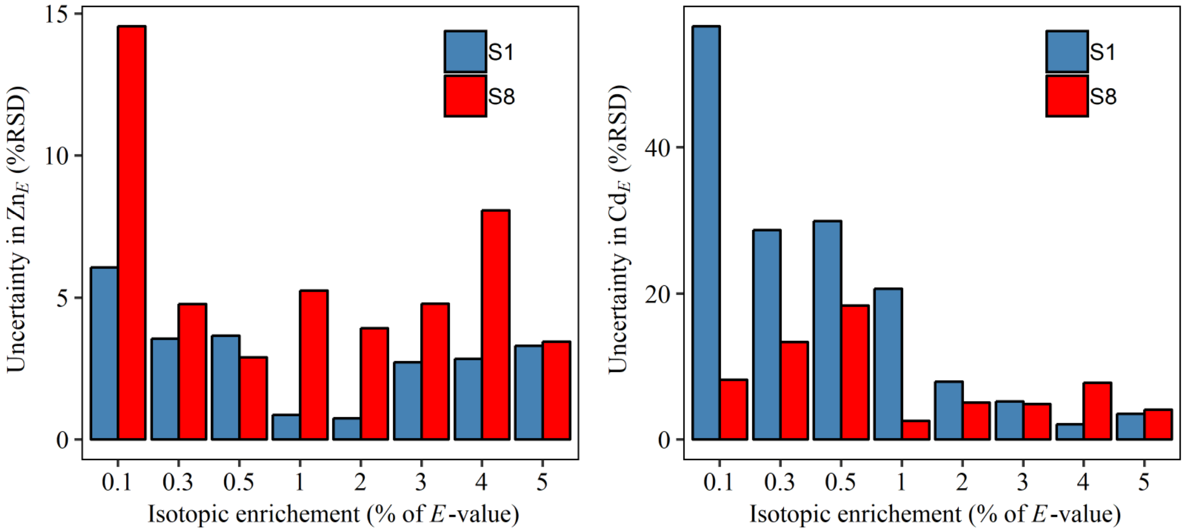

Figure S3. Uncertainty in determining *E*-value for Cd and Zn as a function of isotopic tracer addition

Reference:

Nolan, A.L., Ma, Y., Lombi, E., McLaughlin, M.J., 2004. Measurement of labile Cu in soil using stable isotope dilution and isotope ratio analysis by ICP-MS. Anal. Bioanal. Chem. 380, 789–797. https://doi.org/10.1007/s00216-004-2816-6