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Biological and chemical assessment of zinc ageing in field soils

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**ABSTRACT**

As zinc (Zn) is both an essential trace element and potential toxicant, the effects of Zn fixation in soil are of practical significance. Soil samples from four field sites amended with $ZnSO_4$ were used to investigate ageing of soluble Zn under field conditions over a 2-year period. Lability of Zn measured using $^{65}Zn$ radioisotope dilution showed a significant decrease over time and hence evidence of Zn fixation in three of the four soils. However, 0.01 M $CaCl_2$ extractions and toxicity measurements using a genetically modified lux-marked bacterial biosensor did not indicate a decrease in soluble/bioavailable Zn over time. This was attributed to the strong regulatory effect of abiotic properties such as pH on these latter measurements. These results also showed that Zn ageing occurred immediately after Zn spiking, emphasising the need to incubate freshly-spiked soils before ecotoxicity assessments.

**Capsule:** Ageing effects were detected in Zn-amended field soils using $^{65}Zn$ radioisotope dilution as a measure of lability, but not with either $CaCl_2$ extractions or a lux-marked bacterial biosensor.

**Key words –** attenuation; aging; isotopic dilution; lability; lux biosensor

**INTRODUCTION**

Zinc (Zn) is an essential trace element, ubiquitous in soils, and fundamental to the healthy functioning of biological systems. It is also a potential toxicant when present at elevated concentrations. Consequently, the dynamics of Zn in soils are of widespread interest, both in relation to crop nutrient deficiencies and associated impacts on farm yield and economy (Brown et al., 1993; Alloway, 2003) and in terms of soil health and toxicity (McLaughlin et al., 2000; Warne et al., 2008a). The distribution, mobility and bioavailability of Zn in soils is controlled by a range of physico-chemical characteristics, including the nature and heterogeneity of the soil constituents, the surface charge of soil colloids, and variations in soil pH and redox status. This paper focuses on the sorption and fixation of Zn in a range of soils with differing physico-chemical characteristics. The general term 'sorption' is used throughout, due to the difficulties in differentiating between adsorption and precipitation under common experimental conditions (Sposito, 1984).

Previous research investigating the kinetics of soluble metal sorption in soils has shown that the process can essentially be divided into two steps, with an initial stage of relatively rapid sorption followed by a secondary stage that can continue over weeks, months or even years (Barrow, 1986; Smolders and Degryse, 2007). Furthermore, studies investigating metal desorption and/or chemical extractability have typically shown considerable hysteresis, with a negative correlation between desorption/extractability and the residence time of the metal in the soil (Barrow, 1986; Sparks, 1998). This gradual, ongoing process of sorption and fixation has become known as 'ageing' or 'natural attenuation'. Greater understanding of ageing could aid in the modelling and prediction of long-term changes in metal lability and bioavailability. This would be useful for environmental risk assessment and decision making (e.g. in

relation to 'safe' metal loading rates) and could also enable land managers to maximise the benefits following application of trace metal fertilisers to land.

Although there is substantial evidence of Zn ageing in soils (e.g. Barrow, 1986; Ma and Uren, 1997a and 1997b; Tye et al., 2003; Degryse et al., 2004), the mechanisms and soil properties controlling this are only superficially understood, and much of the evidence comes from laboratory studies conducted under controlled conditions, leaving considerable uncertainty as to the kinetics and practical significance of ageing in the field. In order to understand the implications of ageing for environmental management and risk assessment this knowledge gap must be addressed, for although variations in soil properties and experimental conditions in field studies can mask effects and make data interpretation difficult, the confirmation of hypotheses under field conditions is strongly indicative of a meaningful effect. To elucidate the practical consequences of metal fixation there is also a need for more research using biological endpoints to measure the effects of ageing. To date, most of the research investigating ageing reactions in the toxic concentration range has taken a chemocentric approach, prompting Lombi et al. (2007) to highlight the importance of integrated biological and chemical assessment in revealing both the mechanisms and the effects of metal ageing.

The experiment reported in this paper used soil samples from four Australian field trials, each with a wide range of Zn loadings as $ZnSO_4$, to investigate the ageing of soluble Zn under field conditions over a two-year period. Both chemical and biological based assessments were used to investigate Zn ageing, including: 0.01 M $CaCl_2$ extractions; isotopic dilution with $^{65}Zn$ ($E_a$- and $E_e$-values); and the acute toxicity response of a genetically modified lux-marked bacterial biosensor (*Escherichia coli* HB101 pUCD607). Lux biosensors present a novel approach for investigating the

effects of Zn ageing, and this study is one of the first to use the method for this application (see Paton et al., 1997 for further information on the use of microbial biosensors for soil toxicity testing). The use of the lux-biosensor to measure changes in acute toxicity has a major advantage over more traditional microbial ecotoxicity endpoints (e.g. substrate induced respiration or nitrification) as the lux-marked bacteria are not indigenous to the soils and results thus cannot be confounded by adaptation of the soil microbial community.

## MATERIALS AND METHODS

### *Experimental design, field sites, and field-aged soil samples*

The field trials used in this study were established as part of the Australian National Biosolids Research Program (NBRP), a large-scale research program designed to investigate the potential benefits and risks of recycling biosolids to agricultural land (McLaughlin et al., 2006; Broos et al., 2007; Warne et al., 2008a; Warne et al., 2008b). Metal-salt trials (cadmium (Cd), copper (Cu) and Zn) were also established at many of the NBRP field sites and the work presented here used $ZnSO_4$-amended samples from four of these sites, chosen to cover a range of soil properties. Samples that had been spiked (11 $ZnSO_4$ treatments per site) and aged in the field for up to two years were compared with freshly spiked samples established using 'control' soils from the same four sites. Two field replicates from each Zn treatment were analysed for the field-aged soils, and two experimental replicates for the matching freshly spiked treatments. Field-aged samples were collected after the first (T1) and second (T2) crop harvests at each field site. Hence, depending on the site, T1 samples were aged in the field for 7-12 months, and T2 samples for 17-24 months after addition of soluble $Zn^{2+}$. For a detailed description of the NBRP field trial establishment and subsequent soil sampling, preparation and storage refer to Broos et al. (2007).

*Preparation of the freshly spiked soil samples*

Freshly spiked (T0) samples were prepared in the laboratory using control soils from each of the four field sites to give approximately the same range of Zn concentrations as were present in the field samples (T1 and T2). For each Zn treatment duplicate 150 g soil samples were placed in plastic containers. The appropriate aliquot (ranging from 40 µL to 4500 µL) of $ZnSO_4$ stock solution (containing 659.6 g $ZnSO_4.7H_2O$ $L^{-1}$) was diluted with deionised (DI) water to give a spiking solution volume of 30 mL per sample, then added to the soil and mixed thoroughly by hand. After a 16-hour equilibration period, the samples were leached with several pore volumes of 'Artificial Rain Water' (ARW) containing $10^{-3}$ M $CaCl_2$ and $5 \times 10^{-4}$ M $K_2SO_4$ (Broos et al., 2004) until the electrical conductivity (EC) of the leachate was $< 2$ mS $m^{-1}$. The leached samples were air-dried at 40 °C and sieved (2 mm).

*Chemical and physical characterisation of soils*

Soil pH was measured using 1:5 soil:0.01 M $CaCl_2$ extracts ($pH_{CaCl2}$)and EC was measured in 1:5 soil:water extracts. Clay content ($< 0.002$ mm) was determined by the pipette method. Organic carbon (OC) was determined as the difference between total carbon and carbonate carbon, with total carbon concentration measured by ignition with a Leco CNS elemental analyser and carbonate carbon determined by measuring pressure increases after addition of HCl to the soil in closed containers (Sherrod et al., 2002).

Soil samples (2.5 g) were extracted for determination of exchangeable cation concentrations and cation exchange capacity (CEC) (1 M $NH_4Cl$, pH 7.0) using a mechanical leaching device based on the method of Rayment and Higginson (1992). Samples were analysed for $Ca^{2+}$, $Mg^{2+}$, $Na^+$ and $K^+$ using a GBC 906AA Atomic

Absorption Spectrophotometer, and for $NH_4^+$ using an Alpkem segmented flow autoanalyser. Pre-treatment to remove carbonates was not required.

The methods used for the determination of dithionite-citrate extractable Fe and acid ammonium oxalate extractable Fe were those of Blakemore et al. (1987). Total Zn and Fe concentrations were determined by aqua regia digestion. Elemental analysis was by inductively coupled plasma - optical emission spectroscopy (Spectroflame Modula ICP-OES).

### 0.01 M CaCl₂ extractions

For the 0.01 M $CaCl_2$ extractions, 5 g samples of soil were equilibrated for 16 hours with 25 mL of 0.01 M $CaCl_2$ on an end-over-end shaker. The samples were then centrifuged at 2200 $g$ for 15 minutes and an aliquot was used for pH measurement (completed within 2 hours of extraction). The remaining sample volume was filtered (0.45 μm) and acidified to pH 1 with 5M $HNO_3$, before being analysed for Zn by ICP-OES.

### Isotopic dilution with $^{65}$Zn (E-values)

Isotopic dilution with $^{65}$Zn was used to monitor changes in the labile Zn fraction over time. The procedure was based on the method of Young et al. (2000) but 0.1 M $CaCl_2$ was used instead of 0.1 M $Ca(NO_3)_2$ and all solutions were filtered (0.45 μm) before analysis. The activity of the $^{65}$Zn spike was adjusted for different soil types (20 - 40 kBq $^{65}$Zn per sample) to ensure that a suitable gamma-counting rate would be obtained. Analysis of Zn concentrations in solutions was by ICP-OES and a Wallac 1480 Wizard$^{TM}$ 3" Automatic Gamma Counter was used for radio-assay.

The labile or 'isotopically available' Zn fraction (henceforth referred to as the $E_a$-value) was calculated using Equation 1 (Hamon et al., 2002a):

$$E_a = \frac{Zn_{sol}}{Zn^*_{sol}} \times R \times \frac{V}{W} \qquad \text{[Eqn. 1]}$$

where $E_a$ is the concentration of labile Zn in the soil (mg kg$^{-1}$), $Zn_{sol}$ is the concentration of non-radioactive Zn in solution (mg L$^{-1}$), $Zn^*_{sol}$ is the concentration of radioisotope remaining in solution after the 3 d equilibration time (Bq mL$^{-1}$), $R$ is the total amount of $^{65}$Zn added to the sample (Bq mL$^{-1}$), $V$ is the solution volume (L) and $W$ is the mass of the soil sample (kg).

All $E_a$-values calculated for this experiment were corrected for background Zn and the lability results are thus expressed in terms of the percentage of total added Zn. This was calculated using Equation 2:

$$\% E(Zn^{added}) = \frac{E_a - E_a^{control}}{Zn^{total} - Zn^{controltotal}} \times 100 \qquad \text{[Eqn. 2]}$$

where $E_a$ is the sample $E_a$-value, $E_a^{control}$ is the mean $E_a$-value for the matching control soil, $Zn^{total}$ is the total Zn concentration of the sample, and $Zn^{control\,total}$ is the mean total Zn concentration for the matching control soil. All values are expressed as mg kg$^{-1}$.

The 'isotopically exchangeable' Zn fraction ($E_e$-value) was also calculated for each sample. This fraction represents Zn on soil surfaces that is in equilibrium with the soil solution (Hamon et al., 2002b). The $E_e$-value differs from the $E_a$-value by excluding the solution Zn component as shown in Equation 3 (with all terms expressed in mg kg$^{-1}$).

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$$E_e = E_a - Zn_{sol} \hspace{5cm} \text{[Eqn. 3]}$$

### *E.coli. HB101 pUCD607 lux biosensor assay*

The lux biosensor was applied as an acute toxicity assay to all samples. The sample solutions used in the assays were 1:1 soil:water extracts (25 g soil: 25 mL DI water, equilibrated for 4 hours on an end-over-end shaker, centrifuged at 2500 $g$ for 45 minutes and filtered (0.45 µm)). Duplicate 900 µL aliquots from each sample solution were transferred into 5 mL luminometer sample tubes for the bioassay. The remaining solution was divided into 2 subsamples, with one aliquot acidified in preparation for ICP-OES analysis and the other frozen (-19 ºC) until analysed for dissolved organic carbon (DOC) using a Scalar Formacs HT TOC Analyser.

Freeze-dried cultures of the *E. coli* HB101 pUCD607 lux biosensor were obtained from Remedios Ltd., UK. For each assay a vial of *E. coli* was rehydrated with 10 mL of sterile 0.1 M KCl whilst gently agitating on an orbital shaker for 60 minutes at 25 ºC. Immediately following resuscitation, 100 µL of the cell suspension was added at 15-second intervals to each of the 5 mL sample tubes containing 900 µL aliquots of sample solution and mixed by pipette. Each sample was left to stand for exactly 15 minutes before the light output was measured (again at 15-second intervals) on a Junior LB 9509 luminometer (spectral sensitivity range 380-630 nm). The results were recorded in relative light units (RLU). As soon as the bioluminescence measurements were completed, the pH of the inoculated samples ($pH_{H2O}$) was measured using a Thermo Orion ROSS 420A+ pH microprobe.

All sample solutions were measured in duplicate and the mean results used for all further analysis. The lux response for six matching control samples (i.e. no added Zn) for each soil type were also measured as part of each assay, and the mean control value was used to convert the measured bioluminescence results from RLU to 'percentage of control luminescence'. This conversion made the results of different assays comparable. The converted results were used to calculate effective concentration ($EC_x$) values and their 95 % confidence intervals (95 % CIs) for each of the soil type/sampling time combinations investigated. The $EC_x$ of a substance is the concentration that causes a defined magnitude of response (x) in a given system. Biological evidence for a decline in Zn availability due to ageing would be indicated by increasing $EC_x$ values (based on the total Zn concentration) from T0 to T2.

Results from anomalously dark sample solutions ($< 2$ % of samples) were not included in the $EC_{50}$ calculations as darker solution colours can cause quenching of the sample luminescence, thereby confounding the results (Ivask et al., 2004). The biosensor results for the Dutson Downs field site were also excluded from analysis and are not presented in this paper as their $pH_{H2O}$ values were below the optimum biosensor pH range (pers. comm., Dr. G. Paton, University of Aberdeen). A preliminary experiment (results not shown) confirmed that $pH_{H2O} < 5.5$ adversely affected the biosensor.

*Statistical analyses*

All statistics were calculated using Microsoft Office Excel and XLStatistics Version 5 (Carr, R., XLent Works, Australia). The significance of ageing effects was tested for each soil type using linear regression analysis between the chemical response variables (i.e. $CaCl_2$-extractable Zn and $E_a$-values) and the added Zn concentration (i.e. measured total concentration of each soil sample minus the average total Zn concentration of the

matching control samples). The concentrations of added soil Zn that caused a 50, 20 and 10 % reduction in bioluminescence (i.e. $EC_{50}$, $EC_{20}$ and $EC_{10}$) and their 95 CIs were calculated by fitting a log-logistic distribution according to the method of Barnes et al. (2003). Where 95 % CIs did not overlap they were considered to be significantly different (Barr, 1969; Lo, 1994; Nelson, 1989). However, when 95 % CIs were overlapping statistical significance can not be inferred (Barr, 1969; Lo, 1994; Nelson, 1989) and the standard error of the difference test (Sprague and Fogels, 1977) was used to test for differences between the T0 and T2 $EC_{50}$ values. As the number of comparisons was very small and no significant differences were claimed on this basis, Bonferroni adjustment was not applied to these results.

## RESULTS AND DISCUSSION

### *Characteristic soil properties*

A summary of selected soil properties is given in Table 1. Soil $pH_{CaCl2}$ ranged from 3.9 at Dutson Downs to 6.6 at Spalding. Electrical conductivity (1:5 soil:water extracts) was relatively low, ranging from 0.06 to 0.1 dS m$^{-1}$. Organic carbon content was $\leq$ 2 % in all soils except Dutson Downs (5.6 %). Clay content ranged from 4 % (Dutson Downs) through to 32 % (Kingaroy), and CEC (pH 7) varied from 7.9 cmol$^+$ kg$^{-1}$ (Dutson Downs) to 17.7 cmol$^+$ kg$^{-1}$ (Spalding). Cation exchange sites were dominated by $Ca^{2+}$ in all soils. Kingaroy, a Ferrosol, was the only soil with substantial Fe content (14.5 % total Fe).

Total Zn concentrations in the control soils ranged from 11 mg kg$^{-1}$ (Dutson Downs) to 90 mg kg$^{-1}$ (Kingaroy) and the total Zn ranges in the amended samples also varied for different soil types and sampling periods (Table 1). The differences occurred partly because the amendments for each soil type were chosen on the basis of plant toxicity

data and were thus soil specific (Warne et al., 2008a; Broos et al., 2007), and partly because the T0 samples were not sampled directly from the field plots but were amended (and leached) in the laboratory. Furthermore, the lower total Zn concentration in the T2 soils compared with matching treatments in the T1 soils (i.e. earlier samples from the same field plots) suggests that some ongoing leaching, erosion, or dilution by soil mixing occurred between the T1 and T2 sampling periods. However, as measured rather than nominal Zn concentrations were used for all calculations, the differences in total Zn concentrations do not complicate the interpretation of results. Total added Zn (remaining) was calculated for each individual sample by subtracting the mean background total Zn for the relevant control soil from the total Zn measured in the sample. This ensured that all ageing results were related directly to the soluble added Zn rather than the native Zn already in the soil system, as in contrast to the added Zn, native Zn would not have been 100 % labile at the start of the experiment.

### *0.01 M CaCl$_2$ extractions*

Linear regression analysis of the 0.01 M CaCl$_2$ extractable added Zn vs. total added Zn for each soil type and sampling period (Table 2) ($R^2$ ranged from 0.79 to 0.99, $p < 0.01$) revealed that although the soils showed some significant differences ($p \leq 0.05$) in extractability for the investigated time periods, there was no consistent trend of decreasing extractability with time, making it unlikely that any changes in slope were due to ageing. These results are not in keeping with those of previous studies indicating progressive fixation of soluble Zn added to soils (e.g. Boawn et al., 1960; Brown et al., 1964; Follett and Lindsay, 1971; Armour et al., 1989; Tye et al., 2003; Degryse et al., 2003). However, given the large impact that soil properties can have on extractability and metal partitioning, it is possible that the inherent field variability in key properties such as pH may have complicated and masked trends in these results. Measured pH$_{CaCl2}$

values were not consistent across sampling times and treatments, and linear regression analysis comparing the mean control soil pH for each sampling period and soil type against the slopes of graphs of $CaCl_2$ extractable Zn vs. total Zn (not shown) showed significant differences ($p < 0.01$, $R^2 = 0.86$), indicating that the $CaCl_2$ extraction results would be highly affected by any variations in sample pH. It is well established that pH is a master variable affecting metal partitioning in soil systems (Sauvé et al., 2000; Impellitteri et al., 2001). These findings thus suggest that soil properties may have played a greater role than contact time (i.e. ageing) in determining Zn extractability by 0.01 M $CaCl_2$.

**Labile Zn ($E_a$ values)**

There were linear relationships between labile added Zn and total added Zn for the different sampling times ($R^2$ ranged from 0.98 to 1.00, $p < 0.01$) and the decreases in lability over time observed for the Dookie, Kingaroy and Spalding soils (i.e. decreasing slopes for labile vs. total added Zn regressions) were statistically significant ($p \leq 0.05$) (Table 3). For both Kingaroy and Spalding the changes in slope indicated that fixation was more extensive in the early stages of the experiment, with both soils showing a significant decrease in lability from T0 to T1, but no significant difference between T1 and T2. This observation of decreasing fixation over time also concurs with the results of Tye et al. (2003).

Although pH is also known to affect the lability of added soluble Zn in soils (Degryse et al., 2004), the pH dependency of the labile and salt extractable fractions differs (Nakhone and Young, 1993) and small variations in pH apparently have relatively little effect on Zn lability at pH values < 6 (Tye et al., 2003). As all of the samples used in this experiment, with the exception of the Spalding T0 samples, had $pH_{CaCl2}$ values < 6,

it can be assumed that differences in soil pH between sampling times had a relatively minor impact on lability. This was confirmed by linear regression of the mean control soil pH for each sampling period and soil type versus the slopes of graphs of labile Zn vs. total Zn (not shown) ($R^2 = 0.04$, $p > 0.05$).

For all four soils the labile Zn expressed as a percentage of total added Zn at T0 varied from 70 – 90 % for most Zn treatments, although in the low Zn treatments (< 100 mg kg$^{-1}$ total added Zn) at Kingaroy this value was as low as 40 % (Figure 1). Theoretically, it can be assumed that upon addition of the Zn salts (i.e. at the true time zero) the added Zn would have been 100 % labile. Thus, by the time the T0 samples had been spiked, mixed, leached, dried and sieved ready for analysis (i.e. 6 days), up to 60 % of the added Zn had already been fixed by the soil. Nevertheless, the leaching step, which was the major cause of this delay, was considered an essential part of the experiment, as the samples were also used to measure ecotoxicity with the lux biosensor. Leaching not only removes much of the added counter ion and reduces the ionic strength of the soil solution towards its natural state, it also replenishes the system with calcium and potassium (Stevens et al., 2003; Fait et al., 2006). Leached samples are thus more representative of field conditions (where leaching occurs naturally), and less likely to confound ecotoxicity data by elevated salt concentrations contributing to the adverse effects reflected in EC$_x$ values (Stevens et al., 2003; McLaughlin et al., 2004).

### *Zn buffer capacity – relationship between E$_e$ values and solution Zn*

Sorption curves, with the isotopically exchangeable Zn fraction (E$_e$-value) plotted against the solution Zn fraction, are presented in Figure 2. Linear regression analysis of the log-log transformed data indicated a small but significant time-dependent decrease

in the quantity of isotopically exchangeable Zn for all soils except Dutson Downs

(Table 4). Decreases in $E_e$ over time for corresponding quantities of solution Zn

indicates a net movement of Zn from the labile exchangeable pool to the fixed (non-

labile) pool, thus providing further evidence for Zn ageing.

Generally, the shape of a sorption curve is considered to indicate the bonding strength

or affinity of the sorbate for the soil surface (Tom-Petersen et al., 2004). In the curves

shown in Figure 2 steeper slopes reveal greater partitioning of Zn to the solid phase, and

the slopes decrease in the order Spalding > Kingaroy > Dookie > Dutson Downs. For a

given quantity of surface exchangeable Zn, the soils with greater slopes maintain

correspondingly lower concentrations of solution Zn. Likely characteristics accounting

for these differences in Zn retention include soil pH, clay content, type of clay mineral

present, and surface charge characteristics (see Tiller et al., 1984; Barrow, 1987; Sauvé

et al., 2000; Harter and Naidu 2001; Degryse et al., 2003). Notably, both the pH and

CEC of the different soil types decreased in the same soil order as that specified above,

whilst Kingaroy, Spalding and Dookie also had higher clay contents than the Dutson

Downs soil, and Kingaroy contained a much higher proportion of free Fe-oxide

minerals (indicated by high citrate-dithionite extractable Fe) than the other soil types

(Table 1).

### *Zn toxicity to E. coli HB101 pUCD607*

The $EC_{50}$ values for each combination of soil and sampling time and their 95 % CIs are

presented in Figure 3. Overall, there was no consistent temporal trend in toxicity. This is

contrary to the hypothesis that Zn ageing processes would give rise to higher total Zn

$EC_{50}$ values for the aged samples than for the freshly spiked samples, due to reductions

in the extractable/bioavailable/labile Zn fraction caused by increasing fixation over time. Standard error of the difference testing (Sprague and Fogels, 1977) confirmed that there was no significant difference between the T0 and T2 $EC_{50}$ values for Dookie and Spalding, whilst the significant difference between the Kingaroy T0 and T2 $EC_{50}$ values signalled by non-overlapping CIs (Nelson, 1989) indicated a decrease in $EC_{50}$ rather than an increase.

The lux biosensor responds to the bioavailable Zn in the solution it is exposed to (Tandy et al., 2005) which is largely dependent on the Zn speciation (Nolan et al., 2003). In this experiment, the lack of a clear pattern in water-extractable Zn $EC_{50}$ values over time (results not shown) suggests there was no systematic change in the speciation of the soil-water extracts as a result of ageing. However, differences in the speciation of the test solutions due to variations in pH, DOC, and various soil-derived ligands, together with the effects of competing co-ions on cell uptake, may well account for some of the variation in the calculated $EC_{50}$ values. In fact, it is possible that the reliance on soil-water extracts for exposure of the biosensor may have impeded the detection of ageing effects, because any differences in extractability due to differences in sample pH or other soil properties would also be reflected in the bioassay results.

Across all soil types, significantly higher DOC concentrations were recorded for T0 control samples than for T0 treatment samples (t-test, $p < 0.01$). This was probably due to the increased salt concentrations in the freshly spiked samples. The differences between control and treatment samples diminished over the course of the experiment and were not significant for T1 and T2 samples (t-test, $p > 0.05$), probably due to decreasing soluble salt contents as a result of ongoing leaching. Given that Zn in solution may bind to DOC and hence become less bioavailable, the finding of lower

DOC in the freshly spiked samples than in the aged samples is important, showing that ageing effects had not been masked by decreases in DOC over time. On the other hand, differences in sample pH may well have played a role in obscuring any ageing effects, as a simple linear regression through the lux biosensor (total added Zn) $EC_{50}$ values for all soil types and sampling times was highly significant ($p < 0.01$) (Figure 4) with soil $pH_{CaCl2}$ alone explaining 79% of the variation in Zn toxicity to the lux biosensor. In any case, these results indicate the potential drawbacks of using blanket regulatory limits for all soil types based on total metal contents without taking into consideration the modulating effects of key soil properties on Zn toxicity.

Previous studies using microbial endpoints to investigate Zn ageing are relatively limited and must also be interpreted with respect to a range of potentially confounding factors affecting both metal partitioning and ecotoxicity over time. Indeed, Lombi et al. (2007) noted several examples (e.g. Doelman and Haanstra, 1984; Kelly et al., 1999; Smolders et al., 2003) where apparent evidence of decreasing toxicity of added Zn over time may also have been affected by adaptation of the soil microbial community (Rusk et al., 2004; Fait et al., 2006) and/or by leaching of excess salts (McLaughlin et al., 2004). Taking into account the published results together with the present experiment, it must be concluded that the significance of Zn ageing as a factor affecting ecotoxicity remains uncertain. However, this is not to say that ageing does not play a role. Except in high pH soils, ageing processes are only expected to produce small changes in metal lability/toxicity over relatively long periods of time (Tye et al., 2003), and natural biological variability combined with changes in soil parameters affecting metal partitioning could make detection of ageing processes using biological endpoints quite difficult (Lombi et al., 2007). In situ ecotoxicity monitoring using fibre optic linked

membrane bound biosensor probes (Paton et al., 1997; Nivens et al., 2004) and/or experiments facilitating the direct extraction of soil porewater for testing may prove to be superior in this respect.

In our study, significant reductions in Zn lability between spiking and after leaching/drying (a period of only 6 days) indicates that soil treatment after spiking using soluble metal salts (prior to assessment of ecotoxicity) is crucial in defining metal bioavailability and hence ecotoxicity thresholds. It also strengthens the argument that metal toxicity and availability assessed in soils spiked with soluble metal salts without a post-spiking leaching and ageing treatment are grossly overestimated (Stevens et al., 2003; Smolders et al., 2004).

**CONCLUSIONS**

The identification of ageing under field conditions is an important part of establishing the practical significance of metal fixation, with effects being more difficult to detect due to the increased variability over laboratory conditions, but correspondingly more meaningful when apparent. In this experiment, the expected decrease in $CaCl_2$-extractable Zn over time was not detected. This was attributed to the strong effect of soil pH on this measurement, and the finding that pH varied over time at most sites. As most of the soils were acidic, it is also possible that fixation was not particularly extensive and hence difficult to detect by this method. Measurements using radioisotope dilution ($E_a$ and $E_e$ values) demonstrated a significant effect of time on Zn lability, and indicated that most of the fixation proceeded quite rapidly after Zn spiking, with a considerable decrease in lability occurring even before Zn lability and ecotoxicity inthe freshly spiked samples could be determined (i.e. in the 6 days after spiking during which soils were leached and dried). The *E.coli* HB101 pUCD607 lux biosensor was used as a

microbial toxicity assay (unaffected by adaptation) to assess whether changes in lability over time would be detectable by changes in toxicity, but produced no evidence to suggest that Zn ageing processes had led to decreased bioavailability of Zn between 6 days and 2 years. On the other hand, Zn sorption and bioavailability were shown to vary according to soil type on the basis of both chemical and biosensor results. This finding indicates the importance of soil properties in modulating the toxicity of added soil Zn. Further work is needed to assess the importance of long-term ageing in terms of bioavailability and toxicity, and whether or not this process is significant enough to warrant inclusion in risk assessment, predictive models and the setting of safe limits for Zn in the environment.

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**FIGURE LEGENDS**

Figure 1: Percentage of labile added Zn presented as a function of total added Zn.

Figure 2: Isotopically exchangeable Zn ($E_e$) as a function of the solution Zn concentration.

Figure 3: Lux biosensor $EC_{50}$ values calculated for each soil type and time period on the basis of total added Zn concentration. The error bars represent the 95% confidence intervals.

Figure 4: Lux biosensor $EC_{50}$ values (total added Zn) for all soils and sampling times as a function of soil pH.  The soil pH values are the mean pH (0.01 $M$ CaCl$_2$) for each bioassay. X error bars are the SE of the mean for the soil pH. Y error bars indicate the 95% confidence intervals for the measured $EC_{50}$ values.

**TABLE LEGENDS**

Table 1: Selected chemical and physical properties of the 4 experimental soils, expressed on an oven dry (105°C) basis. Where applicable, standard errors are given in brackets (n = 4 for total Zn and Fe; n = 3 for pH measurements, EC and Fe extractions. Other values presented (CEC, exchangeable cations, and particle size) are the means of duplicate samples.

Table 2: Slopes of linear regression lines and significance testing results for concentrations of $CaCl_2$-extractable added Zn against total added Zn at each time period. Intercepts were constrained to the origin. Significant differences in slope between T0 (freshly spiked), T1 (after 1st harvest) and T2 (after 2nd harvest) data for each soil type are indicated by differing superscripts.

Table 3: Slopes of linear regression lines for labile added Zn against total added Zn. Intercepts were constrained to the origin. Significant differences in slope between T0, T1 and T2 data for each site are indicated by different superscripts.

Table 4: Intercepts of linear regression equations derived from log-log transformed data from Figure 2 (labile exchangeable Zn against solution Zn). Significant differences in intercept between T0, T1 and T2 data for each site are indicated by different superscripts.

Table 1: Selected chemical and physical properties of the 4 experimental soils, expressed on an oven dry (105°C) basis. Where applicable, standard errors are given in brackets (n = 4 for total Zn and Fe; n = 3 for pH measurements, EC and Fe extractions. Other values presented (CEC, exchangeable cations, and particle size) are the means of duplicate samples.

| | Dookie | Dutson Downs | Kingaroy | Spalding |
|---|---|---|---|---|
| Soil type[a] | Dermosol | Podosol | Ferrosol | Chromosol |
| pH (0.01 M $CaCl_2$) | 5.0 (0.01) | 3.9 (0.01) | 5.3 (0.02) | 6.6 (0.04) |
| Electrical Conductivity (dS $m^{-1}$) | 0.1 (0.003) | 0.08 (0.001) | 0.06 (0.003) | 0.08 (0.003) |
| Organic C (%) | 2.0 | 5.6 | 1.8 | 1.9 |
| CEC ($cmol^+$ $kg^{-1}$) | 10.7 | 7.9 | 16.9 | 17.7 |
| Exchangeable cations ($cmol^+$ $kg^{-1}$) | Ca: 5.8 | Ca: 3.2 | Ca: 8.9 | Ca: 11.0 |
| | Mg: 1.1 | Mg: 0.9 | Mg: 2.1 | Mg: 3.6 |
| | Na: 0.1 | Na: 0.1 | Na: 0.1 | Na: 0.1 |
| | K: 1.2 | K: 0.2 | K: 1.0 | K: 2.2 |
| Particle size distribution | 22 % clay | 4 % clay | 32 % clay | 23 % clay |
| | 21 % silt | 5 % silt | 39 % silt | 35 % silt |
| | 57 % sand | 91 % sand | 29 % sand | 42 % sand |
| Total Fe (%) | 3.3 (0.10) | 0.1 (0.00) | 14.5 (0.06) | 3.0 (0.06) |
| Citrate-dithionite Fe (%) | 2.1 (0.02) | 0.1 (0.00) | 10.2 (0.17) | 1.5 (0.01) |
| Ammonium oxalate Fe (%) | 0.4 (0.00) | 0.1 (0.00) | 0.4 (0.02) | 0.1 (0.00) |
| Total Zn in control soil (mg $kg^{-1}$) | 25.0 (0.48) | 11.0 (0.07) | 89.4 (0.52) | 48.6 (0.72) |
| Range of total Zn concentrations determined (mg $kg^{-1}$) | | | | |
| T0 (freshly spiked) | 25 – 1550 | 10 – 900 | 90 – 2350 | 50 – 2550 |
| T1 (after 1st harvest) | 25 – 1950 | 15 – 1300 | 95 – 1250 | 60 – 4250 |
| T2 (after 2nd harvest) | 25 – 900 | 10 – 600 | 90 – 1050 | 60 – 3650 |

[a] Australian Soil Classification System (Isbell, 1996)

Table 2: Slopes of linear regression lines and significance testing results for concentrations of $CaCl_2$-extractable added Zn against total added Zn at each time period. Intercepts were constrained to the origin. Significant differences in slope between T0 (freshly spiked), T1 (after 1st harvest) and T2 (after 2nd harvest) data for each soil type are indicated by differing superscripts.

|    | Dookie | Dutson Downs | Kingaroy | Spalding |
|----|--------|--------------|----------|----------|
| T0 | 0.36[a] | 0.53[a] | 0.33[a] | 0.15[a] |
| T1 | 0.44[b] | 0.55[a] | 0.29[a] | 0.27[b] |
| T2 | 0.37[a] | 0.46[b] | 0.30[a] | 0.25[b] |

Table 3: Slopes of linear regression lines for labile added Zn against total added Zn. Intercepts were constrained to the origin. Significant differences in slope between T0, T1 and T2 data for each site are indicated by different superscripts.

|     | Dookie | Dutson Downs | Kingaroy | Spalding |
| --- | --- | --- | --- | --- |
| T0 | 0.82[a] | 0.77[a] | 0. 94[a] | 0.81[a] |
| T1 | 0.81[a] | 0.72[b] | 0.76[b] | 0.72[b] |
| T2 | 0.74[b] | 0.76[a] | 0.76[b] | 0.73[b] |

Table 4: Intercepts of linear regression equations derived from log-log transformed data from Figure 2 (labile exchangeable Zn against solution Zn). Significant differences in intercept between T0, T1 and T2 data for each site are indicated by different superscripts.

| | Dookie | Dutson Downs | Kingaroy | Spalding |
|---|---|---|---|---|
| T0 | 1.03[a] | 0.24[a] | 1.46[a] | 1.92[a] |
| T1 | 0.91[b] | 0.37[a,b] | 1.40[a,b] | 1.60[b] |
| T2 | 0.83[b] | 0.41[b] | 1.34[b] | 1.63[b] |