

Measurement of isotopically-exchangeable Zn in Zn-deficient rice soil

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Summary

The changes in soil chemistry following submergence of a soil for rice production result in zinc (Zn) being immobilized in very insoluble forms. Consequently, Zn deficiency is widespread in rice crops and in human populations that subsist on rice. We explored the use of stable isotopic dilution assays for assessing Zn dynamics in submerged rice soil with two types of strongly Zn-deficient rice soil from the Philippines. We optimized the isotope enrichment, electrolyte and equilibration time to measure isotopically-exchangeable Zn (E -values) without changing redox conditions. Available Zn was rapidly and strongly immobilized following submergence, controlled by CO₂ accumulation. Addition of the isotopic tracer before submergence produced unreliable E -values because irreversible immobilization of the tracer progressed faster than isotopic exchange. Addition of the tracer to already reduced soil produced stable E -values for tracer–soil contact of up to one week. Longer periods produced unreliable E -values because of continuing irreversible fixation of the tracer. We discuss the implications for applications of isotopic dilution methods to measure trace-element dynamics in submerged soil.

Introduction

Zinc deficiency is the most widespread micronutrient disorder in rice plants; it affects up to 50% of the soil in lowland rice production globally (Dobermann & Fairhurst, 2000) and results in reduced growth, delayed maturity and diminished yields (van Breemen *et al.*, 1980). Zinc is also often deficient in human populations with rice-based diets (IRRI, 2006). Consequently, there are currently major international efforts to breed varieties of rice with a tolerance to Zn deficiency and with large grain Zn contents for human nutrition (IRRI, 2006).

The prevalence of Zn deficiency in rice is linked to redox changes in the soil following submergence. Permanent or prolonged submergence as a result of poor drainage

41 and high pH, large organic matter contents and dissolved bicarbonate (HCO_3^-) contents have
42 been reported to depress Zn availability in soil and restrict Zn uptake by rice plants (Forno *et*
43 *al.*, 1975; van Breemen *et al.*, 1980). Zinc itself does not undergo redox transformations, but
44 its mobility is affected by the reductive dissolution and re-precipitation reactions that take
45 place following submergence. Precipitation of mixed Zn carbonates and possibly Zn
46 sulphides is the most likely explanation for the very low solubility of Zn in many types of soil
47 used for rice cultivation. To understand the mechanisms of Zn uptake and differences
48 between varieties of rice in support of breeding efforts, measures of the dynamics of plant-
49 available forms of Zn in the soil following submergence are needed.

50 Various extractants have been used to estimate the plant-available pool of Zn in soil,
51 including calcium (Ca) and magnesium (Mg) salts, ethylenediaminetetraacetic acid (EDTA),
52 diethylene triamine pentaacetic acid (DTPA), hydrochloric acid (HCl) and nitric acid (HNO_3)
53 (Ponnamperuma *et al.*, 1981; Sinaj *et al.*, 2004; Römkens *et al.*, 2009; Impa & Johnson-
54 Beebout, 2012). These extractions might correlate with plant Zn uptake in the particular
55 circumstances for which they have been calibrated. Simple soil extraction schemes, however,
56 do not measure solid phase-solution equilibria of soil under the true conditions of plant
57 growth, and so are of limited use for studies of uptake mechanisms. Isotopic dilution
58 techniques have a stronger mechanistic basis because they measure the element concentration
59 in equilibrium with the soil solution (Young *et al.*, 2000), which is the pool that can be drawn
60 upon by plant roots in the absence of other root-induced changes in the soil that affect
61 element solubility. The technique is based on adding a known amount of enriched isotope to a
62 soil suspension in equilibrium. The resulting change in isotopic ratios gives an indication of
63 the isotopically exchangeable concentration of metal (*E*-value). Isotopically exchangeable Zn
64 has been recognized as a major source of Zn for plants (Sinaj *et al.*, 2004). The reader is

referred to Midwood (2007) and Hamon *et al.* (2008) for discussion of the principles of the technique.

Isotopic dilution has been used extensively to study trace-element dynamics in aerobic soil, but rarely in submerged anaerobic soil. Exceptions are the early research from Tiller *et al.* (1979) in Zn deficient soil, and more recent research on As (Stroud *et al.*, 2011) and Zn in contaminated soil where Zn is strongly available (Marzouk, 2012). Potential difficulties include the maintenance of anaerobic conditions during the isotopic exchange, the effect of changing redox conditions following submergence on the behaviour of the isotopic tracer and, in our case, the very small concentrations of reactive Zn. The present research is, to our knowledge, the first attempt to use stable isotopic dilution to investigate Zn availability in soil used for rice cultivation.

The aim of our research was to develop methods for measuring isotopically-exchangeable Zn (*E*-value) in Zn-deficient soil with the specific objectives: (i) to optimize isotope equilibration procedures for both aerobic and anaerobic soil where the element of interest is present in very small reactive concentrations, (ii) to develop protocols to ensure constant reducing conditions during the measurements on anaerobic soil, with widely-available laboratory equipment, (iii) to assess the dynamics of *E*-values over time following submergence and (iv) to examine whether soil extractions can be used effectively as a non-isotopic estimate for the determination of *E*-values.

It is worth noting that the experimental design used in this study may be sufficient for this methodological study, but does not allow any generalization of the results to a field scale or even a comparison of the two sites.

Materials and methods

Soil types and isotopic tracer solutions

We investigated two soil types that are used for growing rice, and are typical of the young, alluvial, perennially-wet soil types where Zn deficiency in the rice crop is common. One is a Tropaquept (USDA Soil Taxonomy, 1999) from Bay, Laguna, Philippines; the other is a Hydraquent (USDA Soil Taxonomy, 1999) from Tiaong, Quezon, Philippines.

Sixteen containers of ≈ 40 kg wet soil ($\approx 70\%$ moisture content) were taken randomly from 0–25 cm depth at both the Tiaong and Bay field sites. The soil samples were transported to IRRI (International Rice Research Institute) where they were air-dried and mixed manually every day to aid the drying process. After drying, organic materials such as dried leaves, twigs and snail shells were removed and the samples were then disaggregated to pass through a 2-mm sieve with a modified Rukuhia soil grinder (Day & Dixon, 1965). A representative portion of 10 kg of this soil was then shipped to Cranfield University for further analyses. Relevant properties of the sieved soil are given in Table 1. The total concentration of Zn in the samples was determined by weighing soil into PFA vials and adding concentrated, analytical grade hydrofluoric (HF), HNO_3 and perchloric (HClO_4) acids, followed by a stepped heating programme to 170°C overnight. The dry residue was reconstituted with Milli-Q water, HNO_3 and hydrogen peroxide (H_2O_2). Reference materials (NIST SRM2710, BGS102 and BCR-2, $< 4\%$ error with respect to the certified values) and blanks were all prepared in a similar way.

Enriched ^{67}Zn (89.6% ^{67}Zn) was obtained from ISOFLEX and dissolved in 2 M HNO_3 to obtain a solution of ~ 820 mg $^{67}\text{Zn} \text{ l}^{-1}$. From this stock solution, a range of fresh solutions with measured concentrations of between 0.38–1.90 mg $^{67}\text{Zn} \text{ l}^{-1}$ and ^{67}Zn abundances of 86.0–86.6% were produced for each experiment.

Preparation of anaerobic soil

Portions of 3.5 kg soil were mixed thoroughly with 1% w/w ground rice straw and transferred to plastic basins to give a soil depth of 11 cm. The soil was then mixed by hand with

deionized water to produce a slurry. After 24 hours, further deionized water was added to give 2.5-cm depth of standing water. The basins were incubated at 26°C, tapped periodically to aid release of entrapped gas and the > 2-cm depth of standing water was maintained. Soil pH and redox potential were monitored throughout the experiment by a pH–Eh probe with silver:silver chloride reference electrode, inserted to a depth of 8 cm. Reducing conditions stabilized after three weeks of submergence at $E_h \approx -220$ mV and $\text{pH} \approx 6.5$. To sample the anaerobic soil for soil extractions and isotopic dilution assays, soil cores were taken with an adapted 1.5 cm internal diameter plastic syringe with a Zn-free plunger inserted to the bottom of the soil. Sections of cores equivalent to about 2.5 g dry soil were transferred quickly into polypropylene centrifuge tubes; the upper 2 cm of soil was discarded because it was likely to be partially oxidized.

Extractable Zn

We compared three extraction schemes commonly used to assess metal bioavailability in soil: the standard 0.05 M NH_4 –EDTA with 10 l kg^{-1} L/S (liquid/solid ratio) and 1 hour shaking time (Quevauviller, 1998); 0.005 M DTPA with L/S = 2 l kg^{-1} and 2 hours shaking (Lindsay & Norvell, 1978); 0.43 M HNO_3 with L/S = 10 l kg^{-1} and 2 hours shaking time. The extractions were done by shaking 2–5 g air-dry soil with the extractant, and were replicated four times. Blanks and the standard reference soil BCR 484 (errors <5% with respect to copper (Cu), lead (Pb) and Zn certified values) were prepared in a similar way. For reduced soil, the extractant solutions were made with deoxygenated water and the L/S ratios and electrolyte strengths were corrected to account for moisture content. After adding the extractant solutions, the tubes were capped with Zn-free Versilic[®] silicone stoppers (Saint-Gobain Performance Plastics). Headspace air was displaced with N_2 gas with a syringe needle and contact between the needle and solution was avoided. The tubes were submerged in water during the assays to prevent the access of oxygen through the walls.

Analyses of solutions

The total Zn in the extracts was determined with a quadrupole ICP-MS Perkin Elmer Elan 9000, Boston, MA, USA). Response suppression or enhancement effects were corrected with rhodium as the internal standard. The calibration standards were analysed every 12 samples to check for possible drift during analysis and the data were corrected for reagent contribution.

To determine the isotopic composition of extracts, isotopic measurements were made with 10 replicates per sample, 1000 sweeps per integration and dwell times of 2, 2, 3, 2 and 4 mseconds for ^{64}Zn , ^{66}Zn , ^{67}Zn , ^{68}Zn and ^{70}Zn , respectively. The residual standard deviations (RSD) of the ^{66}Zn : ^{67}Zn ratios were typically < 0.5%. Correction for mass bias was derived from Zn standard solutions assumed to have fixed natural isotopic ratios (de Laeter *et al.*, 2003). The standard solutions were analysed every 3–6 samples and diluted to give similar concentrations (80–115%) to all the samples.

Determination of E-values

In the aerobic soil samples we examined the effect of different electrolytes and isotopic enrichment on the *E*-values. For anaerobic soil, we tested the effect of equilibration time and strength of electrolyte. In addition, the isotope exchange kinetics were studied.

Aerobic soil. Unbuffered salt solutions such as calcium chloride (CaCl_2) or calcium nitrate ($\text{Ca}(\text{NO}_3)_2$) are used widely in isotopic dilution assays, but they might not dissolve sufficient Zn for analytical purposes in soil where Zn is strongly bound (Izquierdo *et al.*, 2013a). Chelating agents such as EDTA are better suited to such soil, therefore we investigated the efficacy of 0.01 mM, 0.05 mM EDTA and 0.1 mM DTPA (Lindsay & Norvell, 1978; Degryse *et al.*, 2004; Atkinson *et al.*, 2011; Izquierdo *et al.*, 2013a). Portions of 2 ± 0.03 g of soil were shaken with 20 ml of electrolyte for 3 days, and then the equivalent to $0.3 \mu\text{g } ^{67}\text{Zn g}^{-1}$ soil

was added to three laboratory replicates of each set. This amount of isotopic tracer represents negligible amounts with respect to the total pool of Zn (Table 1) or to any rates of Zn applied as fertilizer. Further tracer-free tubes were prepared in triplicate to measure the natural isotopic abundances of Zn. All the suspensions were shaken to re-equilibrate for 3 days, and then centrifuged and filtered through 0.2 μm cellulose acetate filters to minimize sub-micron colloids in solution. The pH of the suspensions was measured to determine any acidification because of addition of the tracer.

We also examined the sensitivity of E -values to enrichment of the tracer. The aim was to optimize the isotope addition to soil where the metal is mainly non-labile, and therefore the addition of large amounts of isotope in soluble form is precluded. Portions of 2 ± 0.03 g of soil were shaken with 20 ml of 0.05 mM EDTA. After 3 days, a known amount of isotopic tracer between 0.02–0.45 $\mu\text{g } ^{67}\text{Zn g}^{-1}$ soil was added to the suspensions. These amounts correspond to 5, 10, 25, 50 and 100% of the labile ^{67}Zn (E -value) determined in the above experiment. The suspensions were re-equilibrated for a further 3 days and then centrifuged, filtered and analysed.

Anaerobic soil. A preliminary experiment in which sections of anaerobic soil cores were prepared as above was carried out. The samples were equilibrated with the ^{67}Zn tracer as for the aerobic soil, i.e. 3 days of pre-equilibration with electrolyte followed by addition of the tracer and 3 days of isotopic exchange. Although air in the headspace was displaced with N_2 before pre-equilibration and after addition of ^{67}Zn , and the tubes were submerged in a water bath to prevent the entry of O_2 , we observed an increase in the E_h from about -100 mV initially to $+150$ mV at the end of the experiment. This indicates a failure to maintain reducing conditions and it invalidated the assay, therefore, these results are not reported. In view of these observations, to determine the E -values in this research the assay time was

decreased and pre-equilibration before tracer addition was omitted to minimize oxidation of the soil.

In subsequent isotopic dilution assays, sections of cores of reduced soil were taken, transferred to centrifuge tubes and mixed gently with 2 ml deoxygenated ultrapure water before the addition of a solution containing the equivalent of $0.07 \mu\text{g } ^{67}\text{Zn g}^{-1}$ soil. The tubes were capped and air in the headspace was displaced as described above. The samples were then incubated in a water bath at 26°C for 2 days to allow isotopic exchange, and stirred on a vortex mixer at intervals. After 2 days of equilibration, the tubes were uncapped and 15 ml deoxygenated EDTA (0.2, 0.5 or 1 mM) were added. They were then re-capped, the air was displaced, and they were submerged in a water bath and shaken for 24 hours. Further tracer-free samples were used to measure the natural isotopic abundances of Zn and changes in E_h . The residual soil was dried at 105°C to determine the dry weight of soil. The redox potential of soil extracts remained consistent at about -100 mV throughout the experiment, which showed that reducing conditions were maintained.

Isotopic exchange kinetics. Two replicate sets of laboratory pots of submerged Bay soil were prepared by placing 200 g soil mixed with 1% wt rice straw in polypropylene containers and flooded with deionized water to give 2-cm depth of standing water as above. In 10 pots, a solution containing $0.1 \mu\text{g } ^{67}\text{Zn g}^{-1}$ soil was added after 2 hours of flooding, and in another 10 pots the isotopic tracer was added to the soil after 8 weeks of incubation at 26°C by which time the E_h had stabilized at -220 mV and pH at 6.7. The isotope was added as 10 ml solution applied in the standing water and mixed thoroughly into the soil by stirring with a spatula. Replicate laboratory tracer-free pots were also prepared as control samples for short-term isotopic exchange experiments. The pots were then re-incubated in a water bath at 26°C . Soil pH and E_h were monitored with probes inserted to a depth of 6 cm. After 1, 2, 4, 7, 11, 16 and

21 days, soil cores were taken as above, shaken with 12 ml 0.5 mM EDTA for 24 hours after purging with N₂ and analysed for isotopic abundances as described above. One core was extracted from five different random pots for each sampling.

To study the effect of shorter isotopic exchange times, tracer-free control pots were sampled by extracting cores after 3 and 18 days of incubation. These cores were transferred to tubes and the equivalent of 0.1 µg ⁶⁷Zn g⁻¹ soil was added. The tubes were incubated in a water bath for 2 days to allow isotopic exchange, and were stirred frequently on a vortex mixer. After this period, the samples were shaken with 12 ml 0.5 mM EDTA for 24 hours and filtered before analysis.

Calculation of E-value. The labile pool or *E-value* (mg kg⁻¹) was determined with Equation (1):

$$E - value = \left(\frac{M_{Zn-nat}}{W} \right) \left(\frac{C_{tracer} M_{tracer}}{M_{Zn-tracer}} \right) \left(\frac{{}^{67}Zn_{tracer} - {}^{66}Zn_{tracer} R_{ss}}{{}^{66}Zn_{natural} R_{ss} - {}^{67}Zn_{natural}} \right) \quad (1)$$

where M_{Zn-nat} is the average atomic mass of Zn, W is the weight of dry soil (kg), C_{tracer} is the ⁶⁷Zn concentration (mg kg⁻¹) in the solution containing the tracer, M_{tracer} is the mass of solution containing the ⁶⁷Zn tracer added to the soil (g), M_{Zn-nat} and $M_{Zn-tracer}$ are the atomic mass of Zn in the non-labelled and the labelled soils respectively, ${}^{xx}Zn_{xx}$ denotes isotopic abundance of a particular isotope in the soil supernatant and R_{ss} is the ⁶⁷Zn:⁶⁶Zn ratio in the soil suspension containing the tracer.

Results and discussion

Effect of redox status on extractable Zn

The two chelating agents, EDTA and DTPA, both extracted far smaller amounts of Zn from both soil types when conditions were anaerobic rather than aerobic, which reflects the

decrease in Zn availability following the development of anaerobic conditions (Table 2). The proportionate decrease under anaerobic conditions was far greater with DTPA. An important difference between EDTA and DTPA is that EDTA may dissolve some Zn associated with carbonates (Quevauviller, 1998), whereas DTPA contains CaCl_2 and it is buffered at pH 7.3 so that there is an equilibrium that prevents the release of elements occluded in carbonates. The greater extraction of Zn by EDTA under anaerobic conditions suggests that much of the solid-phase Zn is bound to carbonates. These observations suggest that conventional soil extractions to assess bioavailable Zn in anaerobic soil can give inaccurate results if the soil is examined under different redox conditions.

By contrast, the amount of Zn extracted with 0.43 M HNO_3 was insensitive to redox conditions, and the difference between the two soil types was far larger; very little Zn was extracted from Tiaong soil whether conditions were aerobic or anaerobic. The Tiaong soil has a large carbonate content (Table 1) that is sufficient to neutralize most of the acidity of HNO_3 . This would restrict the dissolution of Zn compared to the less-well buffered Bay soil. The pool of Zn extracted by 0.43 M HNO_3 therefore depends more on the soil pH buffer capacity than on its redox status.

Determination of E-values in aerobic soil

Electrolyte optimization. The *E*-values obtained for Bay soil (Figure 1a) were largely independent of the strength and type of electrolyte (mean and standard deviation: $10.6 \pm 0.1 \text{ mg kg}^{-1}$, coefficient of variation (CV) $<2.5\%$, $n = 9$). The values for Tiaong soil were slightly larger with DTPA than EDTA. In all cases, the concentrations of Zn extracted ($2\text{--}9 \text{ mg kg}^{-1}$) were smaller than the *E*-values by 22–75%. This confirms that EDTA and DTPA at the concentrations and L/S ratio used did not dissolve non-labile Zn. Among the three

electrolytes, 0.05 mM EDTA gave consistently smaller standard deviations, and so it was selected for further experiments.

Tracer acidification. Sterckeman *et al.* (2009) reported that an excess of acidified solution containing the isotopic tracer changed the soil-solution pH in carbonate-bearing soil. The addition of 0.3 $\mu\text{g } ^{67}\text{Zn g}^{-1}$ soil caused an average decrease in the pH of the aerobic Bay soil from 7.4 to 7.1 and from 8.0 to 7.6 in Tiaong soil. The decrease in pH did not exceed 0.5 pH units for any sample ($n = 9$). This isotopic enrichment level was not expected to dissolve non-labile Zn or disrupt the equilibrium in soil.

Tracer optimization. Although the amounts of isotope added varied by an order of magnitude, *E*-values were consistently in the 10–12 mg kg^{-1} range (Figure 1a). The smallest CV ($< 2\%$) was obtained with an amount of tracer equivalent to 25–75% of the labile ^{67}Zn that occurs naturally in the soil (Figure 1b). Even the smallest tracer additions (0.02 $\mu\text{g } ^{67}\text{Zn g}^{-1}$ soil) caused an analytically measurable response, although this was at the expense of a larger error. This is probably because of the weaker analytical resolution combined with increased error when handling amounts of an isotope that are too small. Over-application of the tracer ($> 0.3 \mu\text{g } ^{67}\text{Zn g}^{-1}$ soil) did not improve the accuracy and it might have disturbed the soil equilibrium, which the increase in the lability of Zn suggests (Figure 1a).

Measured E-values. The labile pool of Zn was $\approx 11 \text{ mg kg}^{-1}$ for all data ($n = 24$) for both soil types (Table 2), which is similar to that in other non-polluted agricultural soils (Ayoub *et al.*, 2003; Sinaj *et al.*, 2004; Nazif *et al.*, 2015). Only 9% and 14% of the total Zn in the Bay and Tiaong soils, respectively, is labile, which is similar to the values for polluted and unpolluted soil (10–33%) in the literature (Young *et al.*, 2000; Degryse *et al.*, 2004; Gabler *et al.*, 2007; Izquierdo *et al.*, 2013a).

Determination of E-values in anaerobic soil

Electrolyte optimization. The electrolyte strength and L/S ratio were adjusted for the anaerobic soil where less labile Zn was anticipated. Strengths of 0.2–1 mM EDTA and L/S = 6 l kg⁻¹ extracted sufficient Zn to make accurate measurements, i.e. with small standard deviations, without dissolving non-labile Zn (Figure 2). Both Bay and Tiaong soils contained about 2 mg kg⁻¹ labile Zn under reducing conditions, with good reproducibility. The *E*-values decreased only very slightly with increasing strength of the EDTA solution.

Measured E-values. An average *E*-value of 2.4 mg kg⁻¹ (Table 2) was obtained for both soil types. Flooding and induced reducing conditions depressed the lability of Zn by 80% compared with aerobic soil. Of the total Zn 2–3% only was labile in the anaerobic soil.

Comparison of E-values and extractable Zn

There is good agreement between 0.05 M EDTA extractable Zn and the *E*-values for both soil types regardless of their redox status (Table 2). Although there is no mechanistic basis for this relationship, it is likely that both methods access a similar pool of Zn i.e. non-silicate-bound soil phases. Figure 3 shows our data together with those from other soils used for rice cultivation in the Philippines (unpublished results) and uncontaminated alluvial soil from the UK (Izquierdo *et al.*, 2013a) with small concentrations of available Zn. Although our dataset is limited, it seems that a 0.05 M EDTA extraction could provide a simple non-isotopic estimate of the geochemically active Zn in soil where it is poorly available across a range of redox potentials. Gabler *et al.* (2007) also found good agreement between the two assays for aerobic soil and 0.025 M EDTA. The HNO₃-extractable Zn showed no relation with the *E*-values. The 0.005 M DTPA extraction underestimated the labile fraction of Zn, suggesting that there is Zn associated with carbonates that is still accessible for the isotopic exchange. Nazif *et al.* (2015) also reported the occurrence of labile Zn bound to carbonates in Zn deficient soil in Pakistan.

317 Isotopic tracer added to initially aerobic soil

318 Figure 4a–c shows rapid changes in soil pH and redox conditions, which reflects the
319 electrochemical changes in soil following submergence as anaerobic microbes use alternative
320 terminal electron acceptors for respiration following exhaustion of O₂ (Ponnamperuma,
321 1972). Within 4 days, E_h decreased to –183 mV, followed by a further slow decrease to –252
322 mV after 21 days. The soil pH decreased to a minimum of 6.3 after 4 days because of
323 accumulation of CO₂ from soil respiration; typically CO₂ pressures in the range 5–20 kPa
324 develop after submergence (Ponnamperuma, 1972). Because most reduction reactions
325 consume protons, the pH increased slowly after the minimum for the rest of the experiment.

326 The addition of ⁶⁷Zn tracer before submergence ensured thorough mixing of the
327 isotope with the soil as indicated by the small error bars in Figure 4a. However, the E -values
328 varied widely over time. Significant isotopic exchange takes place within 24 hours following
329 submergence, and some exchange would have occurred while the soil was still oxidized. One
330 might expect, therefore, that the E -value measured within the first 2 days would be similar to
331 that for the fully aerobic soil, or slightly smaller because of the rapid onset of immobilization
332 reactions associated with redox changes. However, the E -value increased from 11 mg kg^{–1} in
333 aerobic soil to 15 mg kg^{–1} within 4 days of incubation and peaked at between 4 and 7 days,
334 concomitant with a minimum in soil pH and a decrease in E_h to –200 mV (Figure 4c). A
335 possible explanation for this initial increase in E -value could be the reductive dissolution of
336 soil–solid phases, such as Fe and Mn oxides, and release of associated Zn into solution. This
337 is not reflected in the extractable Zn concentration, however, which decreased from 8 mg kg^{–1}
338 in the aerobic soil to 0.1 mg kg^{–1} within 7 days, indicating immobilization of Zn, not its
339 release. The E -values were negatively correlated with the extracted Zn measured in solution
340 over the first 4 days ($r = -0.94$, $P < 0.001$, $n = 23$). These observations suggest that Zn was

occluded in carbonate phases formed when anaerobic conditions developed after soil submergence, and this was driven by the accumulation of CO₂. After 7 days, the *E*-value decreased to 6 mg kg⁻¹ and showed little variation thereafter, which reflected changes in the pH and *E*_h.

Zinc immobilization seems to have progressed faster than isotopic exchange during the first 4 days of submergence and favoured the more readily available Zn, i.e. the soluble ⁶⁷Zn tracer. Therefore, there was preferential removal of ⁶⁷Zn from solution over ⁶⁶Zn during the first 4 days. The concentration of ⁶⁷Zn in the equilibrating solution relative to its concentration after addition of the tracer was 4% smaller than that of ⁶⁶Zn (Figure 5). This resulted in a change in the ⁶⁶Zn:⁶⁷Zn ratio towards natural ratios, which have larger apparent *E*-values. Smaller differences occurred with longer equilibration times as the isotopic exchange progressed. After 7 days there was no evidence of preferential ⁶⁷Zn fixation. Presumably by this stage a greater proportion of the labile metal was labelled and both natural and tracer isotopes precipitated more evenly.

Further evidence of immobilization of the isotopic tracer was found in the control soil cores extracted from tracer-free pots after 3 days of incubation (Figure 4a). The tracer was added when the fixation processes were fully operative and the redox potential was decreasing rapidly. Under these very unstable conditions there was little chance for the added isotope to equilibrate and exchange with the native isotopes before fixation, which resulted in large and unrealistic apparent *E*-values (up to 20 mg kg⁻¹). Precipitation or irreversible sorption or both occurs in hotspots, which are somewhat random and would account for large error bars. This is in sharp contrast with the *E*-value < 2 mg kg⁻¹ obtained for short-term equilibration of the control soil extracted and labelled after 18 days of incubation when the redox potential was stable and the conditions were not dissimilar to those of the pre-incubated soil. Unrealistically large *E*-values caused by tracer-derived artefacts (Hamon *et al.*, 2008)

have been reported for Zn in alkaline media (Tiller *et al.*, 1972; Sinaj *et al.*, 2004; Izquierdo *et al.*, 2013b) and attributed to the tracer undergoing irreversible sorption or precipitation.

Formation of zinc sulphide (ZnS) might be responsible for immobilization of Zn in submerged soil (Sajwan & Lindsay, 1986; Bostick *et al.*, 2001), and large concentrations (about 30 mg l⁻¹) of sulphide ions (S²⁻) have been found in anaerobic Bay soil solution. This is unusual because in most submerged soil the concentration of Fe²⁺ in solution is sufficient for S²⁻ to precipitate as amorphous ferrous sulphide (Kirk, 2004). Figure 4a shows marked changes in the soil–solution equilibria that are concomitant with increasing CO₂ in soil within the first few days of submergence. When CO₂ accumulates following submergence and with the pH buffered to near neutral, changes in the carbonate equilibria result in immobilization of Zn by occlusion or sorption. Past research with Tiaong soil (van Breemen *et al.*, 1980; Scharpenseel *et al.*, 1983) concluded that formation of (Ca,Mg,Zn)CO₃ solid solutions is responsible for the extreme insolubility of Zn in this soil when it is submerged. It also explains the observed association between Zn deficiency in rice and soil with large Mg:Ca ratios (van Breemen *et al.*, 1980).

Isotopic tracer added to initially anaerobic soil

The *E*-value changed during the experiment (Figure 4b); it shows a minor but steady increase from 2 to > 4 mg kg⁻¹ over time. The variability among laboratory replicates was greater than for soil labelled before flooding (CV < 17% against < 10%, *n* = 5). This could be attributed partly to poorer mixing of the isotope in reduced soil; the increase in the error bars after 5 days suggests other processes might also cause an uneven distribution of the tracer throughout the soil as time passes.

The extractable Zn concentrations were < 0.1 mg kg⁻¹ throughout the experiment; Figure 6 shows a small decrease after 21 days. The extractable Zn, pH and *E*_h remained

almost constant during incubation (Figures 4b and 6), and it would be reasonable to assume that the increasing trend in E -value reflects the longer equilibration times which enables migration of the added isotope into less labile pools in soil. It is well known that the tracer equilibration time affects the E -value (Oliver *et al.*, 2006), which shows typically a very rapid increase that produces a distinguishable labile pool followed by a slow rate of increase (asymptotic) or a plateau (Young *et al.*, 2005). Three days are acknowledged to be typical for the equilibration time to obtain a good estimate of the labile pool (Ayoub *et al.*, 2003; Young *et al.*, 2005; Hamon *et al.*, 2008), although slow isotopic exchange by lattice diffusion of the introduced tracer can occur for years. Slow diffusion might occur in the soil samples studied, but longer soil-tracer equilibration times might not explain the fast rates of increase in E -value after 10 days and greater dispersion in the measurements. These are unlikely to result from a real acceleration in the isotopic exchange rate because there were no corresponding step changes in soil pH or E_h . The increase in apparent E -values combined with greater variability and very small extractable Zn concentration would suggest irreversible fixation of the isotopic tracer; the larger error bars reflect the variation in which this occurs throughout the soil profile. This is supported by the results from tracer-free control soil sampled after 3 days and 18 days of incubation and assayed for short-term equilibration with the isotope. The decrease in E -values from the sample of day 3 (2.4 mg kg⁻¹) to that of day 18 (1.4 mg kg⁻¹) combined with a decrease in the Zn extracted after 3 weeks (Figure 6) suggests that mechanisms of Zn immobilization similar to those reported for soil labelled before flooding would still be active, but at much slower rates because of more stable redox conditions.

Implications for measurement of E-values

Our results suggest that techniques of isotopic dilution have potential limitations for estimating the lability of Zn in submerged soil when the redox status changes rapidly because

of the lack of equilibrium. Redox conditions should be stable before the addition of the isotope tracer to soil because it must remain freely exchangeable at all times. This appears to invalidate the use of these techniques in the transition between aerobic and anaerobic conditions in soil where Zn precipitation is likely.

Even in strongly reduced soil and apparently stable redox conditions, Zn immobilization may continue and interfere with the isotopic exchange. Nevertheless, this affected the *E*-values much less than in the aerobic-anaerobic transition. Although reasonably long tracer–soil contact times are required for sufficient isotopic exchange to occur, particularly in unstirred systems, prolonged exposure of the isotope to anaerobic conditions might give unreliable results. For example, this might complicate comparisons of *E*-values with *L*-values (pool of Zn that an organism can draw from soil, as described by Hamon *et al.*, 2008) obtained by growing plants in labelled soil to measure the mobilization of non-labile Zn in the rhizosphere. In summary, interpretation of the results of isotopic dilution research in geochemical environments where metal immobilization is a dominant process can be misleading.

Conclusions

Zinc deficiency is a widespread micronutrient disorder in rice, therefore, there is need to understand the mechanisms that control phytoavailability of this element in soil used for rice cultivation. We observed rapid changes in pH and redox conditions following submergence. The labile Zn was strongly and rapidly immobilized within the first 5 days of submergence. Our observations indicate that the fixation of Zn is largely controlled by the accumulation of CO₂ because of soil respiration and that it occurs primarily at the early stages of submergence. This is likely to cause a change in the carbonate equilibria and result in the

occlusion or sorption of Zn, which accounts for the Zn deficiency observed in soil used for rice cultivation.

When soil was labelled with a tracer before flooding, the rapid transfer of labile Zn to the non-labile pool on submergence appeared to progress faster than isotopic exchange. This caused the removal of some tracer from the soil-solution equilibria, with the result that the *E*-values were inaccurate. The addition of isotopic tracer to soil where anaerobic conditions are already stabilized proved to be more reliable. We found that precipitation processes of Zn may continue and interfere with the isotopic exchange, although to a lesser extent. Our results demonstrate that isotopic dilution assays may have some limitations when the element of interest is poorly available and the geochemical environment is favourable to fixation. From our observations, we recommend avoidance of aerobic–anaerobic transitional phases and long exposures (>1 week) of the isotopic tracer to determine metal lability in anaerobic systems where precipitation of the element of interest is likely.

We tested several single batch extractions to assess their suitability as a non-isotopic estimate of the geochemically reactive Zn. Although DTPA and HNO₃ failed to provide a reasonable estimate of the *E*-value, our results of *E*-values with 0.05 M EDTA extraction were in accord regardless of the redox status.

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Government's Department for International Development (DFID) and the Bill & Melinda Gates Foundation.

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562 **Table 1** Properties of the soil types studied

	Bay	Tiaong
Clay/%	49.9	42.2
Silt/%	43.3	39.2
pH (1:5 H ₂ O)	7.3	8.5
CEC/cmole kg ⁻¹	10.6	9.0
Organic C/g kg ⁻¹	54.3	72.8
Carbonate/g kg ⁻¹	1.6	95.9
Total Zn/mg kg ⁻¹	114.3	83.2

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Table 2 Extractable Zn and Zn *E*-values in the soil types studied for aerobic and anaerobic ($E_h = -220\text{mV}$) conditions.

Assay	Bay soil		Tiaong soil	
	Aerobic	Anaerobic	Aerobic	Anaerobic
0.05 M EDTA	14.7 ± 0.9	3.9 ± 0.2	11.6 ± 0.3	1.6 ± 0.1
0.005 M DTPA	7.8 ± 0.2	0.031 ± 0.004	8.5 ± 1.0	0.07 ± 0.01
0.43 M HNO_3	28.3 ± 1.9	34.6 ± 0.6	0.02 ± 0.01	0.5 ± 0.2
<i>E</i> -value	10.9 ± 0.7	2.4 ± 0.3	11.5 ± 0.7	2.3 ± 0.5

Data (mg kg^{-1}) are means $\pm$ 1 standard deviation (SD) ($n = 4$ for extractions; $n = 24$ and 13 for *E*-values in aerobic and anaerobic soil, respectively)

576 **FIGURE CAPTIONS**

577 **Figure 1** Effect of electrolyte type and concentration (mM), ^{67}Zn tracer addition (mg kg^{-1})
578 on: (a) E -values (mg kg^{-1}) and (b) on the coefficients of variation for three laboratory
579 replicates in aerobic soil.

580 **Figure 2** Effect of electrolyte concentration (mM) on E -values in anaerobic soil. The
581 concentrations of Zn extracted are also shown. Data (mg kg^{-1}) are means $\pm$ 1 standard
582 deviation (SD) (error bars).

583 **Figure 3** Comparison of E -values and 0.05 M EDTA-extractable Zn in Bay and Tiaong
584 aerobic and anaerobic soils. For comparison, results for other lowland rice soils from the
585 Philippines (unpublished results) and relatively uncontaminated alluvial soil from UK
586 (Izquierdo *et al.*, 2013a) are plotted.

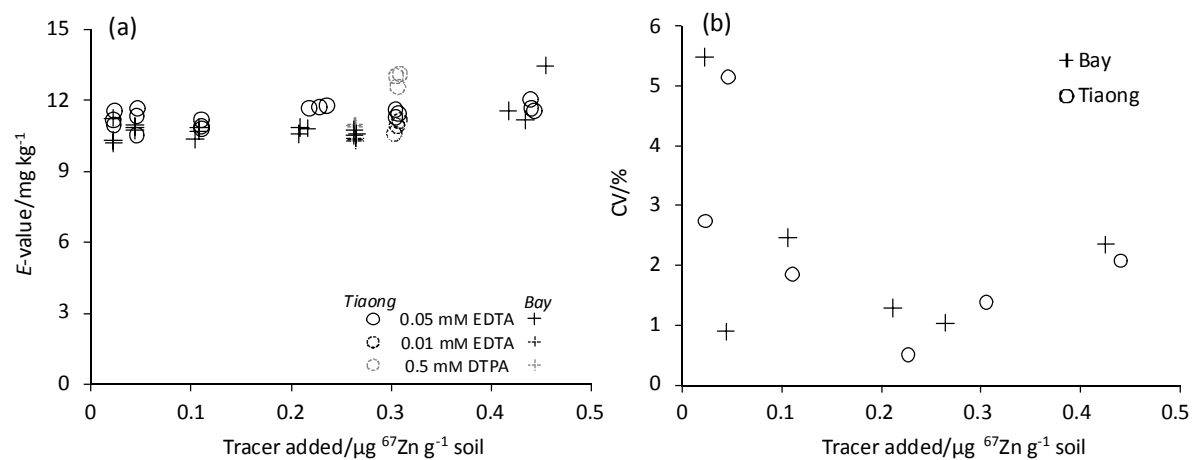
587 **Figure 4** Changes over time in soil redox potential, E -values and Zn extracted (in the
588 equilibrating 0.5 mM EDTA solution) following addition of the isotope tracer to Bay soil that
589 was either (a) dry and then submerged immediately before adding the ^{67}Zn tracer (aerobic
590 soil) or (b) submerged for 8 weeks before adding the tracer (anaerobic soil). Values at
591 incubation time = 0 days were measured in aerobic soil. Data (mg kg^{-1}) are means $\pm$ 1
592 standard deviation (SD) (error bars). Incubation times for the long-term isotopic dilution
593 assays are determined by combining the sampling time (days) followed by 1 day of extraction
594 with 0.5mM EDTA. For short-term isotopic dilution assays, incubation times combine the
595 sampling time (days) followed by 2 days of equilibration and 1 day of extraction with EDTA.

596 **Figure 5** Changes over time in the concentrations of ^{66}Zn and ^{67}Zn relative to their initial
597 values, and their ratios in equilibrating solutions following addition of the ^{67}Zn tracer to Bay
598 soil at the same time as flooding.

Figure 6 Changes over time in Zn extracted in 0.5 mM EDTA in Bay soil submerged for 8 weeks before ^{67}Zn tracer addition (data from Figure 5b). Data (mg kg^{-1}) are means $\pm$ 1 standard deviation (SD) (error bars).

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FIGURE 1

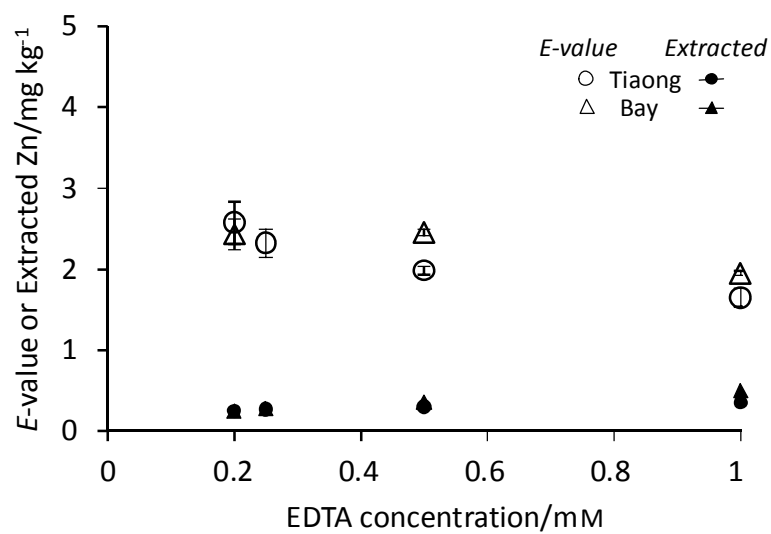


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FIGURE 2



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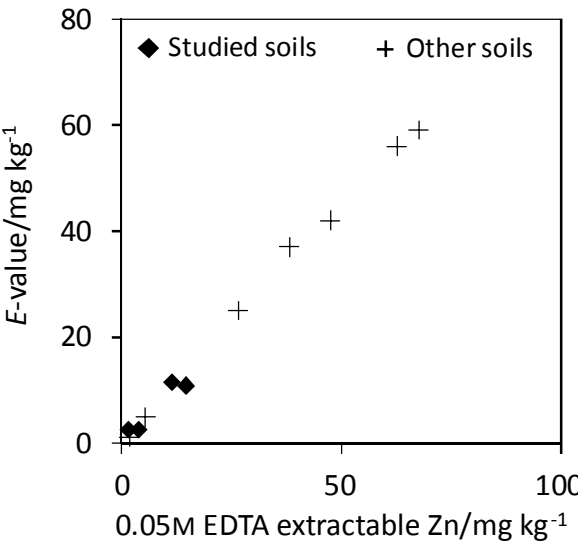
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FIGURE 3



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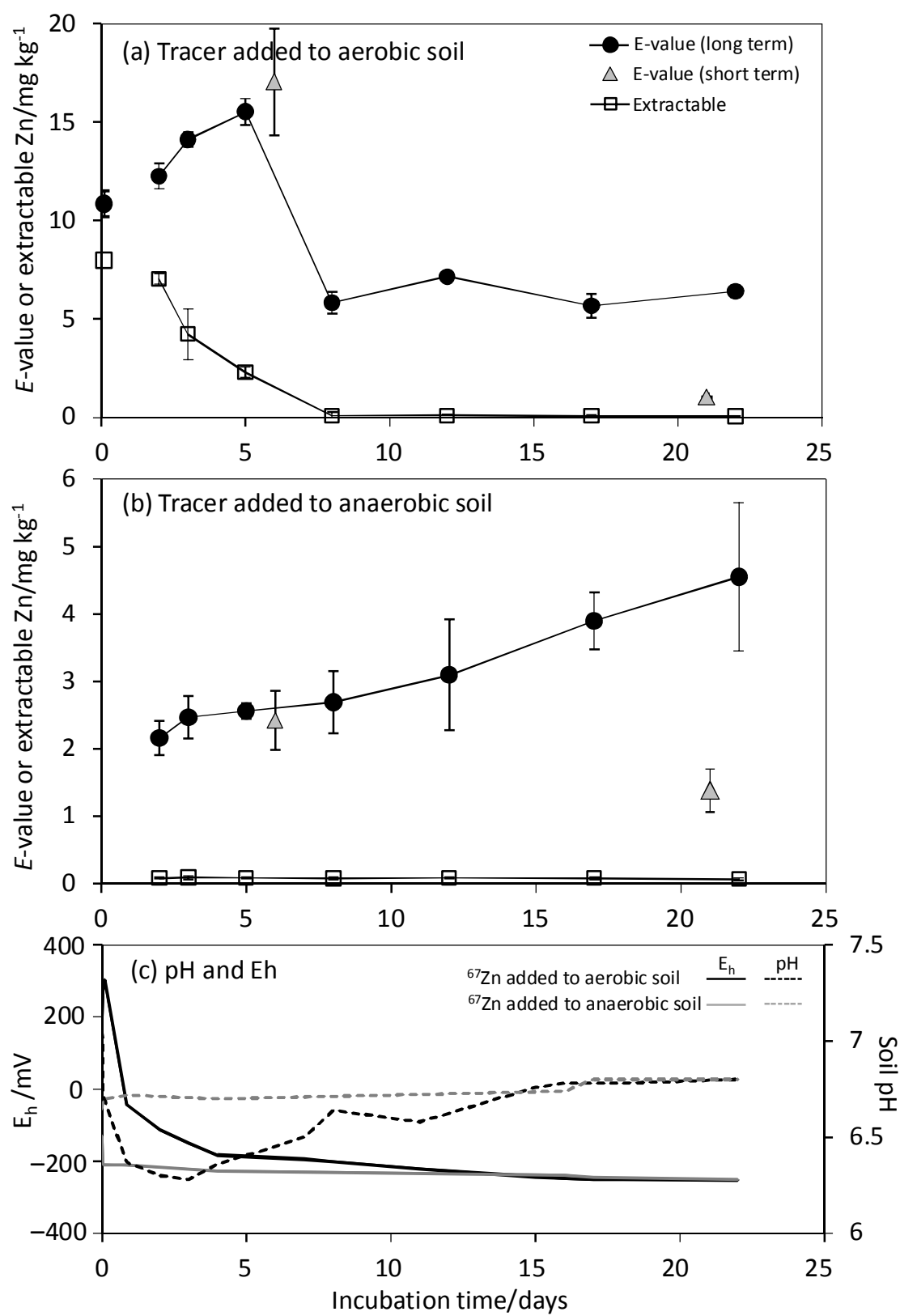
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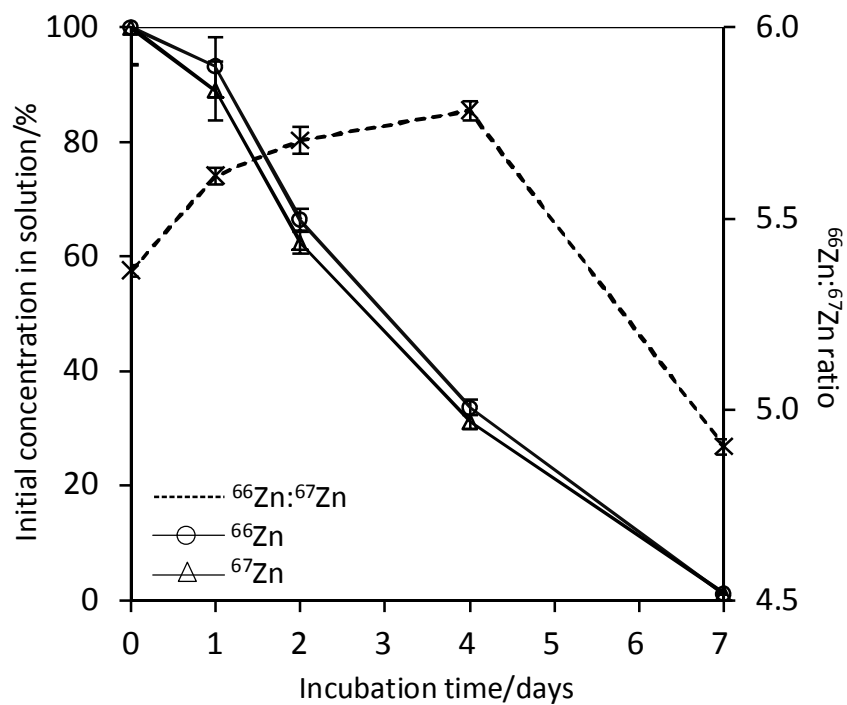
FIGURE 4



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FIGURE 5

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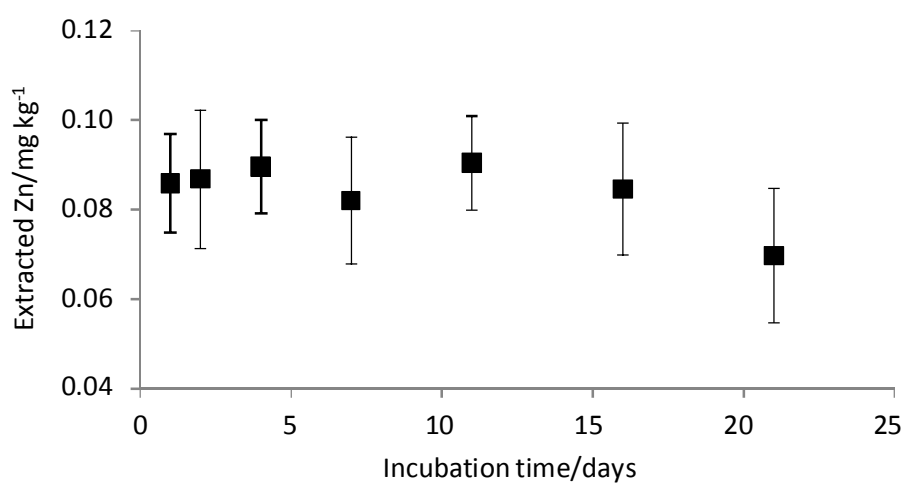


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FIGURE 6



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Measurement of isotopically-exchangeable Zn in Zn-deficient paddy soil

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