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

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16 **Summary**

17 The changes in soil chemistry following submergence of a soil for rice production result in
18 zinc (Zn) being immobilized in very insoluble forms. Consequently, Zn deficiency is
19 widespread in rice crops and in human populations that subsist on rice. We explored the use
20 of stable isotopic dilution assays for assessing Zn dynamics in submerged rice soil with two
21 types of strongly Zn-deficient rice soil from the Philippines. We optimized the isotope
22 enrichment, electrolyte and equilibration time to measure isotopically-exchangeable Zn (*E*-
23 values) without changing redox conditions. Available Zn was rapidly and strongly
24 immobilized following submergence, controlled by CO_2 accumulation. Addition of the
25 isotopic tracer before submergence produced unreliable *E*-values because irreversible
26 immobilization of the tracer progressed faster than isotopic exchange. Addition of the tracer
27 to already reduced soil produced stable *E*-values for tracer–soil contact of up to one week.
28 Longer periods produced unreliable *E*-values because of continuing irreversible fixation of
29 the tracer. We discuss the implications for applications of isotopic dilution methods to
30 measure trace-element dynamics in submerged soil.

31

32 **Introduction**

33 Zinc deficiency is the most widespread micronutrient disorder in rice plants; it affects up to
34 50% of the soil in lowland rice production globally (Dobermann & Fairhurst, 2000) and
35 results in reduced growth, delayed maturity and diminished yields (van Breemen *et al.*,
36 1980). Zinc is also often deficient in human populations with rice-based diets (IRRI, 2006).
37 Consequently, there are currently major international efforts to breed varieties of rice with a
38 tolerance to Zn deficiency and with large grain Zn contents for human nutrition (IRRI, 2006).

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

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

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

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

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

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

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

87

88 **Materials and methods**

89 *Soil types and isotopic tracer solutions*

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

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

108 Enriched ^{67}Zn (89.6% ^{67}Zn) was obtained from ISOFLEX and dissolved in 2 M HNO_3
109 to obtain a solution of ~820 mg ^{67}Zn l^{-1}. From this stock solution, a range of fresh solutions
110 with measured concentrations of between 0.38–1.90 mg ^{67}Zn l^{-1} and ^{67}Zn abundances of
111 86.0–86.6% were produced for each experiment.

112 *Preparation of anaerobic soil*

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

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

126
127 *Extractable Zn*

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

141
142 *Analyses of solutions*

143 The total Zn in the extracts was determined with a quadrupole ICP-MS Perkin Elmer Elan
144 9000, Boston, MA, USA). Response suppression or enhancement effects were corrected with
145 rhodium as the internal standard. The calibration standards were analysed every 12 samples
146 to check for possible drift during analysis and the data were corrected for reagent
147 contribution.

148 To determine the isotopic composition of extracts, isotopic measurements were made
149 with 10 replicates per sample, 1000 sweeps per integration and dwell times of 2, 2, 3, 2 and 4
150 mseconds for ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn and ^{70}Zn, respectively. The residual standard deviations
151 (RSD) of the ^{66}Zn:^{67}Zn ratios were typically < 0.5%. Correction for mass bias was derived
152 from Zn standard solutions assumed to have fixed natural isotopic ratios (de Laeter *et al.*,
153 2003). The standard solutions were analysed every 3–6 samples and diluted to give similar
154 concentrations (80–115%) to all the samples.

155
156 *Determination of E-values*

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

160 *Aerobic soil.* Unbuffered salt solutions such as calcium chloride ($CaCl_2$) or calcium nitrate
161 ($Ca(NO_3)_2$) are used widely in isotopic dilution assays, but they might not dissolve sufficient
162 Zn for analytical purposes in soil where Zn is strongly bound (Izquierdo *et al.*, 2013a).
163 Chelating agents such as EDTA are better suited to such soil, therefore we investigated the
164 efficacy of 0.01 mM, 0.05 mM EDTA and 0.1 mM DTPA (Lindsay & Norvell, 1978; Degryse
165 *et al.*, 2004; Atkinson *et al.*, 2011; Izquierdo *et al.*, 2013a). Portions of 2 ± 0.03 g of soil were
166 shaken with 20 ml of electrolyte for 3 days, and then the equivalent to 0.3 μg ^{67}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 μg ^{67}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

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

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

205

206 *Isotopic exchange kinetics.* Two replicate sets of laboratory pots of submerged Bay soil were
207 prepared by placing 200 g soil mixed with 1% wt rice straw in polypropylene containers and
208 flooded with deionized water to give 2-cm depth of standing water as above. In 10 pots, a
209 solution containing 0.1 μg ^{67}Zn g^{-1} soil was added after 2 hours of flooding, and in another 10
210 pots the isotopic tracer was added to the soil after 8 weeks of incubation at 26°C by which
211 time the E_h had stabilized at –220 mV and pH at 6.7. The isotope was added as 10 ml solution
212 applied in the standing water and mixed thoroughly into the soil by stirring with a spatula.
213 Replicate laboratory tracer-free pots were also prepared as control samples for short-term
214 isotopic exchange experiments. The pots were then re-incubated in a water bath at 26°C. Soil
215 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 N_2 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 ^{67}Zn 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):

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

where $M_{\text{Zn-nat}}$ is the average atomic mass of Zn, W is the weight of dry soil (kg), C_{tracer} is the ^{67}Zn concentration (mg kg^{-1}) in the solution containing the tracer, M_{tracer} is the mass of solution containing the ^{67}Zn tracer added to the soil (g), $M_{\text{Zn-nat}}$ and $M_{\text{Zn-tracer}}$ 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 ^{67}Zn:^{66}Zn 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

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

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

257

258 *Determination of E-values in aerobic soil*

259 *Electrolyte optimization.* The *E*-values obtained for Bay soil (Figure 1a) were largely
260 independent of the strength and type of electrolyte (mean and standard deviation: 10.6 ± 0.1
261 mg kg^{-1}, coefficient of variation (CV) <2.5%, n = 9). The values for Tiaong soil were slightly
262 larger with DTPA than EDTA. In all cases, the concentrations of Zn extracted (2–9 mg kg^{-1})
263 were smaller than the *E*-values by 22–75%. This confirms that EDTA and DTPA at the
264 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 μg ^{67}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 μg ^{67}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 μg ^{67}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 ≈ 11 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

290 *Electrolyte optimization*. The electrolyte strength and L/S ratio were adjusted for the
291 anaerobic soil where less labile Zn was anticipated. Strengths of 0.2–1 mM EDTA and L/S =
292 6 l kg^{-1} extracted sufficient Zn to make accurate measurements, i.e. with small standard
293 deviations, without dissolving non-labile Zn (Figure 2). Both Bay and Tiaong soils contained
294 about 2 mg kg^{-1} labile Zn under reducing conditions, with good reproducibility. The *E*-values
295 decreased only very slightly with increasing strength of the EDTA solution.
296 *Measured E-values*. An average *E*-value of 2.4 mg kg^{-1} (Table 2) was obtained for both soil
297 types. Flooding and induced reducing conditions depressed the lability of Zn by 80%
298 compared with aerobic soil. Of the total Zn 2–3% only was labile in the anaerobic soil.

299
300 *Comparison of E-values and extractable Zn*

301 There is good agreement between 0.05 M EDTA extractable Zn and the *E*-values for both soil
302 types regardless of their redox status (Table 2). Although there is no mechanistic basis for
303 this relationship, it is likely that both methods access a similar pool of Zn i.e. non-silicate-
304 bound soil phases. Figure 3 shows our data together with those from other soils used for rice
305 cultivation in the Philippines (unpublished results) and uncontaminated alluvial soil from the
306 UK (Izquierdo *et al.*, 2013a) with small concentrations of available Zn. Although our dataset
307 is limited, it seems that a 0.05 M EDTA extraction could provide a simple non-isotopic
308 estimate of the geochemically active Zn in soil where it is poorly available across a range of
309 redox potentials. Gabler *et al.* (2007) also found good agreement between the two assays for
310 aerobic soil and 0.025 M EDTA. The HNO_3-extractable Zn showed no relation with the *E*-
311 values. The 0.005 M DTPA extraction underestimated the labile fraction of Zn, suggesting
312 that there is Zn associated with carbonates that is still accessible for the isotopic exchange.
313 Nazif *et al.* (2015) also reported the occurrence of labile Zn bound to carbonates in Zn
314 deficient soil in Pakistan.

315

316 *Isotope exchange kinetics*

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 O_2 (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 CO_2 from soil respiration; typically CO_2 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 ^{67}Zn 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 CO_2. 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 ^{67}Zn tracer. Therefore, there was preferential removal of ^{67}Zn from solution over ^{66}Zn during the first 4 days. The concentration of ^{67}Zn in the equilibrating solution relative to its concentration after addition of the tracer was 4% smaller than that of ^{66}Zn (Figure 5). This resulted in a change in the ^{66}Zn:^{67}Zn 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 ^{67}Zn 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)

366 have been reported for Zn in alkaline media (Tiller *et al.*, 1972; Sinaj *et al.*, 2004; Izquierdo
367 *et al.*, 2013b) and attributed to the tracer undergoing irreversible sorption or precipitation.
368 Formation of zinc sulphide (ZnS) might be responsible for immobilization of Zn in
369 submerged soil (Sajwan & Lindsay, 1986; Bostick *et al.*, 2001), and large concentrations
370 (about 30 mg l^{-1}) of sulphide ions (S^{2-}) have been found in anaerobic Bay soil solution. This
371 is unusual because in most submerged soil the concentration of Fe^{2+} in solution is sufficient
372 for S^{2-} to precipitate as amorphous ferrous sulphide (Kirk, 2004). Figure 4a shows marked
373 changes in the soil–solution equilibria that are concomitant with increasing CO_2 in soil within
374 the first few days of submergence. When CO_2 accumulates following submergence and with
375 the pH buffered to near neutral, changes in the carbonate equilibria result in immobilization
376 of Zn by occlusion or sorption. Past research with Tiaong soil (van Breemen *et al.*, 1980;
377 Scharpenseel *et al.*, 1983) concluded that formation of $(Ca,Mg,Zn)CO_3$ solid solutions is
378 responsible for the extreme insolubility of Zn in this soil when it is submerged. It also
379 explains the observed association between Zn deficiency in rice and soil with large Mg:Ca
380 ratios (van Breemen *et al.*, 1980).
381
382 Isotopic tracer added to initially anaerobic soil
383 The *E*-value changed during the experiment (Figure 4b); it shows a minor but steady increase
384 from 2 to > 4 mg kg^{-1} over time. The variability among laboratory replicates was greater than
385 for soil labelled before flooding (CV < 17% against < 10%, n = 5). This could be attributed
386 partly to poorer mixing of the isotope in reduced soil; the increase in the error bars after 5
387 days suggests other processes might also cause an uneven distribution of the tracer
388 throughout the soil as time passes.
389 The extractable Zn concentrations were < 0.1 mg kg^{-1} throughout the experiment;
390 Figure 6 shows a small decrease after 21 days. The extractable Zn, pH and E_h remained

391 almost constant during incubation (Figures 4b and 6), and it would be reasonable to assume
392 that the increasing trend in *E*-value reflects the longer equilibration times which enables
393 migration of the added isotope into less labile pools in soil. It is well known that the tracer
394 equilibration time affects the *E*-value (Oliver *et al.*, 2006), which shows typically a very rapid
395 increase that produces a distinguishable labile pool followed by a slow rate of increase
396 (asymptotic) or a plateau (Young *et al.*, 2005). Three days are acknowledged to be typical for
397 the equilibration time to obtain a good estimate of the labile pool (Ayoub *et al.*, 2003; Young
398 *et al.*, 2005; Hamon *et al.*, 2008), although slow isotopic exchange by lattice diffusion of the
399 introduced tracer can occur for years. Slow diffusion might occur in the soil samples studied,
400 but longer soil-tracer equilibration times might not explain the fast rates of increase in *E*-
401 value after 10 days and greater dispersion in the measurements. These are unlikely to result
402 from a real acceleration in the isotopic exchange rate because there were no corresponding
403 step changes in soil pH or E_h. The increase in apparent *E*-values combined with greater
404 variability and very small extractable Zn concentration would suggest irreversible fixation of
405 the isotopic tracer; the larger error bars reflect the variation in which this occurs throughout
406 the soil profile. This is supported by the results from tracer-free control soil sampled after 3
407 days and 18 days of incubation and assayed for short-term equilibration with the isotope. The
408 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})
409 combined with a decrease in the Zn extracted after 3 weeks (Figure 6) suggests that
410 mechanisms of Zn immobilization similar to those reported for soil labelled before flooding
411 would still be active, but at much slower rates because of more stable redox conditions.
412
413 *Implications for measurement of* E-*values*
414 Our results suggest that techniques of isotopic dilution have potential limitations for
415 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 CO_2 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

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

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

453 We tested several single batch extractions to assess their suitability as a non-isotopic
454 estimate of the geochemically reactive Zn. Although DTPA and HNO_3 failed to provide a
455 reasonable estimate of the *E*-value, our results of *E*-values with 0.05 M EDTA extraction
456 were in accord regardless of the redox status.

457

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466

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559 sequential extraction and isotopic dilution. *Soil Use and Management*, **21**, 450–458.
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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_2O)	7.3	8.5
CEC/$cmol_c$ 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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566 **Table 2** Extractable Zn and Zn *E*-values in the soil types studied for aerobic and anaerobic (E_h = –
567 220mV) conditions.
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	Bay soil		Tiaong soil	
Assay	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
E-value	10.9 ± 0.7	2.4 ± 0.3	11.5 ± 0.7	2.3 ± 0.5

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

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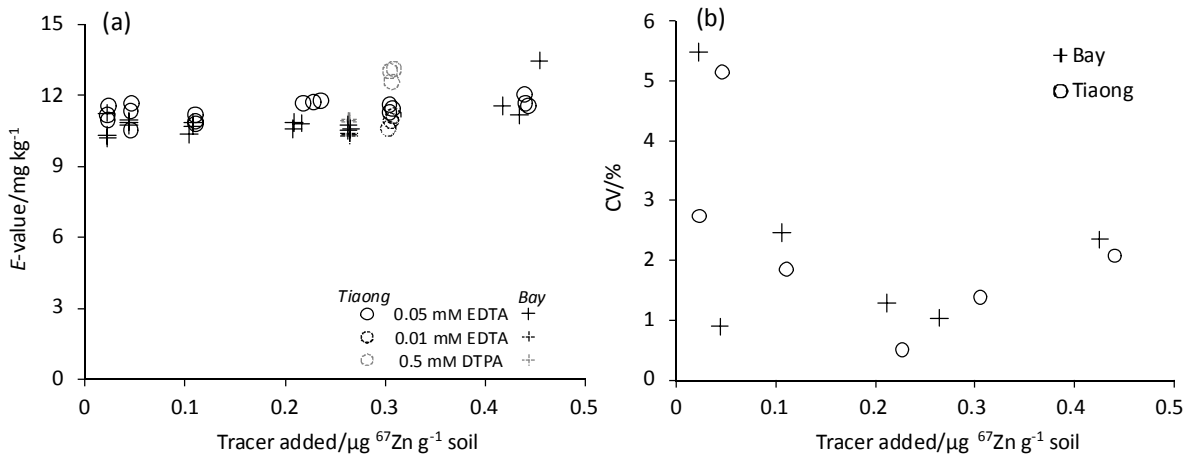
618 FIGURE 1



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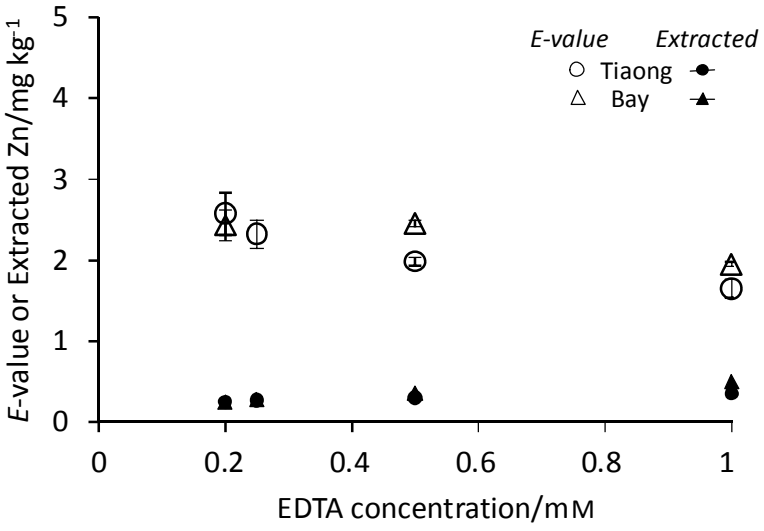
621 FIGURE 2



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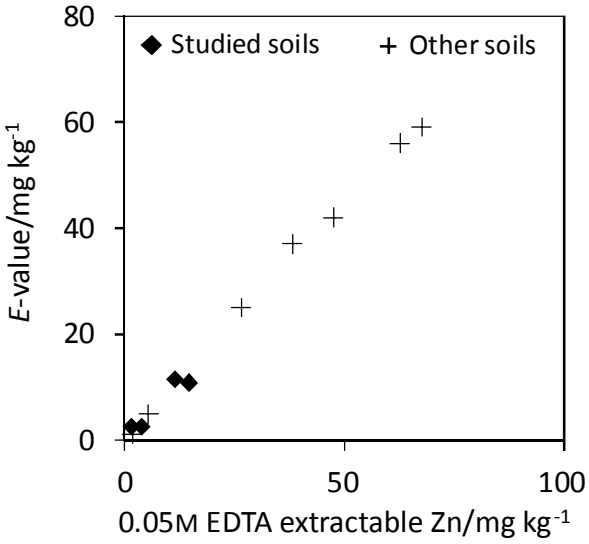
627 FIGURE 3



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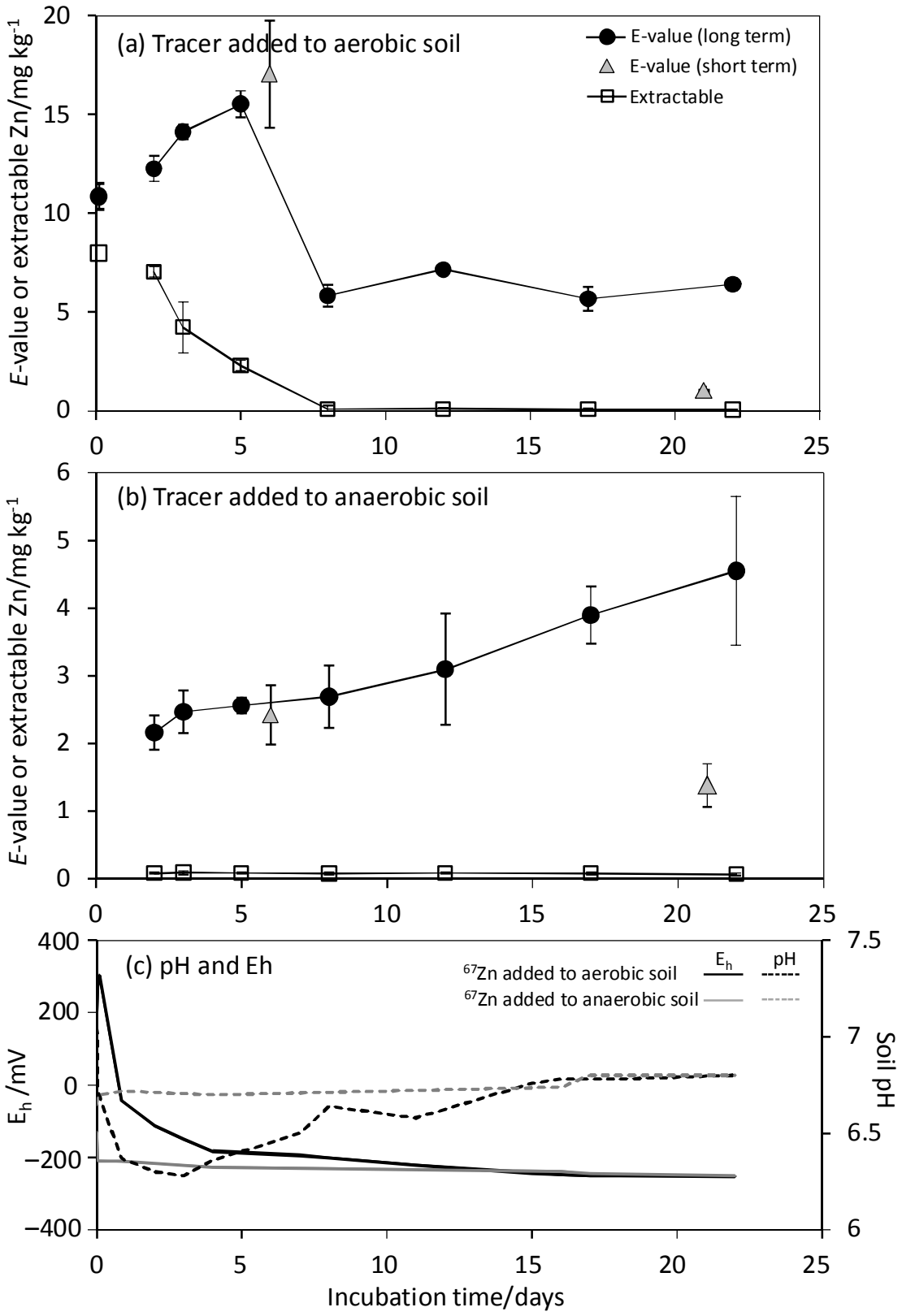

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643 FIGURE 5

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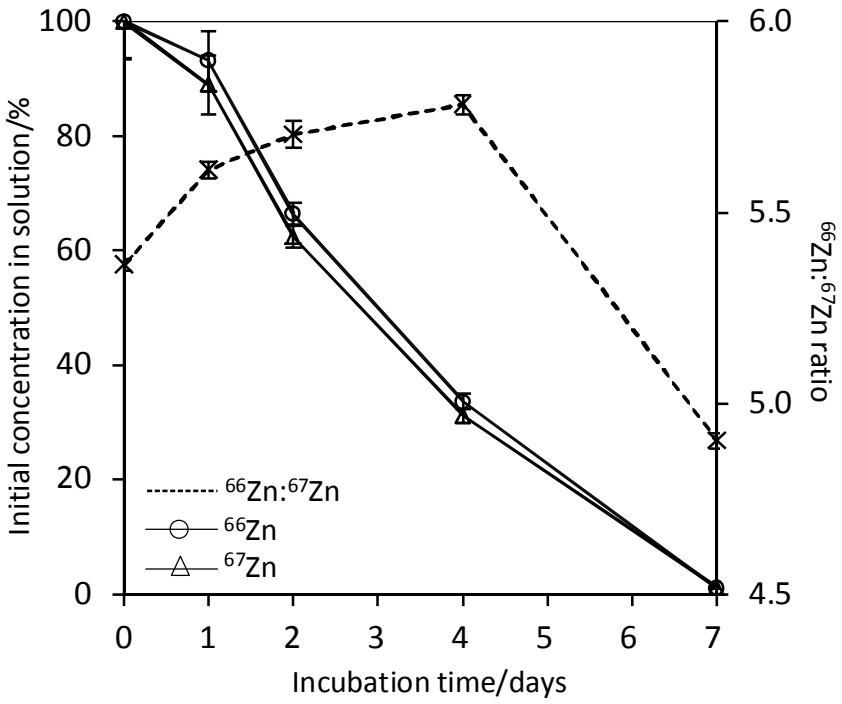

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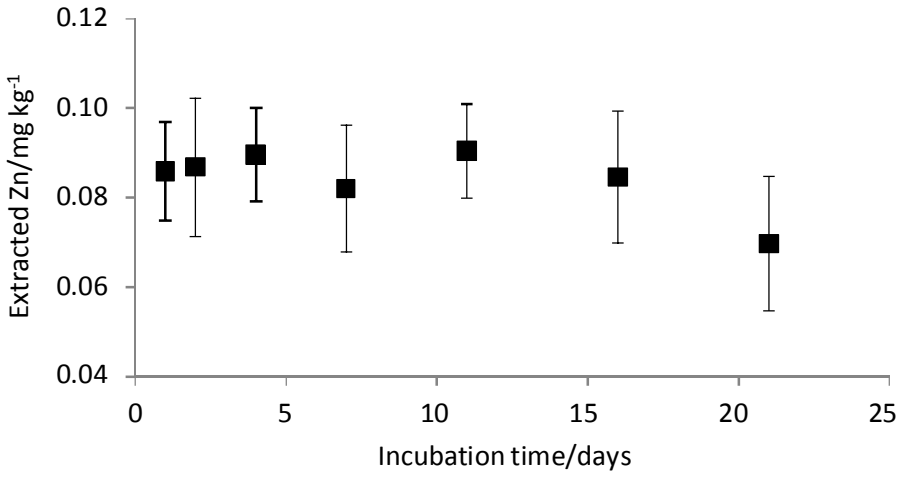
647 FIGURE 6



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

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