Title: Iron and zinc isotope fractionation during uptake and translocation in rice (Oryza sativa) grown in oxic and anoxic soils

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Abstract: Stable isotope signatures are emerging quickly as a powerful technique to study metal uptake and translocation in plants. Fundamental to this development is a thorough understanding of the processes that lead to isotope fractionation under differing environmental conditions. In this study, we investigated zinc (Zn) and iron (Fe) isotope fractionation in rice grown to maturity in anaerobic and aerobic soils under greenhouse conditions. The overall Zn isotope fractionation between the soil and above-ground plant material was negligible in aerobic soil but significant in anaerobic soil with isotopically lighter Zn in the rice plant. The observed range of fractionation was in line with previously determined fractionations of Zn in rice grown in hydroponic solutions (Weiss et al., 2005, New Phytol. 165, 703-710) and submerged soils (Arnold et al, 2010, Plant Cell Environ. 33, 370-381) and emphasizes the effect of taking up different chemical forms of Zn, most likely free and organically complexed Zn, on the isotopic signature found in the plant. The Zn in the grain was isotopically lighter than in the rest of the above-ground plant material grown in aerobic and anaerobic soils alike. This demonstrates that in the course of the grain loading and during translocation within the plant important biochemical and/or biophysical processes occur. The isotope fractionation observed in the grains would be consistent with a unidirectional controlled transport from shoot to grain fractionation with a fractionation factor of $\alpha \approx 0.9994$. Iron isotopes showed an isotopic lighter signature in shoot and in the leach of both soil environments alike. The negative direction of isotopic fractionation is consistent with possible changes in redox state of the Fe during uptake and translocation. The isotope fractionation pattern between shoots and grain material are different for Zn and Fe which finally suggests that different mechanisms operate during translocation and grain loading in rice for these two key micronutrients.
Iron and zinc isotope fractionation during uptake and translocation in rice (*Oryza sativa*) grown in oxic and anoxic soils

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Abstract

Stable isotope fractionation is emerging quickly as a powerful novel technique to study metal uptake and translocation in plants. Fundamental to this development is a thorough understanding of the processes that lead to isotope fractionation under differing environmental conditions. In this study, we investigated Zn and Fe isotope fractionation in rice grown to maturity in anaerobic and aerobic soils under greenhouse conditions. The overall Zn isotope fractionation between the soil and above-ground plant material was negligible in aerobic soil but significant in anaerobic soil with isotopically lighter Zn in the rice plant. The observed range of fractionation was in line with previously determined fractionations of Zn in rice grown in hydroponic solutions (Weiss et al., 2005, *New Phytol*. 165, 703-710) and submerged soils (Arnold T et al., 2010, *Plant Cell Environ*. 33, 370-381) and emphasizes the effect of taking up different chemical forms of Zn, most likely free and organically complexed Zn. The Zn in the grain was isotopically lighter than in the rest of the above-ground plant in rice grown in aerobic and anaerobic soils alike. This suggests that in the course of the grain-loading and during translocation within the plant important biochemical and/or biophysical processes occur. The isotope fractionation observed in the grains would be consistent with a unidirectional controlled transport from shoot to grain fractionation with a fractionation factor of $\alpha \approx 0.9994$. Iron isotopes showed an isotopic lighter signature in shoot and grain compared to the bulk soil or the leachate in aerobic and anaerobic soils alike. The negative direction of isotopic fractionation is consistent with possible changes in redox state of the Fe during uptake and translocation. The isotope fractionation pattern between shoots and grain material are different for Zn and Fe which finally suggests that different mechanisms operate during translocation and grain loading in rice for these two key micronutrients.

1 Introduction

Soils deficient in zinc (Zn) and iron (Fe) constrain rice (*Oryza sativa*) production in large parts of the world (Dobermann and Fairhurst 2000) and deficiencies of Zn and Fe in human populations with rice-based diets causes major health problems for millions of people (Graham, 2007). Identifying nutrient efficient rice lines, applying appropriate breeding techniques, and under-
standing the underlying mechanisms of Zn and Fe efficiency, are important for tackling this large societal challenge (Wissuwa et al., 2008).

Recent work demonstrated significant isotope fractionation during uptake and translocation of trace metals including Fe, Cu and Zn from soils to plants in a wide variety of plant species and this suggests that natural stable isotope compositions provide a powerful technique to study the chemical and biological processes that control micronutrient movement from soils into plants (Alvarez-Fernandez et al., 2014). Isotopic fractionations have been found to differ between plant species and between genotypes of the same species, as well as with soil and nutritional conditions (Arnold et al., 2010a; Aucour et al., 2011; Deng et al., 2014; Tang et al., 2012; Weiss et al., 2005).

Work on the isotopic fractionation during Zn uptake in hydroponic cultures showed that plant shoots are enriched in $^{64}$Zn relative to $^{66}$Zn in comparison to the source, consistent with a uni-directional process during transport of free Zn$^{2+}$ across cell membranes (Aucour et al., 2011; Deng et al., 2014; Weiss et al., 2005). Plants grown in natural soils, however, show negligible or slight heavy isotope enrichment in the shoots (Arnold et al., 2010a; Tang et al., 2012; Viers et al., 2007). Arnold and co-workers suggested that uptake of a Zn(II)-phytosiderophore (PS-Zn) complex could account for an observed heavy isotopic fractionation in Zn uptake by rice (Arnold et al., 2010a). Likewise Smolders and co-workers explained heavy isotopic fractionation during Zn uptake by tomato plants in resin-buffered hydroponics by the uptake of a PS-Zn complex (Smolders et al., 2013). Investigations of Zn complexation by organic molecules using experiments in the laboratory (Jouvin et al., 2009) and ab initio calculations (Fujii and Albarède, 2012) support this model. Few studies determined the stable isotope fractionation of Zn during translocation in higher plants but enrichment of light isotopes in leaves with increasing distance from the root (Caldeias et al., 2011; Moynier et al., 2009; von Blankenburg et al., 2009) and during the translocation from root to shoot (Caldeias et al., 2011; Jouvin et al., 2012; Tang et al., 2012) has been reported.

To date, Fe isotope fractionation has not been studied in rice but a significant body of work exists for graminacae (Guelke and von Blankenburg, 2007; Kiczka et al., 2010b; von Blankenburg et al., 2009). The observed range of isotope fractionation is around 2.25 ‰ per atomic mass unit (amu). Initial pot studies found enrichment of light Fe during plant uptake in strategy I plants as opposed to enrichment of heavy isotopes in strategy II (Guelke and von Blankenburg, 2007). These observation are in line with known plant uptake and isotope frac-
tionation mechanism, whereby a reduction reaction and subsequent uptake of ferrous iron is responsible for the enrichment in light Fe isotopes in strategy I plants, and the complexation of Fe\(^{3+}\) by organic ligands and the subsequent uptake of the PS-Fe complex is responsible for the enrichment of heavy isotopes in strategy II plants. Subsequent work found significant variation in isotopic fractionation among different graminaceous species, showing positive and negative isotope signatures suggesting that the controls are far more complex (Guelke-Stelling and von Blanckenburg, 2012; Kiczka et al., 2010b). One possible explanation is that Strategy II plants possess transporters for ferrous Fe and thus can take up Fe\(^{2+}\) directly (Cheng et al., 2007; Kim and Guerinot, 2007). Kiczka and co workers (2010b) suggested two consecutive stages during Fe uptake to explain light Fe enrichment in graminacae, and in particular processes preceding active transport such as mineral dissolution which favours removal of light isotopes (Chapman et al., 2009; Kiczka et al., 2010a; Kiczka et al., 2011; Weiss et al., 2014; Wiederhold et al., 2006; Wiederhold et al., 2007a; Wiederhold et al., 2007b) and selective Fe uptake at the plasma membrane level. These more recent observations emphasize the possible effect of mixing of different chemical forms on the Fe isotope signature found in stems, grains and leaves during the transport in phloem and xylem (Guelke-Stelling and von Blanckenburg, 2012; Moynier et al., 2013). Although Fe speciation during root uptake is a major contributor to the final isotopic signature, possible mechanisms that influence isotope distribution within plants include successive oxidation and reduction steps during translocation, ligand exchange reactions, remobilisation from older plant tissues and mixing effects of short- (phloem) and long-distance (xylem) transport (Alvarez-Fernandez et al., 2014)(Yoneyama, 2010 #3739).

The aim of the present study was to investigate the controls of stable isotope fractionation of Zn and Fe in rice (\textit{Oryza sativa}) grown under aerobic and anaerobic soil conditions. We conducted experiments in a nutrient-sufficient soil with a widely-grown rice genotype. We analysed soil, leachable fractions, shoots and grains, and we discuss the observed fractionation patterns in light of the possible mechanisms outlined above.

2 Materials and Methods

2.1 Plant growth experiments

Rice (\textit{Oryza sativa} L. cv. Oochikara) was grown in pots in a greenhouse at Rothamsted Research under anaerobic (flooded) and aerobic conditions as described elsewhere (Xu et al., 2008). The soil was taken from the plough layer (0–20 cm depth) of an arable field at Rothamsted. It is a
moderately well-drained Aquic Paleudalf (USDA classification) or Luvisol (FAO classification) with silty clay loam texture (26% clay, 53% silt, 21% sand), 2% organic C, 0.2% total N, 5.93 g kg\(^{-1}\) amorphous Fe oxides (by ammonium oxalate extraction) and pH 6.4. The soil was air-dried, sieved to < 8 mm and homogenized. Two watering treatments (aerobic and flooded) with four pots per treatment were randomly arranged on a bench inside a greenhouse (day/night temperatures 28/25 °C, light period 16 h per day with natural sunlight supplemented with sodium vapour lamps to maintain light intensity of >350 \(\mu\)mol m\(^{-2}\)s\(^{-1}\)). Five pre-germinated rice seeds were planted in each pot. To grow rice under aerobic conditions, plastic pots with holes at the bottom were placed on saucers and filled with 1 kg of soil, while for anaerobic conditions pots without holes were used. Fertilisers (120 mg N kg\(^{-1}\) soil as \(\text{NH}_4\text{NO}_3/(\text{NH}_4)_2\text{SO}_4\), 30 mg P kg\(^{-1}\) and 75.5 mg K kg\(^{-1}\) soil as \(\text{K}_2\text{HPO}_4\)) were added to the soil and mixed thoroughly. Deionised water was added to full saturation with 2 cm of standing water for anaerobic rice, and to 70% of the soil’s water holding capacity for aerobic rice. The two watering regimes were maintained throughout the experiment by daily additions of deionised water. Two further doses of 50 mg of N (\(\text{NH}_4\text{NO}_3\)) were added to each pot at 33 and 70 days after planting. The plants were harvested at grain maturity (117 days after planting). Stems were cut at 2 cm above soil surface, rinsed with deionised water, and dried at 60 °C for 48 h. The samples were separated into straw and grain (including husk), ground, pooled in one sample per pot and then aliquots were processed as described below. Root material was not collected for analysis because a large fraction of the roots had died and degenerated when the plants reached maturity. Only the aboveground shoot materials were used.

2.2 Reagents, sample preparation, ion exchange chromatography and mass spectrometry

Sample preparation was carried out in Class 10 laminar flow benches within class 1,000 clean room facilities. Mineral acids except for HF were purified from Analar grade by sub boiling distillation in quartz stills. Distilled HF (~28 M Ultrapure grade) and \(\text{H}_2\text{O}_2\) (~30% v/v Aristar grade) were purchased from VWR. All acid dilutions used >18.2 MΩ cm\(^{-1}\) grade \(\text{H}_2\text{O}\) from a Millipore purification system.

Oven-dried plant samples were ground using a porcelain pestle and mortar with liquid nitrogen and passed through a 0.5 mm\(^{2}\) sieve. Plant samples were digested using a CEM Microwave Accelerated Reaction System MARS with 100-ml XP-1500 Teflon vessels. 5 ml of 9 M \(\text{HNO}_3\), 3 ml 30% \(\text{H}_2\text{O}_2\), and 0.6 ml 28 M HF were added to approximately 0.35 g of material.
The mixture was ramped to 210 °C and 250 psi and held for 90 min. Soil samples (50 mg) were digested using the same protocol. Soil leaches were used to estimate the composition of the Zn pool available to the plants. 30 ml 0.1 M HCl was added to 0.3 g of air-dried soil, and the resulting suspension was stirred continuously for 48 h at 25 °C. The suspension was centrifuged, and the supernatant was collected.

After digestion, samples were evaporated to dryness and the residue was dissolved in ~1 ml 7 M HCl with 0.05 ml 30% H₂O₂ added to prevent reduction of ferric Fe. The solutions were dried again and re-dissolved in 2.5 ml 7 M HCl containing 0.001 % H₂O₂ by volume. These solutions were split into three aliquots for Zn and Fe concentration measurements, for isotopic analysis, and for archive. The 0.5-ml aliquots for concentration measurement were diluted to 3.5 ml and made up to 1 M HCl and the measured using a Varian quadrupole ICP-MS.

The separation of Zn and Fe from the matrix for subsequent isotope ratio measurements utilized Bio-Rad Poly-Prep columns filled with 2 ml of AG MP-1 100-200 mesh anion exchange resin. The resin was conditioned with 7 M HCl (10 ml) and successively washed with 0.1 M HNO₃ (10 ml), H₂O (10 ml), 0.1 M HNO₃ (10 ml) and H₂O (10 ml). The resin was re-conditioned with 7 M HCl–0.001% H₂O₂ (7 ml) and the samples loaded (1 ml solution in 7 M HCl-0.001% H₂O₂). The majority of matrix elements were eluted using 30 ml 7 M HCl-0.001% H₂O₂. This was followed by elution of Fe in 17 ml 2 M HCl and Zn using 12 ml 0.1 M HCl. The resin was then washed with 10 ml 0.5 M HNO₃, after which it was used again for separations. The Zn and Fe fractions were dried down, dissolved in 0.2 ml 15 M HNO₃, and dried to drive off residual HCl. Finally, 1 ml 0.1 M HNO₃ was added and the samples refluxed for >2 hours to ensure complete dissolution. The solutions were then ready to be analysed with the Nu Plasma MC-ICPMS.

The total procedural blank contributions, accounting for the dissolution and ion exchange procedures, were below 1 % of the total Zn and Fe for the samples with the lowest concentrations. The effect of these blank contributions on the final isotopic results is therefore negligible compared to the measurement uncertainty and no blank correction was applied.

Zinc isotopes were analysed using the double spike method (Arnold et al., 2010b). An isotope spike enriched in ⁶⁴Zn and ⁶⁷Zn was added to the sample aliquot to achieve a total of 1000 ng of Zn and a spike: sample mass ratio of 1. Values are reported as \( \delta^{66}\text{Zn} \) relative to the widely used standard JMC Lyon, i.e. \( \delta^{66}\text{Zn}_{\text{JMC-Lyon}} = \left[ \frac{[^{66}\text{Zn}]_{\text{sample}}}{[^{66}\text{Zn}]_{\text{JMC-Lyon}}} - 1 \right] \times 10^3 \). The Fe isotope analyses were performed by standard-sample bracketing using the reference material IRMM-014 as the standard, as detailed elsewhere (Chapman et al., 2009). Values are re-
ported as $\delta^{56}\text{Fe}$ relative to the IRMM-14 standard, $\delta^{56}\text{Fe}_{\text{IRMM-014}} = \left(\frac{^{56}\text{Fe}^{54}\text{Fe}}{^{54}\text{Fe}}\right)_{\text{sample}} / \left(\frac{^{56}\text{Fe}^{54}\text{Fe}}{^{54}\text{Fe}}\right)_{\text{IRMM-014}} - 1 \times 10^3$.

Certified reference materials (rye grass BCR-281 and blend ore BCR-027) and single element industrial solutions (Johnson Mattey and Puratronic Alpha Aesar) were analysed to determine accuracy and precision of the isotope measurements (see Table 1 for details).

3 Results and Discussion

3.1 Zinc content and isotopic ratios of plants and soil

The average Zn concentration of the shoot tissue grown in aerobic soil is 75.5 ± 12.3 μg g⁻¹ (mean±1SD, n=4) and approx. 2.3 higher than in rice grown in anaerobic soil (32.6 ± 3.5 μg g⁻¹, ±1 SD, n=4, Table 1). All measured concentrations are well above the threshold for deficiency, which is below 10 μg g⁻¹ (Dobermann and Fairhurst, 2000; Smolders et al., 2013), suggesting the plants were Zn sufficient in both soils. This conclusion is supported by the shoot and grain biomasses, which were not significantly different for the two water regimes, despite the difference in Zn concentration of the shoot (Table 1). The lower Zn uptake under anaerobic conditions is expected because of the reduced Zn solubility in submerged soils (Kirk, 2004). Under aerobic soil conditions, the shoots, i.e. stems and leaves, contain a greater proportion of the total plant Zn (75 % versus 63 % under anaerobic conditions). The total Zn concentration of the soil is 105 μg g⁻¹ whilst the 0.1 M HCl soil leach extracted 26 μg g⁻¹.

The Zn isotope compositions of the rice shoots, grains and the total above-ground plant material are reported in Table 1. The total above-ground plant value was calculated from the mass of the shoot and grain material and their corresponding Zn contents and isotope compositions. We find that the shoots of the rice plants grown under both soil regimes are isotopically significantly heavier than the bulk soil or leach (Figure 1). The grains, in contrast, are isotopically light. The calculated Zn isotope composition of the total above-ground plant material is closer to that of the shoot and soil leach and 0.19 ‰ heavier in the rice grown under aerobic conditions (Table 1). This difference is small but significant within the 95% confidence interval {Harris, 2010 #3434}.

We note a small difference in the isotope ratios in rice grown in aerobic or anaerobic soils. The shoots of rice grown in the aerobic soil yield a heavier isotope composition than the Zn in the labile soil pool. This is balanced by a bias towards light isotopes of Zn between shoot and grain, resulting in a small net negative bias between Zn in the total plant and the Zn in the soil.
leach. Under anaerobic soil conditions, the enrichment of heavy isotopes between soil leach and shoot transfer is smaller, and the net light bias between total above ground plant and soil solution is larger (Figure 1).

Bulk and soil leach fractions show isotopic compositions ($\delta^{66}\text{Zn}_{\text{JMC-Lyon}}$) of 0.36±0.04 and 0.59±0.02‰ (mean±2SD, n=2), respectively (Table 1). By mass balance, the Zn fraction remaining in the solid phase has a $\delta^{66}\text{Zn}_{\text{JMC-Lyon}}$ of ca. 0.28‰. This lies well within the range of igneous rocks and/or the inferred range of geological materials (Cloquet et al., 2008; Kelley et al., 2009; Weiss et al., 2014). It is possible that the 0.1 M HCl leachable fraction represents Zn from polluted atmospheric deposition, which can account for the heavier isotopic signature compared to the bulk soil (Dolgopolova et al., 2006; Gioia et al., 2008; Mattielli et al., 2005; Weiss et al., 2007).

3.2 Possible isotope fractionation processes of Zn during plant uptake and translocation

The isotopic fractionation between the different reservoirs is assessed employing the $\Delta^{66}\text{Zn}$ notation calculated using the following equation (Weiss et al., 2013):

$$\Delta^{66}\text{Zn}_{A-B} = \delta^{66}\text{Zn}_A - \delta^{66}\text{Zn}_B$$

where $\Delta^{66}\text{Zn}_{A-B}$ is the isotopic fractionation between reservoirs A and B (i.e. soil leach, bulk soil, grain, shoot or above-ground plant) and $\delta^{66}\text{Zn}_{A,B}$ are the isotopic signatures of the reservoir A and B. The results are given in Table 2.

The observed fractionations between the total above ground plant and the soil leach (-0.27‰ in anoxic soils and -0.08‰ in oxic soils) are well in line with that found in rice grown in hydroponic solution (Weiss et al., 2005)[Smolders, 2013 #3557] and in submerged soils (Arnold et al., 2010a). The signatures in these previous studies were explained by the kinetically controlled uptake of free Zn$^{2+}$ in the hydroponic studies and by the uptake of PS-Zn complexes in the submerged soils. Complexation to a phytosiderophore ligand in the soil solution prefers isotopically heavy Zn as shown by experimental and computational work (Fujii and Albarède, 2012; Fujii et al., 2014; Jouvin et al., 2009). Zinc has low solubility in aerobic and anaerobic soils and graminaceous plant roots, including rice, therefore secrete phytosiderophores to absorb the resulting Zn-phytosiderophore complexes (Reid et al., 1996; von Wiren et al., 1996; Widodo et al., 2010). We expect that phytosiderophore secretion is more pronounced in aerobic soil to solubilise Fe(III) and this indeed can account for the higher Zn capture and the incorporation of heavier isotopes under aerobic soil conditions observed in this study (Figure 1). We
note that under Zn depletion in the soil solution, the isotopic composition of the total plant
above the surface would trend towards that of the initial soil reservoir because the amount
taken up approaches the total amount available in solution. The increased uptake of Zn ob-
served under aerobic conditions and the isotopic composition of the total plant above the sur-
face being closer to that of the soil leachate would be in line with this interpretation. However,
mass balance calculations suggest that the rice extracted only a small fraction of the Zn that
was available for extraction from the soil. The soil per pot contained approximately 26 mg Zn
compared to 1.26 mg and 0.62 mg that were present in the plants under aerobic and anaerobic
soil conditions, respectively (Table 1).

The grains show a significant enrichment of light Zn isotopes relative to the shoot material
in rice grown under aerobic and anaerobic conditions alike (Table 2). The Zn budget of the
shoot is accumulated over most of the growth season, whereas the grains largely accumulate
their Zn during the grain-filling period of approximately 30 days. Previous work suggests that
the rapid accumulation of Zn during the relatively short grain filling period is controlled by the
remobilisation of Zn from various parts of the plant and not increased up-take from the soil
medium (Nishiyama et al., 2012; Yoneyama et al., 2010). Our data indeed agrees with a iso-
tope fractionation model for a uni-directional process such as translocation from shoot to grain
(Figure 2). The Rayleigh model is valid assuming that (i) the Zn is remobilized from the shoot
during grain loading and the soil as additional source is excluded, (ii) the Zn pool of the shoot is
not significantly replenished from the soil during this time period, and (iii) the Zn transport is
not quantitative. The uni directional model is given by:

\[
F^{(a \rightarrow b - 1)} = \frac{(1000 + \delta^{66}Zn_{A})}{(1000 + \delta^{66}Zn_{A,0})}
\]

, where \( \alpha \) is the isotopic fractionation factor between shoot and grain, \( F \) the fraction of Zn in
the shoot, \( \delta^{66}Zn_{A,0} \) the initial isotopic composition of the shoot prior to the grain filling period
and \( \delta^{66}Zn_{A} \) the isotopic composition of the shoot corresponding to the fraction, \( F \), which re-
 mains in the shoot. Our experimental results suggest an initial offset between grain and shoot
of -0.6 ‰ (\( \alpha \approx 0.9994 \)) followed by a slight decrease to the measured values. Within the
framework of this model, a greater extent of remobilisation (\( F < 0.6 \)) would result in even larger
isotopic effects. While we note the good fit of the model in our and previous studies testing the
fractionation of stable Zn isotopes in Zn hyper accumulators and Zn tolerant plants (Aucour et
al., 2011; Deng et al., 2014; Tang et al., 2012) given the boundary conditions imposed, we ac-
knowledge that the mechanisms for Zn transport into the root and subsequently into the grain via xylem and/or phloem are highly complex (Alvarez-Fernandez et al., 2014; Nishiyama et al., 2012; Yamaji and Ma, 2014; Yoneyama et al., 2010) and our one-source one-sink model is therefore most likely far too simplistic. The xylem is the only vascular tissue that reaches the developing grain and the grain tissue is symplastically isolated from the parent plant (Patrick and Offler, 2001). During the transport via the phloem, Zn must enter/re-enter the symplasm at least three times to reach the grain, i.e., at the root prior to the endodermis, during the subsequent transition into the phloem and during the crossover from the phloem into the grain tissue (Broadley et al., 2007). This repetitive transport across plasma membranes probably is one of the many processes that control the magnitude of the isotopic fractionation observed.

3.3 Iron content and isotopic ratios of the plants and soil

Plant Fe concentrations and the total amount of Fe do not differ significantly between the rice plants grown in the two contrasting soil environments (Table 1). In both treatments, the shoot Fe concentrations are well above the threshold range for deficiency, i.e., 50 to 100 μg g⁻¹ (Dobermann and Fairhurst, 2000). The Fe isotopic composition of the 0.1 M HCl leachate is about 0.3 ‰ lighter in δ⁵⁶FeIRMM-014 than the bulk soil (Table 1). This is likely due to partial leaching of selected mineral phases with an isotopically light signature and/or a kinetic mechanism whereby the lighter isotopes are readily removed (Chapman et al., 2009; Kiczka et al., 2010a; Wiederhold et al., 2006). The δ⁵⁶FeIRMM-014 value of the soil leachate is -0.27 ‰ (Table 1).

3.4 Isotope fractionation of Fe during uptake, translocation and grain filling

The fractionations between plant tissues and the Fe source are calculated relative to the bulk soil as this enables us to compare our results with previous Fe isotope work (Guelke and von Blankenburg, 2007; Guelke-Stelling and von Blankenburg, 2012). We find an enrichment of isotopically light Fe between bulk soil and shoot of up to 0.5 ‰ (expressed using δ⁵⁶FeIRMM-014) in aerobic and anaerobic soils alike (Table 2). The largest difference in Δ⁵⁶FeIRMM-014 value is between shoot and bulk soil of plants grown on aerobic soils (0.45 ‰). The Fe taken up into the shoots has a significant lighter isotopic composition compared to the bulk soil but only insignificantly lighter relative to the soil leachate (Table 1 and Figure 1).
A notable finding is the similarity in the Fe isotopic composition between rice grown in aerobic and anaerobic soils, and the small fractionation between reservoirs. This suggests that the different physical-chemical conditions in the soils, i.e. redox potential, oxygen fugacity, solubility, do not affect the isotope signature in the plant, despite of the different Fe redox states (Fe(III)/Fe(III)) and the variable amounts of Fe(III)-PS complexes likely present. The extent of enrichment in light isotopes in the shoot material observed is significantly smaller than that found in previous work (Guelke and von Blankenburg, 2007; Kiczka et al., 2010b) and this favours uptake of Fe in its reduced form. Ferrous Fe is taken up without the need for preceding phytosiderophore-facilitated solubilisation in the rice rhizosphere (Ishimaru et al., 2006). The rice does not show significant enrichment of light isotopes in the grain in disagreement with previous work on oat (Guelke and von Blankenburg, 2007; Guelke-Stelling and von Blanckenburg, 2012). At present, this difference is not easily explainable and further work is needed. Iron, similar to Zn, is transported into the symplasm via the apoplasm before grain loading. The lack of isotopic fractionation could suggest a quantitative transport of complexed Fe(III) from the soil solution to the grain.

4 Conclusions

We find large negative fractionation of Zn during the translocation of from shoots into grain. The direction and magnitude of the shoot to grain fractionation is consistent with other studies and with the concept of enrichment in light isotopes into higher levels of the food chain. The different extend of isotope fractionation between Zn in the soil leach and the total above ground plant suggests that a mixture of different Zn species are taken up depeing on the soil conditions, likely including free Zn2+ and PS-Zn complexes. Within experimental error, Fe isotopes showed the same isotopic pattern between the different plant reservoirs and in rice grown in the two different soil environments. Finally, the different isotope patterns observed for Fe and Zn suggest that the remobilisation and subsequent transport does not occur via the same mechanism.

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1. Zinc (a) and Fe (b) isotope variations, expressed as $\delta^{66}\text{Zn}_{\text{JMC-Lyon}}$ and $\delta^{56}\text{Fe}_{\text{IRMM-014}}$, respectively, between bulk soil, soil leachate, shoot, grain and total above ground material.

2. Isotope fractionation model for uni-directional translocation of Zn from shoot to grain (expressed in fraction of above ground Zn in grain; see text for details regarding the model). The two data points represent the average $\Delta^{66}\text{Zn}$ values between grains and shoot for rice grown under anaerobic and aerobic conditions. Full arrows: data form this study, open arrows: data from literature including transfer from (1) seawater into algae (John et al., 2007), (2) seed to leaf in lentils and tuber to leaf in bamboo (Moynier et al., 2009), and (3) solution into tomato plant (Weiss et al., 2005). Only data from controlled systems are included, where sources and sinks can be identified.
Figure 1
Figure 2

- Solution into diatom (low [Zn])
- Seed to leaf within lentils
- Solution into tomato plant
- Tuber to leaf within bamboo
- Solution into diatom (high [Zn])

Aerobic soil into rice plant

Anaerobic soil into rice plant

Shoot to grain within rice plant

Fraction of total above ground Zn in grain

$\Delta\%Zn_{sink-source}$ (‰)
1. Dry mass, concentrations and δ-values for Zn and Fe in shoots, grain and total material above ground of rice plants grown in aerobic and anaerobic soil. Plants were harvested 117 days after sowing. Also shown are the isotopic compositions of soil measured in the 0.1 M HCl extract and in bulk soil and in standard reference materials measured during this and previous studies (Caldelas et al., 2011; Chapman et al., 2006; Chapman et al., 2009)

2. Isotopic fractionations, expressed as Δ^{66}Zn and Δ^{56}Fe, between shoot, grain, total shoot and soil extract in 0.1 M HCl.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>Comment</th>
<th>n</th>
<th>Zn / µg g⁻¹</th>
<th>δ⁶⁶Zn_{soil,sys} / ‰</th>
<th>Fe / µg g⁻¹</th>
<th>δ⁶⁶Fe_{soil,sys} / ‰</th>
<th>Dry mass / g</th>
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<tbody>
<tr>
<td>Rice</td>
<td>Anaerobic soil</td>
<td></td>
<td></td>
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<tr>
<td>Rice</td>
<td>Shoot 4</td>
<td>Measured</td>
<td>4</td>
<td>32.6 ± 3.5</td>
<td>0.61 ± 0.09</td>
<td>95.2 ± 5.2</td>
<td>-0.32 ± 0.16</td>
<td>11.9 ± 1.8</td>
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<tr>
<td>Rice</td>
<td>Grain 4</td>
<td>Measured</td>
<td>4</td>
<td>28.5 ± 0.5</td>
<td>-0.18 ± 0.10</td>
<td>13.8 ± 0.6</td>
<td>-0.39 ± 0.19</td>
<td>8.0 ± 1.6</td>
</tr>
<tr>
<td>Rice</td>
<td>Total plant above ground</td>
<td>Calculated by mass balance</td>
<td>4</td>
<td>0.32 ± 0.09</td>
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<tr>
<td>Rice</td>
<td>Aerobic soil</td>
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<tr>
<td>Rice</td>
<td>Shoot 4</td>
<td>Measured</td>
<td>4</td>
<td>75.5 ± 12.3</td>
<td>0.73 ± 0.12</td>
<td>96.1 ± 11.2</td>
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<td>12.5 ± 2.6</td>
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<td>33.9 ± 3.6</td>
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<td>0.05 ± 0.01</td>
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<td>Measured</td>
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<td>-0.27 ± 0.02</td>
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<tr>
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<td>This study Caldeñas et al (2011)</td>
<td>3</td>
<td>0.40 ± 0.09</td>
<td></td>
<td></td>
<td>0.32 ± 0.07</td>
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<tr>
<td>Reference</td>
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<td>This study Chapman et al (2006)</td>
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<td>This study Chapman et al (2009)</td>
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<td>Reference</td>
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<td>This study Chapman et al (2009)</td>
<td>7</td>
<td>0.12 ± 0.13</td>
<td></td>
<td></td>
<td>0.49 ± 0.09</td>
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</table>

Note: Results are given as mean±2SD for all samples. The plants were harvested after 117 days.
Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$\Delta^{66}\text{Zn}_{\text{shoot-soil leach}} / %$</th>
<th>$\Delta^{66}\text{Zn}_{\text{grain-shoot}} / %$</th>
<th>$\Delta^{66}\text{Zn}_{\text{plant above ground-soil leach}} / %$</th>
<th>$\Delta^{56}\text{Fe}_{\text{shoot-soil}} / %$</th>
<th>$\Delta^{56}\text{Fe}_{\text{grain-shoot}} / %$</th>
<th>$\Delta^{56}\text{Fe}_{\text{plant above ground-soil leach}} / %$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaerobic</td>
<td>0.02 ± 0.09</td>
<td>-0.79 ± 0.16</td>
<td>-0.27 ± 0.09</td>
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<td>Aerobic</td>
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<td>-0.45 ± 0.07</td>
<td>0.14 ± 0.09</td>
<td>-0.40 ± 0.07</td>
</tr>
</tbody>
</table>
Monday, May 11, 2015

Dear Professor Chabaux:

We hereby submit the revised version of our paper entitled
Iron and zinc isotope fractionation in rice (Oryza sativa) grown in oxic and anoxic soils

We have addressed the reviewers’ comments and have revised the paper accordingly. Please find our comments attached.

We hope it is now acceptable for publication and we thank you very much for your efforts.

With best wishes

Dominik Weiss
Reader in Environmental Geochemistry
Reviewer 1

50-51: add references
We have added the references.

64: « maze »; do you mean « maize »?
Yes, it was a typo.

80-82: describe briefly the meaning of strategies I and II.
We have added this information.

87: « Recent evidence challenge the idea that ligand facilitated ... »: something is missing in the sentence; please re-phrase.
We did rephrase the sentence.

158: would you precise if you took 0.35 g of plant material from one seed/pot or did you mix the material from the 5 original seeds/pot and take an aliquot?
We combined the 5 plants from one pot. So the 0.35 g of plant material represent the pooled batch from one pot. Each treatment had 4 pots. We hope it is clearer in the revised version.

218, 237, 262: table 3 is lacking
We now fixed the numbering

226: table 3 is lacking as well as the test used and p-value
We used the (±2standard deviation) to compare the various samples.
We have not applied any further statistical tests as the number of data points is far too small to enable that.

238; 272: again p-values for significance should be given
See above.

297-298: replace « d » by « <delta> »; where is <alpha> in the equation?
We fixed the equation

Figure 2: The legend of the abscissa does not match the definition given in the txt (297); should be modified
We clarified now the part

307; 320; 323; 324; 326: again p-values for significance should be given
See comment above

Reply to reviewer 2 comments

The first concern the table that is not always referenced properly at least in the 3.5 section table 4 is cited and there is no table corresponding to 3 or 4.
We have addressed now the numbering.
Maybe authors should consider to cite few more papers somewhere i.e. Aucour et al. 2011, Tang et al. 2012, Deng et al. 2014, Jouvin et al 2012 (why not?)......
We apologize for missing out these papers as they are indeed of relevance.
We have revised all the recent literature and incorporated them in the new version.

These papers could enhanced the dataset presented and maybe support their finding and their figure 2. Indeed, they build a Rayleigh type diagram and discuss just a little on that, I think that part could be better discussed. I taking into account their argue that they used only controlled systems studies but I think it will give better clues with more discussion on that part.
We have now included the findings and approaches of the other papers and discuss them as far as this is reasonable. It is important to note that the data set used in our study to develop the model is different from that in the papers cited by the reviewer (i.e. we do not use the Zn content in the root as starting point). Therefore that clearly limits the inter-comparison.
In addition, the Zn accumulation and storage in rice is very different to that of hyper accumulators and we cannot assume the same pool from where Zn would be mobilized. Finally, the Rayleigh model – with its very basic assumptions - is likely far to simplistic and excludes other fractionation mechanisms (e.g., changes in speciation etc.).
Iron and zinc isotope fractionation during uptake and translocation in rice (Oryza sativa) grown in oxic and anoxic soils

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# Corresponding author, e-mail: d.weiss@imperial.ac.uk

Revised version submitted for publication to CR Geosciences
Abstract

Stable isotope signatures are emerging quickly as a powerful technique to study metal uptake and translocation in plants. Fundamental to this development is a thorough understanding of the processes that lead to isotope fractionation under differing environmental conditions. In this study, we investigated zinc (Zn) and iron (Fe) isotope fractionation in rice grown to maturity in anaerobic and aerobic soils under greenhouse conditions. The overall Zn isotope fractionation between the soil and above-ground plant material was negligible in aerobic soil but significant in anaerobic soil with isotopically lighter Zn in the rice plant. The observed range of fractionation was in line with previously determined fractionations of Zn in rice grown in hydroponic solutions (Weiss et al., 2005, New Phytol. 165, 703-710) and submerged soils (Arnold et al, 2010, Plant Cell Environ. 33, 370-381) and emphasizes the effect of taking up different chemical forms of Zn, most likely free and organically complexed Zn, on the isotopic signature found in the plant. The Zn in the grain was isotopically lighter than in the rest of the above-ground plant in rice grown in aerobic and anaerobic soils alike. This demonstrates that in the course of the grain loading and during translocation within the plant important biochemical and/or biophysical processes occur. The isotope fractionation observed in the grains would be consistent with a unidirectional controlled transport from shoot to grain fractionation with a fractionation factor of $\alpha \approx 0.9994$. Iron isotopes showed an isotopic lighter signature in shoot and grain compared to the bulk soil or the leach in both soil environments alike. The negative direction of isotopic fractionation is consistent with possible changes in redox state of the Fe during uptake and translocation. The isotope fractionation pattern between shoots and grain material are different for Zn and Fe which finally suggests that different mechanisms operate during translocation and grain loading in rice for these two key micronutrients.

1 Introduction

Soils deficient in zinc (Zn) and iron (Fe) constrain rice (Oryza sativa) production in large parts of the world (Dobermann and Fairhurst 2000) and deficiencies of Zn and Fe in human populations with rice-based diets causes major health problems for millions of people (Graham, 2007). Identifying nutrient efficient rice lines, applying appropriate breeding techniques, and under-
standing the underlying mechanisms of Zn and Fe efficiency, are important for tackling this
momentous societal challenge (Wissuwa et al., 2008).
Recent work demonstrated significant isotope fractionation during uptake and translocation of
trace metals including Fe, Cu and Zn from soils to plants in a wide variety of plant species. This
suggests that natural stable isotope compositions provide a powerful technique to study the
chemical and biological processes that control micronutrient movement from soils into plants
(Alvarez-Fernandez et al., 2014). Isotopic fractionations have been found to differ between
plant species and between genotypes of the same species, as well as with soil and nutritional
conditions (Arnold et al., 2010a; Aucour et al., 2011; Deng et al., 2014; Tang et al., 2012; Weiss
et al., 2005).
Work on the isotopic fractionation during Zn uptake in hydroponic cultures showed that
plant shoots are enriched in $^{64}$Zn relative to $^{60}$Zn in comparison to the source, consistent with a
uni-directional transport of free Zn$^{2+}$ across cell membranes, most likely kinetically controlled
(Aucour et al., 2011; Deng et al., 2014; Weiss et al., 2005). Plants grown in natural soils, in con-
trast, show negligible or slight heavy isotope enrichment in the shoots (Arnold et al., 2010a;
Tang et al., 2012; Viers et al., 2007). Arnold and co-workers suggested that uptake of a Zn(II)-
phytosiderophore (PS-Zn) complex could account for the observed heavy isotopic fractionation
in Zn uptake by rice tolerant to Zn deficiency (Arnold et al., 2010a). Likewise Smolders and co-
workers explained heavy isotopic fractionation during Zn uptake by tomato plants in resin-
buffered hydroponics by the uptake of a PS-Zn complex (Smolders et al., 2013). Investigations
of Zn complexation by organic molecules using experiments in the laboratory (Jouvin et al.,
2009) and ab initio calculations (Fujii and Albarède, 2012) support this hypothesis. Few studies
determined the stable isotope fractionation of Zn during translocation in higher plants but en-
richment of light isotopes in leaves with increasing distance from the root (Caldelasa et al.,
2011; Moynier et al., 2009; von Blankenburg et al., 2009) and during the translocation from
root to shoot (Caldelasa et al., 2011; Jouvin et al., 2012; Tang et al., 2012) have been reported.
To date, Fe isotope fractionation has not been studied in rice but a significant body of
work exists for graminacae (Guelke and von Blankenburg, 2007; Kiczka et al., 2010b; von
Blankenburg et al., 2009). The observed range of isotope fractionation is around 2.25 ‰ per
atomic mass unit (pmu). Initial pot studies found enrichment of light Fe during plant uptake
in strategy I plants as opposed to enrichment of heavy isotopes in strategy II (Guelke and von
Blankenburg, 2007). These observation are in line with known plant uptake and isotope frac-
tionation mechanism, whereby a reduction reaction and subsequent uptake of ferrous iron is responsible for the enrichment of light Fe isotopes in strategy I plants, and the complexation of ferric iron by organic ligands and the subsequent uptake of the PS-Fe(III) complex is responsible for the enrichment of heavy isotopes in strategy II plants. Subsequent work with different graminaceous species revealed positive and negative isotope signatures in the plants suggesting that the controls are far more complex than previously thought (Guelke-Stelling and von Blanckenburg, 2012; Kiczka et al., 2010b). One possible explanation is that Strategy II plants possess transporters for ferrous Fe and thus can take up Fe$^{2+}$ directly (Cheng et al., 2007; Kim and Guerinot, 2007). Kiczka and co workers (2010b) suggested two consecutive stages during Fe uptake to explain light Fe enrichment in graminaceae, and processes preceding active transport such as mineral dissolution which favours removal of light isotopes (Chapman et al., 2009; Kiczka et al., 2010a; Kiczka et al., 2011; Weiss et al., 2014; Wiederhold et al., 2006; Wiederhold et al., 2007a; Wiederhold et al., 2007b) and selective Fe uptake at the plasma membrane level. These more recent observations emphasize also the possible effect of mixing of different chemical forms on the Fe isotope signature found in stems, grains and leaves during the transport in phloem and xylem (Guelke-Stelling and von Blanckenburg, 2012; Moynier et al., 2013). Although Fe speciation during root uptake is a major contributor to the final isotopic signature, possible mechanisms that influence isotope distribution within plants include successive oxidation and reduction steps during translocation, ligand exchange reactions, remobilisation from older plant tissues and mixing effects of short- (phloem) and long-distance (xylem) transport (Alvarez-Fernandez et al., 2014; Yoneyama et al., 2010).

The aim of the present study was to investigate the controls of stable isotope fractionation of Zn and Fe during uptake and translocation in rice (Oryza sativa) plants grown under aerobic and anaerobic soil conditions. We conducted experiments in a nutrient-sufficient soil with a widely-grown rice genotype. We analysed soil, leachable fractions, shoots and grains, and we discuss the observed fractionation patterns in light of the possible mechanisms outlined above.

2 Materials and Methods

2.1 Plant growth experiments

Rice (Oryza sativa L. cv. Oochikara) was grown in pots in a greenhouse at Rothamsted Research under anaerobic (flooded) and aerobic conditions as described elsewhere (Xu et al., 2008). The soil was taken from the plough layer (top 20 cm) of an arable field at Rothamsted. It is a mod-
erately well-drained Aquic Paleudalf (USDA classification) or Luvisol (FAO classification) with silty clay loam texture (26 % clay, 53 % silt, 21 % sand), 2 % organic C, 0.2 % total N, 5.93 g kg\(^{-1}\) amorphous Fe oxides (by ammonium oxalate extraction) and pH 6.4. The soil was air-dried, sieved to <8 mm and homogenized. Two watering treatments (aerobic and flooded) with four pots per treatment were randomly arranged on a bench inside a greenhouse (day/night temperatures 28/25 °C, light period 16 h per day with natural sunlight supplemented with sodium vapour lamps to maintain light intensity of >350 μmol m\(^{-2}\) s\(^{-1}\)). Five pre-germinated rice seeds were planted in each pot. To grow rice under aerobic conditions, plastic pots with holes at the bottom were placed on saucers and filled with 1 kg of soil, while for anaerobic conditions pots without holes were used. Fertilisers (120 mg N kg\(^{-1}\) soil as NH\(_4\)NO\(_3\)/(NH\(_4\))\(_2\)SO\(_4\), 30 mg P kg\(^{-1}\) and 75.5 mg K kg\(^{-1}\) soil as K\(_2\)HPO\(_4\)) were added to the soil and mixed thoroughly. Deionised water was added to full saturation with 2 cm of standing water for anaerobic rice, and to 70 % of the soil’s water holding capacity for aerobic rice. The two watering regimes were maintained throughout the experiment by daily additions of deionised water. Two doses of 50 mg of N (NH\(_4\)NO\(_3\)) were added to each pot at 33 and 70 days after planting. The plants were harvested at grain maturity (117 days after planting). Stems were cut at 2 cm above soil surface, rinsed with deionised water, and dried at 60 °C for 48 h. The samples were separated into straw and grain (including husk), ground, pooled in one sample per pot and then aliquots were processed as described below. Root material was not collected for analysis because a large fraction of the roots had died and degenerated when the plants reached maturity. Only the aboveground shoot materials were used.

2.2 Reagents, sample preparation, ion exchange chromatography and mass spectrometry

Sample preparation was carried out in Class 10 laminar flow benches within class 1,000 clean room facilities. Mineral acids except for HF were purified from Analar grade by sub boiling distillation in quartz stills. Distilled HF (~28 M Ultrapure grade) and H\(_2\)O\(_2\) (~30% v/v Aristar grade) were purchased from VWR. All acid dilutions used >18.2 MΩ cm\(^{-1}\) grade H\(_2\)O from a Millipore purification system.

Oven-dried plant samples were ground using a porcelain pestle and mortar with liquid nitrogen and passed through a 0.5 mm\(^2\) sieve. Plant samples were digested using a CEM Microwave Accelerated Reaction System MARS with 100-ml XP-1500 Teflon vessels. 5 ml of 9 M HNO\(_3\), 3 ml 30 % H\(_2\)O\(_2\), and 0.6 ml 28 M HF were added to approximately 0.35 g of material.
The mixture was ramped to 210 °C and 250 psi and held for 90 min. Soil samples (50 mg) were
digested using the same protocol. Soil leaches were used to estimate the composition of the Zn
pool available to the plants. 30 ml 0.1 M HCl was added to 0.3 g of air-dried soil, and the result-
ing suspension was stirred continuously for 48 h at 25 °C. The suspension was centrifuged, and
the supernatant was collected.

After digestion, samples were evaporated to dryness and the residue was dissolved in ~1 ml
7 M HCl with 0.05 ml 30% H₂O₂ added to prevent reduction of ferric Fe. The solutions were
dried again and re-dissolved in 2.5 ml 7 M HCl containing 0.001 % H₂O₂ by volume. These solu-
tions were split into three aliquots for Zn and Fe concentration measurements, for isotopic
analysis, and for archive. The 0.5-ml aliquots for concentration measurement were diluted to
3.5 ml and made up to 1 M HCl and measured using a Varian quadrupole ICP-MS.

The separation of Zn and Fe from the matrix for subsequent isotope ratio measurements
utilized Bio-Rad Poly-Prep columns filled with 2 ml of AG MP-1 100-200 mesh anion exchange
resin. The resin was conditioned with 7 M HCl (10 ml) and successively washed with 0.1 M
HNO₃ (10 ml), H₂O (10 ml), 0.1 M HNO₃ (10 ml) and H₂O (10 ml). The resin was re-conditioned
with 7 M HCl–0.001% H₂O₂ (7 ml) and the samples loaded (1 ml solution in 7 M HCl-0.001%
H₂O₂). The majority of matrix elements were eluted using 30 ml 7 M HCl-0.001% H₂O₂. This was
followed by elution of Fe in 17 ml 2 M HCl and Zn using 12 ml 0.1 M HCl. The resin was then
washed with 10 ml 0.5 M HNO₃, after which it was used again for separations. The Zn and Fe
fractions were dried down, dissolved in 0.2 ml 15 M HNO₃, and dried to drive off residual HCl.
Finally, 1 ml 0.1 M HNO₃ was added and the samples refluxed for >2 hours to ensure complete
dissolution. The solutions were ready to be analysed with the Nu Plasma MC-ICPMS.

The total procedural blank contributions, accounting for the dissolution and ion exchange
procedures, were below 1 % of the total Zn and Fe for the samples with the lowest concentra-
tions. The effect of these blank contributions on the final isotopic results is therefore negligible
compared to the measurement uncertainty and no blank correction was applied.

Zinc isotopes were analysed using the double spike method (Arnold et al., 2010b). An isotope
spike enriched in $^{64}$Zn and $^{67}$Zn was added to the sample aliquot to achieve a total of 1000 ng of
Zn and a spike: sample mass ratio of 1. Values are reported as $\delta^{66}$Zn relative to the widely
used standard JMC Lyon, i.e. $\delta^{66}$Zn$_{JMC-Lyon} = \left(\frac{^{66}Zn/^{64}Zn}_\text{sample}}{^{66}Zn/^{64}Zn}_\text{JMC-Lyon} - 1\right) \times 10^3$. The
Fe isotope analyses were performed by standard-sample bracketing using the reference mate-
rial IRMM-014 as the standard, as detailed elsewhere (Chapman et al., 2009). Values are re-
reported as $\delta^{56}\text{Fe}$ relative to the IRMM-14 standard, $\delta^{56}\text{Fe}_{\text{IRMM-014}} = [(^{56}\text{Fe}/^{54}\text{Fe})_{\text{sample}} / (^{56}\text{Fe}/^{54}\text{Fe})_{\text{IRMM-014}} - 1]\times10^3$.

Certified reference materials (rye grass BCR-281 and blend ore BCR-027) and single element industrial solutions (Johnson Mattey and Puratronic Alpha Aesar) were analysed to determine accuracy and precision of the isotope measurements (see Table 1 for details).

3 Results and Discussion
3.1 Zinc content and isotopic ratios of plants and soil

The average Zn concentration of the shoot tissue grown in aerobic soil was 75.5 ± 12.3 µg g⁻¹ (mean±1SD, n=4) and approx. 2.3 higher than in rice grown in anaerobic soil (32.6 ± 3.5 µg g⁻¹, mean±1SD, n=4, Table 1). All measured concentrations are well above the threshold for deficiency, which is below 10 µg g⁻¹ (Dobermann and Fairhurst, 2000; Smolders et al., 2013), suggesting the plants were Zn sufficient in both soils. This conclusion is supported by shoot and grain biomasses, which were not significantly different for the two water regimes, despite the difference in Zn concentration of the shoot (Table 1). The lower Zn uptake under anaerobic conditions is expected because of the reduced Zn solubility in submerged soils (Kirk, 2004).

Under aerobic soil conditions, the shoots, i.e. stems and leaves, contain a greater proportion of the total plant Zn (75 % versus 63 % under anaerobic conditions). The total Zn concentration of the soil is 105 µg g⁻¹ whilst the 0.1 M HCl soil leach extracted 26 µg of Zn g⁻¹ soil.

The Zn isotope compositions of the shoots, grains and the total above-ground plant material are reported in Table 1. We find that the shoots of the rice plants grown under both soil regimes are isotopically significantly heavier than the bulk soil or leach (Figure 1). The grains, in contrast, are isotopically light. The total above-ground plant value was calculated from the mass of the shoot and grain material and their corresponding Zn contents and isotope compositions. The isotope signature of the rice grown under aerobic conditions is closer to that of the shoot and soil leach and 0.19 % heaver (Table 1). This difference is small but significant within the 95% confidence interval (Harris, 2010).

We note slightly different isotope ratios of the various reservoirs tested between rice grown in aerobic and in anaerobic soils. The shoots of rice grown in the aerobic soil for example yield a heavier isotope composition than the Zn in the labile soil pool. This is balanced by a bias towards light isotopes of Zn between shoot and grain, resulting in a small net negative bias between Zn in the total plant and the Zn in the soil leach. Under anaerobic soil conditions, the
enrichment of heavy isotopes in the shoots relative to the soil leach is smaller, and the net light bias between total above ground plant and soil solution is larger (Figure 1).

Bulk and soil leach fractions show isotopic compositions ($\delta^{66}$Zn$_{JMC-Lyon}$) of 0.36±0.04 and 0.59±0.02 ‰ (mean±2SD, n=2), respectively (Table 1). By mass balance, the Zn fraction remaining in the solid phase has a $\delta^{66}$Zn$_{JMC-Lyon}$ of ca. 0.28 ‰. This lies well within the range of igneous rocks and/or the inferred range of geological materials (Cloquet et al., 2008; Kelley et al., 2009; Weiss et al., 2014). It is possible that the 0.1 M HCl leachable fraction represents Zn from polluted atmospheric deposition, which can account for the heavier isotopic signature compared to the bulk soil (Dolgopolova et al., 2006; Gioia et al., 2008; Mattielli et al., 2005; Weiss et al., 2007).

3.2 Possible isotope fractionation processes of Zn during plant uptake and translocation

The isotopic fractionation between the different reservoirs was assessed using the $\Delta^{66}$Zn notation calculated employing the following equation (Weiss et al., 2013):

$$\Delta^{66}\text{Zn}_{A-B} = \delta^{66}\text{Zn}_A - \delta^{66}\text{Zn}_B$$

where $\Delta^{66}\text{Zn}_{A-B}$ is the isotopic fractionation between reservoirs A and B (i.e. soil leach, bulk soil, grain, shoot or above-ground plant) and $\delta^{66}\text{Zn}_{A,B}$ are the isotopic signatures of the reservoirs A and B being compared. The results are given in Table 2.

The observed fractionations between the total above ground plant and the soil leach (-0.27 ‰ in anoxic soils and -0.08 ‰ in oxic soils) are well in line with that found previously in rice grown in hydroponic solution (Smolders et al., 2013; Weiss et al., 2005) and in submerged soils (Arnold et al., 2010a). The signatures in these previous studies were explained by the kinetically controlled uptake of free Zn$^{2+}$ in the hydroponic studies and by the presence and uptake of PS-Zn(II) complexes in the submerged soils. Complexation to a phytosiderophore ligand in the soil solution prefers isotopically heavy Zn as shown by experimental and computational work (Fujii and Albarède, 2012; Fujii et al., 2014; Jouvin et al., 2009). Zinc has low solubility in aerobic and anaerobic soils and graminaceous plant roots, including rice, secrete phytosiderophores to absorb the resulting Zn-phytosiderophore complexes (Reid et al., 1996; von Wieren et al., 1996; Widodo et al., 2010). We would expect that phytosiderophore secretion is more pronounced in aerobic soil to solubilise Fe(III) and this indeed can account for the higher Zn capture and the incorporation of heavier isotopes under aerobic soil conditions observed in this study (Figure 1). We note that under Zn depletion in the soil solution, the isotopic compo-
sition of the total plant above the surface would trend towards that of the initial soil reservoir because the amount taken up approaches the total amount available in solution. The increased uptake of Zn as observed under aerobic conditions and the isotopic composition of the total plant above the surface being closer to that of the soil leach could account for the observed pattern. However, mass balance calculations suggest that the rice extracted only a small fraction of the Zn that was available for extraction from the soil. The soil per pot contained approximately 26 mg Zn compared to 1.26 mg and 0.62 mg that were present in the plants under aerobic and anaerobic soil conditions, respectively (Table 1).

The grains show a significant enrichment of light Zn isotopes relative to the shoot material under aerobic and anaerobic conditions alike (Table 2). The Zn budget of the shoot is accumulated over most of the growth season, whereas the grains largely accumulate their Zn during the grain-filling period of approximately 30 days. Previous work suggests that the rapid accumulation of Zn during the relatively short grain filling period is controlled by the remobilisation of Zn from various parts of the plant and not the increased up-take from the soil medium (Nishiyama et al., 2012; Yoneyama et al., 2010). We find that our data indeed agrees with an isotope fractionation model for a uni-directional process such as translocation from shoot to grain (Figure 2). The Rayleigh model is valid assuming that (i) the Zn is remobilized from the shoot during grain loading and the soil as additional source is excluded, (ii) the Zn pool of the shoot is not significantly replenished from the soil during this time period, and (iii) the Zn transport is not quantitative. The Rayleigh model is given by:

$$ F^{(\alpha_{\text{A}} \cdot -1)} = \frac{(1000 + \delta^{66}\text{Zn}_{\text{A}})}{(1000 + \delta^{66}\text{Zn}_{\text{A},0})} $$

where $\alpha$ is the isotopic fractionation factor between shoot and grain, F the fraction of Zn in the shoot, $\delta^{66}\text{Zn}_{\text{A},0}$ the initial isotopic composition of the shoot prior to the grain filling period and $\delta^{66}\text{Zn}_{\text{A}}$ the isotopic composition of the shoot corresponding to the fraction, F, which remains in the shoot. Our experimental results suggest an initial offset between grain and shoot of -0.6‰ ($\alpha \approx 0.9994$) followed by a slight decrease to the measured values. Within the framework of this model, a greater extent of remobilisation ($F < 0.6$) would result in even larger isotopic effects. While we note the good fit of the model in our and in previous studies testing the fractionation of stable Zn isotopes in Zn hyper accumulators and Zn tolerant plants (Aucour et al., 2011; Deng et al., 2014; Tang et al., 2012) given the boundary conditions imposed, we acknowledge that the mechanisms for Zn transport into the root and subsequently into the
grain via xylem and/or phloem are highly complex (Alvarez-Fernandez et al., 2014; Nishiyama et al., 2012; Yamaji and Ma, 2014; Yoneyama et al., 2010) and our one-source one-sink model is therefore most likely too simplistic. The xylem is the only vascular tissue that reaches the developing grain and the grain tissue is symplastically isolated from the parent plant (Patrick and Offler, 2001). During the transport via the phloem, Zn must enter/re-enter the symplasm at least three times to reach the grain, i.e., at the root prior to the endodermis, during the subsequent transition into the phloem and during the crossover from the phloem into the grain tissue (Broadley et al., 2007). This repetitive transport across plasma membranes probably is one of the many processes that control the magnitude of the isotopic fractionation observed.

3.3 Iron content and isotopic ratios of the plants and soil

Plant Fe concentrations and the total amount of Fe do not differ significantly in the rice plants grown in the two contrasting soil environments (Table 1). In both treatments, the shoot Fe concentrations are well above the threshold range for deficiency, i.e., 50 to 100 μg g⁻¹ (Dobermann and Fairhurst, 2000). The Fe isotopic composition of the 0.1 M HCl leach is about 0.3 ‰ lighter than the bulk soil as expressed in δ⁵⁶FeIRMM-014 (Table 1). This is likely due to partial leaching of selected mineral phases with an isotopically light signature and/or a kinetic mechanism whereby the lighter isotopes are readily removed (Chapman et al., 2009; Kiczka et al., 2010a; Wiederhold et al., 2006). The δ⁵⁶FeIRMM-014 value of the soil leachate is -0.27 ‰ (Table 1).

3.4 Isotope fractionation of Fe during uptake, translocation and grain filling

The fractionations between plant tissues and the Fe source were calculated relative to the bulk soil as this enables us to compare our results with previous Fe isotope work (Guelke and von Blankenburg, 2007; Guelke-Stelling and von Blankenburg, 2012). We find an enrichment of isotopically light Fe between bulk soil and shoot of up to 0.5 ‰ (expressed using δ⁵⁶FeIRMM-014) in aerobic and anaerobic soils (Table 2). The largest difference in δ⁵⁶Fe value is between shoot and bulk soil of plants grown on aerobic soils (0.45 ‰). The Fe taken up into the shoots has a significant lighter isotopic composition compared to the bulk soil but only insignificantly lighter relative to the soil leachate (Table 1 and Figure 1).

A notable finding is the similarity in the Fe isotopic composition between rice grown in aerobic and anaerobic soils, and the small fractionations between the various reservoirs. This suggests

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that the different physical-chemical conditions in the soils, i.e. redox potential, oxygen fugacity, solubility, do not affect the isotope signature in the plant, despite of the different Fe redox states and the variable amounts of Fe(III)-PS complexes likely present. The extent of enrichment in light isotopes in the shoot material observed is significantly smaller than that found in previous work (Guelke and von Blankenburg, 2007; Kiczka et al., 2010b) and this favours the idea that the uptake of Fe is in its reduced form. Ferrous Fe is taken up without the need for preceding phytosiderophore-facilitated solubilisation in the rice rhizosphere (Ishimaru et al., 2006).

The rice does not show significant enrichment of light isotopes in the grain in disagreement with previous work on oat (Guelke and von Blankenburg, 2007; Guelke-Stelling and von Blanckenburg, 2012). At present, this difference is not easily explainable and further work is needed. Iron, similar to Zn, is transported into the symplasm via the apoplasm before grain loading. The lack of isotopic fractionation could suggest a quantitative transport of complexed Fe(III) from the soil solution to the grain.

4 Conclusions

We find large negative fractionation of zinc during the translocation of from shoots into grain. The direction and magnitude of the shoot to grain fractionation is consistent with the concept of enrichment in light isotopes into higher levels of the food chain. The different extend of isotope fractionation between Zn in the soil leach and the total above ground plant suggests that a mixture of different Zn species are taken up depending on the soil conditions, likely including free Zn$^{2+}$ and PS-Zn(II) complexes. Within experimental error, iron isotopes showed the same isotopic pattern between the different plant reservoirs in rice grown in the two different soil environments. Finally, the different isotope patterns observed for iron and zinc suggest that the remobilisation and subsequent transport does not occur via the same mechanism.

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Response to zinc deficiency of two rice lines with contrasting tolerance is determined by root growth maintenance and organic acid exudation rates, and not by zinc-transporter activity. New Phytol.


Figure captions

1. Zinc (a) and Fe (b) isotope variations, expressed as $\delta^{66}\text{Zn}_{\text{JMC-Lyon}}$ and $\delta^{56}\text{Fe}_{\text{IRMM-014}}$, respectively, between bulk soil, soil leach, shoot, grain and total above ground material.

2. Isotope fractionation model for uni-directional translocation of zinc from shoot to grain (expressed in fraction of above ground zinc in grain; see text for details regarding the model). The two data points represent the average $\Delta^{66}$Zn values between grains and shoot for rice grown under anaerobic and aerobic conditions. Full arrows: data from this study, open arrows: data from literature including transfer from (1) seawater into algae (John et al., 2007), (2) seed to leaf in lentils and tuber to leaf in bamboo (Moynier et al., 2009), and (3) solution into tomato plant (Weiss et al., 2005). Only data from controlled systems are included, where sources and sinks can be identified.
Figure 1
Solution into diatom (low [Zn])
Seed to leaf within lentils
Solution into tomato plant
Tuber to leaf within bamboo
Solution into diatom (high [Zn])

Figure 2
1. Dry mass, concentrations and δ-values for Zn and Fe in shoots, grain and total plant above ground of rice plants grown in aerobic and anaerobic soil. Also shown are the isotopic compositions of zinc measured in the 0.1 M HCl extract and in bulk soil and in standard reference materials measured during this and previous studies (Caldelas et al., 2011; Chapman et al., 2006; Chapman et al., 2009)

2. Isotopic fractionations, expressed as Δ⁶⁶Zn and Δ⁵⁶Fe, between shoot, grain, total plant above ground and soil leach in 0.1 M HCl.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>Comment</th>
<th>n</th>
<th>Zn / μg g⁻¹</th>
<th>δ¹⁸OZn,BOC-1pm / ‰</th>
<th>Fe / μg g⁻¹</th>
<th>δ⁶⁹Fe,IRMM-2014 / ‰</th>
<th>Dry mass / g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice</td>
<td>Anaerobic soil</td>
<td>Shoot</td>
<td>Measured</td>
<td>4</td>
<td>32.6 ± 3.5</td>
<td>0.61 ± 0.09</td>
<td>95.2 ± 5.2</td>
<td>-0.32 ± 0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grain</td>
<td>Measured</td>
<td>4</td>
<td>28.5 ± 0.5</td>
<td>-0.18 ± 0.10</td>
<td>13.8 ± 0.6</td>
<td>-0.39 ± 0.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total plant above ground</td>
<td>Calculated by mass balance</td>
<td>4</td>
<td>0.32 ± 0.09</td>
<td></td>
<td></td>
<td>-0.33 ± 0.16</td>
</tr>
<tr>
<td>Rice</td>
<td>Aerobic soil</td>
<td>Shoot</td>
<td>Measured</td>
<td>4</td>
<td>75.5 ± 12.3</td>
<td>0.73 ± 0.12</td>
<td>96.1 ± 11.2</td>
<td>-0.40 ± 0.07</td>
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<tr>
<td></td>
<td></td>
<td>Grain</td>
<td>Measured</td>
<td>4</td>
<td>33.9 ± 3.6</td>
<td>-0.00 ± 0.07</td>
<td>14.8 ± 1.3</td>
<td>-0.23 ± 0.16</td>
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<tr>
<td></td>
<td></td>
<td>Total plant above ground</td>
<td>Calculated by mass balance</td>
<td>4</td>
<td>0.51 ± 0.04</td>
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<td>-0.35 ± 0.07</td>
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<tr>
<td>Soil</td>
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<td>Bulk</td>
<td>Measured</td>
<td>2</td>
<td>0.36 ± 0.04</td>
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<td>0.05 ± 0.01</td>
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<tr>
<td></td>
<td></td>
<td>0.1 M HCl extract</td>
<td>Measured</td>
<td>2</td>
<td>0.59 ± 0.02</td>
<td></td>
<td>-0.27 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Reference Material</td>
<td>Rye Grass</td>
<td>This study</td>
<td>Caldelas et al (2011)</td>
<td>3</td>
<td>0.40 ± 0.09</td>
<td></td>
<td>0.32 ± 0.07</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>BCR 027 Blend Ore</td>
<td>This study</td>
<td>4</td>
<td>0.50 ± 0.10</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>Johnson Mattey</td>
<td>Chapman et al (2006)</td>
<td>3</td>
<td>0.25 ± 0.06</td>
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<tr>
<td></td>
<td></td>
<td>Puratronic Alpha Aesar</td>
<td>Chapman et al (2009)</td>
<td>7</td>
<td>0.33 ± 0.07</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Isotope data are given as mean±2SD and all the other data as mean±1SD. The plants were harvested after 117 days.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>$\Delta^{66}$Zn$_{\text{shoot-soil leach}}$ / ‰</th>
<th>$\Delta^{66}$Zn$_{\text{grain-shoot}}$ / ‰</th>
<th>$\Delta^{66}$Zn$_{\text{plant above-ground-leach}}$ / ‰</th>
<th>$\Delta^{56}$Fe$_{\text{shoot-soil}}$ / ‰</th>
<th>$\Delta^{56}$Fe$_{\text{grain-shoot}}$ / ‰</th>
<th>$\Delta^{56}$Fe$_{\text{plant above-ground-leach}}$ / ‰</th>
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</thead>
<tbody>
<tr>
<td>Anaerobic</td>
<td>0.02 ± 0.09</td>
<td>-0.79 ± 0.16</td>
<td>-0.27 ± 0.09</td>
<td>-0.37 ± 0.16</td>
<td>-0.06 ± 0.10</td>
<td>-0.38 ± 0.16</td>
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<tr>
<td>Aerobic</td>
<td>0.14 ± 0.12</td>
<td>-0.67 ± 0.17</td>
<td>-0.08 ± 0.04</td>
<td>-0.45 ± 0.07</td>
<td>0.14 ± 0.09</td>
<td>-0.40 ± 0.07</td>
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</tbody>
</table>