

IMPERIAL COLLEGE LONDON

**ZN EFFICIENCY IN RICE:
THE ROLE OF 2'-DEOXYMUGINEIC ACID IN
ZN COMPLEXATION AND UPTAKE**

by

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Declaration of Originality

The content of this thesis in its entirety is the result of my own independent research under the supervision of Professor Dominik Weiss and Professor Ramon Vilar. Appropriate references have been provided wherever use has been made of the work of others

Tamara Markovic

10/05/2016

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Abstract

The focus of this thesis is physicochemical characterisation of Zn complex with a natural phytosiderophore ligand – 2'-deoxymugineic acids (DMA), in light of its possible role in the root Zn uptake in staple crop species, such as rice. Mechanisms behind efficient Zn acquisition in rice cultivars are not yet clearly identified. Yet, this information is vital for the progress of biofortification programs of staple food crops.

The work shown in this thesis examines the stability of the [Zn(DMA)] complex in view of the required traits for the fulfilment of the phytosiderophore role. To this end, the ligand in question was synthesised in laboratory conditions. Subsequent tests have shown that the Zn binding affinity of DMA is substantial in comparison to the other rhizosphere present organic molecules. Therefore, solubilisation by DMA is a promising Zn acquisition mechanism in plants.

The studies following this investigate stable isotope partitioning upon Zn complexation with DMA by means of experiments, as well as by using computational chemistry calculation methods. Both methods yielded the same conclusion: Zn complexation by DMA favours the heavy ^{66}Zn isotope ($\Delta^{66}\text{Zn} \sim 0.3 \text{ ‰}$), and hence, can introduce a significant isotopic fractionation into the environment.

The results of a study on field grown rice are also presented. In this study heavy isotopic enrichment is demonstrated under Zn-deficient conditions in two rice genotypes differing in their susceptibility to Zn-deficient soils; where A69-1 and IR26 are low-Zn tolerant and sensitive cultivars used, respectively. The heavy fractionation factors ($(\Delta^{66}\text{Zn})$ 0.21 ‰ and $\sim 0.30 \text{ ‰}$ for the tolerant and sensitive rice, respectively) are consistent with Zn solubilisation and uptake by

phytosiderophore ligands, and the magnitude of the measured factors corresponds well with the values identified in the preceding experimental and theoretical studies.

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Table 6.4 Total bioavailable Zn in Zn-deficient (-Zn) and Zn-sufficient (+Zn) plots. The difference between the treatments in quantities of Zn leached were statistically significant $p < 0.001^{***}$ (Student's t-test). However, values for total Zn content leached in -Zn plot indicate borderline Zn deficiency (~ 30 mg/kg).	147
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Table 6.5 Average Zn concentrations $[\text{Zn}^{2+}]$, measured using ICP-AES technique, and $\delta^{66}\text{Zn}_{\text{London}}$ isotopic composition of the measured plant samples. The data indicate significant difference between Zn concentrations in IR26 plants grown in (-Zn) and (+Zn) soils. The difference was tested using Student's t-test and the determined level of significance was $p < 0.05^*$ in both 1 pph(*) and 8 pph (*).	152
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List of Abbreviations

abs	Absolute
AcOH	Acetic acid
AsA	Ascorbate peroxidase
ATP	Adenosine triphosphate
B3	Becke's three-parameter non-local exchange potential
Boc	<i>Tert</i> -butyloxycarbonyl group
CA	Carbonic anhydrase
CAT	Catalase
CCDC	Cambridge Crystallographic Data Centre
Chl	Chlorophyll
cit	Citric acid, citrate
CyDTA	(1,2-)Diaminocyclohexanetetraacetic acid
DCC	Dicyclohexylcarbodiimide
DCM	Dichloromethane
DFOB	Desferrioxamine B
DFT	Density Functional Theory
DMA	(2'-)Deoxymugineic acid
DMAP	(4-N,N-)Dimethylaminopyridine
DTPA	Diethylenetriaminepentaacetic acid

DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
ES-MS	Electro spray-mass spectrometry
EtOH	Ethanol
Et ₂ O	Diethyl ether
EtOAc	Ethyl acetate
GSSD	Glutathione reductase
GDBJ	Grimme's dispersion with Becke-Johnson damping empirical scheme
HA	Humic acid
HMA	Heavy metal-transporting P-type ATPase
3'-HMA	(3'-)Hydroxymugineic acid
HPLC	High performance liquid chromatography
HRM	House reference material
IDS	Iron-deficiency specific (dioxygenase)
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
ICP-MS	Inductively coupled plasma mass spectroscopy
IRRI	International Rice Research Institute
IRT	Iron-regulated transporter
LC-MS	Liquid chromatography–mass spectrometry
LMWOA	Low molecular weight organic acids
LYP	Lee-Yang and Parr non-local functionals
MA	Mugineic acid
mal	Malic acid, malate

MAs	The family of mugineic acids
MC-ICP-MS	Multi-collector inductively coupled plasma mass spectrometer
MeOH	Methanol
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
MT	Metallothionein
MTBE	Methyl-tert-butyl ether
MTP	Metal tolerance protein
MW	Molecular weight
NA	Nicotinamine
NAAT	Nicotinamine aminotransferase
NICA	Non-ideal competitive adsorption
NMR	Nuclear Magnetic Resonance
NTA	Nitriloacetic acid
oxal	Oxalic acid, oxalate
PER	Guaiacol peroxidase
PHA	Purified peat humic acid
pph	Plants per “hill”
ppm	Parts per million, mg/L or mg/kg
PS	Phytosiderophore
SAM	S-adenosyl-methionine
SD	Standard deviation
SOD	Superoxide dismutase
SRFA	Suwannee River fulvic acid

SSB	Standard Sample Bracketing
satd	Saturated
TLC	Thin-layer chromatography
TM	Trans membrane (domain)
TMDTA	Trimethylenediaminetetraacetic acid
UV	Ultra violet
WHO	World Health Organisation
YSL	Yellow stripe like
ZIP	Zinc-regulated transporter iron regulated transporter-like protein

Chapter 1

Introduction

1.1. Overview

Zinc (Zn) is an essential trace element in all living organisms, which plays a multitude of structural and regulatory roles. When Zn in soil is unavailable, Zn malnutrition quickly becomes widespread. Insufficient Zn content in plants cascades through the food chain thus subsequently causing Zn malnutrition in humans. Mechanisms by which plants obtain Zn from Zn-deficient soils are not very well understood, although recent technological advances contributed considerably to unravelling processes happening at the plant-soil interface (von Wiren, 2011; Wiederhold, 2015). Differences in response to low Zn conditions have been observed between plant species and between genotypes of the same species. As a result, plants with greater ability to grow and yield under deficient Zn conditions are referred to as *Zn-tolerant*, while the less successful ones are known as *Zn-sensitive*.

Numerous research groups believe that the success of the tolerant cultivars is due to efficient Zn uptake from the soil. Studies proposed that small non-protein amino acids with high affinity for transition metals (so called *phytosiderophores* (PS)) are excreted by the roots in order to solubilise and transport Zn from soil solution into the plants. When Zn uptake in rice was investigated in both radiogenic (Suzuki et al., 2006) and stable Zn isotope studies (Arnold et al., 2010a) members of the

mugineic acid family were highlighted as potential phytosiderophore transporters of Zn in crops. Mugineic acids were shown to be excreted by plants from the family of grasses, including major crops such as rice and barley, under Zn-deficient condition, and intriguingly, in higher quantities in Zn-tolerant cultivars (Widodo et al., 2010).

Nevertheless, phytosiderophore-facilitated Zn uptake mechanism is still insufficiently understood. There is no direct evidence to suggest the involvement of phytosiderophores (PS), in the Zn-efficiency trait of plants. If shown relevant, this mechanism could be further explored and utilised for the biofortification of major crops, such as rice. Rice that is more nutritious and less susceptible to low Zn conditions, could be vital to eradicate issues associated with Zn-deficiency in developing countries.

The aim of this thesis is to gain further insight into the coordination of Zn by MAs and to obtain a better understanding of its role in Zn efficiency. This is achieved in this work by tracing changes in the distribution of stable Zn isotopes imposed upon complexation with MAs in three different systems: aqueous solution with synthetic 2'-deoxymugineic acid (DMA) under laboratory conditions, computational modelling (using quantum mechanical calculations) of Zn complexation with various physiologically-relevant ligands, and measuring isotopic effect in field-grown rice plants.

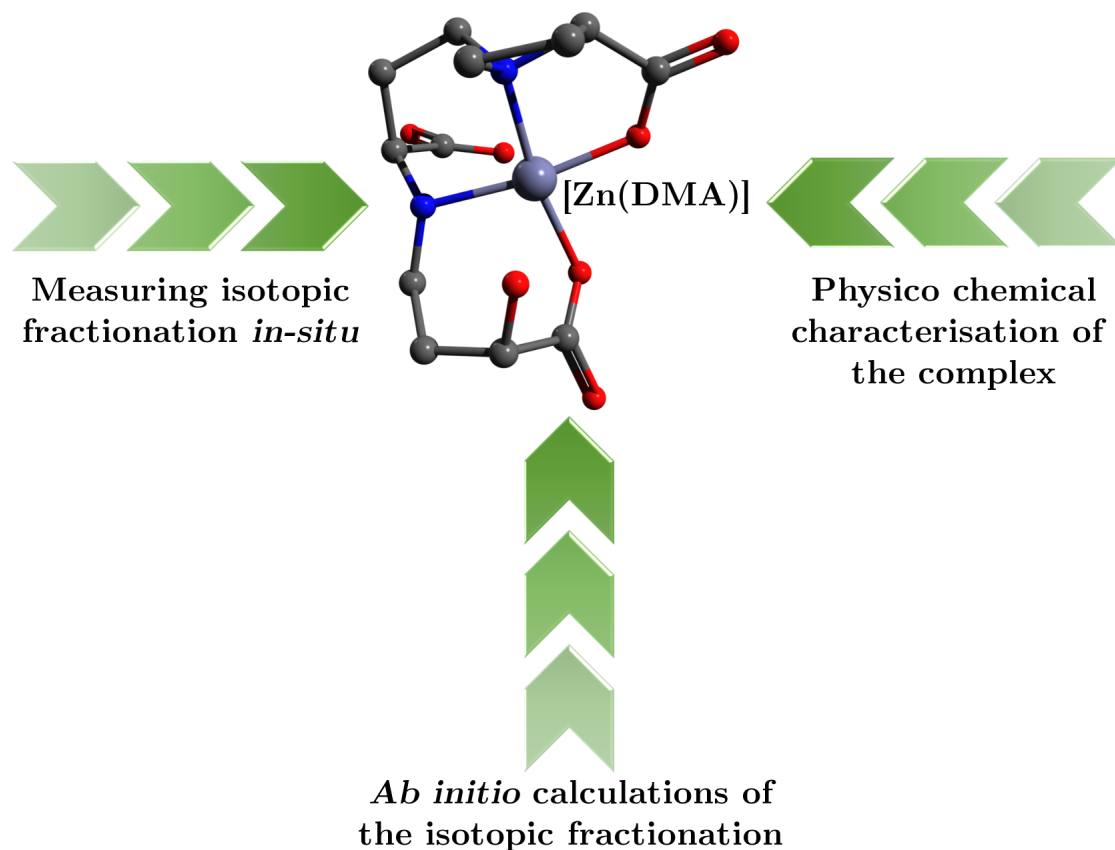


Figure 1.1 Multidisciplinary approach adopted in this work aimed at gaining comprehensive understanding about the coordination chemistry of the [Zn(DMA)] complex and its role in rice Zn efficiency. The [Zn(DMA)] molecule shown in the centre of the figure was constructed using GaussView (Gaussian Inc.) software. Colours denote: C (grey), N (blue), O (red), H (white) and Zn (iris).

1.2. Thesis structure

The current chapter (Chapter 1) provides an overview of the problem investigated in this body of work as well as the structure and the key points of individual chapters presented in this thesis.

Chapter 2 provides an extensive review of studies addressing the role of metal chelating ligands (*i.e.* phytosiderophores) in Zn sequestration and plant uptake, with respect to possible breeding targets.

Chapter 3 presents studies aimed at understanding the formation of [Zn(DMA)]. Descriptions of the complex in previous studies were based on ligands isolated from root washings of plants. However, effective methods for quantitative separation of

different mugineic acids from the isolated material became available a decade after the affinity constants MAa complexes with transition metals were published. Consequently, the currently available thermodynamic values could be inaccurate due to contamination issues. In order to ensure accuracy of the data shown, it is important to use PS ligands in their purest form. Hence, in Chapter 3, phytosiderophore 2'-deoxymugineic acid (DMA) was synthesised and purified before assessing the binding affinity between Zn and DMA ($\log K_{\text{ZnDMA}}$). Information about complex stability of the Zn-MAs complexes is of great importance for soil dynamics modelling therefore the objective of this chapter is to determine the affinity constant of $[\text{Zn}(\text{DMA})]$ complex ($\log K_{\text{ZnDMA}}$), using the pure DMA ligand which has been synthetically obtained.

Chapter 4 investigates one of the theories proposed by Arnold et al. (2010a) using the synthetic DMA ligand. In their Zn fractionation study in field grown rice plants, preferential uptake of the isotope ^{66}Zn over ^{64}Zn in efficient rice genotype was identified as opposed to the sensitive genotype (Arnold et al., 2010a). The isotopic pattern reported was explained by the involvement of PS-facilitated Zn uptake mechanisms, as complexation of Zn with organic ligands was proposed to favour the heavier isotope (Jouvin et al., 2009). Additionally, the authors of the above study proposed DMA as a likely candidate for the phytosiderophore role in rice. To test this, isotopic fractionation was measured upon formation of the $[\text{Zn}(\text{DMA})]$ complex. The objective of this chapter was to test if the observed isotopic fractionation in field-grown plants matches that of Zn complexation by DMA. To do so, a new method was developed, and is reported here, in order to quantitatively separate free Zn^{2+} from complexed Zn species in the same sample.

Measuring isotopic fractionation in laboratory conditions is cumbersome and time-consuming. Theoretical modelling, based on quantum mechanical calculations, offers an effective alternative to estimate isotopic effects in natural systems and guide further experimental efforts. In Chapter 5, a computational model to determine isotopic fractionation in Zn complexes was adopted. Then, the isotopic fractionation in various physiologically relevant Zn complexes was estimated and the results discussed with respect to possible contributing factors such as complex geometry.

Errors associated with this method were addressed by comparing experimental data (Chapter 4) with computational estimates (Chapter 5) and by drawing conclusions about the precision and accuracy of the theoretical methods.

Once the properties of the [Zn(DMA)] are established in the laboratory, the hypothesis about the role of PS in Zn uptake was finally tested in a field experiment by measuring stable isotope fractionation factors. Two rice genotypes differing in their susceptibility to Zn-stress were grown on Zn-fertilised and Zn-deficient soils. The results of isotopic fractionation in both soil and plants under varying Zn conditions are shown in Chapter 6. The magnitude of the measured isotopic fractionation in rice plants is discussed with respect to possible differences in the Zn uptake mechanisms between rice genotypes differing in their Zn-efficiency.

Chapter 7 summarizes the conclusions of the thesis and discusses the overall state of knowledge about ZnMAs complexes and their role in Zn-efficiency. Overall, the data reported in this thesis contribute significantly to our understanding of the phytosiderophore role in Zn uptake in plants. This body of work encourages and guides further studies of PS dynamics in the complex biogeochemical cycling of transition metal elements.

Finally, Chapter 8 provides possible directions and future exploration topics to untwine the complex Zn dynamics occurring at the plant-soil interface.

Chapter 2

Zinc Efficiency Mechanisms: Literature Review

2.1. Motivation

2.1.1. The problem of Zn deficiency in crops

Zinc is an essential nutrient for all biological systems, and a crucial component in a number of regulatory proteins. The size, reactivity and a single oxidation state make an atom of Zn highly advantageous in biological structures. Consequently, Zn is the only metallic cofactor in all six classes of enzymes (oxido-reductases, transferases, hydrolases, lyases, ligases and isomerases) (Alloway, 2009). Zinc is essential in biochemical processes that take part in carbohydrate and protein metabolism, growth regulation, maintenance of membranes integrity, reproduction and pathogen resistance (Alloway, 2008; Marschner, 1995; Prasad, 2009). Despite its vital roles in structure and function at the subcellular level, Zn can be toxic when present in high concentrations. Therefore, improving our knowledge and understanding of Zn cycling in the environment is of great importance to ensure optimal nutrition and minimise risk associated with Zn pollution and consequent toxicity.

Although necessary only in small quantities, Zn deficiency in humans is a widespread problem recognised by the WHO (2002). Hotz and Brown (2004) estimate that 33 % of world population are under risk of Zn malnutrition due to poor diet, with the majority residing in developing countries of Africa and Far-East Asia (Figure 2.1).

Deficiency of certain vitamins and minerals in staple foods have been recognised as a global problem and the concept of *hidden hunger* was introduced describing the effect of foods with very low nutrient per calorie ratio (Bashir et al., 2013). Insufficient Zn uptake is especially dangerous for children, causing growth retardation, hypogonadism, immune dysfunction and cognitive impairment (Prasad, 2009).

The problem of Zn deficiency in human population is a direct consequence of low Zn content of staple foods, such as major crops. The concentration and bioavailability of micronutrients, such as Zn, in world soils has a great impact on agriculture and thus global economy. When bioavailability of nutrients in soil is limited, plants, especially the more sensitive cultivars, suffer from decreased yields and lower nutrient content. Over 30 % of agricultural soils contain 0.8 mg/kg or less of plant-available Zn (Sillanpää, 1990) and are marked as Zn-deficient (Dobermann and Fairhurst, 2000). Plant-available Zn is the fraction of soil Zn pool where Zn is weakly bound to surfaces of organic and inorganic particles, associated with soluble inorganic compounds or complexed with soluble organic matter (Alloway, 2009; Impa and Johnson-Beebout, 2012). The main conditions that may limit plant-available Zn are: low concentrations of Zn and other trace elements in the soil solution, soil pH and redox conditions, content of bicarbonate (HCO_3^-), salt and organic matter, variability and activity of rhizospheral microbial communities, concentrations of macronutrients and the soil moisture status (Alloway, 2009). The dominant factor governing Zn availability to plants is soil pH. For example, by increasing pH, absorptive capacity for Zn increases, and hence, Zn precipitates in iron oxides and chemisorbes on calcium carbonates. On the other hand, presence of organic matter may increase available Zn concentrations in soil due to formation of soluble organic complexes.

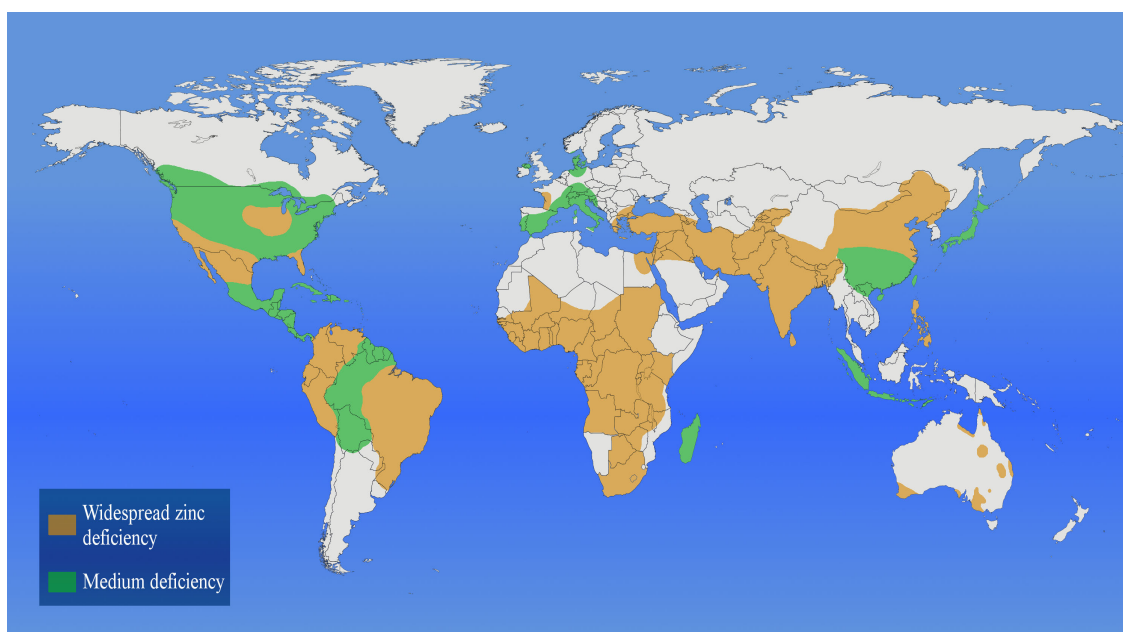


Figure 2.1 Global distribution of Zn deficiency in crops (based on Alloway (2009)).

Application of Zn fertilisers is not necessarily a beneficial strategy in mitigating the problem of Zn-deficiency, as it poses agronomic (*i.e.* soil nutrient misbalance), economic (*i.e.* price growth and unaffordability to small farms) and environmental (*i.e.* pollution) risks. A more sustainable solution to eradicate Zn-deficiency is cultivation of crops that are less susceptible to Zn-deficient conditions. Selection and breeding of cultivars with the ability to extract Zn from soil with low Zn concentration is a positive long-term strategy, not only because of the increase in crops' nutrient content but also due to benefits of reduced fertiliser use.

2.1.2. Crop biofortification programs

All plant species are susceptible to Zn stress, however the extent to which plants are sensitive to low Zn conditions is dependent on their genetic traits. Numerous studies reported significant differences between species and genotypes of the same plant species regarding their tolerance to Zn-deficiency in soils (Cakmak et al., 1997; Hacisalihoglu et al., 2003; Hacisalihoglu, 2002; Rengel, 1996). Rice is one of the most Zn-sensitive plants, especially the varieties grown in lowland production systems.

About 50 % of global lowland rice soils are Zn deficient, largely due to the unique chemistry of submerged soils (Kirk, 2004).

Rice is a species with great natural genetic variability (Graham et al., 1999; Gregorio, 2002). Namely, different genotypes vary greatly in their ability to grow and yield under Zn-stress (Quijano-Guerta et al., 2002; Wissuwa et al., 2006). In light of this fact, agricultural scientists and plant breeders recognised the potential of rice biofortification as a promising strategy to eradicate world-wide Zn malnutrition. Breeding selected crop cultivars to increase their nutritional content (*i.e.* crop biofortification) is a sustainable measure to tackle the widespread deficiency, when compared to the conventional fortification during food processing. Rice is one of the most widely consumed staple foods; hence creating more nutritious and highly yielding crops could have a positive impact on global health and economy (Nestel et al., 2006).

Selection and production of more Zn-efficient rice is hampered by poor understanding of the mechanisms behind tolerance to Zn-stress (Mori et al., 2016). Numerous mechanisms have been suggested to play a significant role in Zn-efficiency, as it is discussed below. Once the relevant mechanisms are elucidated and thoroughly understood, they can be utilised in global rice biofortification programmes as breeding targets.

2.1. Potential mechanisms that complement efficiency trait in rice

2.1.1. Zn uptake in rice grown in lowland rice production systems

In submerged soils, Zn is often physiologically unavailable to plants even when concentration of Zn in the substrate is ample. Plants preferentially take up Zn in its ionic (Zn^{2+}) or very rarely hydroxide ($[\text{ZnOH}]^+$) form (Marschner, 1995). High content of organic material and bicarbonates, alkaline pH and reducing conditions render the majority of soil Zn unavailable to plants. Under such conditions, Zn is

strongly adsorbed onto insoluble ferric hydroxide root plaque formed as a result of Fe(II) oxidation by root-released O₂ (Kirk and Bajita, 1995) (Eq. 2.1).



Due to the release of two H⁺ ions per each mole of Fe²⁺, this reaction leads to acidification in the area surrounding roots (Kirk, 2004). Consequently, the concentration of Fe²⁺ near roots decreases which leads to further catalysis of the above reaction (Eq. 2.1) as more Fe²⁺ diffuses in from the bulk soil. In addition, Fe oxidation was shown to increase Zn mobilisation from highly insoluble forms in the soil, which is then re-adsorbed onto ferric hydroxide plaque, as indicated above, and onto organic matter (Kirk and Bajita, 1995). The Zn deposits on the Fe(OH)₃ surface are insoluble and therefore unavailable to plants. On the other hand, Zn associated with organic matter is acid-soluble and thus physiologically available to plants (Kirk, 2004).

In paddy soils, competition effects governed by concentrations of cations at the root surface is suggested (Arredondo, 2006; Cayton et al., 1985; Giordano and Mortvedt, 1974). The reducing environment around plant roots drives the reduction of Fe and Mn hydrous oxide species into their divalent (Fe²⁺, Mn²⁺) forms (Giordano and Mortvedt, 1974). As a consequence, Zn competes with these divalent cations for binding locations at the metal transporters on the root epidermis thus lowering the Zn intake in plants. This uptake of cations is, in turn, balanced out by excess release of H⁺ from roots, to even out the ionic potential near the root plane (Kirk and Bajita, 1995).

Zn deficiency in rice causes a range of symptoms: leaf bronzing, stunted growth, reduced yield and often – high mortality. Most of the symptoms appear 2 - 3 weeks after transplanting to submerged fields. Graham and Rengel (1993) define the Zn-efficiency of a crop genotype as its relative ability to grow and yield under conditions of marginal Zn supply (Eq. 2.2):

$$\text{Zn efficiency} = \frac{(-\text{Zn})\text{yield}}{(+\text{Zn})\text{yield}} \times 100\% \quad (2.2)$$

Based on observed differences in field performance by various rice cultivars grown on Zn-deficient soil, the IRRI (International Rice Research Institute, Los Baños, Philippines) established a classification of rice strains based on their tolerance to Zn-stress (Graham et al., 1999). Strains that quickly show deficiency symptoms and high plant mortality were labelled *Zn-sensitive*. The genotypes that continued to uninterruptedly grow and yield under Zn-stress were labelled *Zn-tolerant*.

Mechanisms controlling Zn-efficiency remain largely unknown and the research of Zn-efficiency trait in plants focuses on the below-listed five mechanisms, which are individually discussed in the further text:

1. Morphological adaptations of the roots,
2. Root-induced processes to increase Zn bioavailability,
3. Intensification in Zn translocation from roots to shoots,
4. Changes in the subcellular compartmentalisation,
5. Increased rates of biochemical utilisation of tissue Zn.

Morphological modifications of the roots have been observed in variety of crop species. Root architecture has been repeatedly shown to influence plant's ability to sustain low Zn supply (Hajiboland et al., 2005). Root systems of efficient genotypes consist of thinner and longer roots compared to the sensitive plants (Rengel, 1996). Tolerant rice genotypes produce more crown roots per plant as shown recently (Mori et al., 2016). Both mechanisms increase the average root surface area therefore allowing higher accessibility and greater absorption rates of necessary nutrients. Some evidence suggests involvement of mycorrhizal fungi to increase the nutrient uptake from the medium (Kothari et al., 1991). However, such claims are largely disputed due to genotypic differences still being observed in hydroponically grown plants (*i.e.* where mycorrhizal colonies do not form) (Hacisalihoglu and Kochian, 2003).

Apart from the morphological modifications in response to low Zn conditions, roots actively modify chemistry of the surrounding rhizosphere in order to access physiologically unavailable Zn. *Root-induced changes in the speciation of the soil*

solution were proposed by various authors (Cayton et al., 1985; Kirk and Bajita, 1995; Wissuwa et al., 2006). Acidification of the rhizosphere by the activity of H^+ ATPase on the plasma membrane reduces the pH at intermediate root plane hence enabling solubilisation and subsequent transport of Zn down the ion gradient. The support for this claim can be found in the study of Kirk and Bajita who found

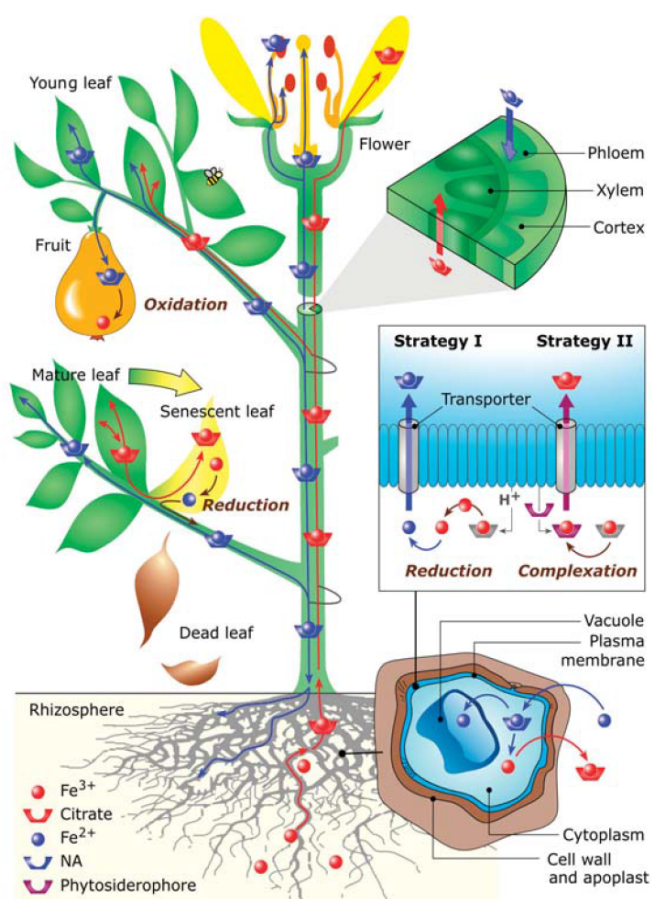


Figure 2.2 (Figure from von Blankenburg et al., 2009, permission acquired). Mechanisms of Fe uptake and translocation have been the most studied and elucidated to date. Under Fe-deficiency, plants utilise two strategies to sequester Fe from its physiologically unavailable $Fe(OH)_3$ form. Reduction by the membrane ATPase activity reduces Fe^{3+} to the soluble Fe^{2+} form; while chelation by phytosiderophore (PS) ligands help uptake Fe^{3+} in a $[Fe^{3+}(PS)]$ form. Further transport of ion within the plant is facilitated by a chain of ligand exchange reaction.

activity (Kirk and Bajita, 1995). Release of acidifying agents into soil is a known strategy to acquire physiologically inaccessible nutrients by mono- and dicotyledonous plants alike (Römheld and Marschner, 1986). Members of the family of grasses (Fam. Graminace) utilise an additional strategy, *i.e.* the secretion of metal-chelating ligands. The secreted ligands, known as *phytosiderophores* (PS), help solubilisation of metals in soil solution by forming complexes, and these complexes are taken up by roots via specialised membrane transporters (Figure 2.2). This mechanism was first shown to play an important role in uptake of Fe (Römheld and Marschner, 1986), however it was later suggested in facilitation of Zn uptake (Arnold et al., 2010a; Ptashnyk et al., 2011; Von Wiren et al., 1996; Widodo et al., 2010; Zhang et al., 1991) as well

as for several other transition elements (Marschner, 1995; Welch and Shuman, 1995). The former, acidification strategy is commonly referred to in the current literature as *strategy I*, whereas the ligand-facilitated micronutrient uptake is marked as *strategy II*, based on Fe uptake study of Romheld and Marchner (1986).

Numerous studies explored effects of *root and shoot Zn translocation rates* on the Zn-efficiency trait. By using radiotracer (^{65}Zn) flux techniques Hocasalihoglu et al. studied concentration-dependent kinetics of Zn^{2+} uptake across the root membrane. Using wheat plants grown on media with a wide range of external Zn^{2+} activities, the group identified two different root Zn uptake systems, where, following Michaelis-Menten kinetics, one was characterised by high velocity and low affinity (where Michaelis constant is $K_m = 2 - 5\mu\text{M}$) and the other one by low velocity but high affinity ($K_m = 0.6 - 2\text{nM}$) (Hacasalihoglu et al., 2001; 2003). The group concluded that the latter system is likely the dominant pathway for root Zn uptake under low soil Zn conditions *i.e.* where the Zn^{2+} activity in the soil solution is low. Farther down the uptake path, loading of transition metals into the xylem has been shown to be of a competitive character. Namely, transporter proteins responsible for loading transition metals into vascular stream do not differentiate between different transition elements therefore competition for the bindings spot occurs. Moreover, in paddy soils where Fe is rarely deficient, the concentrations of the competing Fe cations suppress sufficient Zn uptake and can lead to Zn-deficiency. Study by Wu et al. have shown that existence of mechanisms that support Zn loading into xylem over other divalent cations, can contribute to Zn-efficiency trait (Wu et al., 2011). Nonetheless, little evidence has been published so far to support the role of xylem loading in Zn-efficiency. In the studies of field-grown plants, no significant correlation was observed between genotypes differing in their response to low Zn conditions and their shoot Zn content for different wheat (Erenoglu et al., 1999; Hacasalihoglu, 2002; Kalayci et al., 1999), rye (Erenoglu et al., 1999) and bean (Hacasalihoglu, 2002) cultivars.

The effects of *subcellular Zn compartmentation* on the Zn-efficiency trait is little studied due to complexity of the research model, *i.e.* a living plant. Evidence based on radiogenic ^{65}Zn efflux tracer suggest similar Zn distribution in efficient and inefficient

wheat cultivars (Hacisalihoglu et al., 2003). A group of cellular Zn-reservoir peptides (e.g. metallothioneins) has been proposed to play an important role in Zn homeostasis under Zn-deficient conditions. Expression levels of two (*MT4a* and *MT4b*), out of seven so far characterised genes coding for metallothioneins in *Arabidopsis thaliana*, have shown to be correlated with seed Zn loading and seed germination in low-Zn conditions (Ren et al., 2012).

Evidence for the involvement of *biochemical Zn utilisation rates* in Zn-efficiency were reviewed by Hacisalihoglu and Kochian (2003). Activity of Zn-requiring enzyme carbonic anhydrase (CA) was investigated in various crop species, including wheat (Rengel, 1995) and rice (Sasaki, 1998). Data unanimously show that Zn deficiency decreases the activity of CA in plant. Carbonic anhydrase activity in a sensitive wheat cultivar decreased significantly when grown under Zn deficient conditions, unlike a tolerant genotype, which lost only a smaller percentage of its leaf CA activity. After translocation of test plants into sufficient Zn conditions, CA activity in the tolerant wheat recovered to normal levels while in the sensitive genotype the disturbance caused by Zn-stress was irreversible (Rengel, 1995). Activities of several other enzymes, including Zn/Cu superoxide dismutase (Zn/Cu SOD) (Cakmak et al., 1997; Hacisalihoglu et al., 2003), ascorbate peroxidase (AsA), glutathione reductase (GSSG), catalase (CAT) and guaiacol peroxidase (PER) were tested in connection with Zn deficiency (Cakmak and Marscher, 1993). Apart from PER activity, the activities of all other tested enzymes were significantly reduced. The studies all conclude that Zn deficiency in leaf tissues affects the enzymatic mechanism for neutralising oxidative attack of toxic oxygen radicals including superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2). Higher Zn-enzyme activities in tolerant cultivars maintain higher levels of protection against oxidative damage (Hacisalihoglu and Kochian, 2003) and steady the photosynthetic rate (Rengel, 1995). Activity levels of Zn-enzymes are proposed to be a good indicator of physiologically active Zn pool (Rengel, 1995). Observed differences in activities of various Zn-containing enzymes are possibly a consequence rather than the source of differential Zn efficiency trait between tolerant and sensitive genotypes, as the

observations show that differences in wheat growth rate are apparent before the differences in their CA activity can be identified (Rengel, 1995).

2.1.2. The role of phytosiderophores in plant physiology under Zn-stress

Phytosiderophores are non-protein amino acids with high affinity for binding transition metals (Sugiura et al., 1981). More than three decades ago, Takagi (1976) isolated a range of ‘iron-binding ligands’ from the root washings of oats. These small amino acids were named ‘phytosiderophores’ (Greek: *iron carriers in plants*) due to their function in Fe^{3+} chelation and their secretion was later identified in various crop cultivars, such as barley, wheat, rye, rice, maize and sorghum cultivars (Sugiura et al., 1981). Iron and its translocation in soil and plants has been the most widely investigated micronutrient system (Blindauer and Schmid, 2010), however, increased awareness of the Zn-malnutrition problem and its scale, have sparked interest of the scientific community to gain more understanding and provide sustainable solutions.

Romheld and Marchner (1986) distinguish two strategies used by plants to secure iron acquisition (Figure 2.2). Non-graminace plants acquire iron as Fe^{2+} (*Strategy I*), while graminace are able to take up Fe^{3+} complexed with phytosiderophores [Fe^{3+}PS] (*Strategy II*). The latter strategy plays a major role in response to low Fe conditions (Romheld and Hohenheim, 1991). Numerous studies theorised that grasses use similar mechanism to facilitate Zn acquisition in Zn-deficient conditions (Arnold et al., 2010a; Romheld and Hohenheim, 1991; Welch and Shuman, 1995; Widodo et al., 2010). Among a wide range of organic ligands that have been proposed to play a PS role are low molecular weight organic acids (e.g. citrate, oxalate, malate) (Hoffland et al., 2006), amino acids (especially histidine and cystein), phytosiderophore nicotinamine, and its derivatives (i.e. mugineic acids) in graminaceous plants (Blindauer and Schmid, 2010).

2.1.3. The family of Mugineic acids

2.1.3.1. (Bio)synthesis of mugineic acids

Mugineic acids (MAs) are a family of amino acids with various hydroxylation patterns (Figure 2.3). The structure of these amino acids is characteristic for its three carboxylic groups and an azetidine ring (4-membered ring with nitrogen as its heteroatom).

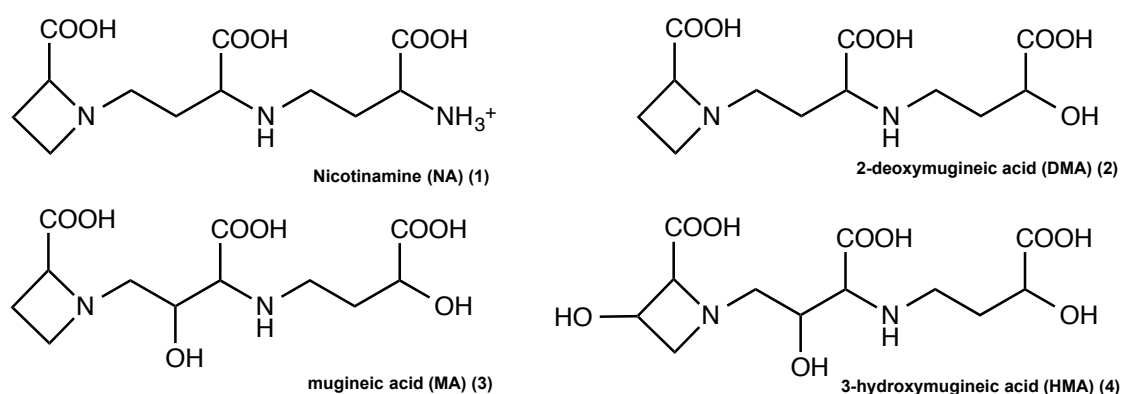


Figure 2.3 The difference between mugineic acids (2-4) and nicotinamine (1) (their biosynthetic precursor), in the affinity for Zn originates from the coordination with different apical donors. While NA coordinates to Zn with its terminal NH_2 group, MAs forms an apical bond to Zn with its terminal OH.

The biosynthesis of MAs is closely related to the methionine cycle (Ma et al., 1995). The biosynthetic pathway of MAs starts with L-methionine (Mori et al., 1987; Suzuki et al., 2006). Three molecules of L-methionine, which are first converted to S-adenosyl-methionine (SAM) by SAM synthetase, form a molecule of nicotinamine (NA). Further conversion of NA into a 3'-keto acid by the involvement of NA aminotransferase and subsequent reduction leads to the creation of 2'-deoxymugineic acid (DMA). DMA then serves as substrate for dioxygenases IDS2 and IDS3 (Kobayashi et al., 2001) in the process of constructing other MAs such as MA and epiMA (Figure 2.4). Nicotinamine (NA) is a molecule with a major role in metal trafficking in plants (Nishiyama et al., 2012). However its derivatives from the mugineic acid family have higher metal-binding affinities and thus are considered more efficient phytosiderophores. Zn-deficiency increases the expression of enzymes involved in both methionine cycling and MA synthesis in roots (Suzuki et al., 2006).

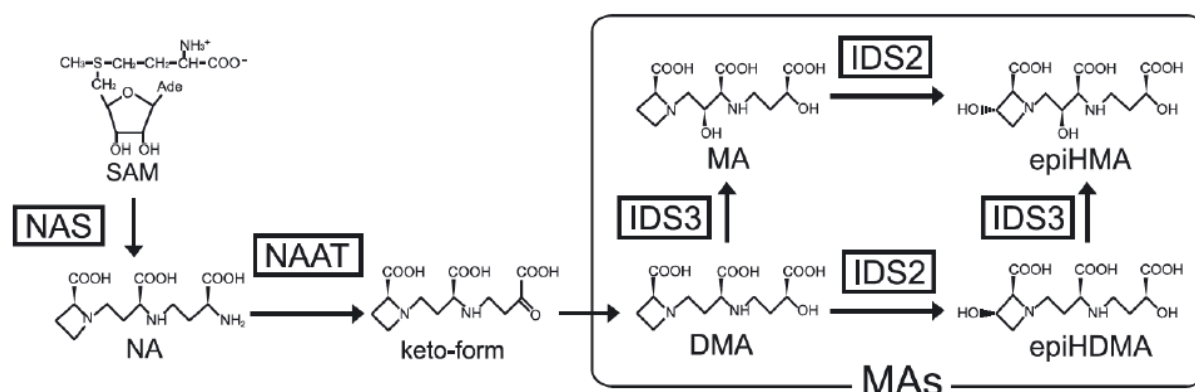
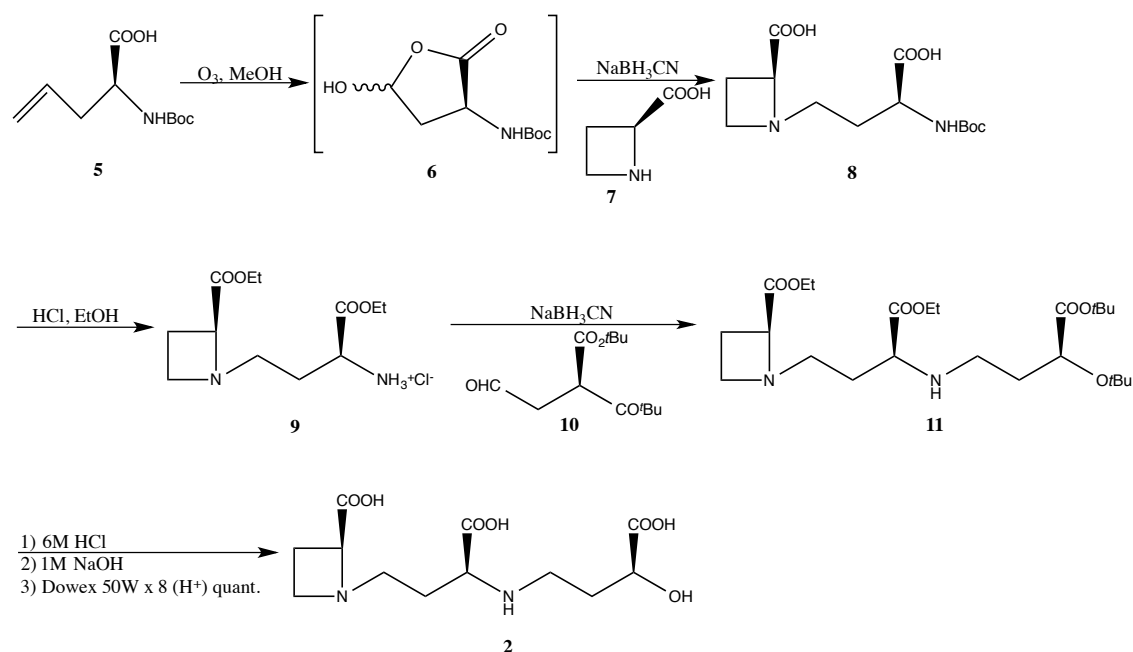


Figure 2.4 (Figure from Suzuki et al. 2006, permission acquired) Biosynthetic pathway of mugineic acids from L-methionine.

Increased interest in studying biological roles of phytosiderophores has prompted development of protocols to isolate and/or synthesise mugineic acids and related molecules. Multiple protocols were developed to synthesise pure mugineic acids in laboratory conditions (Fushiya, 1981; Jung and Kim, 1999; Klair S. S., 1998; Matsuura et al., 1992; Matsuura et al., 1994; Miyakoshi et al., 2001; Ohfune, 1981; Shioiri, 1997; Singh et al., 2005).

In 2007, Namba and co-workers developed a ‘one-pot’ synthetic protocol to produce ample quantities of MA molecules for biological studies (Namba et al., 2007). This synthesis method used inexpensive building blocks (α -hydroxybutyric acid, α -aminobutyric acid and azetidine-2-carboxylic acid) coupled using successive reductive amination steps to construct DMA and subsequently other mugineic acids (Namba and Murata, 2010; Shioiri, 1997). The practicality and efficacy of this protocol is largely due to the minimal number of column chromatography purification steps required, compared to other methods. Recently, Walter et al., (2014) reviewed the protocol of Namba et al. (2007) and suggested improvements towards obtaining higher yields of final products, particularly in the domain of the parallel synthesis of aldehyde **10** (Scheme 2.1).



Scheme 2.1 Synthesis of 2'-deoxymugineic acid using reductive amination steps and commercially available building blocks, as reported by (Namba et al., 2007).

2.1.3.2. Crystal structure of metal complexes with MAs.

Mugineic acids chelate preferentially divalent metal cations in 1:1 ratio, however complexation of Fe^{3+} by MA is of great importance for plant physiology (Römheld and Marschner, 1986). The crystal structures of mugineic acid complexes with cobalt(III) (Mino et al., 1983) and copper(II) (Mino et al., 1981; Nomoto, 1981) have been reported. All mugineic acids bind transition metals in a nearly octahedral configuration where the azetidine nitrogen (N^1), secondary amine nitrogen (N^2) and oxygens of both terminal carboxylic acids (O^1 and O^5) coordinate as basal planar donors whereas the hydroxyl oxygen (O^8) and intermediate carboxylate oxygen (O^3) are axial donors (Mino et al., 1983) (Figure 2.5). Neither C^3 and C^4 of the azetidine ring, nor the differentiating hydroxyl group of mugineic acids take part in metal complexation. However, the terminal alcoholic group plays an important role in complexing metal, considering the large difference in the biological activity between 2'-deoxymugineic acid and its precursor – nicotinamine (Mino et al., 1983), as it will be discussed in the further text.

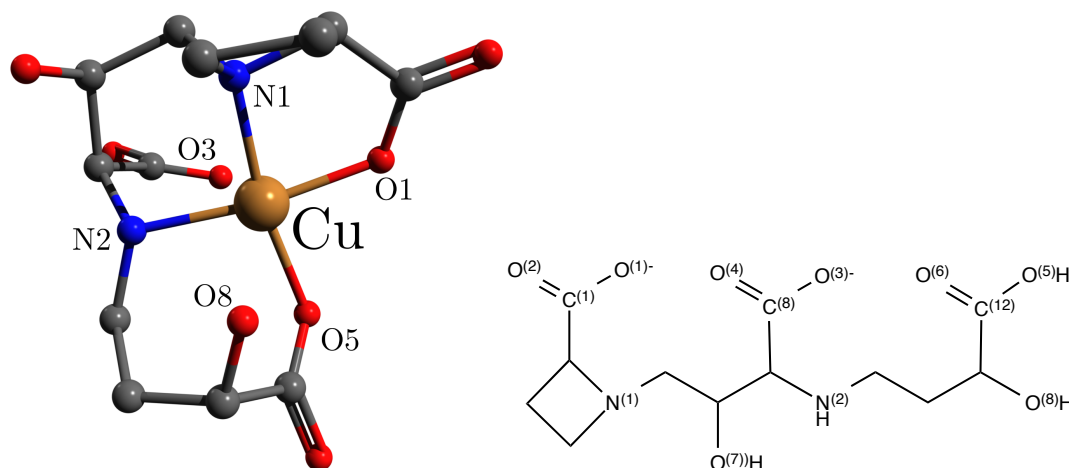


Figure 2.5 (Left) Crystal structure of $[\text{Cu}(\text{MA})]^{2-}$ complex determined by X-ray diffraction (Mino et al., 1981). The image has been generated using Avogadro software (from crystal structure deposited in the CCDC database, ID: MUNGCU). **(Right)** Mugineic acid coordinates to metals by forming an octahedral complex where the azetidine (N^1), secondary amine nitrogen (N^2) and oxygens of both terminal carboxylic acids (O^1 and O^5) coordinate as basal planar donors whereas the hydroxyl oxygen (O^8) and intermediate carboxylate oxygen (O^3) are axial donors.

The phytosiderophore role of mugineic acids arises from their ability to chelate metal cations and form very stable complexes (Namba and Murata, 2010; Shioiri, 1997). The affinity constants ($\log K$) for 1:1 complexes with physiologically essential cations are given in Table 2.1. As can be seen from this table, the stability of metal complexes with mugineic acids are significantly higher in comparison with average values for natural ligands (Bowles et al., 2006). The difference in molecular structure between NA and DMA is only in the terminal functional group (Figure 2.3). Instead of the axial hydroxyl donor group of DMA (**2**), NA (**1**) forms bond via its terminal amino group. As a consequence of the altered molecular geometry, the metal binding affinities (shown in Table 2.1) are different for the two ligands.

The MAs ligand affinity for metals elements decreases in the following order: $\text{Ca}^{2+} < \text{Mn}^{2+} < \text{Fe}^{2+} < \text{Ni}^{2+} < \text{Cu}^{2+} > \text{Co}^{2+} \geq \text{Zn}^{2+}$ (Murakami et al., 1989), which is consistent with Irving-Williams series of divalent first-row transition metals (Irving and Williams, 1953).

The data available originates from potentiometric studies conducted during the 1980s using ligands isolated from the plant root washings (Table 2.1). Although isolation from plant material used to be a standard method to obtain phytosiderophore ligands, the downside of this approach is that the purity of the ligands was not very

high and a cross contamination between different mugineic acids is possible (Hiradate and Inoue, 1996; Neumann et al., 1999). High purity of ligand samples is essential when determining affinity constants of a ligand for a given metal (Blindauer and Schmid, 2010). Hence, the inconsistencies in published stability constants ($\log K$) can be explained by the insufficient purity of the ligand sample used, *e.g.* a mixture of more than one ligand from the mugineic acid family as a consequence of a low sensitivity of the separation method (Hiradate and Inoue, 1996; Neumann et al., 1999). Accurate affinity constants, however, are essential for modelling metal solubilisation behaviour in natural systems. Therefore, it is imperative to confirm the values of the above stability constants (Table 2.1) by running high-precision potentiometric titrations using pure synthetic ligands.

Table 2.1 Stabilities of metal complexes with phytosiderophores from the mugineic acid family and their precursor – nicotinamine expressed as log K . All studies listed in this table used ligands isolated from root washings and thus the probability of ligands cross-contamination is high. Neumann et al. (1999) developed for the first time a high-resolution separation procedure to quantitatively separate different mugineic acids from the same sample (particularly, MA from DMA).

Ligand	Metal	Log K	Reference
Nicotinamine	Zn^{2+}	15.4	Anderegg and Ripperger ,1989
		14.7	Beneš et al. 1983
	Fe^{2+}	12.3	Anderegg and Ripperger ,1989
		12.1	Beneš et al. 1983
	Fe^{3+}	18.1	Beneš et al. 1983
Mugineic acid (MA)	Zn^{2+}	10.7	Sugiura et al., 1981
		12.69	Murikami et al., 1989
	Fe^{3+}	18.1	Sugiura et al., 1981
		17.71	Murikami et al., 1989
	Fe^{2+}	8.1	Sugiura et al., 1981
		10.14	Murikami et al., 1989
2'-deoxymugineic acid (DMA)	Al^{3+}	13.4	Yoshimura et al., 2011
	Zn^{2+}	12.8	Murikami et al., 1989
		10.45	Murikami et al., 1989
		18.38	Murikami et al., 1989
	Fe^{2+}	12.43	Murikami et al., 1989
		10.02	Murikami et al., 1989
3-epi- hydroxymugineic acid (2'-HMA)	Fe^{3+}	15.49	Murikami et al., 1989

2.2. Stable isotopes as novel approach to tracing the fate of metals in the environment

The development of multi collector plasma source mass spectrometers has opened up the door to study metal dynamics in the plant-soil systems as isotope ratios can be determined with the precision needed to resolve small changes in the distribution of stable isotopes. Observed stable isotope fractionations can then be successfully used to infer chemical processes.

The theory of stable-isotope fractionation is well established (Bigeleisen and Mayer, 1947; Hoefs, 2009; Urey, 1947). The differences in physicochemical properties arising from variations in mass are called *isotope effects*, while the partitioning of isotopes between two substances with different isotope ratios is referred to as *isotope fractionation*. Stable-isotope variations of elements above 40 amu are small and hence expressed using the *per mill* (δ) notation, expressing the deviation from the isotope composition of a reference standard:

$$\delta^i_j X_{A,B} = \left(\frac{\text{isotopic ratio of sample}}{\text{isotopic ratio of standard}} - 1 \right) \times 1000 \quad (2.1)$$

where i and j are the isotopes assessed.

The reference standard used is either a certified isotopic standard (IRMM-072) or a widely used industrial single-element standard (Lyon Zn, Romil Zn, Imperial Zn).

The partitioning of an element's stable isotopes between two molecules or phases (A and B) is given by the isotopic fractionation factor (α):

$$\alpha_{A-B} = \frac{R_A}{R_B} \quad (2.2)$$

where R_A and R_B are isotope ratios in A and B, respectively.

As $\alpha_{A-B} \approx 1$, then:

$$10^3 \ln \alpha_{A-B} \approx \delta^i X_A - \delta^i X_B \cong \Delta^i X_{A-B} \quad (2.3)$$

where $\Delta^i X_{A-B}$ (or commonly $\Delta^i X_{A-B}$) is the fractionation between substances A and B.

Isotopes of the same element have different physicochemical properties, which then lead to different behaviour on a sub-molecular scale. For example, due to the lower zero-point energy, lighter isotopes form bonds more quickly compared to heavy isotopes, however their bonds are weaker. Conversely, bonds formed by heavy isotopes are stronger and the reactions involving heavy isotopes occur at a slower pace. As a consequence, mass-dependant isotopic fractionation occurs during equilibrium and kinetic controlled chemical reactions.

Equilibrium isotopic fractionation occurs upon isotopic exchange when the rates of forward (k_f) and backward (k_b) reactions are identical:

$$K_{\text{equil}} = {}^i K / {}^j K = ({}^i k_f / {}^i k_b) / ({}^j k_f / {}^j k_b) \quad (2.4)$$

where i and j are heavy and light isotopes, respectively; and K is the equilibrium constant.

The relative isotopic abundance is governed by energy differences in bonding environments of reaction partners at isotopic equilibrium. Such reaction favours heavy isotopes, as they often rely on formation of stronger and more stable bonds. As a result the isotopic fractionation is a positive value ($\delta^{i/j} X_{A-B} > 0$).

On the other hand, upon unidirectional reactions, where forward and backward reactions are dissimilar ($k_f \neq k_b$), *kinetic isotopic fractionation* occurs. Examples of unidirectional processes are evaporation, diffusion and most biological reactions (e.g. photosynthesis). Kinetic processes discriminate against the heavy, and in favour of the light isotope, thus generating a negative isotopic fractionation ($\delta^{i/j} X_{A-B} < 0$).

2.2.1. Isotope fractionation during $[M^{n+}L]$ complex formation

Measurements of isotopic fractionation in the natural systems can advance our knowledge of plant metal uptake and translocation, hence providing essential data

for crop biofortification programs. The distribution of stable isotopes in the soil-plant environment is a valuable indicator of physicochemical mechanisms taking place during the uptake of Zn into the plant. To this end, however, it is important to quantify extent and direction of isotope fractionation during the relevant physicochemical processes.

2.2.1.1. Experimental determination of isotopic fractionation in metal complexes with organic ligands

In order to study the cycling of the metallic elements in nature, relevant mass-biased reactions that commonly occur in the environment, *e.g.* metal binding by organic molecules, first need to be assessed in the laboratory, in view of isotopic fractionation factors possibly introduced to the environment. The major obstacle, however, in the experimental determination of isotopic partitioning in metal-ligand complexes $[M^{n+}L]$ is separating free (M^{n+}) from complex ($[M^{n+}L]$) species without inducing fractionation during the separation process (Wiederhold, 2015). Methods applied to study fractionation during complexation include phase separation (such as precipitation of insoluble species) (Bigalke M., 2010; Dideriksen et al., 2008), dialysis bags (Morgan et al., 2010) and Donnan-membrane system (Jouvin et al., 2009; Ryan, 2014).

Due to demanding and cumbersome experimental approach, very limited data is available on isotopic effects of Zn complexation. Isotopic partitioning in Zn complexes with humic acids was tested by Jouvin et al. (2009) using NICA-Donnan model to separate free Zn^{2+} on one side, from Zn bound to PHA ($[Zn(PHA)]$) on the other side of the Donnan membrane, at equilibrium. They found that heavy ^{66}Zn is preferentially incorporated into $[Zn(PHA)]$ complex relative to the ^{64}Zn isotope (by 0.24 ‰). The study confirms the theoretical postulations of Urey (1947) that metal complexes with organic ligands should be enriched in the heavier isotope at equilibrium. A similar conclusion was reached in studies exploring isotopic partitioning in organic complexes of other metals such as Mg (Black, 2007), Cu (Bigalke M., 2010; Ryan, 2014) and Fe (Dideriksen et al., 2008; Morgan et al., 2010).

Magnesium is essential for maintaining normal plant tissue functions, particularly the photosynthetic apparatus. During the biosynthetic pathway of chlorophyll molecules, Mg is incorporated in the centre of a porphyrin ring. This reaction was shown to fractionate heavy ^{26}Mg into the chlorophyll complex. The magnitude of the isotopic partitioning caused is 0.60 ‰ and 0.17 ‰, in Mg complexes with chlorophyll-a (Chl-a) and chlorophyll-b (Chl-b) respectively (Black, 2007).

Copper is essential micronutrient and takes various roles in plant metabolism, such as electron transport during photosynthesis and lignin formation (Burkhead et al., 2009). Bigalke et al. (2010) and Ryan et al (2014) used semi-permeable membranes for the separation of different Cu species in order to determine the equilibrium isotope fractionation in Cu complexes with natural and synthetic ligands. Following the equilibration of insolubilized humic acid (IHA) with Cu donor species, enrichment of ^{65}Cu over ^{63}Cu was found in the $[\text{Cu}(\text{IHA})]$ complex ($\delta^{65}\text{Cu}_{[\text{Cu}(\text{IHA})]} - \text{free Cu} = 0.26 \text{ ‰}$) (Bigalke M., 2010). As the organic component of soil mostly consists of humic acids, knowing the exact magnitude of isotopic fractionation in $[\text{Cu}(\text{IHA})]$ provides essential information for soil speciation modelling. Furthermore, heavy ^{65}Cu was preferentially found in 1:1 (M:L) organic complexes with synthetic hexadentate chelators CyDTA and EDTA ($\Delta^{65}\text{Cu}_{[\text{Cu}(\text{CyDTA})]} - \text{free Cu} = 0.62 \text{ ‰}$ and $\Delta^{65}\text{Cu}_{[\text{Cu}(\text{EDTA})]} - \text{free Cu} = 0.51 \text{ ‰}$, respectively); tetradentate ligand nitriloacetic acid (NTA, $\Delta^{65}\text{Cu}_{[\text{Cu}(\text{NTA})]} - \text{free Cu} = 0.44 \text{ ‰}$) and Suwannee River fulvic acid (SRFA; $\Delta^{65}\text{Cu}_{[\text{Cu}(\text{SRFA})]} - \text{free Cu} = 0.14 \text{ ‰}$) (Ryan, 2014). The highest fractionation during Cu binding was found in the Cu complex with the microbial siderophore, desferoxamine-B (DFOB; $\Delta^{65}\text{Cu}_{[\text{Cu}(\text{DFOB})]} - \text{free Cu} = 0.84 \text{ ‰}$) (Ryan, 2014).

Fractionation during the complexation of desferoxamine B complex with Fe was tested both experimentally (Dideriksen et al., 2008; Morgan et al., 2010), and by using theoretical calculations (Domagal-Goldman et al., 2009). Dideriksen et al. (2008) and Morgan et al. (2010) suggested that DFOB preferentially complexes heavier Fe isotope. Dideriksen et al. (2008) showed that metal coordination with DFOB yields equilibrium isotopic factor of 0.60 ‰ ($\Delta^{56}\text{Fe}_{[\text{Cu}(\text{DFOB})]} - \text{free Fe}$). Conversely to the experimental values, theoretical estimations of isotopic partitioning

in [Fe(DFOB)] predicted preference for the light isotope (by 0.3 ‰) (Domagal-Goldman et al., 2009).

Isotopic factors introduced to the environment during metal complexation by organic ligands is significant when taking into consideration the isotopic variability of the observed elements in nature (~ 1.8 ‰ for Zn (Cloquet et al., 2008; Weiss et al., 2008), ~ 1.5 ‰ for Cu (Zhu et al., 2002) and 3 ‰ for Fe (Beard, 1999)). Therefore, identification of isotopic effects upon complexation events in natural systems is an important milestone for geochemistry research of complex processes at the plant-soil interface.

2.2.1.2. Theoretical estimations of complexation induced fractionation factors

Quantum mechanical calculation methods such as *ab initio* modelling have shown a great potential for estimating isotopic effects introduced to the environment during physico-chemical reactions. *Ab initio* approach takes into consideration the first principles of quantum mechanics to calculate zero-energy of the observed species. The theoretical background of this methodology is well established through the works of Urey (1947) and Bigeleisen and Mayer (1947), however the mathematical foundation of this concept was first laid out by Hohenberg and Khon (1964).

Quantum mechanical methods can predict chemical properties of a chemical system (atoms, molecules, *etc.*) by calculating system's energy functions. On this basis, calculations can provide estimations of isotope enrichment factors in different substances, based on the differences in the electronic states of the isotopologues (Fujii et al., 2014). Further details on modelling isotopic fractionation can be found in the introduction section of the Chapter 5 of this thesis.

Theoretical studies focused on modelling isotopic effects of solution speciation and complexation are available for a range of different elements including Mg (Black, 2007; Schauble, 2011), Cr (Schauble et al., 2004), Fe (Domagal-Goldman and Kubicki, 2008; Domagal-Goldman et al., 2009; Fujii et al., 2014; Moynier et al., 2013; Ottonello and Vetusch Zuccolini, 2008), Ni (Fujii et al., 2014; Fujii et al., 2011), Cu (Fujii et al., 2013; Fujii et al.; Seo et al., 2007), Mo (Tossell, 2005) and Cd (Yang et

al., 2015). In most of the studies published to date, Zn complex geometries were modelled in vacuum, *i.e.* in the absence of a solution. However, the solvation sphere can alter fractionation effects significantly (Black et al., 2011) and therefore should be considered if the computational cost is permissible. Rudolph and Pye (1999) derived optimal solvation shell geometries of Zn^{2+} , and reported that an octahedrally coordinated first shell consists of six water molecules bound to the Zn via oxygen atoms of water molecules. Studies have also shown that addition of more water molecules to the solvation sphere decreases the fractionation factors by $\sim 0.2\text{‰}$ at 25 °C (Black et al., 2011; Fujii et al., 2014; Fujii et al., 2011). Therefore, careful considerations of molecular geometries are necessary when comparing theoretical estimations.

Fractionation factors in complexes of both inorganic salts (Black et al., 2011; Fujii and Albarede, 2012; Fujii et al., 2014; Fujii et al., 2011) and organic complexes of Zn (Fujii and Albarede, 2012; Fujii et al., 2014) were investigated using computational methods. Fujii and co-workers conducted several studies focused on determination of isotopic partitioning in Zn species relevant to low temperature geochemistry and biology. Using the *ab initio* approach, the group has shown that aqueous sulphate and carbonate Zn species were enriched (up to $\sim 1\text{‰}$) in heavy ^{66}Zn , while the observed Zn sulphides and chlorides were marginally depleted (Fujii et al., 2014; Fujii et al., 2011). By simulating fractionation effects upon Zn complexation with naturally present organic ligands, Fujii and Albarede (2012) offered an explanation for the Zn isotopic distribution observed in plants. The group has modelled fractionation values in selected Zn complexes, previously identified by Sarret et al (2002), and concluded that heavy ^{66}Zn enrichment in plant roots is due to complexation to phosphates present in the below-ground plant organs (Fujii and Albarede, 2012). Similarly, calculations identified that the light isotopic enrichment in the areal plant organs is due to malate and citrate Zn species present in those tissues (Sarret et al., 2002), which were shown to preferentially incorporate light ^{64}Zn (Fujii and Albarede, 2012). Coordination plays an important role in estimations of fractionation values, as shown by Black et al (2011). The group modelled different

geometries of hydrated Zn-citrate complex and predicted an opposite fractionation effect to those of Fujii and Albarede (2012).

Only recently, theoretical studies attempted to predict isotopic partitioning in metal complexes of a greater molecular weight. Moynier et al. (2013) modelled for the first time fractionation effects upon Fe complexation with phytosiderophore ligands. However, no studies attempted similar calculations with phytosiderophore complexes of Zn.

Ab initio modelling is a very powerful tool to test the origin of isotopic signatures found in the environment. However before interpreting data gathered by theoretical modelling, great care has to be taken in terms of computational method verification, rationale for the molecular geometries selected and, finally, solvation environment chosen. Moreover, Schauble (2004) indicated that error propagation associated with *ab initio* calculations yields high levels of uncertainty. Yet, no studies published thus far reported error approximations for the calculated values, nor this issue was discussed in depth in the current literature.

2.2.2. Interpretation of stable isotope signatures in the environment

As discussed earlier, distribution of stable isotopes in the environment is a helpful guide to understand processes taking place during biogeochemical cycling of relevant elements. Stable isotopes have repeatedly been suggested as valuable tracers of biogeochemical processes in nature (Arnold et al., 2015; Deng et al., 2014; Houben et al., 2014; Kiczka et al., 2010; Tang et al., 2016; Tang et al., 2012; von Blanckenburg et al., 2009; von Wiren, 2011; Weiss et al., 2008; Wiederhold, 2015).

Plants contribute to isotopic signatures during Zn cycling in the environment. Yet, very significant contributors to Zn natural isotopic variability are geochemical processes altering the soil Zn reservoir; these include complexation with organic particles, sorption onto inorganic particles of different mineral species (*e.g.* oxides and hydroxides), dissolution, precipitation etc. (Cloquet et al., 2008; Wiederhold, 2015). Figure 2.6 (based on Cloquet et al. (2008)) outlines fractionation factors of a range of both geochemical and biological reactions involving Zn.”

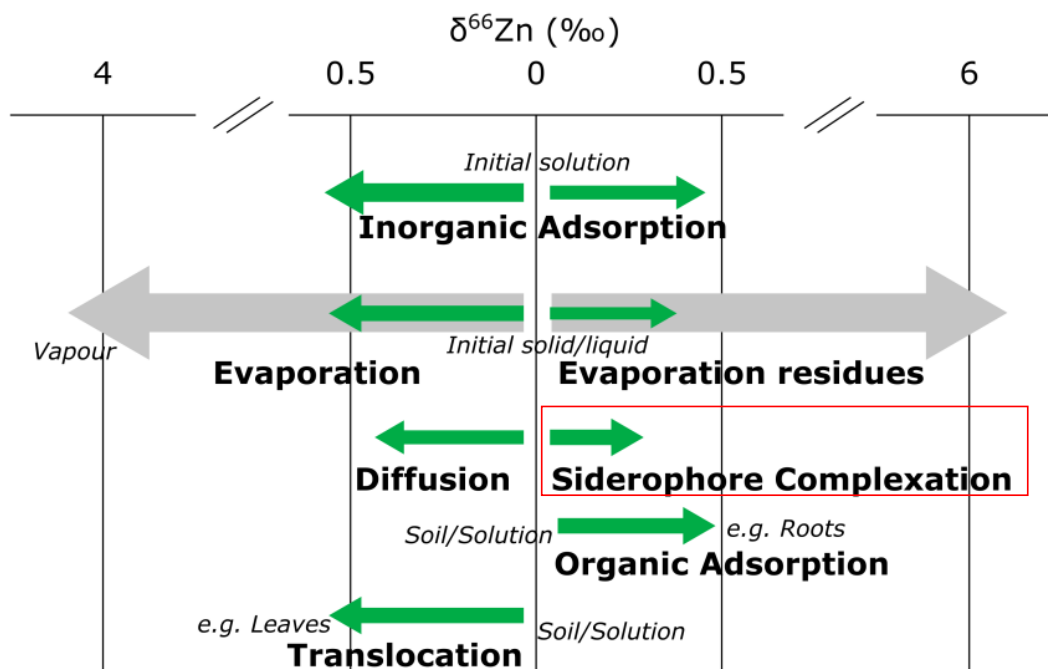


Figure 2.6 (Figure based on Cloquet et al. (2008)) Zn isotopic variability in the environment is affected by a range of biological and geochemical reaction occurring during Zn cycling. Complexation with phytosiderophore ligands (framed) could potentially introduce a significant isotopic effect in plant Zn reservoirs.

Motion of metals between various reservoirs in the environment creates traceable signatures that can be used to explain plant nutrition strategies. Significant metal stable isotope fractionation in higher plants have been demonstrated for a range of elements including Fe (Guelke and von Blanckenburg, 2007; Kiczka et al., 2010), Zn (Arnold et al., 2010a; Arnold et al., 2015; Caldelas et al., 2011; Deng et al., 2014; Houben et al., 2014; Jouvin et al., 2012; Moynier et al., 2009; Smolders et al., 2013; Tang et al., 2016; Tang et al., 2012; Viers et al., 2007; Weiss et al., 2005), Mg (Black et al., 2008; 2007) and Cu (Jouvin et al., 2012; Ryan et al., 2013; Smolders et al., 2013; Weinstein et al., 2011).

While investigating isotopic patterns in plants, Guelke and von Blankenburg (2007) discovered that Fe isotopic partitioning in graminaceous and non-graminaceous plants is consistent with the Fe uptake strategies described earlier by Romheld and Marchner (1986). The study identified two possible mechanisms utilised by plants to obtain Fe from soil. Dicotyledonous and non-graminaceous monocotyledonous plants mobilise Fe via rhizosphere acidification process, which drives reduction of insoluble

Fe^{3+} species to the more soluble Fe^{2+} (*strategy I*, Figure 2.2). Gramineaceous species, on the other hand, developed an uptake mechanism to sequester Fe^{3+} via the chelating activity of root-secreted phytosiderophore ligands (*i.e. strategy II*) (Römheld and Marschner, 1986). Considering that ligand complexation is expected to favour heavy isotopes (Urey, 1947), Guelke and von Blanckenburg (2007) explained the heavy fractionation found in graminaceous species by preferential Fe uptake in as $[\text{Fe}^{3+}(\text{PS})]$ complex. Moreover, in all non-graminaceous plants observed, (Guelke and von Blanckenburg, 2007) found that Fe isotopic fractionation was in line with Fe^{2+} uptake or *strategy I* mechanisms as described by Romheld and Marchner (1986).

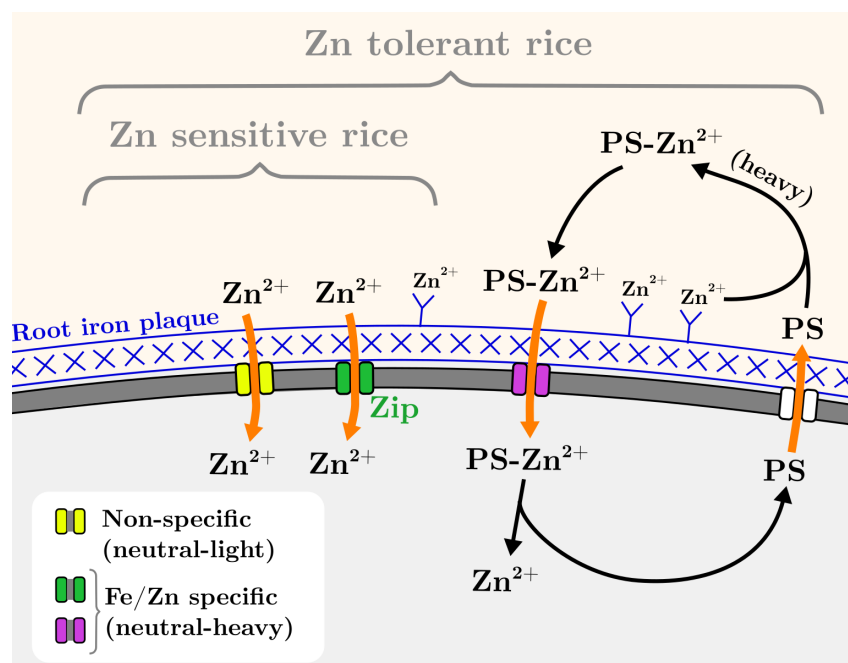


Figure 2.7 Summary of the proposed mechanisms in the basis of the genotypic differences in rice cultivars. At alkaline pH conditions, Zn is adsorbed onto iron oxides that form plaque-like coating on the root surface, and thus is physiologically unavailable to plants. Two mechanisms used by rice to solubilise Zn have been described to date. In addition to acidification of rhizosphere that would in turn stimulate cation uptake across plasma membrane, a PS-facilitated Zn uptake mechanism was suggested (Arnold et al., 2010a). The heavy fractionation in tolerant rice was explained by Zn complexation and uptake by PS ligands under Zn-deficient conditions. *Indications in brackets refer to processes leading to isotopic fractionation.

Zinc reservoirs of both hydroponically (Weiss et al., 2005) and soil-grown plant (Arnold et al., 2010a; Arnold et al., 2015; Viers et al., 2007) have shown a systematic redistribution of stable Zn isotopes when compared to their natural ratios (Rosman

and Taylor, 1998). The $\delta^{66}\text{Zn}$ values of rice and tomato shoots show depletion of heavy ^{66}Zn isotope (by 0.2 – 0.4 ‰) compared to the nutrient solution the plants were grown on (Weiss et al., 2005). Similarly, Viers et al. (2007) reported a gradient of ^{66}Zn depletion along the vertical axis of plants. The predominance of ^{64}Zn in the upper parts of the plant was explained by long-distance transport of Zn via xylem, which favours lighter and thus more mobile isotope (Tang et al., 2012; Viers et al., 2007; Weiss et al., 2005). On the other hand, the enrichment in heavy ^{66}Zn in roots and belowground organs of various plant species (Aucour et al., 2011; Caldelas et al., 2011; Houben et al., 2014; Moynier et al., 2009; Tang et al., 2016; Tang et al., 2012; Viers et al., 2007; Weiss et al., 2005) was justified by equilibrium controlled isotope fractionation during Zn binding events, including adsorption onto the roots and plaques (Weiss et al., 2005), formation of complexes with organic ligands (Aucour et al.; Jouvin et al., 2012; Smolders et al., 2013), such as organic anions (e.g. citrate), amino acids (Arnold et al., 2010a) or phytochelatines in dicotyledonous species (Caldelas et al., 2011).

In a recent study by Arnold et al. (2010a), heavy Zn isotopic enrichment in Zn-deficient rice has been justified by ligand-facilitated uptake. Phytosiderophore 2'-deoxymugenic acids was suggested as a likely candidate for Zn-complexation in deficient rice plants, as it was previously demonstrated that eluted DMA levels are considerably higher in tolerant compared to intolerant cultivars (Suzuki et al., 2006). Although complexation with DMA has been suggested as an important contributor to the heavy $\Delta^{66}\text{Zn}$ found in plants, no attempts have been made to identify, in laboratory conditions or theoretical studies, the isotopic effect upon $[\text{Zn}(\text{DMA})]$ binding. Once the magnitude of isotopic partitioning during this interaction is clear, further progress towards understanding the Zn uptake mechanism facilitated by phytosiderophore molecules is possible.

2.2.3. Identification of efficient seed filling mechanisms as a final step towards identifying Zn biofortification targets

The ultimate goal of Zn biofortification programs is to ensure higher grain yields as well as higher Zn content of the grains. In addition to obstacles upon Zn entering the

plant tissues, several stages during Zn translocation within the plant are highlighted as possible bottlenecks for sufficient Zn supply. During Zn transport from roots to grain, Zn is thought to exit and re-enter the symplast at least twice: during xylem loading in the roots and during phloem unloading in fruits (Palmgren et al., 2008) in most plants. At the end of its translocation via symplast towards the root's stele, Zn is actively loaded into the apoplastic xylem (Sondergaard et al., 2004). Xylem loading (Figure 2.8) was shown to be one of the key contributing mechanisms to metal distribution within the plant (Hussain et al., 2004; Zhu et al., 2007). The reason for this is not only allowing necessary nutrients to travel to the aboveground parts of the plant, but also to restrict the intake of possible toxic elements, such as Cd (Mills et al., 2005).

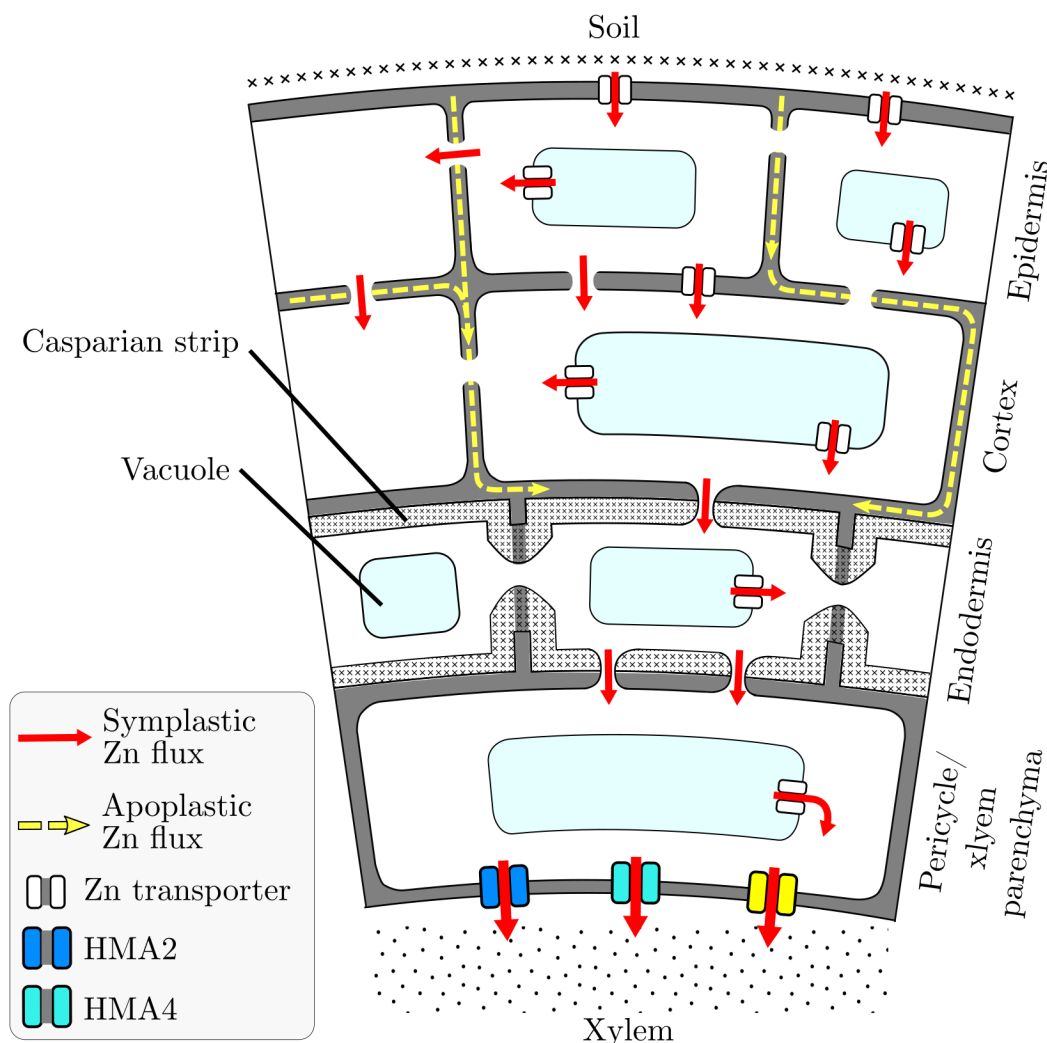


Figure 2.8 Once Zn passes the membrane barrier at the root epidermis, it travels down the apoplastic or symplastic pathways towards the root stele. The suberized layer of endodermis cells called the Casparian strip disables the progression of apoplastically traveling Zn ions, hence the apoplastic Zn is transported into the cytoplasm of the next cell where it joins the symplastic stream via plasmodesmata connections. Zinc is then loaded into the xylem by the activity of specialised heavy metal transporters (e.g. HMA4 and HMA2 in *A. thaliana*)

HMA2 and HMA4 (Heavy Metal transporting ATPase) Zn pumps located in the root xylem parenchyma were shown to play a significant role in Zn homeostasis of *Arabidopsis sp.* (Hussain et al., 2004; Nouet et al., 2015; Yoneyama et al., 2015). Xylem parenchyma of *A. thaliana* contains proton pumps on its plasma membrane, however double mutant *hma2hma4*, with disrupted HMA2 and HMA4 (Heavy Metal transporter ATPase) activity, displays Zn-deficiency symptoms such as chlorosis and shoot stunting (Hussain et al., 2004). This double mutant suffers inadequate Zn transport towards shoots and other aboveground organs, while accumulating Zn in

the roots. Cadmium was also shown to be loaded into xylem by the activity of HMA4 pump (Mills et al., 2005). Cadmium is an element that mimics Zn in transporters' active sites and hence can be harmful for the organism if balanced xylem loading is not maintained. HMA4 has been suggested to play an important role in efficient Zn translocation in dicots (Hussain et al., 2004), however it is not clear whether this transporter is relevant for Zn homeostasis in monocots. Metal transporters are currently best known in *Arabidopsis sp.* but some evidence has been presented for cereal species (Yoneyama et al., 2015). HMA1 gene has been proposed to control for Zn xylem loading (Williams et al., 2005) but further studies are needed to completely enlighten this mechanism.

Zinc travels from roots to shoot via xylem, whereas the redistribution of Zn from senescing to new leaves and seeds occurs via phloem (Page and Feller, 2005). The pathways of Zn remobilisation are still not completely understood. Thus, stable isotope studies could help untwine these mechanisms by monitoring the Zn isotopic fractionation along the Zn uptake path from roots to seeds.

During their long-distance transport via xylem, Zn and other essential metals are primarily in their complex form. Metals in xylem sap are chelated by a range of organic ligands. Further transport of micronutrients into the leaf tissue requires active involvement of the vessel-associated cells that surround the xylem. Once actively taken up by these cells, metal ions move using symplastic pathways until reaching tissues in leaf where they are utilised or stored.

Grain filling is a dynamic stage during which nutrients are loaded from the phloem as well as remobilised from the senescing leaves. Xylem does not reach cereal grains, except in rice cultivars (Impa and Johnson-Beebout, 2012), therefore long-distance transport of nutrients to seeds is achieved by their unloading from xylem and subsequent active transport into the phloem. This complex translocation process is not fully understood to date, however some recent evidence provide a valuable insight into the mechanisms supporting this process (Yoneyama et al., 2015). Transport to seeds via phloem is an important supply path of both Fe and Zn in rice (Yoneyama et al., 2010). Considering high concentrations of phosphate species

(Fukumorita, 1983) and an alkaline pH of the phloem sap (Fukumorita and Chino, 1982; Fukumorita, 1983), translocation of metals complexed to organic ligands is a plausible explanation for maintaining a steady metal ion supply. Nishiyama et al. (2012) investigated speciation of Fe and Zn in phloem sap and demonstrated that these metals are complexed to DMA and NA, respectively, while being transported via phloem vessels.

Mechanisms of Zn uptake, translocation and deposition remain to be further tested before any breeding and/or transgenic biofortification targets are selected. Recently, studies using mutant genotypes identified a number of relevant transporter families and their corresponding regulatory genes. Nevertheless, little data is available on their metal binding properties (Blindauer and Schmid, 2010). The work in this thesis contributes towards better understanding of Zn uptake from soil and crossing the soil-root barrier. In addition, Zn homeostasis involves mechanisms that take place in different part of the plants as well as different development stages. Long and studious observations of these mechanisms can provide answers relevant to future biofortification efforts.

Chapter 3

Complexation of Zn by DMA

3.1. Introduction

3.1.1. Properties of ZnMAs complexes relevant for understanding their role in crop biology

Since phytosiderophore (PS) ligands were first isolated from the root washings of oats (Takagi, 1976), they were an object of intensive experimental (Suzuki et al., 2006) and theoretical research (Kato et al., 2011; Moynier et al., 2013). Mugineic acids are regarded as important contributors to Zn homeostasis, however little concrete evidence is available. To completely understand function of mugineic acids as metal-carriers in plants, it is important to first comprehend their metal-complexation properties.

The molecular structures of mugineic acid (MA) complexes with cobalt(III) (Mino et al., 1983; Nomoto, 1981) and copper(II) (Mino et al., 1981; Nomoto, 1981) were determined using single-crystal X-ray diffraction analysis. Despite slight differences in the coordination environment of metal ions, both complexes display nearly octahedral coordination geometry. Similarities in the binding character of copper(II) and zinc(II) ions suggest that MA would bind Zn in a similar complexation fashion as it does in $[\text{Cu}^{2+}(\text{MA})]$. Nonetheless, apart from a few computational studies that

provide some insight into the geometries of Zn^{2+} and Fe^{2+} complexes with MAs (Kato et al., 2011), no studies available to date have experimentally investigated in detail the coordination geometry of these biologically relevant complexes.

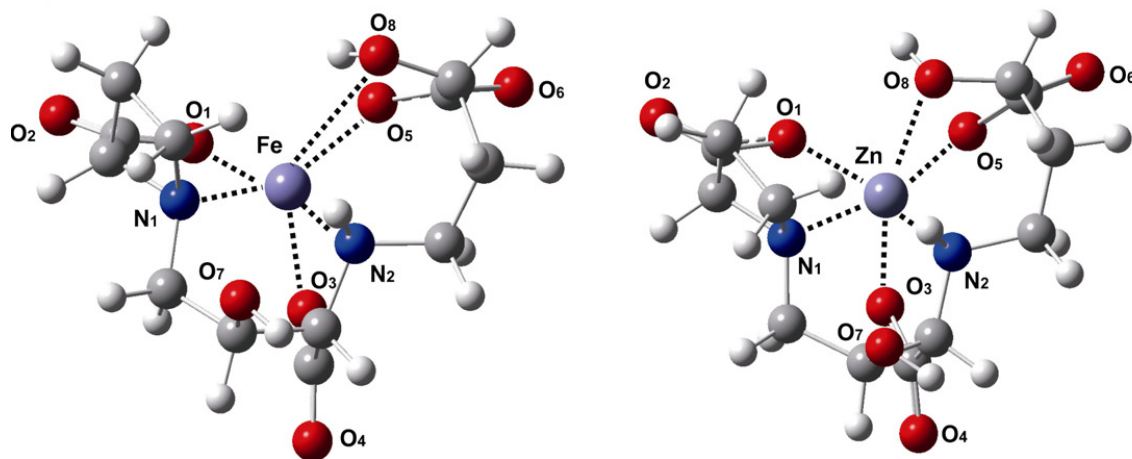


Figure 3.1 (Figure from (Kato et al., 2011); permission acquired) Calculated gas-phase geometries of $[\text{M}^{2+}(\text{HMA})]^-$ complexes, where $\text{M} = \text{Fe}$ (left) and Zn (right).

The role of phytosiderophores in plants is unequivocally linked to an ability to sequester metal ions from various chemical species in soil solution, particularly ones that ‘lock away’ essential nutrients thus making them physiologically unavailable. Phytosiderophores are typically ligands with high affinity for metal ions and hence, form strong and stable complexes. Several studies measured affinity constants of mugineic acids towards various metal ions. These include essential metallic nutrients such as ferric (Murakami et al., 1989; Sugiura et al., 1981) and ferrous Fe (Murakami et al., 1989; Sugiura et al., 1981), Zn^{2+} (Murakami et al., 1989), Cu^{2+} (Murakami et al., 1989) and Mn^{2+} (Murakami et al., 1989); and some elements of a more phytotoxic character, *e.g.* Al^{3+} (Yoshimura et al., 2011). Measured thermodynamic parameters indicate that in aqueous solution mugineic acids, and their precursor nicotamine (NA), form very stable metal complexes ($\text{Log } K > 12$), which are considerably stronger than with any other low-molecular weight organic acids (*e.g.* malate, citrate, oxalate) (Table 3.1). Molecules with metal-carrier role are characteristic not only for their ability to tightly bind metal ions, but also to release them under defined conditions (Blindauer and Schmid, 2010). Mugineic acids have been identified in various segments of plant organism, including both xylem (Kawai,

2001) and phloem (Nishiyama et al., 2012). Therefore, understanding their metal complexation chemistry is important towards understanding the dynamics of both metal uptake from the soil solution and their movement through the plant via a chain of ligand exchange reactions.

Table 3.1 Affinity constants ($\log K$) published for biologically relevant 1:1 (M:L) complexes of phytosiderophore ligands from the mugineic acid family and nicotinamine (NA) – a precursor in their biosynthesis. All studies listed in the table used ligand material isolated from root washings to conduct measurements of complex stabilities.

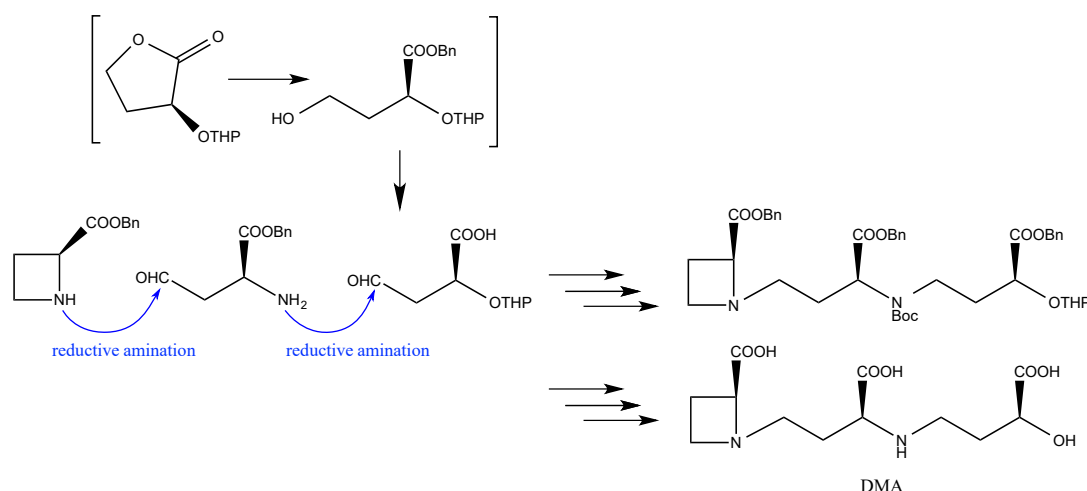
Ligand	Metal	Log K	Reference
Citric acid	Zn ²⁺	4.5	Bjerrum et al.,1964
Malic acid	Zn ²⁺	2.8	Bjerrum et al.,1964
Cysteine	Zn ²⁺	9.8	Bjerrum et al.,1964
Histidine	Zn ²⁺	6.63	Bjerrum et al.,1964
Nicotinamine	Zn ²⁺	15.4	Anderegg and Ripperger ,1989
		14.7	Beneš et al. 1983
	Fe ²⁺	12.3	Anderegg and Ripperger ,1989
		12.1	Beneš et al. 1983
	Fe ³⁺	18.1	Beneš et al. 1983
Mugineic acid (MA)	Zn ²⁺	10.7	Sugiura et al., 1981
		12.69	Murikami et al., 1989
	Fe ³⁺	18.1	Sugiura et al., 1981
		17.71	Murikami et al., 1989
	Fe ²⁺	8.1	Sugiura et al., 1981
		10.14	Murikami et al., 1989
	Al ³⁺	13.4	Yoshimura et al., 2011
2'-deoxymugineic acid (DMA)	Zn ²⁺	12.8	Murikami et al., 1989
	Fe ²⁺	10.45	Murikami et al., 1989
	Fe ³⁺	18.38	Murikami et al., 1989
3-epi- hydroxymugineic acid (2'-HMA)	Zn ²⁺	12.43	Murikami et al., 1989
	Fe ²⁺	10.02	Murikami et al., 1989
	Fe ³⁺	15.49	Murikami et al., 1989

3.1.2. Accuracy of studies reporting metal affinity constants ($\log K$) of mugineic acids to date

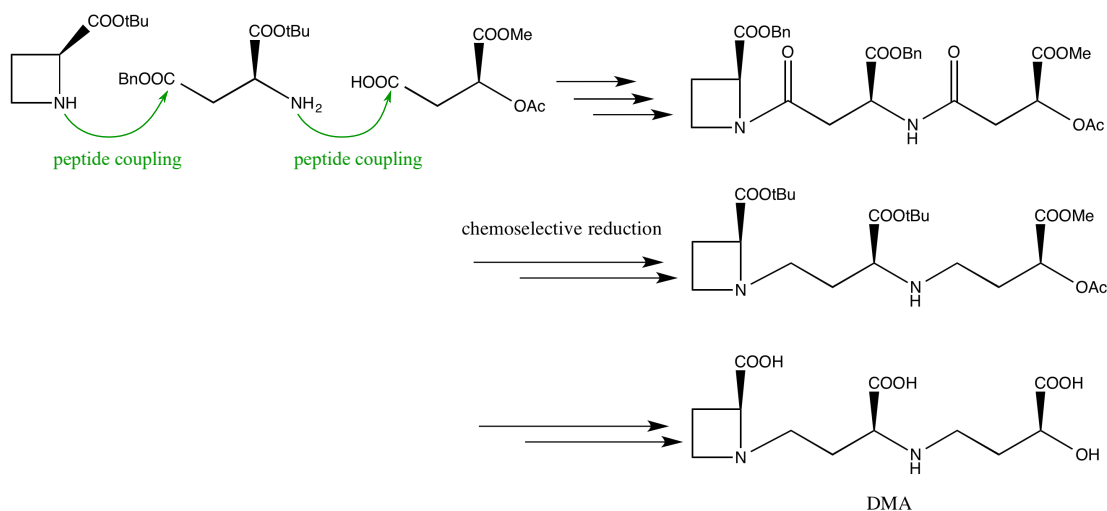
Understanding the fluxes of Zn^{2+} from soil to plant is highly dependent on the accurate determination of both thermodynamics and kinetics of Zn^{2+} binding to the biologically relevant molecules (Blindauer and Schmid, 2010). However, metal affinities of some relevant biomolecules, acting as metal carriers and membrane transporters, are often ill-defined in the current literature. Metal affinity properties of mugineic acids were previously described in studies that used ligand material isolated from root washings rather than synthesized in the lab. Although these reports provoked an important breakthrough in elucidating the function of MAs in plants, possible contamination issues associated with the isolation procedure are the plausible origin of the inconsistencies in the data reported (Table 3.1). Isolation of complexing ligands from root washings yields a sample that contains multiple members of the same molecular family, which cannot be effectively distinguished by available separation techniques. In the literature, studies attempted resolving this problem by developing various separation protocols that utilise TLC on cellulose layers (Kawai et al., 1988), metal complexation properties followed by colorimetric (Shenker et al., 1995), or UV-detection studies (Howe et al., 1999), and finally, high-pressure-liquid-chromatography (HPLC) (Hiradate and Inoue, 1996; Kawai et al., 1987; Mori et al., 1987; Neumann et al., 1999; Takagi, 1993). However, the first high-precision separation technique able to quantitatively segregate mugineic acids, particularly MA and DMA (Neumann et al., 1999) was developed a decade after thermodynamic studies on metal-mugineic acid complexes were published. All above mention considered, it is likely that complexation constants in use today are insufficiently accurate due to cross contamination issues between different mugineic acids in the sample. The published reports on ligand affinities for metal ions can be taken as good estimates of the stability of MAs complexes. However, accurate determination would require the use of individual synthetic ligands (rather than mixtures of MAs) with high level of purity.

3.1.3. Synthetic procedures to obtain mugineic acids

To date, several protocols have been published to synthesise mugineic acids (Matsuura et al., 1992; Namba et al., 2007; Shioiri, 1997; Singh et al., 2005). Nevertheless, most of the reported methods follow a common strategy. All members of the mugineic acids family can be constructed from three basic fragments interconnected via a nitrogen atom (α -hydroxybutyric acid, α -aminobutyric acid and azetidine-2-carboxylic acid). In essence, all synthetic methods gradually build the three fragments independently and then use either the reductive alkylation at the nitrogen atom (Ohfuné et al, (1981); Scheme 3.1), or peptide coupling followed by chemoselective reduction of peptide bonds (Kitahara et al, (1998); Scheme 3.2) to couple the building blocks together into a functional unit.



Scheme 3.1 Ohfuné et al., (1981) uses reductive amination to couple the three building blocks into an inactive phytosiderophore molecule, which can then be activated by removing protective groups from the functional groups of the molecule.



Scheme 3.2 Kitahara et al., (1998) reported a protocol to synthesise mugineic acids by peptide coupling of the building blocks (α -hydroxybutyric acid, α -aminobutyric acid and azetidine-2-carboxylic acid).

The above-described approaches (Scheme 3.1 – 3.2) successfully yield pure MAs, however both procedures are cumbersome due to a significant number of transformation and purification steps. By reducing the number of purification steps, Namba and co-workers (2007) reported a practical method for synthesis of 2'-DMA, MA and 2'-epi-MA (see Scheme 3.3). Due to its effectiveness, this nearly 'one-pot' synthetic procedure has stimulated further physiological studies of mugineic acids. As discussed in the following section, the latter method was adapted to synthesise pure DMA to carry out further Zn-binding studies.

3.1.4. Aim of the study

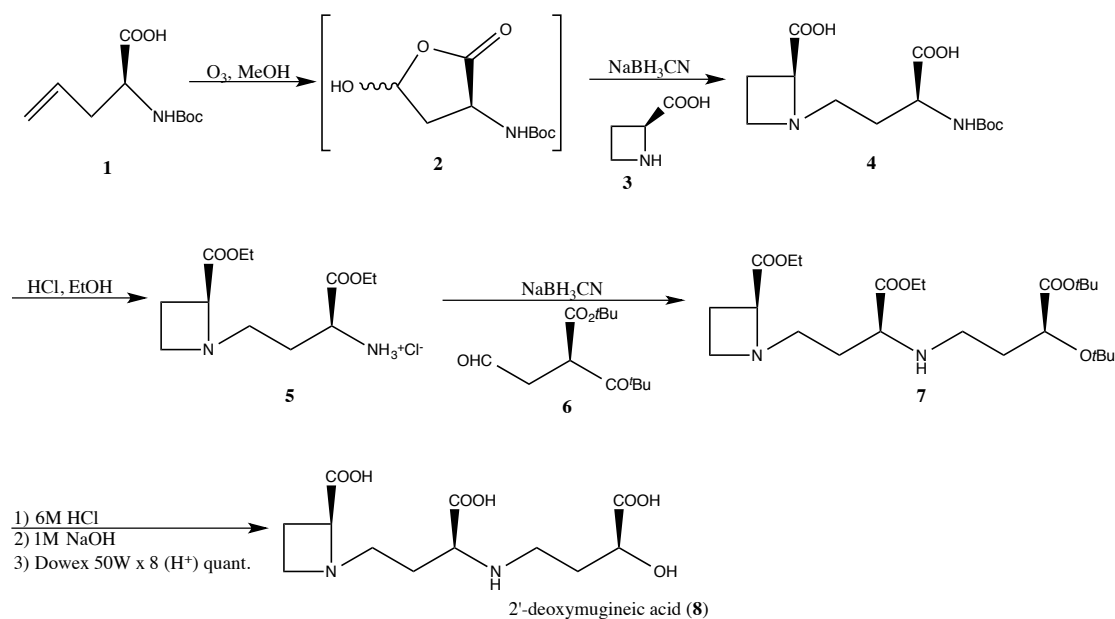
The aim of the work described in the present chapter is to determine the Zn binding affinity of synthetic DMA ($\log K_{\text{ZnDMA}}$) and compare the obtained value with the one previously determined using ligand material extracted from root washings (Murakami et al., 1989). In order to do this, the phytosiderophore DMA was synthesised in the lab following the experimental protocol developed by Namba et al (2007). Starting from commercially available building blocks, DMA synthesis procedure was conducted to produce sufficient quantities of pure ligand material necessary for the complexation study reported in the current chapter, and isotopic fractionation studies (for further details see Chapter 4).

The results on the [Zn(DMA)] complex's stability are presented and discussed to determine whether the character of this complexation event fulfils the requirements necessary for the phytosiderophore role.

3.2. Materials and Methods

3.2.1. DMA Synthesis (modified from Namba et al.,(2007))

For the purposes of conducting experimental studied shown in this thesis, phytosiderophore DMA was synthesised by following the strategy of Ohfuné et al. (1981) (Scheme 3.1) as described in the protocol of Namba et al. (2007) (Scheme 3.3). Commercially available starting materials (Boc-L-azetidine-2-carboxylic acid and L-allyl-glycine) were used in two successive reductive amination steps to gradually build functional 2'-deoxymugineic acid.

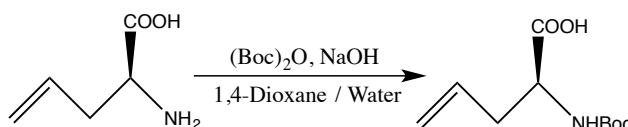


Scheme 3.3 The main of the synthetic procedure to contract DMA(8) from the three building blocks: α-aminobutyric acid derivate (1), azetidine- 2-carboxylic acid (3), and α -hydroxybutyric acid derivate (6).

The main synthesis streamline shown in Scheme 3.3 is preceded by the preparation of starting compounds:

- (Boc)-protection of l-allyl-glycine to produce N-Boc-allyl-glycine (**1**; Scheme 3.4);
- Removal of Boc protection group from commercially available Boc-L-azetidine carboxylic acid to obtain L-azetidine-2-carboxylic acid (**3**; Scheme 3.5);
- Synthesis of S-tert Butyl-2(tert-butoxy)-4-oxobutanoate (**6**; Scheme 3.6)

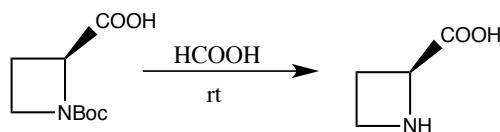
All starting materials and reagents were purchased from commercial sources and used without further purification. The progress of the synthesis was monitored by ^1H NMR spectroscopy recorded at 297 K in the solvent indicated, using Bruker AC300 spectrometer employing standard Bruker software. The spectra were calibrated with respect to tetramethylsilane and the residual solvent peaks as indicated in the individual spectra.



Scheme 3.4 Protection of the primary amine using Boc group

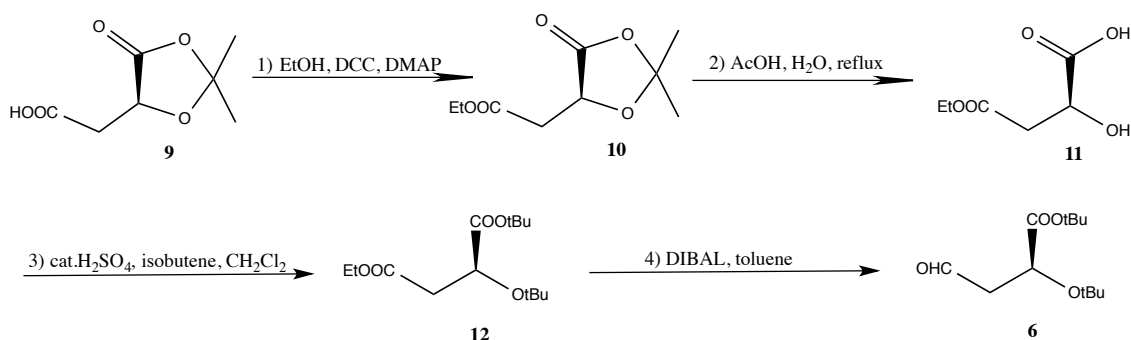
N-Boc-allyl-glycine (1). Although commercially available, n-Boc-allyl-glycine was synthesized from its unprotected form by reacting with di-tertbutyl dicarbonate ((Boc)₂O) in presence of NaOH (Scheme 3. 4) (Kaul et al., 2005; Passarella et al., 2010). L-allyl-glycine (0.5 g, 4.34 mmol) of was dissolved in 1,4-Dioxane (10 ml) and cooled to 0 °C. To this mixture a solution of NaOH (347.2 mg, 8.68 mmol) in 15 ml of water was added. (Boc)₂O (1.135 g, 5.2 mmol) was dissolved in 10 ml 1,4-Dioxane and then added, within 15 min, to the cooled mixture of L-allyl-glycine (flow rate ~30 ml/h) while stirring. The reaction mixture was further stirred for 18 h at room temperature. The organic phase was first evaporated and the remaining water phase was acidified to pH 4 using 3 M HCl solution. The mixture was then extracted using 100 ml Et₂O (x3) and the combined organic layers were dried over Na₂SO₄, filtered and concentrated under reduced pressure to yield a thick colourless oil (938 mg, 98

%). $^1\text{H-NMR}$ (600 MHz, CDCl_3) δ = 5.75-5.6 (m, 1H), 5.18 (m, 2H), 5.05 (m, 1H), 4.42-4.39 (m, 1H), 2.59-2.52 (m, 2H), 1.46 (s, 9H).



Scheme 3.5 Removal of the Boc protection group to obtain L-azetidine-2-carboxylic acid

L-azetidine-2-carboxylic acid (3). A solution of Boc-L-azetidine carboxylic acid (250 mg, 1.24 mmol) was dissolved in formic acid (1.5 ml, 4.97 mmol) and stirred overnight at room temperature under an inert atmosphere (N_2) (Couty et al., 2005; Hanessian and Fu, 2001). After evaporation under reduced pressure, the crude was precipitated using a small amount of diethyl-ether and evaporated to dryness to obtain a white solid material (200 mg, 99 %). $^1\text{H-NMR}$ (600 MHz, D_2O) δ = 4.78 (m, 1H), 4.10-4.04 (m, 1H), 3.94-3.88 (m, 1H), 2.81-2.73 (m, 1H), 2.57-2.48 (m, 1H), 1.48 (s, 9H).



Scheme 3.6 the four-step protocol to obtain S-tert Butyl 2(tert-butoxy)-4-oxobutanoate (**6**), the final building block in the synthesis of DMA

S-tert Butyl 2(tert-butoxy)-4-oxobutanoate. 6: To a solution of (S)-(+)-2,2-Dimethyl-5-oxo-1,3-dioxolane-4-acetic acid (**9**) (3 g, 17.23 mmol) in absolute EtOH (2.01 ml) dry DCM (35.49 ml) was added. While cooling on an ice bath, 1.75 mg DMAP was added to the reaction mixture, along with a solution of DCC (3.91 g, 18.95 mmol) in dry DCM (5.64 ml) and stirred overnight at room temperature. The suspension was filtered through a plug of Celite[®] and the filtrand was washed with 75 ml DCM. Combined filtrates were washed consecutively with 200 ml saturated NaHCO_3 , 200 ml (0.5 N) HCl and 150 ml half saturated NaCl_{aq} . The organic layer

was dried over anhydrous Na_2SO_4 , filtered and concentrated under reduced pressure. The precipitate formed was dissolved with 20 ml Et_2O /Hexane (1:1) and filtered over a cotton plug before finally concentrated under reduced pressure. The solution of the residue in a $\text{AcOH}/\text{H}_2\text{O}$ (4:1) mixture was heated under reflux at 110°C for 40 min and concentrated under reduced pressure, co-evaporated under toluene azeotropy and vacuumed for 3 h. The obtained reaction crude was dissolved in dry DCM (17.23 ml) and isobutene gas was bubbled through the solution under vigorous stirring. 30 min after the bubbling had started the first dose of conc. H_2SO_4 (0.05 ml) was added to the reaction mixture. Over the following 4 h the rest of the concentrated H_2SO_4 (0.17 ml) was added. 1 h after the last dose of acid was added isobutene bubbling was stopped, the flask was well sealed with the para-film and stirring continued for another 16 h. The reaction mixture was quenched with saturated NaHCO_3 , followed by EtOAc work up (3 x 75 ml). The combined organic layers were washed with brine (100 ml), dried over anhydrous Na_2SO_4 , filtered and concentrated under reduced pressure. The crude was purified by flash column chromatography on silica gel (elution using $\text{EtOAc}/\text{Hexane}$ (1.5 : 8.5)). The purified product (2.54 g, 54 %) was kept in the freezer until continuation of the synthesis **^1H NMR** (400MHz, CDCl_3): δ = 4.45-4.34 (dd, J = 8.2, 5.2 Hz, 1H), 4.23-4.10 (m, 1H), 2.69-2.57 (m, 3H), 1.45 (s, 9H), 1.24-1.21(t, J = 7.2 Hz, 3H), 1.19 ppm (s, 9H). **^{13}C NMR** (75 MHz, CDCl_3): δ = 172.4, 170.4, 81.2, 75.3, 69.0, 60.6, 39.5, 27.8, 27.8, 14.2 ppm; **LC-ES+**: m/z calculated for $\text{C}_{14}\text{H}_{28}\text{O}_5$ [M-H]: 276.37, found: 275.19.

To the previously purified product (2.16 g, 7.89 mmol) dry toluene was added (28 ml). The mixture was stripped with nitrogen in duration of ca. 20 min, followed by cooling on an acetone/ CO_2 bath until the solution reached the temperature of -78°C . To the cooling mixture, 9.47 ml (1 M) DIBAL-H was added dropwise (the drops were ran down the inner wall of the flask, in order to pre-cool the reactant before reaching the mixture). After adding the whole amount of the DIBAL-H, the reaction mixture was stirred for 1 h at -78°C . The reaction mixture was then quenched (at -78°C) with 4 ml MeOH and 5 ml satd. NH_4Cl . Both MeOH and NH_4Cl were added slowly dropwise (flow rate ~ 25 ml/h). Right after the quenching, 30 ml of methyl-tert-butyl ether (MTBE) was added and the heterogeneous solution continued to

stir. After 15 min of stirring, the cooling bath was removed and within the next 10 min the solution temperature reached 0 °C, after 30 min it had reached room temperature. The mixture was stirred for an additional 2 h at rt. After this, 2 g anhydrous MgSO₄ was added and the mixture was stirred for further 30 min. The mixture was filtered, and the filtrate was evaporated under reduced pressure (1st fraction). The remaining solid was mixed with additional 20 ml of MTBE and 1.5 g anhydrous MgSO₄ and stirred for further 30 min. This mixture was then filtered and concentrated under reduced pressure (2nd fraction). The two fractions were then combined and co-evaporated until thick colourless oil remained in the flask. The obtained crude was then purified using a flash column chromatography on silica-gel with EtOAc/Hexane = 1.5/8.5 to 2.5/7.5 yielding the pure product (559 mg, 30.8 %). ¹H NMR (400MHz, CDCl₃): δ = 9.82-9.72 (t, *J* = 2.0 Hz, 1H), 4.45-4.34 (dd, *J* = 7.8, 4.8 Hz, 1H), 2.76-2.63 (m, 2H), 1.49-1.45 (s, 9H), 1.21-1.17 ppm (s, 9H). ¹³C NMR (75 MHz, CDCl₃): δ = 200.0, 172.6, 81.9, 75.8, 67.7, 47.5, 28.1, 28.0 ppm; LC-ES+: *m/z* calculated for C₁₂H₂₃O₄ [M-H]: 231.31, found: 231.16.

Once sufficient amount of basic building blocks was obtained, the DMA synthetic procedure was conducted following the protocol published by Namba et al (2007) (Scheme 3.3).

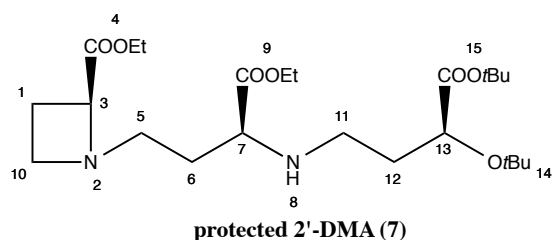


Figure 3.2 Molecular structure of the protected 2'-DMA (7) C₂₄O₇N₂H₄₄ (MW:472.6)

Protected 2'-deoxymugineic acid (DMA). The main synthetic process starts by bubbling ozone (O₃) through a solution of Boc-L-allylglycine (654 mg, 3.04 mmol) in 22 ml of dry methanol at -78 °C. Once the solution was saturated with O₃ (after about 30 min) and light blue in colour, it

was bubbled with nitrogen gas until complete discoloration. At this stage, L-azetidine-2-carboxylic acid (307 mg, 3.04 mmol) and NaBH₃CN (191 mg, 3.04 mmol) were added to the solution and stirred at room temperature continuously for 2 h, followed by concentration under reduced pressure. The residue was dissolved in cooled anhydrous HCl/EtOH (prepared from 2.5 ml acetyl chloride and 53 ml dry EtOH_{abs}) and stirred for 2.5 h at 0 °C after which the ice bath was removed and the

stirring continued at room temperature for the next 18 h. The reaction mixture was then evaporated, dehydrated under toluene azeotropy and dried under vacuum for 4 h. The resulting residue in methanol (30 ml), was reacted with aldehyde **8** (700 mg, 3.04 mmol) and NaBH₃CN (191 mg, 3.04 mmol) and stirred for 4 h at room temperature, quenched with saturated NaHCO₃ and extracted with EtOAc (3x50 ml). The combined organic layers were dried over anhydrous Na₂SO₄, filtered and concentrated under reduced pressure. The residue was purified by a flash column chromatography on silica gel. The product was eluted with hexane/ethyl acetate (1:3) → ethyl acetate (100%) (containing 0.1 % triethylamine) to give protected DMA **9** (512.6 mg, 35.65 %) as colourless oil. ¹H NMR (400MHz, CDCl₃): δ= 4.28-4.13 (m, 4H, 2 x -OCH₂CH₃ (-C⁴OOEt; -C⁹OOEt)), 4.0-3.92 (t, *J* = 6.4Hz, 1H, H¹³), 3.67-3.54 (q, *J* = 8.8Hz, 1H, H³), 3.51-3.35 (t, *J* = 6Hz, 1H, H^{10a}), 3.34-3.18 (t, *J* = 6Hz, 1H, H⁷), 2.91-2.8 (q, *J* = 7.6 Hz, 1H, H^{10b}), 2.78-2.64 (m, 2H, H^{5a}, H^{11a}), 2.60-2.48 (m, 2H, H^{5b}, H^{11b}), 2.42-2.21 (m, 2H, H^{1a}, H^{1b}), 1.91-1.61 (m, 4H, H^{6a}, H^{6b}, H^{12a}, H^{12b}), 1.49-1.43 (s, 9H, -C¹⁵(CH₃)₃), 1.37-1.24 (td, *J* = 7.2 Hz, 6H, 2 x -OCH₂CH₃ (-C⁴OOEt; -C⁹OOEt)), 1.25-1.14 (s, 9H, -C¹⁴(CH₃)₃); ¹³C NMR (100 MHz, CDCl₃): δ = 174.4, 173.4, 172.1, 80.1, 74.1, 69.7, 64.8, 60.2, 60.1, 59.2, 55.1, 50.7, 43.7, 33.9, 30.65, 27.6, 27.5, 21.0, 14.0, 13.9 ppm; LC-ES+: *m/z* calculated for C₂₄H₄₄N₂O₇ [M-H]: 472.61, found: 473.22.

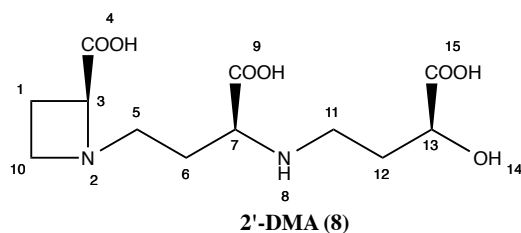


Figure 3.3 Molecular structure of the active 2'-DMA (**8**) with protective groups removed C₁₂O₇N₂H₁₉ (MW: 303.1)

De-protection to obtain active DMA

(**2**). Purified material (470 mg, 0.99 mmol) was suspended in cold 6 M HCl (9 ml) solution and stirred overnight at room temperature. The mixture was then concentrated under reduced pressure, dehydrated twice with toluene and re-

suspended in 1 M NaOH (9 ml). This mixture was stirred for 10 h at room temperature, neutralised to pH 7 using 1 M HCl and concentrated under reduced pressure. The residue was purified on ion separation column Dowex 50Wx8 (H⁺) twice. The sticky residue was dissolved in 2-3 ml of MQ water and added onto the resin before the column was washed with 100 ml of MQ water. The purified material

was eluted (H_2O to 1 M NH_4OH) to recover de-protected DMA material (292 mg, 44%) after lyophilisation. The material was further triturated with dry MeOH (2 ml) for 2 h and isolated by centrifugation. The process was repeated twice before lyophilisation and recovery of pale white crystals of DMA (139 mg, 46 %). ^1H NMR (400MHz, D_2O) δ = 4.63 (t, $J=9.5\text{Hz}$, 1H, H^3), 4.04 (dd, $J=7.3, 4.5\text{ Hz}$, 1H, H^{13}), 3.97 (td, $J=9.9, 4.5\text{ Hz}$, 2H, H^{10a} , H^{10b}), 3.84 (q, $J=9.6\text{ Hz}$, 1H, H^3), 3.65 (dd, $J=8.6, 4.6\text{ Hz}$, 1H, H^{5a}), 3.28 (dddd, $J=41.4, 12.9, 9.3, 6.0\text{Hz}$, 2H, H^{5a} , H^{11a}), 3.07 (qdd, $J=12.5, 8.3, 6.3\text{ Hz}$, 2H, H^{5b} , H^{11b}), 2.63 (dtd, $J=12.0, 9.5, 4.4\text{ Hz}$, 1H, H^{1a}), 2.42 (dq, $J=12.0, 9.4\text{Hz}$, 1H, H^{1b}), 2.16-1.82 (m, 4H, H^{6a} , H^{6b} , H^{12a} , H^{12b}). **ES-MS** m/z calculated for $\text{C}_{12}\text{H}_{19}\text{N}_2\text{O}_7^-[\text{M-H}]^-$: 303.120, found: 303.119.

The purity of synthetic materials in the previous procedures, was assessed by NMR spectroscopy, mass spectrometry (ES-MS) and elemental analysis. The spectra and the obtained results can be found in Appendix I. Purified compound **8** was used for the experimental studies shown in this thesis, including potentiometric titrations (this chapter), and isotopic fractionation measurements (Chapter 4).

3.2.2. Potentiometric titrations

Potentiometric studies of DMA were carried out using Titrand 905 (Metrohm, Switzerland) potentiometer paired with Pentium Dual-Core E5300 personal computer. The burette control and data acquisition were performed with the PASAT computer program (Fontanelli, 1990). For measuring pH changes glass electrodes were used, which were calibrated prior to each titration. This was done by titrating a known volume of standardised HCl solution with a CO_2 -free NaOH solution. The equivalent point was determined by the method described by Gran (1952), which precisely calculates the standard potential (E^0). GLEE program (Protonic Software, UK) was used to fit the Gran plots and determine E^0 as well as the apparent ion product of water (pK_w).

A Wilhelm bridge filled with 0.5 M NaCl was used to separate the glass electrode (Crison 52 50 Ag/AgCl) and the reference electrode (Crison 52 40 Ag/AgCl in 0.5 M NaCl solution). The experimental system was stabilised at $25 \pm 0.1^\circ\text{C}$ ($298.1 \pm 0.1\text{K}$) and under Ar atmosphere. Potentiometric titrations were carried out using I =

0.15 M KCl as the supporting electrolyte. The potentiometric titrations were carried out for the ligand solution only, in the first part of the experiment; followed by the titrations of complex in 1:1 molar ratio. The pH range investigated was 2.5 - 11.0 and the corresponding ion and ligand concentrations ranged from 1×10^{-3} to 5×10^{-3} M.

To fit the protonation and stability constants the Hyperquad program (Protonic Software, UK) was used (Gans, 1996). The different titration curves for each system were treated as separated curves. When more than one model fits the experimental data, the most reliable chemical model was chosen by performing *F* tests at the 0.05 confidence level (Bologni, 1983; Hamilton, 1964). Finally, the sets of data were merged together and treated simultaneously to give the final stability constants. The HYSS programme was used to plot the distribution diagrams of species (Alderighi, 1999).

3.3. Results and Discussion

3.3.1. Synthetic DMA ligand

The synthetic efforts yielded sufficient quantities of DMA ligand by following the protocol reported by Namba et al. (2007) and introducing minor changes to the synthesis of S-tert Butyl-2(tert-butoxy)-4-oxobutanoate (compound **6**). Namely, the first step towards synthesis of the compound **6** (Scheme 3.6) was optimised by applying Steglich esterification protocol (Walter et al., 2014) rather than *via* the alkylation with ethyl iodide, as stated in the Namba et al. (2007) procedure.

The final purification step of the chosen synthetic protocol includes ion exchange separation on wet Dowex 50x8 (H^+ form) (Dow Chemical Company, Michigan, US) resin, where elution of the ligand (using H_2O to 1M NH_4OH) requires formation of ammonium salts. Although the subsequent steps (repetition of ion exchange purification and water elution, lyophilisation and trituration with dry MeOH; for details see section 3.2) are aimed at minimising the level of impurities, including ammonium counterions, the elemental analysis (Appendix I) showed that the ratio of C-N is less than the theoretical C-N ratio of the molecule in question. This implies higher nitrogen content to the one theoretically anticipated and hence, minor

presence of ammonium counterion in the synthetic ligand material. The presence of ammonium, *i.e.* its content as per results of elemental analysis, was considered when running potentiometric titrations, however no effect on Zn complexing behaviour was noted, as can be deduced from the results shown below.

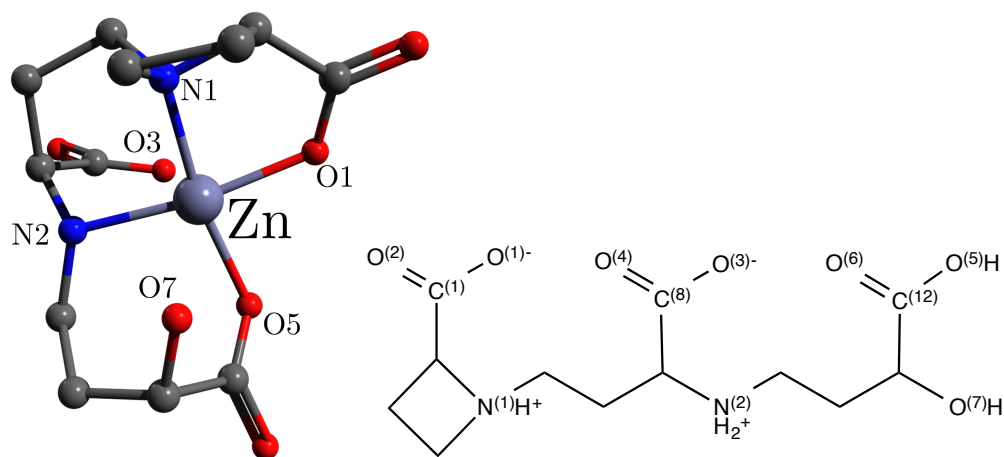


Figure 3.4 (left) [Zn(DMA)] structure obtained using Avogadro software; (right) Molecular structure of DMA ligand ($C_{12}H_{20}N_2O_7$)

The NMR as well as the ES-MS spectra of the final compound (Appendix I) identify the product as the 2'-DMA. In the lower ppm section of the ^1H NMR spectrum, protons of the methylene bridges ($-CH_2-$) on each side of N^8 atom are identified as multiplets at $\delta=2.16-1.82$ ($-C^{6,12}H_2-$), and $\delta=3.65-3.07$ (for $-C^{5,11}H_2-$ where the chemical shift is higher due the bond with N atoms). The de-shielded protons of the azetidine ring reflect the presence of the heteroatom N in the ring as well as the closeness to the carboxylic group ($-C^4OOH$). Hence the ^1H NMR spectrum of this substructure is divided into three: a very de-shielded H^3 signal ($\delta = 4.63$) split into a triplet by the neighbouring $-C^1H_2-$ group (multiplet, $\delta = 2.63-2.42$), and the dt signal belonging to $-C^{10}H_2-$ group ($\delta = 3.97$). Molecular weight of the 2'-DMA, based on the empirical formula $C_{12}H_{19}N_2O_7[M-H]^-$, was calculated to be 303.120. The ES-MS result of the final synthetic material was m/z : 303.119 (Appendix I), which overall, confirms that the synthetic compound is DMA.

3.3.2. Potentiometric titration study to determine the ZnDMA affinity constant

Firstly, titration of a known volume of DMA ligand solution was titrated using the automated system (described in section 3.2.) to determine the buffer regions, which

are the indicators of the protonation constants (pK). The logarithms of the stepwise protonation constants of DMA are given in Table 3.2. As it can be seen from this table, the results obtained closely resemble the values reported by Murakami et al., (1989).

Table 3.2 Comparison of the measured protonation constants (pKa) of 2'-deoxymugineic acid (DMA) reported in this study (using the synthetic ligand) and the study of Murakami (1989), verifies previously reported data.

Reaction		This study L	[Murakami et al., 1989] pKa
$\text{NH}_3 + \text{H} \rightleftharpoons \text{NH}_4$		9.399(5) ^b	
$\text{H}_2\text{L} + \text{H} \rightleftharpoons \text{H}_3\text{L}$	pKa	3.10(1)	3.193(4)
$\text{HL} + \text{H} \rightleftharpoons \text{H}_2\text{L}$	pKa	7.969(9)	8.25(2)
$\text{L} + \text{H} \rightleftharpoons \text{HL}^{\text{a}}$	pKa	10.935(7)	10.00(1)
$\log \beta^{\text{c}}$		22.00	

^a Charges omitted for clarity. ^b Numbers in parentheses are standard deviations in the last significant figure. ^c Cumulative basicity constant $\beta = \text{SK}_{\text{HjL}}$.

The value pKa 1 corresponds to the proton dissociation of the terminal carboxylic group (atom no. 14, Figure 3.3) while the second (pKa 2) and the third protonation constants (pKa 3) refer to dissociation of protons from the secondary amine (atom no. 7, Figure 3.3) and the tertiary amine of the azetidine ring (atom no. 2, Figure 3.3), respectively (Murakami et al., 1989). Figure 3.5 demonstrates the speciation behaviour of DMA over a range of pH conditions, with respect to the measured pKa constants. As it can be seen from the figure, within the physiological pH range, DMA is present as $[\text{H}_2(\text{DMA})]^{2-}$ and $[\text{H}(\text{DMA})]^{3-}$ species.

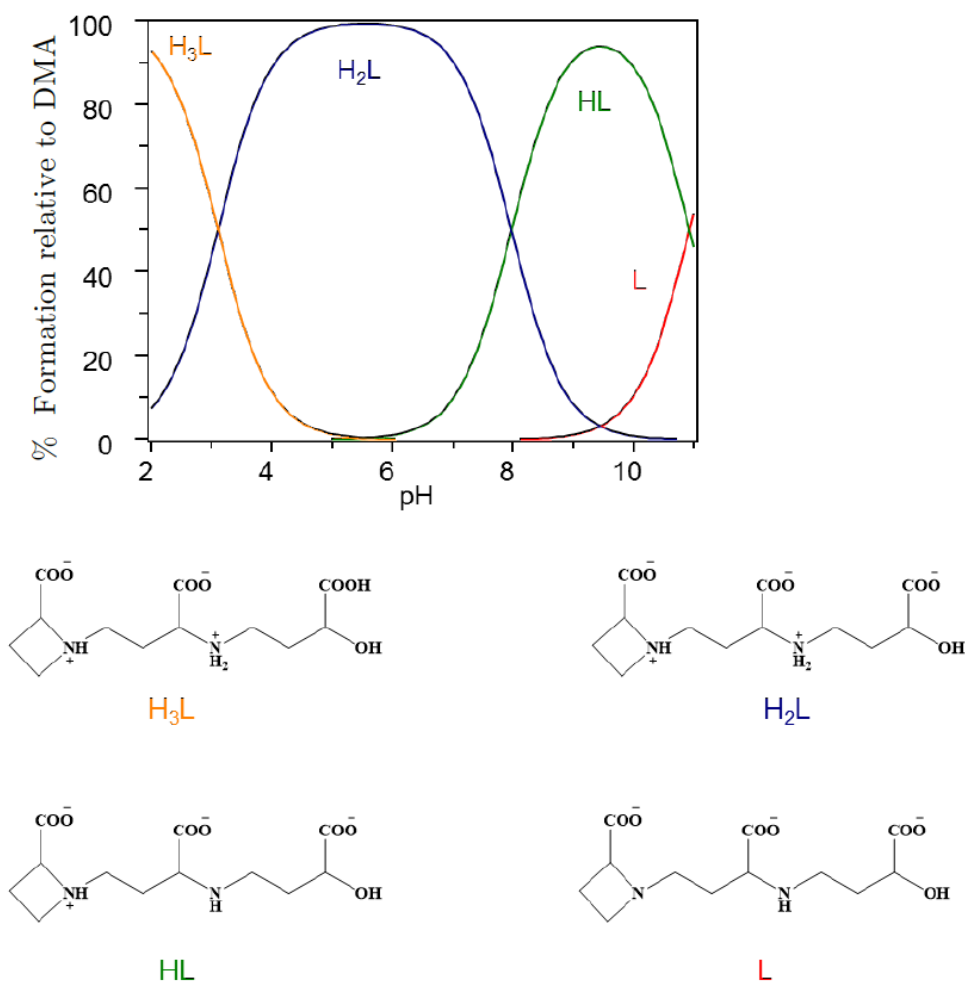


Figure 3.5 (top) Distribution diagram for the protonation of the DMA ligand (L) in 0.15 M KCl at $25 \pm 0.1^\circ\text{C}$; $[\text{L}] = 0.0001 \text{ M}$. The speciation was plotted using HySS software (Protonic Software) to simulate speciation for the experimental conditions given. **(bottom)** Protonation states of L as presented in the speciation plot above.

In the presence of bivalent metal ions, the ligand completes dissociation after releasing the three protons, as described above. (Murakami et al., 1989) identified that the fourth proton, which belongs to the terminal alcoholic group (atom no. 13, Figure 3.3), is released in the presence of trivalent cations (e.g. Fe^{3+} or Al^{3+}). These claims are in agreement with X-ray diffraction analysis of Co^{3+} complex with MA (Mino et al., 1983).

Potentiometric titrations of the (1:1) $[\text{Zn}^{2+}(\text{DMA})]$ complex identified high affinity ($\log K_{\text{ZnDMA}}$) of this association. As shown in Table 3.2, the $\log K_{\text{ZnDMA}}$ constant determined in this work is in line with the one previously reported by Murakami et

al. (1989). This shows that the log K constants published in the study of Murakami et al. (1989) are sufficiently accurate.

Table 3.3 Logarithms of the stability constants for the formation of Zn^{2+} complexes with the DMA ligand determined in 0.15 M KCl at $25.0 \pm 0.1^\circ\text{C}$

Reaction	This study	Murakami et al. [1989]
$\text{M} + \text{L} \rightleftharpoons \text{ML}^{\text{a}}$	13.14(1) ^b	12.842(6)
$\text{ML} + \text{H}_2\text{O} \rightleftharpoons \text{ML}(\text{OH}) + \text{H}$	-10.86(3)	

^a Charges omitted for clarity. ^b Numbers in parentheses are standard deviations in the last significant figure.

During calculations of the protonation constants (pK), the data treatment had taken into consideration the protonation constant of ammonium. Under the stated experimental conditions, formation of $[\text{Zn}^{2+}(\text{NH}_3)]$ did not occur. Therefore, no interfering effects have noted from the small quantity of ammonium counterions present in the synthetic ligand material. Figure 3.6 indicates the distribution of the $[\text{Zn}^{2+}(\text{DMA})]$ species over the examined pH range.

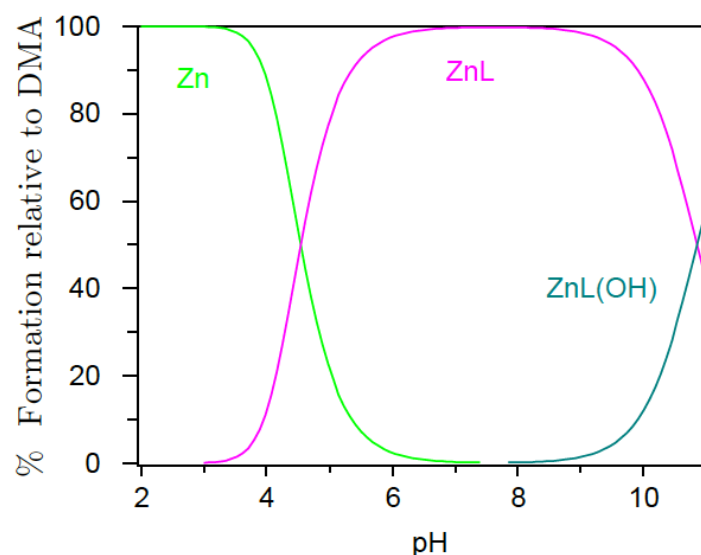


Figure 3.6 Distribution diagram for the formation of Zn(II)-L complexes determined in 0.15 M KCl at 298.1 ± 0.1 K. $[L] = [Zn] = 0.001$ M. The figure was constructed using HySS software (Protonic Software) to simulate speciation for the experimental conditions stated. Natural pH conditions relevant for Zn uptake range between mildly acidic to acidic pH in soil solution ($\sim$ pH 5.5), occurring as a result of acidification by plant roots to promote Zn root uptake, to mostly neutral in plant organs ($\sim$ pH 7; (Staal et al., 2011)), to alkaline in parts of plant vascular system, *i.e.* phloem (pH 8; (Olsen and Palmgren, 2014)).

3.3.3. Implications for the biological relevance of DMA

The $[Zn^{2+}(DMA)]$ binding affinity ($\log K_{ZnDMA}$) determined in this work, as well as previously published data, show that DMA is able to sequester Zn from other chemical species and form a complex of adequate stability for long-distance transport. The binding affinity between Zn and DMA is evidently higher than for most of the natural organic ligands (Bowles et al., 2006).

The efficiency of PS ligands as a Zn carrier in rhizosphere is influenced by concentrations of other transition metal elements and their binding affinities. Currently available data show that binding affinities between M^{n+} and phytosiderophore ligands are consistent with the Irving-Williams series ($Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II)$) of the relevant stabilities of metal complexes (Benes et al., 1983; Dell'mour et al., 2010; Murakami et al., 1989). The intricate combination of physicochemical reactions and balance of ions in plants' rhizosphere remains to be elucidated. Modelling interactions of transition metal species under relevant conditions could provide answers regarding Zn absorption

mechanisms and the circumstances they are governed by. For accurate modelling of complex metal cycling in natural environments, affinity constants ($\log K$) of PS complexes with a range of metal elements need to be accurately determined, particularly Fe. Currently, two partially conflicting studies report affinity constants of iron complexes with mugineic acids (Table 3.3). While stability of $[\text{Fe}^{3+}(\text{MA})]$ complex ($\log K_{\text{Fe}^{3+}+\text{MA}}$) was shown to be similar in the Sugiura et al. (1981) and Murakami et al. (1989) reports, the estimated affinity constants for $[\text{Fe}^{2+}(\text{MA})]$ complex were not. The published $\log K_{\text{Fe}^{2+}+\text{MA}}$ values are 8.1 and 10.1, in studies of Sugiura et al. (1981) and Murakami et al. (1989), respectively. The discrepancy between the two studies is also reflected in the $\log K_{\text{Zn}^{2+}+\text{MA}}$ values reported. Higher values for $\log K_{\text{Zn}^{2+}+\text{MA}}$ are reported by Murakami et al. (1989) ($\log K_{\text{Zn}^{2+}+\text{MA}} = 12.7$), compared to those by Sugiura et al., (1981) ($\log K_{\text{Zn}^{2+}+\text{MA}} = 10.7$). The inconsistencies in the current literature need to be further investigated by reassessing the published data in new potentiometric studies using synthetic ligand material.

3.4. Conclusions

The work outlined in this chapter was focused on reassessing the coordination chemistry of $[\text{Zn}(\text{DMA})]$ complex, and validating the binding affinity of DMA for Zn via potentiometric titration using chemically synthesised DMA. The potentiometric analysis confirmed the previous findings of Murakami et al. (1989) that DMA forms a very stable complex with Zn, and therefore could have an important biological role as a Zn carrier.

The phytosiderophore role of DMA in rice is plausible when considering the physicochemical properties of the complex observed, however the functional aspect of DMA as a Zn carrier in rhizosphere cannot to be observed outside the scope of the dynamic soil environment. Thus, to accurately model the elaborate and sophisticated interactions in the rhizosphere system, it is necessary to rely on a trustworthy database of thermodynamic constants. At the moment such reliable database does not exist due to a number of inconsistent reports published. The synthetic method suggested and the results reported in this chapter contribute to generating such an

important database. Comprehending the binding dynamics in complex systems such as rhizosphere is vital for our understanding of micronutrient supply in plants.

Chapter 4

Experimental Determination of Equilibrium Isotopic Fractionation between Free Zn^{2+} and Zn Complexed to a Natural Phytosiderophore and Its Structural Analogues $[\text{ZnL}]^{2-}$

4.1. Introduction

4.1.1. Isotope geochemistry in environmental studies

Stable isotope chemistry has shown to be a powerful tool in environmental chemistry studies (von Blanckenburg et al., 2009). With improvements in high-precision mass spectrometry, such as multi collector inductively coupled plasma-mass spectrometry (MC-ICP-MS), it is possible to observe fine isotopic signatures in the environment and trace back to the underlying physicochemical processes. Growing body of evidence shows that metal-ligand interactions have significant effects on the extent and direction of stable isotope fractionation of metals in geological (Matthews, Zhu et al. 2001) and biological (Dideriksen, Baker et al. 2008, Black, Epstein et al. 2008)

systems alike. Complexation is one of the core chemical processes in chemical cycles both in biosphere as well as lithosphere and thus an essential driver in formation of isotopic signatures in nature.

When studying isotopic preference upon complexation of metals one of the major practical challenges is to adequately separate free metal (M^{n+}) from that complexed to organic ligands ($[ML]$) (Wiederhold, 2015). Among very few studies to investigate metal isotopic fractionation in organic complexes, Jouvin et al. (2009) and Bigalke et al. (2010) used NICA-Donnan model to separate Zn bound to the humic acid (HA), an analogue of soil organic matter, in laboratory conditions. By combining a Donnan-type model for nonspecific electrolytes binding and specific binding features of non-ideal competitive adsorption (NICA) model Kinniburgh et al. developed a method to investigate a multicomponent competitions during binding reactions between ions and humic substances under various pH and free metal concentration conditions (Kinniburgh et al., 1996). The same set up was used in the studies of Jouvin et al (2009) and Bigalke et al. (2010) to estimate the isotopic behaviour of Zn^{2+} and Cu^{2+} respectively, in a system simulating an environment (soil and/or water systems) enriched in organic matter. The system, consisting of two parts divided by a negatively charged Donnan membrane, was penetrable by free Zn^{2+} ions from either side (Figure 4.1). Purified peat humic acid (PHA) however, was deposited only on one side of the membrane (donor side) and could not pass the barrier. Hence the complexation of free Zn by PHA could only occur in that part of the system.

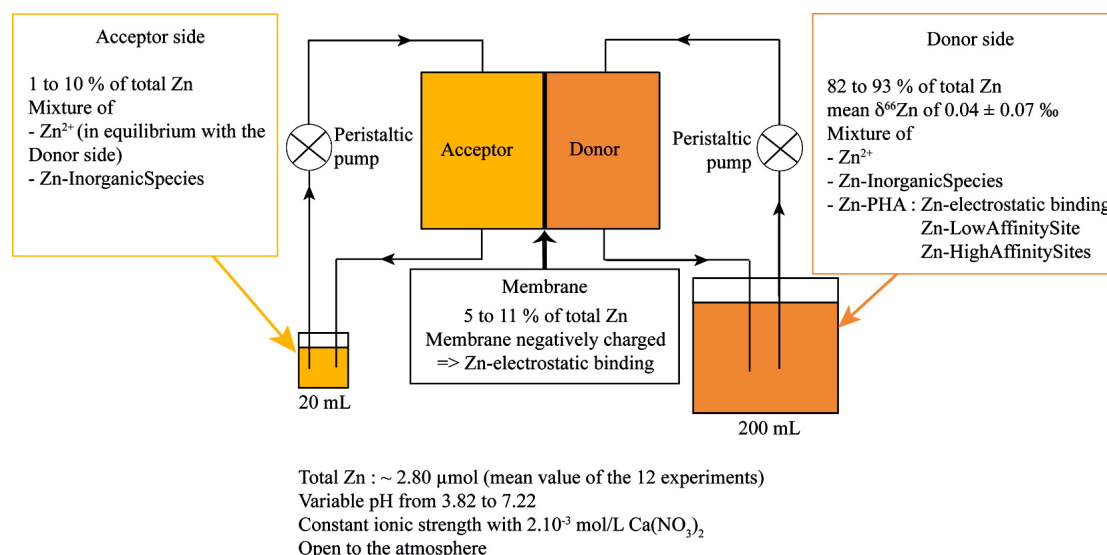


Figure 4.1 (Figure from Jouvin et al., (2009), permission acquired) NICA^{*}-Donnan system used by Jouvin et al. (2009) to equilibrate free Zn^{2+} and $[\text{Zn(PHA)}]$ complex. At the beginning of the experiment (initial $\delta\text{Zn}_{\text{source}} = +0.04 \pm 0.07 \text{ ‰}$), Zn containing species are located only in the donor side but free Zn^{2+} ions can travel across the negatively charged Donnan membrane, unlike the Zn bound to PHA. ^{*} stands for non-ideal competitive adsorption model (Kinniburgh et al., 1996).

After a long equilibration period (2 - 3 days) during which free Zn^{2+} ions travelled across the membrane and down the ion gradient, it was possible to measure isotopic fractionation in the two parts of the system. Using this model, Jouvin et al. (2009) quantified for the first time the Zn partitioning in an experimentally studied complexation reaction with an organic acid. They reported preferential binding of heavy ^{66}Zn isotope ($\Delta^{66}\text{Zn}_{[\text{Zn(PHA)}] - \text{free Zn}^{2+}} = 0.24 \pm 0.04 \text{ ‰}$) as it is expected following the rules of thermodynamics applied to equilibrium reaction. By using a similar system Bigalke et al., (2010) measured the isotopic fractionation of Cu^{2+} during sorption on insoluble humic acid (IHA). The total observed Cu isotope fractionation at established equilibrium between the solution and the $[\text{Cu(IHA)}]$ complex was $\Delta^{65}\text{Cu}_{[\text{Cu(IHA)}] - \text{free Cu}^{2+}} = 0.26 \pm 0.11 \text{ ‰}$ which is in close correlation with the earlier findings of Jouvin et al. (2009). Compared to the natural stable isotope variation of these elements ($\sim 1.8 \text{ ‰}$ for Zn (Cloquet et al., 2008) and $\sim 1.5 \text{ ‰}$ for Cu (Zhu et al., 2002)) it is evident that complexation by organic acids is an important contributor to isotopic signatures in the environment. Observations from both groups strongly support the claims that complexation by organic ligands would affect Zn speciation, mobility and bioavailability in the environment.

Despite its importance in these pioneering studies, while the NICA-Donnan model is robust it is cumbersome and that could potentially introduce artefacts, which interfere with the high-precision measurements such as MC-ICP-MS. Equilibration of the system before the sample can be taken lasts between 2 and 3 days for ligands with high binding affinity for cations (*e.g.* humic acids), and possibly longer for weaker ligands. Jouvin et al. (2009) identified $\delta^{66}\text{Zn}$ of -0.14 ‰ produced at the Donnan membrane's surface for which no adequate explanation was offered.

4.1.2. Aims of the study

In this chapter we present a newly developed method to separate free Zn^{2+} from Zn coordinated to polydentate ligands, namely 2'-deoxymugineic acid, (DMA) and its structural analogues - ethylenediaminetetraacetic acid (EDTA), trimethylenediaminetetraacetic acid (TMDTA), and diaminocyclohexanetetraacetic acid (CyDTA) (Figure 4.2). This new method makes use of commercially available cation-exchange resins to separate different Zn species from the mixed sample. The isotopic fractionation upon complexation with the selected ligands was measured using MC-ICP-MS technology.

Furthermore, a connection between the complex binding affinity ($\log K$) and isotopic fractionation is discussed with respect to isotope fractionation data obtained for phytosiderophore DMA and the three commercially-available ligands under study (EDTA, TMDTA and CyDTA). The ligands used in this study were selected due to the similarity in the Zn coordination sphere with the phytosiderophores from the mugineic acid family (Figure 4.2).

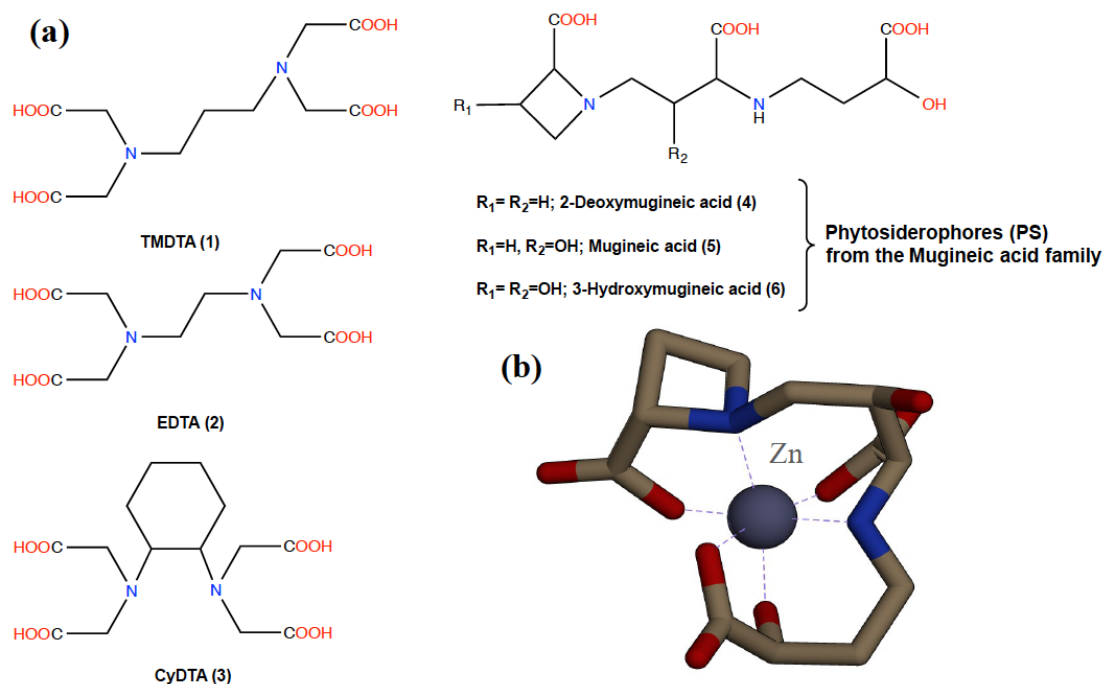


Figure 4.2 Chemical structures of the four organic ligands used in this study (1-4) and the corresponding affinity constants for Zn^{2+} ; (b) molecular structure of the Zn-MA complex modelled with molecular mechanics using ChemBio3D, where colours of the atoms refer to N (blue), O (red) and Zn (iris, central atom).

4.2. Methods

4.2.1. Modelling Zn complexation using GEOCHEM-EZ

Using GEOCHEM-EZ software (Shaff et al., 2010) to model Zn^{2+} speciation in solution we confirmed that a complete complexation is established upon mixing equimolar amounts of $\text{Zn}(\text{OAc})_2$ and L^{4-} (1 mM) solutions (where L^{4-} refers to the deprotonated version of the tested ligands EDTA, TMDTA, CyDTA and DMA) at pH 6.2 as shown in Table 4.1 (a-b).

Table 4.1 Conditions given and GEOCHEM-EZ modelled speciation (expressed in percentages (%)) of complexes formed. Speciation was calculated in mixtures of equimolar amounts of $\text{Zn}(\text{OAc})_2$ and test ligands EDTA **(a)** and CyDTA **(b)**, buffered to pH 6.2 with 0.5 M KMes buffer. In both tested cases all available Zn is bound to the chelating ligands. As GEOCHEM-EZ does not recognise TMDTA and DMA in its database these ligands were not modelled. Nevertheless, due to the similarity in Zn bonding environment and Zn binding affinity ($\log K$) the same speciation pattern was anticipated, (and further confirmed when measuring Zn distribution in different mol fractions).

(a)

Conditions:			
$\text{Zn}(\text{OAc})_2$	0.5 mM	pH	6.2
EDTA^{-4}	0.5 mM		
KMes	0.5 M		
Metal		Ligand	
Zn^{2+}	100% complexed with EDTA	EDTA	100% complexed with Zn
		OAc	97.92% as a free ligand 2.08% complexed with H^+
K^+	100% as a free ion	MES	54.20% as a free ligand 45.80% complexed with H^+

(b)

Conditions:			
$\text{Zn}(\text{OAc})_2$	0.5 mM	pH	6.2
CDTA^{-4}	0.5 mM		
KMes	0.5 M		
Metal		Ligand	
Zn^{2+}	100% complexed with CDTA	CDTA	100% complexed with Zn
		OAc	97.92% as a free ligand 2.08% complexed with H^+
K^+	100% as a free ion	MES	54.20% as a free ligand 45.80% complexed with H^+

4.3. Sample synthesis

All experimental work was carried out in Class 10 laminar flow hoods in Class 1000 Clean Laboratory. All samples were prepared in Teflon Savillex vials (Savillex, MN, USA) throughout the experiment. Acid solutions were prepared using 18 mΩ*cm Mili-Q water (Bedford, MA, USA) and AnalaR grade HCl (6 M) and HNO₃ (15.6 M) both sub-distilled in the laboratory. Commercial solutions of Cu (ROMIL Ltd., Cambridge, UK) and Zn (ROMIL Ltd., Cambridge, UK) were used as dopant solution for instrumental mass bias correction and for quality control of the isotope measurement on MC-ICP-MS, respectively. Zn(OAc)₂ dihydrate, CyDTA monohydrate and TMDTA were purchased from Sigma-Aldrich, Na₄EDTA from Fisher Scientific and MES monohydrate from VWR.

DMA was synthesised in the laboratory by following the modified protocol of Namba et al. (2007). The details on this protocol are reported in Chapter 3 of this thesis. The obtained pure DMA ligand material was used in this study to make sample solutions as indicated below.

A Zn(OAc)₂ (1 mM) solution at pH 6.3 was prepared by dissolving Zn(OAc)₂·2H₂O (0.11 g, 0.5 mmol) in 500 ml of MQ H₂O. Na₄EDTA solution (1 mM) was prepared by dissolving Na₄EDTA dihydrate (0.095 g, 0.25 mmol) in 250 ml of MQ H₂O and stirring until complete dissolution. Similarly, 250 ml of (1 mM) stock solutions of TMDTA (0.077 g, 0.25 mmol) and CyDTA monohydrate (0.091 g, 0.25 mmol) were prepared. KMES buffer solution (potassium 2-(*N*-morpholino)ethanesulfonate) (0.5 M) was prepared by dissolving Mes monohydrate (26.66 g, 125 mmol) in 250 ml of MQ H₂O and stirring at 60 °C until complete dissolution was achieved. The pH of the solution was adjusted to 6.2 by the addition of KOH (3 M).

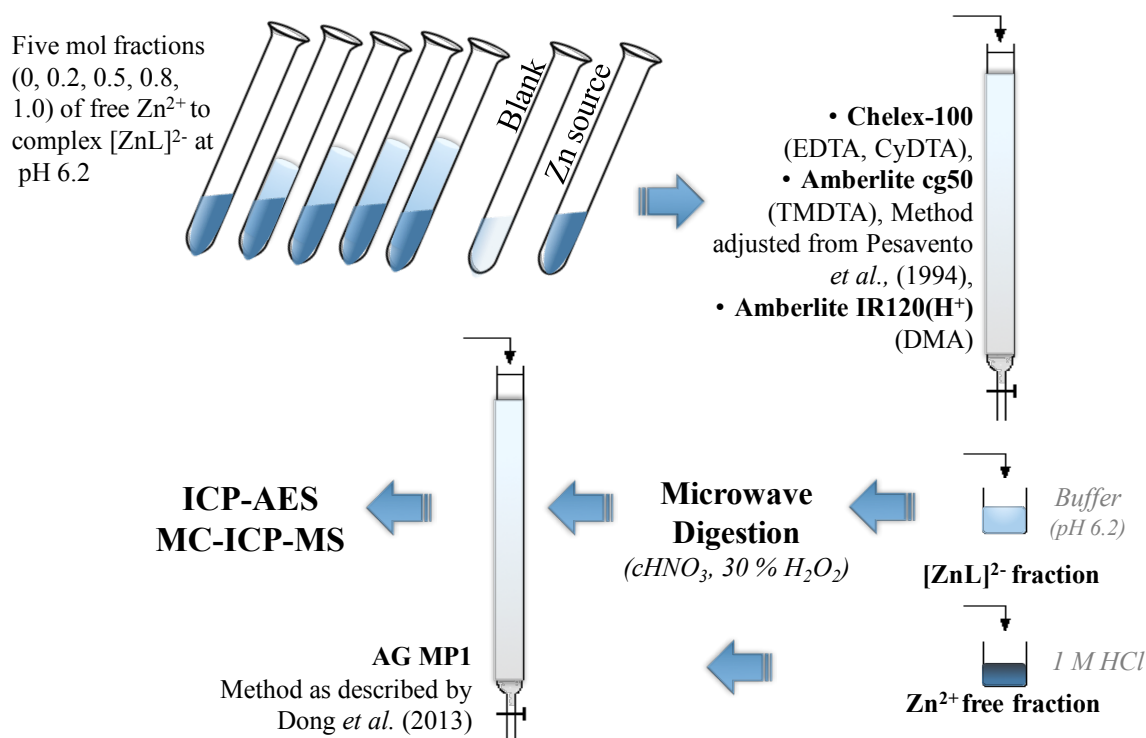


Figure 4.3 Schematic representation of the sample preparation and column exchange protocol used to separate free Zn^{2+} and $[\text{ZnL}]^{2-}$ fractions from the initial mixtures. This method enables quick and straightforward determination of $\delta^{66}\text{Zn}$ isotope preference upon complexation with organic ligands, when compared to NICA-Donnan method used by (Jouvin *et al.*, 2009).

Five different mol fractions (0, 0.2, 0.5, 0.8 and 1) of free Zn^{2+} to total Zn were prepared by adding 10 ml $\text{Zn}(\text{OAc})_2$ (1 mM) to a range of volumes (10, 8, 5, 2 and 0 ml, respectively) of ligand solutions (1 mM) (EDTA, TMDTA, CyDTA and DMA) (as shown in Figure 4.3). All reagents were prepared using 18.2 $\text{m}\Omega\cdot\text{cm}$ Mili-Q water. The solutions were buffered to pH 6.2 using KMes (0.5 M) buffer and equilibrated overnight before proceeding to ion exchange separation.

4.3.1. Ion Exchange Chromatography

Three different cation exchange resins were used to separate complex $[\text{ZnL}]^{2-}$ from free Zn^{2+} fractions based on their complexation strength. The separation was achieved using the cation exchange chromatography procedure summarised in Figure 4.3. Namely, Chelex-100 (BioRad, Na^+ form, 100-200 mesh), a strong chelating resin was used in combination with the reported protocol to separate $[\text{ZnL}]^{2-}$ complex fractions with strong synthetic ligands EDTA and CyDTA; Amberlite cg50 (Dow,

H⁺ form, 100-200 mesh) effectively separated free Zn²⁺ from a [Zn(TMDTA)]²⁻, whereas Amberlite IR-120 (H) (Alfa Aesar, H⁺ form, particle size 0.62 - 0.83 mm) was used in separation of free Zn²⁺ from [Zn(DMA)]²⁻. All resins were prepared and cleaned according to the manufacturers' recommendations and then loaded onto BioRad PolyPrep columns. The ion exchange chromatographic protocol reported in Table 4.2 was followed.

Table 4.2 Cation exchange separation protocol. Modified ion exchange chromatography procedure for the separation of free Zn²⁺ from [Zn-L]²⁻ complex (Kingston et al., 1978); digestion of complex fraction and finally, purification of Zn fraction from the rest of the matrix and other transition elements to enable high precision isotope ratio measurements of ⁶⁶Zn/⁶⁴Zn (Dong et al., 2013).

Procedure	Comment	Resin/Method	Step	Medium	Volume (ml)
Cation Exchange Procedure	To separate free Zn ²⁺ from [ZnL] ²⁻ (after Kingston <i>et al.</i> , 1978)	Chelex-100 , Na ⁺ form, 200-400 mesh (in case of ZnEDTA and ZnCyDTA)	Resin Loading	H ₂ O	1 - 2
			Cleaning	2M HCl	5 x 2ml
			Conditioning	H ₂ O	3 x 2
		Amberlite cg50 , H ⁺ form, 100-200 mesh (in case of ZnTMDTA)	Equilibration	KMES buffer (pH 6.3)	3 x 2
			Sample loading	H ₂ O (pH 6.3)	5 x 2 up to 10 x 2
			Matrix elution	KMES buffer (pH 6.3)	2 x 2
		Amberlite IR-120 , H ⁺ form (in case of ZnDMA)		H ₂ O	3 x 2
				/ZnL complex elution/	
			Zn elution	1M HCl	5 x 2
				/free Zn ²⁺ elution/	
			Cleaning	1M NaOH	2 x 2
				H ₂ O	3 x 2
Complex digestion	To break the organic complexes in Zn-L fractions eluted after cation exchange separation	Microwave Accelerated Reaction System	Sample preparation	Dried complexed fractions dissolved in 5 ml sub-boiled (15.6M) 3ml 30% H ₂ O ₂	
			Digestion method	gradual heating to 210°C and 1.3 kPa (90 min)	
Anion Exchange Procedure	To separate free Zn ²⁺ from interfering material (after Dong <i>et al.</i> , 2013)	AG MP1 , BioRad, Cl ⁻ form, 100-200 mesh	Resin Loading	0.5M HNO ₃	1 - 2
			Cleaning	0.5M HNO ₃	5 x 6
			Conditioning	H ₂ O	5 x 3
				6M HCl	4 x 1
			Sample loading	6M HCl	1 x 1
			Matrix elution	6M HCl	3 x 3
				2M HCl	2 x 3.5
			Zn elution	0.1M HCl	2 x 3.5
			Cleaning	0.5M HNO ₃	5 x 2
				H ₂ O	5 x 1

4.3.2. Cation exchange column procedure (Chelex-100, Alberlite cg50, Amberlite IR120 (H))

Three different resins were applied according to their Zn binding strength. Chelex-100 resin (BioRad, Na⁺ form, 100-200 mesh) is a strong chelating resin with high preference for divalent cations like Zn²⁺. Here, it was used to separate free Zn from a range of mixtures with two strongly binding ligands – EDTA and CyDTA. It has shown to be unsuitable for separation of weaker complexes such as natural organic ligands (Bowles et al., 2006) and/or [Zn(TMDTA)]²⁻ due to evident Zn scavenging from the complex by the resin's strong chelating arms under the conditions stated (Table 4.4). Alternatively, Amberlite cg50 (Dow, H⁺ form, 100-200 mesh) was used. As shown in the elution profiles (Table 4.4), this weakly acidic cation exchanger does not interfere with metal-ligand complexes such as [Zn(TMDTA)]²⁻. Additional challenges emerged when separation of free Zn²⁺ fraction from mixture with [Zn(DMA)]²⁻ complex was tested. This complex is considered very stable based on its affinity constant ($\log K \sim 13$, as shown in Chapter 3), however, most of the commercially available cation separation resins are too strong and thus can break the complex. After testing multiple resin systems, Amberlite IR120 (H) was selected because of its optimal recovery and quick elution, to be used with the above described method (Table 4.2) to separate free Zn²⁺ and complexed [Zn(DMA)]²⁻ fractions from initial mixtures.

Initially, pre-cleaned resins were soaked in 100 ml of MQ H₂O per 5 g of resin and then pipetted into BioRad PolyPrep (Bio-Rad Laboratories, CA, USA) columns (i.d. 8 mm). The chosen resin was then cleaned with 2 M HCl and equilibrated with the 0.5 M KMES buffer (pH 6.2). The buffered samples were then loaded on to the column. [ZnL]²⁻ complex was collected straight away as the samples ran down the column. The resin was further equilibrated with the buffer in order to elute any remaining complexed Zn. Washing column with 1 M HCl eluted all free Zn²⁺ initially exchanged with the resin matrix. After collecting both free and complexed Zn, all the collected samples were evaporated to dryness. Subsequently, Zn fractions were diluted in 1 ml (6 M) HCl in preparation for the anion exchange separation. Prior to

this step, complexed fraction were first digested with a mixture of 15.6 M HNO_3 and 8.8 M H_2O_2 using microwave wet-ashing protocol to break the organic component of the sample (for more details see section 2.3.2 of this chapter). Once digested, the $[\text{ZnL}]^{2-}$ complexed samples were evaporated, refluxed in 6 M HCl for 1 h, re-evaporated and then dissolved in 1 ml (6 M) HCl same as the fractions containing free Zn^{2+} .

4.3.2.1. Digestion of the $[\text{ZnL}]^{2-}$ fractions

In order to measure isotopic composition in fractions containing the $[\text{ZnL}]^{2-}$ complexes, the organic matrix (*i.e.* the corresponding ligand) was broken down using microwave digestion method. Dried samples were diluted in a mixture of 5 ml (15.6 M) HNO_3 and 3 ml (8.8 M) H_2O_2 , and then the organic content was digested using a microwave dry-ashing protocol (210 °C, 1.7 kPa, 90 min).

4.3.2.2. Anion exchange separation on AG MP1 resin

Samples for both free Zn^{2+} and complex $[\text{ZnL}]^{2-}$ fraction diluted in 1 ml (6 M) HCl were passed down PolyPrep columns containing 0.7 ml AG-MP1 (Bio-Rad, Cl^- form, 100-200 mesh) anion-exchange resin (Table 4.2.). Anion exchange chromatography was used to isolate Zn fractions and eliminate matrix components and other trace elements that could possibly cause interferences when measuring Zn isotope composition on MC-ICP-MS.

All collected samples were evaporated to dryness, refluxed in 15.6 M HNO_3 . After final evaporation, dry samples were re-dissolved in 2.5 ml (0.5 M) HNO_3 to determine the Zn concentration and the isotopic composition of Zn in each sample.

4.3.3. Determination of $[\text{Zn}^{2+}]$ concentration

The samples were taken for analysis of their Zn, Cu and Fe concentration by inductively coupled plasma atomic emission spectroscopy (ICP-AES) using a Thermo scientific iCAP 6500 duo view ICP-AES at the Natural History Museum (London, UK). Elements were calibrated using a mixture of single element solutions to give concentrations between 0.05 ppm and 50 ppm and were matrix matched with the

sample to give 4 % HNO_3 (v/v). The samples were aspirated through a sea-spray[©] nebuliser and lofted into the plasma using an argon carrier gas. The carrier gas flow was at 0.7 L/min. The plasma settings are detailed in Table 4.3. Element calibration lines were linear using a minimum of four standards and a correlation coefficient of >0.9999 . Sample solutions were diluted to ensure elements were within the calibrated range. One wavelength of light was used for each element and was selected based on the absence of observable interferences, sensitivity and suitability for the sample matrix/concentrations.

Table 4.3 Thermo Scientific ICP-AES operating parameters

AP 6500 duo-view parameters:	
Power	1250 W
Aux gas flow	1.0 L/min
Nebuliser gas flow	0.7 L/min
Cool gas flow	14 L/min

All blanks measured in this study have concentrations of Zn below quantification limits for the ICP-AES method used. Both lab acid blanks and procedural blanks contained $[\text{Zn}^{2+}] \ll 2 \mu\text{g/L}$ ($\text{Zn}^{2+}_{\text{total}} \ll 20 \text{ ng}$), which is negligible with respect to concentration of the samples (Table 4.5).

4.3.4. Zn isotope composition determinations

The purified Zn fractions from separated samples were dissolved in 0.1 M HNO_3 to final Zn concentration of 100 ppb. Isotope ratios were measured using MC-ICP-MS (Nu instruments, UK) and expressed in parts per thousand (per mill, ‰) using the $\delta^{66}\text{Zn}$ notation (Eq. 4.1):

$$\delta^{66}\text{Zn} = \left[\frac{\left(\frac{^{66}\text{Zn}}{^{64}\text{Zn}} \right)_{\text{sample}}}{\left(\frac{^{66}\text{Zn}}{^{64}\text{Zn}} \right)_{\text{standard}}} - 1 \right] \times 1000 \text{ [‰]} \quad (4.1)$$

The empirical external normalisation method (Mason et al., 2004b) was used to correct for instrumental mass bias and the measurements were bracketed with the in-house standard London Zn (Arnold et al., 2010a; Mason et al., 2004b). Accuracy and

precision of the isotope measurements were assessed by analysing two standard single-element solutions (IRMM-3702 Zn (Mason et al., 2004a; Mason et al., 2004b) and Romil Zn (Moeller et al., 2012) during each measurement session.

For every ligand system tested, the $^{66/64}\text{Zn}$ isotope ratios of the initial solution (*i.e.* $\text{Zn}(\text{OAc})_2$), the free (*i.e.* Zn^{2+}) and the complexed (*i.e.* $[\text{ZnL}]^{2-}$) fractions were determined and a mass balance calculated to further test the integrity of the data.

To quantify the isotope effect introduced on complexation of Zn with the test ligands, the isotopic fractionation ($\Delta^{66}\text{Zn}_{[\text{ZnL}]^{2-} - \text{free Zn}^{2+}}$) was calculated as:

$$\Delta^{66}\text{Zn}_{[\text{ZnL}]^{2-} - \text{free Zn}^{2+}} = \delta^{66}\text{Zn}_{[\text{ZnL}]^{2-}} - \delta^{66}\text{Zn}_{\text{free Zn}^{2+}} \quad (4.2)$$

where L refers to the tested ligands (L= ETDA, TMDTA, CyDTA and DMA).

Zn isotopic composition was measured in both free Zn^{2+} and corresponding $[\text{ZnL}]^{2-}$ fractions, as well as the source Zn solution ($\text{Zn}(\text{OAc})_2$), in order to confirm closed mass balance.

The controlled design of the experimental solutions allows for the mathematical determination of the $\delta^{66}\text{Zn}$ in $[\text{ZnL}]^{2-}$ complex fractions. Based on the mass conservation principle we used $\delta^{66}\text{Zn}_{\text{free Zn}^{2+}}$ trend and the isotopic signature of the Zn source solution ($\delta^{66}\text{Zn}_{\text{Zn}(\text{OAc})_2 \text{ source}}$) to calculate the Zn partitioning in $[\text{ZnL}]^{2-}$ complex fractions ($\delta^{66}\text{Zn}_{[\text{ZnL}]^{2-}}$). The calculated $\delta^{66}\text{Zn}_{[\text{ZnL}]^{2-}}$ trend, determined from the Zn isotopic ratios in the source solution and in the Zn free fraction ($\delta^{66}\text{Zn}_{\text{free Zn}^{2+}}$) was plotted over the measured $\delta^{66}\text{Zn}_{[\text{ZnL}]^{2-}}$ complex data, and discussed with regards to accuracy of the method and possible interfering effects of organics in the $[\text{ZnL}]^{2-}$ fractions.

4.4. Results and Discussion

4.4.1. Ion exchange separation of free Zn^{2+} from $[\text{ZnL}]^{2-}$

In order for this approach to be successful, no Zn exchange should occur between $[\text{ZnL}]^{2-}$ complexes and the cation exchange resin (Figure 3.1). Therefore, an essential

part of the resin selection process is to ensure that the resin's active groups interact only with free Zn^{2+} , while no Zn is de-coordinated from the corresponding $[\text{ZnL}]^{2-}$ complex. To this end, resins used in this study were selected based on their affinity for Zn^{2+} . Chelating resin Chelex-100 was used for experiments with EDTA and CyDTA which are the strongest ligands (highest Zn affinity constants - $\log K_{[\text{Zn}(\text{EDTA})]} = 16.4$ and $\log K_{[\text{Zn}(\text{CyDTA})]} = 18.5$, (Bjerrum et al., 1964)). Alternatively, Amberlite cg50 and Amberlite IR-120 were used for TMDTA ($\log K_{[\text{Zn}(\text{TMDTA})]} = 15.8$, (Bjerrum et al., 1964)) and DMA ($\log K_{[\text{Zn}(\text{DMA})]} = 13.1$, Chapter 3), respectively (Table 4.4).

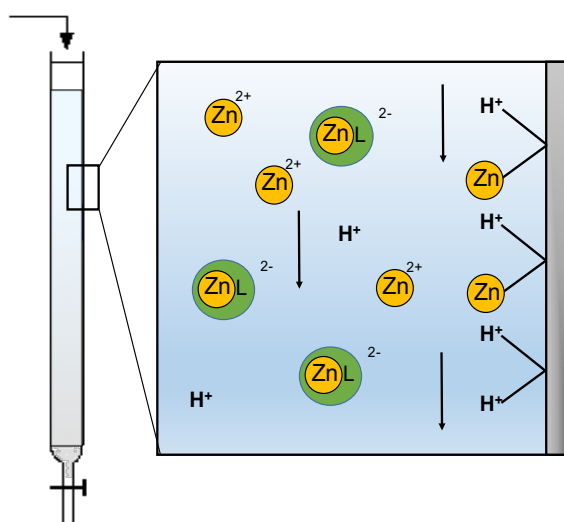


Figure 4.4 Ion exchange chromatography. The resins selected retain free Zn^{2+} from the sample but do not interact with the $[\text{ZnL}]^{2-}$ complex which is eluted immediately. The retained Zn^{2+} is eluted only upon acidification *i.e.* protonation of resins' functional groups.

During the elution sequence summarised in Tables 4.2, the Zn complexes were instantly eluted from the column loaded with the corresponding resin, whereas free Zn^{2+} was retained on the resin and only stripped from it on addition of HCl. Figures 4.5 (a-c) show representative elution profiles for three samples with different molar ratios of free Zn^{2+} to total Zn. Although the elution behaviour is only presented for $[\text{Zn}(\text{CyDTA})]^{2-}$, the other ligand systems showed the same pattern (Table 4.4).

At 0 mol fraction of free Zn^{2+} , the $[\text{ZnL}]^{2-}$ complex was eluted with the buffer solution instantly (Figure 4.5c). For the 0.5 mol fraction, the complexed $[\text{ZnL}]^{2-}$ was eluted with the buffer solution and the free Zn^{2+} was eluted with 1 M HCl (Figure 4.5b). Finally, with the 1 mol fraction, no Zn^{2+} was eluted with the buffer solution, whereas upon the addition of 1 M HCl, elution of free Zn^{2+} was instantaneous, explaining the sharp peak. For all ligands tested, average Zn recovery was calculated

to be 96 – 105 % of Zn (Figure 4.5a). The chelating resin Chelex-100 successfully separated free Zn^{2+} in mixtures with $[\text{Zn}(\text{EDTA})]^{2-}$ and $[\text{Zn}(\text{CyDTA})]^{2-}$ at pH 6.2, whereas using Amberlite resins, free Zn^{2+} was completely separated from mixtures with $[\text{Zn}(\text{TMDTA})]^{2-}$ complex (Amberlite cg50) and $[\text{Zn}(\text{DMA})]^{2-}$ (Amberlite IR-120) under the same experimental conditions.

If the resin's chelating environment overpowers the ligand's ability to maintain the complex with Zn, then the complex will dissociate and the resin will sequester both free and complexed Zn present in the solution (*e.g.* EDDA attempted separation using Amberlite cg50 resin – see Table 4.4). As a result, typical elution pattern shows that all Zn is recovered in the acid wash step while none would be eluted in the initial buffer elution.

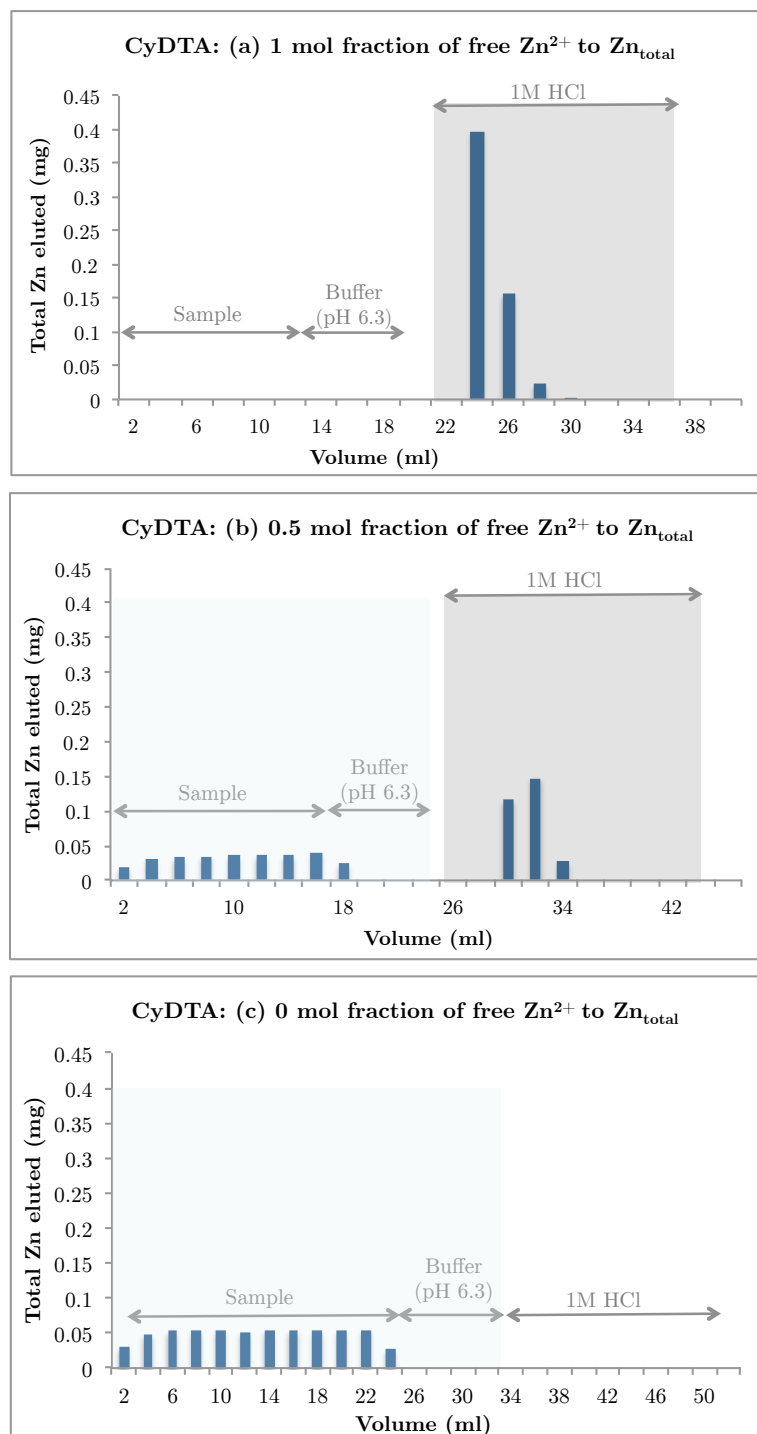


Figure 4.5 Elution profiles of solutions containing three different mol fractions (1, 0.5, 0) of free Zn^{2+} to complexed $[\text{ZnL}]^{2-}$ at pH 6.2. Elution profiles are shown for CyDTA, yet the same pattern was observed for the other ligands (see Table 4.4). (a) 1 mol fraction shows complete elution of Zn^{2+} in presence of 1 M HCl. (b) 0.5 mol fraction of $[\text{ZnL}]^{2-}$ is eluted instantly with 0.5 M KMes whereas for eluting free Zn^{2+} 1 M HCl was used. (c) In 0 mol fraction, all Zn was eluted instantly in complexed form. No free Zn^{2+} was detected with subsequent elution with acid, as visible from the profiles after addition of 1 M HCl to the columns.

Table 4.4 Separation behaviour observed upon elution of samples containing different mol fractions of free Zn^{2+} to total Zn. Selection of an adequate chelating resin to isolate free Zn^{2+} from a mixture with its complex form is tested against the affinity constant ($\log K$) of the complex. Unlike Amberlite IR-120(H), strong chelating resins, such as Chelex-100 and Amberlite cg50, were not adequate for separation of complexes with complexation strength $\log K < 13$, due to dissociation of complexes.

Ligand sytem	Log K _[Zn-L]	Resin	Mol fraction free Zn ²⁺ to total Zn	Concentration [Zn ²⁺] measured						New mol fraction:
				Complexed fraction [Zn-L] ²⁺ :			Free fraction [Zn ²⁺]:			
				ppm	mg	%	ppm	mg	%	
EDDA	11.2	Amberlite cg50	0.5	0.00	0.000	0.0	0.59	0.004	100.0	<i>complex broken</i>
			0	0.04	0.000	6.1	0.64	0.004	93.9	<i>complex broken</i>
EDTA	16.4	Chelex-100	1	0.00	0.000	0.0	77.14	0.463	100.0	1.00
			0.5	43.07	0.258	46.5	49.46	0.297	53.5	0.53
			0	86.27	0.518	97.6	2.13	0.013	2.4	0.02
TMDTA	15.6	Chelex-100	1	0.00	0.000	0.0	101.68	0.610	100.0	1.00
			0.5	21.81	0.131	21.0	81.92	0.492	79.0	<i>complex broken</i>
			0	39.21	0.235	35.6	71.06	0.426	64.4	<i>complex broken</i>
		Amberlite cg50	0.5	0.28	0.002	44.2	0.35	0.002	55.8	0.56
			0	0.55	0.003	88.2	0.07	0.000	11.8	0.12
CyDTA	18.5	Chelex-100	1	0.00	0.000	0.0	0.97	0.580	100.0	1.00
			0.5	48.19	0.289	49.8	48.54	0.291	50.2	0.50
			0	96.72	0.580	100.0	0.00	0.000	0.0	0.00
DMA	12.8	Amberlite cg50	0.5	0.02	0.010	3.6	0.43	0.256	96.4	<i>complex broken</i>
			0	0.03	0.019	7.0	0.41	0.248	93.0	<i>complex broken</i>
		Amberlite IR120 (H)	0.5	0.21	0.127	67.1	0.10	0.062	32.9	0.33
			0	0.29	0.175	85.6	0.05	0.029	14.4	0.14

The method used in this study circumvents experimental challenges associated with measuring isotopic ratios of Zn originating from different ionic species in the same sample (free Zn^{2+} and Zn complexed to organic ligands). The protocol listed in Table 4.2 lasts on average 2-3 h in total, while for the same process the method employing NICA-Donnan model requires multiple days of equilibration (Jouvin et al., 2009). Also, possible isotopic fractionation upon ion adhesion to the Donnan membrane has to be considered and corrected for appropriately (Jouvin et al., 2009). Hence, additional control experiments are needed which makes the NICA-Donnan method significantly longer and more cumbersome.

4.4.2. Isotopic fractionation between complexed and free Zn^{2+} and comparison with theoretical, experimental and field observations

The results shown in Figure 4.6 demonstrate a heavy isotope bias during Zn complexation with all the ligands tested, and the magnitude of fractionation between

free and complexed Zn ($\Delta^{66}\text{Zn}_{[\text{ZnL}]2- - \text{free Zn}^{2+}$) was independent of the Zn:Ligand ratio. This suggests the fractionation was in all cases at thermodynamic equilibrium. The average values of $\Delta^{66}\text{Zn}_{[\text{ZnL}]2- - \text{free Zn}^{2+}$ across the mol fractions tested were $0.42 \pm 0.09 \text{ ‰}$ (2SD, n=6) for [Zn(EDTA)], $0.47 \pm 0.05 \text{ ‰}$ (2SD, n=3) for [Zn(TMDTA)], $0.60 \pm 0.14 \text{ ‰}$ (2SD, n=6) for [Zn(CyDTA)], and $0.26 \pm 0.18 \text{ ‰}$ (2SD, n=9) for [Zn(DMA)] (Figure 4.6, Table 4.5).

Our results suggest therefore that the fractionation occurring upon complexation by the observed ligands is at thermodynamic equilibrium and the heavier Zn isotope is preferentially complexed by ligands. The heavy bias is in agreement with equilibrium reaction dynamics during formation of strong bonds between metal and ligand (Bigeleisen and Mayer, 1947). The same conclusions were reached in experimental studies of organic complexes of Fe ($\Delta\text{Fe}_{[\text{Fe}(\text{DFOB})] - \text{FreeFe}} = 0.6 \text{ ‰}$, (Dideriksen et al., 2008)), Zn ($\Delta\text{Zn}_{[\text{Zn}(\text{PHA})] - \text{FreeZn}} = 0.24 \text{ ‰}$, (Jouvin et al., 2009)) and Cu ($\Delta\text{Cu}_{[\text{Cu}(\text{HA})] - \text{FreeCu}} = 0.26 \text{ ‰}$, (Bigalke M., 2010)).

Leaching soils with EDTA or DTPA solution is often used to determine quantities and isotopic composition of soils (Dobermann and Fairhurst, 2000). The results presented here unequivocally demonstrate that chelating ligands, such as EDTA, introduce isotopic fractionation into a system. This is an important finding, when considering the extent to which chelating ligands are used in environmental geochemistry studies. Chelating ligands were previously used in isotope geochemistry studies to *e.g.* buffer hydroponic solutions for plant growth (Black et al., 2008; Jouvin et al., 2012) and/or to assess the extractable soil Zn (Widodo et al., 2010) with presumption that they do not exhibit fractionation behaviour.

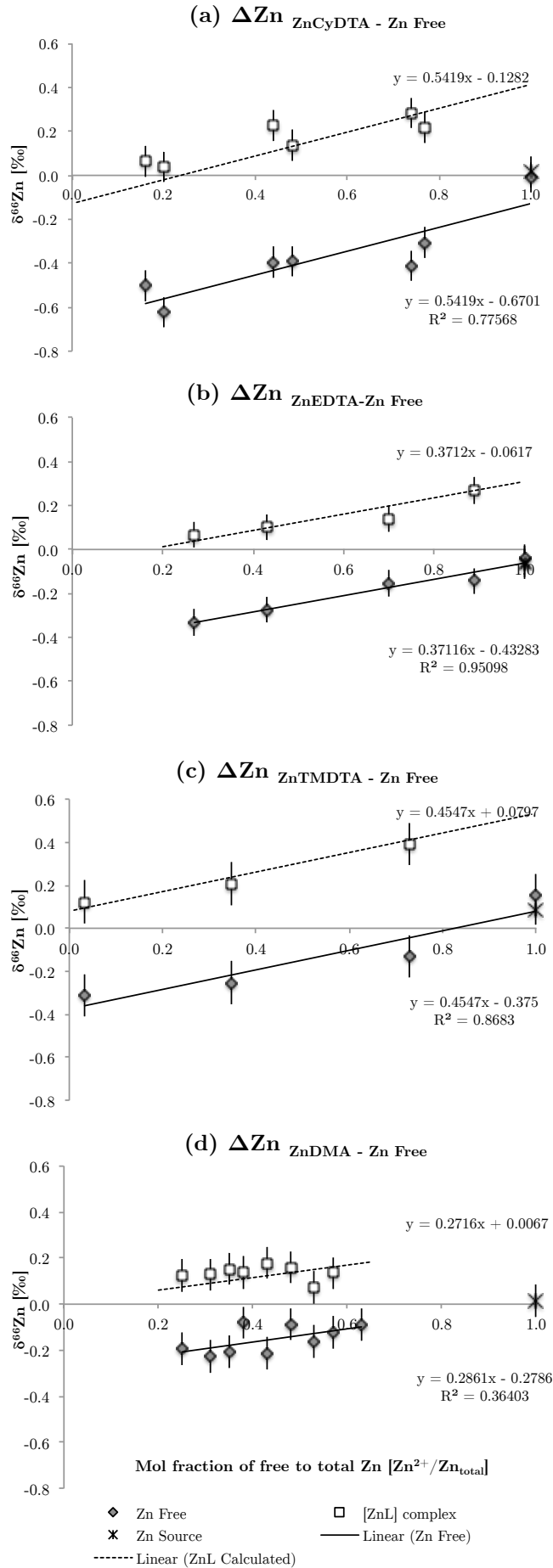


Figure 4.6 Experimentally determined $\delta^{66}\text{Zn}$ in free Zn^{2+} and $[\text{ZnL}]^{2-}$ complex fractions for the various mol fractions of free to total Zn $[\text{Zn}^{2+}/\text{Zn}_{\text{total}}]$ (indicated on X-axis). Data points represent average measured with errors corresponding to the analytical errors. The isotopic fractionation factor ($\Delta^{66}\text{Zn}_{[\text{ZnL}]^{2-} - \text{free Zn}^{2+}}$, Eq. 4.1) are within error the same for all the mol fractions, confirming the fractionation factors determined are for systems at equilibrium. The four experimental fraction factors for the complexation of Zn within $[\text{ZnL}]^{2-}$ are : $0.60 \pm 0.14 \text{ ‰}$ (2SD, $n = 6$), in $[\text{Zn}(\text{CyDTA})]$, $0.42 \pm 0.09 \text{ ‰}$ (2SD, $n = 6$) in $[\text{Zn}(\text{EDTA})]$, $0.47 \pm 0.05 \text{ ‰}$ (2SD, $n = 3$) in $[\text{Zn}(\text{TMDTA})]$, and, $0.26 \pm 0.18 \text{ ‰}$ (2SD, $n = 9$) in Zn complexes with DMA. (The $\delta^{66}\text{Zn}_{[\text{ZnL}]}$ trend, indicated in the figures as dashed lines, was calculated from the isotopic ratios identified in the initial system ($\delta^{66}\text{Zn}_{\text{Zn-source}}$) and the measured $\delta^{66}\text{Zn}_{\text{free Zn}^{2+}}$ trend. Our measured data match the $\delta^{66}\text{Zn}_{[\text{ZnL}]}$ trend estimated based on the mass conservation principle.)

Table 4.5 $[\text{Zn}^{2+}]$ concentration and $\delta^{66}\text{Zn}$ isotopic composition measured in the samples containing different ratios of free to complex Zn. (a) Quantitative recovery of Zn found in all systems observed (99 %, 105 %, 96 % and 98 % in TMDTA, EDTA, CyDTA and DMA samples, respectively). (b) Calculated fractionation factors induced upon the Zn complexation with the tested organic ligands for each mol fraction tested. The fractionation factors of the various mol fractions agree within 2SD errors, suggesting that the system is at equilibrium. Consequently, the equilibrium ΔZn was calculated for the complexation reaction using the individual $\Delta^{66}\text{Zn}$ from each mol fraction.

EDTA	(a)						(b)						
	Zn Complexed	Zn Free	Total Zn	[Zn] (mg)	Expected	Recovered (%)	Zn Complexed	2SD	n	Zn Free	2SD	n	$\Delta^{66}\text{Zn}$ (‰)
				Mol fraction calculated (Free Zn ²⁺ /total Zn)									
Repetition 1													
0	0.617	0.021	0.638	0.03	0.583	109.35	0.28	0.03	2	n.d.	n.d.		
0.2	0.468	0.114	0.581	0.20	0.583	99.67	n.d.	n.d.		-0.33	0.07	3	
0.5	0.275	0.359	0.634	0.57	0.583	108.77	0.22	0.02	2	-0.35	0.02	2	0.56
0.8	0.114	0.397	0.511	0.78	0.583	87.60	0.09	0.01	2	-0.39	0.06	4	0.48
1	0.000	0.488	0.488	1.00	0.583	83.73	n.d.	n.d.		0.04	0.03	4	
Repetition 2													
0	0.421	0.157	0.579	0.27	0.578	100.18	0.07	0.03	4	-0.33	0.08	4	0.40
0.2	0.327	0.249	0.576	0.43	0.578	99.74	0.10	0.04	3	-0.27	0.07	4	0.38
0.5	0.174	0.398	0.572	0.70	0.578	99.06	0.14	0.09	3	-0.15	0.06	4	0.29
0.8	0.061	0.515	0.576	0.89	0.578	99.77	0.27	0.24	4	-0.14	0.15	5	0.41
1	0.000	0.564	0.564	1.00	0.578	97.66	n.d.	n.d.		-0.04	0.01	3	
Zn source:	0.000	0.578	0.578	1.00		Mean:				-0.06	0.01	3	Mean: 2SD
Blank:	0.000	0.000	0.000			98.55							0.42 0.09
TMDTA	Zn Complexed	Zn Free	Total Zn	Mol fraction calculated	Expected	Recovered (%)	Zn Complexed	2SD	n	Zn Free	2SD	n	$\Delta^{66}\text{Zn}$ (‰)
Repetition 1													
0	0.790	0.009	0.799	0.01	0.739	108.07	0.47	0.19	3	n.d.	n.d.		
0.2	0.774	0.026	0.800	0.03	0.739	108.27	0.12	0.07	4	-0.31	0.05	2	0.43
0.5	0.505	0.270	0.775	0.35	0.739	104.84	0.21	0.18	4	-0.25	0.11	4	0.46
0.8	0.208	0.557	0.766	0.73	0.739	103.60	0.39	0.17	4	-0.13	0.03	2	0.52
1	0.000	0.746	0.746	1.00	0.739	100.94	n.d.	n.d.		0.15	0.00	1	
Zn source:	0.000	0.739	0.739	1.00		Mean:				0.09	0.09	3	Mean: 2SD
Blank:	0.000	0.000	0.000			105.14							0.47 0.05
CyDTA	Zn Complexed	Zn Free	Total Zn	Mol fraction calculated	Expected	Recovered (%)	Zn Complexed	2SD	n	Zn Free	2SD	n	$\Delta^{66}\text{Zn}$ (‰)
Elution profile													
0	0.580	0.013	0.593	0.02	0.583	101.70	n.d.	n.d.		n.d.	n.d.		
0.5	0.289	0.291	0.580	0.50	0.583	99.48	n.d.	n.d.		n.d.	n.d.		
1	0.000	0.580	0.580	1.00	0.583	99.42	n.d.	n.d.		n.d.	n.d.		
Repetition 1													
0	0.545	0.003	0.548	0.01	0.583	93.99	n.d.	n.d.		n.d.	n.d.		
0.2	0.453	0.086	0.539	0.16	0.583	92.31	0.06	0.01	5	-0.50	0.16	3	0.57
0.5	0.299	0.239	0.537	0.44	0.583	92.10	0.14	0.01	2	-0.39	0.10	3	0.53
0.8	0.110	0.461	0.571	0.81	0.583	97.82	0.22	0.04	4	-0.31	0.28	2	0.52
1	0.000	0.456	0.456	1.00	0.583	n.d.	n.d.	n.d.		-0.01	0.02	5	
Repetition 2													
0	0.613	0.002	0.615	0.00	0.583	105.39	-0.02	0.00	1	n.d.	n.d.		
0.2	0.437	0.109	0.546	0.20	0.583	93.51	0.04	0.02	3	-0.62	0.01	3	0.66
0.5	0.283	0.266	0.550	0.48	0.583	94.23	0.23	0.00	3	-0.40	0.02	3	0.62
0.8	0.115	0.341	0.456	0.75	0.583	n.d.	0.28	0.02	2	-0.41	0.01	2	0.69
1	0.000	0.528	0.528	1.00	0.583	90.50	n.d.	n.d.		0.05	0.01	5	
Zn source:	0.000	0.583	0.583	1.00		Mean:				0.01	0.02	4	Mean: 2SD
Blank	0.000	0.000	0.000			96.41							0.60 0.14
DMA	Zn Complexed	Zn Free	Total Zn	Mol fraction calculated	Expected	Recovered (%)	Zn Complexed	2SD	n	Zn Free	2SD	n	$\Delta^{66}\text{Zn}$ (‰)
Repetition 1													
0	0.347	0.114	0.461	0.25	0.414	111.29	0.12	0.14	2	-0.19	0.03	4	0.32
0.2	0.300	0.133	0.434	0.31	0.414	104.77	0.13	0.01	3	-0.23	0.07	2	0.36
0.5	0.218	0.201	0.419	0.48	0.414	101.16	0.16	0.03	4	-0.09	0.03	5	0.25
0.8	0.179	0.203	0.382	0.53	0.414	92.36	0.07	0.03	4	-0.16	0.06	5	0.24
1	0.126	0.216	0.342	0.63	0.414	82.60	-0.19	0.41	4	-0.09	0.07	9	0.10
Repetition 2													
0	0.258	0.142	0.400	0.35	0.414	96.57	0.15	0.06	4	-0.21	0.01	4	0.36
0.2	0.267	0.166	0.433	0.38	0.414	104.67	0.14	0.01	4	-0.08	0.04	2	0.22
0.5	0.233	0.174	0.407	0.43	0.414	98.26	0.18	0.06	4	-0.21	0.14	4	0.39
0.8	0.165	0.216	0.381	0.57	0.414	91.92	0.13	0.03	4	-0.12	0.09	5	0.26
1	n.d.	0.229	/	/	0.414	/	n.d.	n.d.		-0.09	0.07	9	
Zn source:	0.000	0.414	0.414	1.00		Mean:				0.01	0.04	4	Mean: 2SD
Blank:	0.000	0.000	0.000			98.18							0.28 0.18

The magnitude of the isotopic fractionation reported in this study is in close correlation with isotopic patterns previously indicated using NICA-Donnan approach (Jouvin et al., 2009). Both data by Jouvin et al. (2009) and results presented here emphasise the importance of complexation by organic matter and the contribution of the isotope effect created upon complexation mechanisms to the Zn isotopic signals in the environment. Although this effect in soil solution is often masked by occurrence of other physiological processes, an increasing number of studies supports the idea that complexation with organic ligands plays an important role in speciation at the plant-soil level and can introduce heavy isotope signatures in the environment. Yet, the link between these signatures and complexation chemistry is insufficiently explored.

Our results for the four hexadentate ligands, with similarities in their Zn complexing fashion, allow us to explore the link between isotope signatures and the complex structure. Despite the similar Zn-coordinating donor groups, the differences in the exact geometries of the $[\text{ZnL}]^{2-}$ complexes result in a range of affinity constants (K_{ZnL}). Figure 4.7 indicates a correlation between the ligand's affinity for Zn (expressed as $\log K_{\text{ZnL}}$) and the isotopic bias found in this experiment. The figure indicates an increase in heavy bias with increasing complexation strength. Such a trend has been inferred in the theoretical work of Black et al. (2011), who conducted a computational study of environmentally relevant Zn complexes. Their findings suggested that preference for heavy isotopic composition is likely due to formation of shorter and presumably stronger bonds between the Zn^{2+} centre and the donor atoms of the corresponding ligands. The empirical relationship obtained in Figure 4.7 can be used to provide initial predictions of isotopic fractionation in ligands with similar Zn complexing environments and affinity constants, such for most phytosiderophores. The correlation found indicates that stronger preference for Zn (*i.e.* higher $\log K_{\text{ZnL}}$ value) yields heavier $\delta^{66}\text{Zn}$ signature, as Black et al. (2011) have implied previously using computational bond strength analysis.

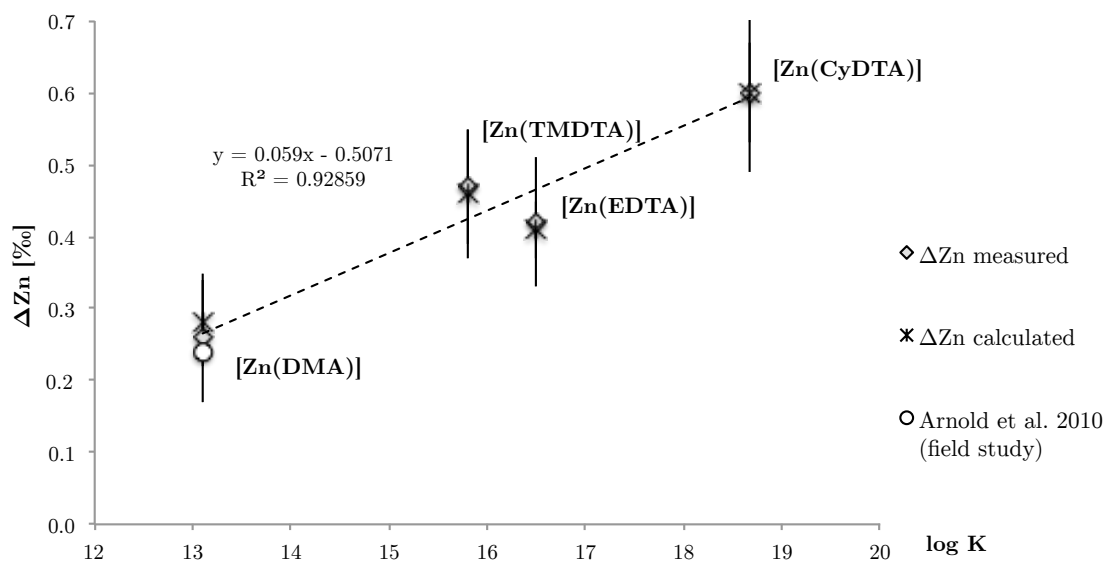


Figure 4.7 Measured Zn isotopic fractionation upon complexation by organic ligands as a function of their stability constants ($\log K_{ZnL}$). Data represent measured means $\pm 2SD$ ($n = 4$). Also shown on the graph are values from the Zn studies of Arnold et al. (2010a) in the Zn-deficient soil of Philippine rice plots. The relation between the isotopic fractionation ($\Delta^{66}Zn_{[ZnL] - free Zn^{2+}}$) and $\log K_{ZnL}$ is linear, as suggested for $\Delta^{56}Fe$ (Page and Feller) – free Fe^{3+} (Morgan et al., 2010) and $\Delta^{65}Cu$ (Kitahara et al.) – free Cu^{2+} (Ryan, 2014). Uncertainty of the slope (± 0.01) and the y-intercept (± 0.18) were calculated using Student's t-test on the trend line.

4.5. Conclusions

The method reported in this study successfully circumvents experimental challenges associated with measuring isotopic ratios of Zn originating from different ionic species in the same sample. The ion exchange chromatography protocol shown here successfully separated free Zn^{2+} from $[ZnL]^{2-}$ complex species, before isotopic fractionation in the separated fractions was measured. The method reported here shows great potential to be used in studies with natural chelating ligands, and their ability to complex essential metallic nutrients in the environment.

To our knowledge, this is the first study to report direct measurements of isotopic partitioning of Zn on complexation with a phytosiderophore. The work reports $\Delta^{66}Zn_{[ZnDMA]^{2-} - free Zn^{2+}} = 0.26 \pm 0.18$ ‰ measured in lab conditions, which is in agreement with conclusions of Arnold et al. (2010a) based on their rice field study. Considering the range of Zn isotope variation observed to date in the terrestrial environments

(Cloquet et al., 2008), the extent of fractionation for Zn observed during complexation with phytosiderophores and other organic ligands is significant.

The strength of Zn binding is correlated with the extent of isotopic fractionation. Namely, stronger binding between Zn and the corresponding organic ligand (the higher $\log K_{\text{ZnL}}$ value) implies greater preference for the heavier Zn. This can be explained by the tendency of the heavy isotope to form shorter and stronger bonds (Black et al., 2011).

Chapter 5

Ab initio Modelling of $\Delta^{66}\text{Zn}$ [ZnL] - $\text{Zn}(\text{H}_2\text{O})_6$ from First Principles of Quantum Mechanics.

5.1. Introduction

5.1.1. Computational studies as an alternative method to model isotopic fractionation

Computational modelling is becoming an important approach in geochemistry studies to test initial hypotheses and guide research efforts, especially when technical obstacles hamper experimental progress. Experimental data on isotopic effects caused by coordination of metals to ligands is limited. To this end, theoretical calculations were carried out to test isotopic fractionation in metal complexes as an attempt to rationalise experimental data reported in Chapter 4 of this thesis, as well as to predict isotopic effects of similar physiologically relevant ligands.

Computational methods require significantly less time and resources, therefore their wider application in the environmental research is highly anticipated. Quantum-mechanical methods such as *ab initio* modelling have shown a great potential for

estimating isotopic effects introduced to the environment during physicochemical reactions. *Ab initio* studies take into consideration the first principles of quantum mechanics to calculate the zero-energy of the observed species. The theoretical background of this approach, particularly the Density Functional Theory (DFT) modelling is very well established in the works of Urey (1947) and Bigeleisen and Mayer (1947), however the mathematical foundation of this concept was first laid out by Hohenberg and Khon (1964).

With improvements and wider availability of computational facilities in the last decade, a number of mathematical methods and computer codes became available for use as practical techniques for solving quantum chemistry problems. To date, numerous studies using computational *ab initio* methods were published aiming to predict the direction and the extend of isotopic fractionations of different metals in complexes with organic ligands, including Fe (Domagal-Goldman et al., 2009; Moynier et al., 2013), Zn (Black et al., 2011; Fujii and Albarede, 2012; Fujii et al., 2014), Mg (Black, 2007), Cu (Fujii et al., 2013) and Ni (Fujii et al., 2014).

5.1.2. Density Functional Theory – background and applications

Density functional theory (DFT) is used to model electronic structures of atoms, molecules and solids by approximating the structure of the electron density cloud. For this, the DFT theory simplifies the many-body Schrödinger equation (Eq. 5.1 – 5.2) by utilising the 1st Kohn-Sham principle (1965).

Following the laws of quantum mechanics all information about the system is contained in its wave function.

$$\hat{H}\Psi = E\Psi \quad (5.1)$$

where H represents a Hamiltonian operator, Ψ is a wave function and E is energy.

The total energy of the system, and thus the Hamiltonian is described as the sum of its kinetic energy (T), potential energy (V) and the energy of the inter-electron interactions (U) due to Coulombic attraction or repulsion of particles:

$$\hat{H}\Psi = [\hat{T} + \hat{V} + \hat{U}] \Psi = \left[\sum_i^N \left(-\frac{\hbar^2}{2m_i} \right) \nabla_i^2 + \sum_i^N V(\vec{r}_i) + \sum_{i<j}^N U(\vec{r}_i, \vec{r}_j) \right] \quad (5.2)$$

where ∇_i^2 is the Laplacian operator acting on particle i ; m_i - mass of the particle i ; q_i - charge of the particle i ; r_{ij} - distance between particles i and j .

Hohenberg and Kohn (1964) developed a simplification to define a property of the system not by using its complex wave function but a functional of its electron density (ρ). In quantum mechanics, electron density is described as a probability of locating a particle in a defined position in space:

$$\hat{\rho}(\vec{r}) = |\Psi(\vec{r})|^2 \quad (5.3)$$

where $\vec{r}$ is a vector function of the particle (electron) observed.

The reference system in DFT, *i.e.* the zero energy system, corresponds to hypothetical state where, following the Born-Oppenheimer approximation, electrons in the observed molecule are static and at an infinite distance from the nuclei (Jensen, 1999). This way the computations are limited to motions of electrons only. Therefore, the above Eq. 5.1 becomes the Kohn-Sham single electron wave function:

$$\hat{H}\Psi = \left[-\sum_i^N \left(\frac{\nabla_i^2}{2} \right) - \sum_i^N \sum_j^N \frac{V_i}{r_{ij}} + \sum_{i<j}^N \frac{1}{r_{ij}} \right] \Psi \quad (5.4)$$

where the first term $(-\sum_i^N (\frac{\nabla_i^2}{2}))$ is an expression for the kinetic energy of the electrons; the second summa $(-\sum_i^N \sum_j^N \frac{V_i}{r_{ij}})$ represents the electrons-nuclei attraction, and the final term $(+\sum_{i<j}^N \frac{1}{r_{ij}})$ is the repulsion between the electrons in the electron cloud.

Thermodynamic analysis using quantum mechanistic methods based on the electronic density cloud can, by estimating system's various energy functions, predict specific chemical properties of the observed system (*e.g.* a molecule).

5.1.3. Calculations of equilibrium stable isotope fractionation using Density Functional Theory (DFT)

Equilibrium stable isotope fractionation can be successfully modelled based on the small energy differences between isotopologue substances. This variability in energy

is caused by mass-dependant differences in vibrational energies of the observed species, and leads to an uneven partitioning of isotopes at equilibrium conditions.

DFT is a valuable tool in calculating isotopic partitioning in different chemical species. It is a preferred computational method for structures containing transition metals since it takes into consideration static electron correlations with minimal computational cost (Cramer and Truhlar, 2009). Optimized DFT outputs are widely preferred over the results of Hartree-Fock calculations, which is an alternative method to approximate solution of the Schrödinger equation. However, inability to improve results systematically as well as neglecting van der Waals interactions within a molecule makes DFT unsuitable for certain types of studies (Jensen, 1999).

DFT provides estimates on isotope partitioning at thermodynamic equilibrium by calculating energy of different isotopologues. This information is then coupled with partition coefficient function (Q) to determine the isotope partitioning. Statistical mechanics defines partition function as:

$$Q = \sum_n e^{-\epsilon_n/kT} \quad (5.5)$$

where ϵ_n represents the energies of the molecular states, k is the Boltzmann constant ($1.3806 \times 10^{-23} \text{ m}^2\text{kg s}^{-2}\text{K}^{-1}$) and T is the temperature expressed in K.

While the potential energies of the isotopologues are similar, the kinetic energies differ among the molecules incorporating different isotopes thus causing a quantum mechanical effect, which can be quantified via reduced partition function ratios ($\ln \beta$) as it is shown in the further text.

Partition function (Q), and therefore the ratios of the partition functions for two isotopologues (X and Y), is a function of symmetry numbers and their masses (Eq. 5.6):

$$\frac{Q_X}{Q_Y} = \frac{s_X}{s_Y} \left(\frac{M_X}{M_Y} \right)^{\frac{3}{2}} \quad (5.6)$$

where Q_x and Q_y refer to isotopologues containing heavy and light isotope respectively, s_x and s_y refer to symmetry numbers of a heavy and a light

isotopologue respectively, and M_x and M_y represent masses of isotopologue molecules.

Bigeleisen and Mayer (1947) define a mass-dependant factor β as the ratio of the equilibrium constants for the dissociation of the two isotopic molecules into atoms. In this study, Bigeleisen and Mayer derived and discussed in detail calculations to determine isotope enrichment factor ($\ln \alpha$) from the reduced partition function ratio ($\ln \beta$) (Bigeleisen and Mayer, 1947). Namely, the difference between the reduced partition function ratios (RPFR, calculated as $1000 \cdot \ln \beta$) of the isotopologue molecules is approx. equal to the predicted equilibrium isotope fractionation between them:

$$\delta M_{\text{isotopologue } x - \text{isotopologue } y} \approx 1000 \cdot \ln \beta \text{ [‰]} \quad (5.7)$$

where x represents a molecule containing heavier isotope and y refers the same molecule with the lighter isotope substituted.

From here, isotope enrichment factor is therefore the difference in $\ln \beta$ values between the observed chemical species:

$$\Delta M_{[\text{Specie A} - \text{Specie B}]} = \ln \alpha \approx \ln \beta^A - \ln \beta^B \quad (5.8)$$

where A and B mark two chemical species, between which isotopic fractionation occurs.

5.1.4. Computationally predicted isotopic effects in natural systems

In one of the early DFT studies, behaviour of isotopic fractionation during Fe adsorption on a series of mineral phases suggested light enrichment in Fe-mineral complexes both *in vacuo* and in solution when the oxidation state of the system was held constant (Domagal-Goldman and Kubicki, 2008; Domagal-Goldman et al., 2009). Although the experimental studies of the same group disagreed with the predicted isotopic trends (Domagal-Goldman and Kubicki, 2008), the results demonstrate that ligand exchange in the environment is an important contributor to the overall isotopic patterns.

Black and co-workers published several papers (Black et al., 2008; Black, 2007) describing isotopic partitioning of Mg during activation of chlorophylls a and b. At the end of the chlorophyll biosynthesis process, Mg is incorporated into the central chlorin ring. The group showed computationally (Black, 2007) and experimentally (Black, 2006) that this process fractionates in favour of the heavy isotope. Similarly, a computational approach was used in a study by Fujii and Albarede (2012) to explain the isotopic patterns found in various plant species (Aucour et al., 2011; Moynier et al., 2009; Viers et al., 2007; Weiss et al., 2005). They estimated isotopic partitioning in complexed Zn species previously identified in metallophyte *Arabidopsis halleri* using XAFS spectra (Sarret et al., 2002). The group concluded that complexation with small organic acids in both the above- and below-ground organs can cause the characteristic distribution of stable Zn isotopes, as identified in the study of Weiss et al. (2005). Namely, Zn complexes with citrates and malates are enriched in light ^{64}Zn thus generating a light Zn isotope signal in plant's shoots and leaves. Conversely, Zn-phosphate species were found to be enriched in the heavy ^{66}Zn , hence explaining the positive isotopic fractionation measured in roots and below-ground organs.

Using DFT approach, speciation of Fe (Fujii et al., 2014; Moynier et al., 2013), Cu and Ni (Fujii et al., 2014) was found relevant for creating isotopic distribution patterns in plants. Moynier et al (2013) calculated that even when redox changes are neglected, the change in the Fe speciation counts for up to 1.5 ‰ of the observed isotopic signal in higher plants. The same group used a computational approach to untwine mechanisms driving isotopic behaviour in different conditions relevant for environmental geochemistry and biology (Fujii et al., 2013; 2014). These findings consolidate our understanding of uptake and translocation of vital micronutrients such as Zn and Fe.

Computational studies are often used to understand and justify isotopic partitioning in natural systems. However, these methods are still not fully verified. Hence, significant efforts are being invested to develop more robust computational approaches. It is imperative to validate computationally generated data against experimentally determined values. This is important because it helps define the

accuracy and precision of theoretical estimates, before any attempts are made to interpret isotopic signatures in complex natural systems.

5.1.5. Aims of the study

The aim of the work presented in this chapter was to develop a computational method able to identify isotopic effects imposed upon Zn complexation with a range of physiologically relevant ligands, particularly phytosiderophores from the mugineic acid family. The computational model was structured to incorporate the latest computational code and the largest basis set attainable, with respect to its computational cost. This is to achieve the optimal precision available.

Moreover, to assess the accuracy and precision of theoretical method in general, the objective of the study was to verify the estimated fractionation factors, against the fractionation data obtained experimentally (as presented in Chapter 4).

5.2. Methods

5.2.1. Physiologically relevant Zn complexes and their 3D Crystal Structures

To ensure the use of adequate complex structures that are present and stable in nature under relevant physiological condition model molecules were selected from the literature and their 3D structures were obtained from the Cambridge Crystallographic Data Centre (CCDC) Database. Molecular optimisation of single cluster molecules was modelled *in vacuo* without any forced symmetry. The solubilisation effect of the complexes beyond the first coordination sphere was not considered.

Based on the study of Rudolph and Pye (1999), we assumed that the coordination number of Zn is six. In some cases when Zn coordinates with multidentate ligands, a decrease in the coordination number due to stereochemical restrictions can occur. Hydrated Zn ion is surrounded by six water molecules though some evidence points otherwise. Pavlov et al (1998) have found that for $[\text{Zn}(\text{H}_2\text{O})_4](\text{H}_2\text{O})_2$ complex, where Zn coordinates to four water molecules in its first coordination shell while being stabilised by two additional water molecules in its second shell (Figure 5.1), has a

lower energy than the $[\text{Zn}(\text{H}_2\text{O})_6]$ complex. Both hydrated Zn structures were tested using computational model described in detail below and, conversely to the study of (Pavlov et al., 1998), zero energy of $[\text{Zn}(\text{H}_2\text{O})_6]$ complex was lower by ~ 0.009 kJ/mol.

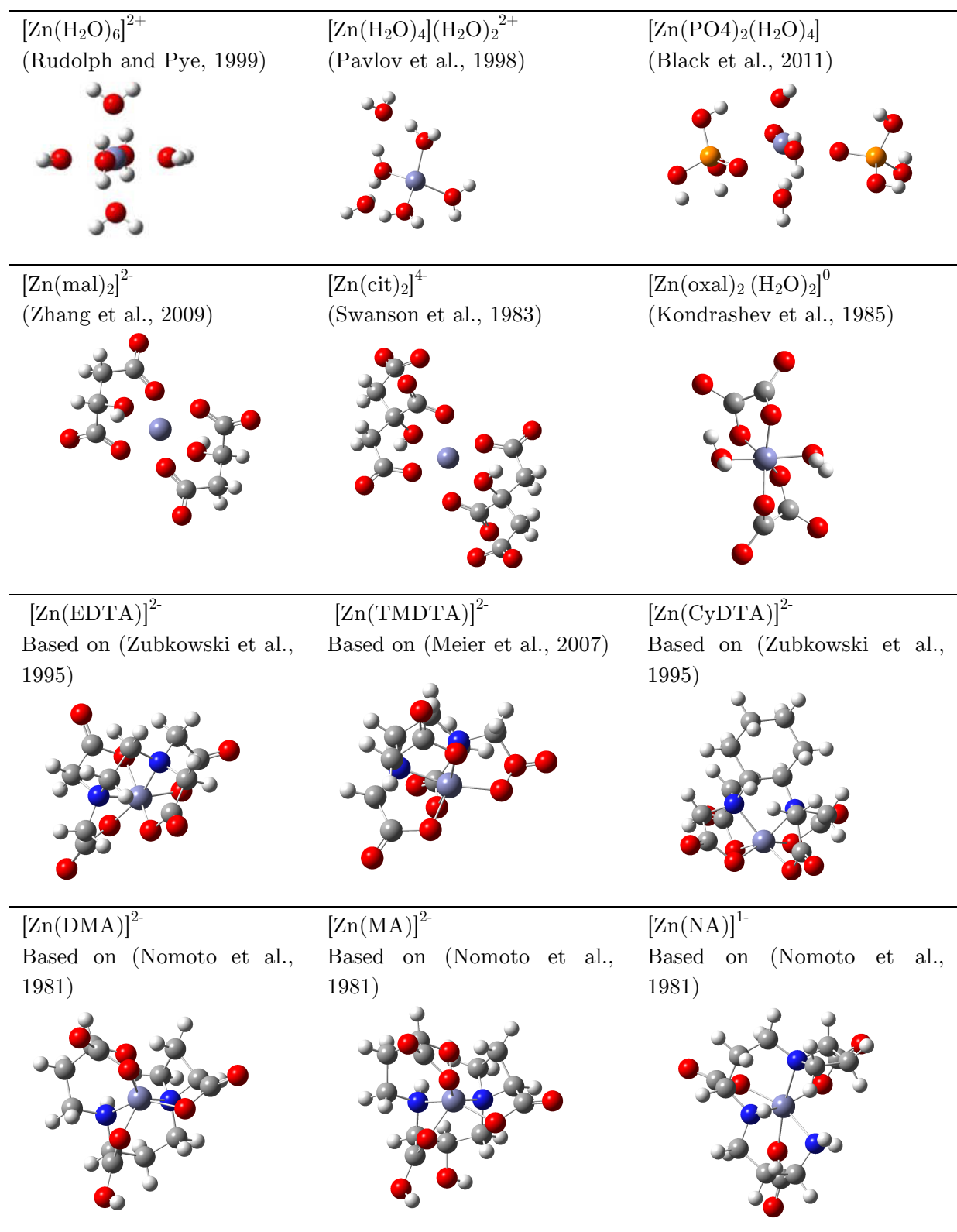


Figure 5.1 Molecular structures of Zn complexes used in the present work to calculate isotopic enrichment factors. All structures are reported in the literature as Zn species present under physiologically relevant pH conditions. Their electronic structure was obtained from Cambridge Crystallography Database Centre (CCDC), and further optimised using Gaussian software (Gaussian Inc.) Symbols colours refer to: Zn (iris), O (red), C (grey), H (White), N (blue) and P (orange).

5.2.2. Computational details and RPFR calculations

Orbital geometries and vibrational frequencies of Zn complexes with a range of physiologically relevant ligands were computed using DFT by implementing Gaussian 09 d.01 code (Frisch et al., 2009) and finally compared to those of hexa-aqua Zn specie as a reference complex.

A hybrid density functional was used, which incorporated Becke's three-parameter non-local hybrid exchange potential (**B3**) (Becke, 1988) and Lee-Yang and Parr (**LYP**) (Lee et al., 1988) non-local functionals. The level of theory (**B3-LYP**) was combined with heterogeneous basis sets: the **6-31G(p)** all-electron basis set (applied for H, C, N and O atoms) and an effective core potential **Lanl2DZ** basis set was considered in the case of Zn (Hay and Wadt, 1985). The above stated combination of the level of theory and basis sets were selected based on the study of Black et al. (2011), as the most effective method to achieve high accuracy and lower the computational cost. In addition, a dispersion scheme was applied to account for pseudopotentials of the atoms' non-valent electrons. This correction for core electron pseudopotential was done using **GDBJ** empirical scheme, which is a D3 version Grimme's dispersion with Becke-Johnson damping.

Frequencies for both ^{66}Zn and ^{64}Zn isotopologues were extracted using Gauss View (Gaussian Visualisation) Software and reduced partition function ratios ($\ln \mathbb{f}$) were calculated for each isotopologue as indicated in the further text.

5.3. Results and Discussions

5.3.1. Method verification

Our computational model was tested against the findings of Black et al (2011) and Fujii et al (2011). As shown in Figure 5.2 our data computed for hexa-aqua Zn complex $[\text{Zn}(\text{H}_2\text{O})_6]^+$ closely correlate to the values reported in studies by Black et al. (2011) and Fujii et al., (2012; 2011) for the same coordination complex. The small

differences in absolute $\ln \beta$ values arise from variations in basis set size and the use of dispersion scheme corrections. As expected, the differences between individual $\ln \beta$ calculated decrease with higher temperature conditions.

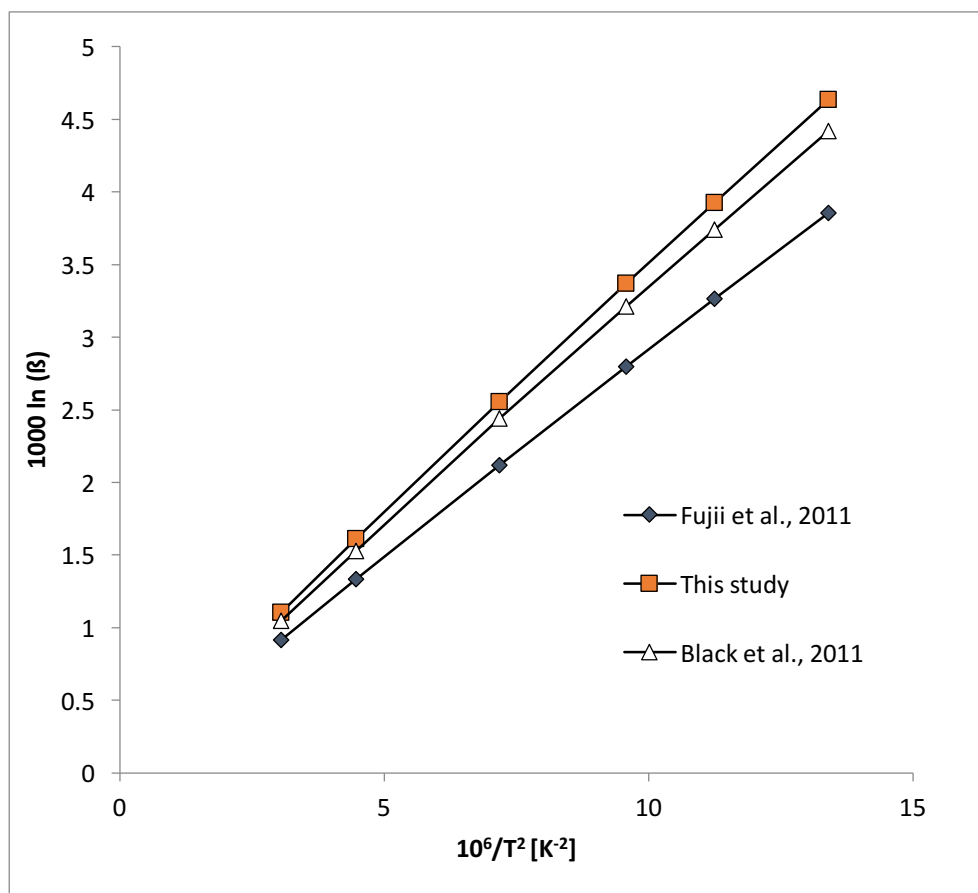


Figure 5.2 Comparative $\ln \beta$ values for the three studies of the same complex ($[Zn(H_2O)_6]^+$) using similar computational methods. Fujii et al. (2012; 2011) used 6-31 G* basis set for H, O, C and Zn. Both this study and the one by Black et al. (2011) employed a combination of Lanl2DZ basis set for Zn and 6-31 G for H, O and C. Results indicate variations of up to 0.8 ‰ when different calculation set up is used.

5.3.2. Complexation induced stable Zn isotopic signatures calculated using DFT method

The adopted computational model was further used to generate estimates of fractionation factors in organic complexes with three groups of organic molecules:

- (1) phytosiderophores from the family of mugineic acids (MA, DMA and NA, a precursor in the MAs biosynthesis);

- (2) low molecular weight ligands derived from organic acids (malate, citrate, oxalate) and phosphate;
- (3) synthetic structural analogues of phytosiderophore ligands (EDTA, TMDTA and CyDTA).

Data in Table 5.1 shows logarithmic values of reduced partition function ratios ($1000 \cdot \ln \mathfrak{f}$) for $^{66}\text{Zn}/^{64}\text{Zn}$ isotopologues of the marked species. Molecular structure of the selected species can be found in Figure 5.1. Isotope enrichment factors ($\ln \alpha$) upon complexation (expressed in per mill notation, ‰) were calculated by subtracting $\ln \mathfrak{f}$ value for the hydrated Zn complex ($[\text{Zn}(\text{H}_2\text{O})_6]^{2+}$) from the $\ln \mathfrak{f}$ values calculated for individual $[\text{ZnL}]$ species (see Eq. 5.8).

Table 5.1 DFT calculated data for the logarithm of the reduced partition function ($\ln \mathfrak{f}$) expressed in per mill (‰), for the $^{66}\text{Zn}/^{64}\text{Zn}$ isotopologues of the listed species.

Complex	Specie	Temperature (°C)					
		0	25	50	100	200	300
$\text{Zn}(\text{H}_2\text{O})_6$	$[\text{Zn}(\text{H}_2\text{O})_6]^{2+}$	4.6340	3.9270	3.3690	2.5550	1.6100	1.1050
$\text{Zn}(\text{H}_2\text{O})_4(\text{H}_2\text{O})_2$	$[\text{Zn}(\text{H}_2\text{O})_4(\text{H}_2\text{O})_2]^{2+}$	5.2160	4.4350	3.8150	3.8150	2.9050	1.2670
ZnEDTA	$[\text{Zn}(\text{EDTA})]^{2-}$	4.2615	3.6008	3.0817	2.3297	1.4629	1.0023
ZnTMDTA	$[\text{Zn}(\text{TMDTA})]^{2-}$	4.4747	3.7808	3.2359	2.4466	1.5370	1.0535
ZnCyDTA	$[\text{Zn}(\text{CyDTA})]^{2-}$	4.1040	3.4650	2.9630	2.2380	1.4040	0.9610
Zn-Malate	$[\text{Zn}(\text{mal})_2]^0$	4.2013	3.5672	3.0654	2.3323	1.4768	1.0167
Zn-Citrate	$[\text{Zn}(\text{cit})_2]^0$	4.4398	3.6837	3.1530	2.3840	1.4972	1.0257
Zn-Oxalate	$[\text{Zn}(\text{oxal})_2(\text{H}_2\text{O})_2]^0$	4.6122	3.5742	3.0569	2.3084	1.4474	0.9906
Zn-Phosphate	$[\text{Zn}(\text{PO}_4)_2(\text{H}_2\text{O})_4]$	4.1221	3.4914	2.9942	2.2708	1.4322	0.9840
ZnMA	$[\text{Zn}(\text{MA})]^{2-}$	4.8441	4.1069	3.5247	2.6761	1.6896	1.1612
ZnDMA	$[\text{Zn}(\text{DMA})]^{2-}$	4.9739	4.2193	3.6232	2.7541	1.7429	1.2003
ZnNA	$[\text{Zn}(\text{NA})]^{1-}$	4.6055	3.8947	3.3354	2.5240	1.5868	1.0878

The analysis of isotopic enrichment factors ($\ln \alpha$), as shown in Table 5.1 and Figure 5.3, have identified mugineic acids as the only group of ligands tested that shows consistent enrichment in the heavy isotope. Namely, in the physiological temperature range (green area on Figure 5.3) the $\ln \alpha_{250\text{C}}$ values were 0.32 ‰ and 0.44 ‰ for Zn complexes with DMA and MA, respectively, and 0.11 ‰ for Zn complex with NA, a precursor in the biosynthesis of MAs.

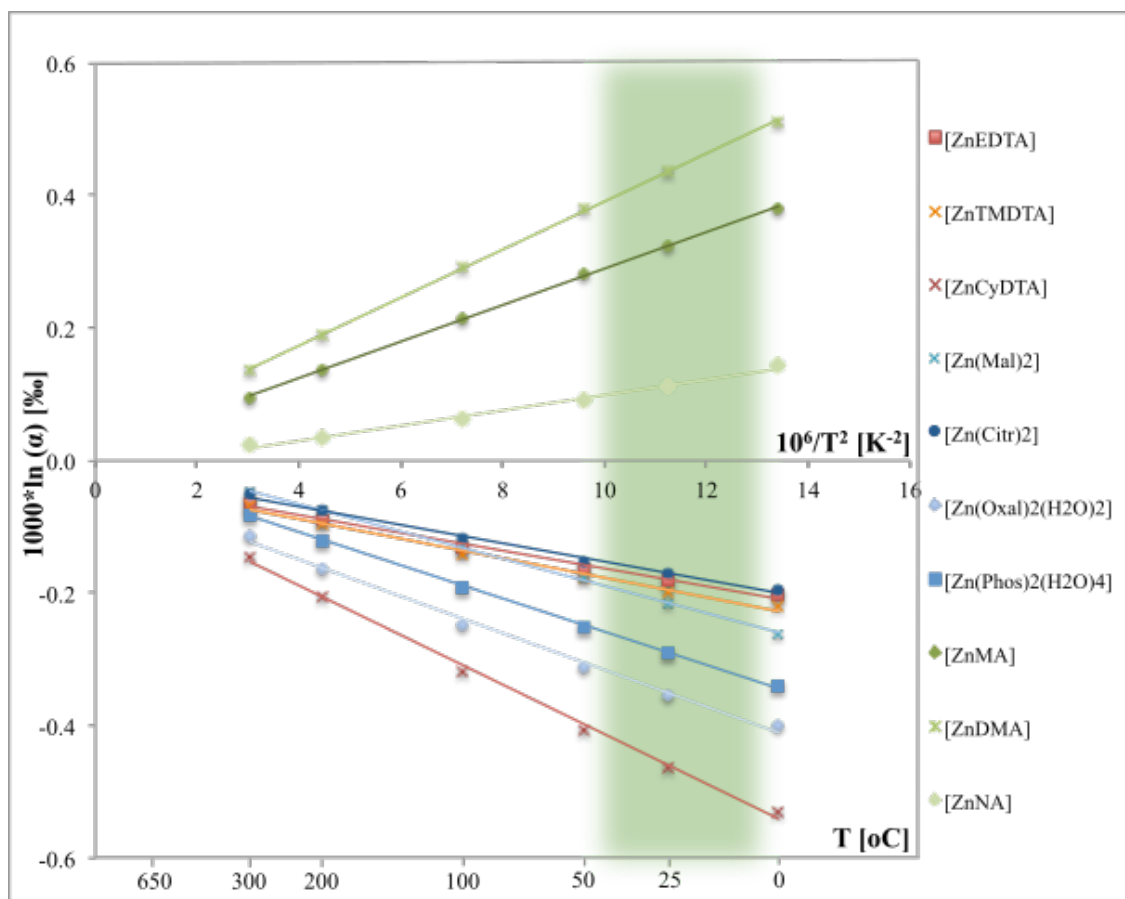


Figure 5.3 Change in ΔZn values over a range of temperatures (0-300 °C). The three trend lines in the positive region of the $1000 \cdot \ln(\alpha)$ scale, belong to data gathered for the two tested mugineic acids (MA, DMA) and their precursor (NA). These three ligands are the only ones among the tested to preferentially complex the heavy Zn isotope (0.2 - 0.3 ‰ at the physiologically optimal range of temperatures, shown in green shade). The blue lines define a group of LMWOA-type of ligands. All four tested LMWOA preferentially incorporate light ^{64}Zn into their complexes. Similarly, all Zn complexes with the synthetic ligands (EDTA, TMDTA and CyDTA) exhibit enrichment in light Zn of up to -0.53 ‰ (CyDTA) at 25 °C.

In Chapter 4, we experimentally determined that complexation of Zn by DMA yields heavy isotopic enrichment of 0.26 ± 0.18 ‰ (2SD, $n=9$). This considered, here developed computational model provides a close estimation ($\ln \alpha_{[\text{Zn}(\text{DMA})] - [\text{Zn}(\text{H}_2\text{O})_6]} = 0.32$ ‰) for isotopic enrichment factor upon complexation with this phytosiderophore ligand. Furthermore, these values are in line with the conclusions of Arnold et al. (2010a) who suggested that Zn complexation by DMA is the source of a distinctive stable Zn isotope signature (0.18 - 0.24 ‰) in the field grown rice plants.

Conversely, complexation with any other tested ligands indicates light isotopic bias. Both, the synthetic structural analogues of MAs (EDTA, TMDTA and CyDTA) and LMWOAs show preferential complexation of light ^{64}Zn .

Synthetic ligands EDTA, TMDTA and CyDTA bind Zn in the same hexadentate fashion as the phytosiderophores from the mugineic acid family. Contrary to our experimental observations (Chapter 4), isotopic enrichment factors ($\ln \alpha$) calculated using the DFT model report light isotopic enrichment (Table 5.1, Figure 5.3) in all three Zn complexes with synthetic ligands: -0.15 ‰ in $[\text{Zn}(\text{TMDTA})]$, -0.34 ‰ in $[\text{Zn}(\text{EDTA})]$ and -0.46 ‰, in $[\text{Zn}(\text{CyDTA})]$. Not only that the calculated estimations are offset by up to ~ 1 ‰, but also the estimated trends propagate in the opposite direction compared to the laboratory observations.

The $\ln \beta$ values calculated for the selected organic acids from the group of LMWOA show light isotopic fractionation with magnitudes between -0.29 ‰ and -0.10 ‰ (Table 4.1, Figure 4.3). In the Fujii and Albarede (2012) study, depending on the selected specie of Zn-phosphate complex, $\ln \beta$ ranges between 0.16 and 1.1 ‰ in favour of the heavy ^{66}Zn . Data shown in the presented work, to the contrary, indicate a preference for the light ^{64}Zn (-0.29 ‰) during formation of a phosphate complex. Nevertheless, the opposing conclusions between two studies are not in direct disagreement as Zn-phosphate complexes can take various different geometries thus influencing the final isotopic effect. Moreover, light isotopic enrichment calculated in Zn complexes with citrate and malate (-0.10 ‰ and -0.21 ‰, respectively) are in line with conclusions of Fujii and Albarede (2012), when considering the differences in the Zn coordination fashion between the corresponding ligand species. In other words, the way a ligand binds to the metal centre defines the geometry of the complex and strength of the coordination bonds; therefore the isotopic fractionation induced varies in the complexes of the same species but different molecular geometry. Constructing molecules using GaussView software, such as done in Fujii and Albarede (2012) has an element of subjectivity, which can cause significant variations among studies of the same chemical species. Starting from crystallographic data, deposited in the CCDC database, is a way to minimise uncertainty upon determining geometry of relevant molecules.

5.3.3. Comparison between experimental and calculated isotopic effects

Ligands that complex Zn by coordinating with, on average, shorter bonds, form stronger complexes (with higher $\log K$ values) (Bigeleisen and Mayer, 1947; Urey, 1947). In the previous chapter (Chapter 4), isotopic effects upon Zn complexation with four organic hexadentate ligands (DMA, TMDTA, EDTA and CyDTA) were measured in laboratory conditions, and a correlation between the magnitude of isotopic fractionation and the binding affinity of the $[\text{ZnL}]^{2-}$ complex ($\log K_{[\text{ZnL}]}$; where $L = \text{DMA, TMDTA, EDTA or CyDTA}$) was described (Figure 3.5).

Upon plotting computationally modelled data against the experimentally determined values, it is evident that the DFT model provides sensible estimates for smaller complexes with lower $K_{[\text{ZnL}]}$ constants, however it does not reflect our lab observations for the three complexes with the highest stability, namely, TMDTA ($\log K_{[\text{Zn(TMDTA)]}} = 15.8$; (Bjerrum et al., 1964)), EDTA ($\log K_{[\text{Zn(EDTA)]}} = 16.5$; (Bjerrum et al., 1964)) and CyDTA ($\log K_{[\text{Zn(CyDTA)]}} = 18.7$; (Bjerrum et al., 1964)).

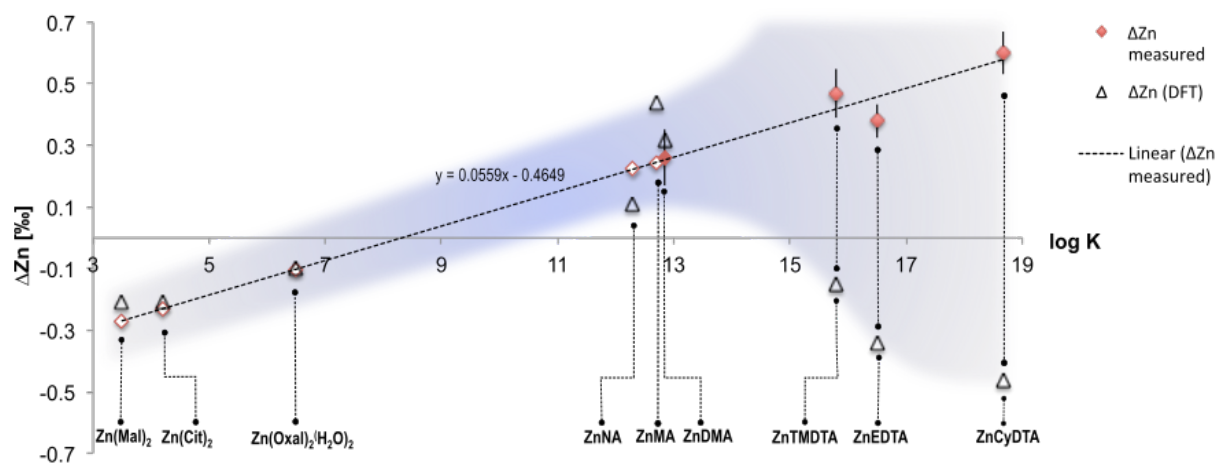


Figure 5.4 Combined data estimated by means of DFT calculations (triangles) and measurements in lab conditions (red diamonds). The trend determined by data points measured in the lab was extrapolated further to provide an estimate for smaller LMWOA molecules ($\log K < 12.5$, white diamonds). Errors produced using DFT calculations are far too great for this type of data to be used as valid predictions of absolute fractionation values (here shaded using Inkscape software to illustrate discrepancies observed in this study). Nonetheless, when data is analysed carefully and used responsibly this method can provide a valuable initial estimations for use in environmental science disciplines.

The computational model correctly predicted, within error limits, the isotopic fractionation introduced upon formation of $[\text{Zn}(\text{DMA})]$ complex. The same method was insufficiently accurate when identifying isotopic effects of complexation by synthetic ligands used in this study. The calculated values for isotopic fractionation in $[\text{Zn}(\text{TMDTA})]$, $[\text{Zn}(\text{EDTA})]$ and $[\text{Zn}(\text{CyDTA})]$ were significantly offset from the experimental values (by 0.62 ‰, 0.76 ‰ and 1.06 ‰, respectively).

Based on the data for the measured isotopic fractionation in four different Zn complexes (Figure 5.4, red diamonds) and their $\log K_{\text{ZnL}}$ constants, a correlation trend was plotted and then extrapolated to estimate isotopic enrichment factors for the ligands of lower complexation strength (white diamonds). DFT modelled data (triangles) plotted on Figure 5.4 show that the modelled values in the lower section of $\log K_{\text{ZnL}}$ axis match the expectations from the experimentally obtained trend, however for $\log K_{\text{ZnL}} > 13$, the model generates a systematic error. To illustrate further the observed errors, the offset between calculated and measured isotopic effects (including standard deviation of the measurements) was accentuated by an indigo-shaded area in Figure 5.4. Our results indicate that for bigger ligands, and in particular those with higher affinity for Zn, the DFT-based calculations introduce a significant error (up to 1 ‰ for $\Delta^{66}\text{Zn}$, or 0.5 ‰ *pamu*).

These systematic errors upon computational estimations of fractionation factors were observed previously. By comparing isotopic partitioning between three different Fe-organic complexes, Morgan et al. (2010) indicated a correlation with their Fe binding activity. However, the inconsistency observed between $\Delta\text{Fe} = f(\log K)$ trend lines for different ligand pair combinations was explained by a systematic deviation introduced by DFT calculation method. In other words, increased difference in the $\log K$ constants between ligands tested deviate DFT estimated values further away from experimental fractionation factors (Morgan et al., 2010).

5.3.4. Limitations of the computational method and error propagation

Inconsistencies between experimental and theoretical values can be found in the current literature. In the previously published studies, computationally generated data predicted fractionation values with magnitudes often greater than that

experimentally observed (Table 5.2). This offset has been noted before (Black, 2007; Morgan et al., 2010) but not investigated in depth.

Table 5.2 lists isotopic partitioning of several key transition metals in the biosphere (Fe, Zn and Mg) obtained experimentally and by computational simulations. As it can be seen in the table, early computational studies provided partitioning estimates that were significantly greater than the values measured experimentally. Extensive analyses by Black et al. (2007) have shown a large discrepancy between theoretically calculated and experimentally determined values for Mg incorporated in the chlorin ring of a chlorophyll molecule. Calculated values correctly predicted the direction of isotopic fractionation in the observed system but, nonetheless, overestimated the extent of the fractionation several fold. Similarly, Domagal-Goldman et al. (2009) utilised *ab initio* approach to show a depletion in heavy ^{56}Fe upon complexation by a strong chelating siderophore - desferrioxamine B (DFOB). Their findings disagreed with the experimental conclusions of Dideriksen et al. (2008) and, later Morgan et al. (2010), regarding not only the extent of the fractionation but also its direction. The theoretically estimated isotopic effect was lighter by 0.45 ‰ pamu compared to the values reported in the experimental conditions.

An average offset of ~ 0.5 ‰ pamu has also been identified between an experimental (Dideriksen et al., 2008) and a theoretical study (Domagal-Goldman et al., 2009) of equilibrium fractionation factors upon formation of Fe^{3+} -DFOB complex. Calculations of Domagal-Goldman et al. (2009) predicted a light isotopic enrichment in a range of mineral phases, yet previously published experimental results (Domagal-Goldman and Kubicki, 2008) demonstrated that their theoretical predictions were overestimated by 0.4 ‰ pamu.

Table 5.2 Fractionation values reported for relevant coordination complexes by both experimental and computational studies. Results of the computational modelling predict higher magnitudes of Δ values for the observed metal-ligand complexes when compared to the measured data obtained in experimental studies.

Isotope Fractionation	Reference	$\Delta M_{\text{Reservoir A -}}$	Comment
		Reservoir B (pamu)	
$[^{56}\text{Fe-DFOB}]^{2-}-^{56}\text{Fe}^{2+}$	Dideriksen et al., 2008	0.3	Aqueous solution
$[^{56}\text{Fe-DFOB}]^{2-}-^{56}\text{Fe}^{2+}$	Domagal-Goldman et al., 2009	-0.2	<i>Ab initio</i> calculations
$[^{56}\text{Fe-DFOB}]^{2-}-^{56}\text{Fe}^{2+}$	Morgan et al., 2010	>0	Aqueous solution
$[^{56}\text{Fe-Nicotinamine}]^{-}-^{56}\text{Fe}^{2+}$	Moynier et al., 2013	-0.03	<i>Ab initio</i> calculations
$[^{56}\text{Fe-Phytosiderophore}]^{-}-^{56}\text{Fe}^{3+}$	Moynier et al., 2013	0.5	<i>Ab initio</i> calculations
$[^{66}\text{ZnCl}_4]^{2-}-^{66}\text{Zn}^{2+}$	Black et al., 2011	-0.5	<i>Ab initio</i> calculations
$[^{66}\text{Zn-citrate}]^{-}-^{66}\text{Zn}^{2+}$	Black et al., 2011	0.07 to 0.25	<i>Ab initio</i> calculations
$[^{66}\text{ZnPHA}]-^{66}\text{Zn}^{2+}$	Jouvin et al., 2009	0.1	Aqueous solution
$[^{66}\text{Zn}_3(\text{PO})_4]-^{66}\text{Zn}^{2+}$	Fujii and Albarede (2012)	0.5	<i>Ab initio</i> calculations
$[^{66}\text{Zn-malate}]^{-}-^{66}\text{Zn}^{2+}$	Fujii and Albarede (2012)	-0.2 to 0.1	<i>Ab initio</i> calculations
$[^{66}\text{Zn-citrate}]^{-}-^{66}\text{Zn}^{2+}$	Fujii and Albarede (2012)	-0.4 to 0.6	<i>Ab initio</i> calculations
$[^{25}\text{Mg-ChlA}]-^{25}\text{Mg}^{2+}_{\text{leaf}}$	Black et al., 2006	0.15	<i>Hedera helix</i>
$[^{25}\text{Mg-ChlA}]-^{25}\text{Mg}^{2+}$	Black et al., 2007	0.79 to 1.42	<i>Ab initio</i> calculations
$[^{25}\text{Mg-ChlA}]-^{25}\text{Mg}^{2+}$	Black et al., 2007	-0.13 to -0.17	Cyanobacteria <i>S. elongatus</i>
$[^{26}\text{Mg-ChlA}]-^{26}\text{Mg}^{2+}_{\text{leaf}}$	Black et al., 2006	0.3	<i>Hedera helix</i>
$[^{26}\text{Mg-ChlA}]-^{26}\text{Mg}^{2+}$	Black et al., 2007	1.5 to 2.76	<i>Ab initio</i> calculations
$[^{25}\text{Mg-ChlB}]-^{25}\text{Mg}^{2+}_{\text{leaf}}$	Black et al., 2006	0.03	<i>Hedera helix</i>
$[^{25}\text{Mg-ChlB}]-^{25}\text{Mg}^{2+}$	Black et al., 2007	0.6	<i>Ab initio</i> calculations
$[^{26}\text{Mg-ChlB}]-^{26}\text{Mg}^{2+}_{\text{leaf}}$	Black et al., 2006	0.08	<i>Hedera helix</i>
$[^{26}\text{Mg-ChlB}]-^{26}\text{Mg}^{2+}$	Black et al., 2007	1.2	<i>Ab initio</i> calculations
$[^{26}\text{Mg-ChlA}]-^{26}\text{Mg}^{2+}$	Black et al., 2006	-0.26 to -0.35	Cyanobacteria <i>S. elongatus</i>

Errors associated with theoretical approximations are becoming smaller with improvements in the computational methods and implementation of relevant corrections. The discrepancy between results obtained by DFT calculations and those experimentally determined has previously been addressed in the study of (Morgan et al., 2010), where the large differences in fractionation factors were interpreted by a specific deficiency in DFT models of the reference molecule - hydrated Fe complex ($[\text{Fe}^{3+}(\text{H}_2\text{O})_6]$). The present work shows that the aforementioned discrepancy does not originate in inaccurately postulated reference molecule. The calculations for the small LMWOA molecules, shown in this study, correlate well with our experimental expectations. However, for bigger molecules, with a greater number of relevant

vibrational modes, the error accumulation is more pronounced and thus such data has to be carefully assessed. This point was previously illustrated by Schauble (2004) who calculated that for an arbitrary error of 1 cm^{-1} in calculated isotopic shifts of a single 500 cm^{-1} vibrational mode, the error propagates to a 1 ‰ offset in isotopic enrichment factor α , at $0\text{ }^{\circ}\text{C}$. Taking into account the growing number of vibrational modes with an increase in size and complexity of a molecule, accumulation of errors is likely and, above all, significant.

The study shows that for ligands with high affinity for Zn, calculations using DFT models produce results with high uncertainty levels. The errors identified in this study ($\sim 0.5\text{ ‰}$, pamu) are far too large to accurately estimate and/or interpreting isotopic effects in natural systems. Improved understanding of the limitations of DFT models is imperative if such methods are to be widely used in the area of environmental geochemistry.

5.3.5. Further implications for the use of computational models in environmental geochemistry studies

While the use of DFT calculations to determine isotope enrichment factors is valuable, the limitations of the method are to be taken into consideration when interpreting natural isotopic signatures. Numerous approximations integrated in this method can yield significant errors. Therefore, careful considerations of the accuracy and limitations of this approach are essential before discussing possible implications for isotope distribution in natural systems. Nevertheless, this approach is highly beneficial when quick initial estimations of isotopic effects are required, particularly when experimental measurements are unavailable, too expensive or too cumbersome.

Studies comparing experimental and theoretical fractionation data on basic physicochemical processes are largely missing. In order to validate computational models and ensure the accuracy of the produced data, more studies comparing theoretical and experimental data need to be conducted. Until then, responsible and careful data analysis of the computed data has to be undertaken before presenting any implications in the natural systems. Further laboratory measurements of fractionation factors are essential not only for determine a scope within which

computational methods can be accurately and effectively used, but also to fully understand the isotope dynamics upon formation of organic complexes.

5.4. Conclusions

Among all tested ligand groups, phytosiderophores from the mugineic acid family (MA, DMA and NA) were shown to fractionate in favour of the heavy ^{66}Zn by 0.11 – 0.44 ‰. The magnitude of this isotopic effect is consistent with both our previous experimental observations (Chapter 4) and those outlined in the field study of Arnold et al. (2010a). The study lends support to previous explanations of heavy isotopic enrichment in plants – that heavy bias observed in plants is likely a result of complexation with ligands such as plant-synthesised phytosiderophores.

Applied DFT model successfully reproduced expectations for the isotopic fractionation in Zn complexes with both phytosiderophores from the mugineic acid family and small organic acids (LMWOA ligands) as shown in Figure 5.4, thus demonstrating a potential of the computational approach. However, the isotopic effects calculated upon Zn complexation with the strong synthetic ligands (TMDTA, EDTA and CyDTA) were offset from the experimental values by ~ 1 ‰. The error distribution, which is indicated in Figure 5.4 as difference between the real experimental values and the calculated estimates, illustrates the observed limitations of this method. Considerable error accumulation was identified in theoretical estimates of Zn complexes with bigger and more tightly binding ligands ($\log K_{\text{ZnL}} > 13$).

Errors associated with theoretical estimations of isotopic effects have not yet been extensively studied. Very few studies obtained both experimental and theoretical datasets, and hence were unable to critically examine the accuracy of theoretical models. In this work a pattern to the error propagation of DFT-based models was found that sheds a new light to the application of theoretical studies in environmental geochemistry, particularly its limitations. Gathering more experimental values and comparing them to relevant theoretical predictions could greatly help further develop computational techniques for future use. In the

meantime, computationally generated data should be analysed critically and with care, especially when intention is to describe mechanisms that take place in intricate natural systems.

Chapter 6

Isotopic Fractionation in Rice Supports Hypothesis of Phytosiderophore-facilitated Zn Uptake

6.1. Introduction

6.1.1. The problem of Zn deficiency in rice production systems

All plant species are susceptible to Zn-stress, however different species, and even genotypes, respond differently to the same soil conditions. Plants such as clover, carrot, alfalfa and multiple crop species *e.g.* oats, rye and wheat are relatively tolerant to zinc deficiency. On the other hand, beans, rice, sorghum and maize are highly sensitive to the low zinc soil content. Zn deficiency is one of the greatest challenges in rice production, due to the particular soil chemistry of paddy soils (Kirk, 2004). Despite being present in sufficient concentrations, reducing

environment and high iron content lead to formation of insoluble Zn species, which are physiologically unavailable to plants. When Zn supply is limited, plants can suffer stunting or reduced growth, leaf reduction, chlorosis and bronzing, leaf amorphism and/or roseting (Alloway, 2008).

As one of the economically most significant crops, major efforts have been made to understand and disclose possible mechanisms to overcome Zn-deficiency in rice. Rice is a species with a large genetic variability. It has been shown that rice genotypes differ greatly in their ability to grow and yield under Zn-stress (Graham et al., 1999; Gregorio, 2002), which showcase a great potential for biofortification and breeding studies. Overcoming Zn deficiency in rice grains would imply a significant decrease in widespread health problems originating in Zn malnutrition (Nestel et al., 2006), especially in south and south-east Asia where rice is a staple food.

Plants from the gramineae group, which includes rice, are reported to utilise two different uptake strategies to secure essential metals supply (Römheld and Marschner, 1986). In *strategy I*, increased activity of H^+ -ATPase in the plasma membrane lowers the rhizosphere pH thus changing the speciation in the soil solution and consequently the bioavailability of metals such as Fe, Zn and Cu. While *strategy I* exists in both graminaceous and non-graminaceous plants, the release and sequestration of metal ions by organic ligands (*i.e. strategy II*) is considered a characteristic of graminaceous species only (Römheld and Marschner, 1986). These organic ligands, *i.e.* phytosiderophores (PS), are considered to play an important role in uptake of, not only Fe (Römheld and Marschner, 1986), as originally thought but also Zn (Arnold et al., 2010a; Suzuki et al., 2006; Widodo et al., 2010) and Cu (Schmidt et al., 1997; Welch et al., 1993). More specifically, under deficient condition small non-protein amino acids from the mugineic acids family are released from the roots into the substrate where by means of complexation Zn is extracted from the soil solution and made physiologically available to the plant. Plant species that release PS-like ligands in higher quantities are more abundant in Zn, thus the release of phytosiderophores has been linked to crops' tolerance to Zn-stress.

Chelation-based Zn uptake strategy has initially been proposed only in graminaceous family of monocots (Takagi, 1976). The release of chelating ligands (phytosiderophores) is an important adaptation as it helps sequester Zn from otherwise insoluble Zn species (Kirk and Bajita, 1995; Welch and Shuman, 1995). Organic ligands have been isolated from the root washings of various crop species (Takagi, 1976; Zhang et al., 1998) and their excretion rates have been positively correlated to the levels of tolerance (Hajiboland et al., 2005; Hoffland et al., 2006). Nevertheless, it is suspected that biosynthesis of phytosiderophores is upregulated by Fe-deficiency (Bashir et al., 2006; Inoue et al., 2008; Nagasaka et al., 2009; Palmer and Guerinot, 2009). Taking that paddy rice soils are rarely deficient in Fe (Dobermann and Fairhurst, 2000), it explains a significantly lower PS excretion in rice compared to other crops (Oburger et al., 2014).

To completely understand strategies employed by rice and other crops to overcome low Zn availability in the substrate, further testing in isolated (laboratory-based) and natural systems is required. Nevertheless, further analytical developments are necessary to allow identification of Zn-efficiency mechanisms *in situ*. To this end, stable isotope chemistry has shown to be a powerful tool in environmental chemistry studies (von Blanckenburg et al., 2009) due to its metal visualising ability in the environment. Latest technological advances in the area of environmental geochemistry, especially the advent of multi collector inductively-coupled plasma mass spectrometry (MC-ICP-MS), offer a valuable insight into the mechanism of Zn uptake and transport under limiting soil conditions. This powerful technique has already yielded significant results and offered an insight that can help further explain the origins of Zn-efficiency trait.

6.1.2. Regulation of Zn transport within plants

Transport of ion solutes across the root cortex towards the stele with vascular vessels follow two parallel pathways, where the first one occurs via cell walls and intercellular gaps and the other follows plasmodesmata connections of cells' symplasm (for more information see the Chapter 2). Zn in its ionic form (Zn^{2+}) has a moderate root apoplastic mobility, however further transport is mostly obstructed by

the impermeable Casparian strip, a suberized band of cells in the endodermal layer. The second transport path across plasma membrane into the symplast and, further, loading to the xylem vessels is done via active transporters (Figure 6.1). Transport of Zn into the symplast of the epidermis is believed to be governed by the activity of Zn-regulated transporter iron regulated transporter-like proteins (ZIPs) (Guerinot, 2000; Li et al., 2013) same as Fe (review by Curie et al. (2009)), Mn (Pedas et al., 2009) and Cd (Grotz et al., 1998). These highly conserved proteins have eight transmembrane domains that vary in length and function. The cytoplasmic side, consisting of transmembrane domains TM-3 and TM-4, are rich in histidine residues therefore being an effective metal-binding site. Upon inducing mutation in a histidine rich site, Rogers et al. (2000) reported an obstruction in the transport function. Several ZIP transporters in rice have been characterised (*OsIRT1*, *OsIRT2*, *OsZIP1*, *OsZIP3*, *OsZIP4* and *OsZIP5*) (Ishimaru et al., 2011), where the most important regulators of root Zn uptake and homeostasis are *OsZIP1* and *OsZIP3* (Ramesh, 2003).

Heavy metal transporters HMA2 and HMA4 control Zn efflux into the xylem and from there on – its long distance transport. Mutations in these transporters create an imbalance in Zn distribution in plants, represented by accumulation of Zn in roots and decreased Zn in its shoot (Hussain et al., 2004). *OsZIP4* has been shown to play an important role in Zn translocation within the plant (Ishimaru et al., 2007), especially into the vascular bundles and meristematic tissues (Ishimaru et al., 2005). Transgenic plants that over-express *OsZIP4* and/or *OsZIP5* accumulate Zn in their roots while their shoot Zn concentrations are significantly lower (Ishimaru et al., 2011; 2007). Therefore *OsZIP4* is likely involved in the xylem unloading and limiting Zn transport to shoots. Interestingly, the same pattern of shoot gene expression is found in the *OsZIP4*-transgenic rice and rice labelled as Zn-sensitive (Ishimaru et al., 2011).

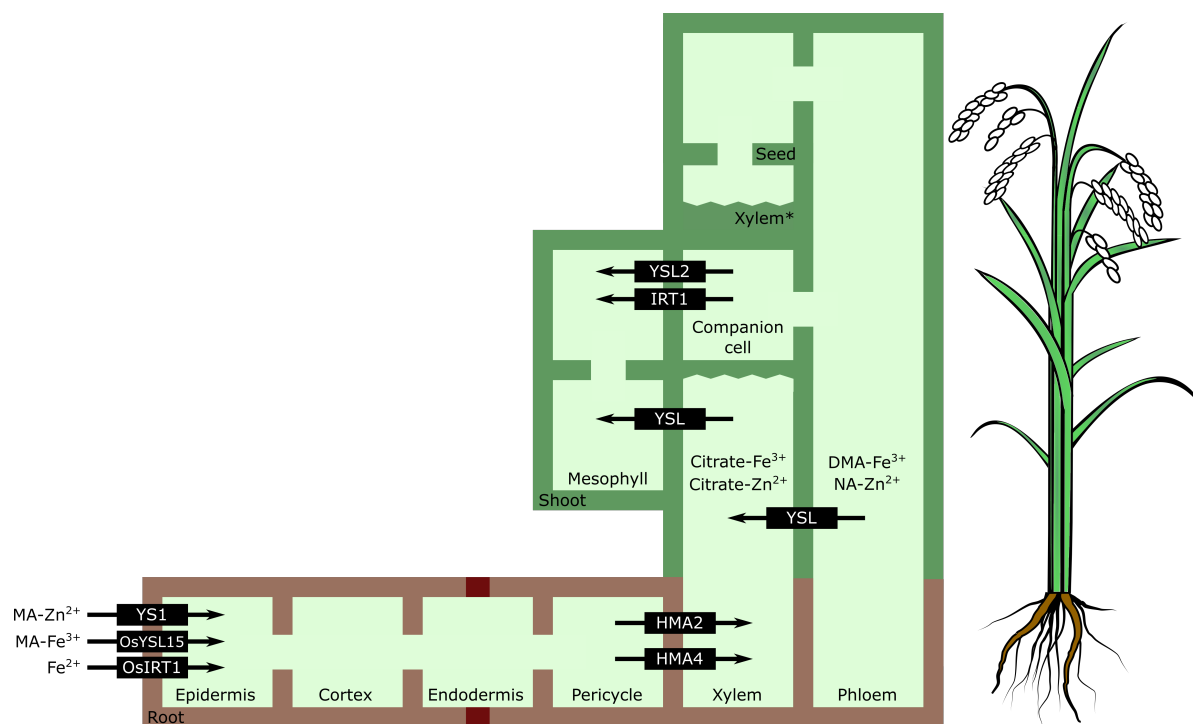


Figure 6.1 (Figure based on Palmer and Gueriot (2009)), permission acquired) In addition to the uptake of metal ions (Fe^{2+} , Zn^{2+}) via non-selective IRT (*Iron-Regulated Transporter*) proteins, monocots take up Fe and Zn in form of a complex (*i.e.* $[\text{Fe}^{3+}(\text{MA})]$, $[\text{Zn}^{2+}(\text{MA})]$) via the YSL (*Yellow Stripe-Like*) transporters on the epidermis membrane. Loading of Zn into vascular vessels is supported by active transport, *i.e.* HMA (Heavy Metal ATPase) proteins. In xylem and phloem, Zn is thought to be mainly bound to organic ligands such as citrate and nicotianamine, respectively (Yoneyama et al., 2015). Translocation of Zn from vascular tissues to different plant organs is supported by the activity of different transporters from the YSL group. *continuous xylem; MA, mugineic acids; DMA, 2'-deoxymugineic acid; NA, nicotianamine.

YSL (Yellow Stripe-Like) family of transporters play an important role in metal distribution between different tissues of the plant (Palmer and Gueriot, 2009; Waters et al., 2006). Metals are being transported via symplastic pathways to pericycle where they are loaded into the xylem and then, carried by the transpiration stream to the shoot. Unlike wheat and barley, xylem is not discontinued at the base of the rice grain and hence, Zn is directly transported to the nuclear epidermis (Figure 6.1; (Impa and Johnson-Beebout, 2012)). However, youngest leaves in rice do not have a developed xylem - therefore the phloem is the key nutrient source. Transporter proteins involved in the phloem unloading are still insufficiently known. Nevertheless, it is believed that member of the YSL group are the likely candidates, as some of the well characterised transporters (YSL1 and YSL3) are located to the shoot vasculature, pollen grains and possibly, seeds. Some recent evidence suggested

that YSL1 and YSL3 transporters are involved in redistribution of Fe, Zn and Cu from leaf tissue (Waters et al., 2006).

6.1.3. Stable Zn isotope signatures in higher plants

Growing body of evidence shows that metal-ligand interactions have significant effects on the extent and direction of stable isotope fractionation of metals in geological (Matthews, Zhu et al. 2001) and biological (Dideriksen, Baker et al. 2008,

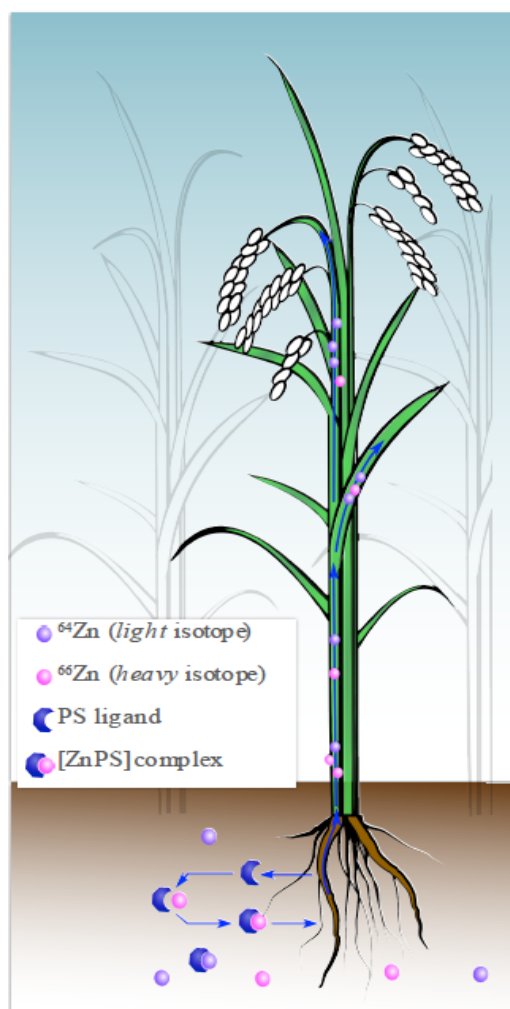


Figure 6.2 Rice is likely to utilise ligand-facilitated Zn uptake strategy under deficient Zn conditions. Promising Zn chelating compounds to fulfil the phytosiderophore role are small amino acids from the mugineic acids family.

Black, Epstein et al. 2008) systems alike. Metal complexation is one of the core chemical processes in nature and thus an essential driver in formation of isotopic signatures in the environment.

Studies of stable Zn isotope partitioning in plants have revealed a distinct isotopic pattern. Passive trans-membrane transport, such as uptake of Zn^{2+} via root membranes does not fractionate Zn isotopes (Weiss et al., 2005). Yet, a systematic depletion in the heavy ^{66}Zn isotope has been observed along the Zn uptake path in plants, starting from roots which are the most enriched. Initially, Weiss et al. (2005) identified preference for light ^{64}Zn over ^{66}Zn isotope in hydroponically grown plants and thus demonstrated the presence of unidirectional transport mechanisms. However, plants grown in their natural environment (*i.e.* soil medium) were shown to fractionate in favour of the heavy ^{66}Zn compared to the Zn-available pool in soil (Viers et al., 2007; Arnold

et al., 2010a). Xylem Zn loading and long distance transport in plant shoots involve kinetic reactions therefore following a kinetic isotope fractionation pattern. By

observing the isotopic partitioning in a range of plant species, Viers and co-workers (2007) found light ^{64}Zn enrichment in leaves and upper plant parts but not in shoot or roots. They report a correlation between the height of the plant and the extent of the isotopic fractionation thus implying a kinetic control of reactions involved in the Zn shoot transport. Similarly, observations of Moynier et al. (2009) suggest an increase in light Zn bias with the translocation distance.

In contrast, Arnold et al. (2010a) found distinct heavy isotopic fractionation in soil grown tolerant rice and justified these findings by the involvement of ligand facilitated Zn uptake (Figure 6.2), as Zn complexation by ligands, being an equilibrium-based reaction, introduce heavy isotopic effects (Bigeleisen, 1947). Furthermore, solubilisation modelling has shown that PS release and subsequent complexation of Zn is sufficient to increase Zn uptake in lowland rice production systems (Arnold et al., 2010a). These findings are in line with conclusions of Suzuki et al. (2006) who claimed that 2'-deoxymugineic acid is an important contributor to overcoming Zn-stress in rice plants. Their later study imply that formation of $[\text{Zn}(\text{DMA})]$ complex is a vital factor in Zn translocation to rice leaves (Suzuki et al., 2008).

6.1.4. Aims of the study

The aim of the experiments presented in this chapter is to test the hypothesis whether PS ligands, like DMA, are involved in the Zn uptake in rice. The objective of the study is to measure isotopic fractionation in tolerant and sensitive rice genotypes grown on Zn-deficient soil, similarly to the design of Arnold et al. (2010a), and correlate the isotopic fractionation measured in the field with the isotopic effects determined during DMA complexation of Zn in the laboratory (Chapter 4).

The study of Arnold et al (2010a) have found heavy (0.18 - 0.24 ‰) isotopic enrichment in shoots of Zn-tolerant rice genotypes and those findings were justified by an involvement of PS-ligand facilitated Zn-uptake, as DMA is the dominant phytosiderophore in rice (Nozoye et al., 2011). Moreover, Zn uptake is significantly intensified in the presence of DMA (Suzuki et al., 2008). In Chapter 4 of this thesis isotopic partitioning upon formation of $[\text{Zn}(\text{DMA})]$ complex was identified ($0.26 \pm$

0.18 ‰) in laboratory conditions, and the value reported closely correlate to the one found by Arnold et al. (2010a). If chelation-based Zn uptake is a strategy present only in tolerant genotypes, isotopically heavy Zn should be more abundant in these plants due to the complexation preference for heavy ^{66}Zn . Conversely, sensitive rice plants are assumed to predominantly uptake Zn in its ionic form therefore no isotopic fractionation is expected.

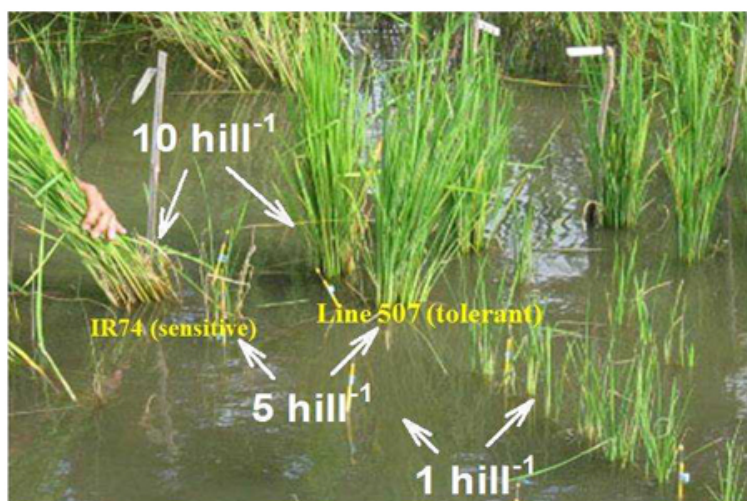


Figure 6.3 (Figure adopted from Mori et al. (2016), permission acquired.) Growth of tolerant (right) and sensitive (left) rice genotypes when planted in groups of different densities per ‘hill’. Plants grown at higher planting densities have been reported to grow better and have higher yields (Hoffland et al., 2006; Ptashnyk et al., 2011); which can be correlated with higher concentrations of plant-excreted ligands.

Previous studies have shown that plants grown in higher densities are less sensitive to Zn stress (Hoffland et al., 2006; Mori et al.). This was then linked to higher average concentrations of PS ligands in the rhizosphere (Hoffland et al., 2006; Ptashnyk et al., 2011). To simulate different concentrations of the excreted ligands, rice genotypes were grown in two planting densities - 1 plant per hill and 8 plants per hill (where ‘hill’ refers to a group of seedlings planted together; Figure 6.3). The aim this experiment was to further test whether the tolerance to Zn deficiency is correlated to the higher concentrations of the excreted PS ligands.

6.2. Methods

6.2.1. Lowland production system and soil conditions

Two genotypes of rice (*Oryza sativa* L.) were cultivated in lowland production systems at the International Rice Research Institute (IRRI) in Los Baños, Laguna (Philippines) between February and March 2014. The substrate where the experimental culture was grown consists of perennially wet, montmorillonitic, calcareous Hydraquent soil (USDA Soil Taxonomy) from Tiaong, Quezon province of Philippines. The soil is characteristic for its high-bicarbonate content thus being prone to Zn-deficiency. Additional information about the soil status, as described by IRRI-ASL are given in Table 6.1 (Izquierdo et al., 2016).

Three weeks before planting, the soil plots were submerged and this lowland production system was maintained throughout the experiment. Submerged soil system was divided into two treatment plots, where the first plot received additional Zn in a form of a ZnSO_4 fertiliser (15 kg/ha) (+Zn or Zn sufficient plot) and the other half of the system was untreated (-Zn or Zn-deficient plot). Both (-Zn) and (+Zn) plots were fertilised with a standard recommended dose (136 kg/ha) of NPK (14-14-14).

Table 6.1 Chemical characterisation of the lowland soil system (Izquierdo et al., 2016) used in the present study

Aerobic pH (1:5 H_2O)	8.5
Carbonates (g/kg)	95.9
Total Organic Carbon (TOC) (g/kg)	72.8
CEC ($\text{cmol}_\text{c}/\text{kg}$)	9.0
Clay (%)	42.2
Silt (%)	39.2
Available Zn (0.1 M HCl) (mg/kg)	0.1
Total Zn (mg/kg)	83.2

6.2.2. Genotype selection and plant growing conditions

The two rice genotypes (IR26 and A69-1) were chosen for their phenotypic differences when exposed to Zn-stress. While IR26 (Zn-sensitive) plants are known to be more susceptible to Zn deficiency, often resulting in a reduced growth, lowered yield and delayed maturity, A69-1 genotype is more resistant and thus regarded as Zn-tolerant (Rose et al., 2012).

Seeds of the selected rice genotypes were surface-sterilised for 1 minute before being subjected to 50 °C heat of a convection oven for 72 h in order to break the dormancy. Following this step, seeds were soaked in deionised water for 24 h and then aired in net bags for further 24 h. This period was necessary to prepare seeds for the 20-days seedling process where young plants were grown in seedling trays. Following this process, 20-day old seedlings were carefully transplanted into the submerged soil.

Plants were grown in groups referred as ‘hills’ consisting of either 1 or 8 plants. Four replica plots for each plant genotype and density system were assembled. In total, 4 rows were assembled per genotype with 6 hills in each row. Within the row, 20 cm of space was allowed between each hill as well as between adjacent rows. Observing different plant densities permits factoring for different concentrations of excreted phytosiderophores. In the further text the author refers to the experimental units defining density as ‘one plant per hill’ (1 pph) and ‘eight plants per hill’ (8 pph). It is expected that plants grown in higher densities will experience higher influx of Zn due to higher average utilisation of PS ligands excreted, compared to groups consisting of a single plant (Hoffland et al., 2006; Mori et al., 2016).

6.2.3. Zn concentration and Zn isotopes analysis

In this study, the isotopic fractionation measured was assessed in the light of physiological processes happening under specific field conditions.

To avoid contamination and ensure clean environment, all work described here was conducted in the Class 100 laminar hoods inside Class 1000 clean lab (MAGIC facilities, Imperial College London).

All acids used were supplied from VWR international and purified from Analar grade by sub-boiling distillation. 7 M HCl used for ion exchange procedure (for details see Section 2.3.3) was achieved by mixing sub-boiled 6 M and Merck Suprapur (12 M) in required amounts. All dilutions used in the presented protocols were made by diluting purified acids with MiliQ H₂O (>18.2 MΩ/cm grade) from a Milipore purification system.

6.2.3.1. Soil leachates and extractable Zn

A typical range of Zn concentrations in soils is between 10 and 300 mg Zn/kg, whereas the overall mean total Zn concentration in soil, reported in literature is approx. 55 mg Zn/kg (Alloway, 2009).

Soil leaching using 0.1 M HCl solution provides a good estimate of the plant available Zn content (Dobermann and Fairhurst, 2000). Therefore, soil extracts were prepared by leaching 0.3 g air-dried soil in 30 ml of 0.1 M HCl, for 48 h. Ten Zn-deficient and, corresponding ten Zn-sufficient soils were leached following this method.

After 48 h of stirring and equilibration, samples were filtered using acid-cleaned syringes (max. volume 10 ml) and 0.25 µm pore-size filters. Filtrates were collected in clean Teflon beakers before evaporating to dryness and re-diluting in 1 ml (7 M) HCl in preparation for the ion exchange chromatography. All samples were passed down the anion exchange column using the protocol described in Section 2.3.3. After purification of the leached Zn on AG MP1 resin, Zn concentrations as well as $\delta^{66}\text{Zn}$ isotope compositions were measured as described below.

6.2.3.2. Plant sample digestions

After the harvest, plants were oven-dried at 60 °C for 48 hours. Aerial parts of the oven-dried plants were separated and prepared for further digestion.

Leafs and shoots were lyophilised and crushed using pestle and mortar to fine particles. Homogenised plant material and standard reference material were than

digested using a CEM Microwave Accelerated Reaction System MARS-X (Milestone Ethos) as described below.

350 mg of plant material were weighed inside Teflon vessels (100 ml, XP-1500) followed by an addition of 5 ml (16 M) HNO_3 , 3 ml H_2O_2 and 0.6 ml (20 M) HF . Standard reference material used was Rye Grass international standard (BCR-281), further supported by intra-institutional standards Rice IR36-4wt and Mixed Grass Standard (HRM-14). Digestion protocol was programmed to gradually increase the temperature and pressure to 210 °C and 1.7 kPa, respectively; and then hold these conditions constant for another 90 minutes (Arnold, 2009).

The sample digestion using microwave protocol resulted in full breakdown of organic material. In all cases, solid plant material was dissolved into a colourless phase with viscosity identical or nearly identical to the acids used. After digestion in the microwave samples were evaporated and dissolved in 1 ml (0.3 M) HNO_3 . 20 % aliquot or 0.2 ml of each sample was further diluted in 5.8 ml (0.3 M) HNO_3 and submitted for the Zn concentration measurements. The results were used as a reference for determining Zn recovery after anion exchange column, to confirm quantitative recovery of Zn after passing the sample down the column.

After re-evaporating the HNO_3 , remaining samples were refluxed in 7 M HCl , evaporated again and then re-dissolved in 1 ml (7 M) HCl .

6.2.3.3. Anion Exchange Chromatography

Before proceeding to isotope composition measurements, Zn in plant samples needs to be isolated from the digested matrix and other transition elements, which might interfere with the isotope ratio measurements. It is essential to recover majority of Zn from the sample while simultaneously ensuring complete elimination of all other components that could potentially interfere with mass-spectrometric analysis and/or possibly cause matrix effects.

Here, a strongly basic anion-exchange resin AG-MP 1 was used to purify Zn from the digested plant material. 10 ml-capacity Poly-Prep Columns (Bio-Rad, CA, USA) were loaded with pre-cleaned 0.7 ml AG MP 1 anion-exchange resin (Bio-Rad, 100-

200 mesh, Cl⁻ form). The full ion-exchange chromatography protocol is shown in Table 6.2.

Firstly, the resin was cleaned successively with 0.5 M HNO₃ (10 ml) and MiliQ H₂O (10 ml) three times before proceeding to conditioning with 7 M HCl (4 ml). Samples (1 ml in HCl) were slowly loaded onto the resin. Elements of matrix and Cu were eluted first using 7 M HCl (12 ml), followed by Fe elution with 2 M HCl (7 ml) and finally, Zn fraction was collected after adding 7 ml of 0.01 M HCl onto the column.

To wash the resin, 10 ml of 0.5 M HNO₃ and MiliQ H₂O were successively loaded onto the resin. The columns containing the resin were then stored in vials filled with MiliQ water acidified with a few millilitres of both 16 M HNO₃ and 6 M HCl.

Table 6.2 Sample treatment to isolate Zn from dry plant material and pre-clean samples for subsequent Zn concentration and Zn isotope ratio measurements. Anion-separation protocol for quantitative Zn elution using AG-MP1 resin is described in detail by Dong et al. (2013).

Procedure	Resin/Method	Step	Medium	Volume (ml)
Matrix digestion	Microwave Accelerated Reaction System (MARS)	Sample preparation	Dried complexed fractions dissolved in 5ml sub-boiled (15.6M) 3ml 30% H ₂ O ₂	
		Digestion method	Heating to 210°C at 1.3 kPa pressure (90 min)	
Anion Exchange procedure	AG MP1, BioRad Cl ⁻ from, 100-200 mesh (from Dong <i>et al.</i> , 2013)	Resin Loading	0.5M HNO ₃	1 - 2
		Cleaning	0.5M HNO ₃	5 x 6
		Conditioning	H ₂ O	5 x 3
			6M HCl	4 x 1
		Sample loading	6M HCl	1 x 1
		Matrix elution	6M HCl	3 x 3
			2M HCl	2 x 3.5
		Zn elution	0.1M HCl	2 x 3.5
		Cleaning	0.5M HNO ₃	5 x 2
			H ₂ O	5 x 1

The collected Zn fractions were evaporated to dryness and refluxed in 16 M HNO₃. After changing the matrix to HNO₃ and re-evaporation, the dry samples were finally diluted to 2.5 ml (0.3 M) HNO₃. From each sample 1 ml aliquot was further diluted

in 5 ml (0.3 M) HNO_3 and then submitted for the final Zn concentration analysis using ICP-AES.

When using the Sample-Standard Bracketing (SSB) method (Peel et al., 2008) (details in section 2.3.5) to determine stable isotope partitioning, a quantitative recovery of $\geq 98\%$ after a column chromatography is necessary to eliminate possible mass-dependant isotopic fractionation imposed upon adsorption and elution from an ion-exchange resin (Cloquet et al., 2008). A comparison of the Zn concentrations between content of the samples before and after the purification on the anion exchange columns indicates a complete Zn recovery. The average recovery of Zn after the chromatographic separation yielded values between 97.5 – 104.9 % (for more details see Table 6.5).

6.2.3.4. Blanks

In order to ensure that no contamination occurred and to control for the procedural precision during the anion exchange chromatography several types of blanks were used. Starting from the digestion protocol, every set of samples was microwaved together with a standard reference material as well as an acid blank. The acid blank was further subjected to the anion exchange chromatography as a procedural blank.

As shown in Table 6.4, Zn concentrations measured in soil leaching blanks yielded negligible values. For both (–Zn) and (+Zn) soils, Zn contributions were well below quantification limits of ICP-AES meaning that $\text{Zn}_{\text{total}} < 20$ ng, which compared to average Zn concentrations of (–Zn) and (+Zn) soil leachates ($11 \pm 4\mu\text{g}$ and $60 \pm 15\mu\text{g}$, respectively) is $\ll 1\%$ of Zn present in the samples.

6.2.3.5. Zn isotope composition: bulk sample and acid leachates

Zn isotope ratios were measured using Nu (Nu instruments, UK) multi collector inductively-coupled plasma mass spectrometer (MC-ICP-MS) at the MAGIC facilities (Imperial College London, UK). The isotopes ^{62}Ni , ^{63}Cu , ^{64}Zn , ^{65}Cu , ^{66}Zn , ^{67}Zn and ^{68}Zn were measured simultaneously. External Normalisation method (enSSB) was used in combination with an inter-elemental correction previously

reported in detail by Peel et al. (2008). Any drifts in the instrumental mass bias during the measurement sessions were corrected using Cu ERM-AE633 as an external dopant. Presence of any interfering elements was checked by scanning for the relevant isotope masses.

The bracketing standard used in all the measurement sessions was an in-house Zn isotope standard London Zn, previously characterised by Arnold et al. (2010b). Zn ratios were expressed in $\delta^{66}\text{Zn}$ notation as the ratio of isotopic abundances of ^{66}Zn to ^{64}Zn in a measured sample compared to the average value of $^{66}\text{Zn}/^{64}\text{Zn}$ abundance ratio of London Zn in-house standard analysed before and after each sample:

$$\delta^{66}\text{Zn}_{\text{London}} = \left[\frac{(^{66}\text{Zn}/^{64}\text{Zn})_{\text{sample}}}{\text{average } (^{66}\text{Zn}/^{64}\text{Zn})_{\text{London}}} - 1 \right] \times 1000 \quad (6.1)$$

Essential part of establishing the accuracy and reproducibility of measurements whose object is natural isotopic variability – is comparison with an internationally recognised Zn isotope standard reference material. Single certified international Zn isotope standard reference material does not exist (Cloquet et al., 2008), however several widely used standards with inter-laboratory certification are in use as a reference material. Due to the limited availability, one of the previously most extensively reported reference material, widely known as ‘Lyon Zn’ (a Johnson Matthey (JMC) Zn standard solution batch 3-0749L, originally from the Lyon-CNRS laboratory) is lately being replaced by a mono-elemental isotopic standard solution denoted IRMM-3702. Reported offset in ^{66}Zn isotope composition between IRMM-3702 Zn ($\delta^{66}\text{Zn}_{\text{IRMM}}$) with respect to Lyon Zn ($\delta^{66}\text{Zn}_{\text{JMC-Lyon}}$) is $0.32 \pm 0.16 \text{ ‰}$ (Cloquet et al., 2008). London Zn solution, used in this study was calibrated against both standards, and the average measured Zn isotope composition of London Zn relative to both Lyon Zn and IRMM 3702 are given in Table 6.3, along the data reported in previous studies.

Table 6.3 International standard reference materials and their Zn content verified internationally in studies using MC-ICP-MS techniques. *Conversion of $\delta^{66}\text{Zn}_{\text{IRMM}}$ values reported in relation to JMC Lyon was done according to Hoefs (2009) and using conversion factor $\delta^{66}\text{Zn} (\text{IRMM})_{\text{JMC-Lyon}}$ as measured by Cloquet et al., (2008).

Reference material analysed:	Publication details:	Measured value ($\delta^{66}\text{Zn}_{\text{JMC-Lyon}}$):	Measured value ($\delta^{66}\text{Zn}_{\text{IRMM}}$)*:	n
IRMM Zn	Cloquet et al., 2008	$0.32 \pm 0.16 \text{ ‰}$		
	This study	$0.25 \pm 0.1 \text{ ‰}$		
BCR-281 (Rye Grass)	Arnold et al., 2010b	$0.38 \pm 0.09 \text{ ‰}$	$0.06 \pm 0.09 \text{ ‰}$	7
	Caldelas et al., 2010	$0.5 \pm 0.1 \text{ ‰}$	$0.18 \pm 0.1 \text{ ‰}$	5
	This study	$0.34 \pm 0.15 \text{ ‰}$	$0.08 \pm 0.1 \text{ ‰}$	12
Romil Zn	Mason et al., 2004	$-9.01 \pm 0.08 \text{ ‰}$	$-9.33 \pm 0.08 \text{ ‰}$	6
	Weiss et al., 2007	$-8.98 \pm 0.07 \text{ ‰}$	$-9.30 \pm 0.07 \text{ ‰}$	/
	Arnold et al., 2009	$-9.00 \pm 0.07 \text{ ‰}$	$-9.32 \pm 0.07 \text{ ‰}$	/
	Caldelas et al., 2010	$-9.10 \pm 0.1 \text{ ‰}$	$-9.42 \pm 0.1 \text{ ‰}$	12
	This study	-8.91 ± 0.1	-9.16 ± 0.1	10
London Zn	Arnold et al., 2009	$0.08 \pm 0.04 \text{ ‰}$	$-0.24 \pm 0.04 \text{ ‰}$	10
	Arnold et al., 2010b	$0.11 \pm 0.04 \text{ ‰}$	$-0.21 \pm 0.04 \text{ ‰}$	12
	Caldelas et al., 2010	$0.10 \pm 0.06 \text{ ‰}$	$-0.22 \pm 0.06 \text{ ‰}$	9
	This study	$0.03 \pm 0.04 \text{ ‰}$	$-0.22 \pm 0.04 \text{ ‰}$	11
Imperial Zn	Arnold et al., 2010b	$0.11 \pm 0.07 \text{ ‰}$	$-0.21 \pm 0.04 \text{ ‰}$	6
	This study	$0.02 \pm 0.09 \text{ ‰}$	$0.23 \pm 0.09 \text{ ‰}$	14
Southampton Zn	This study	$-7.01 \pm 0.14 \text{ ‰}$	$-7.26 \pm 0.14 \text{ ‰}$	10

Precision and accuracy of the isotopic measurements during each analytical session were evaluated by repeated measurements of certified reference material BCR-281 (Rye Grass) and the three in-house single-element solutions Romil Zn (Romil Ltd, UK), Southampton Zn and Imperial Zn. Data on the reproducibility of these measurements is shown in Table 6.3. The values for the measured single-element solutions agree well with the previously published data.

Every sample was measured in quadruplicate, and the means with the corresponding standard deviations (2SD, 95 % certainty interval) were reported.

6.3. Results and Discussion

6.3.1. Soil conditions, bioavailable soil Zn pool and its isotopic composition

Fundamental soil characterisation, including the soil moisture content was done at IRRI (Philippines) immediately after sampling (0 WAS) and then repeated three weeks later after all plants had been harvested (3 WAS). The average residual moisture content in oven-dried (-Zn) soils was 4.2 ± 3.0 % (1 SD, n=12) at 0 WAS. Three weeks later (3 WAS) the average moisture content in the same plots stabilised at the mean value of 4.5 ± 0.6 % (1SD, n=12). Values for soil moisture in (-Zn) plots were very similar for those in (+Zn) soil, namely $(4.1 \pm 0.6$ % (1SD, n=12) and 5.5 ± 2.9 % (1SD, n=12) for 3 WAS and 0 WAS, respectively).

Zn leaching procedure described in detail in the section 2.3.1 is a norm when assessing soil Zn availability in plants such as rice (Dobermann and Fairhurst, 2000). Therefore, it is expected that leachates collected are genuine representations of Zn bioavailability status in tested soils. Concentrations obtained after leaching both Zn-deficient (-Zn) and Zn-sufficient (+Zn) soils can be found in the below table (Table 6.4).

Table 6.4 Total bioavailable Zn in Zn-deficient (-Zn) and Zn-sufficient (+Zn) plots. The difference between the treatments in quantities of Zn leached were statistically significant $p < 0.001^{***}$ (Student's t-test). However, values for total Zn content leached in -Zn plot indicate borderline Zn deficiency (~ 30 mg/kg).

Sample No.	Treatment:	Total [Zn ²⁺], mg/kg	Treatment:	Total [Zn ²⁺], mg/kg
1	- Zn	31.50	+Zn	213.74
2	- Zn	32.50	+Zn	194.67
3	- Zn	32.52	+Zn	218.81
4	- Zn	43.78	+Zn	174.73
5	- Zn	42.95	+Zn	174.39
6	- Zn	31.40	+Zn	192.76
7	- Zn	30.94	+Zn	182.37
8	- Zn	32.71	+Zn	157.67
9	- Zn	29.56	+Zn	189.59
10	- Zn	43.82	+Zn	243.28
Blank	-Zn Blank	<0.0001	+Zn Blank	<0.0001

By using the acid leaching method described above, ~6 times less Zn was extracted from Zn-deficient soils (-Zn) compared to (+Zn) plots. The average concentrations for the -Zn and +Zn soils were $35 \pm 11 \mu\text{g/g}$ (2 SD, n=10) and $194 \pm 50 \mu\text{g/g}$ (2 SD, n=10) respectively. Although a statistically significant difference ($p < 0.001^{***}$) in Zn presence was observed between the treatments, the deficiency in (-Zn) plots can be described only as marginal (Sillanpää, 1990).

Measurements of Zn composition in the soil leachates describe a rough trend towards lighter isotope presence in the Zn-deficient soils (Figure 6.4). All data are expressed in parts per thousand (‰, per mill) as the ratio of ^{66}Zn to ^{64}Zn relative to an in-house standard (London Zn).

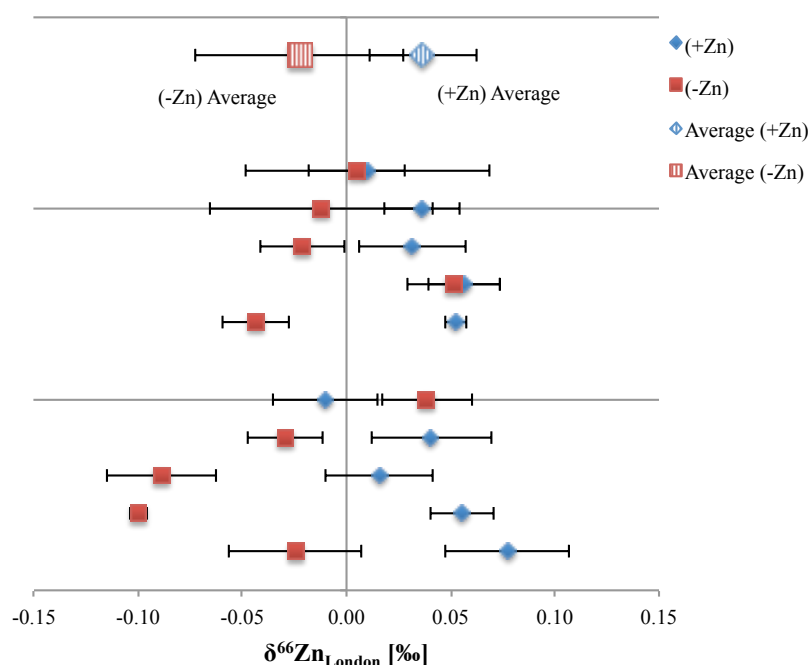


Figure 6.4 Plant available Zn isotope composition in Zn-deficient (-Zn) and Zn-sufficient (+Zn) soils leached in 0.1 M HCl. The plot shows comparisons of corresponding 10 plots in each treatment as well as the average value per treatment. The data suggest small but significant difference in isotopic compositions between the experimental plots ($p < 0.01^{**}$).

The $\delta^{66}\text{Zn}_{\text{London}}$ in the (+Zn) soils had a mean value of $0.04 \pm 0.08 \text{ ‰}$, which is in agreement with the combined composition of the native soil and the Zn fertiliser ($\delta^{66}\text{Zn}_{\text{London}} = -0.23 \pm 0.03 \text{ ‰}$) (Arnold, 2009). In the (-Zn) soils, the isotopic fractionation was predominantly deviating towards slightly lighter $\delta^{66}\text{Zn}$ values with average $\delta^{66}\text{Zn}_{\text{London}} = -0.02 \pm 0.1 \text{ ‰}$ (2 SD, n = 10).

The difference examined using standard Student's t-test have shown $p < 0.001^{***}$ significance level for the difference in total Zn content between the treated plots (Table 6.4) and $p < 0.01^{**}$ level of significance for the Zn isotopic composition ($\delta^{66}\text{Zn}$) between the Zn-deficient and Zn-sufficient soils (Figure 6.4).

The isotopic content of the bioavailable Zn fraction in the two tested plots (Figure 6.4) demonstrates a significant depletion in the heavy ^{66}Zn in the Zn-deficient plot compared to the fertilised one. Such pattern can be interpreted by the activity of Zn uptake mechanisms with preferential removal of the heavier ^{66}Zn . The observation is in agreement with the previously proposed ligand-facilitated uptake in Zn-deficient conditions. Excretion of chelating ligands that preferentially complex and uptake ^{66}Zn from the soil result in an isotopically light soil Zn pool.

6.3.2. Plant growth and shoot Zn content in sensitive IR26 and tolerant A69-1 rice genotypes

A subsample of harvested plants was analysed at IRRI to obtain shoot dry weight (DW, g/plant) and average Zn shoot content (mg/kg plant material). The results of analyses, shown on Figures 6.5 and 6.6, illustrate the difference between the tolerant (A69-1) and the sensitive (IR26) genotype in the ability to produce more plant material and shoot Zn uptake, respectively. Due to combining the plant material into a single sample (repetitions of 1 pph density treatment) in order to perform the analytical test, statistical significance was only calculated for the 8 pph density, as enough material was available for repeated analyses.

A significant decrease in produced shoot dry weight was observed in both tolerant A69-1 (Student's t-test, $p < 0.05^*$) and sensitive IR26 rice ($p < 0.05^*$). In both Zn-deficient (-Zn) and Zn-sufficient (+Zn) conditions, the tolerant genotype (A69-1) was more successful at producing shoot biomass compared to the sensitive IR26. At 1 pph planting density, A69-1 produced 31 % more shoot DW compared to the sensitive IR26 under Zn-stress; and 33 % more DW when Zn was in ample supply (Student's t-test, $p < 0.05^*$). However, the difference in the shoot biomass decreased when rice was grown in groups of 8 plants. At this planting density, A69-1 genotype

produced 24 % and 21 % more dry shoot weight while growing on $-Zn$ and $+Zn$ soils, respectively (Figure 6.5).

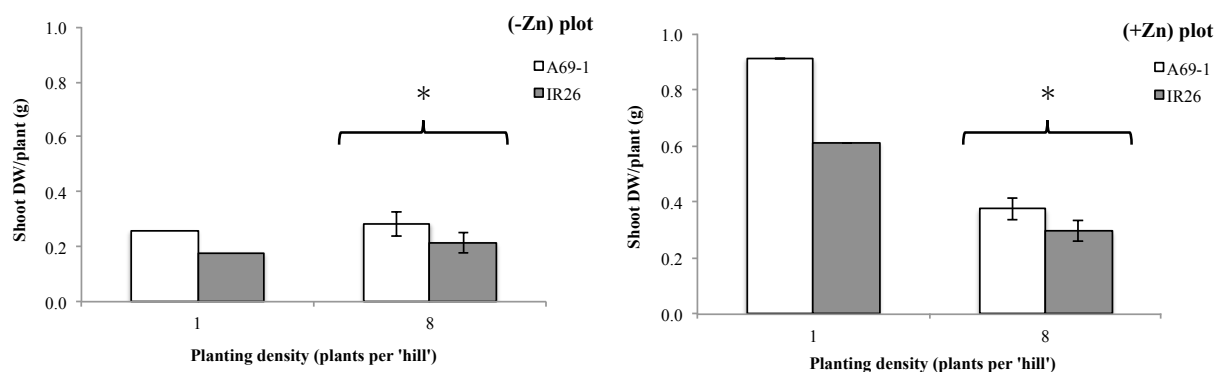


Figure 6.5 The effect of planting density on rice shoot dry weight for two genotypes (tolerant (A69-1) and sensitive (IR26)) when grown on Zn-deficient ($-Zn$) and Zn-sufficient ($+Zn$) plots. Both genotypes were affected by the Zn-stress in the ($-Zn$) plot, where significant decrease in the produced DW was observed ($p < 0.05^*$). The tolerant rice grew better and produced more DW (21 - 30 %) on both plots compared to the sensitive IR26 (Student's t-test, $p < 0.05^*$).

Similarly to the shoot DW, concentrations of Zn in shoots decreased significantly in both genotypes when exposed to Zn-stress ($p < 0.001^{***}$). A69-1 rice contained 18 % more shoot Zn when grown on fertilised soil at 1 pph density. However, that difference increased to 33 % more Zn in A69-1 rice when plants were exposed to Zn-stress. Both genotypes had very similar shoot Zn content when grown in groups of 8, regardless of soil Zn-status.

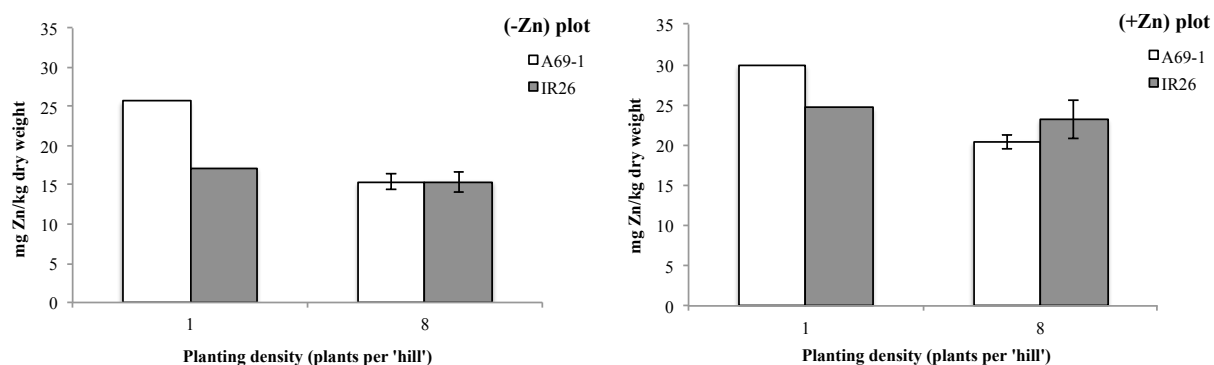


Figure 6.6 Concentrations of shoot Zn in rice plants in both sensitive and tolerant rice are significantly different (Student's t-test, $p < 0.001^{***}$) when grown exposed to Zn stress ($-Zn$) compared to the plants from the fertilised plot ($+Zn$). At 1 pph density, tolerant A69-1 genotype accumulated more Zn in its shoots compared to the sensitive IR26 genotype under the same Zn conditions, and this difference became greater under Zn-stress. At high planting density (8 pph), no difference between genotypes was observed.

The data indicate that, even when Zn is not a limiting factor (+Zn plot), both A69-1 and IR26 suffer reduced growth by 59 % and 51 %, respectively, when plants are grown at 8 pph density compared to 1 pph. This is likely due to competition between plants within the same group. The competition for nutrients within the group could also explain a reduction in the shoot Zn content (Figure 6.6) in rice grown in the (+Zn) plot. Namely, both genotypes have taken up less Zn when grown in hills of 8 plants as opposed to a hill of a single plant – 6 % in sensitive IR26 rice and 32 % in tolerant A69-1 genotype.

Increasing planting density was previously shown to have a positive effect on plant Zn uptake (Hoffland et al., 2006; Mori et al., 2016). This phenomenon has been explained by an increase in Zn bioavailability due to a concentration-governed rhizosphere effect, likely a Zn solubilisation by excreted organic acids. The results presented above demonstrate that at a high planting density (≥ 8 pph), even with sufficient Zn-supply, plants can sustain reduced growth and decreased Zn uptake (Figure 6.5 and 6.6), probably as a result of a competition.

6.3.3. Genotype-based differences in Zn content and isotopic composition of bulk rice plants

The values for Zn content measured in the aboveground rice organs are shown in Table 6.5. In summary, the concentrations of Zn varied between 20.3 ± 0.6 mg/kg (in IR26 grown on (-Zn) soils at 1 pph density) and 36.3 ± 7.9 mg/kg (in A69-1 grown on (+Zn) soil at 1 pph density). No significant difference in the Zn concentrations were observed between the tolerant and the sensitive genotype for either planting density tested (Student's t-test; $p > 0.05$). The aboveground Zn content of A69-1 rice plants was similar in samples from Zn-deficient and those from Zn-fertilised plots (Student's t-test; $p > 0.05$ in both 1 pph and 8 pph). Conversely, Zn-deficiency (-Zn plot) caused significant decrease in Zn content between IR26 rice grown on Zn-fertilised and Zn-depleted soil, both in 1 pph (Student's t-test; $p < 0.05^*$) and 8 pph (Student's t-test; $p < 0.05^*$) planting density (Table 6.5).

Table 6.5 Average Zn concentrations $[\text{Zn}^{2+}]$, measured using ICP-AES technique, and $\delta^{66}\text{Zn}_{\text{London}}$ isotopic composition of the measured plant samples. The data indicate significant difference between Zn concentrations in IR26 plants grown in (-Zn) and (+Zn) soils. The difference was tested using Student's t-test and the determined level of significance was $p < 0.05^*$ in both 1 pph(*) and 8 pph (*).

Genotype	Treatment	Density	Repetitions	Total [Zn] /mg plant (kg):		Recovery (%)		Zn isotopic composition:	
				Average	SD	Average	SD	$\delta^{66}\text{Zn}_{\text{London}}$	SD
IR26	(-Zn)	1pph	4	20.3*	0.6	100.9	1.2	0.27	0.15
IR26	(+Zn)	1pph	4	26.7*	0.3	101.2	2.2	-0.03	0.07
A69-1	(-Zn)	1pph	4	24.9	7.1	103.1	/	0.03	0.13
A69-1	(+Zn)	1pph	4	36.3	7.7	105.0	1.9	0.06	0.09
IR26	(-Zn)	8pph	4	23.0*	6.6	95.9	6.2	0.32	0.06
IR26	(+Zn)	8pph	4	35.3*	11.1	97.5	6.7	0.03	0.05
A69-1	(-Zn)	8pph	4	30.9	6.7	99.4	9.2	0.13	0.09
A69-1	(+Zn)	8pph	4	29.8	2.5	98.6	8.3	0.02	0.13

Table 6.5 also lists mean stable Zn isotopic composition ($\delta^{66}\text{Zn}_{\text{London}}$) for the two rice genotypes under varying Zn stress and planting density conditions. Compared to the soil on which the plants were grown, a distinct isotopic pattern was observed in both 1 pph (Figure 6.7) and 8 pph (Figure 6.8) growth densities. All rice plants (both IR26 and A69-1) grown in Zn-sufficient (+Zn) conditions have shown negligible Zn fractionation relative to the soil Zn composition ($\Delta^{66}\text{Zn}_{\text{Rice plant} - (+\text{Zn}) \text{ soil}} \sim -0.06$ to 0.03 ‰).

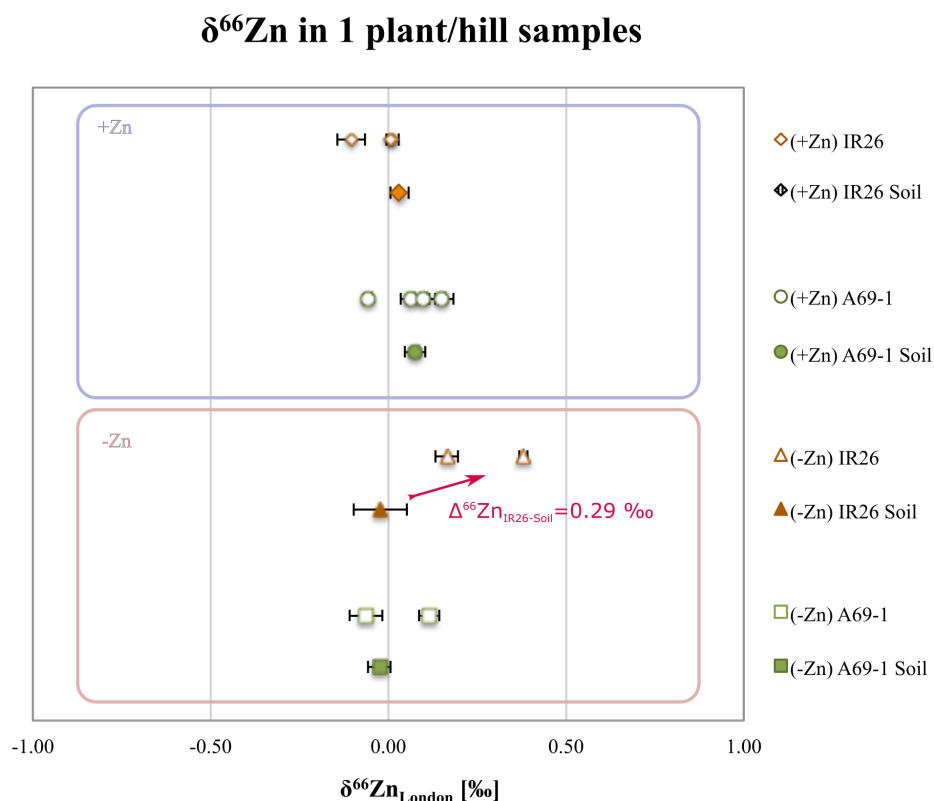


Figure 6.7 Stable Zn isotope fractionation ($\Delta^{66}\text{Zn}$) measured between soil and rice plants grown at 1 plant per ‘hill’ planting density. Upon sufficient Zn supply (+Zn) no isotopic discrimination was observed for rice genotypes tested. Under deficient condition (-Zn) measurements of the Zn isotope ratios in the sensitive (IR26) genotype samples have shown a heavy enrichment (the same pattern was observed the 8 pph density, Figure 6.8), unlike the tolerant A69-1 where no difference in the isotopic composition was found compared to the relevant soil sample.

In (-Zn) soil measured isotopic ratios identified a heavy drift in tolerant A69-1 rice at 8 pph density and sensitive IR26 rice regardless of the planting density. Tolerant A69-1 plants were enriched in the heavy ^{66}Zn compared to the (-Zn) soil ($\Delta^{66}\text{Zn}_{\text{A69-1 rice} - (-\text{Zn}) \text{ soil}} = 0.21\text{‰}$) when grown in hills consisting of 8 plants (Figure 6.8). This heavy ^{66}Zn enrichment at 8 pph density signals the involvement of chelation-based Zn uptake mechanisms, which are usually expressed under deficient soil conditions. It is likely that plants grown at 8 pph density were competing for the locally available Zn, therefore the effect of Zn-deficiency was on average stronger per plant compared to the 1 pph planting density treatment. For both densities tested, samples of IR26 plants were isotopically heavier compared to the (-Zn) soils. The magnitudes of the isotopic fractionations in both 1 pph and 8 pph samples were similar (0.29 ‰ and 0.32 ‰, respectively) (Figure 6.7 - 6.8).

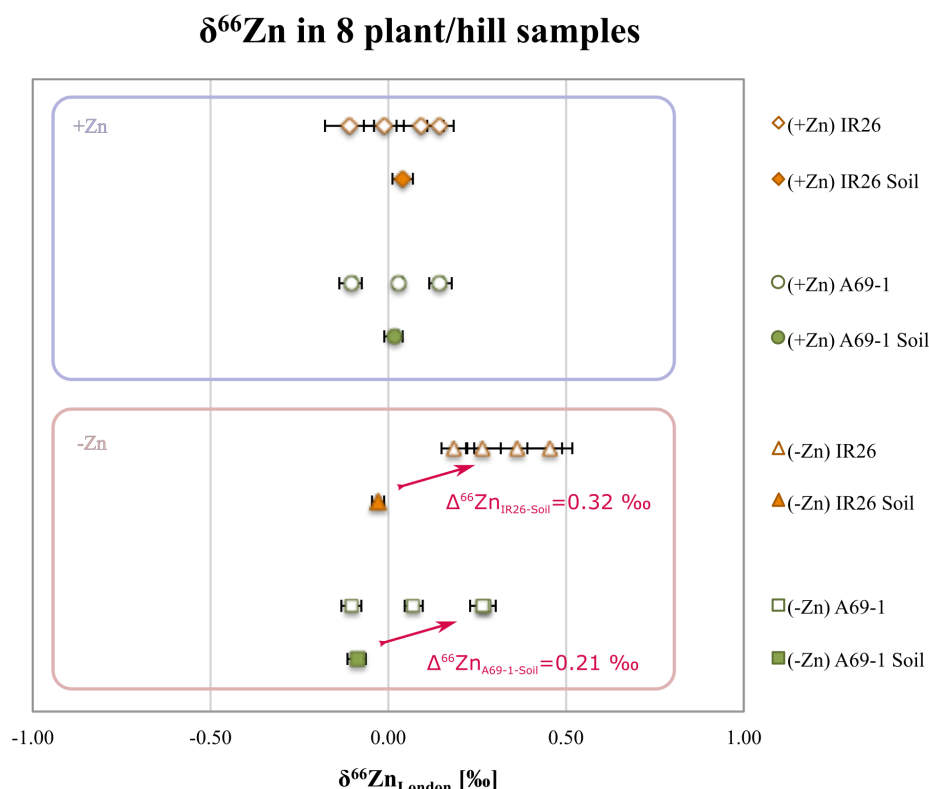


Figure 6.8 Stable Zn isotope fractionation ($\Delta^{66}\text{Zn}$) measured between soils and rice plants grown at 8 plants per hill density. For both 8 pph and 1 pph (Figure 6.6) density systems under sufficient Zn supply (+Zn) no isotopic discrimination was observed for rice genotypes tested. Under deficient condition (-Zn) measurements of the sensitive (IR26) genotype demonstrated a distinct isotopic pattern regardless of planting density. Namely, IR26 plants were systematically enrichment in heavy ^{66}Zn by $\sim 0.3 \text{ ‰}$ compared to (-Zn) soil). This is consistent with ligand-facilitated uptake of Zn. The tolerant (A69-1) genotype expressed similar behaviour only when planted in groups of 8 plants. It is likely that in the case of higher density the competition between the plants grown in the same group intensified the effect of deficiency.

The magnitude of fractionation factors found ($\Delta\text{Zn}_{\text{IR26 Rice} - (-\text{Zn}) \text{ soil}} \sim 0.3 \text{ ‰}$) is in line with the observations of Arnold et al. (2010a), and lends support that complexation by ligands (e.g. phytosiderophores) could be the mechanism behind the isotopic fractionation observed. However, in contrast to the study of Arnold et al (2010a), the results of this study clearly show that mechanisms producing heavy isotopic fractionation are present not only in the tolerant but also in the sensitive genotypes.

Isotopic fractionation upon Zn complexation by phytosiderophore ligands is expected to introduce negative isotopic fractionation, as indicated above. In Chapter 4 of this thesis, the author presents the work on laboratory determination of isotopic effects of Zn complexation by organic ligands. One of the key conclusions drawn is that the

fractionation occurring is at equilibrium. Therefore, regardless of the ratio between complexed and free Zn species, the value for isotopic enrichment in [ZnL] complex remains the same. Taking the findings of the Chapter 4 into consideration, it can be deduced that heavy isotopic shifts observed in both genotypes grown on deficient plots are indicative of a release of Zn-binding ligands. However, due to the equilibrium character of this fractionating process, the extent of the isotopic fractionation does not provide us with information about ligand concentrations and/or fluxes. In order to fully examine the involvement of Zn-chelating ligands in overall Zn efficiency, it is necessary to conduct studies and quantify the excretion rates of PS-like ligands from plant roots under a controlled environment.

All things considered, if complexation by PS ligands is the reason for the observed ^{66}Zn enrichment under Zn deficient conditions, the isotopic data presented here demonstrate that PS-facilitated Zn uptake is not a genotype-specific mechanism.

6.3.4. Implications for possible involvement of PS-facilitated Zn uptake in rice Zn efficiency trait

Mechanisms behind Zn tolerance are still poorly understood, however several theories exist. Majority of researchers support the claim that soil Zn uptake rates are of vital importance for plant's Zn status (Erenoglu et al., 1999; Marschner, 1995; Wissuwa et al., 2006), although evidence to support the relevance of Zn utilisation rates have been presented (Hacisalihoglu et al., 2003). Tolerance to Zn-deficiency in rice has repeatedly been shown to depend on mechanisms taking place in the rhizosphere. The effect these mechanisms likely involve increased Zn solubilisation and/or neutralising Zn uptake hindering agents, such as HCO^{3-} or Fe^{2+} (Mori et al., 2016).

Release of PS-ligands from mugineic acids family has been described as a highly efficient mechanism to increase Zn supply in various graminaceous plants (Arnold et al., 2010a; Cakmak, 1996; Ptashnyk et al., 2011; Suzuki et al., 2008; Walter et al., 1994; Weiss et al., 2005; Widodo et al., 2010; Zhang et al., 1998). Although root washings of rice contain much lower concentrations of these ligands (Oburger et al., 2014) compared to other crop species, modelling of Zn solubilisation by 2'-deoxymugineic acid (DMA) have indicated that even at such small excretion rates,

Zn uptake has been significantly improved (Ptashnyk et al., 2011). The results described in this chapter stand by conclusions of Arnold et al. (2010a) that PS, and likely DMA, is responsible for the heavy fractionation observed. Isotopic fractionation reported in both this study and that of Arnold et al. (2010a) correspond well with the magnitude of isotopic fractionation identified upon Zn complexation by DMA in laboratory conditions (0.26 ± 0.18 ‰; Chapter 4 of this thesis). Yet, the results of the current chapter disagree with conclusions of Arnold and co-workers (2010a) and demonstrate that the Zn-chelating strategy is utilised by both sensitive and tolerant rice under Zn-stress (Figure 6.6 and 6.7).

Nevertheless, efflux rates of phytosiderophore DMA could still be an important factor in overcoming Zn-deficiency in rice and thus a potential breeding target. The results of the current chapter suggest that the ability to secrete Zn-binding ligands is not the determining factor of Zn tolerance. However, it is possible that plant tolerance is proportional to efflux rates of PS ligands. Tolerance to Zn-deficiency has previously been linked to the increased efflux of MAs from rice genotypes grown in nutrient solution. Widodo et al. (2010) have shown that the rate of DMA exudation in tolerant RIL46 rice are more than double than those of sensitive IR74 genotype at sufficient Zn supply, and more than three times higher under Zn-deficient conditions (Widodo et al., 2010). Still, no studies have directly measured efflux of mugineic acid ligands in soil solution.

6.4. Conclusions

In this study, mechanisms of Zn acquisition from submerged soils were investigated in both tolerant and sensitive rice grown on Zn deficient soils, by means of measuring plant growth, Zn uptake and isotopic composition of Zn pools in both soil and plants. While no isotopic fractionation ($\Delta\text{Zn}_{\text{Rice} - (+\text{Zn}) \text{ soil}} \sim 0$ ‰) was found between Zn-fertilised soils and observed plants, plants grown on Zn-deficient soils were enriched in the heavy ^{66}Zn ($\Delta\text{Zn}_{\text{Rice} - (-\text{Zn}) \text{ soil}} = 0.21 - 0.32$ ‰) when compared to the plant available Zn in the soil. Previous studies have identified such heavy fractionation only in tolerant rice genotypes, and suggested that the underlying

mechanism, likely the PS-facilitated Zn uptake, contributes significantly to the Zn-efficiency trait. Data gathered in the current chapter as well as Chapter 4 of this thesis support the claims that the observed isotopic enrichment is due to complexation by PS ligands and subsequent uptake of Zn in a form of [ZnPS] complex. However, a distinct heavy isotopic shift was demonstrated in sensitive rice genotype, which shows that the heavy fractionation inducing mechanisms are not specific for tolerant genotypes.

Although it was expected that plants grown in larger groups would benefit from higher concentration of organic ligands excreted to the rhizosphere, the data indicate a decrease in plants' efficiency *i.e.* a reduction in dry weight production and shoot Zn content. Feasible reason for the observed phenomenon is an increased competition between plants of the same group for the necessary nutrients obtainable from the soil solution.

Investigations of Zn isotopic distribution in plants have provided valuable data for the crop biofortification research. Mechanisms in the basis of Zn efficiency remain largely unknown, however a potential breeding target has been suggested. Tolerance to Zn-deficiency possibly correlates with phytosiderophore efflux rates from the plant roots. Studies on differential PS efflux in relevant genotypes could provide valuable information on the genotypic differences in the Zn-efficiency trait.

Chapter 7

Conclusions

7.1. Thesis summary

In this thesis, the association between Zn and 2'-deoxymugineic acid (DMA) was explored, in the light of its possible role in Zn uptake by major crops, such as rice. The work conducted in this body of research followed a bottom-up approach, where first, the stability of the $[\text{Zn}(\text{DMA})]$ complex ($\log K_{\text{ZnDMA}}$) was reassessed before isotopic partitioning during $\text{Zn} + \text{DMA}$ complexation reaction was tested in laboratory conditions and, subsequently, within a natural system. A computational model to estimate isotopic fractionation upon Zn complexation was adopted in order to complement experimentally determined values without the need for further cumbersome and costly laboratory procedures.

7.2. Main conclusions and outcomes of the thesis

- In comparison to naturally present organic ligands such as low molecular weight organic acids (LMWOA), DMA forms a strong complex with Zn. This is implied by the affinity constant determined upon potentiometric titration studies (Chapter 3). The high stability of the $[\text{Zn}(\text{DMA})]^{2-}$ complex suggests that DMA has the ability to scavenge Zn in soil solution, if present in chemical species unavailable to plants, and form a strong association.

Therefore, the process of Zn complexation by DMA has the potential to successfully solubilise Zn in the rhizosphere. In other words, DMA and its complex with Zn possess the chemical properties necessary for the phytosiderophore role in plants.

- Understanding and unveiling the mechanisms in plant's rhizosphere is hampered by a lack of experimental methods to investigate individual processes at the plant-soil interface. The problem of separating free Zn from its [ZnL] complex is resolved by using a cation exchange chromatography, as reported in Chapter 4. The reported chromatography method allows for measuring isotopic fractionation in different species of the same sample and hence, is a valuable step towards generating more experimental data about isotopic effects caused by naturally occurring processes.
- The process of Zn complexation by strong organic ligands is an equilibrium reaction. Therefore, heavy isotopic enrichment is introduced during the formation of Zn complexes. The magnitude of the fractionation induced is correlated with the ligand's affinity for Zn. The higher the Zn affinity constant ($\log K$) and thus, the more stable is the complex between Zn and the ligand in question, the higher the preference for the heavier isotope.
- Isotopic fractionation during physiochemical processes relevant in nature can be investigated by means of computational modelling. Although the theoretical approach to estimate isotopic effects is becoming increasingly popular, very little computational data have been verified against laboratory measurements. Comparison of the experimental (Chapter 4) and the computational (Chapter 5) data gathered in this thesis suggest that during computational calculations errors rapidly accumulate, and this can lead to significant offset in estimates compared to the real, *i.e.* measured isotopic factors, particularly in larger molecules.

- The trend identified between the logarithmic value of ligands' affinity constants ($\log K_{\text{ZnL}}$) and the fractionation factors induced by Zn complexation suggest that the magnitude of Zn fractionation found in plants matches the isotopic effects of ligands with $\log K_{\text{ZnL}} \sim 11-13$. Considering that Zn affinity constants of mugineic acids belong to this interval it is feasible that complexation with members of the MA family contributes significantly to the observed isotopic signatures in rice plants.
- Under Zn-deficient conditions, rice cultivars tolerant to low Zn supply grow better (*i.e.* produce more shoot dry weight) and contain more shoot Zn. However, the difference between two cultivars is becoming smaller with greater planting density of seedlings. Although it has been shown that increased planting density aids average Zn uptake per plant, our observations suggest that effects of competition within the group can outweigh benefits of planting in larger groups. In the presented study, eight plants grown together experienced Zn stress more intensely, even the tolerant genotype.
- In Zn-deficient rice, Zn uptake is mediated by a mass-biased mechanism. Isotopically heavy Zn pool in plants under deficient conditions suggests that Zn has been transported from soil in a form of a complex. This claim is furthermore supported by the depletion in the heavy isotope in leachable soil Zn pool.
- The ability to acquire Zn by complexation with organic ligands is not a unique property of tolerant rice genotypes as previously thought. The heavy ^{66}Zn enrichment in sensitive and tolerant rice cultivars indicate that both genotypes have the ability to excrete organic ligands with aim to help solubilise soil Zn and transport it into roots. Considering the fractionation value determined upon Zn complexation with the rice phytosiderophore DMA,

the extent of the fractionation in rice plants implies that majority of its Zn is acquired via ligand-facilitated uptake mechanism.

- All above taken into consideration, isotopic signatures found in soil-grown rice plants can be explained by the activity of PS-supported Zn uptake. The contribution of this mechanism to the Zn efficiency trait is not yet clear, however further direct studies of DMA in soil solution could provide more useful details, as further discussed in the Chapter 8.

Chapter 8

Prospective Research Directions Towards Elucidating Zn-efficiency

The present thesis sheds new light onto the mechanism of rice Zn uptake and its involvement in the Zn-efficiency trait. However, the scope of this work was insufficient to answer all relevant questions, and future studies to extend this work are expedient. Results presented here complement previously gathered knowledge and provide a foundation for future research endeavours.

The ultimate goal of Zn biofortification programs is to ensure higher grain yields as well as higher Zn content of the grains. In addition to obstacles upon Zn entering the plant tissues, several stages during Zn translocation within the plant are highlighted as possible bottlenecks for sufficient Zn supply. Mechanisms of Zn uptake, translocation and deposition remain to be further tested before any breeding and/or transgenic biofortification approaches are attempted. Zinc homeostasis involves mechanisms that take place in different part of the plants as well as different development stages. The work in this thesis contributes towards better understanding of Zn uptake from soil and crossing the soil-root barrier. Long and studious observations of all mechanisms contributing to Zn homeostasis can provide answers relevant to future biofortification effort.

8.1. Possible areas for future research

8.1.1. Composing a database of $[M^{n+}(MA)]$ binding affinity constants

The thermodynamic constants providing information about stability of metal-ligand complexes are widely used in studying interactions in larger systems, *e.g.* modelling of uptake and/or compartmentation behaviour in biological models. Accurate determination of these constants using potentiometric titrations is vital.

As briefly discussed in Chapter 3, the values published during 1980s for MAs affinity constants might have been affected by insufficiently pure ligand material used. Hence, this could be the reason behind the inconsistencies in the log K constants reported. Intensive efforts over the past decade resulted in development of synthetic protocols that yield mugineic acids of substantial purity. The protocols have been continuously optimised and adapted to make them more convenient and accessible to a wider research community. Hence, the conditions are met to re-evaluate previously reported affinity constants of MAs, and construct a reliable database of thermodynamic constants using contemporary, high precision methods. Testing ligands of synthetic, rather than of plant origin is essential for achieving the adequate accuracy.

8.1.2. Determination of isotopic effects of a single biochemical reaction

Experimental techniques allowing measurements of fractionation factors caused by a single mass-biased reaction are rare. Not only that designing such experiments is demanding, but also it is challenging to ensure that measured fractionation values reflect only the target reaction, without input of any possible interference. Therefore, the chromatography protocol, reported in Chapter 4 of this work, was designed to initiate and encourage data collection on isotopic effects upon complexation events. Enlarging the pool of laboratory determined fractionation data is essential for untwining and gaining a thorough understanding of physicochemical reactions taking place in the environment. Any new methodological developments in this area could

have a major impact on our understanding of isotopic partitioning during metal cycling in nature.

In recent years, theoretical modelling of physicochemical processes is becoming a widely-used approach to bridge the gap created by the insufficient database of experimentally determined fractionation values, as discussed above. This alternative method is particularly favourable due to its time- and resource-effectiveness, however concerns have been raised whether theoretically calculated data can adequately substitute laboratory measurements. In the view of this work, current theoretical data can only be regarded as estimations of the real, laboratory measurements. In Chapter 5 propagation of uncertainty during calculations of isotopic effects is shown to steadily accumulate errors. Further efforts invested towards improvement of the computational methods are beneficial for the improvement of accuracy and precision of not only the generated data but, more importantly, the conclusions implied on the basis of theoretical calculations. *Ab initio* methods are now commonly used to decipher isotopic patterns in research areas such as pollution studies and cancer research. Recent increase in studies using computational means to interpret isotopic signals in nature, urges research community to investigate the problem of accuracy of *ab initio* models. At the moment, it is necessary to analyse and present computational data carefully, so that any implications derived reflect the true nature of the investigated problem.

8.1.3. Observing the behaviour of mugineic acids in large biological systems

In this body of work, isotopic fractionation during formation of a Zn complex with a phytosiderophore (2'-deoxymugineic acid, DMA) was measured in laboratory conditions for the first time. The measured value was then taken into account, when isotopic signatures were observed in a natural system, where previously DMA-facilitated Zn uptake was suggested. Stable isotope analysis is a valuable tool to trace fate of metal elements in nature. However, tracing isotopic signatures in the environment is not a simple task. In biological systems isotopic signatures are often complex due to overlapping effects of various mechanisms taking place. As the observed biological system (*i.e.* soil-grown rice plant) is a sophisticated and dynamic

system, measured isotopic patterns are likely to be a sum of effects arising from multiple mass-biased mechanisms. Although the study has provided a valuable insight into the physiological responses to varying Zn conditions, for unequivocal confirmation of the involvement of phytosiderophores in the Zn-efficiency trait, direct observation of phytosiderophore activity has to be carried out.

To verify the involvement of PS-supported Zn-uptake in rice, it is necessary to perform a study where the fate of both Zn and PS ligands would be monitored simultaneously. Based on the knowledge and experience gathered during the work with field-grown rice plants, an expanded experiment similar to the set up described in Chapter 6 is proposed. Namely, in addition to measuring isotopic fractionation in both plants and the medium, simultaneous measurements of phytosiderophore effluxes (*e.g.* DMA in rice) is highly informative. Under such set up, the positive $\Delta^{66}\text{Zn}$ shift in isotopic signals between the medium and plants is an indicator of complexation-based Zn uptake mechanisms, while the ligand efflux information helps specify the responsible carrier ligands. In its essence, the idea behind such experimental setup is simple, however challenging to design. The first question arising regards the medium for plant growth. Hydroponic culture is a simplified and more manageable system, particularly for studying efflux rates of secreted ligands. Nevertheless, studies warn that hydroponically grown plants do not accurately represent the real system, as far as the Zn-efficiency trait goes. On the other hand, methods to allow measurement of PS efflux rates into soil solution are still in the process of development, although some progress has been made. Nevertheless, technical difficulties with measuring mugineic acids *in situ* are significantly hampered by their biology. Some of the relevant crop species, such as rice, excrete only a small amount of PS ligands; hence, very sensitive detection methods have to be used. The experimental observations are additionally hampered by a subtle diurnal cycle according to which plants secrete mugineic acids. Namely, plants excrete MAs only in the early morning hours (Takagi, 1976), approximately 4 h after the onset of the light (Zhang et al., 1991). This trait further complicates any experimental strategy by putting time restrictions for conducting *in situ* observations. This is just one of

many technological challenges that needs to be addressed before we are able to collect data on the behaviour of mugineic acids *in situ*.

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Appendix I

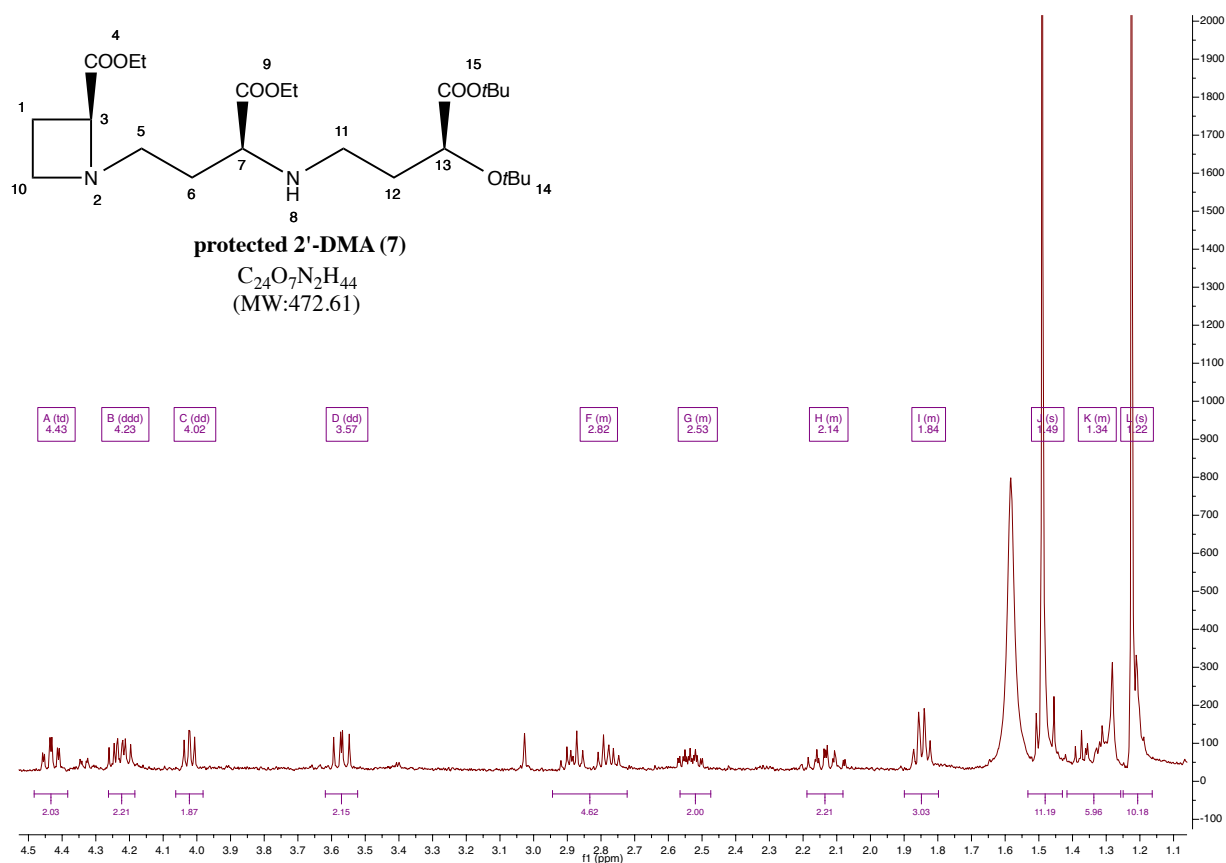
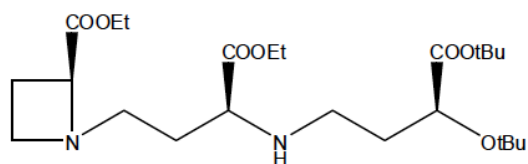


Figure A 1 ^1H NMR spectrometry of the protected 2'-deoxymugineic acid. ^1H NMR (400 MHz, Chloroform- d) δ 4.43 (td, $J = 8.8, 2.2$ Hz, 2H), 4.23 (ddd, $J = 10.4, 9.2, 6.3$ Hz, 3H), 4.02 (dd, $J = 6.9, 5.6$ Hz, 2H), 3.57 (dd, $J = 10.3, 8.1$ Hz, 2H), 2.94 – 2.72 (m, 5H), 2.57 – 2.47 (m, 2H), 2.19 – 2.08 (m, 2H), 1.90 – 1.80 (m, 3H), 1.49 (s, 11H), 1.42 – 1.26 (m, 6H), 1.22 (s, 10H).



Chemical Formula: $C_{24}H_{44}N_2O_7$

Molecular Weight: 472.62

Elemental Analysis: C, 60.99; H, 9.38; N, 5.93; O, 23.70

WEIGHT MGS	% CARBON	% HYDROGEN	% NITROGEN	% OTHER
1.8950	59.98	9.17	6.00	
1.4634	60.04	9.23	5.97	

Figure A 2 Elemental microanalysis of the synthetic product – 2'-DMA with protecting groups attached. For the expected percentages of the carbon (C, 60.99 %), nitrogen (N, 5.93 %) and hydrogen (H, 9.38 %), the elemental analysis have positively identified the substance tested as the protected DMA (C, 60.0 %; N, 6.0 %; H, 9.2 %).

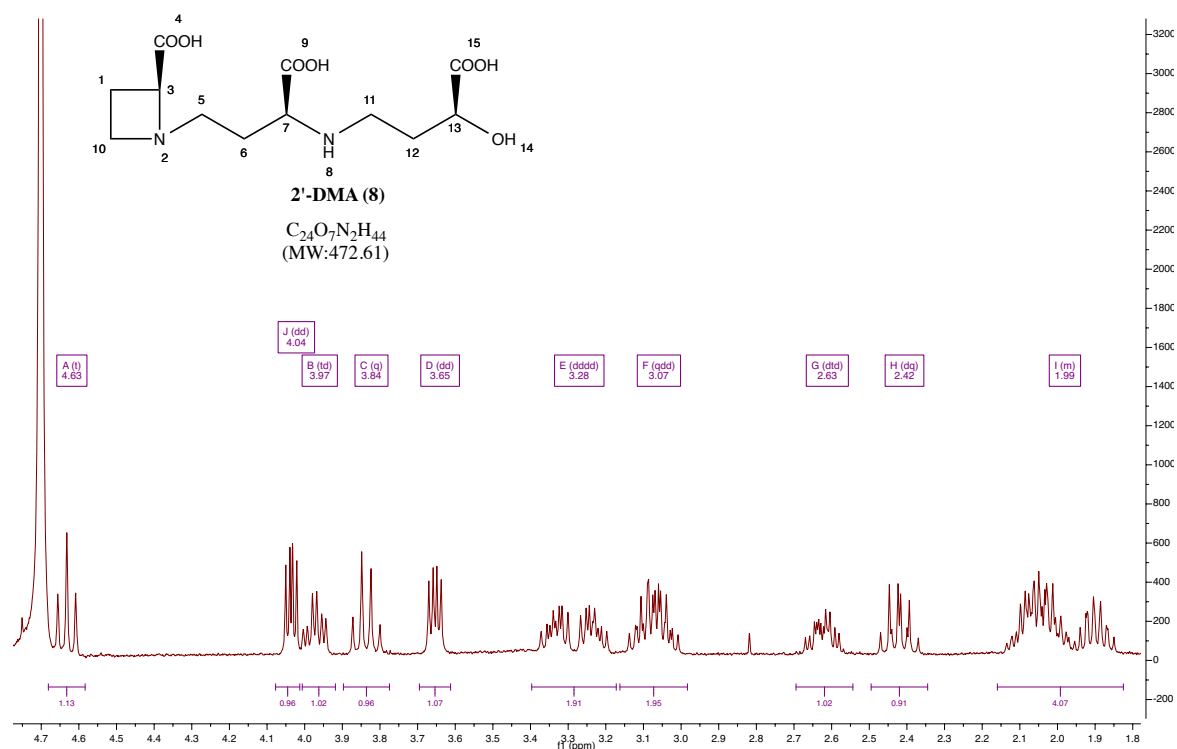


Figure A 3 ^1H NMR spectrometry of 2'-deoxymugineic acid (DMA) synthesized using Namba *et al.* (2007) protocol. ^1H NMR (400MHz, D_2O) δ = 4.63 (t, J =9.5Hz, 1H, H^3), 4.04 (dd, J =7.3, 4.5 Hz, 1H, H^{13}), 3.97 (td, J =9.9, 4.5 Hz, 2H, H^{10a} , H^{10b}), 3.84 (q, J =9.6 Hz, 1H, H^3), 3.65 (dd, J =8.6, 4.6 Hz, 1H, H^{5a}), 3.28 (dddd, J =41.4, 12.9, 9.3, 6.0Hz, 2H, H^{5a} , H^{11a}), 3.07 (qdd, J =12.5, 8.3, 6.3 Hz, 2H, H^{5b} , H^{11b}), 2.63 (dtd, J =12.0, 9.5, 4.4 Hz, 1H, H^{1a}), 2.42 (dq, J =12.0, 9.4Hz, 1H, H^{1b}), 2.16-1.82 (m, 4H, H^{6a} , H^{6b} , H^{12a} , H^{12b}).

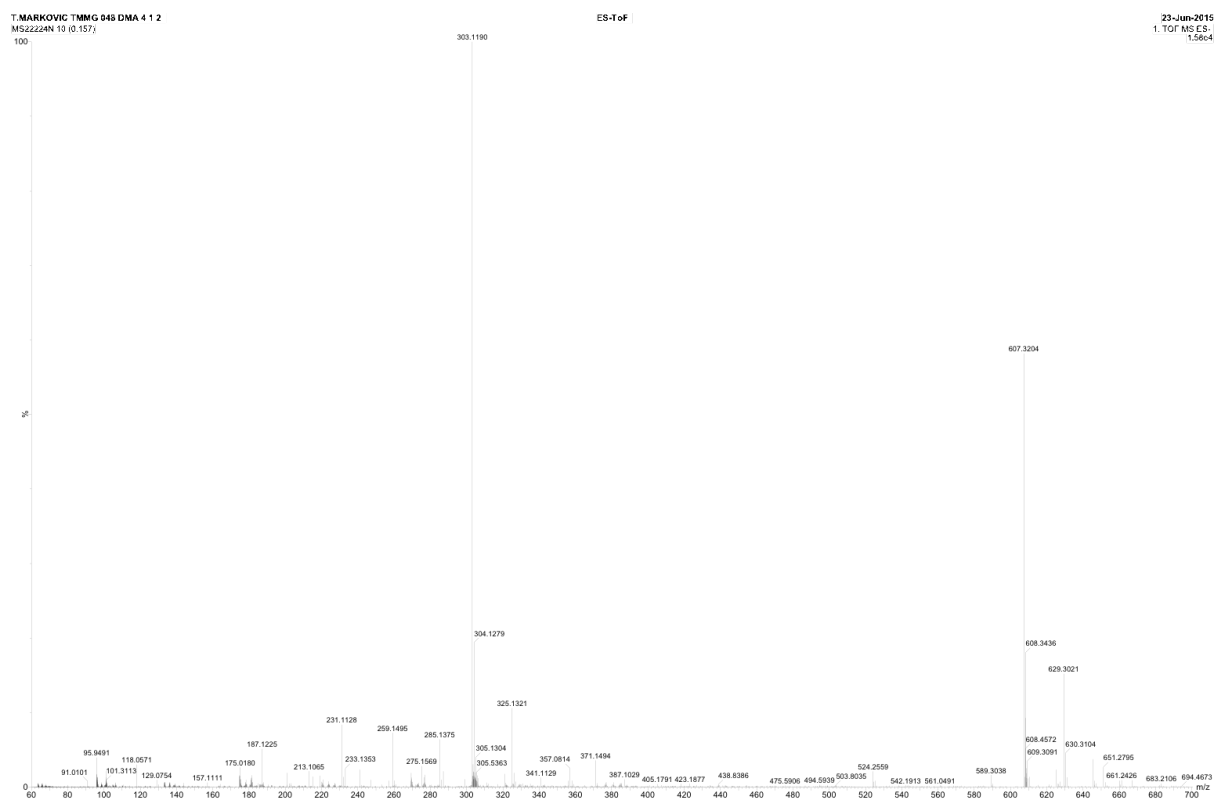
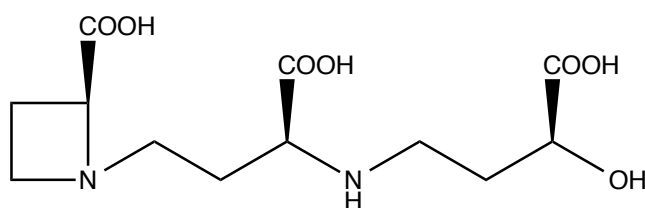


Figure A 4 2'-deoxymugineic acid synthesized using Namba et al. (2007) protocol: ES-MS: m/z calculated for $C_{12}H_{19}N_2O_7^-$ [M-H] $^-$: 303.120, found 303.119.



Chemical Formula: $C_{12}H_{20}N_2O_7$

Molecular Weight: 304.30

Elemental Analysis: C, 47.37; H, 6.63; N, 9.21; O, 36.80

WEIGHT MGS	% CARBON	% HYDROGEN	% NITROGEN	% OTHER
1.4828	42.70	7.71	11.04	
0.9853	41.53	7.53	11.02	

Figure A 5 Elemental microanalysis of the synthetic product – 2'-deoxymugieneic acid (DMA). For the expected percentages of the carbon (C, 47.4 %), nitrogen (N, 9.2 %) and hydrogen (H, 6.63 %), the elemental analysis have positively identified the substance tested as the DMA (C, 42.0 %; N, 11.0 %; H, 7.6 %). However, the slightly higher content of nitrogen is explained by the presence of NH_4^+ counterions as a result of column purification step involving elution with ammonia solution.

Appendix II



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Appendix III

Iron and zinc isotope fractionation during uptake and translocation in rice (*Oryza sativa* L.) grown in oxic and anoxic soils

T. Arnold, T. Markovic, G.J.D. Kirk, M. Schönbächler, M. Rehkämper, F.J. Zhao
and D.J. Weiss

C.R. Geosciences (2015) 347: 397-404

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 is in line with previously determined fractionations of Zn in rice grown in hydroponic solutions and submerged soils 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 the translocation within the plant important biochemical and/or biophysical processes occur. The isotope fractionation observed in the grains would be consistent with an unidirectional controlled transport from shoot to grain 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 the redox state of Fe occurring during the uptake and translocation processes. 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.

Experimental determination of equilibrium Zn isotopic fractionation in complexes with 2'-deoxymugeneic acid (DMA) and its structural analogues, and implications for uptake mechanisms in plants

T. Marković, S. Manzoor, E. Humphreys-Williams, G. J. D. Kirk, R. Vilar and D. J. Weiss

Under revision for publication in Environmental Science and Technology

Abstract

Stable isotope signatures of Zn and other trace metals are increasingly used to study trace metal fate and behaviour in the environment. In most natural systems, Zn is to some extent complexed with organic ligands; therefore it is important to know the corresponding isotope fractionations to interpret the overall fractionation. We here develop methods to quantitatively separate relevant Zn-ligand (ZnL) complexes from free Zn^{2+} using cation exchange chromatography, and we determine the extent of isotope fractionation at a range of Zn:L ratios. We apply the methods to a phytosiderophore (2'-deoxymugeneic acid, DMA), which we synthesise, and three commercially-available ligands (EDTA, TMDTA and CyDTA). For all the ligands, we find a preference for heavy ^{66}Zn in the complexed metal, which we attribute to equilibrium fractionation in coordination bonding. The extent of fractionation is independent of the Zn:L ratio for all the ligands, indicating isotopic equilibrium. We find a linear relation between the extent of isotope fractionation ($\Delta^{66}\text{Zn}_{[\text{ZnL}]2- - \text{free Zn}^{2+}}$) and the log of the [ZnL] stability constant (K_{ZnL}). This relation could be used to estimate fractionations for other organic ligands with well-defined [ZnL] stability constants.

Fractionation of Zn upon complexation with physiologically relevant organic ligands in plants

T. Markovic, A. Simperler, J. Harvey, R. Vilar and D.J. Weiss

Poster Presentation no.2013. 25th Goldschmidt Conference, Prague, Czech Republic (2015)

Abstract

The contribution of ligand-facilitated uptake of Zn in graminaceous plants, such as rice, is studied with the purpose to better understand the mechanism of Zn efficiency. Previous work suggested that the formation of Zn complexes with naturally available organic ligands introduces an enrichment in heavy ^{66}Zn isotope, analogous to the mechanisms shown for Fe uptake [1]. Organic compounds that have been associated with a Zn transport role in plants include low molecular weight organic acids such as citrate and malate, small amino acids e.g. cysteine, as well as strong chelators from the group of phytosiderophores (PS) and peptide based metallothioneins.

This study quantifies isotopic fraction upon Zn^{2+} complexation with three synthetic ligands (i.e. EDTA, TMDTA and CyDTA) similar in reactivity and structure to 2'-deoxymugineic acid (DMA), a phytosiderophore from the group of mugineic acids utilised by rice to facilitate Zn translocation. Under the experimental conditions used, we observe heavy isotope enrichment within the complex (expressed as $\Delta^{66}\text{Zn}$) of $0.52 \pm 0.06\text{‰}$, $0.48 \pm 0.04\text{‰}$ and $0.63 \pm 0.08\text{‰}$ for EDTA, TMDTA and CyDTA respectively.

Heavy ^{66}Zn enrichment (0.18 to 0.22‰), previously reported by our group in field-grown rice [2], was explained by involvement of DMA in Zn uptake mechanisms under Zn deficient conditions. To estimate Zn partitioning when complexed by DMA, we calculate $\delta^{66/64}\text{Zn}$ using computational methods (i.e. DFT) as well as extrapolate the anticipated value from the experimental results shown above. Both methods agree in the direction of bias and the extent of isotopic fractionation, where $\delta^{66/64}\text{Zn}_{\text{free-Zn} - [\text{ZnDMA}]_2} \sim 0.32\text{‰}$.

Our results demonstrate advances in the application of DFT as a fast and powerful tool in future environmental and stable isotope studies. Nevertheless, further efforts in optimising analytical techniques are necessary to overcome the challenges of measuring Zn fractionation *in situ*.

**Zn partitioning in coordination complexes relevant for plant physiology:
a comparative experimental and *ab initio* study**

T. Markovic, A. Simperler, J. Harvey, R. Vilar and D.J. Weiss

*Geochemistry Group Research in Progress Meeting, National Oceanography Centre
Southampton, United Kingdom (2015)*

Abstract

Isotope fractionation of zinc in dynamic environment such as the plant-soil interface has been widely discussed. It has been suggested that the zinc uptake mechanism and subsequent isotope fractionation follows the same pattern observed for iron, namely, one of the uptake paths, possibly essential for the normal growth and yielding of crops living under deficient conditions, is the complexation of metals by carrier ligands called phytosiderophores (PS). While acting as the carriers for Zn^{2+} , phytosiderophores likely introduce an enrichment in heavy isotope.

In order to examine this phenomenon, isotopic fractionation upon Zn^{2+} complexation with a range of polydentate ligands has been studied experimentally and theoretically using *ab initio* methods. The extent of the heavy ^{66}Zn enrichment relative to ^{64}Zn has been experimentally determined in complexes with three artificial ligands similar in reactivity and structure to phytosiderophores, i.e. EDTA, TMDTA and CyDTA. The observed extent of fractionation ranges between $+0.48 \pm 0.04 \text{ ‰}$ and $+0.63 \pm 0.08 \text{ ‰}$, which correlates well with the zinc complexation constants of the ligands in question. Although exhibiting the similar trend, data obtained utilising *ab initio* approximations estimate preference for light ^{64}Zn with $\Delta^{66/64}\text{Zn}_{\text{aq-complex}}$ estimated between -0.33 ‰ and -0.46 ‰ .

Advances in the application of the *ab initio* methods have a strong tendency to become an extensively useful tool in stable isotope studies. Nevertheless, current state of the art calls for a conscientious and responsible data analysis. Further optimisation of quantum chemistry based methods is necessary to provide a reliable and unambiguous tool for predicting an underlying partitioning of stable isotopes in the environment.

Determination of Isotopic Fractionation between free Zn^{2+} and Zn-EDTA²⁻ and its implication for Zn uptake in plants

T. Markovic, S. Manzoor, R. Vilar and D.J. Weiss

1st WiBioSE conference, Arandjelovac, Serbia (2014)

Abstract

Several recent studies have reported isotopic fractionations during metal uptake by plants, thought to be due to complexation reactions involving root-released complexing agents called phytosiderophores (PS), and suggesting isotopic fractionation as a novel method for studying metal uptake mechanisms. To test this idea, we determined for the first time the extent of isotopic fractionation between free Zn^{2+} and Zn-EDTA, the latter serving as a model ligand complex for Zn-PS. This was achieved by (i) preparing varying molar fractions of free and complexed Zn^{2+} ; (ii) establishing an ion exchange procedure to separate quantitatively Zn-EDTA complex from free Zn^{2+} using Chelex-100 resin, and (iii) determining the extent of isotope fractionation in a range of mole fractions using multi-collector inductively coupled plasma mass spectrometry. The magnitude and direction of the experimentally determined equilibrium fractionation are consistent with field and laboratory observations for Zn^{2+} and other metal cations, providing strong support for the use of isotopic fractionation in studies of metal uptake by plants.