The Isotopic Discrimination of Copper in Soil-Plant Systems: Examining Sources, Uptake and Translocation Pathways

A thesis submitted to the University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy

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CONTENTS

ABSTRACT

Copper (Cu) is an essential micronutrient for plants and many microorganisms, playing a key role in electron transport during photosynthesis, lignin formation and cell wall metabolism. However, when Cu is present at elevated concentrations it can cause toxicity with impacts on the growth, reproduction and survival of aquatic and terrestrial organisms. The biogeochemical cycle of Cu in aquatic and terrestrial environments can be influenced by numerous biological (e.g. root rhizosphere) and physicochemical (e.g. redox, pH) properties. A better understanding of Cu biogeochemical cycling is required to ensure optimal Cu supply to organisms. As such, there is an increasing need for the development of new analytical tools that can be used in complex environmental systems to examine this.

This thesis investigates the use of Cu stable isotopes to yield new information on the behaviour of Cu in soil-plant systems. Copper has two stable isotopes, ^{63}Cu and ^{65}Cu, and the different partitioning of these two isotopes between Cu pools (known as fractionation) can provide information on the reactions and mechanisms involved in Cu transport from one pool to another. Stable isotope data from plant growth studies were coupled with solid phase speciation and dialysis solution speciation to yield a better understanding of the isotopic signature of bioavailable Cu and the mechanisms by which Cu is absorbed into, and translocated throughout, plants.

The effect of Cu complexation by soluble organic matter was quantified to assess whether the isotopic signature of bioavailable 'free' Cu differed to that of the total soil solution Cu. This is important as fractionation between soil solution and plants cannot be accurately measured if the isotopic signature of

the available pool of Cu is not accurately known. Copper isotope fractionation was examined in solutions of both synthetic organic ligands and Suwannee River fulvic acid (SRFA) using Donnan dialysis to separate the free and complexed Cu pools. The results showed that Cu contained within the organic complex was enriched in the heavy isotope, with the magnitude of fractionation proportional to the strength of the Cu-ligand bond. These results highlight the importance of determining the isotopic signature of the bioavailable 'free' Cu when looking at plant uptake mechanisms, as the isotopic signature of the total solution Cu is different to that of free Cu if Cu is partly complexed with organic ligands, as is usually the case in environmental samples.

When using Cu isotope fractionation to assess root absorption mechanisms, it is important to consider the contribution of Cu adsorbed to the cell wall. In order to assess the isotope fractionation involved with Cu adsorption onto plant cell walls, four-week old plants and seedlings of Fe-acquisition Strategy I and Strategy II species were exposed to various concentrations of Cu for short periods of time. Adsorbed Cu was then desorbed from four-week old tomato and oat plants, using 3 different desorption techniques to determine which root washing technique quantitatively released adsorbed Cu while not extracting symplastic Cu. The results showed that the root wash procedure based on cation exchange using La and Ca was the best extractant to exclusively target the apoplastic Cu, while EDTA and HCl extractants showed signs of symplastic Cu removal. No significant isotope fractionation was found during adsorption onto the surface of monocotyledonous (monocot, Strategy II) plant roots, but adsorption onto the surface of dicotyledonous (dicot, Strategy II) plant roots yielded Cu isotope fractionations on the order of that seen during Cu complexation with fulvic acid ($\Delta^{65}Cu_{root\text{-}solution}$ = *ca.* 0.2‰). The results suggested that a difference in the type and/or strength of Cu binding sites on the cell walls exists for monocot and dicot species, and highlight the importance of root washing when assessing isotope fractionation due to root absorption.

The fractionation of Cu stable isotopes during uptake into plant roots and translocation to shoots was used to gain new information on Cu acquisition mechanisms by plants. Copper isotope fractionation values were coupled with intact tissue speciation techniques (X-ray absorption spectroscopy, XAS) to examine the uptake, translocation and speciation of Cu in a dicot (tomato) and monocot (oat) plant species. Plants were grown in solution culture where Cu was maintained as free Cu by regular replacement of the nutrient solution, so that complexation-induced isotope fractionation in the solution did not complicate the determination of fractionation due to plant uptake. The iron (Fe) conditions were varied to test whether the stimulation of Fe acquiring mechanisms can affect Cu uptake in plants. The results showed that isotopically light Cu was preferentially incorporated into tomatoes ($\Delta^{65}Cu_{\text{whole plant-solution}}$= *ca.* -1‰), whereas oats showed minimal isotopic fractionation, with no effect on isotope fractionation with changing Fe conditions in either species. The presence of isotopically light Cu in tomatoes was attributed to a reductive uptake mechanism. The heavier isotope was preferentially translocated to shoots in tomato, while oat plants showed no significant fractionation during translocation. The translocation fractionation observed for tomatoes was suggested to be linked to an oxidation and organic complexation with nicotianamine, as both of oxidation and complexation processes lead to heavy isotope enrichment. The majority of Cu in roots and leaves of both species existed as sulphur-coordinated Cu(I) species indicating glutathione/cysteine-rich proteins. The lack of isotopic discrimination in oat plants suggests that Cu uptake and translocation was not redox-selective and different translocation pathways exist between monocot and dicot plant species.

The results presented in this thesis provide significant new information on the behaviour of Cu isotopes in soil-plant systems. For the first time it has been shown that Cu complexation with soluble organic matter and adsorption onto plant roots can cause notable isotopic fractionation, with the organic

complex or root surface enriched in the heavy isotope. The most significant findings of this research relate to differences observed between Cu uptake and translocation mechanisms between monocot and dicot species, elucidated from Cu isotope fractionations and XAS analysis. These data open the door to future research into Cu source tracing using isotopic signatures, further investigations into Cu behaviour in soil solutions in-situ, as well as field studies looking at Cu uptake and translocation mechanisms in plants grown in soil environments.

DECLARATION

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PUBLICATIONS ARISING FROM THIS THESIS

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STATEMENT OF AUTHORSHIP

Components of the research described in this thesis have been published or have been submitted for publication (as listed below). The contribution of each author to these works is described below.

Chapter 3: Environmental Science and Technology; **DOI:** 10.1021/es500764x

Chapter 4: Environmental Science and Technology; submitted

Chapter 5: New Phytologist; 2013, 199: 367-378

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STRUCTURE OF THESIS

This thesis is presented as a combination of papers that have been published, accepted or submitted for publication.

Chapter 1 provides an overview of the literature on Cu in soil-plant systems, as well as stable isotope geochemistry and its application to tracing element biogeochemical cycling. This chapter also includes the proposed objectives of the research presented throughout this thesis.

Chapter 2 provides an overview of the sample preparation methods developed for three key sample types analysed throughout this thesis: plant tissues, solutions exposed to plant roots, and dialysis solutions of Cu in a strontium nitrate background electrolyte. This chapter outlines the procedures that were trialled in order to obtain an optimised digestion, purification and isotope analysis procedure for Cu.

Chapter 3 describes how Donnan dialysis was used to separate free and complexed soluble Cu to determine the isotopic fractionation resulting from Cu complexation with soluble organic matter. This work has been accepted for publication in Environmental Science and Technology.

Chapter 4 details an investigation into the Cu isotope fractionation arising from root adsorption in the apoplast in various Strategy I and Strategy II plant species. This work also highlights the importance of

selecting an appropriate root washing technique to avoid symplastic nutrient release. This chapter has been prepared as a manuscript and submitted to Environmental Science and Technology for publication.

Chapter 5 describes a comprehensive study using Cu isotope ratios and X-ray Absorption Spectroscopy to examine Cu uptake and translocation mechanisms in hydroponically grown plants. This work has been published in New Phytologist.

Chapter 6 provides a summary of the findings and implications of the research presented in this thesis and includes some key recommendations and future research arising from this work.

CHAPTER 1

REVIEW OF THE LITERATURE

1. COPPER IN THE SOIL ENVIRONMENT

Copper is a naturally occurring element that is an essential micronutrient for plants and animals. Copper concentrations in soils are often related to the Cu content of the parent rock, with soils derived from felsic igneous rocks (e.g. granite) or coarse-grained materials (e.g. sands and sandstones) containing less Cu than those derived from mafic igneous rocks (e.g. basalt) or fine grained sedimentary rocks (e.g. clays and shales) (Jarvis, 1981). Copper can exist in the environment in three oxidation states: Cu(II), Cu(I) or Cu(0) (Jarvis, 1981). Reimann and Caritat (1998) published the Cu content of various rocks, with basalt having the highest Cu concentration at 90 mg kg^{-1}, and sandstone having the lowest at 2 mg kg^{-1}. In soils that have been subject to intense weathering, such as in tropical areas, or heavily weathered landscapes, such as Australia, this pattern of Cu distribution may be modified due to remobilisation and secondary distribution of Cu (Jarvis, 1981). The average natural Cu content of soils across the world ranges from 2 - 250 mg kg^{-1}, while studies of a number of agricultural soils from several countries found an average of 5 – 100 mg kg^{-1} (Jarvis, 1981). However, it is important to mention that there are also anthropogenic sources of Cu. According to Nriagu (1979), anthropogenic contributions of Cu to the environment exceed natural ones by approximately 300%. A more recent study looking at global Cu production throughout history suggests that over the past 100 years Cu fluxes, as measured by deposition in Greenland ice cores, are around an order of magnitude higher than natural background levels (Hong *et al.*, 1996). Sources include, but are not limited to: mine tailings, metal production, fertiliser and algaecide/fungicide application, sewage sludge application, coal, oil and wood combustion (Nriagu, 1979; Martins & Mourato, 2006). The largest discharge of anthropogenic Cu waste is from the electronics industry with > 10% of Cu used in this industry ending up in waste (*ca.* $2x10^9$ kg Cu $year^{-1}$) (Graedel *et al.*, 2004).

Copper is a versatile trace element, with an ability to interact strongly with both inorganic and organic soil components, form precipitates with sulphides, carbonates, hydroxides and other ions, as well as

existing as elemental Cu. Copper may be found in the solid-phase of the soil bound with a number of components, with associations ranging from weak Van der Waal forces to strong covalent bonds, co-precipitated with iron (Fe)/aluminium (Al) or ferromanganese oxides or incorporated into clay crystal lattices (fixation) (Flemming & Trevors, 1989). In the inorganic fraction, manganese (Mn) oxides show the highest specificity towards Cu^{2+} ions and correlations have been found between Mn oxide content in soils and the amount of adsorbed Cu^{2+} (McBride, 1981; Flemming & Trevors, 1989). Despite the ability of Cu to interact strongly with inorganic substrates, it is the organic fraction of soils that generally sorbs the largest quantity of Cu, with up to 98% of total Cu in soils found to be associated with the organic fraction (Jarvis, 1981; Flemming & Trevors, 1989). The organic matter in soils and sediment is believed to regulate the mobility and bioavailability of Cu. Petruzzelli *et al.* (1978) studied the influence of organic matter on Cu adsorption and found that when the organic matter fraction was degraded, the adsorption of Cu was significantly reduced. The Cu^{2+} ion is unique in its ability to form inner sphere complexes with soil organics in the acidic pH range, usually bonding directly with two or more organic functional groups (mainly carboxylic, carbonyl and phenolic groups) (McBride, 1981). Organic solid phases hold Cu^{2+} in a form that is kinetically available but thermodynamically stable (McBride, 1981).

The concentration of Cu contained in the solution phase of soils is highly important, as it determines the mobility, bioavailability and potential toxicity of soil Cu. The speciation of soluble Cu is also important for understanding soil Cu behaviour (Lepp, 2005). In most soils the concentration in solution is controlled by sorption/desorption phenomena associated with the various soil colloidal materials, predominantly organic components (Jarvis, 1981), with the quantity of Cu contained in the solution phase (typically ranging from 0.05 - 1 µM (McLaren *et al.*, 1981)) much smaller than total Cu held by clay and humus colloids or as precipitates (Stevenson & Fitch, 1981). The solubility of Cu is controlled by the competition between solid phase adsorption, which reduces the soluble concentration, and complexing of Cu by soluble ligands, which prevent its adsorption and maintain Cu in solution. Thus,

Cu^{2+} bound to soluble organic matter in soils is considered to be the largest contributor to the plant available fraction in soils, despite ferromanganese oxides and phyllosilicates having a greater exchangeability of Cu^{2+} (McBride, 1981). In a study by Nolan *et al*. (2004) isotopically exchangeable Cu in the tested soils accounted for 3.7-52% of the total soil Cu, confirming previous research that suggested Cu exists mostly as non-labile (non-exchangeable) species. The highest exchangeable Cu percentage was found in Cu contaminated soils, demonstrating the difference in Cu availability in soils with predominantly endogenous Cu, where it resides in immobile forms such as mineral crystal lattices, and soil with contaminant sources of Cu which exist in the exchangeable pool. Copper association with organic matter has been shown to increase the Cu in the labile (exchangeable) pool (Kline and Rust, 1996). McLaren and Crawford (1974) also found that the speed at which the bulk of the labile Cu pool reached isotopic equilibrium suggested that the exchange between the surface and solution Cu in the soil is rapid. Hence, equilibrium would be restored quickly if any disruption to the system occurred, such as that induced by plant uptake of Cu. A schematic of the dynamic equilibrium system between solid-phase Cu and solution-phase Cu is shown in Figure 1.

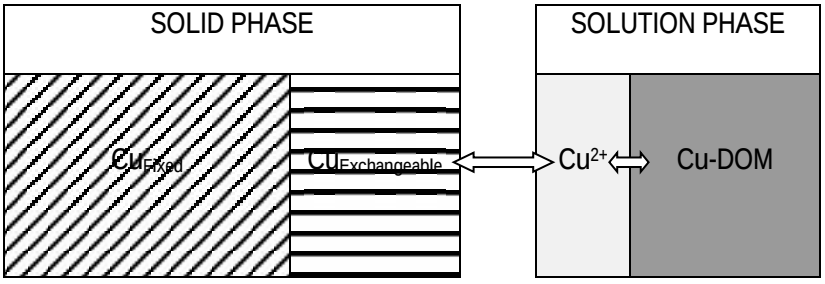

Figure 1 Schematic of the equilibrium that exists between exchangeable, free ion and dissolved organic matter (DOM) complexed Cu. The fixed pool of Cu is unreactive on timescales that are of importance to the soil-solution equilibrium, and refers to endogenous Cu within mineral crystal lattices and insoluble precipitates. Exchangeable Cu refers to Cu present on the adsorption sites (such as cation exchange sites) of solid matter, such as soil, roots and organic matter. Cu^{2+} refers to the oxidised free Cu ion. Cu-DOM refers to Cu complexed by soluble organic matter.

The effect of pH on Cu behaviour in soils is variable and is also related to soil organic matter. Copper complexation with dissolved organic matter at high pH compensates for the increased solid phase

adsorption observed at high pH, resulting in soluble Cu concentrations being less susceptible to pH changes than other divalent metals (McBride & Blasiak, 1979). However, while the total solution Cu may show less variability than other divalent metals in solution across a wide pH range, the speciation of the dissolved Cu changes with pH. As the pH approaches 7-8 the overall solubility of Cu decreases to a minimum but then increases as the pH rises above 8 as carbonate and anionic hydroxyl complexes become the dominant inorganic species and complexation by dissolved organic matter (DOM) leads to more Cu being solubilised (Figure 2). Organic ligands have greater solubility at higher pH so while the concentration of 'free' Cu^{2+} ions decreases with increasing pH, the amount of Cu associated with soluble DOM increases resulting in ratios of total dissolved Cu to 'free' Cu^{2+} that can exceed 100 (McBride, 1981). Taken in the context of Figure 1, the Cu^{2+} free ion pool decreases due to precipitation and sorption to solid phases as pH is increased, but compensating for this is an increase in soluble organic ligands which strongly complex Cu, maintaining Cu in solution. Hence, overall the proportion of Cu in each of the two soluble pools shown in Figure 1 changes with pH, but the overall size of the 'solution phase' Cu box remains relatively constant. Dudley *et al.* (1986) studied sewage sludge-amended soils and found that the solubility of Cu was determined by the complexation capacity of the DOM, which in turn was affected by the pH of the system. Cavallaro and McBride (1978; 1984) found a strong pH dependence of Cu sorption in both acid clay soils and calcareous soils. DOM has been shown to strongly complex Cu, and as such enhances its solubility and mobility, with DOM with higher aromaticity having a greater affinity for Cu (Amery *et al.*, 2008). In an isotope dilution study examining natural Cu attenutation in soils spiked with high levels of Cu, the labile Cu pool decreased rapidly over 30 days due to preciptation/nucleation of Cu on soil surfaces, Cu occulsion within organic matter and diffusion into micropores, with pH being the major environmental factor controlling Cu attenuation rates (Ma *et al.*, 2006).

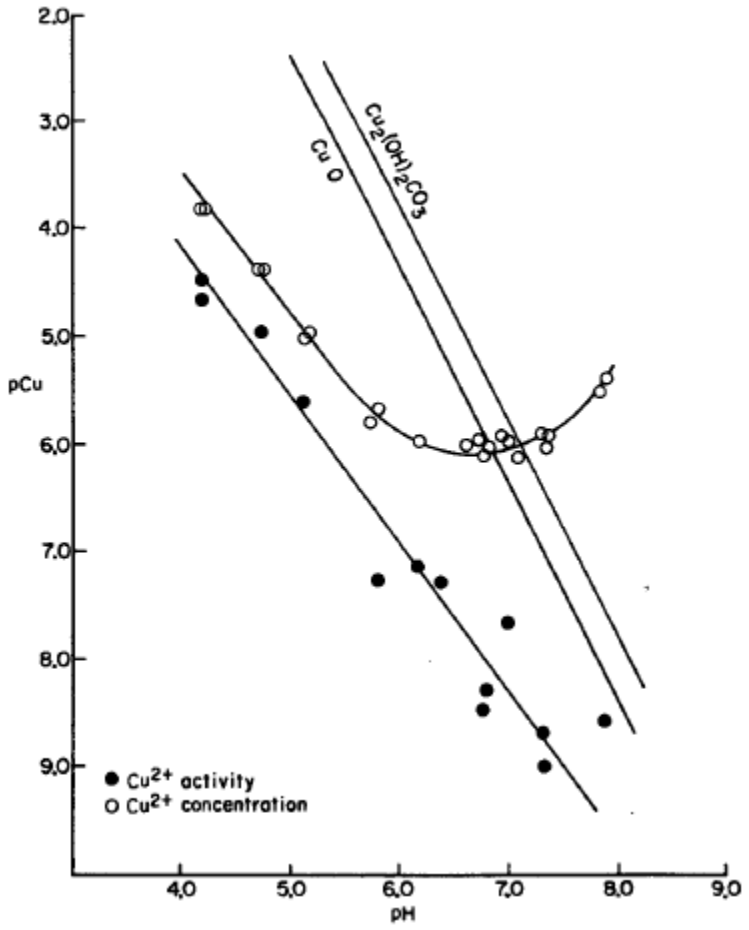

Figure 2. Copper concentration and free ion (Cu^{2+} ion) activity in response to pH. Solubility lines plotted as a function of ion activity. McBride MB, Blasiak JJ. 1979. Zinc and Copper Solubility as a Function of pH in an Acid Soil1. Soil Sci. Soc. Am. J. 43(5): 866-870.

Soil redox conditions can also affect the behaviour of Cu in the soil environment. A decrease in the soil redox potential (E_h) can has a variable effect on concentrations of soluble Cu in soil. Firstly, the solubility of Cu can decrease due its reduction to form elemental Cu^0 or due to precipitation of insoluble, highly stable Cu sulphide minerals (e.g. chalcopyrite $CuFeS_2$ or covellite CuS) (Flemming &Trevors, 1989; Tack *et al.*, 1996). Simpson *et al.* (1998) suggested sulphides are the dominant solid phase controlling soluble concentrations of Cu under anoxic conditions. Upon oxidation, the metal can be released back to a soluble form, although complexation with organic matter and adsorption reactions tend to occur rapidly after release (Simpson *et al.*, 1998). Alternatively, the solubility of Cu has also been shown to increase during anaerobic incubation of soil, due to dissolved organic matter breakdown

and dissolution of metal (hydr)oxides. Grybos *et al.* (2007) found that in a wetland soil under reducing conditions, dissolved Cu concentrations increased by a factor of approximately 10. Chuan *et al.* (1996) suggested that pH was more important than E_h in controlling Cu solubility in a sandy loam paddy soil, but when the pH was held constant lower E_h values did lead to increased solubility of Cu. Biasioli *et al.* (2010) reported that the labile pool of Cu, as measured by isotopic dilution, increased significantly after intermittent submergence to generate anoxic conditions. Under oxidising conditions, it appears that organic matter and metal (hydr)oxides (namely Mn oxides) regulate Cu behaviour, but under anoxic conditions the proportion of Cu associated with these species decreases and the proportion associated with carbonates, insoluble humic species, sulphides and DOM (if present) increases (Olivie-Lauquet *et al.*, 2001; Speelmans *et al.*, 2007). Hence, redox conditions play an important role in determining Cu behaviour and availability in the soil environment.

2. COPPER IN PLANTS

2.1. Plant Copper Uptake and Transport

Copper is an essential element for the metabolic functioning of plants. It is a vital component of proteins used in processes such as photosynthesis, respiration and lignification, and as such, plants must absorb Cu from the surrounding soil (Hall & Williams, 2003). Surface horizons of soils tend to be enriched in organic matter, and thus tend to have greater Cu concentrations than lower horizons, as a result of plant remnants retained in soil matter. This organic layer serves the important role of maintaining Cu in a complexed form that is available for plant uptake, yet is relatively resistant to movement by leaching (Stevenson & Fitch, 1981).

In general, plant uptake of ionic nutrients requires the transport of ions through the soil solution to the root surface where absorption occurs. Mass flow and diffusion are the two key ion transport processes reported in the literature. The process that has the greatest influence on the supply of a given nutrient depends on the solution concentration and the plant requirement (Barber, 1962). Mass flow is generated by the absorption of water into the plant roots, which causes a convective movement of water containing nutrient solutes towards the root surface (McLaughlin *et al.*, 1998). When the plant requires more nutrients than can be supplied by mass flow, diffusion takes over as the controlling nutrient supply process, as depletion of the ion occurs around the root, leading to a diffusion gradient. Checkai *et al.* (1987) found that the maximum concentration of Cu brought to roots by mass flow would only account for <1% of the Cu in the plant. When EDTA was added to the nutrient solution, the accumulation of Cu in tomato plants increased significantly. It was hypothesised that the complexation with EDTA led to increased diffusion of Cu to the unstirred zone around the roots, buffering Cu activities in the vicinity of metabolically active uptake sites (Checkai *et al.*, 1987). The concept of increased solution metal with ligand complexation enhancing metal uptake has been discussed in the literature. The common conclusion is that ligands can enhance metal uptake in plants by enabling greater diffusion to the unstirred zone around the root where the free ion equilibrium is quickly restored after disturbance from metal absorption (McLaughlin *et al.*, 1997a; Degryse *et al.*, 2006; Degryse *et al.*, 2008). Additionally, it is possible that the metal-ligand complex could be absorbed directly through breaks in the root endodermis (Smolders & McLaughlin, 1996; McLaughlin *et al.*, 1997b). As discussed above, the dominant form of soluble Cu in soils is complexed with organic matter (McLaren & Crawford, 1973; Hickey & Kittrick, 1984; McLaughlin *et al.*, 1998). It has been shown that in solutions maintained at constant Cu concentrations, Cu is more rapidly absorbed from solutions of free Cu^{2+} than from solutions of EDTA or DTPA complexed Cu (DeKock & Mitchell, 1957; Dragun *et al.*, 1976). However, in solutions where the free ion concentration of Cu was kept constant, the presence of plant excreted organic ligands enhanced Cu uptake (Degryse *et al.*, 2008). The difference arises from the fact that

complexation in constant concentration solutions leads to a decrease in free ion for absorption at the root interface, while when the free ion concentration is kept the same, the presence of more solution Cu due to organic ligands holding it in solution, enhances the diffusion and Cu free ion buffering at the plant root. Copper from sludge applications, compared to Cu salt applications, led to higher root Cu concentrations, despite lower Cu^{2+} activity in the soil solution (Minnich *et al*., 1987). The sorption of Cu^{2+} to solid-phase exchange sites from the salt application and the increased labile, soluble organically complexed Cu associated with the sludge application could explain this.

The method of Cu uptake into plant roots and cells remains unclear, but several possible mechanisms exist. In order for plants to absorb Cu from soils with low free ion concentrations, such as soils with predominantly endogenous Cu present in non-labile forms, an ion mobilisation step in the rhizosphere is required (Lepp, 2005). Given the lack of in-depth data on Cu uptake mechanisms, it is useful to assess the mechanisms of uptake used for other nutrients, to determine if Cu could potentially be absorbed by similar processes. Iron is an important plant nutrient and two different strategies have been evolved for extracting Fe from the soil environment. Strategy I plants (dicotyledons or non-graminaceous monocotyledons) secrete H^+ and reductants to dissolve precipitated/adsorbed Fe(III). Strategy II plants, or graminaceous monocotyledons, secrete phytosiderophores, which are organic complexants that bind Fe(III) directly and enable its absorption (Marschner & Romheld, 1994; Lepp, 2005). These two strategies are illustrated in Figure 3.

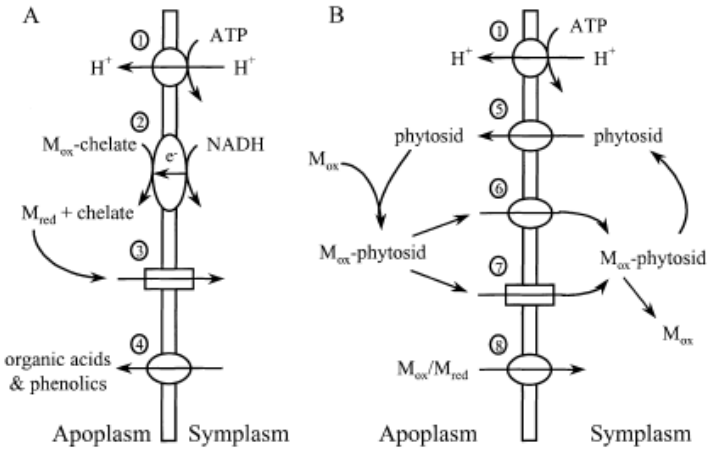

Figure 3. Potential metal uptake mechanisms in (A) Strategy I plants and (B) Strategy II plants: (1) H^+ ATPase that releases H^+ into the rhizosphere increasing the free metal ion activity; (2) release of reductants to reduce oxidised metal ions (M_{ox}) in the rhizosphere; (3) transport proteins (carriers or channels) that facilitate the absorption of metal ions, which may require the reduced ionic form of the metal (M_{red}); (4) transport proteins that release organic acids or chelates that act as either reducing agents or chelating agents; (5) a transport protein which releases phytosiderophores into the rhizosphere; (6) a selective carrier protein for the metal-phytosiderophore complex; (7) a low affinity carrier protein for the metal-phytosiderophore complex; and (8) diffusion driven ion uptake. Figure adapted from Grusak *et al.* (1999).

Strategy I plants have been shown to be capable of reducing Cu^{2+} by secretion of the Fe reductase compounds (Welch *et al.*, 1993). It was even suggested that Cu deficiencies in the particular species of pea studied were capable of inducing the release of the Fe reductase compounds, suggesting that this process may be involved in the uptake and regulation of Cu absorption in Strategy I plants. However, Holden *et al.* (1995) suggested that in soil, where most Cu is bound as organic complexes, the Fe reductase system would be irrelevant, as it is only effective in reducing free solution Cu^{2+} given the strong correlation between reduced Cu and free Cu ion activities observed in isolated tomato membrane vesicles. However, as noted previously, there is a dynamic equilibrium controlling free ion Cu^{2+}, and the reduction of Cu^{2+} to Cu^{+} would deplete the Cu^{2+} pool stimulating replenishment of this free ion from the solid or solution complex pool (Figure 1). Arnold *et al.* (2010) suggested that Zn is taken up in rice (a Strategy II plant) by phytosiderophores in the same way as Fe, given modelling with stable isotope data. Bowen *et al.* (1987) reported that Cu and Zn competitively inhibit one another

during uptake by tomatoes and rice. It was concluded from this work that Cu and Zn must be absorbed into these plants by the same mechanism, which, given the work of Arnold *et al.* (2010), would suggest that Cu is mobilised and taken up by rice through the release of phytosiderophores into the soil. However, Grusak *et al.* (1999) reported that there was no direct evidence to support the hypothesis that phytosiderophores enhance Cu uptake, as uptake in the presence of these compounds was similar to that observed with inorganic salts or synthetic chelates. A possible explanation for this discrepancy may be related to the concentration used by Bowen *et al.* (1987) (~0.05 mM Cu) which is far in excess of the background Cu concentration in soil solutions (0.05 - 1 µM (McLaren *et al.*, 1981)). Hence, the findings of Bowen *et al.* (1987) may not be relevant to true field conditions. However, Chaignon *et al.* (2002) also found a strong correlation between Cu uptake and phytochelatin (often termed phytosiderophore, but this term is technically only for Fe binding compounds) released under Fe deficient conditions in a Cu-contaminated, calcareous vineyard soil. These results are in line with those of Treeby *et al.* (1989) who also found an increase in Cu uptake under Fe deficiency, as well as Zn and Mn, in calcareous soils without Cu contamination.

The acquisition of Cu by phytochelatin complexes is a viable possibility and cannot be ruled out. Murakami *et al*. (1989) reported the affinity of phytochelatin compounds deemed phytosiderophores for various ions, and found a higher affinity for Cu^{2+} than for Fe^{3+}, which would technically have them classed as phytochalcophores. Chaignon *et al.* (2002) noted that the method for measuring phytosiderophore release is not specific to phytosiderophores and it is possible other root exudates are also measured. As such, the results of Murakami *et al*. (1989) are probably better described as a measure of the strong complexing ability of root exudates, rather than phytosiderophores exclusively. The conflicting reports in the literature on Cu uptake mean that more specific studies are required to confirm the mechanisms involved.

After Cu mobilisation in the soil, adsorption to the cell wall cation exchange sites is likely to occur before uptake by the plant. This adsorption may occur as free ions, reduced species or as complexed species (e.g. phytochelatins, as discussed previously). The initial adsorption is important, because Cu is then taken up by selective transport systems. The details of this process remain unclear, but most likely secondary transporters such as channel proteins and H^+-coupled carrier proteins are involved, and there is some evidence suggesting that specific ATPases (enzymes that catalyse energy production and drive the movement of nutrients across the cell membrane) may exist in the plant membranes (Marschner & Romheld, 1994; Williams *et al.*, 2000; Lepp, 2005). There is usually a strong negative membrane potential (-200 mV) at the root surface that drives metal cation uptake by secondary transporters, provided that a diffusion pathway exists and no excess energy is required for absorption (Graham, 1981; Lepp, 2005). Lepp (2005) reported that while the exact mechanism of Cu root absorption is unclear, the ability of roots to absorb Cu exceeds that which is required to fulfil the nutritional needs of the plant, when Cu is abundantly present and bioavailable. Hence, a mechanism to maintain Cu homeostasis in plant vegetative tissue is required to help limit Cu toxicity in soils where excessive Cu root absorption can occur, as plants must be able to limit Cu translocation to an extent. Plant methods of limiting Cu toxicity effects are relatively poorly understood, but complexation with metallochaperones and compartmentalisation in vacuoles in roots is known to occur (Cobbett & Goldsbrough, 2002; Ni *et al.*, 2005).

Copper transport systems in a variety of organisms have been studied, and a highly homologous P-type ATPase has been isolated in yeast, bacteria, and eukaryotic cells (both plant and animal) (Harris & Gitlin, 1996; Andres-Colas *et al.*, 2010). While this system is relatively poorly understood, its importance in Cu transport is evident by its evolutionary conservation (Yruela, 2009). In Wilson's disease, >25% of

the mutations characterised thus far occur within a particular sequence of this protein (Harris & Gitlin, 1996). Wilson's is a rare disease whereby the body cannot effectively eliminate Cu, leading to accumulation and damage to kidneys, brain and eyes. In eukaryotic cells, a group of transporters with high affinity for Cu, known as the CTR family, have been isolated. One example of the CTR family has been isolated from the plant species *Arabidopsis thaliana,* which is a model organism highly studied for its nutrient transporting mechanisms; the specific Cu transporting protein is known as COPT1 and it transports Cu(I) (Sancenon *et al.*, 2003; Sancenon *et al.*, 2004; Andres-Colas *et al.*, 2010). COPT1 has been found to be very important in Cu transport and to be involved in root growth and pollen development (Sancenon *et al.*, 2004; Andres-Colas *et al.*, 2010). However, COPT1 is not believed to have a role in Cu sequestration from the soil, as there was no detectable concentration of COPT1 in *Arabidopsis* roots studied (Kampfenkel *et al.*, 1995). Metallochaperones are also important in Cu transport as they are known to carry and selectively insert Cu into the active sites of given target proteins. Metallochaperones complex Cu, thereby preventing it from binding with the many potential Cu sequestration sites in the cytoplasm (Hall & Williams, 2003).

2.2. Toxicity of Copper in Plants

Copper is an essential micronutrient for plants, as it is contained in a number of important metabolic proteins. A decrease in photosynthesis and respiration, resulting in depressed growth, can occur from insufficient available Cu due to disruption of Cu-containing electron carriers within photosystems (PS) I and II (Baron *et al.*, 1995). However, at concentrations only mildly above nutritional requirements (total Cu solution concentrations > 1 µM or Cu^{2+} free activities on the order of > 10^{-7} – 10^{-8} M (Reichman *et al.*, 2006; Antunes *et al.*, 2007)) phytotoxic effects can be observed, causing notable changes in plant growth, nutrient uptake, chlorophyll content (leading to chlorosis) and enzyme activities (Martins & Mourato, 2006).

Adsorption-desorption processes in the rhizosphere play a key role in determining the bioavailability of Cu in soils, and hence, Cu toxicity. Zhao *et al*. (2006) suggested hydrogen ions (H^+) were able to mitigate the toxicity of Cu^{2+} to tomato and barley plants, as H^+ not only competed with Cu^{2+} for solid phase and organic binding sites in the soil, but they also compete for binding sites on the plant-cell wall surface (Zhao *et al.*, 2006). However, Rooney *et al*. (2006) suggested soil CEC (measured at the soil pH) and exchangeable Ca (which can act as a protective ion) are better predictors for Cu toxicity thresholds. The protective effect caused by other ions in the soil solution hints towards the potential importance of the cell wall adsorption step in the Cu uptake/toxicity mitigation mechanism of plants. In addition, the importance of resupply of Cu from solid phases to plant roots has been shown to be an important limiting step in controlling Cu toxicity, with diffusive gradient thin film (DGT) measurements of soil Cu shown to be the best predictor for plant tissue Cu, according to Zhao *et al.* (2006). The study of Zhao *et al.* (2006) highlights the importance of not only understanding the physical mechanisms of Cu uptake into plant cells to asses toxicity, but also the influence of the soil medium on Cu bioavailability and uptake.

Copper is known to induce oxidative stress and inhibit photosynthesis when present at toxic concentrations (Martins & Mourato, 2006). Martins and Mourato (2006) noted that, in tomatoes, as Cu concentrations were increased peroxide (H_2O_2) concentrations increased; H_2O_2 is a common product of oxidative stress. The tomatoes in their experiments increased the production of guaiacol peroxidises initially, as an early defence mechanism against H_2O_2 damage (Martins & Mourato, 2006). It is believed Cu^+ and Cu^{2+} ions are able to block photosynthetic electron transport in PSI and PSII (Sandmann & Boger, 1980b). Copper is capable of catalysing the formation of harmful free radicals, such as hydroxyl and superoxide radicals, and these species induce oxidative stress, altering metabolic pathways and affecting enzyme functionality (Martins & Mourato, 2006). Copper has also been associated with the destruction of lipids in the chloroplast membranes, due to peroxidation (Sandmann & Boger, 1980a; Bowen, 1987; Baron *et al.*, 1995). The subsequent release of free fatty acids from lipid degradation affects electron transport, mainly on the water oxidation side of PS II (Baron *et al.*, 1995). In addition to this, excess Cu can displace other metals from their ligands in important metalloproteins, hindering their performance. An example is Cu displacing magnesium (Mg) in chlorophyll (Andres-Colas *et al.*, 2010).

Excess Cu has been shown to alter cell membrane properties, chromatin structure and protein synthesis. This can lead to a leakage of ions, such as potassium and phosphate out of the cell, as well as to the inhibition of root elongation and of plant and leaf growth (Woolhouse & Walker, 1981; Devos *et al.*, 1991). The severity of the effect usually increases with increasing time and concentration (Woolhouse & Walker, 1981; Lepp, 2005; Martins & Mourato, 2006). The overall toxic effects of excess Cu on plants usually result in suppressed mitosis in the root meristem (Doncheva, 1998). Martins and Mourato (2006) found that excess Cu concentrations in solution caused decreased root uptake of Fe and Zn, and decreased the leaf concentrations of calcium (Ca), Fe and Zn.

Monitoring Cu uptake and translocation to control deficiency and toxicity issues *in situ* can be difficult. The emerging technologies associated with stable isotope ratio detection, when applied to soil-plant systems, have the potential to answer some of the questions surrounding nutrient uptake that traditional techniques alone struggle to answer.

3. STABLE ISOTOPES

3.1. Background on Stable Isotopes and Fractionation

Isotopes are nuclei of the same element, with the same number of protons and electrons, but with a different number of neutrons in the nucleus, leading to slightly different nuclidic masses. There are two classes of isotopes; stable isotopes with half lives ≥ 10^9 years, and unstable isotopes that radioactively decay over time (Hoefs, 2004; Johnson *et al.*, 2004). Of all the identified elements, only 21 are monoisotopic, while the remainder have at least two known isotopes (Hoefs, 2004). Copper has two naturally occurring stable isotopes, ^{63}Cu which accounts for 69% of all Cu nuclei, and ^{65}Cu, which accounts for the other 31% (Albarede, 2004; Hoefs, 2004), resulting in the standard atomic mass of Cu of 63.546.

The stable isotopes of elements can partition differently between different phases of a substance (e.g. liquid-vapor) or between two different substances. This process is known as isotope fractionation, and it is either mass-dependent or non-mass dependent. Non-mass dependent fractionation is not driven by the proportional difference in atomic mass between isotopes, but instead by other nucleic effects, and has been observed in photochemical processes, such as ozone formation and degradation

(Heidenreich & Thiemens, 1983). Mass dependent fractionations are more routinely observed and are most important for Cu. Mass-dependent fractionations can be equilibrium or kinetic in nature and lead to changes in the ratio of heavy to light isotopes (i.e. $^{65}Cu/^{63}Cu$) for each substance/substrate involved. Factors such as redox changes, changes in coordination environment or bond partner, all allow for the expression of mass dependent fractionation between isotopes of the heavier elements (Schauble 2004). There are two key conditions under which isotope fractionation can occur. The first is equilibrium isotope fractionation, driven mainly by the vibrational energy of molecules, which is affected by atoms with different masses (e.g. Schauble, 2004; Criss, 1999). Equilibrium isotope exchange reactions occur without a net change in species or phase, only a redistribution of isotopes between different chemical species or phases to achieve a complex with the lowest vibrational bond energy. The second is kinetic isotope fractionation, which occurs where reactions are controlled by rates of reaction. Kinetic isotope fractionating reactions are often associated with incomplete, unidirectional processes that do not reach equilibrium, such as dissociation and biologically mediated reactions (Criss, 1999; Johnson *et al.*, 2004). In general, equilibrium reactions will lead to heavy isotope enrichment in the complex with the strongest bonds, due to the lower vibrational energy between the atoms, and as such, a greater thermodynamic stability of the complex (Criss, 1999; Hoefs, 2004). Processes that are under kinetic control usually lead to a light isotope enrichment in the products, as bonds with lighter isotopes are broken faster and can also diffuse to the site of reaction faster, and therefore are more readily available to react (Criss, 1999; Hoefs, 2004). Redox processes are known to significantly fractionate transition metal isotopes, with light isotopes enriched in reduced products, while heavy isotopes are enriched in the oxidised product (Weiss *et al.*, 2008).

Stable isotope fractionation is usually not reported as absolute ratios, because changes in absolute ratios, particularly of heavy metal isotopes, are extremely low, as they are related to the relative mass difference between isotopes (Criss, 1999). As a result, stable isotope ratios are reported as

dimensionless delta values (δ), which are calculated using the measured isotopic ratio of the sample relative to an accepted standard for the element of interest, as defined by Equation (1). If the δ value is positive, there is an enrichment of the heavy isotope relative to the standard, and if it is negative, there is a light isotope enrichment.

$$\delta_x = 1000\frac{R_x - R_{Std}}{R_{Std}} \quad (‰) \qquad \text{Equation 1}$$

where R_x = isotope ratio of the sample, R_{Std} = isotope ratio of the standard.

3.2. Copper Isotopic Fractionation in the Natural Environment

The fractionation of Cu isotopes within environmental systems is an emerging area of research due to their potential to reveal information on the biogeochemical cycling of Cu. Copper in the environment shows larger isotopic variation than non-redox sensitive metals, such as Zn, with δ^{65}Cu values varying by up to 9‰ in soil samples (Larson *et al.*, 2003) and up to 3‰ in water samples (Fernandez & Borrok, 2009), primarily due to redox changes in the environment. A summary of naturally occurring Cu isotope fractionations reported in the literature are given in Figure 4 and are discussed throughout this section.

Primary Cu(I) minerals (the most common being chalcopyrite, $CuFeS_2$), from most locations, tend to have a narrow isotopic range of ±0.5‰ (Markl *et al.*, 2006). Many soils and sediments studied to date have had δ^{65}Cu values within the ±0.5‰ range (Marechal *et al.*, 1999; Bigalke *et al.*, 2010). Oxidation and reduction reactions are the source of the largest Cu isotope fractionation, with reduction products being strongly enriched in light isotopes and oxidation products being enriched in the heavier isotope

(Zhu *et al.*, 2002; Larson *et al.*, 2003; Ehrlich *et al.*, 2004; Asael *et al.*, 2007). Hence, large fractionations are usually only associated with significant weathering events that involve redox changes (Larson *et al.*, 2003). Markl *et al*. (2006) reported the post-oxidation remains of Cu(I) ores show light isotopic enrichment, with δ^{65}Cu values down to -2.92‰ (relative to NIST 976). Secondary Cu(II) minerals produced by oxidation are usually isotopically heavier and show high variability between -1‰ and +3‰, with most having values greater than +0.5‰, due to the preferential oxidation of ^{65}Cu (Markl *et al.*, 2006). In both laboratory and field studies, positive δ^{65}Cu values in solution have been attributed to Cu derived from the oxidative weathering of primary Cu(I) minerals. Laboratory studies have shown oxidative mineral dissolution favours heavy Cu by up to 3‰ as light isotopes preferentially remain in the reduced Cu(I) mineral (Mathur *et al.*, 2005). In the field, $\Delta^{65}Cu_{solution\text{-}mineral}$ values up to +1.6‰ have been observed (Kimball *et al.*, 2009). However, after dissolution, Cu can be further fractionated by the local solution chemistry, e.g. pH conditions, complexation or further redox changes.

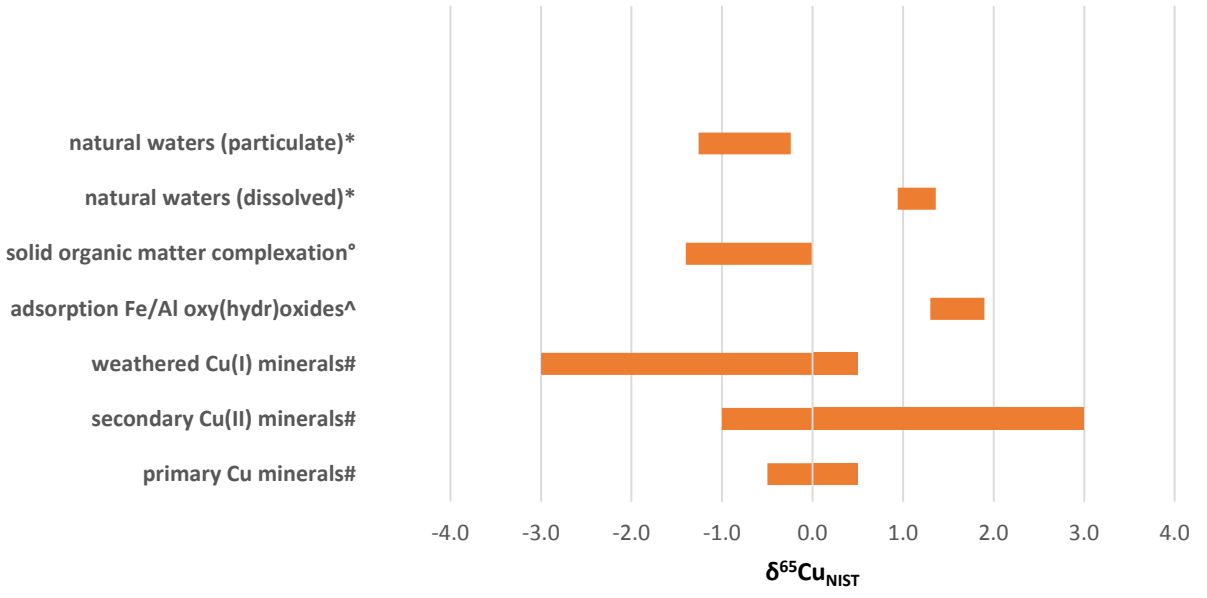

Figure 4 **Summary of the range of Cu isotope ratios, relative to the NIST 976 (δ^{65}Cu)** reported for various natural samples or processes. ⁑ Naverette *et al*. (2011); •Jouvin *et al*. (2012); ‡Weinstein *et al*. (2011); *Vance *et al*. (2008); °Bigalke *et al*. (2010b); †Pokrovsky *et al*. (2008); ^Ehrlich *et al*. (2004); #Markl *et al*. (2006).

Analysis of Cu isotope fractionation in soils has shown that Cu isotopic signatures are strongly influenced by soil properties, including Fe content, redox potential and pH. Bigalke *et al.* (2013) noted the δ^{65}Cu value varied systematically with depth between -0.02 to +0.16‰ down an intertidal soil profile, and Cu concentrations were related to Fe and nitrogen (N) contents. Seasonal fluctuations in redox potential were suggested as an explanation for the six-monthly cycle of oscillating isotope values. A decrease in redox potential stimulates the reductive dissolution of Fe(III) precipitates, which may also release co-precipitated Cu and stimulate Cu(II) reduction. A study of hydromorphic soils found greater δ^{65}Cu variability (up to 0.6‰ within an individual soil) and a correlation between Cu and Fe behaviour that was attributed to changing redox conditions (Bigalke *et al.*, 2010a). All soils studied to date have also suggested light isotope enrichment in the organic fractions which can be attributed to preferential uptake and cycling of light Cu in plant species or deposition of light enriched Cu from the atmosphere (Bigalke *et al.*, 2010a; Bigalke *et al.*, 2011). Soils that underwent oxic weathering showed a slight tendency toward lighter isotopes down the profile (Bigalke *et al.*, 2011), the opposite trend to that observed for hydromorphic and intertidal soils exposed to changing redox conditions (Bigalke *et al.*, 2013). The largest apparent isotope fractionating mechanism in soils under the influence of oxic weathering appeared to be from organic matter complexation and subsequent transport throughout the profile (Bigalke *et al.*, 2011) (Figure 5).

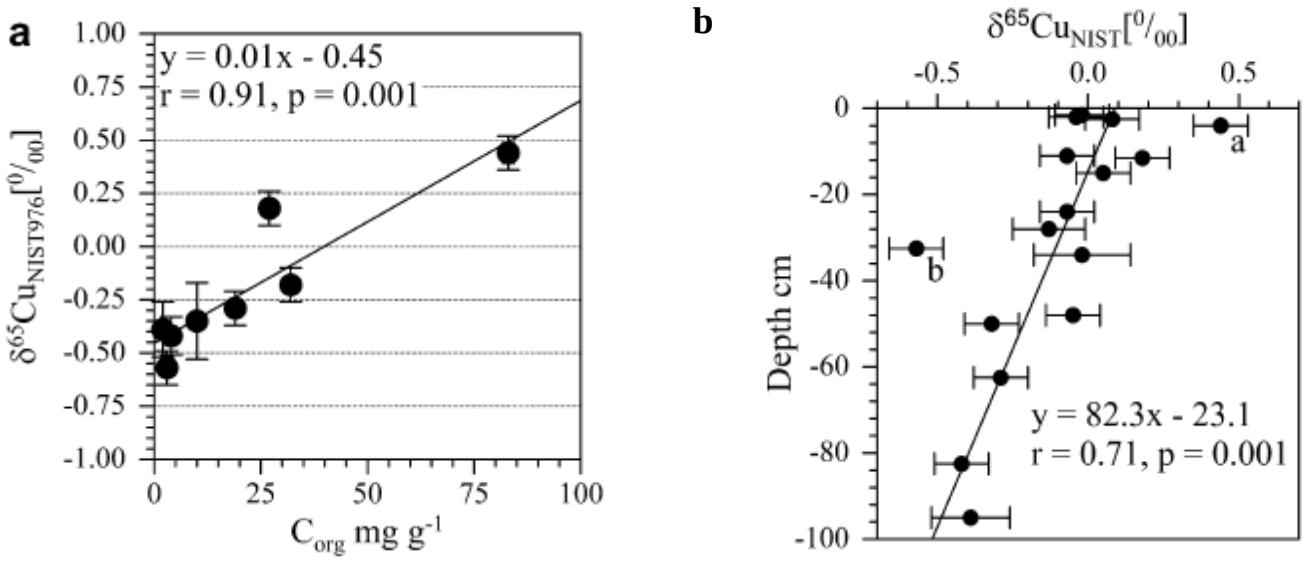

Figure 5. The relationship between organic carbon concentrat**ion and δ**^{65}Cu values of a Haplic Podzol soil (a), and the depth dis**tribution of δ**^{65}Cu values for a Skeleti Cambisol, Dystric Cambisol and two Haplic Podzol soils (b), as published by Bigalke *et al.* (2011).

Complexation and adsorption of Cu in the environment are key processes influencing the behaviour and fractionation of Cu isotopes. Ehrlich *et al.* (2004) found heavy isotope enrichment on the surfaces of Fe and Al oxy(hydr)oxides with $\Delta^{65}Cu_{(solid\text{-}solution)}$ = +0.6 to +1.3‰ ($\Delta^{65}Cu = \delta^{65}Cu_{solid} - \delta^{65}Cu_{solution}$), confirming previous work by Mathur *et al.* (2005) and Balistrieri *et al.* (2008), who also found heavy enrichment on metal hydroxide precipitates. This heavy enrichment is brought about by the greater thermodynamic stability of shorter ^{65}Cu – O bonds and the lower vibrational energies of the complex with the heavy isotope (Ehrlich *et al.*, 2004; Hoefs, 2004; Balistrieri *et al.*, 2008).

Understanding how organic matter complexation of Cu affects its isotopic signature is important given how readily Cu partitions to the organic phase. Bigalke *et al*. (2010) found that Cu complexation by insolubilised humic acid followed the previously established trend for Fe and Zn, with heavy isotope enrichment in the organic complex (Dideriksen *et al.*, 2008; Jouvin *et al.*, 2009; Morgan *et al.*, 2010). The isotope fractionation associated with adsorption of Cu to bacteria was found to be pH dependent,

with only weak fractionation between pH 4-8, but light enrichment at acidic pH values (1.8-3.3) with $\Delta^{65}Cu_{(bacteria\text{-}solution)}$ = - 0.9 to - 1.4‰ (Pokrovsky *et al.*, 2008). In most soils soluble organic complexes and solid phase adsorption sites will co-exist, and heavy Cu will partition to the strongest bonding environment, which is usually the dissolved organic complex, due to the formation of strong inner-sphere complexes. This was observed in the field by Vance *et al*. (2008) in a study of dissolved Cu in oceans and streams. Light Cu was found to partition to the particulate phase, while heavy Cu was held in soluble complexes. Fujii *et al*. (2013) used *ab initio* calculations to model equilibrium Cu complexation with sulphates and carbonates in solution, showing heavy Cu partitioned to the inorganic complexes. These authors suggested that this may be an explanation for the heavy Cu isotope enrichment found in oceanic and groundwaters in the absence of strong organic ligands.

3.3. Stable Isotopes as a Tool for Environmental Forensics

Stable isotopes provide a powerful tool in the realm of environmental forensics for assessing sources and behaviour of metals, as well as for more traditional stable isotope elements such as O, Cl and C. Once armed with an understanding of processes affecting metal isotope fractionation in the environment, it becomes possible to use the stable isotope ratios of a given metal to help fingerprint its source. When dealing with large scale contamination, it becomes increasingly important to be able to determine the source of the contamination for two key reasons: firstly, to prevent further environmental damage, and secondly, to assign responsibility to the correct parties, so they can fund and monitor the remediation of the damaged land.

The use of stable isotopes for contaminant source tracing of metals has been demonstrated and successfully exploited for lead (Pb) (Mukai *et al.*, 1993; Whitehead *et al.*, 1997; Gallon *et al.*, 2005). Lead isotopes have proven beneficial in this field due to the fact they do not undergo any measureable

biological, chemical or physical fractionation as they enter and interact with the environment (Gallon *et al.*, 2005; Couture *et al.*, 2010). However, with most metals, including Cu, post-environmental depositional fractionation does occur. Isotope fractionation involved in metal transport (such as diffusion and dissolution) and post-depositional processes (such as adsorption and biological uptake) remains a fundamental problem in the assessment of metal sources using this technique. If the fractionation and direction of these transport and post-depositional processes were quantified and understood, it would allow for more effective modelling and source analysis. Another essential requirement for determining contaminant sources is that the isotope ratio of sources should be distinctly different from that of the natural environment (Weiss *et al.*, 2008). There have been some successful applications of non-traditional stable isotopes, such as Cr, selenium (Se), cadmium (Cd) and Zn, to contaminant source tracing (e.g. Cloquet *et al.*, 2008; Mattielli *et al.,* 2008; Weiss *et al.,* 2008). Bullen *et al.* (2007) showed that Cr isotope ratios could effectively distinguish natural and anthropogenic sources of Cr(VI) contamination, using data collected from the Pacific Gas and Electric Compressor Facility in Hinkley, California.

To date, a limited number of studies have attempted to use Cu isotopes for environmental forensics. Copper isotopes are not typically used for tracing the spread of refinery contamination, as has been done for Zn, as Cu isotopes do not significantly fractionate during smelting (Bigalke *et al.,* 2010). Hence, contaminant Cu retains the isotopic signature of the parent ore minerals, which is often similar to the natural background isotopic signatures. Recently, stable isotopes have been used to assess Cu behaviour during transport through a wetland system. It was shown that heavy Cu was preferentially retained in the wetland, with $\Delta^{65}Cu_{retained\text{-}outlet}$ values up to +0.9‰, and that there was a significant correlation between particulate Cu and Al, and Fe concentrations at the outlet (Babcsányi *et al.*, 2014). This suggested that Cu was transported out of the wetland sorbed on mineral particles. Two recent studies looking at Cu isotope signatures in rivers proximal to vineyards have shown that the organic

particulate fractions strongly reflect the isotopic signature of the $CuSO_4$ fungicide applications ($\delta^{65}Cu$=-0.37‰), suggesting fungicide is strongly partitioning to the organic particulate phase (El Azzi *et al.*, 2013; Petit *et al.*, 2013). In both cases the total dissolved river Cu was shown to be enriched in heavy isotopes (El Azzi *et al.*, 2013; Petit *et al.*, 2013). The light enrichment in the organic particulate phase was attributed to preferential light Cu uptake by microorganisms and/or partitioning of $CuSO_4$ fungicides to the organic phase with no fractionation.

3.4. The Use of Stable Isotope Fractionation to Understand Metal Uptake Mechanisms

Isotope ratios may be useful in ascertaining the dominant form in which a particular metal has entered an organism and may shed light on how plants regulate the uptake of particular nutrients. Biological transport processes throughout the plant would be expected to be driven predominately by unidirectional reactions. A progressively lighter delta value would be expected as the metal is translocated from roots to shoots to reproductive tissues if it is driven by kinetically controlled unidirectional reactions. There are numerous potential metal uptake mechanisms employed by plants (see section 2.1), depending on the metal and the plant species in question. An attempt has been made to better understand plant uptake strategies, particularly for Fe and Zn, by studying metal isotope fractionation patterns (Arnold *et al.*, 2008; von Blanckenburg *et al.*, 2009). This was done by examining the isotope ratios of the metal in the soil and comparing them to the plant isotope ratios to see if fractionation had occurred. The extent of fractionation and whether the plant is enriched with the heavy or light isotope will depend on the nature of the plant uptake strategy, i.e. whether it is under kinetic (diffusion) or equilibrium control (complexation) (von Blanckenburg *et al.*, 2009). The results of Bigalke *et al.* (2010) suggest that surface organic layers contained isotope signatures that reflect the Cu isotopic composition of the plants growing upon it (i.e. light isotope enriched), rather than that present in the lower soil layers, likely due to the recycling of plant materials into the soil. Bigalke *et al*. (2010) found Cu fractionation in organic soil layers differed between soils that grew coniferous vegetation and

those that grew deciduous vegetation. This prompted the suggestion that fractionation of Cu might depend on plant anatomy.

There are limited studies looking at Cu fractionation during plant uptake, but results to date suggest a preferential light Cu uptake in most biological systems. Zhu *et al.* (2010) reported that the Cu accumulator *Elsholtzia splendens* had an overall lighter isotopic signature than the soil, but found that leaves were enriched in heavy isotopes compared to the stem by *ca.* 0.3‰, in contrast with Zn. Weinstein *et al.* (2011) reported monocot species with above-ground tissues enriched in light isotopes, relative to soil Cu, by up to -0.94‰, and the isotopic composition of the sampled leaves became progressively lighter with height. Most recently Jouvin *et al.* (2012) grew a series of plant species in solution culture, and showed significant enrichment in the light Cu isotope in the plants, relative to the growth medium. It was suggested that a reductive uptake mechanism was being employed by the plants to absorb Cu into their roots, resulting in the light isotope enrichment in plant tissues. A consistent result appears to be emerging that suggests plants preferentially take up isotopically light Cu.

There has been more study of Fe uptake in plants than Cu, and comparison may provide some explanations or the mechanisms driving the observed Cu isotope fractionations already reported in plants. Guelke *et al*. (2007) found a difference in Fe isotope ratios between Strategy I plants, which were enriched in light Fe isotopes, and Strategy II plants, that had only a very small enrichment in the heavy isotope. A later study using solution culture as the growth medium again showed light isotope enrichment in Strategy I plants (Guelke-Stelling & von Blanckenburg, 2012) (Figure 6). This was attributed to the fact that Strategy I plants release Fe reductases into the rhizosphere to reduce Fe(III) and then take up Fe(II). Reduction was also attributed to the significant light Cu isotope enrichment in

plants species grown by Jouvin *et al.* (2012). The minimal Fe isotope fractionation observed in Strategy II plants was attributed the fact that limited reductive uptake was occurring and complexation of Fe(III) by phytosiderophores was able to occur in these plants (Figure 6). Arnold *et al.* (2010) performed a similar study examining Zn uptake by rice (a Strategy II plant), and the results supported a similar uptake mechanism as reported for Fe in Strategy II plants by Guelke *et al*. (2007). The authors suggested that Zn associated with Fe oxides, both on root surfaces and in soil mineral phases, was enriched with heavy Zn, consistent with other findings of heavy enrichment in precipitates (Pokrovsky *et al.*, 2005; Jouvin *et al.*, 2009), and that this isotopic signature was being conserved during plant uptake via phytochelatin complexation. It was also concluded that the complex was not dissociated during uptake, as was found by previous authors using radioisotopes (von Wiren *et al.*, 1996), as this would be a kinetic process that would favour light isotope enrichment in plant tissues. While isotope fractionation data alone cannot definitively determine the mechanisms involved in plant nutrient absorption, they can provide vital starting information about the potential processes involved, e.g. whether there is a redox transformation involved or whether complexation is involved.

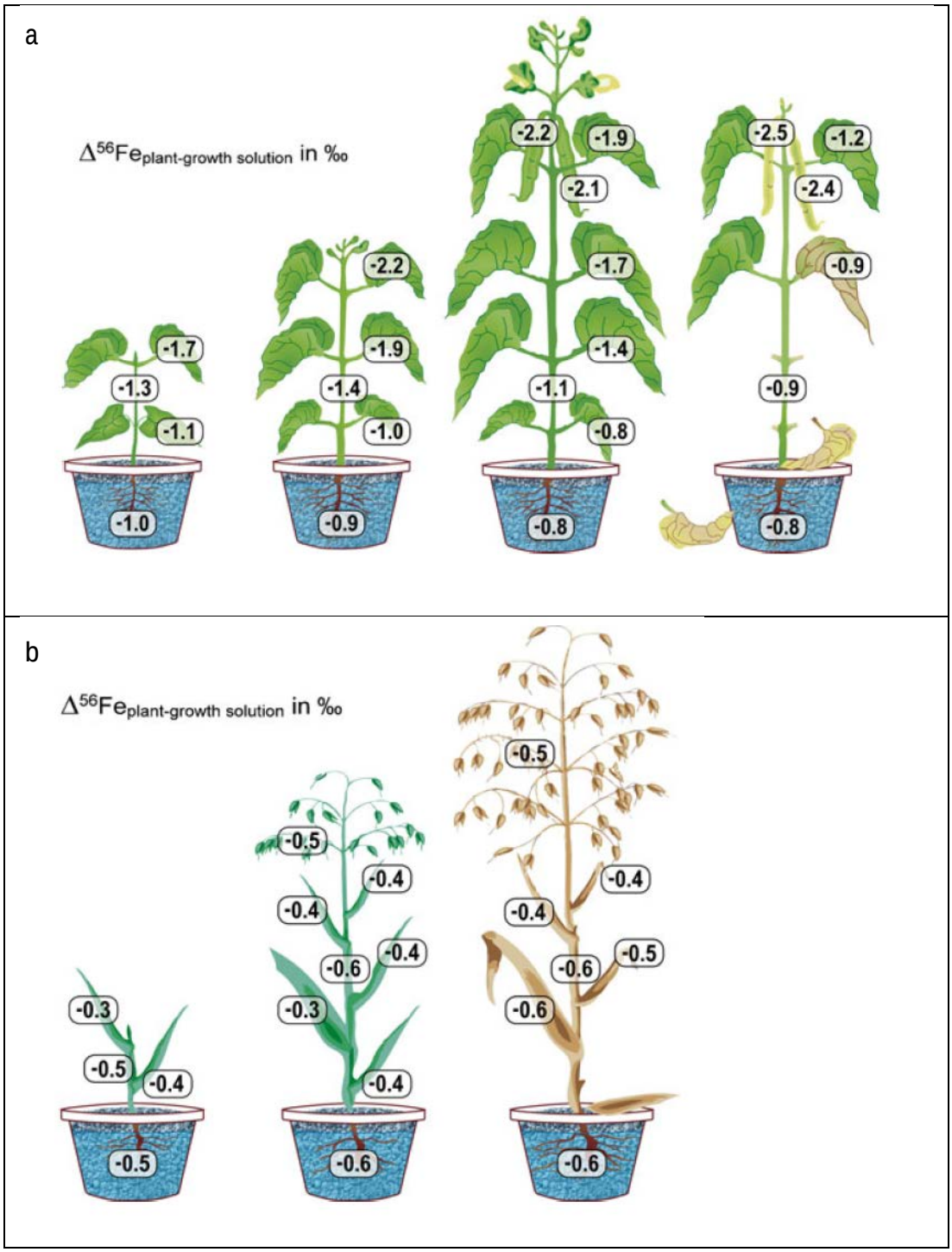

Figure 6 The isotopic fractionation of Fe during uptake and translocation in Strategy I bean plants (a) and Strategy II oat plants (b). Figure taken from Guelke-Stelling and von Blanckenburg (2012).

Assessments of Cu absorption into bacteria and microorganisms have been shown to induce varying degrees of Cu isotope fractionation. Copper uptake into azurin proteins expressed in *Escherichia coli*

and *Pseudomonas aeruginosa* was found to fractionate Cu isotopes between $\Delta^{65}Cu_{protein\text{-}solution}$=-1‰ to -1.5‰, suggesting light ^{63}Cu isotopes are preferentially incorporated into bacteria (Zhu *et al.*, 2002). Copper-Zn superoxide dismutase and Cu-metallothionein from yeast showed $\Delta^{65}Cu_{protein\text{-}solution}$ values between -1.2‰ and -1.7‰, respectively (Zhu *et al.*, 2002), again showing the biological preference for uptake of light isotopes. The larger fractionation involved with metallothionein may be explained by the presence of a reduction step. Copper was added to the growth medium as Cu^{2+}, however, metallothionein incorporates Cu^{+}. Navarrete *et al.* (2011) also found light enrichment of Cu associated with live bacterial cells, with $\Delta^{65}Cu_{solid\text{-}solution}$ up to -1.4‰ for gram negative bacteria and up to -2.6‰ for gram positive bacteria and intracellular incorporation by a natural consortia of bacteria showing $\Delta^{65}Cu_{solid\text{-}solution}$= *ca.* -1.0‰ to -4.4‰, indicating a very strong preference for bacteria to internalise light ^{63}Cu. However, heat-killed cells did not follow this trend, with $\Delta^{65}Cu_{solid\text{-}solution}$ values between -0.3‰ and +0.69‰, which suggested the live bacterial cells were actively absorbing light Cu, while the heat killed cells were adsorbing Cu to organic surface functional groups (Navarrete *et al.*, 2011). As discussed in Section 3.2, Pokrovsky *et al*. (2008) found bacteria exhibited pH dependent Cu isotope fractionation during cell adsorption. Mathur *et al*. (2005) and Kimball *et al.* (2009) suggested the reduction in the heavy isotope signature of aqueous Cu, after oxidative dissolution of CuS minerals, when microbes were added, was due to the preferential sorption of heavy ^{65}Cu by bacterial cells. This may have been due to Fe-(oxy)hydroxides precipitated on the surface of the cells, as adsorption on oxides favours the heavy isotope (Balistrieri *et al.*, 2008). These Cu fractionating mechanisms are summarised in Figure 7 and shows overwhelmingly that biological processes induce a light isotope enrichment in the product, relative to the Cu source.

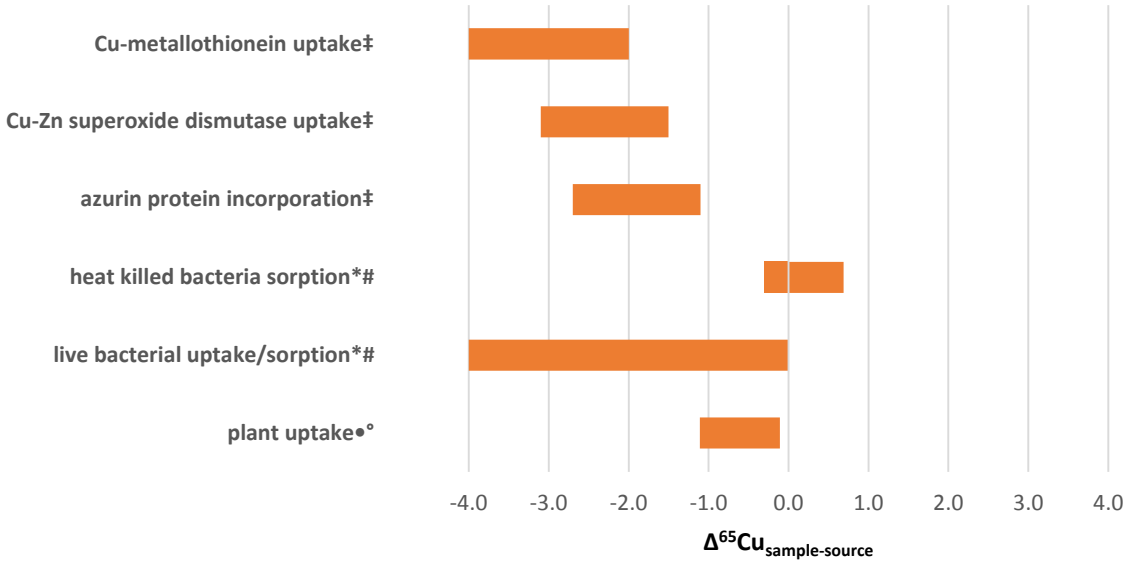

Figure 7 **Summary of reported biologically induced Cu isotope fractionations $\Delta^{65}Cu_{sample\text{-}source} = \delta^{65}Cu_{biological\ sample} - \delta^{65}Cu_{source\ Cu}$**. ‡Zhu *et al*. (2002); #Navarrete *et al*. (2011); *Pokrovsky *et al*. (2008); °Jouvin *et al*. (2012); •Weinstein *et al*. (2011).

Copper isotopes have been used to assess Cu transport up trophic chains, as well as for georeferencing. It was shown that the $\delta^{65}Cu$ values for bones of herbivores did not significantly differ to those of plants, but carnivore bones showed an enrichment in the heavy Cu isotope by up to 1‰, relative to herbivore bones and plants (Jaouen *et al.*, 2013b). A further application of Cu isotopes has been found when trying to distinguish the differences in sex and geographical location in humans/human remains. Copper isotopes have been successfully used to identify the sex of ancient human remains, as male blood and bones show a distinct enrichment in the heavy Cu isotope, relative to females (Jaouen *et al.*, 2012). Most recently, blood samples of a series of volunteers from a remote Russian community showed that enrichment in the light Cu isotope occurred with age (Jaouen *et al.*, 2013a). Furthermore, it was found that the Cu isotope signatures in the blood of these subjects was significantly enriched in the light isotope, relative to other European communities (Jaouen *et al.*, 2013a). This was suggested to be related to the increased metabolic rate associated with the cold stress experienced by this community resulting in an increased turnover of Cu through the body. It is

known that as Cu is processed throughout the body, different mammalian organs can fractionate Cu, with kidneys known to be particularly enriched in heavy Cu for reasons that are still unclear (Cabrera *et al.*, 2008; Jaouen *et al.*, 2013b).

While varying biologically-induced fractionations have been proposed in the literature, a general trend toward light isotopic enrichment seems to be emerging, at least in environmental samples. This may provide a tool for determining the role biological cycling of Cu plays in the environment. Biological uptake can potentially have a significant impact on the Cu isotope signature of the local environment, particularly in areas where the background soil Cu is from crustal sources, which tend to be isotopically unfractionated. In addition, given the essential nature of Cu to many forms of life, it may also provide a tool for understanding how to optimise Cu uptake for efficient micronutrient use.

4. COPPER ISOTOPES IN SOIL-PLANT SYSTEMS: A CONCEPTUAL MODEL

The analysis of Cu isotope ratios can be applied to understand sources, uptake mechanisms and translocation pathways in plants because of the fractionation that has been observed for Cu isotopes in environmental systems, through both biotic and abiotic pathways. In addition, an understanding of Cu isotope ratios in plants and soil solutions may provide an understanding of the exchangeable Cu pool, and hence availability, of Cu in soils, e.g., through long-term aging reactions, reduction in the soil (e.g. to Cu(I) and Cu(0)), complexation with organic matter and complexation with biological exudates. All of these processes are likely to induce a particular fractionation pattern that may be used to understand the uptake of Cu, and coupled with solid phase speciation analysis and modelling, may provide some evidence for a particular Cu uptake mechanism. With an understanding of how Cu is taken up by plants, better management practices can be implemented to enhance the uptake in Cu-deficient plants.

Alternatively, in environments where Cu is present at toxic levels, strategies could be implemented that help reduce Cu uptake.

Based on the literature, a conceptual model of predicted fractionation directions (in plants and soils) is presented in Figure 8. The isotope ratio of Cu present in the labile fraction will be dependent somewhat on the mineral weathering/dissolution processes that are involved in Cu^{2+} release; i.e. whether partial oxidative or reductive dissolution has occurred. However, in terms of the soil-plant system, the adsorption/desorption equilibrium of the labile Cu fraction will be more important. This will predominately involve desorption of Cu from solid phases, likely to be organic in nature (1), as previously discussed, with desorption favouring light Cu isotopes as heavy isotopes are preferentially retained in the strongest bonding environment. From here Cu^{2+} could become adsorbed to cation exchange sites in the cell wall (2). This adsorption could fractionate Cu in either direction depending on whether it is diffusion controlled (light isotope enrichment) and whether the root surface can offer a stronger bonding environment for Cu^{2+} than the soluble Cu-hexaaquo complex (heavy isotope enrichment. If Cu is absorbed as Cu^{+} then a reduction step, likely in the apoplasm, will occur prior to symplastic absorption. This will favour light isotope uptake as reduction products are enriched in the light isotope (3). Copper(II) may be absorbed straight to the symplasm through diffusion controlled mechanisms, likely to result in a light isotope enrichment in the symplasm due to diffusion being a kinetically controlled process (4). It is possible that Cu^{2+} could be complexed with soluble organic matter (SOM) in the soil solution (5), which would be expected to lead to heavy isotope enrichment in the complex if the complex offers a stronger bonding environment for Cu. The organically complexed Cu can then move to the apoplasm (6) where it may be dissociated and the free ion absorbed (7), with dissociation likely resulting in light isotope enrichment. Alternatively, it may be possible for the Cu-SOM complex to be absorbed directly into the symplasm, likely resulting in minimal fractionation or perhaps a small light enrichment caused by diffusion (8). Sorption of Cu^{2+} to Fe plaques on the root surface (9),

followed by reduction of Cu on these surfaces may occur in the soil environment (10). Adsorption of Cu^{2+} to Fe plaques will likely induce a heavy isotope enrichment on the plaque surface, due to the greater thermodynamic stability offered by the stronger bonding environment, but if subsequent reduction of Cu^{2+} occurs, this will likely lead to light isotope enrichment in the reduced products.

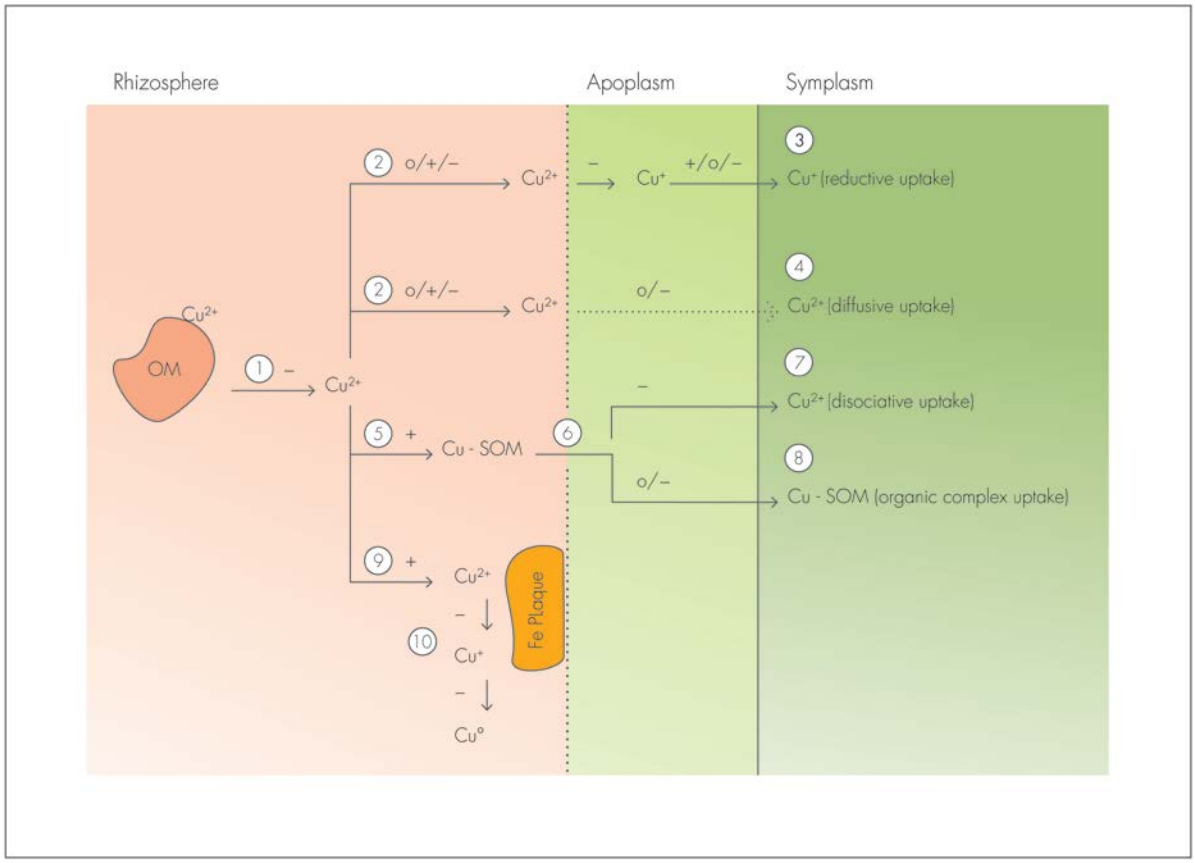

Figure 8. Potential Cu uptake mechanisms and movements in soil, and the hypothesised fractionation direction associated with each pathway. '+' = heavy isotope enrichment, '-' = light isotope enrichment, OM = organic matter and SOM = soluble organic matter.1 - desorption of Cu from the solid phase (shown here as organic matter); 2- cell wall adsorption of $Cu^{2+}$to cation exchange sites on the root cell wall; 3 reduction of Cu^{2+} to Cu^{+} in the apoplasm and subsequent uptake of the reduced species into the symplasm; 4 - diffusion of Cu^{2+}adsorbed on the cell wall into the symplasm; 5 - complexation of Cu^{2+} with SOM in the soil solution; 6 – diffusion of the Cu-SOM complex into the apoplasm, followed by either dissociation and free Cu^{2+} uptake (7) or uptake of the Cu-OM complex into the symplasm (8); 9 - sorption of Cu^{2+} to Fe plaques on the root surface followed by subsequent stepwise reduction to elemental Cu (10).

A hypothesised model of Cu fractionation during translocation in plant tissues is shown in Figure 9. It depicts two main translocation processes: a redox driven process where Cu is cycled through the plant through reduction and oxidation reactions (1), which inevitably lead to a lighter isotopic enrichment in the leaves, or, a complex controlled translocation (2), where the Cu isotope ratio may be conserved or

some slight heavy isotope enrichment may be observed at the leaves, given the slight preference of heavy isotopes to be used in complexation/equilibrium reactions.

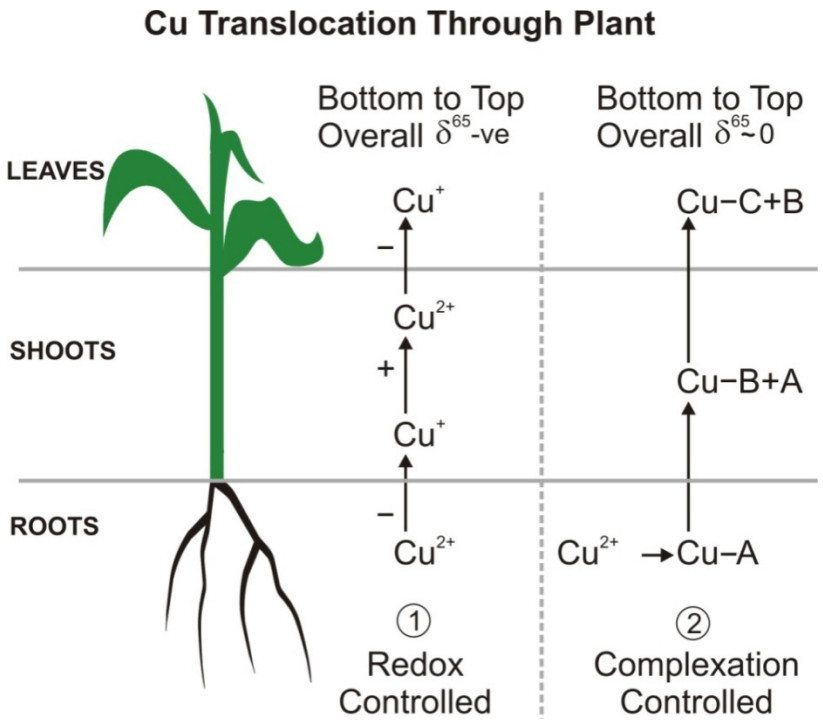

Figure 9. Two potential Cu translocation pathways to move Cu from roots to leaves (redox reactions (1) or complexation reactions (2)) and the predicted isotopic fractionation direction, where '-' indicates light isotope enrichment between source and sink tissues, while '+' indicates heavy isotope enrichment.. '-ve' = overall light enrichment, '0' = overall no isotope fractionation, calculated by subtracting the final Cu ratio in leaves from Cu ratio in roots.

5. CONCLUSIONS

Copper is a key micronutrient in soil-plant systems that has the potential to exert toxicity effects when present in excess. Copper behaviour is strongly dictated by the presence of organic matter, given Cu is able to form strong inner-sphere complexes with organics. In order to develop better management strategies to enhance or suppress Cu uptake, depending on the available soil Cu in production systems, a better understanding is required of the mechanisms employed by plants to absorb Cu. Stable isotopes have proven useful in this field for other heavy elements, i.e. Zn and Fe. Stable isotopes can provide *in-situ* information about Cu transformations and reactions. Recent literature has

suggested stable Cu isotopes can yield important information on the behaviour of Cu in soil-plant systems. Stable isotope techniques will be applied to examine the effect of Cu complexation with soluble organic matter, Cu adsorption to plant roots, and Cu uptake and translocation on the stable Cu isotope ratios of Cu. These findings will provide some key information on the overall biogeochemical cycling of Cu and its isotopes.

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CHAPTER 2

OPTIMISATION OF SAMPLE PREPARATION, COLUMN PURIFICATION AND ISOTOPE RATIO ANALYSIS PROCEDURES

1. INTRODUCTION

Traditional stable isotopes, such as oxygen (O), nitrogen (N) and carbon (C) have commonly been used for decades to source and track the behaviour of these elements in environmental systems (Meyers, 1997; Robinson, 2001). The use of stable isotopes to understand systems involving heavier elements (especially those that are > 40 atomic mass units) has been hampered by the difficulties associated with accurately and precisely determining the small isotope ratio variations observed in natural samples, as there is an inverse correlation between the magnitude of isotope fractionation and atomic mass (Weiss *et al.*, 2008). The development of multi collector – inductively coupled plasma – mass spectrometry (MC-ICP-MS) in the mid-1990s has provided a powerful tool that has enabled the detection of mass dependent isotopic variations down to <0.1‰ in environmental samples for elements from lithium to uranium (Yi *et al.*, 1998; Rehkamper & Mezger, 2000; Ehrlich *et al.*, 2001; Chu *et al.*, 2002). The high ionisation efficiency of the plasma inlet system makes MC-ICP-MS ideal for non-traditional stable isotopes that previously could not be measured due to their high ionisation potentials (Albarède & Beard, 2004).

The use of MC-ICP-MS for high precision measurements of Cu isotope fractionation requires careful sample preparation and purification to ensure that the measured isotope fractionation is inherent to the sample and not an artefact of the sample preparation or measurement method. Quantitative digestion of biological samples is important to ensure that Cu isotope fractionation is not induced due to incomplete digestion of tissues with different Cu isotope signatures. Separation and purification of the digested Cu from matrix elements is important to avoid isobaric and polyatomic interferences during isotope measurements of Cu and elements used for mass bias correction (i.e. nickel (Ni) and zinc (Zn)) during MC-ICP-MS analysis. The separation of Cu needs to be optimised for each sample matrix used, as the ion-exchange chemistry depends strongly on the presence of competing ions. The purification procedure must be optimised to achieve quantitative Cu recovery for mass balance purposes and to

avoid artificial isotope fractionation due to the separation process, as reported by Marechal and Albarede (2002).

The aim of this chapter is to document the steps taken to develop an optimised digestion, purification and MC-ICP-MS analysis procedure for the determination of accurate and precise Cu isotope ratios in plant tissues and the complex solutions examined in this thesis. The data collected using these optimised methods are presented and discussed throughout this thesis and include samples of plant tissues, hydroponic solutions and dialysis solutions containing high concentrations of matrix elements (e.g. calcium (Ca), strontium (Sr), sodium (Na) and potassium (K)).

2. PLANT DIGESTION

A digestion procedure was required that could quantitatively recover Cu from plant matrices in order to assess Cu isotope fractionation in hydroponically grown plants (Chapter 5). The isotope literature has suggested that concentrated solutions of hydrochloric acid (HCl), nitric acid (HNO_3) and hydrofluoric acid (HF) are required for the complete digestion of plant material for isotope analysis (Weiss *et al.*, 2005; Guelke & Von Blanckenburg, 2007). Hydrofluoric acid has been suggested as a requirement for strong acid digestions to ensure the complete breakdown of siliceous materials that may be present in plant tissues (Feng *et al.*, 1999; Dolgopolova *et al.*, 2006).

A series of experiments were performed to optimise a microwave digestion procedure for the complete digestion of plant tissues (roots, stems and leaves) and to determine if HF was necessary for the quantitative recovery of Cu from tissues. To do this two plant standard reference materials (SRMs), tomato leaves (NIST 1573a, 4.7 mg Cu kg^{-1}) and apple leaves (NIST 1515, 5.64 mg Cu kg^{-1}), were digested using three procedures combining strong acids with microwave heating to determine their Cu recovery efficiency for accurate analysis of Cu isotope ratios.

2.1. Procedure 1

In a Class 100 clean laboratory, *ca.* 0.2 g of the freeze-dried SRM materials (n=3-5) were weighed into 55 mL polytetrafluoroethylene (PTFE) digestion vessels with 6 mL of HNO_3 and 2 mL 30% H_2O_2 and cold digested for 90 mins. After cold digestion, 0.8 mL of 50% HF was added into each digestion vessel and the vessel capped. The samples were microwave digested (MARSXpress, CEM) using a temperature program adapted from Dolgopolova *et al.* (2006) (Table 1). The digest solutions were transferred to 25 ml PTFE vials and evaporated to dryness on a hotplate at *ca.* 140°C. The dried

samples were resuspended in 2 mL of 7M HCl and total Cu concentrations determined by inductively coupled plasma- mass spectrometry ICP-MS (Agilent 7700).

Table 1. Temperature and time program for microwave digestion used for digestion procedures 1 and 2.

Stage	Temperature	Time
1	90°C	10 mins
2	120°C	10 mins
3	160°C	10 mins
4	180°C	60 mins

The recovery of Cu from SRMs using Procedure 1 was found to be < 65% for both plant SRMs. Tomato leaves (NIST 1573a) showed a recovery of 59±4% (n=5) and apple leaves (NIST 1515) had a recovery of 65±2% (n=3). It was observed that during cold digestion using this procedure, the plant SRMs oxidised rapidly after the addition of HNO_3 and H_2O_2 causing small amounts of material to be spattered due to the rigorous reaction, and this may have resulted in sample loss from the open digestion vessels leading to low Cu recoveries.

1.1. Procedure 2

A two-stage digestion procedure (Procedure 2) was developed to decrease the potential erratic loss of material during the initial cold digestion stage of Procedure 1. Freeze-dried tomato and apple leaf SRMs (n=6-8) were cold digested for 10 hrs in less acid and peroxide than Procedure 1, with 3 mL of HNO_3 and 1 mL of 30% H_2O_2 added to 0.2g of SRM material in PTFE vials with caps placed loosely on top of each vial to prevent the loss of material during the rapid oxidation stage. The caps were then secured on the vials and refluxed on a hot plate for 2 hr at *ca.* 140°C. The vials were then uncapped and evaporated to dryness at *ca.* 140°C. Samples were resuspended in 2 mL of HNO_3 and transferred to 55 mL PTFE microwave digestion vessels with an additional 4 mL of HNO_3, 2 mL of 30% H_2O_2 and

0.8 mL of 50% HF. The samples were then microwave digested (MARSXpress, CEM) using the same temperature and time program as outlined in Table 1. The digest solutions were transferred to 25 mL PTFE vials and evaporated to dryness on a hotplate at *ca.* 140ºC. The dried samples were resuspended in 2 mL of 7M HCl and total Cu concentrations determined by ICP-MS.

The Cu recoveries were significantly improved to 70-80% using Procedure 2. However, quantitative recovery of Cu from plant SRMs was not achieved using this method and large sample variability was found between replicates, with tomato leaves having recoveries of 70±16% (n=8) and apple leaves having recoveries of 79±15% (n=6). This low Cu recovery and high variability for Procedure 2 may be due to the co-precipitation of Cu with fluoride minerals (e.g. CaF) in samples due to the use of HF (Feng *et al.*, 1999; Aldaco *et al.*, 2007).

2.3 Procedure 3

In order to simplify the digestion procedure and remove the need for hazardous HF that requires specialised laboratories and has unique disposal requirements, a digestion procedure without HF was also examined. Procedure 3 was nearly identical to Procedure 2 but did not use HF. Also, in this procedure after cold digestion the 2 mL of resuspended sample was transferred to a 50 mL PTFE microwave digestion vessel (Ethos E, Milestone) with an additional 5 mL of HNO_3 and 3 mL of 30% H_2O_2. The samples were microwave digested using a modified US EPA 3052 method (1996) (Table 2).

Procedure 3 was found to yield quantitative Cu digest recoveries. The removal of HF from the digestion process led to tomato leaf SRM recoveries of 104±3% and apple leaf SRM recoveries of 103±4%.

Table 2. Temperature and time program for microwave digestion using Procedure 3.

Stage	Temperature	Time
1	Ramp to 100°C	25 mins
2	Ramp to 150°C	25 mins
3	Ramp to 180°C	25 mins
4	Hold at 180°C	15 mins

2.4 Summary

Copper was found to be quantitatively recovered from tomato leaf (NIST 1573a) and apple leaf (NIST 1515) SRMs using HNO_3 and H_2O_2 without HF (Procedure 3). Plant material was cold digested in HNO_3 and H_2O_2, followed by a hotplate reflux for 2 hours before drying down and redissolving in 2 mL of HNO_3. This pre-digested sample was then microwave digested with additional HNO_3 and H_2O_2. Digestion Procedure 3 was selected as the optimum procedure for the digestion of plant tissues in this thesis to ensure quantitative Cu recovery and avoid inducing Cu isotope fractionation during digestion.

3. COPPER COLUMN PURIFICATION

Copper can be efficiently separated from matrix elements in environmental samples using strong anion exchange resins such as AG-MP-1 (BioRad) (Marechal *et al.*, 1999). This purification method requires that the digested sample be dissolved in 7M HCl before loading onto the resin column. Dissolving the sample in high molarity HCl allows formation of anionic metal chloride complexes for those metals capable of forming such species (i.e. Cu, Fe and Zn) (Slaveykova & Wilkinson, 2002). The strength of the negative charge on the anionic metal chloride complex changes as the acid molarity changes, and this dictates the affinity of the complex to anion exchange resin, and as such, its elution profile from the column. Alkali metals, such as Ca, K, Mg, and Na do not form anionic chloride species, hence, they remain cationic and elute from the column without retention. Kraus and Moore (1953) showed that even in high molarity HCl conditions Ni does not form anionic chlorides, and hence, Ni is eluted from columns in the first few millilitres of matrix flush. Copper, Fe and Zn all form strong anionic chloride species (e.g. $CuCl_4^{2-}$) in high molarity HCl, hence, these metals bind strongly to the anion exchange column (e.g. Figures 1-3). As the molarity of HCl is lowered, the negative charge on the Cu-chloride complex decreases as the number of chloride ions bound to the central metal ion changes, as depicted in Figure 1. When the molarity of HCl is lowered from 10M to 5M, Cu speciation changes from being dominated by $CuCl_4^{2-}$ and $CuCl_3^-$ to predominantly $CuCl_2^0$ and $CuCl^+$ species. This change in Cu speciation with HCl molarity can be used to efficiently separate Cu from Fe and Zn on an AG-MP-1 column, as Fe and Zn remain predominantly as anionic species in 5M HCl (Figures 2 and 3). Zinc forms such strong anionic chloride species in HCl that it does not change speciation toward neutral/cationic species until the concentration of HCl is <1M (Figure 3).

Isotope standards of Ni and Zn are often used as dopants for mass bias correction during MC-ICP-MS analysis of Cu due to their similar mass and first ionisation potentials, meaning they require similar

amounts of energy to become ionised in the plasma (e.g. Bigalke *et al.,* 2010; Ehlrich *et al.,* 2004; Li *et al.,* 2009). For this reason the complete removal of Ni and Zn from the Cu fraction in purification columns is essential to ensure that the dopant Ni or Zn isotope standard is not affected by Ni or Zn from the sample matrix or from contamination (e.g. present in acids, vials, tubing, etc) (see Section 4 for additional details).

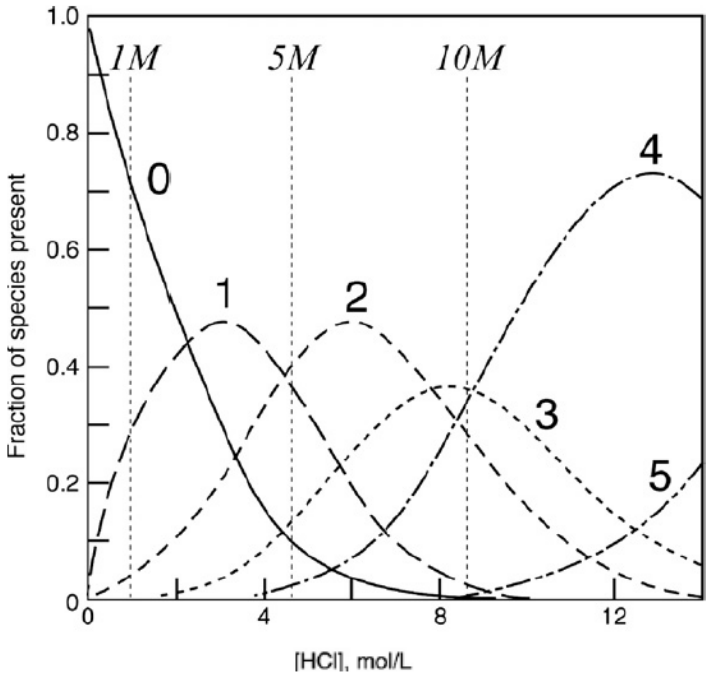

Figure 1. Copper speciation in solutions with increasing HCl molarity. Numbers next to each line denote the number of Cl atoms bound to a central Cu(II) ion. Figure from Borrok *et al.*(2007).

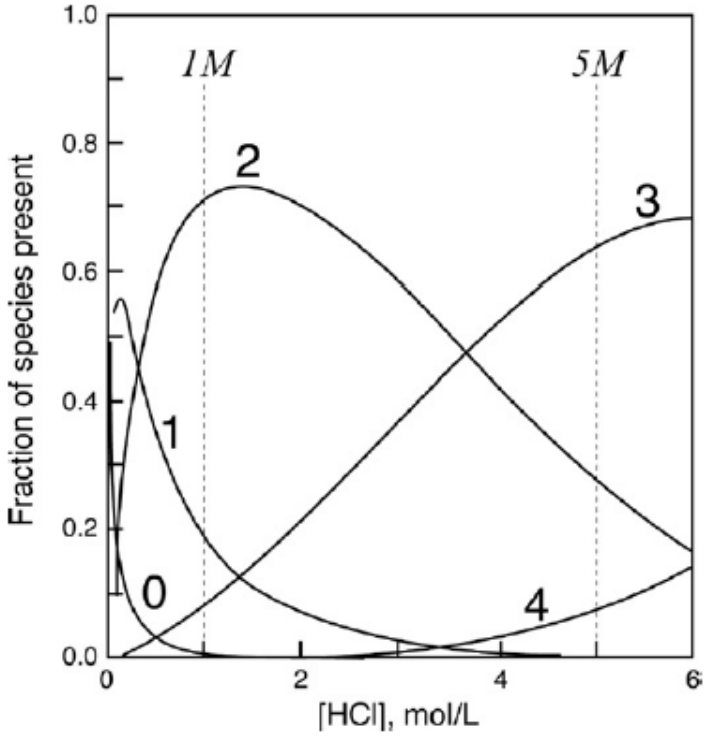

Figure 2. Iron speciation in solutions with increasing HCl molarity. Numbers next to each line denote the number of Cl atoms bound to a central Fe(III) ion. Figure from Borrok *et al.* (2007.

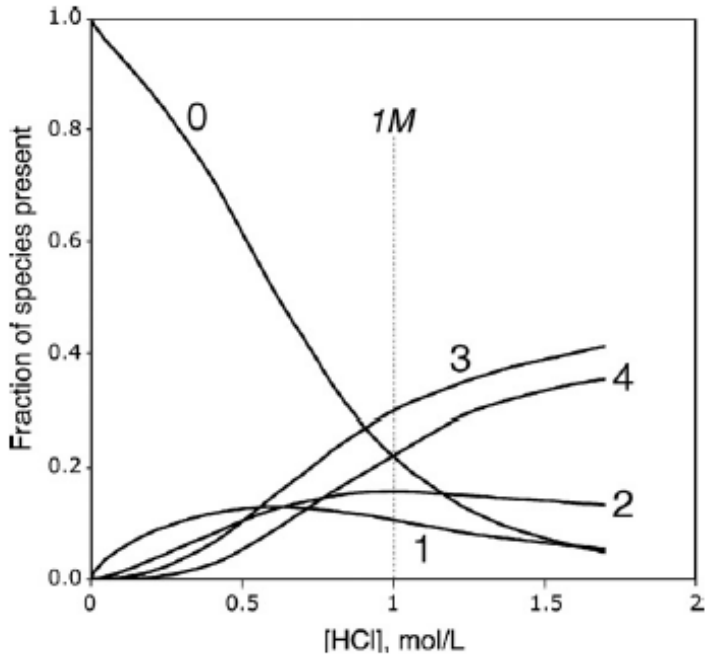

Figure 3. Zinc speciation in solutions with increasing HCl molarity. Numbers next to each line denote the number of Cl atoms bound to a central Zn(II) ion. Figure from Borrok *et al.* (2007

The elution profile of Cu from columns can change slightly depending on the presence of competing elements in the sample matrix. Chapman *et al.* (2006) previously demonstrated that the sample matrix can affect the elution of matrix ions and Cu fractions from the AG-MP-1 anion exchange column (Table 3). The required volume of acid to elute matrix elements and Cu needs to be examined for every new sample matrix to ensure Cu is purified from potential interfering ions and to prevent loss of Cu due to early breakthrough. It is essential that Cu is quantitatively recovered from the columns as the heavy isotope of Cu (^{65}Cu) elutes slightly earlier than the light (^{63}Cu) isotope (Marechal & Albarede, 2002). Thus, if all Cu is not recovered from the column, any isotope fractionation measured in samples cannot be attributed solely to the sample, as it may have been artificially fractionated due to a low Cu recovery from the column. A series of experiments were performed to optimise the AG-MP-1 resin column method for the purification and pre-concentration of Cu in plant digests and the complex solutions generated throughout this thesis.

Table 3. Copper breakthrough volumes for various reference materials and sample matrices using 1.6 mL of AG-MP-1 resin with an 80 mm bed height in glass columns. Taken directly from Chapman *et al.* (2006).

Reference Material	Description	Volume of acid eluted before Cu release	Volume of acid to remove the >99% of matrix (mL)
BCR 32	Phosphate	2	4
SGR-1	Shale	4	4
BCR 143	Over fertilised soil	5	3
NIST SRM 1648	Urbane particulate	6	3
NIST SRM 2710	Soil	6	4
HRM 2	Mixed soil	7	4
GXR-1	Iron rich soil	7	4
BCR 38	Fly ash	7	3
CP-1	Copper porphyry	7	3
NIST SRM 2709	Soil	8	4
NIST SRM 1632a	Coal	9	3

1.1. Column Purification Procedure 1

An initial study was performed using a solution of inorganic ions commonly found in plant matrices (hereafter termed artificial plant matrix) to assess the efficacy of the Marechal *et al.* (1999) separation method(Table 4) to isolate Cu from other matrix ions likely to occur in my plant digests. The makeup of the artificial plant matrix can be found in Table 5 and is based on the approximate concentrations of elements in the NIST 1573a tomato leaves. The columns were prepared by wetting AG-MP-1 (100-200 mesh) resin (BioRad) with ultrapure deionised water (Millipore) to make a slurry. The resin slurry was then added to acid washed BioRad Poly-Prep columns to give *ca.* 1.6 mL of resin. The resin was washed with 5 mL of 0.5M HNO_3 followed by 5 mL of ultrapure deionised water three times and was then conditioned for sample loading with 7 mL of 7M HCl. One millilitre of the artificial plant matrix solution in 7M HCl (n=3) was added to the column. The 7M HCl was added to the column in 1 mL aliquots and each millilitre was collected as it was eluted off the column and analysed for Cu using ICP-MS and matrix elements using ICP-OES (Perkin-Elmer) (Table 5). Copper was found to elute from the column after the addition of 6 mL 7M HCl (Table 5). The majority of matrix ions were eluted in the first 6

mL of 7M HCl addition followed by the Cu fraction in the following 35 mL. The Fe fraction eluted after Cu using 10 mL of 2 M HCl and Zn using 10 ml of 0.5 M HNO_3. All HNO_3 and HCl used in this study were Teflon distilled using Savillex sub-boiling apparatus (DST-100). The HCl used for column purification was double-distilled in a Teflon elbow joint under a heat lamp.

Table 4. Column separation procedure outlined by Marechal et al. (1999) used for Cu purification from plant matrices.

Fraction	Eluant	Volume
Sample Load	7M HCl	1mL
Matrix + Ni	7M HCl	6mL
Cu Fraction	7M HCl	35mL
Fe fraction	2M HCl	10mL
Zn fraction	0.5M HNO_3	10mL

Table 5. An example of Cu separation from other matrix elements in 1 mL of an artificial plant matrix solution loaded onto an AG-MP-1 resin column and purified using the method of Marechal, *et al.* (1999).

Sample Name: *Artificial Plant Matrix*			Digest Totals									
				Cu	Ca	Mg	Na	S	Al	Fe	Mn	P	Zn
Fraction	Eluant	Volume (mL)	Total Added	7.15 ng	708 µg	367 µg	125 µg	184 µg	585 µg	306 µg	222 µg	174 µg	36.0 µg
Sample Loading	7M HCl	1		<0.1	87.68	47.94	17.68	9.48	79.94	<0.10	0.85	<0.10	<0.05
Matrix	7M HCl	1		<0.1	487.58	254.32	88.75	100.88	409.19	<0.10	93.05	44.27	<0.05
Matrix	7M HCl	1		<0.1	124.73	65.08	21.02	76.04	89.55	<0.10	102.63	87.00	<0.05
Matrix	7M HCl	1		<0.1	9.45	4.86	2.47	11.00	5.99	<0.10	21.45	34.52	<0.05
Matrix	7M HCl	1		<0.1	<0.10	<0.10	1.04	3.13	<0.05	<0.10	3.82	5.95	<0.05
Matrix	7M HCl	1		<0.1	<0.10	<0.10	<0.10	1.33	<0.05	<0.10	0.61	<0.10	<0.05
Matrix	7M HCl	1		<0.1	<0.10	<0.10	<0.10	1.29	<0.05	<0.10	<0.05	<0.10	<0.05
Cu	7M HCl	1		20	<0.10	<0.10	<0.10	<0.10	<0.05	<0.10	<0.05	<0.10	<0.05
Cu	7M HCl	1		80	<0.10	<0.10	<0.10	<0.10	<0.05	<0.10	<0.05	<0.10	<0.05
Cu	7M HCl	1		200	<0.10	<0.10	<0.10	<0.10	<0.05	<0.10	<0.05	<0.10	<0.05
Cu	7M HCl	1		380	<0.10	<0.10	<0.10	<0.10	<0.05	<0.10	<0.05	<0.10	<0.05
Cu	7M HCl	1		550	<0.10	<0.10	<0.10	<0.10	<0.05	<0.10	<0.05	<0.10	<0.05
Cu	7M HCl	1		640	<0.10	<0.10	<0.10	<0.10	<0.05	<0.10	<0.05	<0.10	<0.05
Cu	7M HCl	1		750	<0.10	<0.10	<0.10	<0.10	<0.05	<0.10	<0.05	<0.10	<0.05
Cu	7M HCl	1		770	<0.10	<0.10	<0.10	<0.10	<0.05	<0.10	<0.05	<0.10	<0.05
Cu	7M HCl	1		750	<0.10	<0.10	<0.10	<0.10	<0.05	<0.10	<0.05	<0.10	<0.05
Cu	7M HCl	5		2500	<0.10	<0.10	<0.10	<0.10	<0.05	<0.10	<0.05	<0.10	<0.05
Cu	7M HCl	5		500	<0.10	<0.10	<0.10	<0.10	<0.05	<0.10	<0.05	<0.10	<0.05
Cu	7M HCl	5		50	<0.10	<0.10	<0.10	<0.10	<0.05	<0.10	<0.05	<0.10	<0.05
Cu	7M HCl	5		<0.1	<0.10	<0.10	<0.10	<0.10	<0.05	<0.10	<0.05	<0.10	<0.05
Cu	7M HCl	5		<0.1	<0.10	<0.10	<0.10	<0.10	<0.05	<0.10	<0.05	<0.10	<0.05
Fe	2M HCl	5		<0.1	<0.10	<0.10	<0.10	<0.10	<0.05	138.15	<0.05	<0.10	<0.05
Fe	2M HCl	5		<0.1	<0.10	<0.10	<0.10	<0.10	<0.05	128.85	<0.05	<0.10	<0.05
Zn	0.5M HNO_3	5		<0.1	<0.10	<0.10	<0.10	<0.10	<0.05	21.15	<0.05	<0.10	30.61
Zn	0.5M HNO_3	5		<0.1	<0.10	<0.10	<0.10	<0.10	<0.05	<0.10	<0.05	<0.10	3.06
	Total			7170	709.44	372.20	130.96	203.15	584.67	288.15	222.41	171.74	33.67
	% recovered from column			*100.2*	*100.1*	*101.3*	*104.6*	*110.5*	*99.9*	*94.2*	*100.0*	*98.7*	*94.0*

Copper could be quantitatively separated from matrix ions likely to be present in plant tissues, using the anion exchange procedure published by Marechal *et al.* (1999) (Table 5). The column procedure was further examined using real plant samples of SRM 1573a (tomato leaves digested using Procedure 3 – Section 2.3) to determine what effect an organically derived matrix had on the elution efficiency and recovery of Cu from the resin columns. In real plant samples, an extra 1 mL of 7M HCl (total 7 ml) was found to be required to flush matrix elements from the column before quantitative recovery of the Cu fraction (Figure 4). The elution profile of elements from the resin column showed quantitative recovery of Cu and good separation of the Cu fraction from matrix elements (Figure 4) with an average column Cu recovery of 102±5% (n=6). Isotope analysis of purified NIST 1573a digests showed that Cu column recoveries within 100±10% did not affect the accuracy or precision of isotope ratio analysis (δ^{65}Cu =0.63 ± 0.12‰, mean ± 2σ) (Figure 5). Subsequent analyses throughout this thesis confirmed that column recoveries within 100±10% did not cause notable artificial isotope fractionation.

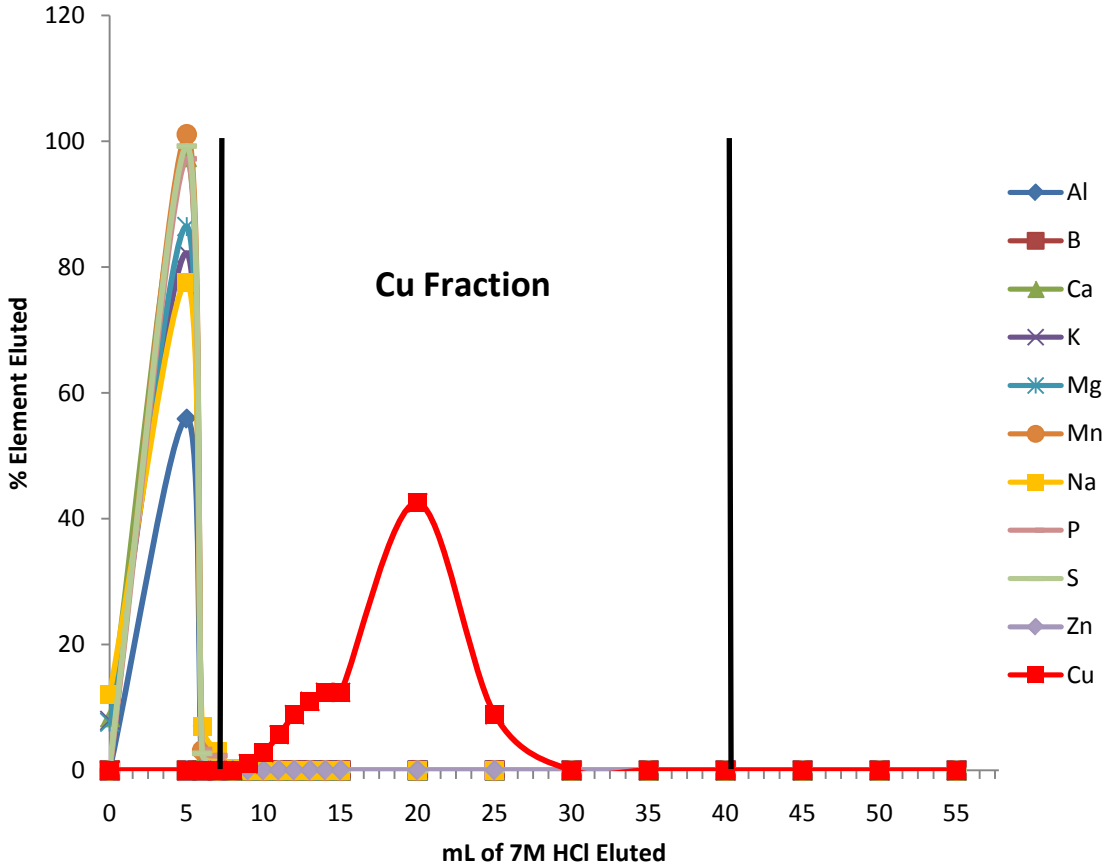

Figure 4. Digested NIST 1573a SRM elution profile from AG-MP-1 resin using the method of Marechal *et al*. (1999).

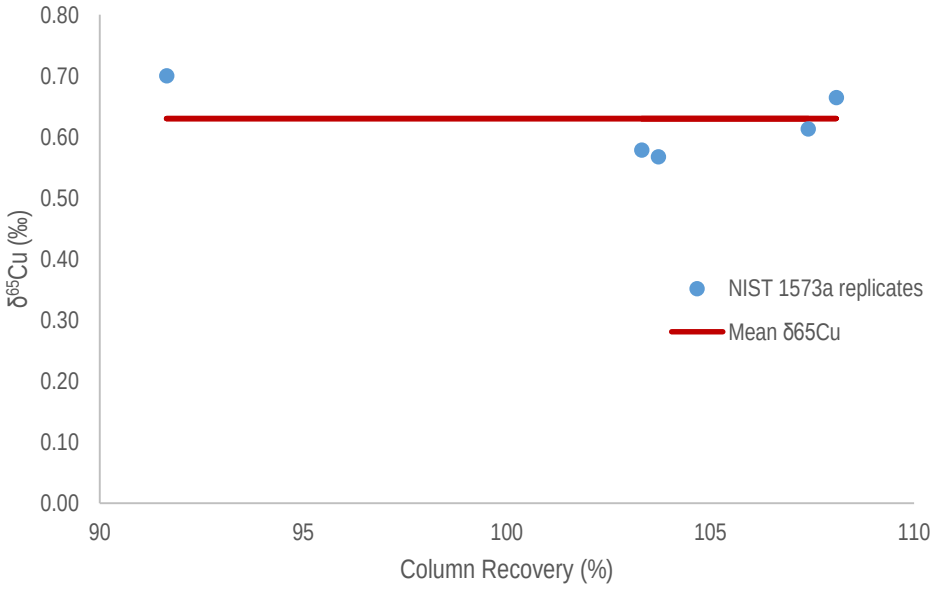

Figure 5. The column recovery and δ^{65}Cu values for 5 NIST 1573a digested replicates.

A final examination of the sample preparation method for the purification of Cu in plant tissues was undertaken using tomato and oat plants grown in a hydroponic medium (the plants used in the uptake experiments presented in Chapter 5). Under hydroponic conditions, plants can access excessive amounts of nutrients, as that they are supplied in a highly bioavailable form. Hence, plants can take up and accumulate very high concentrations of matrix elements, e.g. Ca, K, and Na, that can make Cu purification from these elements difficult (Table 3). Hydroponically grown tomato and oat plants were digested using the optimised microwave method (Procedure 3 – see Section 2.3) and column purified using the procedure published by Marechal *et al.* (1999) (Table 4). It was found that one column purification was not sufficient to separate matrix ions from the Cu fraction (Table 6). This finding was similar to that previously reported by Bigalke *et al.* (2010) for Cu in soil matrices. A second column purification was found to reduce matrix elements in samples to below 2 mg L^{-1} (the threshold concentration determined for interfering matrix elements to avoid isotopic interferences during MC-ICP-MS Cu measurements; see Section 4.3). Digested stem samples were found to require a third column

purification to remove residual Ca and Na from the Cu fraction. It was possible to keep the total Cu recoveries from all 3 column purifications (n=6) at 100±10% for the whole purification process. Matrix elements were hereby reduced to < 2 mg L^{-1} in the final resuspended solutions for MC-ICP-MS analysis.

Table 6. Matrix elements present in the Cu fraction of hydroponically grown tomato and oat plant tissues after a single AG-MP-1 resin column purification following the method of Marechal *et al.* (1999).

Hydroponic Plant Species/Tissues	Sample Concentrations						
	Cu % Recovery	Ca mg/L	K mg/L	Mg mg/L	Mn mg/L	P mg/L	S mg/L
Tomato roots	103.9	16.86	90.66	6.98	2.34	28.40	13.71
Tomato stems	102.1	17.13	66.16	3.65	<0.05	11.49	2.27
Tomato leaves	94.2	25.92	18.79	3.53	<0.05	16.77	9.70
Oat roots	103.4	2.62	40.38	3.50	<0.05	14.29	3.68
Oat stems	97.8	2.60	61.48	<0.1	<0.05	14.56	<0.1
Oat leaves	96.5	7.92	91.60	<0.1	<0.05	23.06	9.29

The column purification procedure elutes Cu in 35 mL of 7M HCl, which is then dried down and the residue redissolved in 1.5 mL 2% HNO_3 for Cu isotope ratio analysis (i.e. a concentration factor of 23.3). Concentrating such a large volume of 7M HCl could lead to a significant concentration of trace level contaminants present in the 7M HCl leading to problematic contamination in the sample (i.e. the blank contribution could become significant). The contamination of samples during digestion and column purification with Cu (as well as Ca, Na, Ni, and Ti – see Section 4) can significantly affect the accuracy of the Cu isotope ratios determined. To avoid this problem, double distilled HCl and single distilled HNO_3 were used throughout the sample preparation and column purification procedures in a Class 100 clean room. In order to confirm that the blank was not adversely affecting the measured isotope ratio of Cu in samples, procedural blanks from the triple column purification procedure were determined (n=3). The average amount of Cu in the blank solutions after 3 column purifications was

determined to be 20 ng (±10 ng) Cu. Comparing the blank Cu concentration to the Cu concentrations in the digested plant tissue samples, which were typically >1 mg for hydroponically grown plants, this represents *ca*. 0.002% of the sample Cu, which is insignificant. Nickel was found to be below the detection limits of ICP-MS analysis in the Cu fraction (0.1 ng mL^{-1}; < 0.2 ng in sample) and hence would not significantly contribute to the Ni isotope signal during MC-ICP-MS analysis (*ca.* < 0.02%).

3.2. Column Purification Procedure 2

In this thesis, experiments were undertaken to examine the effect of Cu complexation by organic ligands using Donnan dialysis (Chapter 3) and Cu adsorption onto plant roots (Chapter 4) on Cu isotope fractionation. These experiments resulted in sample matrices different to those of the digested hydroponic plant tissues used to optimise the Cu column purification procedure. These different sample matrices contained high concentrations (*ca.* 1 mM) of matrix ions such as Ca, K, Na and Sr. The column purification procedure was further assessed in the presence of these complex sample matrices to ensure Cu was quantitatively recovered from columns and separated from matrix ions. It was again found that 2-3 column purifications were required to purify Cu from these matrices using the optimised Marechal *et al.* (1999) column procedure (Table 4). However, the lower Cu concentration in the final solutions of the complexation and adsorption studies (200 - 400 μg Cu L^{-1}) meant that the procedural blank for Cu of 20 ng would now represent 5 – 10% of the total Cu in samples. This Cu contribution from the blank in the sample is significant and does not allow for reliable, accurate isotope ratios to be determined by MC-ICP-MS.

A new purification procedure based on that published by Borrok *et al.* (2007) was developed for the purification of Cu in solutions from the complexation (Chapter 3) and root adsorption (Chapter 4) studies. Borrok *et al*. (2007) developed a method of Cu purification that requires significantly lower

volumes of HCl, with the sample dissolved in 1 mL of 10M HCl and loaded onto the column, then the matrix removed with 4 mL 10M HCl and the sample collected in 6 mL of 5M HCl using the AG-MP-1 strong anion exchange resin (Table 7). The efficacy of this method for quantitative separation of Cu was assessed using solutions generated during the Donnan dialysis and root sorption studies (Figure 6 and Figure 7). The Donnan dialysis solution was made from a dialysis acceptor solution (0.3 mL) containing 1mM $Sr(NO_3)_2$ and 15µM Cu that had been dried down and diluted to 1 mL in 10M HCl (see Chapter 3). The root sorption solution was prepared by drying down 30 mL of a solution of a 50 mL ethylenediaminetetraacetic acid (EDTA) root wash solution that had been exposed to hydroponically grown oat plant roots to desorb apoplastic metals, and redissolving the solution in 1.5 mL of 10M HCl. After a 3 mL matrix flush using 10M HCl, Cu was eluted in 7 ml of 5M HCl (Table 7). This meant that when Cu was resuspended in 1.5 mL of 2% HNO_3, potential contaminants were only concentrated by a factor of 4.6, compared to a factor of 23.3 for the Marechal *et al*. (1999) method. This procedure reduced the procedural blank for Cu to below the detection limits of the ICP-MS (< 0.1 ng Cu mL^{-1}, or <0.05% of the Cu signal). Concentrations of Ni remained below the detection limits of the ICP-MS throughout this column purification procedure (0.1 ng Ni mL^{-1}; <0.02% of the Ni in the sample).

Table 7. Copper purification method showing the original method published by Borrok *et al.* (2007), and the modifications made for the sample matrices from the root adsorption and dialysis experiments.

Fraction	Eluant	Borrok *et al.* (2007)	Root wash/Dialysis Solutions
		mL	mL
Sample Load	10M HCl	1	1
Matrix + Ni	10M HCl	4	3
Cu Fraction	5M HCl	6	7
Fe fraction	1M HCl	4	4
Zn fraction	Deionised Water	4	4

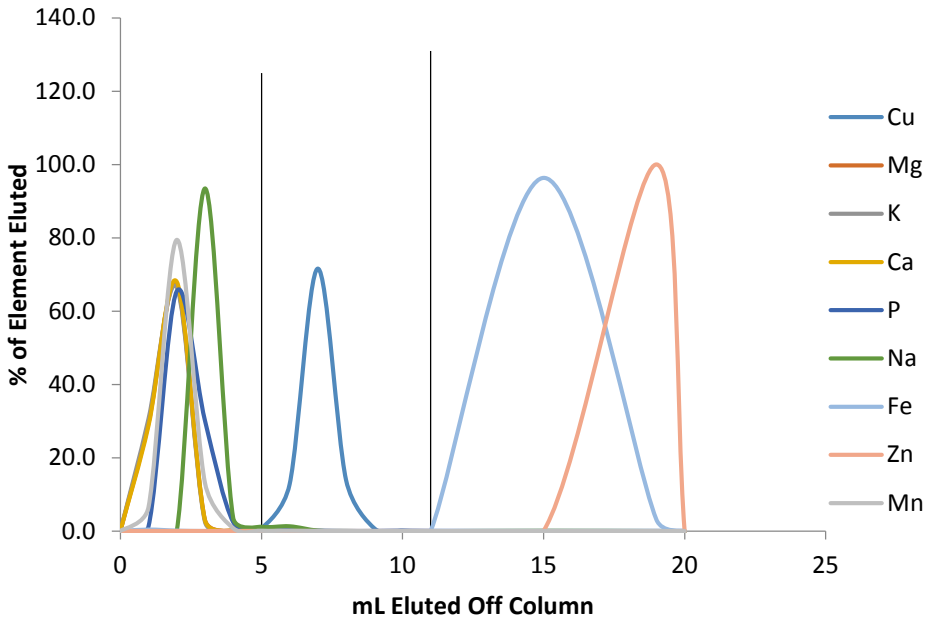

Figure 6. Elution profile of Cu and major matrix elements from an EDTA oat root wash solution, using the method of Borrok *et al.* (2007).

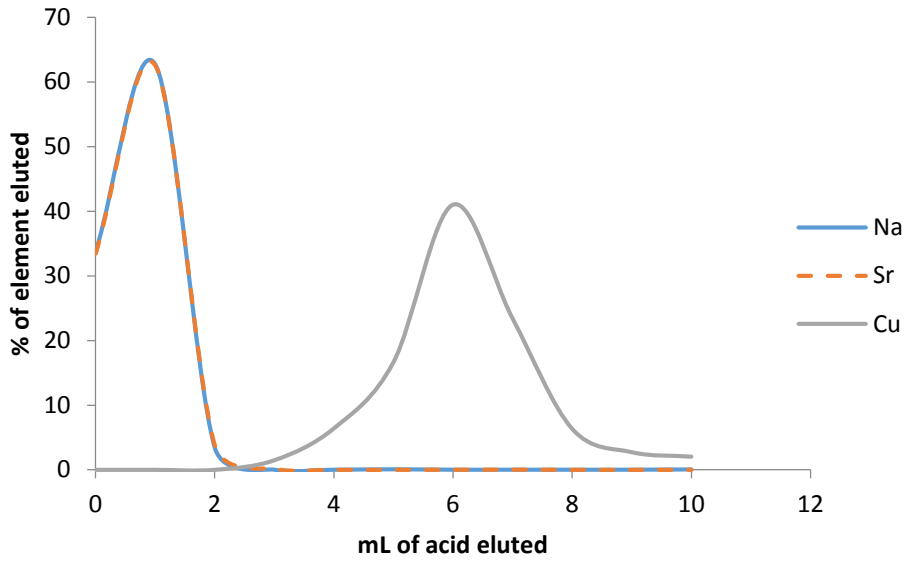

Figure 7. Elution profile of Cu from a Cu-EDTA dialysis donor solution containing 1mM Sr(NO3)2, 5mM MES and 30µM Cu and **15µM EDTA using the method adapted from Borrok** *et al.* (2007).

3.3. Summary

The column purification method published by Marechal *et al.* (1999) was found to be suitable for purifying Cu from NIST 1573a tomato leaf SRM and digest solutions of plant tissues grown in a hydroponic medium where Cu was present at high concentrations. In order to remove major ions such as Ca, K, and Mg from the digests of stem and leaf tissues, 2-3 column purifications were required, but the overall Cu recovered was within 100±10% (n=6). The procedural blank from this method was 20 ng of Cu, and relative to the total Cu in digests of hydroponically grown plant tissues, this was insignificant (<0.01%). However, the Marechal *et al.* (1999) purification method was deemed unacceptable for the sample matrices produced from plant root desorption solutions (Chapter 4) and dialysis solutions (Chapter 3), so a modified column purification method based on that published by Borrok *et al.* (2007) was used for these samples to lower the procedural blank to below ICP-MS detection limits (equivalent to <0.15 ng of Cu in the purified sample). This Cu purification procedure worked extremely well for purifying the lower concentrations of Cu from samples of these matrices with only one column pass, with quantitative Cu recovery (100±10%) (n=4), resulting in Cu eluting in 7 ml 5M HCl. This method of column purification reduced analysis time and column-induced contamination.

4. ISOTOPE ANALYSIS BY MC-ICP-MS

Once samples have been digested and purified, a procedure is required to accurately and precisely measure Cu isotope ratios by MC-ICP-MS. Plasma mass spectrometers favour the transmission of heavier isotopes and this effect needs to be corrected to obtain accurate Cu isotope ratios. In the literature, two methods are proposed to be used in conjunction with one another; standard-sample bracketing and element spiking (Albarède & Beard, 2004; Peel *et al.*, 2008). Sample-standard bracketing involves measuring samples between two bracketing standards of known isotope composition and the drift from the known isotope value observed between the two standards is assumed to encompass the instrumental drift. The resulting isotope ratios are reported as dimensionless $\delta^{65}Cu$ values, as determined by Equation 1. The NIST 976 Cu isotope standard was used as the bracketing standard, as is standard practice in the literature for the reporting of $\delta^{65}Cu$ values (Albarede, 2004; Moeller *et al.*, 2012). In order to quantify the mass bias effects during analysis of samples and standards an isotope standard of another dopant element within the isotopic mass range of the element of interest can be added (e.g. for Cu, Ni or Zn is often used). The exponential law correction method (Equation 2) is commonly used to relate the drift in the dopant element to that of the element of interest within an individual standard or sample measurement (e.g. see Marechal *et al.* (1999), Peel *et al.* (2008) and Mason *et al.* (2004) for full discussions).

$$\delta^{65}Cu = 1000\left(\frac{\text{Rsample}}{\text{Average}\,R_{\text{standard1}},R_{\text{standard2}}} - 1\right)‰ \qquad \text{Equation 1}$$

where R_{sample} is the $^{65/63}Cu$ ratio of the sample, $R_{standard\ 1}$ is the $^{65/63}Cu$ ratio for the standard measured before the sample and $R_{standard\ 2}$ is the $^{65/63}Cu$ ratio measured after the sample.

$$\text{True}\ ^{65/63}Cu = (^{65/63}Cu_{measured} \times \frac{64.92779}{62.92960})^{\beta} \qquad \text{Equation 2}$$

where β is the fractionation factor determined from the external mass bias correction measurement of the dopant element (Ni or Zn in the case of Cu) (see Marechal *et al.* (1999), Peel *et al.* (2008) and Mason *et al.* (2004) for full discussions on β calculation).

The most common elements used for the correction of mass bias during Cu isotope analysis by MC-ICP-MS are Ni and Zn (e.g. Marechal *et al.*, 1999; Ehrlich *et al.*, 2004; Mason *et al.*, 2004; Borrok *et al.*, 2007). Initial experiments were performed using the Zn isotope standard IRMM-3702 ($^{66/64}$Zn = 0.56397) for mass bias correction of Cu isotopes during MC-ICP-MS analysis. Copper isotope ratios were determined in column-purified solutions by MC-ICP-MS (Neptune, Thermo Scientific) at a concentration of 100 µg Cu L^{-1}. Samples were doped with IRMM 3702 Zn to a final concentration of 100 µg L^{-1} for mass bias correction (1:1 Cu:Zn). Copper isotope ratios were analysed under dry plasma conditions using an APEX Q desolvator (ESI), 100 µL min^{-1} PFA nebuliser and Ni sampler and skimmer cones. The ^{65}Cu isotope was measured on the centre cup and sample voltages ranged from 3-6 V between different analytical sessions. Samples were bracketed by a concentration-matched standard of Cu NIST 976 spiked with Zn IRMM 3702. Mass bias correction using to the dopant element was first performed on the individual samples and standards using the exponential law (Marechal *et al*., 1999) (Equation 2), and the drift in the measurement was corrected for by sample-standard bracketing according to Equation 1.

4.1. MC-ICP-MS Methodology

A series of experiments were performed to optimise analytical procedures to precisely and accurately measure Cu isotope ratios by MC-ICP-MS. An initial experiment was performed to determine if the procedural blank from the column purification procedure significantly contributed to the Cu isotope fractionation observed in samples. Samples of pure NIST 976 Cu (600 µg L^{-1}) (n=3) were passed

through the column using the optimised purification procedure outlined in Section 3.1 (Table 4) and the purified sample was then diluted to 100 μg L^{-1}, and doped with IRMM-3702 Zn. The isotope ratio was then measured and the $\delta^{65}Cu$ value of the purified sample was calculated to check for any deviations from 0‰. If the blank does not significantly contribute to the Cu or (doped) Zn signal in the purified sample and there is no column fractionation, the bracketing standards and 'samples' should contain the same Cu isotope ratio, resulting in a $\delta^{65}Cu$ value of 0‰. The determined delta $\delta^{65}Cu$ was 0.12 ± 0.18‰ (n=5) which is shifted from the expected 0‰. The procedural blanks were found to contain elevated Zn concentrations that represented the equivalent of 30% of the Zn isotope signal in samples (Table 8). Zinc contamination in samples may have been coming from acids, columns, resin, vials and tubing and could not be reduced in this study to below 10% of the Zn signal in samples.

Table 8. Average MC-ICP-MS voltages for Cu and Zn in 100 μg L-1 Cu NIST standard spiked with IRMM Zn, procedural blank solutions for single distilled HCl and 2%HNO_3 **and the total % of a 100 μg** L^{-1} sample signal the blank equated to.

	^{63}Cu	^{64}Zn	^{65}Cu	^{66}Zn	^{67}Zn	^{68}Zn
Average MC voltages for standard (Cu = 100 μg L^{-1})	6.66	1.97	3.14	1.18	0.18	0.82
Average MC voltages for single distilled HCl blank	0.19	0.60	0.09	0.36	0.05	0.25
(percentage of standard signal)	2.88	30.63	2.87	30.45	30.58	30.13
Average MC voltages for 2%HNO3	0.01	0.02	0.01	0.01	0.00	0.01
(percentage of standard signal)	0.21	0.97	0.21	0.96	1.00	0.97

The sample analysis procedure was modified to replace the Zn isotope standard IRMM-3702 dopant with Ni (NIST 986 $^{62/60}Ni$ = 0.1386) for Cu mass bias correction. Nickel has previously been used with success for the mass bias correction of Cu isotopes by MC-ICP-MS in soils and biological tissues (Ehrlich *et al.*, 2004; Li *et al.*, 2009; Bigalke *et al.*, 2010). The analysis procedure was further modified to increase analysis concentrations of Cu in samples to 300 μg L^{-1} (from 100 μg L^{-1}) and the sample

introduction system changed from dry plasma to wet plasma conditions. Nickel was spiked into each sample at 1000 µg L^{-1}, providing a 3:10 Cu:Ni ratio to enable approximate voltage matching between Cu and Ni. The ^{63}Cu isotope was detected on the centre cup of the MC-ICP-MS at 6-10 V depending on the analytical session. By removing the desolvator system the amount of tubing that samples were in contact with was reduced, further limiting the potential for sample contamination. Procedural blanks showed that contamination was no longer an issue with blanks having Cu and Ni concentrations below ICP-MS detection limits (<0.1 µg L^{-1}; 0.2 ng Cu and Ni). There was no significant fractionation observed for Cu ($\delta^{65}Cu$=0 ± 0.04‰, n=3) in solutions representing an artificial plant matrix digest that had been spiked with NIST 976 Cu (purified by the column procedure outlined in Section 3.1), analysed at 300 µg Cu L^{-1} spiked with NIST 986 Ni (1000 µg Ni L^{-1}) as a dopant element for mass bias correction. Hence, all subsequent isotope analyses were performed using NIST 986 Ni for mass bias correction.

4.2. Accuracy and Precision of Analysis

The accuracy and precision of each analytical session was assessed by analysing an in-house Cu wire standard bracketed by the NIST 976 Cu isotope standard, which is no longer available, and therefore, in short supply. A long-term reproducibility of 0.04‰ was determined from the 2σ of the wire standard (n=50). If the $\delta^{65}Cu_{wire\ standard}$ deviated from 0.45 ± 0.02‰ (± σ) during an analytical session, then the analytical run was repeated. The overall error of the optimised method (i.e. sample digestion, column purification and isotope analysis) was determined using NIST 1573a SRM (n=5) (tomato leaves). The maximum sample error was for the optimised method was determined to be ±0.12‰ (2σ) (n=5). Furthermore, the reliability of the mass bias correction was assessed by comparing the corrected delta values using the exponential law to the delta values calculated using other mass bias correction methods; sample standard bracketing only (SSB), a modified SSB approach (m-SSB) (Peel *et al.*, 2008) and the exponential sample standard bracketing (en-SSB) technique (Peel *et al.*, 2008). It is expected that there will be some variation between the correction methods, particularly between the

SSB-only correction and the other methods, as SSB alone does not quantify the fractionation effect that is done by the dopant element. However, if the difference between the corrected $\delta^{65}Cu$ values from the various methods varied by more than ± 0.12‰, i.e. the error of the method, the analytical session was repeated.

4.3 Interferences

A number of potential interferences that can occur during MC-ICP-MS analysis of Cu and Ni isotopes have been determined, some of which are outlined in Table 9. The likelihood of many of these interferences occurring in the plasma will be dependent on the elemental composition of the sample matrix. The presence of matrix ions can affect the ionisation of Cu and Ni isotopes in the plasma and reduce the accuracy of the determined isotope ratios. Elements that were identified as recalcitrant in plant matrices, e.g. Ca, K, and Na, were systematically examined for their potential interferences on Cu isotope ratios and critical limits of interference. This was performed by spiking a sample of the Cu bracketing standard (NIST 976 Cu and NIST Ni 986; 300 µg Cu L^{-1}: 1000 µg Ni L^{-1}) with increasing concentrations of potentially interfering elements and checking for any deviation from $\delta^{65}Cu$ = 0 ‰. The presence of Ca and Na at concentrations ≥ 2 mg L^{-1} in sample solutions interfered with the accurate determination Cu isotope ratios by MC-ICP-MS, i.e. outside the external reproducibility of the instrument (0.04‰) (Figure 8). This established a critical limit of 2 mg L^{-1} for Ca and Na in the post-column samples to avoid analysis-induced Cu fractionation being observed in samples. Samples were not analysed for Cu isotope ratios if Ca and Na concentrations could not be reduced to below 2 mg L^{-1} in solutions following column purification.

Table 9. Copper and Ni isotopic abundances and potential interferences occurring in the MC-ICP-MS plasma. (De Laeter et al., 2003; Mason et al., 2004; Petit et al., 2008).

Approximate Atomic Mass	Element Isotope (abundance)	Interferences
60	^{60}Ni (26%)	$^{44}Ca^{16}O^{+}$, $^{23}Na^{37}Cl^{+}$, $^{43}Ca^{16}O^{1}H^{+}$
61	^{61}Ni (1%)	$^{44}Ca^{16}O^{1}H^{+}$, $^{45}Sc^{16}O^{+}$
62	^{62}Ni (4%)	$^{46}Ti^{16}O^{+}$,$^{23}Na^{39}K^{+}$, $^{46}Ca^{16}O^{+}$,$^{31}P^{16}O_2^{+}$, $^{40}Ar^{23}Na^{+}$,$^{47}Ti^{16}O^{+}$, $^{23}Na^{40}Ca^{+}$, $^{46}Ca^{16}O^{1}H^{+}$
63	^{63}Cu (69%)	$^{36}Ar^{12}C^{14}N^{1}H^{+}$, $^{14}N^{12}C^{37}Cl^{+}$, $^{16}O^{12}C^{35}Cl^{+}$
64	^{64}Ni (1%)	$^{32}S^{16}O_2^{+}$, $^{32}S_2^{+}$, ^{64}Zn
65	^{65}Cu (31%)	$^{49}Ti^{16}O^{+}$, $^{32}S^{16}O^{1}H^{+}$, $^{40}Ar^{25}Mg^{+}$, $^{40}Ca^{16}O^{1}H^{+}$, $^{36}Ar^{14}N_2^{1}H^{+}$, $^{32}S^{33}S^{+}$, $^{32}S^{16}O^{17}O^{+}$, $^{33}S^{16}O_2^{+}$, $^{12}C^{16}O^{37}Cl^{+}$, $^{12}C^{18}O^{35}Cl^{+}$, $^{31}P^{16}O^{18}O^{+}$, $^{130}Ba_2^{+}$

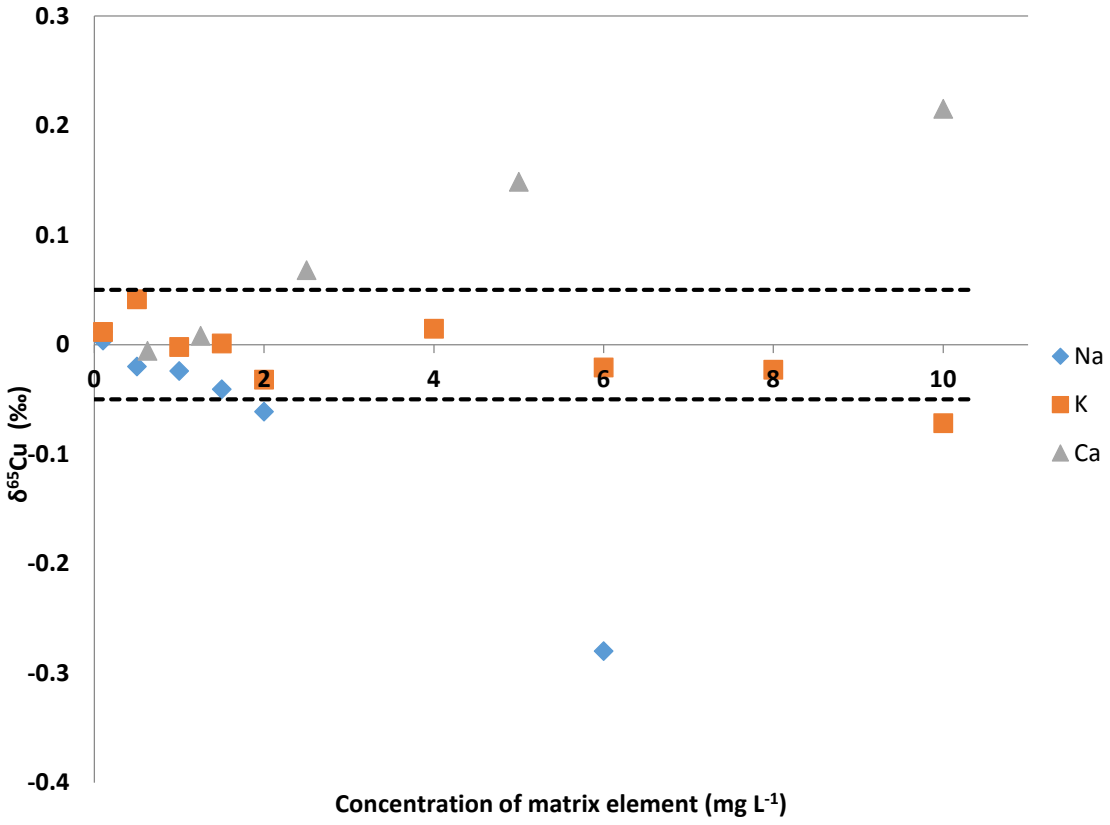

Figure 8. δ^{65}Cu of a NIST 976 bracketing standard spiked with various concentrations of matrix elements. Dashed lines represent 0.05‰, the external reproducibility of the instrument.

4.4. Summary

The optimal method for measuring Cu isotope ratios of purified samples was in solutions at 300 µg Cu L^{-1} spiked with 1000 µg Ni L^{-1} NIST 986. The exponential law correction coupled with sample-standard bracketing was used for mass bias correction. The accuracy and precision of the instrument was checked at the beginning of each analytical session, and every 15 samples thereafter if it was a long analytical session, by measuring an in-house Cu wire standard (δ^{65}Cu=0.45±0.04‰, mean ± 2sd, *n*=50). If the ratio for the Cu-wire standard deviated from the known value by more than 1sd the analytical session was repeated. Analysis of the Cu wire standard spiked with various concentrations of

commonly occurring matrix elements showed that Ca and Na concentrations in purified samples needed to be below 2 mg L^{-1} to avoid interferences on the accurate determination of Cu isotope ratios.

5. CONCLUSIONS

A suitable method for accurate measurement of Cu isotopes ratios for the plant tissue and solution samples generated throughout this thesis was determined, including digestion, purification and MC-ICP-MS analysis.

Digestion: Copper was found to be quantitatively recovered from plant tissues using a two-stage combined hotplate-microwave digestion procedure using HNO_3 and H_2O_2. Organic material was found to be successfully digested after a hotplate reflux with 3 mL HNO_3 and 1 mL H_2O_2, followed by microwave digestion with 7 mL HNO_3 and 3 mL H_2O_2.

Purification: Digested plant tissues were dried down and re-suspended in 7M HCl for column purification, where matrix elements were removed in 7 mL of 7M HCl and Cu was quantitatively eluted in 35 mL of 7M HCl, as per the Marechal *et al.* (1999) method. A more efficient method of Cu purification with a lower procedural blank was adopted for root adsorption and dialysis solutions where the matrix elements were removed in 3 mL of 10M HCl and Cu collected in 7 mL of 10M HCl, as per the Borrok *et al*. (2007) method. Purified samples were analysed for concentrations of matrix elements to confirm they were below interfering concentrations (<2 mg L^{-1}).

Isotope Analysis: Copper isotope ratios were measured using MC-ICP-MS at Cu concentrations of 300 µg L^{-1} with NIST 986 Ni spiked into the samples at 1000 µg Ni L^{-1} for matrix-induced mass bias correction. Mass bias drift was corrected using a combination of sample-standard bracketing and the application of the exponential law. Isotope ratios were analysed using wet plasma conditions with a 100 µL min^{-1} PFA nebuliser. An in-house Cu wire standard was used to measure the instrumental long-term reproducibility using this isotope measurement method. It was found that a consistent value of $\delta^{65}Cu$=0.45 ± 0.04‰ (mean ± 2σ) (n=50) was measured over a 24-month period.

Blanks: Procedural blanks from the efficient Cu purification method of Borrok *et al.* (2007) were found to have Cu and Ni concentrations below ICP-MS detection limits (<0.1 µg L^{-1}; 0.15 ng Cu and 0.15 ng

Ni). These were insignificant in comparison to the 300 and 1000 µg L^{-1} concentrations of Cu and Ni, respectively, contained in the samples.

Errors: The maximum sample error for $\delta^{65}Cu$ in plant materials using the optimised method was determined to be ±0.12‰. This was determined by 2σ value for the isotope analysis of digested, and purified NIST 1573a tomato leaf SRM samples (n=5). It was also found that column recoveries within 100±10% did not induce artificial fractionation of Cu isotopes outside the reproducibility of the method.

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CHAPTER 3

COPPER ISOTOPE FRACTIONATION DURING EQUILIBRATION WITH NATURAL AND SYNTHETIC LIGANDS

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CHAPTER 4

COPPER ISOTOPE FRACTIONATION DURING ADSORPTION TO ROOT CELL WALLS

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Copper isotope fractionation during adsorption to root cell walls

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ABSTRACT

Tracing the biogeochemical cycling of copper (Cu) through changes in its stable isotope composition has received increased attention recently. In particular, Cu isotopes have been used to examine plant uptake and translocation mechanisms. A key process prior to the uptake of Cu is apoplastic root adsorption. This study examined the effect of root adsorption on Cu isotope fractionation in three dicotyledon (dicot) species and two monocotyledon (monocot) species. Each species was analyzed as seedlings and four-week old plants and plant age was found to have no significant effect on the measured isotope ratios. Enrichment in the heavy Cu isotope (^{65}Cu) was observed on the roots of dicot plants, similar in magnitude to that previously reported for Cu-organic matter binding, and no significant fractionation was observed during adsorption to monocot plant roots. An assessment of three different apoplastic root wash extractants (a lanthanum + calcium chloride solution (La+Ca), ethylenediaminetetraacetic acid (EDTA) and hydrochloric acid (HCl)) was undertaken in conjunction with this work to determine a suitable method for removing apoplastic Cu for isotope analysis. The proportion of absorbed Cu released during apoplastic Cu removal increased in the order La+Ca<EDTA<HCl. These results indicate that inclusion of root-adsorbed Cu in Cu isotopic ratios of plant roots of dicot species may obscure the true isotopic ratio of Cu taken up by plants. As such, a suitable root washing procedure should be used to remove apoplastic Cu prior to isotopic analysis.

INTRODUCTION

Copper (Cu) is an essential micronutrient for plant growth as it plays a key role in electron transport during photosynthesis and also functions as a co-factor in many key proteins [1,2]. Plants grown in soils that are deficient in available Cu can suffer from low crop yields, impaired water transport and poor fertility [1]. However, Cu toxicity can cause poor root development, chlorosis of vegetative tissue, and potentially, iron (Fe) deficiencies due to reduced Fe uptake as a result of competition [2]. The mechanisms of Cu mobilisation and absorption are poorly characterised. New analytical tools are needed to complement existing techniques to provide further insights into plant uptake mechanism to manage plant Cu deficiency and toxicity in soils globally.

The emergence of analytical methods for analysing stable isotope ratios of heavier elements has enabled the relatively small isotope fractionations of Cu to be measured accurately, and this has provided information on the reaction mechanisms involved during environmental processes [3]. In a recent study by Jouvin *et al.* [4], the changes in stable Cu isotope ratios during plant uptake were measured to assess Cu absorption mechanisms in plants having different Fe uptake mechanisms (Strategy I plants: dicotyledons and non-graminaceous monocotyledons; and Strategy II plants: graminaceous monocotyledons [5]). These authors found an enrichment of the light Cu isotope (^{63}Cu) in all plant roots studied, but particularly in those of dicotyledon plant species. This result is indicative of Cu(II) reduction to Cu(I) during absorption by Strategy I plants, as reduction reactions strongly favour the light isotope [6]. In the study of Jouvin *et al.* [4], adsorbed Cu was not removed from the roots prior to isotope analysis.

In a recent study, we further examined this absorptive uptake fractionation for Strategy I and II plant species, but attempted to remove Cu adsorbed in the apoplast, as the effect of Cu sorption on root cell

walls on the isotope ratio of Cu is unknown. The plant roots were washed in a solution of calcium chloride ($CaCl_2$) + lanthanum chloride ($LaCl_3$) (Ca+La) before digestion [7]. The results supported the findings of Jouvin *et al*. [4] that suggested Cu was taken up as reduced species in Strategy I plants, although we found no significant isotope fractionation in Strategy II oat plants [7]. The fractionation between solution and plant for tomatoes was larger in our study than in that of Jouvin *et al.* [4]. The observed difference in Cu fractionation between these two studies may be due to the inclusion of adsorbed Cu(II) with a different isotopic signature in the analysis of root Cu isotopes by Jouvin *et al.* [4]. It has been shown that Cu complexation with, or adsorption onto, organic matter can lead to notable isotope fractionation. Sorption of Cu onto insolubilized humic acid showed enrichment in the heavy isotope in the adsorbed phase of +0.26‰ ($\Delta^{65}Cu_{solid\text{-}solution}$) [8], while sorption onto the cell wall of heat killed microorganisms resulted in fractionation factors of between -0.3 to +0.69‰ ($\Delta^{65}Cu_{solid\text{-}solution}$) [9]. More recently Ryan *et al.* [10] reported significant heavy isotope enrichment in organic ligand complexes, with the fractionation factor ($\Delta^{65}Cu_{complex\text{-}freeCu(II)}$) increasing with increasing complex-Cu bond strength. A large amount of Cu associated with roots may be contained within the adsorbed rather than the absorbed pool. For instance, Chaignon *et al.* [11] found that 40-80% of total Cu in roots of soil-grown plants was apoplastically bound (adsorbed) using a 0.01 M HCl desorption solution. Bravin *et al.* [12] found a similar contribution of adsorbed Cu to the total root Cu for hydroponically grown wheat at Cu free ion activities ranging between 0.4 nM to 2.4 µM, using the same desorption method. Hence, if adsorbed and absorbed Cu have a different isotope ratios, the experimentally obtained isotope ratio of root Cu will depend on whether adsorbed Cu is included or not.

Adsorption from the soil solution to the root apoplast is believed to be a key first step in the absorption of Cu into the plant root symplast [13]. The cell wall is comprised of pectic polysaccharides, resulting in free carboxyl groups with negative charges that electrostatically attract cations into the Donnan free space [13]. This, along with the negatively charged mucigel that surrounds the outermost layer of roots,

generates the root cation exchange capacity (CEC) [13]. The root CEC is involved in increasing the concentration of cations in the Donnan free space and from here, membrane uptake processes enable absorption. In general, the CEC of monocotyledons (moncots) is half that of dicotyledon (dicot) plant species [14, 15]. Copper contained in the roots of monocots was found to be linearly related to Cu adsorbed on the root surface [16]. However, such a correlation does not prove that adsorption is a controlling factor in the uptake of Cu, since both adsorption and absorption depend on the free Cu concentration in solution. The role of adsorption in the overall uptake of Cu is still unclear.

Adsorption of Cu to plant roots is affected by the soluble concentration of free Cu, as well as the concentration of competing ions, the pH, and the plant species in question. The soil pH plays an important role in controlling root Cu adsorption. Acidic conditions generally lead to an increase in free Cu ions in the soil solution that can move into the apparent root free space, but this effect can be balanced by increased competition with H^+ for root CEC sites [17]. The affinity of root adsorption sites for Cu is much greater than for most other divalent cations (e.g., Ca, magnesium (Mg) and zinc (Zn)), which is why saturation of roots with Cu has been used as a method of assessing root CEC [18, 19]. Iwasaki *et al.* [20] showed that, at uptake solution ionic strengths between 0.01 and 0.1 M and pH values between 4.5 and 5.5, the roots of ryegrass (monocot) adsorbed between 25-130 µg Cu g^{-1} root fresh weight (RFW), while roots of red clover (dicot) adsorbed 75-260 µg Cu g^{-1} RFW. These experiments were carried out at very high solution Cu concentrations (5 mg Cu L^{-1}), and therefore, are likely an indication of maximal Cu sorption, rather than the amount of Cu sorption under normal growth conditions. However, they do support the conclusion that dicots have higher root CECs than monocots, as reported by numerous authors, e.g., [14, 15].

There are a number of root washing techniques reported in the literature that have been used to separate and quantify apoplastically bound metals from root-absorbed metals. The most common method to assess root CEC uses an acid to replace adsorbed cations with H^+ ions, with some methods directly measuring this H^+ quantity through titration and others replacing the sorbed H^+ with another cation which is measured after another displacement [21]. Other methods for removing adsorbed cations from roots include chelating ligands such as EDTA [22], cation exchange using barium salts and ammonium acetate [23], and La+Ca cation exchange [24]. The root washing method needs to be highly efficient at removing adsorbed metal from the root surface, without breaking the root membrane allowing leaking of absorbed metals. Iwasaki *et al.* [20] reported that 80-90% of adsorbed Cu could be desorbed using 0.01 M HCl for 10 minutes, but extracting for longer periods of time resulted in significant leaching of amino acids believed to be leaking from the symplasm. Potassium (K) leakage is often monitored during apoplastic extractions and excessive K release is a sign of root impairment [25].

The aim of this study was to determine the isotope fractionation induced by adsorption of Cu to plant roots in order to gain a better understanding of Cu isotope behaviour during plant uptake. This was done by depleting a Cu solution by root exposure and measuring the subsequent change in Cu isotope ratio. Root washing was applied to the roots to confirm the Cu removed from solution could be recovered from the root surface to achieve mass balance. The study compared the isotopic fractionation data for Cu solutions exposed to seedlings and four-week old plants of both Strategy I and Strategy II species. Additionally, three previously published root washing methods were assessed to find the most effective method of removing adsorbed Cu without leaching Cu from the symplast.

METHODS

Plant Growth

Three Strategy I species (*Solanum lycopersicum* (tomato), *Brassica napus* (canola), *Vicia faba* (bean)) and two Strategy II species (*Triticum durum* (wheat) and *Avena sativa* (oat)) were used in this study. Plant seeds were germinated on moistened paper towel for 10-14 days and either used as seedlings in adsorption experiments or transplanted into a hydroponic growth solution to continue growing for four weeks, as per the method published by Ryan *et al.* [7]. The hydroponic nutrient solution used to grow plants for four weeks consisted of $Ca(NO_3)_2$ (2 mM), $MgSO_4$ (0.5 mM), KNO_3 (1.2 mM), NaH_2PO_4 (0.1 mM), 2-(N-morpholino)ethanesulfonate (MES) (2.5 mM), NaCl (25 μM), H_3BO_3 (15 μM), $MnSO_4$(5 μM), $(NH_4)_6Mo_7O_{24}$ (0.07 μM), $ZnSO_4$ (1 μM) and $CuCl_2$ (1 μM). To maintain Fe in a soluble form for plant growth, Fe (15 μM) was provided in the chelated form with di-sodium ethylenediaminetetraaceticacid (EDTA) for the Strategy I plants or with N-(hydroxylethyl) ethylenediaminetriaceticacid (HEDTA) for the Strategy II plants. The HEDTA chelate was used for the Strategy II plants as they showed Fe deficiency symptoms when grown with Fe-EDTA. The plants were grown in 10-L pots with 10 plants per pot and maintained at pH *ca.* 5. Nutrient concentrations were monitored for depletion using inductively coupled plasma-optical emission spectroscopy (ICP-OES) and replenished as necessary.

Adsorption to Seedling Roots

Root adsorption was assessed from a solution of 0.2 mM Cu (high Cu loading), made from an in-house Cu wire isotopic standard, in 1 mM KNO_3 background electrolyte, using 10-14 day old seedlings of tomato, bean, canola, wheat and oat plants. Seedlings were combined to get *ca.* 1g root fresh weight (RFW) to enable *ca.* 50% depletion of Cu from the adsorption solution. If depletion sits to close to 100% or 0% the difficulty in measuring a difference in isotope ratio increases, hence, 50% depletion was aimed for in all experiments to optimise the accuracy of isotope ratio analysis. Tomato and canola

plants were placed in 5 mL of Cu solution, while wheat, oat and bean plants were placed in 10-20 mL of Cu solution to obtain an optimal root:solution ratio to reach 50% solution Cu depletion after 15 minutes of root exposure. Adsorption solutions were then filtered through a 0.2-μm membrane filter and analysed for Cu concentration with ICP-OES (Perkin Elmer) and for the Cu isotope ratios with a multi collector-inductively coupled plasma-mass spectrometer (MC-ICP-MS) (Neptune, Thermo Scientific) (see *Isotope Analysis*). The difference between the initial and final Cu concentrations and isotope ratios was attributed to adsorption of Cu by roots.

In order to confirm mass balance was being achieved, a selection of seedling roots were digested in 3 mL HNO_3 + 1 mL H_2O_2 and refluxed on a hot plate at 180°C. Total Cu concentrations in the digest solutions were determined by ICP-OES. The seedlings had not been exposed to Cu during germination and the amount of seed Cu was negligibly small (e.g., 51 ng per tomato seed, 125 ng per wheat seed; average value of 5 replicates) compared to the amount of Cu adsorbed from the 0.2 mM solution (around 30-120 μg, depending on the volume of adsorption solution used). Hence, it was assumed that all Cu recovered from the root digest originated from the adsorption solution. Copper was then purified from the root digest solutions for isotopic analysis as outlined by Ryan *et al.* [10] (see *Copper Purification*).

Adsorption to Four Week Old Plant Roots

In this experiment, the fractionation during adsorption was assessed with four-week old plants (tomato, bean, canola, wheat and oat) at a lower Cu concentration than in the seedling experiment (1 μM Cu – low Cu loading) to assess root adsorption at more realistic Cu concentrations. As the plants were grown in a hydroponic solution containing 1 μM Cu, a desorption step was needed prior to the adsorption experiment to remove Cu already adsorbed to the root surface. The roots were rinsed with deionized

water, and then washed with 5 mM EDTA for 30 s. After this step, the plants were placed for 20 minutes in a 100 mL adsorption solution, which contained 1 μM Cu, made from an in-house Cu wire isotopic standard, in a 0.5 mM $Ca(NO_3)_2$ background electrolyte. Adsorption solutions were then filtered through 0.2-μm membrane filters and analysed for total Cu concentrations by ICP-OES and Cu isotope ratios by MC-ICP-MS (see *Isotope Analysis*). The difference between the initial and final Cu concentrations and isotope ratios was attributed to Cu root adsorption.

Assessment of Root Washing Methods

Three previously published metal desorption methods were examined to determine how effective they were at removing adsorbed (apoplastic) Cu without removing Cu from the symplasm. In Method 1, modified from Weiss *et al.* [26], plant roots were shaken in a 50 mL solution of 1 mM $LaCl_3$ + 0.05 mM $CaCl_2$ for 5 minutes. In Method 2, used previously for extracting adsorbed Cu from algal cells by Hassler *et al.* [27], the roots were shaken with 50 mL of 5 mM EDTA for 30 s. In Method 3, taken from Chaignon *et al.* [11], plant roots were shaken with 100 mL of 1 mM HCl for 3 minutes, followed by the addition of 1 mL of 1 M HCl to increase the HCl concentration to 10 mM, and shaken for a further 5 minutes.

In this experiment, tomato and oat plants were used to compare the different root washing methods. The plants were grown in a hydroponic growth media according to the method outlined by Ryan *et al.* [7], and described above for the adsorption study using four-week old plants. After four weeks of growth, plants were removed from the nutrient solution, the roots were separated from vegetative tissues, rinsed under running deionized water and washed using one of the three root washing methods. Plant roots were removed from wash solutions and solutions were passed through 0.2-μm

membrane filters to remove any fine root material, and total concentrations of released elements were analysed using ICP-OES and isotope ratios analysed on selected samples by MC-ICP-MS.

Copper Purification

The high Cu loadings (0.2 mM) used in the seedling adsorption experiment meant that after diluting to 300 µg Cu L^{-1} for MC-ICP-MS (>40 fold dilution), any matrix ions present in the adsorption solution were diluted below concentrations that cause interferences for Cu isotope analysis (i.e., Na and Ca <2 mg L^{-1}). This meant no Cu purification was required for these samples and they were analysed at 300 µg L^{-1}, without purification, using MC-ICP-MS. However, purification was needed for the digest solutions of the seedling roots, the adsorption solutions from the four-week old plants and the trial solutions from root washing to separate Cu from the matrix elements, as the concentration ratio of matrix elements to Cu was much higher in these samples. The adsorption solutions from the four-week old plants and the root wash solutions also needed to be concentrated to attain high enough Cu concentrations for isotope analysis (*ca.* 300 µg L^{-1}). To concentrate Cu in these samples, 25 mL of the sample solution was added to a PTFE vial, dried down on a hotplate at *ca*. 140°C and resuspended in 1.5 mL of 10 M HCl.

Copper was isolated from other elements using the method published by Ryan *et al.* [10], adapted from the method of Borrok *et al.* [28]. Briefly, columns of *ca.* 1 mL of AG-MP-1 resin (BioRad, 100-200 mesh) were conditioned with 5 mL of 10 M HCl, 1 ml of the sample (in 10 M HCl) was loaded onto the column, matrix elements were rinsed through with 3 mL of 10 M HCl and Cu was eluted in 7 mL of 5 M HCl. The 7 mL Cu fraction was collected into a PTFE vial, and evaporated to dryness at *ca*. 140°C on a hotplate. The sample was then redissolved in 1.5 mL of 2% HNO_3 for isotope analysis. A 0.2-mL aliquot of this purified sample was diluted to 7 mL for ICP-OES analysis to confirm the sample was sufficiently purified

from matrix elements and to confirm the column recovery of Cu fell within the acceptable range of 100±10% to avoid inducing artificial Cu isotope fractionation [10].

Isotope Analysis

Copper isotope ratios ($^{65}Cu/^{63}Cu$) were determined using MC-ICP-MS (Neptune, Thermo Scientific). The samples and standards were measured in 2% HNO_3 at concentrations of between 250 -300 μg Cu L^{-1}. The NIST 986 Ni isotope standard ($^{62/60}Ni$ = 0.1386) was spiked into samples and standards at a 3:10 Cu:Ni ratio to quantify the fractionation effect. Sample-standard bracketing together with external normalization with NIST 986 Ni allowed for offline mass bias correction using the exponential method [7, 10]. Measurements were made in low resolution mode using wet plasma conditions with an uptake rate of 100 μL min^{-1} using a PFA nebuliser and Ni sampler and skimmer cones.

An in-house Cu wire standard with a known value relative to the NIST 976 Cu standard of $\delta^{65}Cu$ = 0.45± 0.04‰ (mean ± 2SD, *n*=50 over 24 months) was used as the bracketing standard. Delta values are reported relative to NIST 976 Cu, according to Equation 1, taking into account the difference of 0.45‰ between the Cu wire standard and the NIST 976 standard:

$$\delta^{65}Cu = \left(\frac{^{65}Cu/^{63}Cu_{sample}}{^{65}Cu/^{63}Cu_{wire}} - 1 \right) + 0.45 \qquad \text{Equation1}$$

where $^{65}Cu/^{63}Cu_{wire}$ is the average of isotope ratio for the wire standard measured before and after the sample.

The analytical precision of the MC-ICP-MS analysis was determined by running the in-house Cu wire standard bracketed by NIST 976 at the beginning and end of each analytical session to confirm that the

$\delta^{65}Cu$ value was measured as 0.45 ± 0.02‰ (mean ± SD). The analytical session was repeated if the wire standard failed to produce this value.

The $\delta^{65}Cu$ value of adsorbed Cu for both seedlings and four-week old plants was calculated using a mass balance equation (Equation 2):

$$\delta^{65}Cu_{adsorbed} = \delta^{65}Cu_{initial} - \left(\frac{f \times \delta^{65}Cu_{final}}{1 - f} \right) \qquad \text{Equation 2}$$

where f is the fraction of Cu remaining in solution, $\delta^{65}Cu_{initial}$ is the isotopic composition of the adsorption solution before root exposure (0.45‰), and $\delta^{65}Cu_{final}$ is the isotopic compositon of the solution after root exposure (measured by MC-ICP-MS).

The seedlings used to assess root adsorption contained negligible Cu prior to exposure to the adsorption solution. Hence, the seedling roots could be digested and analysed by MC-ICP-MS to confirm that the $\delta^{65}Cu_{adsorbed}$ value calculated from Equation 2 was accurate.The four-week old plants would have contained absorbed Cu accumulated during their hydroponic growth, and hence, no confirmation of mass balance could be made by measuring the isotopic signature of Cu of their digested roots.

The results of the adsorption fractionation are reported as the difference between the solution $\delta^{65}Cu$ values and the root adsorbed $\delta^{65}Cu$, according to Equation 3.

$$\Delta^{65}Cu_{adsorbed\text{-}solution} = \delta^{65}Cu_{adsorbed} - \delta^{65}Cu_{solution} \qquad \text{Equation 3}$$

Statistical Analyses

The adsorption solution delta values were compared using a one-way analysis of variance (ANOVA) test in GenStat (v14), with a Tukey multiple comparison test used to ascertain significantly different means. Furthermore, a one-sample t-test was used to test whether the adsorption fractionation values reported for each species were significantly different from zero, i.e., to determine whether fractionation had occurred. Differences between treatment means were assessed at the 5% level of significance.

RESULTS AND DISCUSSION

Root Adsorption

There was no significant difference (α=0.05) in the $\Delta^{65}Cu_{adsorbed\text{-}solution}$ values whether they were measured in the 1 μM Cu solution with four-week old plant roots, or the 0.2 mM Cu solution with seedlings (Table 1). This suggests that the stage of root development and Cu concentration do not significantly affect the degree to which Cu isotopes fractionate when adsorbing to plant roots. Hence, the overall averages for each species, combining the results for the seedlings and four-week old plants, as presented in Table 1, were used for all further comparisons. In the seedling experiment, the amount of Cu removed from the adsorption solutions corresponded within 15% to the amount of Cu recovered in the seedling digests, confirming the mass balance. Furthermore, there was no significant difference (α=0.05) between the fractionation factors calculated from the change in solution isotopic composition (using Equation 2) and those determined from the digested root solutions. Given there was no significant difference in the fractionation factors obtained with the four-week old plants and the seedlings, these data suggest that the seedling method can be used in future studies to provide a fast and accurate method for assessing the effect of root adsorption on isotope fractionation without the need for column purification or long periods of plant growth.

There was a significant difference found between the $\Delta^{65}Cu_{adsorbed\text{-}solution}$ values of Strategy I and Strategy II plants ($p = 0.003$) (Table 1). The Strategy I plant species tested all had $\Delta^{65}Cu_{adsorbed\text{-}solution}$ values significantly different from zero, i.e., fractionation had occurred during sorption; however, the Strategy II plants showed no significant fractionation (Table 1). The difference in isotope fractionation factors may be related to the type and number of cation-binding functional groups on the surface of monocot and dicot plant roots. Keller and Deuel [29] found that 70-90% of root CEC was due to the carboxyl groups of pectins, the primary component of which is galacturonic acid. Furthermore, measurements of the root CEC electrical properties indicate that the main source of negative charge on plant roots is from dissociated weak acids, with pKa values similar to those of polygalacturonic acid [30, 31, 32, 33]. Meychik and Yermakov [33] identified four main ion-exchange groups on the roots of both monocot and dicot plants and classified them as amine (anion exchange), galacturonic acid, carboxyl, or phenolic in nature. These authors reported a significant difference in the proportion of these groups between monocot and dicot species, with dicots having significantly more galacturonic and carboxyl-binding sites. The log K stability constants for the binding of Cu to galacturonic acid are 4.7 and 7.6 for the unprotonated and protonated complexes, respectively [34]. These binding stability constants for Cu-galacturonic acid are similar to those reported for Cu-fulvic acid (log K =6-8, Hirose *et al.* [35]). Under equilibrium conditions, heavier isotopes are known to partition to the strongest bonding environment, due to the lower vibrational energies that result from binding with the heavier isotope [36]. Recently, Ryan *et al*. [10] noted that as the bond strength between Cu and soluble organic ligands increased, the isotope fractionation factor between the free Cu^{2+} and the complex increased, with heavier isotopes accumulating in the stronger bonding organic complex. The isotope fractionation values observed during Cu binding to dicot cell walls in this study are similar to those observed during Cu binding to soluble fulvic acid ($\Delta^{65}Cu_{complex\text{-}solution} = +0.14 \pm 0.11‰$; Ryan *et al.* [10]) and adsorption to insolubilized humic acid ($\Delta^{65}Cu_{complex\text{-}solution} = 0.26‰$; Bigalke *et al.*, [8]). These results are consistent with cation exchange sites in Strategy I plants offering a stronger bonding environment for Cu compared to the

soluble Cu-hexaaquo complex, hence, heavy isotopes partition more readily to the plant cell wall. Furthermore, the similarities in fractionation factors induced by Cu adsorption to Strategy I plant roots and by complexation/adsorption with natural organic matter suggest that Cu is bound to the roots of these plants in complexes with similar association constants [10].

The absence of Cu isotope fractionation observed in Strategy II plant roots may be due to the differential makeup of the functional groups on the plant roots (Table 1). As mentioned previously, monocots have significantly less carboxyl-based strong cation binding sites, compared to dicots, leading to CECs approximately half that of dicot species [33, 37]. As such, there is less of a thermodynamic driver for Cu isotope fractionation during root adsorption.

The fractionation of stable isotopes has emerged as a potential tool for tracing heavy metal cycling in the environment and has been used to ascertain information about nutrient uptake and translocation mechanism in plants (*e.g.,* [4, 7, 38]). Bravin *et al.* [12] and Chaignon *et al.* [11] have shown that >50% of the total root Cu can be associated with the apoplastic pool in the roots. Given the potentially large contribution of adsorbed Cu to total root Cu, root washing is required to obtain accurate $\delta^{65}Cu$ values for Cu absorbed in roots. This would appear particularly important for dicot plants, where heavier isotopes appear to be preferentially sorbed to the root apoplast (Table 1), while absorbed Cu is isotopically light [7]. A schematic showing the potential difference in the $\delta^{65}Cu$ value measured for plant roots with and without root washing is shown in Figure 1, using the absorbed $\delta^{65}Cu_{root}$ values of Ryan *et al.* [7] and assuming 40% of the total root Cu is adsorbed to the surface, which is reasonable assumption given the results of Bravin *et al.* [12] and Chaignon *et al*. [11]. This theoretical calculation shows that by not removing adsorbed Cu from the root, the $\delta^{65}Cu$ value is determined to be

approximately 50% lower than it is in reality, leading to an underestimate of the degree of fractionation induced by reductive uptake (Figure 1).

Root Washing Methods

The three root washing techniques trialled in this study yielded vastly different concentrations of released metals, including Cu, K and phosphorus (P), between extractants, as well as between the tomato and oat species trialled (Table 2). In general, there were higher concentrations of Cu, K and P extracted from tomato roots than oat roots (Table 2). Extracted K and P concentrations were used to determine if symplastic leakage was occurring. Potassium leakage is a common sign of root impairment [25] and P exists predominantly as the phosphate anion, and hence, is not sorbed on apoplastic cation exchange sites. Our results suggested that the HCl and EDTA extractants removed symplastic metals given the exceedingly high K and P removal in comparison to the La+Ca extraction (Table 2).

Since P is not sorbed to cation exchange sites, we tried to distinguish between adsorbed and absorbed Cu released by the extractant assuming that following equation held:

$$Cu_{extracted} = Cu_{adsorbed} + Cu_{absorbed,extracted} = Cu_{adsorbed} + m \times P_{extracted} \qquad \text{Equation 4}$$

with m the ratio of absorbed Cu to absorbed P released from the symplasm during extraction. The value of m was determined as the slope of the relationship between the Cu concentration and the P concentration in the extract (plotted for all extracts and replicates), and was 0.006 mol Cu/mol P for tomatoes and 0.025 mol Cu/mol P for oats. The amount of adsorbed Cu for each replicate of all treatments was then estimated as follows:

$$Cu_{adsorbed} = Cu_{extracted} - m \times P_{extracted} \qquad \text{Equation 5}$$

where $Cu_{extracted}$ and $P_{extracted}$ are the total extracted Cu or P concentrations (expressed as mmol kg RFW^{-1}). The fraction of Cu in the extract that originated from the adsorbed pool ($fCu_{adsorbed}$) was then estimated as:

$$fCu_{adsorbed} = \frac{Cu_{adsorbed}}{Cu_{extracted}} \qquad \text{Equation 6}$$

For both tomatoes and oats, the calculated Cu released from the symplastic absorbed pool increased in the order La+Ca< EDTA <HCl (Figure 2). The apoplastic Cu concentration is likely best reflected in the La+Ca extraction results, given that this process relies solely on cation exchange. It is possible that the use of a strong Cu complexer (like EDTA), or a harsh acidic extractant, can cause cell wall rupturing through complexation or release of cations that play a role in cell wall integrity (e.g., Ca). However, it should be noted that Chaignon *et al.* [11], who used the HCl extractant to desorb plant roots, assessed the plants for K leakage and did not find signs of symplastic leakage. We believe this may be due to the use of more mature plants in the study of Chaignon *et al*. [11], as there was some evidence in our dataset of relatively more K and P leakage when plants were smaller (data not shown). This may be due to lower cell wall structural integrity (thinner cell walls) in smaller plants [39].

A general trend of decreasing $\delta^{65}Cu$ value was observed as the extractant released more absorbed Cu for the dicots, while no significant change in $\delta^{65}Cu$ between extractants was observed for monocots (Figure 3). However, it should be noted that the results showed a high level of variability. There was a significant difference between the isotope ratios in La+Ca and HCl extracts, but EDTA was not significantly different from either of them (α=0.05), as determined by a Tukey multiple comparison test. More data will be required to determine the robustness of these isotope data. The decrease in $\delta^{65}Cu$ value for dicots as more absorbed Cu is released by the extractant are in line with the previous studies

on isotopic fractionation during Cu uptake by plants [4, 7]. It has previously been shown that light Cu isotopes are strongly enriched in Strategy I plants, likely due to absorption of reduced Cu species, as products of reduction strongly favour light isotopes [4, 7]. Hence, as more symplastic Cu is released into the root wash solution, more light Cu isotopes would be expected to be released into solution. However, Strategy II species have shown limited to no Cu isotope fractionation during absorption [4, 7]. Hence, even with an increase in released symplastic Cu from Strategy II species, no significant change in isotope ratio would be expected. The results obtained here are consistent with the theory that Strategy I plants preferentially absorb light Cu while Strategy II plants do not significantly fractionate Cu isotopes during absorption (Figure 3).

Our results indicate that the adsorbed Cu can be significantly fractionated from the plant available δ^{65}Cu in solution and that adsorbed and absorbed Cu can have different δ^{65}Cu values in Strategy I plants. However, Strategy II plants show no significant fractionation during root adsorption. In order to use Cu stable isotopes to assess plant uptake processes, apoplastic Cu needs to be removed first to avoid interference of the adsorbed δ^{65}Cu signal with that of absorbed Cu. An effective root washing method must remove all adsorbed Cu without damaging the root cell walls/membranes. Damage of the cell walls/membranes results in extraction of symplastic Cu which may contain a different isotopic signature than Cu adsorbed on the root surface. This appears to be the case for Strategy I plant species where root adsorption leads to heavy isotope enrichment on the root surface, but root absorption leads to light isotope enrichment inside the symplasm. On the other hand, neither Cu adsorption or absorption seem to induce considerable fractionation for Strategy II plants. This difference in Cu isotope fractionation during root adsorption on monocots and dicots may be linked to differences in the functional group makeup of their respective cell walls. Our results highlight the importance of considering root adsorption when using stable isotopes to study Cu uptake.

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Tables.

Table 1. The fractionation factor between adsorbed and solution Cu for Strategy I and II plant roots, $\Delta^{65}Cu_{adsorbed\text{-}solution}$ (‰) (2SD in brackets), either determined at low Cu loading using four-week old plants or at high Cu loading using seedlings, as well as the average for both methods.

		Four week old plants		Seedlings		Average
	Species	*n*	$\Delta^{65}Cu_{root\text{-}solution}$	*n*	$\Delta^{65}Cu_{root\text{-}solution}$	$\Delta^{65}Cu_{root\text{-}solution}$†
Strategy I	Tomato	5	0.40 (0.26)	3	0.25 (0.17)	0.34 (0.30)^{a*}
	Canola	3	0.33 (0.18)	5	0.18 (0.22)	0.26 (0.32)^{ab*}
	Bean	4	0.15 (0.06)	4	0.14 (0.14)	0.15 (0.10)^{bc*}
Strategy II	Wheat	6	0.02 (0.18)	7	0.03 (0.16)	0.03 (0.12)^{cd}
	Oat	8	-0.03 (0.37)	6	0.05 (0.04)	0.00 (0.29)^{d}

† Different letters indicate significantly different $\Delta^{65}Cu$ values at the 5% significance level (one-way ANOVA, Tukey's post-hoc test). An asterisk (*) indicates $\Delta^{65}Cu$ values significantly different from 0, i.e., significant root adsorption Cu isotope fractionation has occurred.

Table 2. Extracted element concentrations (mmol kg RFW^{-1}) for tomatoes and oats (results from root desorption trial 1 only shown, n=3).

Species	Root Wash Extractant	*n*	Cu	P	K	Ca	Fe	Mg	Mn	Na	Zn
Tomato	La + Ca	3	0.02	0.10	52.7	N/A	0.01	5.49	2.44	2.11	0.10
	EDTA	3	0.07	4.26	72.3	10.8	1.06	5.85	3.36	N/A	0.20
	HCl	3	0.81	135	1917	112	17.5	156	56.0	23.5	4.84
Oat	La + Ca	3	0.04	0.00	13.9	N/A	0.00	2.48	0.60	2.32	0.18
	EDTA	3	0.33	4.68	58.8	6.48	0.39	6.17	3.26	N/A	0.42
	HCl	3	0.23	2.95	99.0	7.68	0.13	9.69	3.24	7.00	0.67

N/A – not analysed as this ion was present in the extract solution.

Figures.

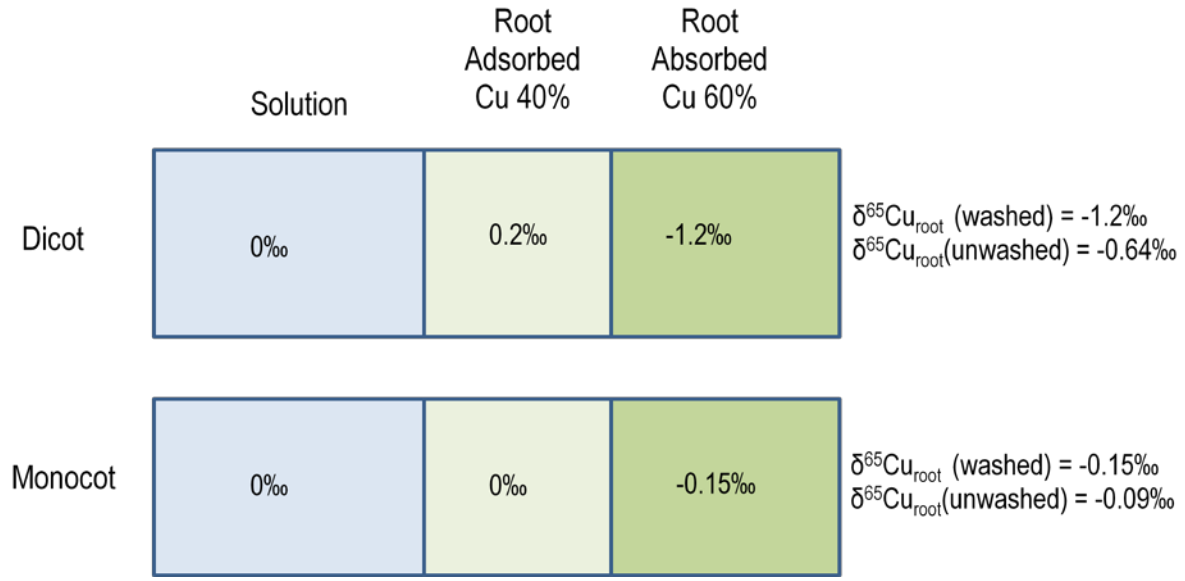

Figure 1. A schematic of how inclusion or exclusion of root-adsorbed Cu affects the measured isotopic signature of root Cu, based on the average values for root-absorbed Cu from Ryan *et al.* 2013 and the average values of root-adsorbed seen in this study. The $\delta^{65}Cu$ value for non-washed roots was calculated from the $\delta^{65}Cu$ of the adsorbed ($\delta^{65}Cu_{ads}$) and absorbed ($\delta^{65}Cu_{abs}$) Cu and the fraction of Cu in adsorbed (fCu_{ads}) and absorbed (fCu_{abs}) pools (assumed to be 40% and 60%, respectively) following: $\delta^{65}Cu_{root}(\text{washed}) = (\delta^{65}Cu_{ads} \times fCu_{ads}) + (\delta^{65}Cu_{abs} \times fCu_{abs})$.

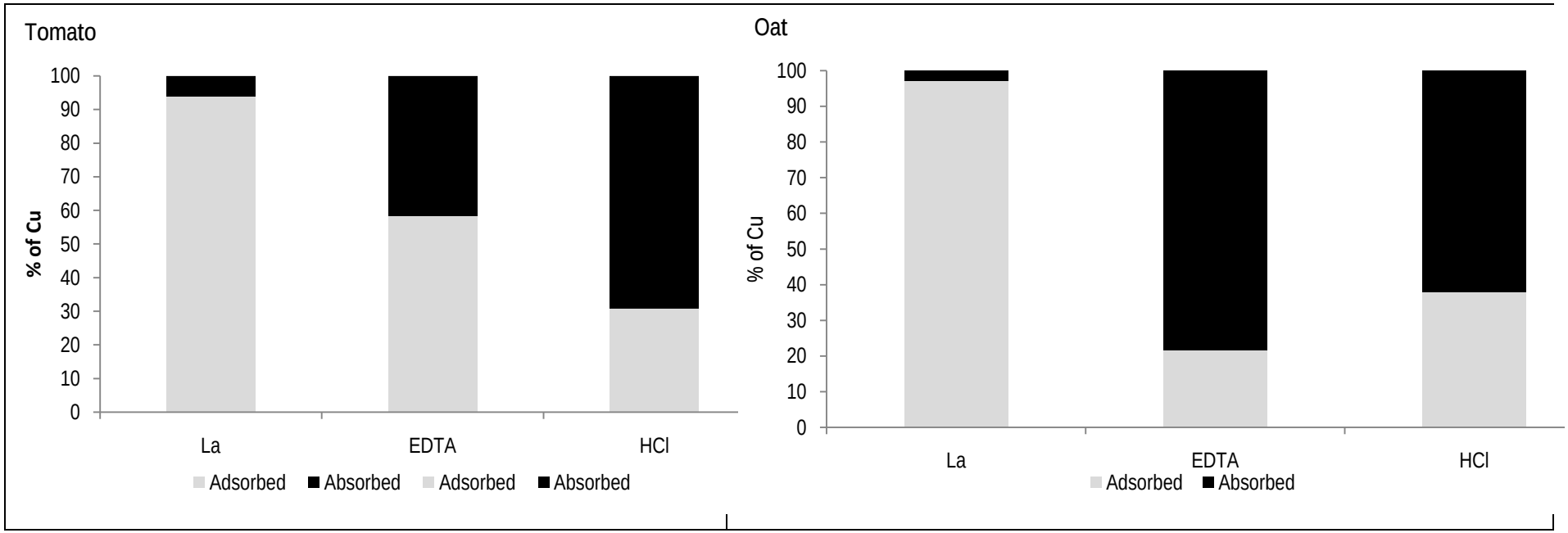

Figure 2. Percentage of Cu estimated to be from the adsorbed and absorbed pools for the three root wash extractants in both trials (n=6) for tomato and oat.

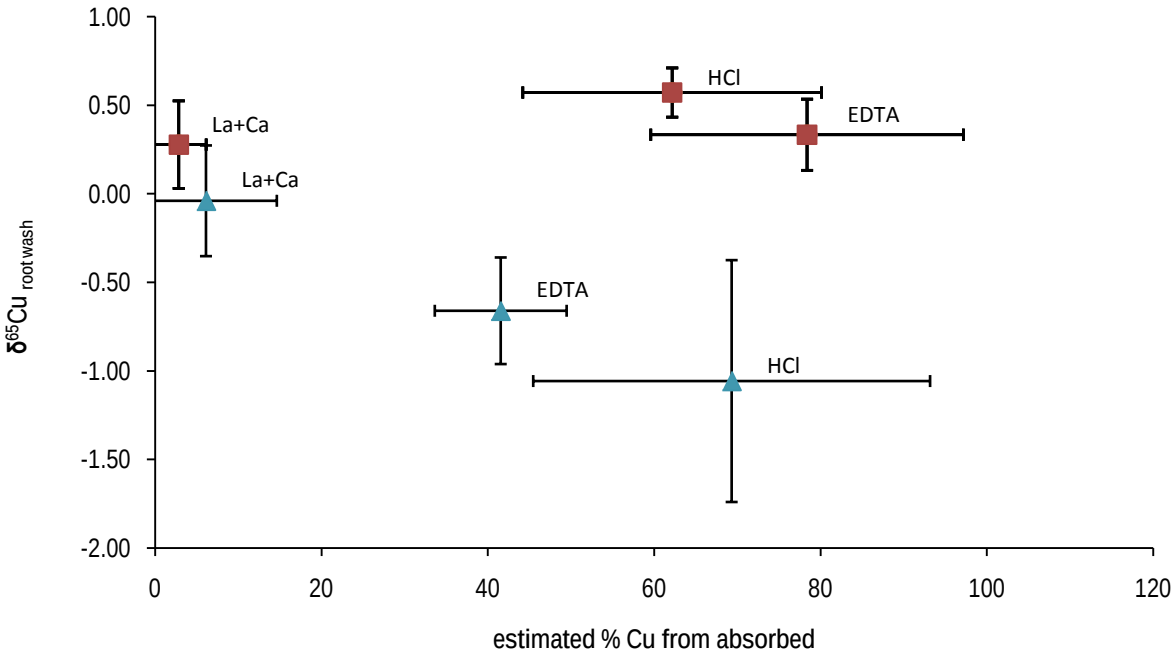

Figure 3. $\delta^{65}Cu$ values for three root wash solutions used to desorb Cu from tomato (closed diamonds) and oat (open triangles) roots against the percentage of Cu in the root wash solution estimated to be extracted from the symplastic (=absorbed) pool. Values presented as average ± 2 SE (standard error) (*n*=3-5).

CHAPTER 5

COPPER SPECIATION AND ISOTOPIC FRACTIONATION IN PLANTS: UPTAKE AND TRANSLOCATION MECHANISMS

The work contained in this chapter has been published in *New Phytologist*.

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Author Contributions

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Name of Principal Author (Candidate)	Brooke Ryan		
Contribution to the Paper	Performed experimental and analysis work, interpreted data and wrote manuscript.		
Signature		Date	

Name of Co-Author	Jason Kirby		
Contribution to the Paper	Assisted with experimental design, solid-state speciation analysis, data interpretation and manuscript evaluation.		
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Signature		Date	28/7/14

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Signature	Date: July 17, 2014

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New Phytologist, *199*(2), 367-378

CHAPTER 6

SUMMARY, CONCLUSIONS AND FUTURE RESEARCH

GENERAL CONCLUSIONS

Copper (Cu) plays an essential role in the functioning of plants as an electron carrier during photosynthesis and is a key component involved in lignin formation (Burkhead *et al.*, 2009). However, Cu is also a prominent environmental contaminant known to exert toxic effects on plants when present at excessive concentrations. Even though the essentiality of Cu and its potential to cause toxicity is well recognised, the processes involved in its mobilisation and uptake by plants are poorly characterised.

The stable isotope fractionation of transition metal elements has recently been used to elucidate information about the plant mobilisation and uptake of these elements (Guelke & Von Blanckenburg, 2007; Arnold *et al.*, 2010). The development of multi-collector inductively coupled plasma mass spectrometry (MC-ICP-MS) technologies has made the detection of the small (sub per mille) isotope fractionation of these elements possible. Arnold *et al.* (2010) concluded from stable isotope fractionation of zinc (Zn) that Zn was absorbed with phytosiderophore complexes into rice. Studies of Fe uptake have shown that distinct Fe isotope fractionations can exist between Strategy I and II plants, both in field- and hydroponically-grown plants (Guelke & Von Blanckenburg, 2007; Guelke-Stelling & von Blanckenburg, 2012). Given the interaction noted in the literature between Fe and Cu uptake, it was hypothesised that Cu isotopes could be used in a similar way to Fe isotopes to elucidate information on potential plant uptake mechanisms.

This thesis has presented the results of experiments focussed on using Cu stable isotopes, coupled with other solid- and solution-state speciation technologies, to gain a better understanding of plant Cu uptake, including the processes occurring in the soil prior to absorption by plants. The results of these investigations showed the following:

1. Copper complexation with natural organic matter and biologically derived ligands (e.g. siderophores) can induce significant isotope fractionation between the soluble free ion and complexed Cu. The heavy isotope was found to partition to the organic complexes, with the magnitude of isotope fractionation correlating with the Cu-ligand complex stability.
2. Adsorption of Cu onto plant roots can induce significant fractionation of Cu isotopes, although this is species dependent. Strategy I plants appear to become enriched in heavy Cu isotopes on root cation exchange surfaces, while Strategy II plants show no discernable fractionation.
3. Copper absorbed by Strategy I plants was found to be enriched with the light Cu isotope, relative to the uptake medium, while Strategy II plants showed minimal isotope fractionation during uptake. This agrees with the results reported for Fe isotopes (Guelke & Von Blanckenburg, 2007; Guelke-Stelling & von Blanckenburg, 2012) and suggests that similar reductive (Strategy I) and complexation (Strategy II) uptake processes are driving Cu and Fe uptake by plants.

The data presented in this thesis have shown that stable Cu isotopes can be useful tools for examining plant uptake and translocation mechanisms. The three key outcomes of this research provide important pieces of information that add to the emerging picture of Cu biogeochemical cycling and the subsequent Cu isotope fractionation involved. After Cu has been released from the solid phase into a mobile form or deposited from an external source into an environment, a number of processes can potentially fractionate Cu isotopes from the original source isotope ratio. Stable isotopes have been suggested as potential tool for examining the biogeochemical cycling of Cu. However, a clear understanding of the isotope fractionation mechanisms associated with various environmental processes is required to enable routine use of stable isotopes for this purpose; e.g. soluble ligand complexation, root adsorption and plant uptake are all important environmental processes that have the potential to fractionate Cu isotopes.

COPPER ISOTOPE FRACTIONATION PRIOR TO PLANT UPTAKE

Copper complexation with soluble organic ligands plays a key role in controlling the behaviour of soluble Cu in environmental systems, with >99% of Cu associated with organic matter in most soils (McBride, 1981). Bigalke *et al.*(2010) previously showed that adsorption of Cu to solid phase organic matter could lead to heavy Cu isotope enrichment in the solid phase. The work presented in this thesis has shown that Cu complexation with dissolved organic matter can also fractionate Cu, with heavy Cu isotopes preferentially partitioning into the organic complex, leaving the free Cu pool enriched in the light isotope. Furthermore, the strength of the Cu-ligand bond appears to drive the magnitude of isotope fractionation. This is a key piece of new information and may prove useful in predicting Cu fractionation in the rhizosphere given the array of potential complexing agents that can exist in soil solutions. Natural waterways also have a variety of organic ligands that can complex Cu, and it has previously been suggested that this complexation drives the fractionation observed in a large scale study of natural waters by Vance *et al*. (2008). When assessing Cu isotope fractionation during biological uptake, the isotopic signature of absorbed Cu is typically compared with that of the total soluble Cu. The information presented in this thesis suggests this method may be an inaccurate approach given the most bioavailable form of Cu, the free ion, may already be significantly lighter than the total soluble δ^{65}Cu value due to soluble organic matter complexation. Without considering the effect of this process it is possible that Cu isotope fractionation measured in plants may erroneously be attributed to uptake processes when in fact they stem from pre-uptake fractionation processes in the soil solution.

Another pre-uptake process that could potentially interfere with the accurate interpretation of plant δ^{65}Cu values is the adsorption of Cu to root cation exchange sites before absorption. The results presented within this thesis suggested Cu adsorption onto root surfaces can significantly fractionate Cu

isotopes, enriching the surface with heavy Cu isotopes. The implications of this are twofold: firstly, if absorption of Cu is dependent on root adsorption as a first step, the Cu available for uptake may be slightly heavier than the free Cu pool, and the true fractionation factor during the absorption process is, hence, different from the one estimated based on the free Cu pool; secondly, when measuring Cu isotopes in plant roots, if this adsorbed Cu is not removed before measurement, the root δ^{65}Cu will be reported as being heavier than it is in reality. This is important when attempting to use isotope mass balance to make inferences about uptake and translocation processes. This was highlighted in Chapter 5 in the discussion around the difference between our translocation fractionation values and those of Jouvin *et al.*(2012) in tomato plants; we found preferential translocation of heavy Cu, while Jouvin *et al.*(2012) did not. While these results may be accurate and related to the different growth conditions of each experiment, it is possible that the lack of root washing by Jouvin *et al.*(2012) led to an artificially heavier δ^{65}Cu root value, hence, when comparing the root and shoot δ^{65}Cu values, a difference could not be detected.

A secondary conclusion from this series of experiments was that some root washing techniques that are routinely used may be too harsh and can extract symplastic Cu, at least in young plants analogous to those used in this study. A $LaCl_3$+$CaCl_2$ extraction was found to be the most reliable technique with low phosphorus (P) and potassium (K) release, while extraction with an ethylenediaminetetraacetic acid (EDTA) solution or with dilute hydrochloric acid (HCl) led to high K and P concentrations in the root wash solutions, suggesting damage of the root membrane and leakage of absorbed nutrients. Isotope data provided further evidence that suggested the EDTA and HCl extractions were removing symplastic Cu, as the extracted Cu was isotopically lighter for tomato plants when using these extractants, compared to the $LaCl_3$+$CaCl_2$extractant. Strategy I plants were shown, both in this thesis and the studies of Jouvin *et al.*(2012) and Weinstein *et al.* (2011), to preferentially absorb light Cu isotopes, most likely due to a reductive uptake process. However, root-adsorbed Cu in Strategy I plants was

found to be enriched in heavy Cu, as shown by the short term solution depletion studies presented in Chapter 4. Hence, the presence of light-enriched Cu in the root-wash solution of Strategy I plant roots would suggest the release of symplastic Cu. This result has important implications for work outside the isotope community, as root washing is required in many plant physiology studies where damage to the root membrane can obscure the measurement of nutrient uptake. For Strategy I plants, where there is a distinct fractionation of Cu isotopes during root absorption, it may be possible to use stable isotope analysis to assess root wash solutions for signs of symplastic Cu leakage, and hence, signs of membrane damage.

COPPER ISOTOPE FRACTIONATION DURING PLANT UPTAKE AND TRANSLOCATION

The most significant finding of this study was that Cu isotopes were differentially fractionated during uptake into Strategy I and II plants. This difference was attributed to the different Fe uptake mechanisms of Strategy I and II species being employed for Cu absorption into roots. A strong enrichment in light isotopes was found in Cu absorbed into tomato (Strategy I) plant roots, which was attributed to reductive uptake via the known Fe reductase mechanism in Strategy I plants. This light isotope enrichment was not found in Strategy II plants (oats) reflecting a lack of reductive uptake, with phytosiderophore/phytochelatin excretion and Cu complexation the suggested uptake mechanism in oats. These results are in accord with the Fe isotope results obtained by Guelke *et al.*(2007) and suggest a strong overlap between Fe and Cu uptake mechanisms. This thesis therefore presents the first evidence that Strategy I and II plants can mobilise and absorb Cu via different mechanisms. However, further research will be required to investigate whether Cu fractionation is influenced by plant nutritional status, as well as plant Fe uptake strategy, as has been suggested for Fe by Moynier *et al.* (2013).

Given the essentiality of Cu as a micronutrient to obtain optimal crop yields, a better understanding of the Cu uptake process may assist with the design of targeted micronutrient fertilisers to enhance Cu absorption in deficient soils. In general, horticultural crops tend to be Strategy I species so the application of Cu as an organically complexed Cu(I) species may enhance root absorption. The literature suggests that soluble organic complexation enhance Cu lability (see Chapter 1). If lability can be enhanced as well as providing Cu in a readily absorbable species, this may help alleviate Cu deficiency in crops more rapidly. However, it remains to be seen if Cu(I) can be provided to an oxic soil environment in a form that is still bioavailable for plant root absorption.

The weathering of Cu mineral deposits can produce strongly fractionated Cu isotope signatures in the soluble fraction that could be used for Cu ore prospecting, either through measurement of solutions or plants. Mathur *et al.* (2009) reported weathered remains of 9 Cu porphyries and found the δ^{65}Cu values ranged from -16.96 to +9.98‰, which is significantly shifted from typical crustal sources of Cu (e.g. 0 ± 0.5‰) (Mathur *et al.*, 2009; Mathur *et al.*, 2012; Mathur *et al.*, 2013). Kimball *et al.* (2009) noted that acid mine drainage from a mineralised watershed was enriched in heavy Cu isotopes by up to +1.6‰. Furthermore, many anthropogenic contaminant Cu sources have isotope signatures different from crustal Cu. It is possible that the assessment of plant Cu isotope ratios could be a preliminary indicator of an anomalous Cu mineral deposit or contaminant source, provided the deposit or contaminant contained an isotopic signature significantly different to the local crustal Cu source.

NEW CONCEPTUAL MODEL OF COPPER ISOTOPE FRACTIONATION

Overall the data in this thesis have aided in the development of a conceptual model for Cu isotope fractionation in soil-plant systems. This model helps with the understanding of what processes are involved in Cu biogeochemical cycling and have shown several commonplace reactions in the

environment can significantly fractionate Cu isotopes. These fractionation processes must be considered moving forward when applying Cu isotope signatures to the understanding of Cu biogeochemical cycling in the natural environment.

At the beginning of this thesis a hypothesised conceptual model of potential Cu isotope fractionating mechanisms in soil-plant systems was presented. This conceptual model is presented again in Figure 1, however, the new fractionation information provided by this thesis has been highlighted in blue, clearly indicating the contribution this work provides to the understanding of Cu isotope biogeochemical cycling in soil-plant systems. The adsorption of Cu to plant root cell walls (process 2) has been shown to enrich the surface of Strategy I plants with heavy isotopes, while not fractionating during adsorption to Strategy II plant roots. The reduction of Cu prior to absorption leads to light isotope enrichment in the reduced species that is absorbed to the symplasm, relative to the growth medium (process 3), although it remains to be seen whether there is isotope fractionation between the reduced species in the apoplasm and those absorbed to the symplasm. Copper complexation with soluble organic ligands has been shown to enrich the complex in the heavy isotope (process 5) and absorption of organically complexed Cu, as was suggested for Strategy II plants in Chapter 5, showed minimal fractionation, relative to the Cu ion in solution (process 8).

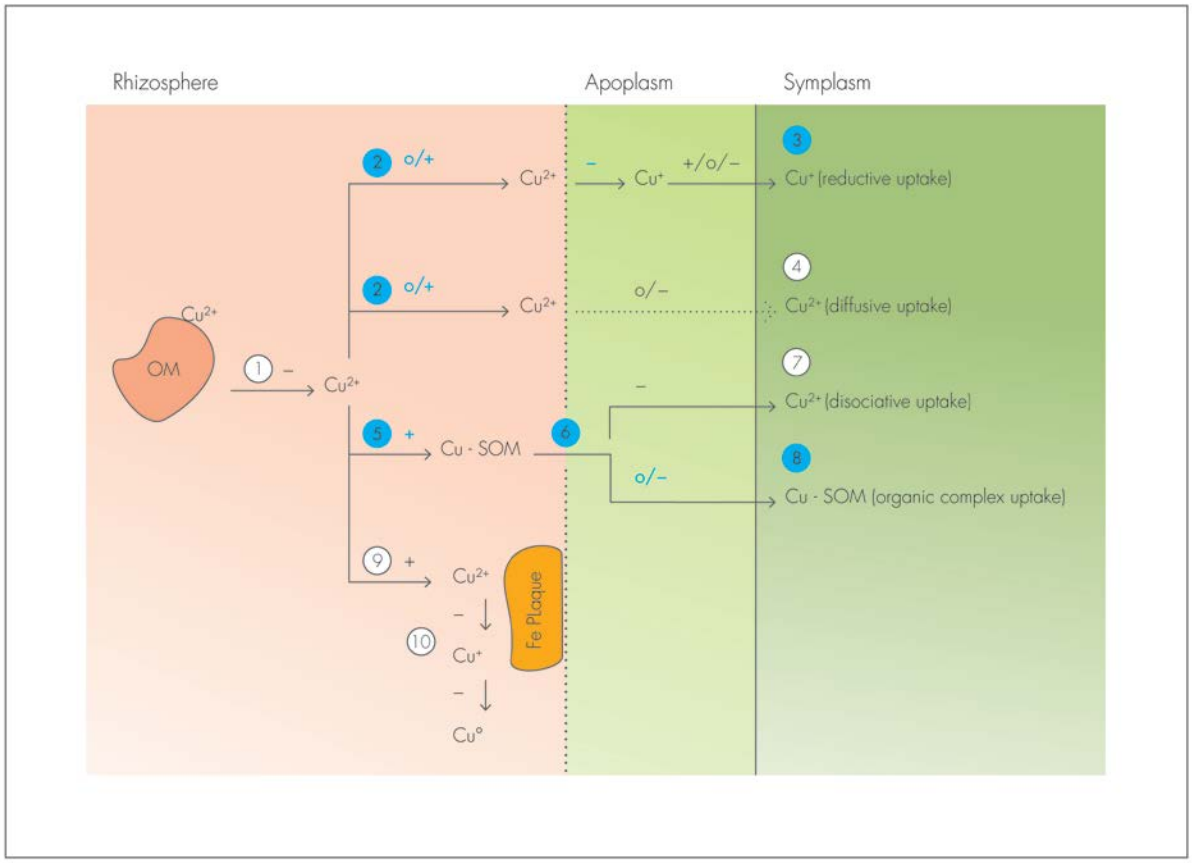

Figure 1.Potential Cu uptake mechanisms and movements in soil, and the hypothesised fractionation direction (black) and measured isotope fractionation direction (blue) associated with each pathway. '+' = heavy isotope enrichment, '-' = light isotope enrichment, OM = organic matter and SOM = soluble organic matter. 1 - desorption of Cu from the solid phase (shown here as organic matter); 2- cell wall adsorption of $Cu^{2+}$to cation exchange sites on the root cell wall; 3 reduction of Cu^{2+} to Cu^{+} in the apoplasm and subsequent uptake of the reduced species into the symplasm; 4 - diffusion of Cu^{2+} adsorbed on the cell wall into the symplasm; 5 - complexation of Cu^{2+} with SOM in the soil solution; 6 – diffusion of the Cu-SOM complex into the apoplasm, followed by either dissociation and free Cu^{2+} uptake (7) or uptake of the Cu-OM complex into the symplasm (8); 9 - sorption of Cu^{2+} to Fe plaques on the root surface followed by subsequent stepwise reduction to elemental Cu (10).

FUTURE RESEARCH PRIORITIES

This work has highlighted a number of questions that should be answered and further research to be completed in order to improve our understanding of how Cu isotopes can be used in environmental systems. Suggestions for future research in this field are:

1. More Strategy I and II plant species should be examined to see if the inferences around Cu isotope fractionation being driven by Strategy I and II Fe uptake mechanisms are valid. This will help determine how robust and variable the results are that were presented in Chapter 5.
2. In order to extend the results presented in Chapter 5 to 'real word' samples, the effect of a soil medium on isotope fractionation induced by plant absorption of Cu should be examined. A variety of Strategy I and II plant species should be grown in soil, and the absorbed Cu isotope ratios should be compared to total soil solution Cu as well as free Cu, isolated by Donnan dialysis. These data are critical to assessing how useful Cu isotopes are for tracing uptake mechanisms and Cu cycling *in situ* in the soil environment.
3. The robustness of the relationship between bond strength and Cu isotope fractionation factors should be examined by assessing more organic ligands with the established Donnan dialysis technique.
4. Chapter 5 presents some preliminary information that suggests redox cycling is driving Cu translocation in Strategy I plants, resulting in Cu isotope fractionation between root and shoot, while a non-fractionating translocation mechanism appears to be present in Strategy II plants. More investigation is required to examine whether the proposed explanation of Cu(I) oxidation to Cu(II) followed by nictotianamine complexation is in fact responsible for Cu translocation in Strategy I species. It may be the case that translocation mechanisms and subsequent isotope fractionation are more related to nutritional status rather than inbuilt into the physiology of the plant nutrient uptake strategy. Furthermore, more data should be collected to examine the effect this translocation process has on the Cu isotope signature present in plant vegetative tissues.
5. The suitability of plant isotope signatures for identifying sites of Cu contamination or anomalous mineral Cu deposits should be further investigated. While mobilisation processes are likely to fractionate Cu to some degree, the $\delta^{65}Cu$ of the soluble Cu fraction will be influenced by the underlying Cu sources. Strategy II plants may be more useful for this task than Strategy I plants,

given it has been shown in this thesis that Strategy II plants induce minimal isotope fractionation between solution and plant tissues during Cu absorption.

6. As an extension to point 5, research should be conducted into the efficacy of using Cu isotopes to trace the movement and sources of Cu contaminants that have isotopic signatures significantly different from background crustal Cu sources.
7. The finding that Strategy I plants absorb Cu as Cu(I) could potentially be exploited to design more bioavailable micronutrient fertilisers. Further studies should be conducted to determine if providing Cu as Cu(I) to the soil is possible, and if so, whether it enhances Cu absorption. In areas of severe micronutrient deficiencies investigations should be undertaken to determine if an adequate Cu(I) fertiliser can be designed that alleviates Cu deficiency more effectively than standard Cu(II) fertilisers.

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