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A Modelling Approach: Effects of Exuded Organic Acids and Exogenous Chelating Ligands on Bioavailability and Uptake of Metals by Radishes (*Raphanus sativus*) Grown in Hydroponics.

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

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Abstract

Metal contamination of soil can be reduced by adding chelators to improve the efficiency of metal uptake in phytoremediation, but optimal concentrations and types of chelators have not been determined. A geochemical model (Visual MINTEQ3.1) was used to estimate the effects of four chelators on the solubility of four metals in hydroponic solution. The model showed that no iron was soluble in the absence of a chelator, while the solubilities of cadmium, copper and zinc were high with or without chelators. Despite low iron uptake in all treatments, symptoms of iron-deficiency were not visible. High concentrations of exuded organic acids in solution had negligible effects on metal solubility because few metal-organic acid complexes formed. The amounts of metals taken up by radish (*Raphanus sativus* L.) varied with the type of chelator provided. EDTA and DTPA maximized cadmium and zinc uptake, respectively.

Keywords

Chelation, cadmium, copper, zinc, uptake, toxicity, modelling, radish, solubility, speciation.

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Chapter 1

1 General Introduction

1.1 Overview

Soil and water are frequently contaminated by metals, some of which are non-essential elements and therefore may be toxic to plants even at low concentrations, such as cadmium and lead; others are essential nutrients that are toxic only at high concentrations, such as iron and magnesium (Ahmad et al. 2014). Low concentrations of these metals are naturally present in the earth; however, anthropogenic sources of contamination such as mining, smelting, car fumes, and fertilizers, among others, have increased the likelihood of finding metals in dangerous amounts. As of 2015, there are reports of more than 10 million sites of soil pollution worldwide; more than half of these are polluted with metals (He et al. 2015). While the United States Environmental Protection Agency (EPA) states that levels of copper, cadmium, lead and zinc in agricultural soil should not exceed 1500, 39, 300 and 2800 mg/kg, respectively (Environmental Protection Agency 2010), concentrations of lead up to 3680 mg/kg have been found in garden soils in the Massachusetts, USA (Clark et al. 2006), and concentrations of other metals are often similarly above permitted limits (Monfared-Heidarey 2011).

Plants have been used to bioremediate contaminated soils (Marques et al. 2009); however, such phytoremediation efforts have had mixed success (Koptsik 2014). One of the most practical methods to predict metal uptake by plants is by using modelling software. Programs such as Visual MINTEQ and Geochemist Workbench can help to determine soluble and insoluble metal quantities in soil solution, as well as their speciation (i.e., chemical form) while they are soluble. By using these models, it is possible to determine what are the best conditions to increase plant metal uptake. The use of software modelling allows the user to obtain accurate predictions and save time and money when selecting the conditions to maximize metal uptake and optimize bioremediation of soil.

1.2 Factors that affect metal bioavailability

Metals may be present in several forms with different levels of solubility, including as dissolved species (free ions, chelated ions, soluble salts), exchangeable species (organic and inorganic compounds) and as precipitates (oxides, phosphates), which are insoluble and cannot be taken up by plants (Zalidis et al. 1999). Bioavailability of metals is affected by many factors, including, but not limited to, pH, organic matter content, cation exchange capacity, microbial activity, and root exudates (Nascimento and Xing 2006; Ruttens et al. 2006; Zeng et al. 2011). In soil and nutrient solution, the most relevant factor is pH, which plays a crucial role in determining metal speciation, solubility and movement, which are all necessary for bioavailability of metals (Zhao et al. 2010). Decreased or increased soil pH may reduce the mobility and bioavailability of metals (Kalra 1995; Badawy et al. 2002; Du Laing et al. 2007) because each element has an optimal pH for solubility. Organic matter can also influence metal uptake for two reasons: (1) it supplies organic chemicals that can serve as chelators, which can increase metal solubility and increase metal uptake (McCauley et al. 2009); and (2) it can serve as a cation exchange site for metal ions (Kennou et al. 2015). Root exudates, which include organic acids and other molecules that can act as chelators, forming metal-chelate complexes that can increase metal solubility (Mehes-Smith et al. 2013).

A single metal ion is held within each chelator molecule by ionic bonds (IUPAC 1994). Whether or not chelation increases or decreases metal ion bioavailability depends on the relative binding affinity of the chelate for the metal ion. Binding affinity is the strength of binding between one molecule and another molecule or ion, and is measured as the chemical equilibrium dissociation constant (K_D). Different chelators have different affinities and may selectively chelate one metal over another.

Microbial activity also plays a big role in phytoextraction processes, especially in soil. Bacteria and fungi at the soil-root interface improve the accumulation of nutrients in plants by either enhanced nitrogen fixation or improved solubilization of nutrient elements, including nutrient metals, by breaking down insoluble organic compounds present in soil (Wood et al. 2016).

1.3 Metals in the plant environment

1.3.1 Effects of metals on plants

Lead and cadmium are non-essential metals that can cause harmful effects on plants. Copper and zinc are essential micronutrients that are required for optimal plant development, but elevated concentrations of these metals have negative effects on overall plant health. One of the most important sources of metal toxicity for plants is agriculture. Organic and inorganic fertilizers, may contain low concentrations of metals (Semu and Singh 1995). These fertilizers are manufactured from rock phosphates, which often contain metal impurities, including cadmium, arsenic, antimony chromium, zinc, copper and nickel (Camelo et al. 1997; Sabiha-Javied et al. 2009). Insecticides and fungicides also contain low concentrations of metals, which are intended to target the pest organisms (Semu and Singh 1995). Many copper-based pesticides are used in agriculture, and have the capacity not only to affect plants in soil, but also to leach into fresh water bodies and cause widespread damage to local fauna (De Oliveira-Filho et al. 2004). Although the concentrations of metals in a single dose of fertilizer or pesticide is relatively low, the repeated application of these products eventually increases the metal concentration in soil and water (Markert 1993).

In this thesis, I will focus on the effects of cadmium, copper and zinc because they are common contaminants in the soil (Clark et al. 2006) and I will examine how exogenous chelators affect the solubility of these metals. Because iron solubility is controlled mostly by chelation (Visual MINTE 3.1) and iron may compete with the previously mentioned metals for the chelators, I will also study how the combination of metal treatment and chelation affects iron solubility. Plants exposed to cadmium toxicity usually present symptoms including chlorosis, growth inhibition, browning of root tips and death (Sanita Di Toppi and Gabbrielli 1999; Wójcik and Tukiendorf 2004; Mohanpuria et al. 2007). Cadmium affects several plant processes including the uptake, transport and use of essential nutrients (calcium, magnesium, phosphorus and potassium) due to competition for the same trans-membrane carriers (Clarkson and Lüttge 1989), and can also reduce the absorption and fixation of nitrogen by inhibiting nitrate reductase activity in the shoots (Hernandez et al. 1996; Balestrasse et al. 2003). Cadmium has a similar atomic

radius to calcium, copper, manganese and zinc, and it can cross the root cell membranes through transporters for these essential elements (Verkleij et al. 2009)

Copper is a micronutrient and has an important role in the respiratory electron transport chain, as well as in CO₂ assimilation and ATP synthesis (Demirevska-Kepova et al. 2004). When present in toxic quantities, copper is cytotoxic because it can produce oxidative stress and reactive oxygen species via the Fenton reaction (Lewis et al. 2001), as well as causing growth retardation and leaf chlorosis by reducing iron uptake and directly interfering with chlorophyll synthesis (Duvign 2000) .

Zinc is also an essential micronutrient for plants that helps with a wide variety of processes, including influencing the activity of hydrogenase anhydrase, stabilization of ribosomal fractions, as well as helping with the maintenance of the integrity of cellular membranes and protein synthesis (Hafeez et al. 2013). Excess zinc causes effects such as chlorosis and retarded growth and hastened senescence (Choi et al. 1996; Ebbs and Kochian 1997). Zinc has a similar atomic radius to iron, copper and manganese, and therefore can substitute for them as a cofactor in enzymes when present in high concentrations and can compete with them for access to transport proteins (Shanmugam et al. 2011), causing deficiency of competing metals and therefore stunting plant growth.

1.4 Use of chelators in phytoremediation

Phytoremediation is based on the use of natural or genetically modified plants capable of extracting hazardous substances from the soil (Adriano 2001). Modern phytoremediation is divided into four methodological approaches: phytoextraction, phytomining, phytostabilization and phytoevaporation, and often uses plants that are classified as hyperaccumulators (Rascio and Navari-Izzo 2011) Hyperaccumulators are classified depending on the minimum concentration of metal in plant dry tissue. This concentration has been established to be 10,000 µg/g for zinc, 300 µg/g for copper and 100 µg/g for cadmium (van der Ent et al. 2013). Hyperaccumulators do not take up excess metals for their own benefit. They protect themselves from metal toxicity by sequestering the metal ions in sites removed from metabolic activity, such as in trichomes, vacuoles, or in the apoplast (Meyers et al. 2008; McNear and Kupper 2014)

Phytoextraction is based on the absorption of metals by roots and subsequently removing those metals with phytomass harvesting. This technique is especially effective when hyperaccumulators are able to translocate high amounts of metals into their shoots. However, remediation is limited by its slow time to take up substantial amounts of metals, as well as an increased risk of leaching of metals into water bodies when applying chelators when compared to plants grown without the presence of chelators (Ghosh and Singh 2005). The use of chelators for this technique is not essential, however, it can help to increase metal uptake by plants (Mehes-Smith et al. 2013). Phytomining is a special case of phytoextraction in which the concentration of a metal in the harvestable plant tissue is high enough such that it is economically feasible to recycle the metal. After harvest, the plant tissues are combusted to obtain bio-ore. In theory, phytomining can be utilized to mine any type of metal in plants, although nickel is the most phytomined metal (Brooks et al. 1998). The environmental advantages and disadvantages of phytomining are the same as for phytoextraction, with the added benefit of obtaining a sellable product (bioore). Its main disadvantage is its limited use due to the few number of hyperaccumulators (Koptsik 2014). Phytostabilization involves reducing the mobility and availability of metals in soils in the rhizosphere – the region of soil that is under the influence of root exudates (Pulford and Watson 2003). Plant-induced chemical changes in the rhizosphere include altered pH, redox potential or increased concentration of chelators (Raskin and Ensley 1999a). Phytostabilization is useful for reducing leaching of metal pollutants to the groundwater but, because phytostabilized metals are not taken up by the plants, it is not an approach that can be used to remove contaminants from the environment. The main advantage of this technique is its low cost due to the lack of need to remove biomass from contaminated areas; however, high concentrations of metals accumulating in the rooting zone may eventually prevent survival of plants (Raskin and Ensley 1999b). Finally phytoevaporation is based on the capacity of plants to absorb organic contaminants (petroleum products, ammunition contaminants and pesticides) and transform them into low-toxicity volatile compounds (Prasad and De Oliveira Freitas 2003). Its main disadvantage is its low versatility, especially since only a few contaminants can be volatilized into harmless compounds. While the vast majority of

metals are not volatile, some species of the Brassicaceae family are effective at removing selenium from soil through this mechanism (Pulford and Watson 2003).

While phytoextraction is less disruptive to the soil and costs less than chemical or physical techniques to remove contaminants (Marques et al. 2009) it has some disadvantages. For example, it can take decades to bring contaminants down to acceptable concentrations. Another main disadvantage of this technique is the plant's inability to absorb certain forms of metals, including insoluble compounds such as hydroxides and sulfides that tend to precipitate and complex with organic materials (Sillanpa and Sihvonen 1997).

Phytoextraction is more efficient after a contaminated soil is treated with a chelator. Since ethylenediamine-tetraacetic acid (EDTA) has been reported to aid in plant nutrient uptake from hydroponic solution since 1949 (reviewed in Klein and Manos 1960), it was one of the first chelators tested for phytoremediation. EDTA and other synthetic chelators, including nitrilotriacetic acid (NTA), diethylenetrinitriolo-pentaacetic acid (DTPA) and N-(2-hydroxyethyl)-ethylenediamine-triacetic acid (HEDTA) have been shown to enhance phytoextraction of metals (Sun et al. 2001). Studies done with diverse plants, such as rattlebush (*Sesbania drummondii*), corn (*Zea mays*), and sunflower (*Helianthus annuus*), among others, have shown that chelators can increase uptake of lead up to 40 times (Ruley et al. 2006) and copper up to 100 times (Luo et al. 2005), while plants such as rice (*Oryza sativa* L.) and oilseed rape (*Brassica napus* var .Ladoga) have shown a positive iron uptake increase of up to 100% (Hasegawa et al. 2011; Bloem et al. 2017)

Currently, synthetic chelators such as EDTA, HEDTA and DTPA are most commonly used to improve plant uptake of elements ; however, new biodegradable chelators such as nitrile-triacetic acid (NTA) have been gaining popularity because they will not linger in the environment due to efficient degradation by microbes (Nancharaiah et al. 2006).

1.5 Chelators exuded from plant roots

Plants usually have large root surface areas with membrane transporters, which are able not only to take up nutrients but also contaminants (Meagher 2000). The non-essential metals can enter through non-specific membrane channel proteins (Raskin et al. 1994). Plants can increase the solubility of essential metals by several mechanisms, including the production of root exudates that either alter the pH of the rhizosphere or chelate metal ions (Mehes-Smith et al. 2013), both of which will affect the bioavailability of essential and non-essential metals as mentioned in Section 1.2. Some common root exudates include carbohydrates, amino acids, organic acids, and proteins. Of these, organic acids are the ones most likely to chelate metal ions (Badri and Vivanco 2009).

Bioavailability could increase or decrease depending on the direction of pH change and the chemistry of the metal-chelate complex, giving the plant some control over nutrient uptake and contaminant exclusion. For example, exudation of organic molecules can be triggered by nutrient deficiency or by stress caused by contaminants (Javed et al. 2013). Low phosphate concentration in soil can cause plants to release extracellular phosphatases, which hydrolyze and mobilize inorganic phosphorus (Duff et al. 1994). Plants can secrete about 20-fold more acid phosphatases from roots when they are subjected to low phosphorus conditions than when compared to sufficient phosphorus conditions (Tadano and Sakai 1991). Exudation of phenolic molecules is also very useful in order to influence iron mobility. Iron-deficient alfalfa (*Medicago sativa*) plants produce phytoalexins, which dissolve ferric phosphate and generates the soluble ferrous iron (Masaoka et al. 1993). Other plants, such as tomato (*Solanum lycopersicum*), can exude caffeic acid, which also solubilizes iron, while cereals and grasses exude phytosiderophores, which can solubilize ferric compounds for uptake by roots (Romheld and Romheld 1987).

Exudates also play an important role in metal detoxification. The exudation of organic acids, such as malate and citrate, is higher in aluminum-tolerant plants than aluminum-sensitive plants. The formation of aluminum-organic acids complexes create a slower transport through the plasmalemma, reducing the uptake of metal throughout the plant's lifespan (Kochian 1995). The production of other exudates, such as phenolic compounds,

help with metal detoxification of aluminum as well, mostly by creating a deprotonation reaction of phenolics in the presence of organic acids, which in turn strengthens the interaction between aluminum ions and organic acid ligands (Driscoll and Schecher 1990).

1.6 Study species

Radish (*Raphanus sativus* L. cv. Crimson Giant Champion) has been previously used in several studies (Georgieva et al. 1997; Garg and Kataria 2009; Hamadouche et al. 2012; Hladun et al. 2015) to investigate its potential use as a phytoremediating agent that targets metals such as cadmium, lead (Hamadouche et al. 2012), copper and zinc (Vamerali et al. 2012). However, its phytoremediation efficiency varies. Under field conditions and when exposed to a pluri-contaminated site, radishes were more effective at extracting zinc than other metals, and showed poor results when extracting cadmium by taking up only 0.63 mg/kg (Vamerali et al. 2010). However another study showed high cadmium extraction (7.26 mg/kg) by radish plants exposed to 10 mg/kg cadmium in soil (Lin et al. 2014)

While radish plants are accumulators of certain metals, the mechanisms for this are still poorly understood. Root exudation of hydrogen ions, as well as molecules such as OH^- and CO_3^{2-} and organic acids, may increase or decrease pH depending on the conditions of the environment, therefore increasing or decreasing metal solubility (Javed 2011). Low molecular weight organic acids (LMWOAs), such as citrate, oxalate, and gluconate, may play a central role in cases of tolerance to metals such as aluminum (Wang et al. 2015), but the exudation of these compounds has not been investigated in radishes. Some studies done with these organic acids show that they may inhibit metal uptake under acidic conditions, but may enhance it under neutral to alkaline conditions by generating aqueous organic complexes (Wang and Mulligan 2013).

Even though radishes have been previously tested as potential phytoremediating agents, their efficacy when combined with the use of chelators is still not clear.

1.7 Role of software modelling

Chelators have different affinities for different metals under specific conditions, but their efficacy in increasing metal solubility still has not been properly determined. One way to determine their potential role in phytoextraction is with the use of modelling software. Computer software such as Visual MINTEQ 3 (Gustafsson 2013) has been used to generate computer-simulated information about the bioavailability of metals in response to changes in solution pH and chelate-metal complexes that are formed in the nutrient solution. However, these models have not been used to assess changing metal bioavailability during plant growth. The information that is needed to improve the models includes plant-induced changes in pH, changes in nutrient and metal concentrations as the elements are taken up by the plants and the concentrations of chelators exuded by the roots. By obtaining experimental data and adding it into the model, it may be possible to increase the accuracy of the model and use it to predict the conditions necessary to maximize metal uptake by radishes.

Chapter 2 contains a detailed description of Visual MINTEQ 3.1, including the parameters that are used to calculate metal solubility, the variables that one can manipulate to model a variety of environmental conditions, as well as the types of output that are generated.

1.8 Objectives

The main goal of this research is to identify and analyze the root exudates produced by radishes grown in hydroponics, while at the same time obtaining information about plant uptake of the key nutrient iron, as well as that of metal contaminants. This information will then be processed using the software Visual MINTEQ 3.1 and the results will help to predict the best way to maximize (or minimize) the uptake of the tested metals by radishes. This goal will be accomplished by completing the following objectives:

1. Determine the direction and rate of change of pH in nutrient solution in which radish is growing in order to establish the pH to be used in modelling,

2. Measure total plant dry mass to determine the effects of exogenous chelators on relative metal tolerance,
3. Quantify metal (copper, iron, cadmium and zinc) uptake by radish to compare the model's predicted metal bioavailability to the amounts actually taken up by the plants,
4. Identify and quantify organic acids in the nutrient solution to determine the concentrations of exudates to be used in modelling,
5. Improve the Visual MINTEQ model by adding concentrations of exuded organic acids to the database,
6. Make recommendations of the best chelators used to increase metal uptake.

1.9 Rationale for experiments

Radishes were selected as a plant of interest because previous studies identified it as a potential accumulator of cadmium, copper and zinc (Georgieva et al. 1997; Garg and Kataria 2009; Vamerli et al. 2010; Hamadouche et al. 2012; Lin et al. 2014; Hladun et al. 2015) and because of its rapid growth rate. Radishes can grow from seed to full maturity in 30 to 40 days, which makes them viable candidates for phytoremediation purposes since they can be grown and harvested several times per season. The selection of a hydroponic system was based on eliminating as many interfering factors as possible, especially microbial interactions. While the concentrations of the metals that will be studied will be lower than those in natural occurring soil, plants (including radishes) take up higher amounts from hydroponic due to the chemical simplicity of the culture (e.g., no microbes, no binding of metals to soil particles, controlled pH, etc.) (Salvatore et al. 2012) as well as the morphology of roots grown in solution (e.g., larger regions of the root where solutes can enter the xylem without having to cross the plasma membrane) (Bloem et al. 2017).

Chapter 2

2 Modelling software: Visual MINTEQ 3.1

A number of software programs, including Visual MINTEQ, PHREEQC, The Geochemist's Workbench, and GEOCHEM-PC, are available that help us to make predictions about the effects of exuded and exogenous ligands (e.g. chelators) on metal availability in solution. In general, these programs use thermodynamic equilibrium constants, compound solubility, as well as other factors such as pH, ionic strength, and aqueous complexation reactions, to estimate the chemical fates of elements in solution. For this project, the modelling program that will be used is Visual MINTEQ 3.1, developed by Jon Peter Gustafsson of the KTH Royal Institute of Technology in Sweden (Gustafsson 2013). Visual MINTEQ 3.1 was chosen due to its robust and updated database, ease of use, as well as its common use by other researchers. Its capacity to calculate speciation of ions and complexes in water, as well as its capacity to simulate changes in chemical composition of a water sample after adding different elements and compounds, make it a great option for this research project.

The information below is intended to provide readers with a better understanding of the chemical databases included in Visual MINTEQ 3.1, the variables one should manipulate to estimate metal solubility in nutrient solution, and the nature of the model's output.

2.1 Visual MINTEQ 3.1

2.1.1 Introduction

Visual MINTEQ uses four different databases to generate accurate modelling results. The **solids database** contains all the solid compounds that can be modeled by the software. These include, but are not limited to, solids that tend to precipitate out of solution such as oxides and phosphates. Chemical information about the solids included in this list has been taken from the NIST (National Institute of Standards and Technology) 2.1.1 database. The main **thermodynamic database** includes all the elements and compounds that can be modeled by the software, and includes information such as charge, ion size (radius), molecular weight, stability constant and enthalpy change (ΔH). The **component**

database includes all the possible elements and compounds that the user can input to the model. This includes organic and inorganic compounds. It is important to note that the component database includes all the exogenous chelators that will be used in this research project, as well as organic acids that are expected to be exuded from the plant roots. Finally, the **DOM complex database** contains information that allows the software to model dissolved organic matter.

The data required by Visual MINTEQ to predict the equilibrium composition of a certain solution, consists of a chemical analysis of the sample, as well as other components such as pH, PE, as well as minerals present in the solution. Visual MINTEQ computes several formulae in order to obtain data such as saturation index (SI), which is required to compute solids in solution. The model starts its calculations using the assumption that all components of the solution are ionic. Since the chelation of metal ions, the formation of metal salts (e.g., Cu SO_4), and precipitation of solids in solution would affect the concentrations of elements available for these and other reactions, Visual MINTEQ performs up to 5000 iterations of the computations in order to calculate the correct equilibrium speciation in solution accurately (Allison et al. 1991)

Visual MINTEQ allows the user to input different values for factors that would affect modelling, including but not limited to: solution pH, alkalinity (capacity of a solution to neutralize an acid) measured as CaCO_3 , solution temperature, solution ionic strength, as well as different components (organic and inorganic) and their respective concentrations. Visual MINTEQ also allows the user to exclude chemical species and to specify solid phases, redox couples, pE (redox potential) and Eh (electron or oxidation potential) as well as CO_2 and other gas (including but not limited to methane, nitrogen and oxygen) pressure.

2.1.2 Settings used for current project

In order to get the most accurate results possible, several settings were optimized. The following sections will explain each of the modifications, and their impact on the modelling results.

2.1.2.1 Saturation of solids

Saturation of solids is one of the most important aspects that must be tuned in order to obtain accurate predictions for metal bioavailability. With the default setting, solids that will tend to precipitate in an aqueous solution are not allowed to precipitate in the model, meaning that 100% of solids such as FeO, FeOH, CuO, ZnOH, PbOH, among others, will be modelled as if they were mixed in solution, therefore being readily available for plant uptake.

Several studies have shown that metals in solution tend to oxidize in alkaline conditions, forming insoluble compounds that will not be readily available for uptake (Chuan et al. 1996; Walker et al. 2003; Shahid et al. 2012). Therefore, if one wants to use the model to estimate bioavailability of metals to plants, it is necessary to force Visual MINTEQ to allow all insoluble compounds to precipitate out of solution.

2.1.2.2 Redox couples

Redox reactions are of great importance for estimating metal bioavailability due to the importance of different charge states of metal ions. Ions with different charges will form different compounds which may affect solubility of metals in solution. While it is possible to input metal compounds in the form that they were added to the nutrient solution (for example, iron as ferric iron, Fe^{3+} , copper as the cupric ion, Cu^{2+} , and the zinc ion, Zn^{2+}), the model needs to take into consideration that redox reactions will happen in solution. Of the metals used in my experiment, cadmium (Cd^{2+}) and lead (Pb^{2+}) are the only ones that will not experience redox reactions, since their oxidation state is the only one possible under my experimental conditions. Above pH 3.5, ferric iron tends to form oxides; only at a very low pH, is it a free Fe^{3+} ion and readily available for uptake (Brumbarova and Bauer 2009). In contrast, ferrous iron, Fe^{2+} , remains soluble up to a pH of about 8, at which point it tends to form oxides. Thus, redox reactions in solution can alter iron availability by converting Fe^{3+} to Fe^{2+} and vice versa. For my modelling experiments, the $\text{Fe}^{3+}/\text{Fe}^{2+}$, $\text{Cu}^{2+}/\text{Cu}^+$ and $\text{Zn}^{2+}/\text{Zn}^+$ couples will be enabled, allowing the program to perform the thermodynamic calculations for these reactions to obtain accurate estimates of metal availability. It is important to note, however, that the Zn^{+1} state is very

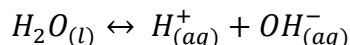
rare, and can be found only under very specific temperature and pressure conditions (Rappoport and Marek 2006). Low pH tends to increase metal solubility and plant uptake because the ions are stable, but can cause toxicity if pH is too low. High pH tends to decrease metal solubility due the formation of oxides and hydroxides. This may cause nutrient deficiency since the formation of hydroxides prevents the plants from taking up vital nutrients (Ross 1994).

2.1.3 Temperature

A solution temperature of 25°C is normally used in chemical modelling unless it is stated otherwise (Beverskog 1997). Temperatures higher or lower than 25°C have an effect on thermodynamic reactions, including Gibbs energy, enthalpy and entropy. At the same time, temperature has a direct effect on pH. In order to understand that, it is important to understand the ionic product for water constant, the water equilibrium formula, and how that relates to pH.

Since water molecules can function both as acids and bases, a water molecule can accept a hydrogen ion from a second water molecule. That way, a water molecule acts as a base (recipient), while the other one functions as an acid (donor) (Geissler et al. 2001).

However, since the hydroxonium ion is a very strong acid, and the hydroxide ion is a very strong base, they recombine in less than 70 µs, forming water again. The formation of water is represented by the following equation:



The ionic product for water, or K_w , is the equilibrium constant for the reaction:

$$K_w = [H^+][OH^-]$$

It is important to note that the ionic product for water, K_w , is temperature-dependent, increasing with temperature (Tabbutt 2001) .

At room temperature (25°C), K_w has a value of 1.00×10^{-14} . However, for each hydrogen ion arising from water, it is necessary to also have a hydroxide ion. At room temperature,

the concentration of hydrogen ions equals the concentration of hydroxide ions and, therefore, it is possible to rewrite the equation as following

$$[H^+]^2 = 1.00 \times 10^{-14}$$

If the equation is solved for [H], then the following is generated

$$[H^+] = 1.00 \times 10^{-7}$$

If we substitute the hydrogen concentration in the pH equation, we get the following

$$pH = -\log_{10}[H^+] = -\log_{10}[1.00 \times 10^{-7}] = 7$$

Therefore, it is possible to understand why pH has a value of 7 when it is neutral, since there is a balance between total hydroxide and hydrogen molecules. Since K_w is temperature-dependent, pH changes depending on temperature, therefore playing a very important factor in the generation of accurate speciation. The model can be adjusted for temperatures between 20 to 60°C, with the model being designed to work best at a temperature of 25°C. This project uses the standard temperature of 25°C even though the temperature of the nutrient solution was 23°C.

2.1.3.1 Pressure

Pressure affects the dissociation of molecules and directly affects the temperature required to change the K_w value (Bandura 2006). Visual MINTEQ 3.1 has been optimized to model water with pressure of 1 atm or very close to 1 atm. For the purposes of my project, a standard 1 atm or 1.01325 bar will be used in the model.

2.1.3.2 Components in the model

Visual MINTEQ requires that all the components (elements and compounds) are entered in their individual chemical forms. For example, instead of entering $FeCl_3$, the program requires the user to input the total concentration of Fe^{3+} and total concentration of Cl^- . In my experiments, a modified Hoagland's nutrient solution (Table 2-1) was used. The original Hoagland solution was designed for a variety of plants grown in hydroponic conditions. One of its main strengths is its high nitrogen content, which makes it suitable

for plants which require nitrogen to build biomass in a short period of time (Hoagland and Arnon 1950). The primary modification made to the nutrient solution was to omit EDTA; the original recipe contains 11 μM EDTA as the only chelator.

Table 2-1: Components of the nutrient solution used to grow radish and as entered in Visual MINTEQ 3.1. The concentrations of compounds added to solution and the concentrations of the resulting component ions in solution are shown.

<i>Original Component</i>	<i>Concentration (μM)</i>	<i>Component as entered in Visual MINTEQ</i>	<i>Concentration (μM)</i>
Ca(NO ₃) ₂	1000	Ca ²⁺	1000.00
CuSO ₄	0.15	Cu ²⁺	0.15
FeCl ₃	10	Fe ³⁺	10.00
H ₃ BO ₃	6	H ₃ BO ₃	6.00
K ₂ HPO ₄	100	K ⁺	800.02
K ₂ SO ₄	100	Mg ²⁺	280.29
KNO ₃	400	Mn ²⁺	2.44
Mg(NO ₃) ₂	280	NO ₃ ⁻	3260.68
MnCl ₂	2.4	PO ₄ ³⁻	100.00
Na ₂ MoO ₄	0.2	SO ₄ ²⁻	100.65
NH ₄ NO ₃	300	Zn ²⁺	0.5
ZnSO ₄	0.5	NH ₄ ⁺	300.06

2.1.4 Modelling output

The results of Visual MINTEQ 3.1 modelling include three main outputs: **equilibrated mass distribution** (distribution of components between dissolved, adsorbed and precipitated phases), **species distribution** (percentage distribution among dissolved and adsorbed species) and the **concentrations and activities of aqueous inorganic species**. An extra output, **amount of finite solids**, is displayed only when solids are formed due to precipitation of compounds.

2.1.4.1 Equilibrated mass distribution

The equilibrated mass distribution is the total and percentage values of dissolved, adsorbed and precipitated phases for each element. For example, the output in Table 2-2 shows that 99.241% of copper in solution is in a soluble form, while the remainder is an insoluble that has formed a finite solid.

2.1.4.2 Species distribution

The species distribution, sometimes also called chemical speciation, refers to the distribution of an element amongst chemical species in a system (VanBriesen and Small 2010). The modelling results show all the components that are dissolved in solution and the percentage of the total amount of each chemical species that was estimated to be formed (Table 2-3). For example, the output in Table 2-3 shows that 99.729% of the NO_3^- is in the free ion form, and the remainder is split between CaNO_3^+ and KNO_3 (aq).

It is important to note that while speciation modelling is useful to estimate all the possible compounds that are dissolved in solution, it can carry a certain degree of uncertainty (Nitzsche et al. 2000). The main source of error when using modelling software comes from kinetic uncertainty. The model does not have the capacity to determine the reaction rate between species (for example, how long does it takes to form a Zn-EDTA complex), and while some kinetic constants have been determined for the most important chelators available, they have not been introduced into most of the geochemical models available (VanBriesen and Small 2010).

2.1.4.3 Amount of finite solids

The amount of finite solids shows all solid phases that are presumed to be present initially or that have precipitated in the solution. All the components that are used to create the solid compounds are initially extracted from the aqueous phase in order to avoid double counting (Allison et al. 1991). Visual MINTEQ also allows for the user to enter initial amount of finite solids if those are previously present in the solution (as would be the case for lake or stream water), but for my project only aqueous compounds are initially included in the model because each solution was made from stock solutions and reverse osmosis water.

Table 2-2: Sample equilibrated mass distribution table as modelled by Visual MINTEQ 3.1. A modified Hoagland's nutrient solution with copper at toxic levels (10 μM) and no chelator is being modeled at pH 6.

<i>Component</i>	<i>Total dissolved</i>	<i>% dissolved</i>	<i>Total sorbed</i>	<i>% sorbed</i>	<i>Total precipitated</i>	<i>% precipitated</i>
Ca^{+2}	0.001	100	0	0	0	0
Cl^{-1}	0.000034	100	0	0	0	0
Cu^{+2}	0.000010	99.994	0	0	6.4882E-10	0.006
Fe^{+3}	4.38E-13	0	0	0	0.00001	100
H^{+1}	0.000027	100	0	0	0	0
H_3BO_3	0.000006	100	0	0	0	0
K^{+1}	0.000800	100	0	0	0	0
Malate^{-2}	0.000016	100	0	0	0	0
Malonate^{-2}	0.000021	100	0	0	0	0
Mg^{+2}	0.000280	100	0	0	0	0
Mn^{+2}	2.62E-08	1.074	0	0	0.00000242	98.926
MoO_4^{-2}	9.84E-08	99.345	0	0	6.4882E-10	0.655
NH_4^{+1}	0.000300	100	0	0	0	0
NO_3^{-1}	0.003260	100	0	0	0	0
Oxalate^{-2}	3.86E-06	100	0	0	0	0
PO_4^{-3}	0.000097	97.58	0	0	0.00000242	2.42
SO_4^{-2}	0.000110	100	0	0	0	0
Zn^{+2}	0.000000	100	0	0	0	0

Table 2-3: Sample species distribution table as modelled by Visual MINTEQ 3.1. A modified Hoagland's nutrient solution with copper at toxic levels (10 μM) and no exogenous chelator is being modeled at pH 6.

<i>Component</i>	<i>% of total concentration</i>	<i>Species name</i>
Oxalate^{-2}	31.812	Oxalate^{-2}
	0.467	H-Oxalate ⁻
	0.572	Zn-Oxalate (aq)
	1.554	$\text{Cu}-(\text{Oxalate})_2^{-2}$
	19.502	Cu-Oxalate (aq)
	26.082	Ca-Oxalate (aq)
	19.83	Mg-Oxalate (aq)
	0.056	$\text{NH}_4\text{-Oxalate}^{-}$
	0.118	K-Oxalate ⁻
NO_3^{-1}	99.729	NO_3^{-1}
	0.226	CaNO_3^{3+}
	0.044	KNO_3 (aq)

Chapter 3

3 Materials and methods

3.1 Plant selection, germination and growth conditions

Seeds of radish (*Raphanus sativus* L. cv. Crimson Giant Champion) were germinated on moist (distilled water) filter paper in Petri dishes and left in the dark for 24-36 hours.

When the radicles were approximately 1 cm long, seedlings were transplanted to 15 cm diameter pots filled with rinsed coarse (≈ 1 mm) sand supplemented with nutrient solution at half strength. The nutrient solution was a modified Hoagland solution that contained the compounds mentioned in table 2.1. Sand culture was necessary until the seedlings were large enough to be supported in the hydroponic system

Seedlings were placed in a growth chamber at an air temperature 21°C, 60% relative humidity with a 16 hr light and 8 hr dark cycle. Daytime solution temperatures, however, were measured and found to be 23°C. Light intensity was $124 \pm 3 \mu\text{mol/m}^2/\text{s}$. After 7 days, plants were large enough to be transferred from the sand into 1 L glass jars filled with aerated nutrient solution. A black plastic lid and foam were used to support the plant. The jars were covered in black cloth to prevent algal growth. Plants grew in the jars for 3 days before adding the experimental treatments, with the main purpose of allowing the plants to acclimatize to their new growth conditions and prevent plant mortality during the first few days of treatment. Plants were harvested after a total of 18 days in the jars.

3.2 Treatments

Five chelator treatments (none, Na₂EDTA, NTA, HEDTA and DTPA) (Figure 3-1) and four metal treatments (no extra metal, cadmium, copper and zinc), in a full factorial design, were used as treatments with 4 replicates per experimental treatment. All growth solutions contained nutrient solution (Table 2-1), which had an initial concentration of 10 μM FeCl₃.

The concentrations of metals used were determined by a series of preliminary experiments in which plants were grown as described in section 3.1 except that 0 to 100 μM CdCl_2 , CuSO_4 or ZnCl_2 were added to the nutrient solution in the 1 L jars. For each of the added metals, the highest concentration that caused sub-lethal symptoms of metal-stress in the radish was 10 μM ; the metal-treated plants showed chlorosis and stunted growth compared to the control plants, but they continued to grow over the period of exposure to the metals. Thus, 10 μM CdCl_2 , CuSO_4 and ZnCl_2 were chosen to be the metal treatments that were used to determine the influence of chelators on metal solubility and uptake.

Although these chelators usually complex metals in a 1:1 ratio, the ratio of exogenous chelators to treatments metals used was 1.1:1. Not only is this the ratio of chelator (EDTA) to iron in the full recipe for Hoagland's nutrient solution (Hoagland and Arnon 1950), but it also ensures that some iron would remain available to the plants given the metal treatment. In other words, even if all of an exogenous chelator was bound to cadmium, copper or zinc, some chelator would remain available to complex with iron. Using a higher amount of chelators may hinder plant growth (Bandiera et al. 2010).

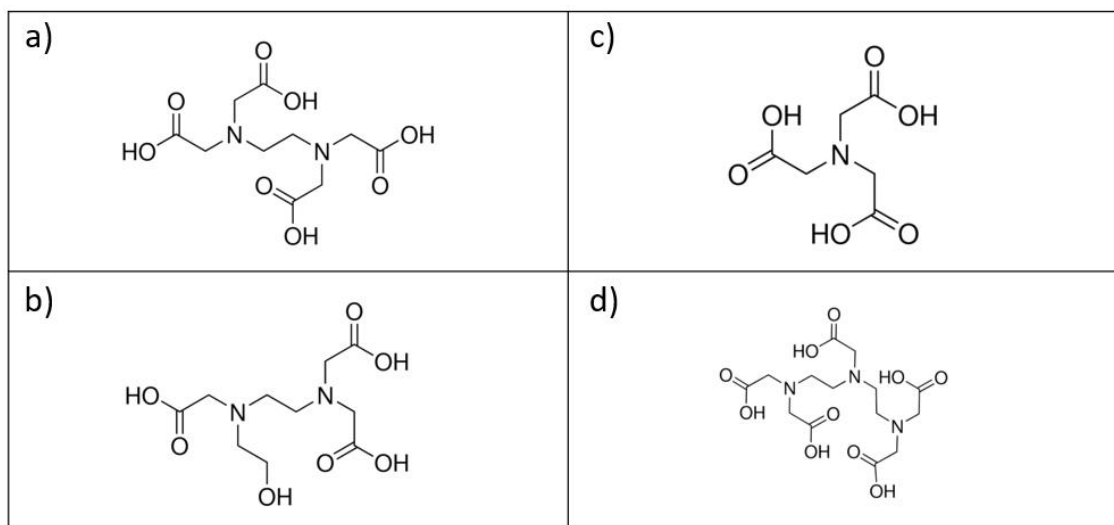


Figure 3-1: Structures of chelators used in this project: A) EDTA, B) HEDTA, C) NTA, D) DTPA.

3.3 Determination of the rate of change of pH levels versus time

The solution pH levels were measured every third day with a portable pH/Cond/TDS/Salinity tester (Hach Pocket Pro+ Multi 2) in order to determine the rate and direction of the change in pH. These measurements were necessary in order to determine which pH range would be modeled by Visual MINTEQ 3.1. The nutrient solution was not buffered with any additives. Solution levels were not replenished to the original one liter and evaporation was considered to be negligible due to the lid and foam cover preventing water from escaping the container. A reduction of the nutrient's solution level was considered normal as the plant requires its uptake to perform its normal processes. Bacterial growth determination

In order to verify the absence of bacteria, which may degrade or produce organic chelators, a 1 mL sample of each growth medium was collected at the end of the growth period and streaked onto Bacto Agar medium in a Petri dish. The plate was placed in an incubator at 30°C and checked after 24 to 48 hours for bacterial colonies (Hauser 2006). Any colonies detected would be considered as possible contamination of the solution.

3.4 Plant biomass determination

At harvest, plants were taken out of their jars and weighed to determine total plant fresh biomass. Samples were then placed in brown paper bags and left in an oven at 60°C for 1 week, when total plant dry biomass was recorded.

3.5 Determination of total metal uptake

Intact radish plants were dried at 60°C for 1 week before preparing samples for ICP-MS (Inductively Coupled Plasma – Mass Spectroscopy) analysis. NIST SRM (National Institute of Standards and Technology Standard Reference Materials 1570A, spinach leaves; 1573A, tomato leaves; and 8412, corn stalks) samples were also dried for ICP analysis to determine the efficiency of the digestion procedure. Blank nitric acid samples were also digested and analysed to verify that there was no contamination.

Dried samples were hand-chopped to ~1 mm pieces and 0.1 g subsamples were weighed into acid-washed test tubes. Afterwards, 1 ml ultra-pure nitric acid (OmniTrace® Ultra™) was added to each tube and a marble was placed on top of the tube to prevent evaporation of acid and loss of sample during digestion. Acid digestion proceeded at room temperature for 24 hr, then at 90°C until the fumes were transparent (3 to 4 hr). After cooling to room temperature, samples filtered through a VWR 413 qualitative filter. Deionized water was used to rinse the test tube contents into the final test tube and the samples were brought down to a final volume of 25 ml. Samples were sent to ALS Environmental for ICP-MS analysis. Due to the influence of chelators on iron solubility, each of cadmium, copper, zinc and iron were measured.

3.6 Determination of exuded organic acids in growth medium with a LC–ESI–TOFMS system

3.6.1 Derivatization

Small volume injection samples (less than 10 µL) as well as low concentration of organic acids in solution prevent possible detection with a regular C18 reverse phase chromatographic column without performing additional steps. A derivatization procedure was required to add benzyl rings to the carboxyl groups in organic acids to use a regular C18 column and obtain high robustness and detection levels. Each 500 µL sample of the growth medium was taken at the end of the treatment and transferred into a 1.5 mL Eppendorf tube. An internal standard of citric acid adjusted to be 10 µM in the 500 µL sample was added to correct for possible instrument inconsistency. Samples were dried in a vacuum evaporation system (SpeedVac SC100, Savant) set to medium temperature for a period of 24-36 hours. Afterwards, 50 µL of benzyl alcohol and 30 µL of TMS (Trimethylsilyl)-chloride were added to the samples, and then the closed Eppendorf tubes were first placed in an ultrasonic bath (0°C temperature, 45 minutes) and then in a hot water bath (75°C, 45 minutes). The reaction was stopped by adding 150 µL of 0.3 mM TMAF (tetramethyl-ammonium-fluoride) and a mixture of 50% acetonitrile and 50% water, bringing the final volume to 500 µL.

3.6.2 LC–ESI–TOFMS analysis parameters and procedure

To separate, identify and quantify exudates in the growth medium, samples (8 μ l) were injected on a Zorbax Extend C-18 column Rapid Resolution HT (3.0 \times 150 mm, 3- μ m, 600 Bar, Agilent Technologies) at 40°C and eluted with a gradient of CH₃CN (Solvent B: 90% CH₃CN in H₂O, containing 0.1% formic acid: HCOOH) in H₂O (Solvent A: containing 0.1% HCO₂H) as follows. The initial condition was 50% B in A, which was held for 2 minutes and sent to waste in order to prevent contamination, this was followed by a linear gradient to 95% solution B over 9 minutes, before returning to initial conditions. The flow rate was set to 0.350 ml/min, and infused into an Agilent 6230 TOFMS through a Dual Spray ESI source with a gas temperature of 325°C flowing at 10 L/min, and a nebulizer pressure of 40 psi. The fragmentor voltage was set to 150 V with a capillary voltage of 4000 V and a skimmer voltage of 65 V. The instrument was set in positive ESI mode. Automated internal calibration was done by injecting a reference mass mix containing purine and 1H,1H,3Htetrafluoropropoxyphosphazene (molecule with hydrogen adduct: (M+H⁺)=121.050873 and 922.009798 Da) continuously into the ESI interface with the reference sprayer. The column was conditioned at 50% B for 8 minutes between samples, and the organic acids were detected as their Na⁺ adducts [M + Na]⁺.

3.7 Modelling

Visual MINTEQ 3.1 was used as the modelling software. Parameters selected for this project are detailed in Chapter 2. Input parameters included pH range, from 6 to 7, exogenous chelators (EDTA, HEDTA, NTA and DTPA) and compounds added in nutrient solution. Two sets of models were run, one including the previously mentioned parameters (nutrient solution and exogenous chelators), and a second one including the chelators that were exuded from the plants (malate, malonate, oxalate, citrate, aconitate, succinate and fumarate).

3.8 Statistical analysis

Data were analyzed using GraphPad Prism 6. The effects of the metals and chelator treatments on solution pH were done by using a repeated measures 2-way ANOVA

followed by Tukey's post hoc test. Effects of biomass and metal concentrations on roots and shoots (analyzed together) were analyzed by using two-way ANOVA followed by Tukey's post hoc test. The differences between treatments for the correlation of total of metal taken up (measured by ICP-MS) to total soluble metal as modelled by Visual MINTEQ 3.1 were analyzed by using one-way ANOVA followed by Tukey's post hoc test.

Chapter 4

4 Results

4.1 pH versus time

While the pH generally increased over the 15-day experimental period, the variance among individuals was high and increased as time went on (Figure 4-1).

The pH of solution for the treatment with no toxic metals but with a standard dose of iron did not vary over time (Figure 4-1). The pH of the solutions increased up to 0.5 pH units by day 12 for the cadmium treatment, and 0.3 pH units for the copper and zinc treatments, which explains the significant interaction between time and chelator treatment (Appendix 2). Since pH 6.0 was the initial value, and the general trend was considered to be towards a more basic pH over time, a range of pH 6.0 to pH 7.0 was selected to model the effect of chelators on metal solubility.

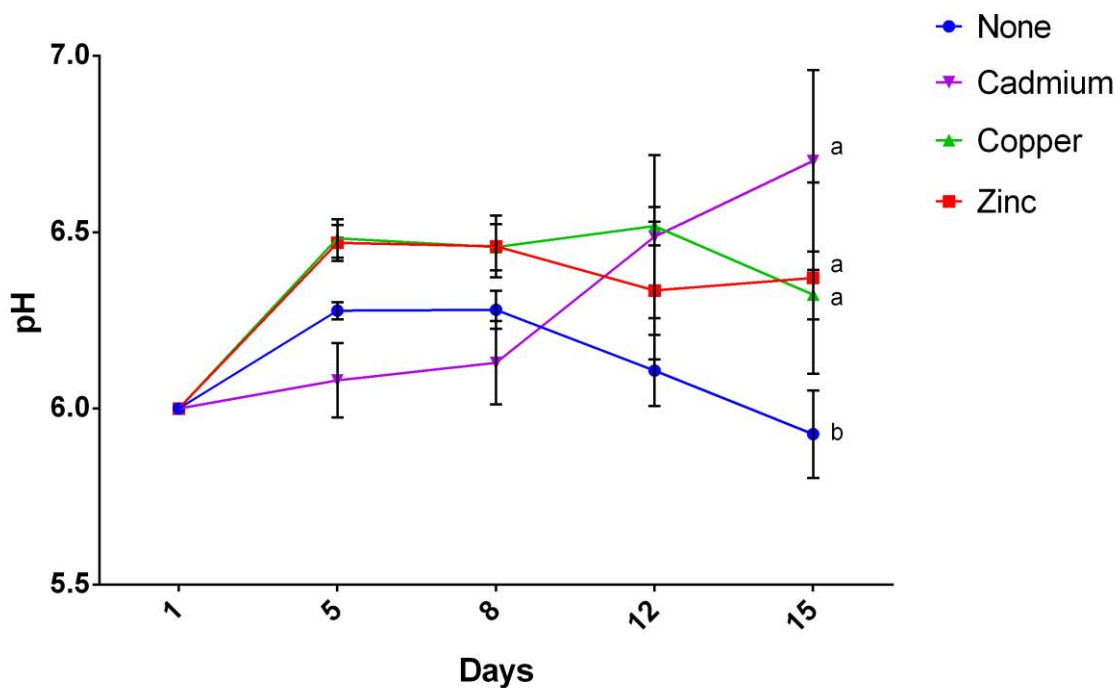


Figure 4-1: pH change over time. Jars with modified Hoagland's nutrient solution and either no metal treatment (blue circle), 10 μM CdCl_2 (purple triangle), 10 μM CuSO_4 (green triangle) or 10 μM ZnCl_2 (red square). pH differences between treatments were analyzed using a repeated measures two-way ANOVA (see Appendix 2) followed by Tukey's post hoc means comparison test. Significant differences are shown only for treatments at day 15. Bars represent the mean \pm standard error of the mean.

4.2 Plant biomass

The dry weights of plants from the various metal and exogenous chelator treatments are shown in Figure 4-2. With only a few exceptions, plants grown with cadmium, copper or zinc had less biomass than the plants grown in the solution to which extra metals had not been added.

Among the control samples (i.e., those with no added metal treatment), the plants with no exogenous chelators had 50% lower dry weight than all plants that were grown with chelators. Biomass of plants control plants that were given EDTA, HEDTA, NTA or DTPA did not statistically differ from each other.

In plants exposed to excess copper, those given HEDTA had a 24-fold weight increase over the control plants, a 14-fold increase over plant grown with DTPA, a 5-fold increase over NTA-grown plants, and an almost 3-fold increase over EDTA samples. Chelator treatments did not affect the biomass of plants subject to either excess cadmium treatment or excess zinc treatment.

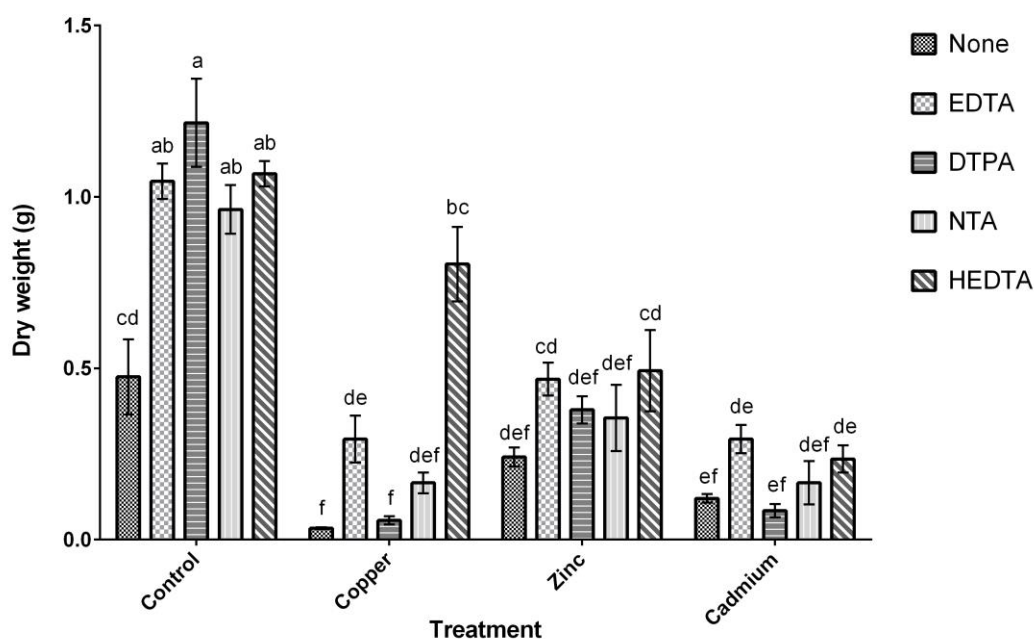


Figure 4-2: Plant dry weight. Radish were grown for 15 days in nutrient solutions containing no extra metal (control) or 10 μM of copper, zinc or cadmium, as well as one of five exogenous chelator treatments: none, EDTA, DTPA, NTA or HEDTA. Dry weight between plants was compared by using two-way ANOVA followed by Tukey's post hoc means comparison test (See Appendix 2). Different lower case letters indicate significant differences ($p \leq 0.05$). Bars represent the mean \pm standard error of the mean.

4.3 Bacterial growth

No bacterial growth was evident on the Petri dishes with nutrient agar after 48 hours.

4.4 Organic acids

Of the seven different organic acids detectable by the LC–ESI–TOFMS system, only three were above detection limits (1 μM) in the solution: malate, malonate and oxalate. Concentrations of exuded organic acid varied depending on the metal treatment (Figure 4-3). In general, concentrations of exuded malate and malonate were four to five times higher than those of oxalate, which remained almost constant at 4 μM .

In the control plants (Figure 4.3A), exuded malate was present in concentrations that ranged between 17 μM (no chelator) and 26 μM (DTPA treatment); however, two-way ANOVA found no effect of chelator treatment on malate exudation. Exuded malonate was 3-fold higher in concentration for plants from the DTPA and NTA treatments compared to the control, and was unaffected by either the EDTA or HEDTA treatment.

Plants from the zinc treatment (Figure 4-3B) exuded malate into the nutrient solution in the range of 16-20 μM , and malate exudation was unaffected by chelator treatment. Exuded malonate was about 30% higher from the plants grown without a chelator than for plants from the EDTA, NTA and HEDTA treatments. DTPA did not affect malonate exudation. Plants from the copper treatment (Figure 4-3C) showed similar results, with malate exudation being unaffected by chelator treatments, but the total concentration of malate measured in the nutrient solution was as much as 20% lower than that of the control plants (Figure 4-3A), topping at a maximum concentration of 21 μM . Malonate exuded from plants in the copper treatment was about 25% less in DTPA- and NTA-treated plants compared to the no-chelator control, and was unaffected by EDTA or HEDTA treatment.

In plants from the cadmium treatment (Figure 4-3D) exudation of malate, malonate and oxalate were unaffected by the chelator that was present in the nutrient solution. However, the concentration of exuded malate was as much as 25% lower than from plants in the other three metal treatments (control, copper and zinc).

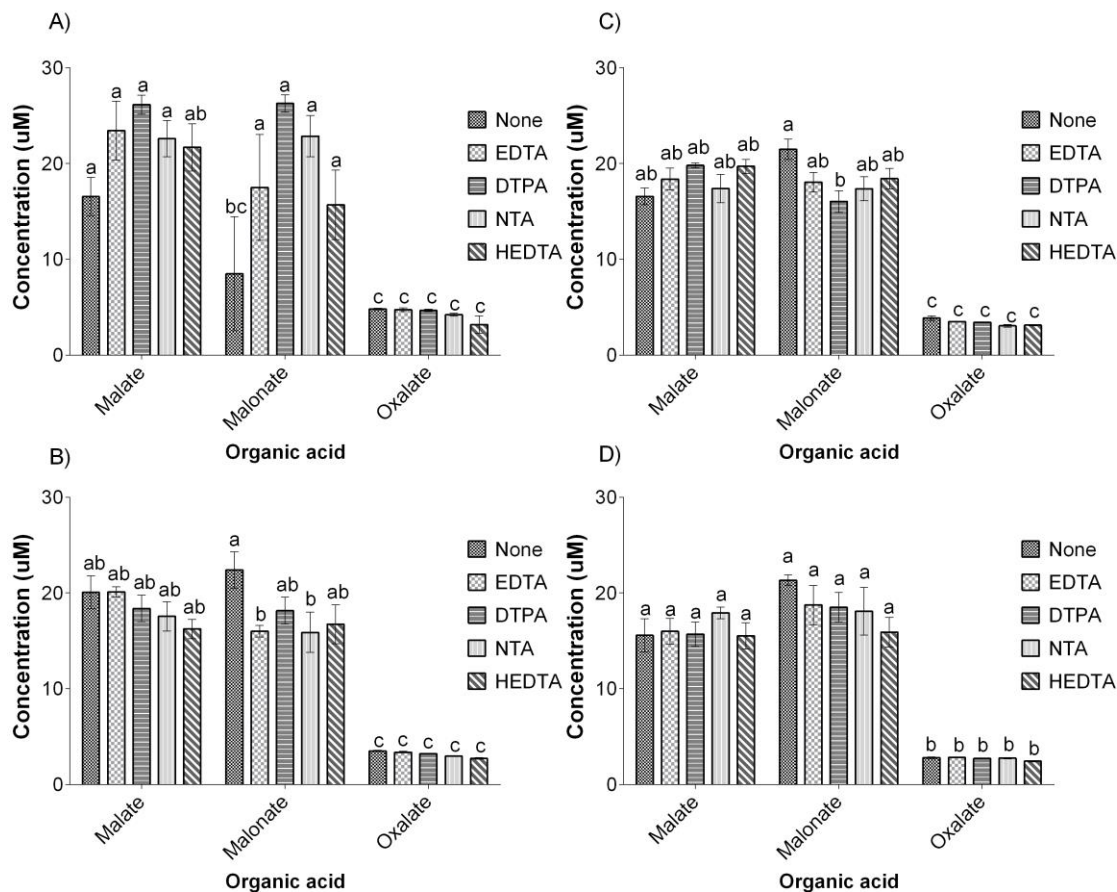


Figure 4-3: Organic acid exudates. Plants were grown in nutrient solution with A) no excess metal (control) or 10 µM excess B) zinc, C) copper or D) cadmium. Differences in organic acid exudate concentration were analyzed using two-way ANOVA followed by Tukey's post hoc means comparison test (See Appendix 2). Different lower case letters indicate significant differences ($p \leq 0.05$). Bars represent the mean \pm standard error of the mean.

4.5 Metal uptake

4.5.1 Concentrations of metals in radish tissue

The concentrations of metals taken up by radish varied depending on the treatments that the plants were subjected to. (Fig 4-4).

In plants from the treatment with no excess metal (Figure 4-4A), concentrations of cadmium, copper and zinc did not vary with chelator treatment. Plants from the treatment with no excess metal grown with the presence of NTA took approximately 75% more iron than plants grown with an absence of an exogenous chelator and plants with added EDTA. Compared to plants with added HEDTA and DTPA, plants with added NTA showed an increase of 110% and 175% uptake of iron respectively.

For plants from the zinc treatment (10 μ M zinc chloride) (Figure 4-4B), concentrations of copper did not vary with chelator treatment and concentrations of cadmium were two-fold higher in plants treated with HEDTA or NTA. The control plants, grown with no chelating agent, had 179% higher concentrations of zinc than plants grown in EDTA, a 449% higher zinc concentration than HEDTA plants and a 237% greater concentration of zinc than plants grown with NTA. Zinc concentrations in plants grown with DTPA did not differ from those in control plants, but were 148% higher than that of plants grown in the presence of EDTA, an almost 5-fold increase over plants grown with HEDTA, and a 3-fold increase over plants grown with NTA.

In the copper treatment, concentrations of cadmium and zinc were low across all chelator treatments (Figure 4-4C). Control plants, or plants with no chelating agent added to the nutrient solution, had a significantly higher uptake of copper per unit of dry mass than the rest of plants grown with synthetic chelating agents, having over a 6-fold increase over plants grown with EDTA and plants grown with NTA, an almost 24-fold increase over plants grown with HEDTA, and an almost 4-fold increase over plants grown with DTPA.

Plants under cadmium toxicity showed no difference in cadmium uptake among plants grown with either no chelating agents, EDTA, HEDTA and DTPA as a chelating agent

(Figure 4-4D), however, plants grown with NTA had 30% less cadmium than control and EDTA grown plants, and 32% less than plants grown with DTPA in the nutrient solution.

4.5.2 Total amount of metal taken up by radish

Metal uptake by radish varied depending on the treatments that the plants were subjected to. As expected, cadmium was present in trace amounts in all but the cadmium treatment, where it was present in high concentrations (Figure 4-5)

Plants from the treatment with no excess metal (Figure 4-5A) took up 25% less iron in the absence of an exogenous chelator (control) compared to plants with added NTA, but contained the same concentration of iron as the plants from the remaining chelator treatments. Zinc and copper concentrations remained unchanged regardless of the chelators that plants were exposed to.

Plants from the zinc treatment (10 μ M zinc chloride) showed a larger response in zinc uptake when they were subjected to either: no exogenous chelator or DTPA (Figure 4-5B). DTPA-exposed plants had a two-fold increase in zinc uptake over EDTA plants, and an almost four-fold increase in zinc uptake over HEDTA and NTA plants. Plants from the 'none' treatment (plants that did not have access to a chelator) had 40% more zinc than EDTA plants, and 250% more zinc than both HEDTA and NTA plants. Iron uptake also proved to be higher in plants that were not exposed to exogenous chelators, as well as in EDTA and HEDTA plants, when compared to NTA and DTPA plants (over 400% more uptake). Total copper taken up was unchanged regardless of the chelator the plant was exposed to in the zinc (10 μ M zinc chloride) treatment.

In the copper treatment (10 μ M copper sulfate) plants showed no difference in uptake of copper when subjected to the presence of different chelators (Figure 4-5C). Zinc uptake was similarly unchanged regardless of the exogenous chelator the plant was exposed to. Iron uptake, however, was increased by 465% in plants from the HEDTA treatment when compared to control.

Plants under cadmium toxicity conditions (Figure 4-5D) contained very high concentrations of cadmium while at the same time showing reduced iron uptake

compared to plants from the other metal treatments. EDTA and HEDTA induced a 2- to 3-fold higher uptake of cadmium compared to plants with no chelator, while the other chelators did not affect cadmium uptake. EDTA proved to be superior to the other chelators by increasing metal uptake over both NTA and DTPA treatments, by 275% and 315%, respectively. HEDTA-treated plants also showed a 2-fold increase in cadmium uptake over NTA-treated plants, and a 1.5-fold increase over DTPA-treated plants. The uptake of the other three metals analysed did not differ among chelator treatments. It is important to note, however, that iron uptake was the lowest in the cadmium solution treatment among all four different metal treatments.

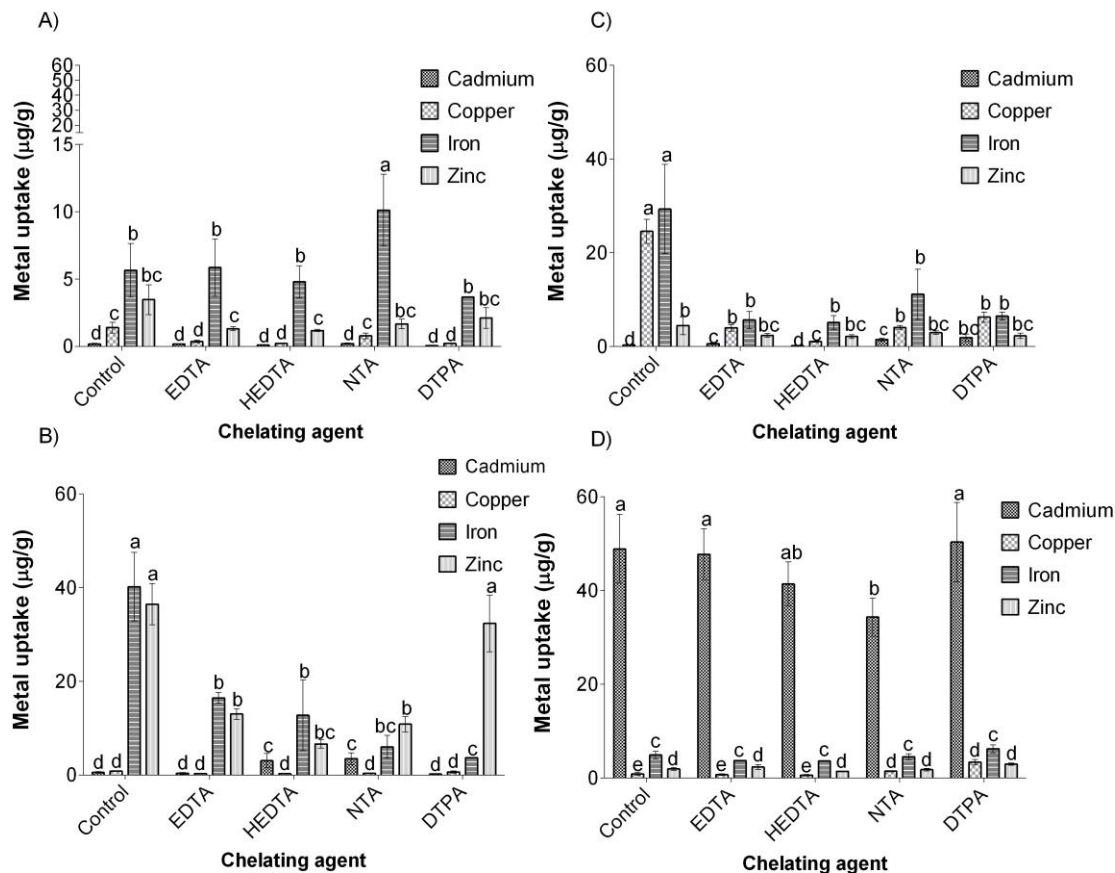


Figure 4-4: Concentrations of metals in radish. Plants were grown in nutrient solution with A) no excess metal (control) or 10 µM excess B) zinc, C) copper or D) cadmium. Differences in concentration of metals in radish were analyzed using two-way ANOVA followed by Tukey's post hoc means comparison test (See Appendix 2). Different lower case letters indicate significant differences ($p \leq 0.05$). Bars represent the mean \pm standard error of the mean.

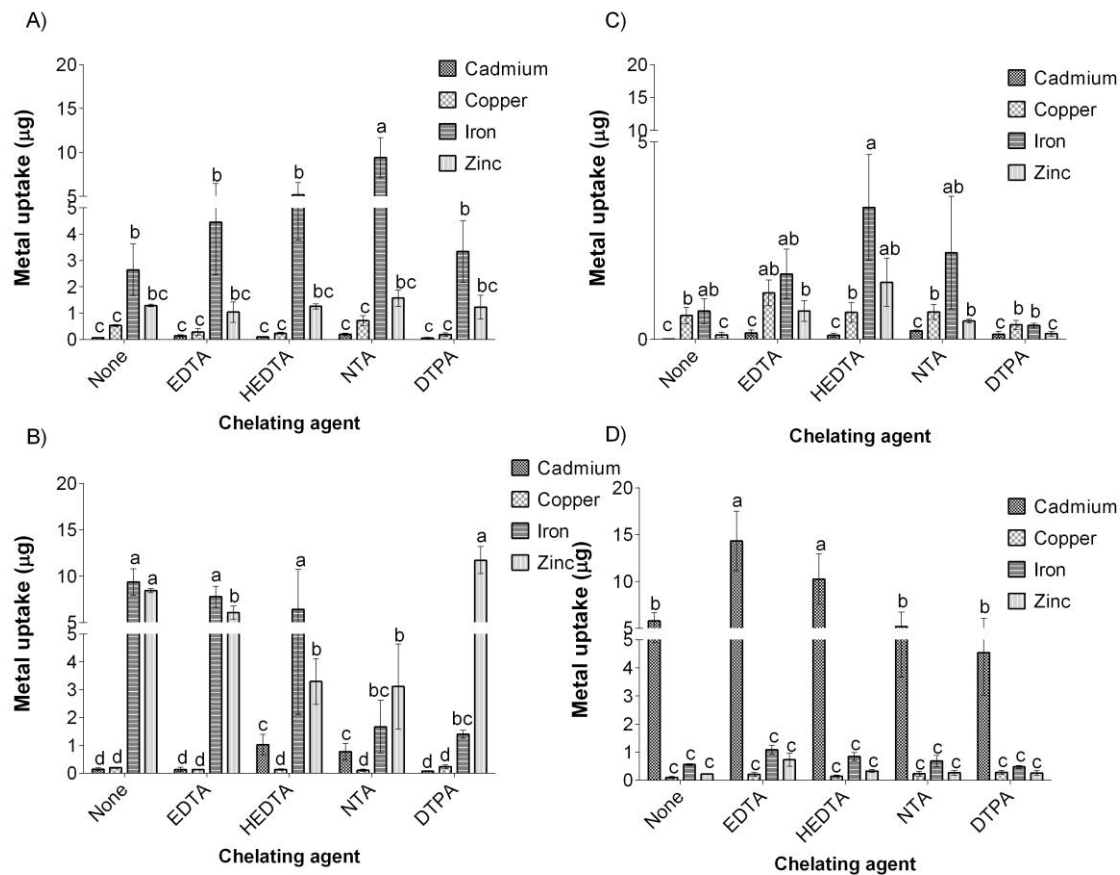


Figure 4-5: Total metal uptake. Plants were grown in nutrient solution with A) no excess metal (control) or 10 μM excess B) zinc, C) copper or D) cadmium. Differences in total metal uptake in radish were analyzed using two-way ANOVA followed by Tukey's post hoc means comparison test (See Appendix 2). Different lower case letters indicate significant differences ($p \leq 0.05$). Bars represent the mean \pm standard error of the mean.

4.6 Visual MINTEQ 3.1

The modelling software Visual MINTEQ 3.1 (Figures 4-6 to 4-9) did not detect differences in metal solubility between the original model and the models that incorporated the exudates obtained by LC-MS analysis (compare the left-side panels to those on the right side of each figure), with the exception of the copper treatment with no chelator in solution (Figure 4-8A-B). While the original model showed solubility of copper falling to 50% at pH 6.5, the model with organic acids showed this same result at pH 6.9, indicating greatly increased solubility of copper for a wider range of pH. The model without added organic acids (Figure 4-8A) also showed copper solubility at pH 7.0 to be only 7.5% of the total copper provided, mostly due to the formation of copper oxides in solution. When taking into consideration exuded organic acids (Figure 4-8B), copper solubility increased, with 40.6% of the total copper added being soluble at pH 7.0. This was due to the formation of malate-copper, malonate-copper and oxalate-copper complexes.

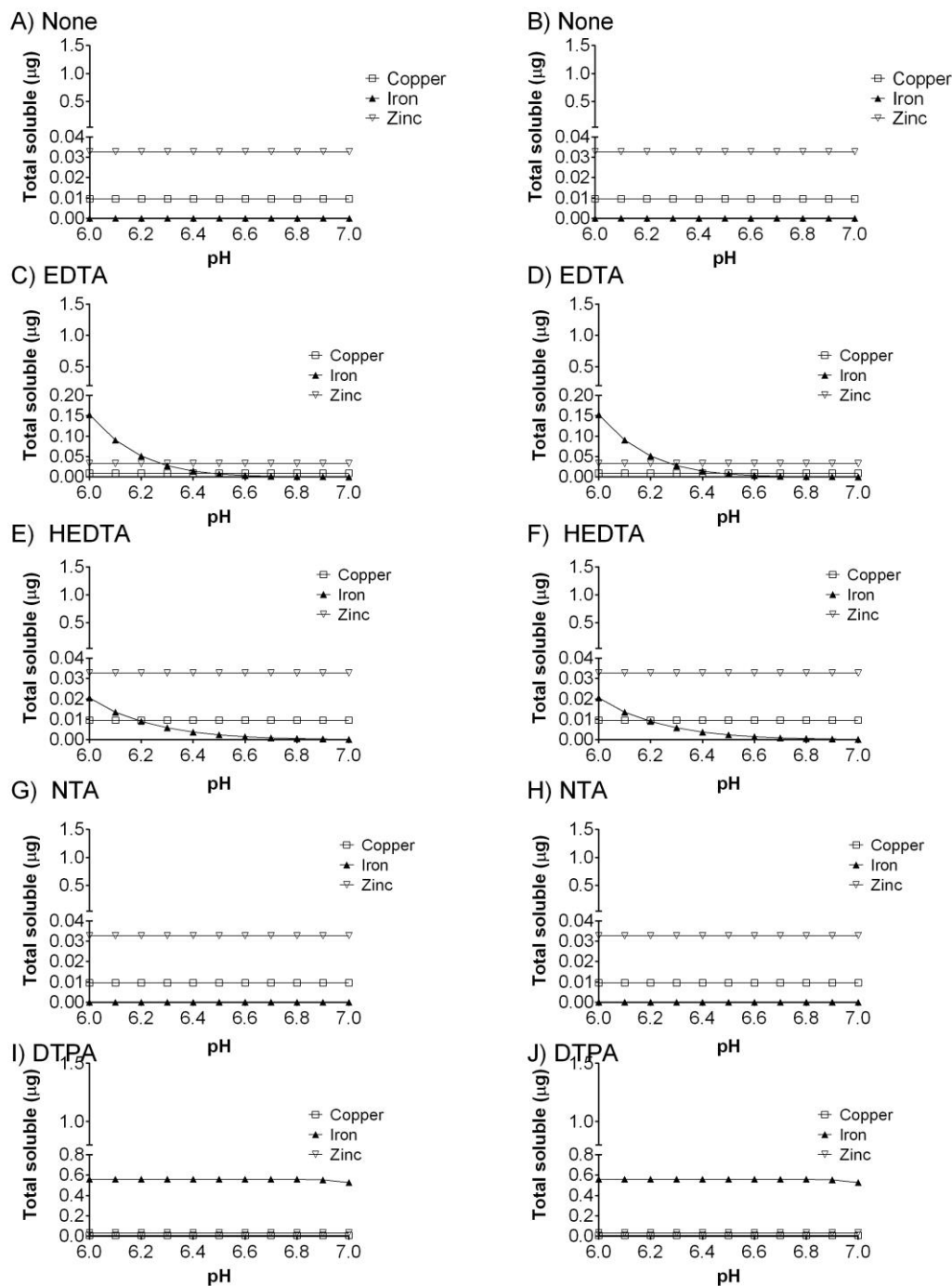


Figure 4-6: Total soluble metals in control solution (solution with no metal treatment) as modeled by Visual MINTEQ 3.1. Solutions with each of five chelator treatments (A, B) no chelator; (C, D) EDTA; (E, F) HEDTA; (G, H) NTA; (I, J) DTPA, were modelled without (on the left) and with (on the right) considering exuded organic acids. Points represent total soluble metal in solution.

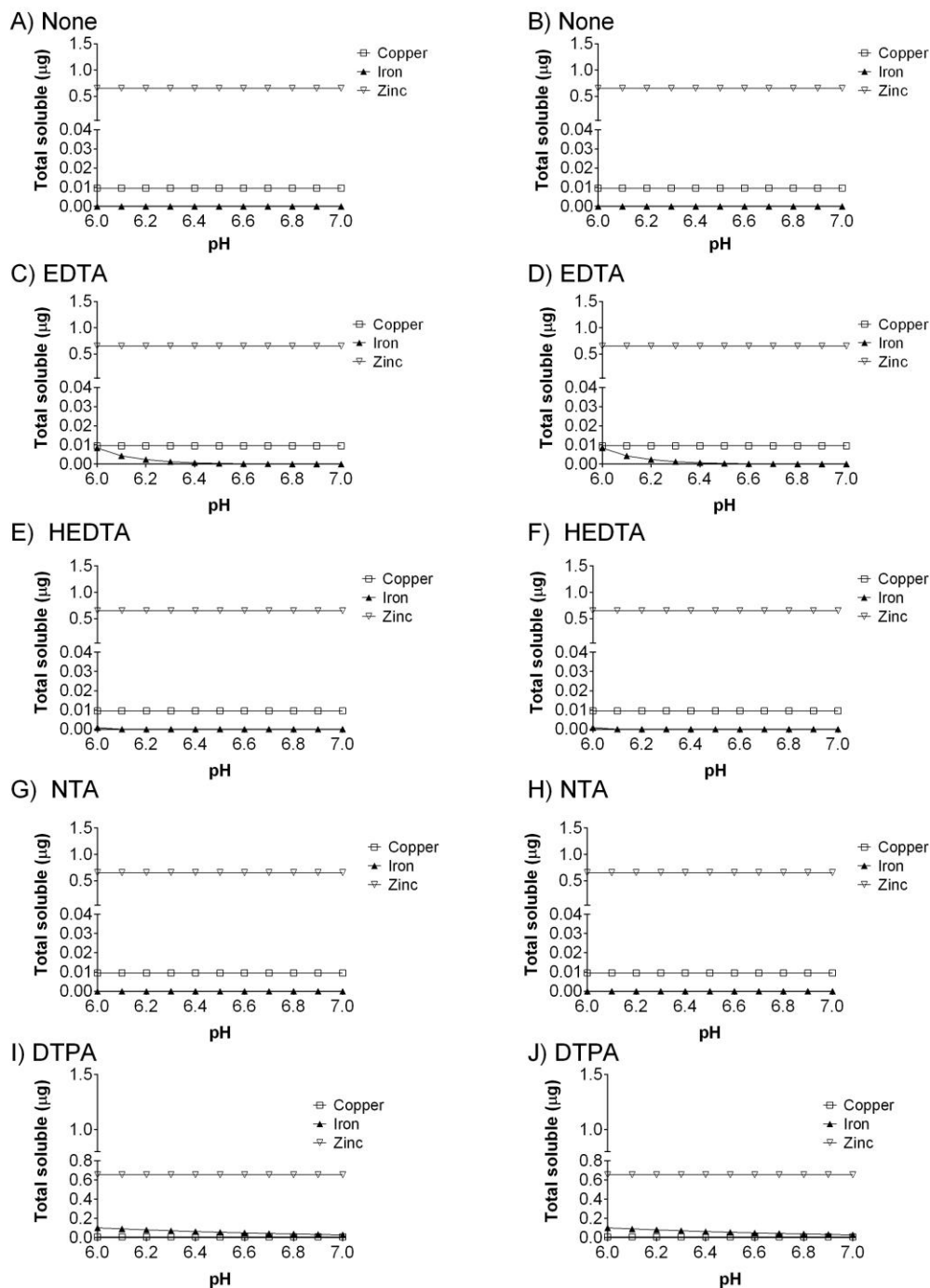


Figure 4-7: Total soluble metals in solution with excess zinc as modeled by Visual MINTEQ 3.1. Solutions with each of five chelator treatments (A, B) no chelator; (C, D) EDTA; (E, F) HEDTA; (G, H) NTA; (I, J) DTPA, were modelled without (on the left) and with (on the right) considering exuded organic acids. Points represent total soluble metal in solution.

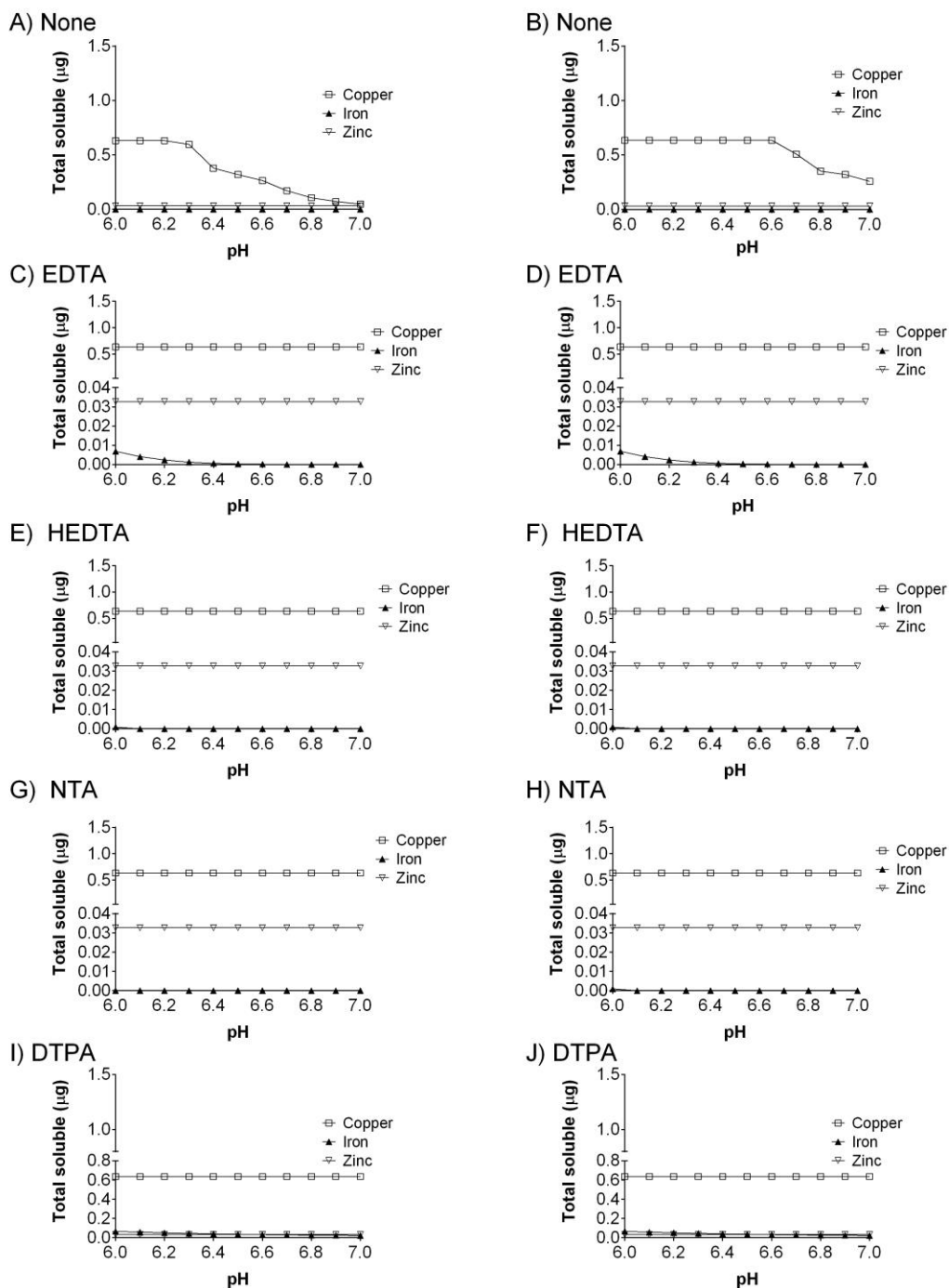


Figure 4-8: Total soluble metals in solution with excess copper as modeled by Visual MINTEQ 3.1. Solutions with each of five chelator treatments (A, B) no chelator; (C, D) EDTA; (E, F) HEDTA; (G, H) NTA; (I, J) DTPA, were modelled without (on the left) and with (on the right) considering exuded organic acids. Points represent total soluble metal in solution.

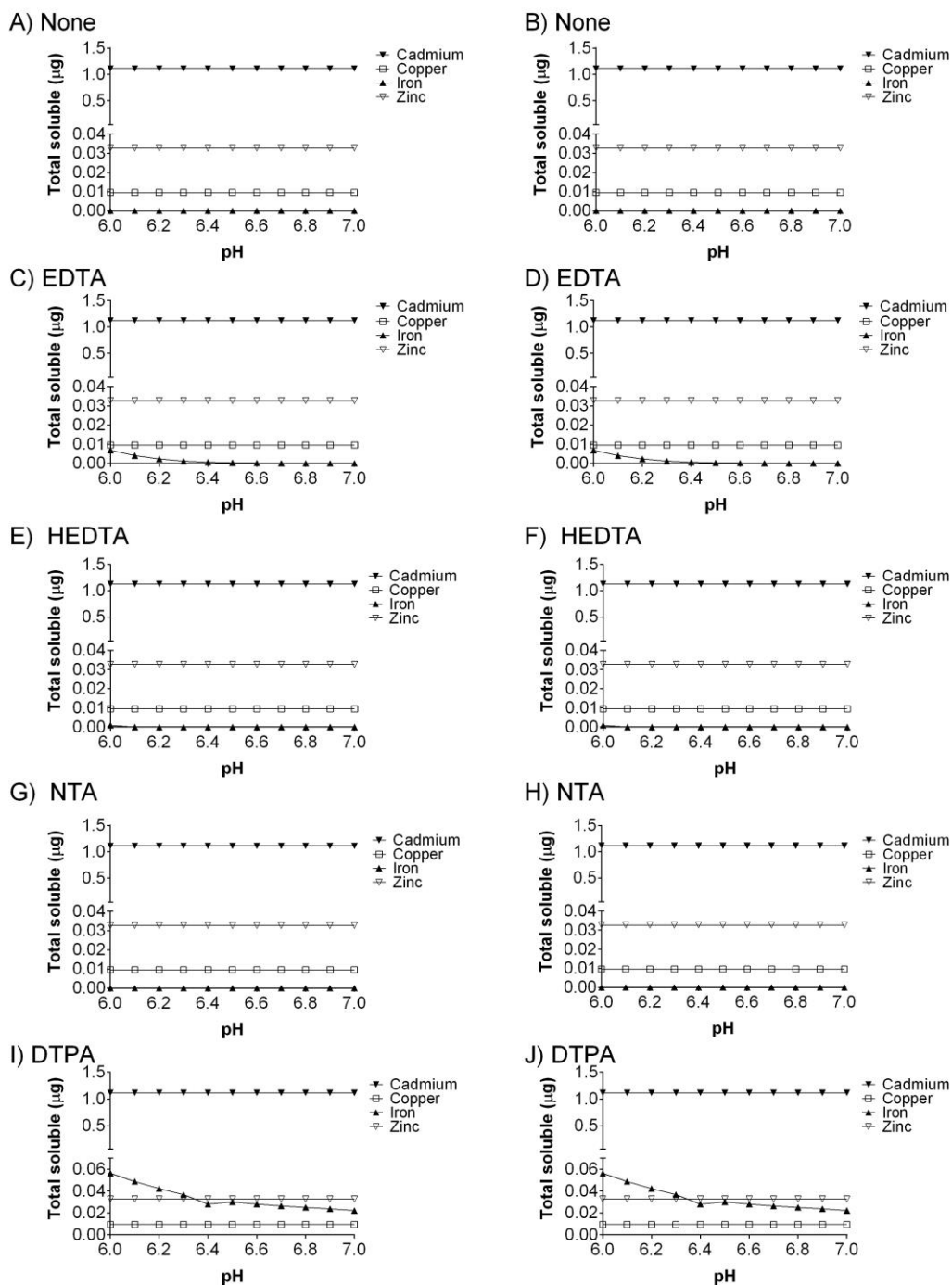


Figure 4-9: Total soluble metals in solution with excess cadmium as modeled by Visual MINTEQ 3.1. Solutions with each of five chelator treatments (A, B) no chelator; (C, D) EDTA; (E, F) HEDTA; (G, H) NTA; (I, J) DTPA, were modelled without (on the left) and with (on the right) considering exuded organic acids. Points represent total soluble metal in solution.

4.7 Metal taken up vs. solubility according to Visual MINTEQ 3.1

In order to evaluate the validity of the model, the percentage of total soluble metal (modelled by Visual MINTEQ 3.1) that was taken up by the plants (measured by ICP-MS) was calculated. An even percentage within chelator treatments would indicate that the model could predict uptake, while differences between these treatments would indicate poor accuracy of the model in this scenario. Since the focus of this project was using modelling software to predict uptake of certain metals under toxic conditions, Figure 4-10 shows only the metal of interest for each metal treatment. For the treatment with no metal in excess, iron is shown because chelators are known to affect iron solubility. In order to perform the calculations, a pH was necessary to model solubility. Since the range of pH varied between 6 and 7 during the duration of the experiment, a pH of 6.5 was selected. Final results were obtained by dividing total amount of metal taken up by the total amount of soluble metal according to Visual MINTEQ 3.1 at pH 6.5.

Results showed a higher percentage of HEDTA being taken up when compared to Visual MINTEQ 3.1 than the EDTA treatment in the control treatment (no excess metal) (Figure 4-10A). Calculations for the 'none' treatment (no chelating agent) could not be made since the model predicted a 0% solubility for iron, while plants took up a certain amount of that metal. Results for plants being grown with excess zinc showed statistical differences between the DTPA and 'none' treatments when compared to the HEDTA and NTA treatments (Figure 4-10B), while no statistical difference was found within any treatments in plants grown with excess copper (Figure 4-10C). Results for calculations made for plants grown with excess cadmium showed a significant increase between 'none', EDTA, HEDTA and NTA when compared to the DTPA treatment.

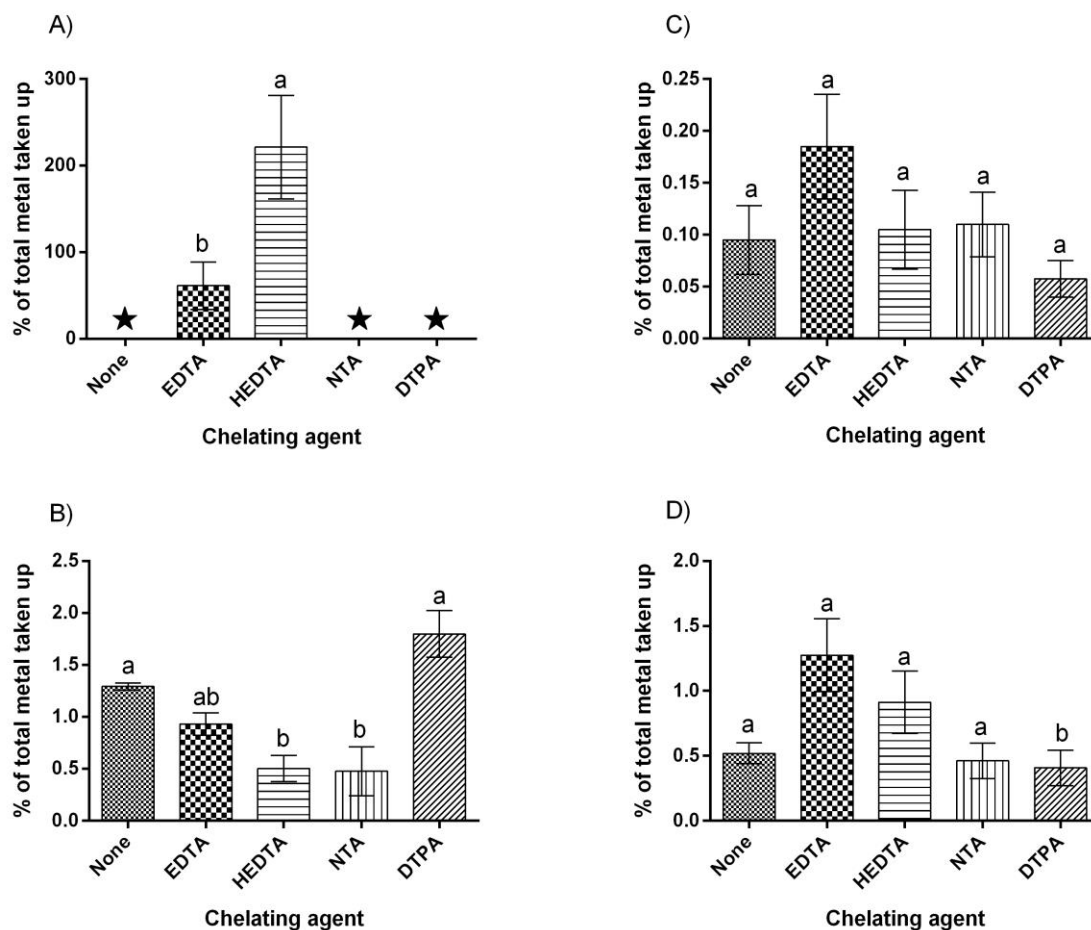


Figure 4-10: Percentage of metal taken divided by total metal solubility at pH 6.5 according to Visual MINTEQ. Only the results for the metal of interest for each treatment is shown. Plants were grown in nutrient solution with A) no excess metal (control) or 10 μM excess B) zinc, C) copper or D) cadmium. Differences in percentage of metal taken up by plants were analyzed using one-way ANOVA followed by Tukey's post hoc means comparison test (See Appendix 2). Different lower case letters indicate significant differences ($p \leq 0.05$). Bars represent the mean \pm standard error of the mean. Stars represent calculations that could not be made due to mathematical limitations.

Chapter 5

5 Discussion

5.1 Plant biomass and organic acid exudates

Plants that were not given chelators ('none' treatment) should not have access to iron under the conditions of this experiment because iron is predominantly present as Fe^{3+} , which tends to form insoluble oxides and hydroxides. Chelators are commonly used to increase solubility of iron and are widely used in agriculture (Sun et al. 2001) and modelling showed an increase in iron solubility when exogenous chelators were added to the nutrient solution, especially in plants with added DTPA (Figure 4.6-I,J and 4.9-I,J). As seen in Figure 4-2, plants with no access to chelators in the control (no added metal treatment) had less biomass than plants with access to chelators. The reduced biomass can partially be explained by reduced iron uptake in the absence of a chelator (Figure 4-5). The lack of response in biomass of plants from the cadmium treatments to the chelator treatments (Figure 4-2) may be a result of near-constant iron solubility in the growth solutions across the chelation treatments (Figure 4-5D). The relationships among biomass, chelator treatment and iron uptake for plants from the copper and zinc treatments were less clear. Other studies have also shown no increase in biomass upon addition of chelators (Habiba et al. 2014; Bloem et al. 2017).

After 15 days of plant growth, malate and malonate were present in the nutrient solution in higher quantities than oxalate. Exudation of organic acids by radish has been studied by others, but the results have varied between two studies. In one, radish exuded high concentrations of succinic, malic and tartaric acid when exposed to phosphorus deficiency conditions (Zhang et al. 1997). In another, high concentrations of malic and succinic acid were exuded in response to cerium oxide nanoparticles (Zhang et al. 2017). The difference in the concentration of these exudates could be explained by different exuding responses (e.g., toxicity, improve nutrition). It is believed that the increased exudation of certain organic compounds under toxicity conditions may happen due to

increased membrane permeability that only occurs on studies with plants under toxicity conditions (Zhang et al. 1997)

While it was expected that plants subjected to metal toxicity would produce higher exudate concentrations, control plants were the ones that had higher exudate concentrations. This reduced exudation in response to excess metal could have been a result of metal toxicity; above a certain threshold, metals can reduce the production of organic exudates (Xie et al. 2013; Montiel-Rozas et al. 2016). Even if the concentrations of metals were not enough to induce such toxicity, the reduced biomass caused by metal exposure might have been a determining factor in the production of organic acids – smaller roots might produce less exudate, however, a correlation between plant size and organic acid exudate was not found. It is also possible that the plants were exuding other acids not measured by this project. It has been reported that plants can shift towards the production of monocarboxylic acids instead of di- and tricarboxylic acids when exposed to metal stress (Westergaard Strobel et al. 1999)

5.2 Modelling and metal uptake

The addition of exuded organic acids did not affect any of the four metals availability as determined by Visual MINTEQ modelling (Figures 4-6 to 4-9) even though organic acids such as malate were in concentrations as high as 30 μM (Figure 4-3). However, in some cases, treatment with exogenous chelators did affect metal availability. For this project, the concentrations of metals in plants (Figure 4-4) is of less interest than the total amounts of metal taken up (Figure 4.5) since the main objective was to manipulate chelators to maximize total metal uptake. Results from the various metal treatments, focusing on total metal uptake, are discussed individually below.

5.2.1 Control treatment

Based on plant dry weights (Figure 4-2) chelators increased biomass compared to having no chelator, and the DTPA-treated plants had a 20% higher biomass compared to the plants from the remaining chelator treatments and a 2.5-fold increase in biomass over the control plants. The model predicted that the nutrient solution with no chelators or with exogenous NTA should have had iron (as Fe^{3+}) completely precipitated as hematite

(Fe_2O_3), making iron unavailable for plant uptake (Appendix 3). Uptake results, however, showed NTA to be the most effective chelator for improving iron uptake (Figure 4-5A). A possible explanation for this may be that the chemistry near the root apex (<1 mm) has different pH and redox conditions, allowing the plant to solubilize iron in this region (Williams et al. 2014).

Several studies (Huang et al. 1997; Vassil et al. 1998; Vadas et al. 2007) have shown that plants may be able to take up limited amounts of chelators, which then allow plants to translocate metals from roots to shoots more effectively at physiological pH. NTA is the smallest molecule of the four chelators that were tested (Figure 3-1), so it is possible that higher amounts of NTA-iron complexes were taken up by the plants, allowing them to increase their iron uptake. While plants with added DTPA should have had access to soluble iron over the entire 6 to 7 pH range, this was not reflected in the uptake results (Figure 4-5A). Again, the large DTPA molecule could be playing a role by chelating iron but then blocking uptake by the plant through the apoplastic pathway.

5.2.2 Copper treatment

In the copper treatment, plants provided with HEDTA had the largest biomass, which may be explained by the results of the ICP-MS analyses results. HEDTA-treated plants had a 3-fold higher iron uptake than control and a 4-fold increase in iron compared to DTPA-treated plants, which were not statistically different from the plants from the NTA and EDTA treatments.

Exogenous chelators did not increase copper uptake (Figure 4-5C). Wei et al. (2007) also showed diminished copper uptake by *Chrysanthemum coronarium* L when exposed to copper toxicity. One possible explanation for the poor performance of chelators in terms of increasing copper availability would be if plants preferentially take up free copper ions (Degryse et al. 2006). The presence of chelators would reduce the free copper ion activity in the solution (Parker et al. 1995).

In addition, it has been shown that chelators such as ethylenediamine-N,N'-disuccinic acid (EDDS) EDDS-Cu can be taken up by the nonselective apoplastic pathway (Tandy

et al. 2006). The Casparian strip, a highly suberized band that halts apoplastic flow, acts as a barrier and forces metals to cross the cell membranes of the endodermis, which prevents the diffusion of metals and metal-chelators from the cortex to the stele (Marschner 1989). In order for intact metal-chelator complex to get into the stele, it would be necessary for them to find a break in the root endodermis and Casparian strip. While it is true that these complexes may find their way around the endodermal barrier due to diffusion to adjacent tissues (Lane and Martin 1977), it would still reduce the efficiency of chelators in their task of increasing metal uptake.

5.2.3 Zinc treatment

For plants in the zinc treatment, DTPA outperformed the remaining chelators as well as the control in terms of zinc uptake. It is important to note, however, that iron uptake was greatly reduced in DTPA-treated plants when compared to control and EDTA plants. Zinc has been shown to interfere with the iron uptake mechanism (Rosen et al. 1977), while the possible reduction in catalase activity can interfere with the plant's ability to uptake iron and other micronutrients (Agarwala et al. 1977). Other studies have shown higher zinc uptake in soybean (*Glycine max* L. cv Klaxon) and lettuce (*Lactuca sativa* CV) treated with DTPA relative to control, and the high stability constant of DTPA (Appendix 1) may be aiding the extraction of zinc by these plants (Vadas et al. 2007; López-Rayó et al. 2015). The exact mechanism of action of DTPA on zinc uptake is, however, still unknown.

5.2.4 Cadmium treatment

Both the EDTA and HEDTA treatments increased cadmium uptake by radishes. This is in contrast to a study with maize that showed a reduction in cadmium content in shoots exposed to a cadmium toxicity treatment, which was proposed to be due to reduced Cd^{2+} activity and increased Cd-EDTA complex activity (Custos et al. 2014). On the other hand, a study with wavy saltbush (*Atriplex undulata*) and quail saltbush (*Atriplex lentiformis*) showed an increase of as much as 117% of cadmium uptake by shoots in response to EDTA. NTA- and DTPA-treated plants had a slightly lower biomass when compared to the EDTA- and HEDTA-treated plants. This is in accordance with a study

showing a reduction of 45% in root and 36% in shoot biomass when plants were subjected to EDTA (Eissa et al. 2014), which could then explain lower uptake due to decreased plant weight. The higher stability constant of EDTA and HEDTA for cadmium when compared to NTA could have played a role in increasing uptake, while the cadmium-DTPA stability constant could have been too high, preventing a dissociation of cadmium into free Cd^{2+} ions which are easily absorbed by the roots (Wang et al. 2007).

5.3 Model and its relation to metal uptake

Regarding the influence of organic acids in the model, Visual MINTEQ showed no effects of exuded malate, malonate and oxalate on the solubility of metals in the nutrient solution. Even with the organic acids acting as chelators, and present in their deprotonated form, their low stability constants makes it hard for them to compete for metals in solution, and hydroxide molecules end up being favored, creating insoluble oxides and hydroxides (Fangueiro et al. 2002). It appears that organic acids would have had a higher influence at an acidic pH, getting closer to their acid dissociation constant, without having to compete for metal ligands with hydroxide molecules.

Organic acids may play a more important role in metal chelation inside the plant, most likely aiding detoxification by binding to metal molecules in the cytosol and transporting them to the vacuole. Physiological pH inside a plant cell is around 5.0, increasing the affinity of organic ligands for metal molecules (Mathys 1977). Organic acids also play a significant role in essential and non-essential nutrient transport inside the plant, with non-essential metals such as cadmium being chelated, most likely by citrate (Rengel 2002; Rascio and Navari-Izzo 2011) Although not fully understood yet, there are indications that some toxic metals could even be transported from xylem-to-phloem without being unloaded into leaf blades, greatly reducing the toxic properties of these metals (Fujimaki et al. 2010).

One of the most interesting results of this experiments was the low uptake of metals in each treatment when compared to the soluble metals that were available according to the model (Figure 4-10A-D). Metal uptake was below 2.5% except in the iron treatment, where the model predicted low solubility of iron available and plants took up a higher

amount than expected. This low uptake could be caused by the selectivity of membrane transporters, preventing metals from being taken up, as well by the effect of the Casparian strip acting as a physical barrier (Meyers et al. 2008; Verkleij et al. 2009). Higher than expected uptake, such as the one obtained in the control treatment, could be caused by the aid of phytosiderophores, which have been shown to aid with iron uptake (Romheld and Marschner 2017).

While it is expected that plants given exogenous chelators would take up more metals, several studies with different chelators, including the ones analysed in this study, and others, including but not limited to hydroxyiminodisuccinic acid (HIDS) and ethylenediaminedisuccinic acid (EDDS) have shown this is not always the case (Blaylock et al. 1997; Walker et al. 2003; Tandy et al. 2004; Hasegawa et al. 2010). Hasegawa et al. (2010) showed that EDTA could increase the uptake of iron (Fe^{3+}) in roots; however, this wouldn't necessarily result in increased uptake in leaves and stems. Since leaves, shoots and roots were pooled in order to get enough mass for the ICP-MS analysis in my study, there is a possibility that the average plant metal uptake obscured differences that might have been found among root or shoot tissues. The production of phytosiderophores, which can solubilize ferric compounds for uptake by roots (Romheld and Romheld 1987), could be responsible for the higher iron uptake than expected in plants that were not given chelators. As mentioned in Section 1.5, production of other compounds, including phenolics and other amino acids, could explain the iron uptake.

While exogenous chelators should continue to be used to enhance phytoremediation, my results indicate that modelling metal-chelator interactions cannot predictably and consistently be used to predict the uptake of metals by plants (Figure 4-10A-D). Indeed, metal uptake was proportional to the predicted amount of soluble metal only for the plants subjected to the excess copper treatment. Different uptake/solubility ratios indicate poor correlation between the model and the experimental results. While Visual MINTEQ 3.1 is a good predictor of metal solubility and speciation, the number of interactions in a biological system become very difficult to predict. Kinetic uncertainty plays a big role, and the 1.1:1 ratio of chelator:metal may have not been enough to ensure a chelator-metal bond. Even though a hydroponic setup was utilized in order to reduce the number of

factors that could affect metal solubility and uptake by radishes, some potentially important variables are not taken into consideration by Visual MINTEQ 3.1. These include possible adsorption to the glass jars, possible production of phytosiderophores by radishes and how the chelators affect their production, as well as the impact of kinetic energy of the constant fluid movement caused by aeration of the nutrient solution. Other studies (Epstein et al. 1999; Cajuste et al. 2000; Walker et al. 2003; Menzies et al. 2007) have found poor correlations in these types of modelling exercises, with low R^2 values for chelator concentrations and metal uptake, as well as different results with different agents depending on plant, metal and growth conditions. In some scenarios, other studies (Athalye et al. 1995; Sahut et al. 2003) have shown that chelators do not have a positive effect on metal uptake and, in some cases, they may even reduce metal uptake by reducing metal activity in the nutrient solution (Alkorta et al. 2004).

Chapter 6

6 Recommendations, limitations and future work

6.1 General recommendations

One of the main differences among the chelators that have been used in this study is their degradation potential. NTA is considered “environmentally friendly” due to its capacity to be degraded by microbial organisms including *Cohnella asacharovorans*, *Chelatobacter heintzii* and *Agrobacterium radiobacter* (Bucheli-Witschel and Egli 2001). These organisms are able to utilize aminopolycarboxylic acids (APCAs), the group to which NTA belongs, as a sole source of carbon, nitrogen and energy (Bucheli-Witschel and Egli 2001). A potential for photodegradation of the Fe(III)-EDTA complex has also been shown; however, its persistence in EDTA-contaminated environments suggest that this is a slow process (Egli 2001). On the other hand, the high stability constant of DTPA prevents any biodegradation and photodegradation, and this complex can remain on site for extended periods of time (Sýkora et al. 2001; Sýkora and Pitter 2001).

Another important factor to consider while utilizing chelators is the concentration that will be applied to help with phytoremediation. In hydroponics, 100 μM EDTA can cause toxicity and therefore reduce plant biomass (Rengel 2002), and in soil experiments it has been shown to cause necrotic lesions on leaves when applying a dose comparable to 550 kg/ha (Bloem et al. 2017). Since areas where solutes can enter the xylem without crossing the plasmalemma membrane are larger in hydroponic roots (Bloem et al. 2017), a higher metal uptake is expected to occur in hydroponic conditions. Therefore, it may be necessary to use a conservative approach when determining how much chelator is applied to detoxify a certain amount of metal contamination. Increasing the chelator:metal ratio (for example, 2:1 or 3:1) may be appropriate, especially in hydroponics where metals such as zinc and copper are already soluble, but it could be necessary to conduct experiments to consider possible toxicity of microelements such as manganese.

Finally, application of chelators on top of the soil may be sufficient to aid with metal extraction. While it may appear intuitive to add any chelator before a crop is planted, the

high mobility of chelators due to rain and plant absorption (Bloem et al. 2017) will allow them to move from the surface of the soil towards a depth of 90+ centimeters.

In cases where there is an immediate need to increase metal absorption due to nutrient deficiency, foliar application of a metal-chelator would be considered a good option, since plants can absorb and export nutrients from the point of application to the point of utilization (Kannan 2010). However, this may not be an optimal approach for large agricultural fields.

6.2 Recommendations per metal

EDTA was shown to be the best chelator to improve cadmium uptake, by increasing metal uptake over both NTA and DTPA treatments by 275% and 315%, respectively. HEDTA was shown to be the second most effective. EDTA was also superior to HEDTA for cadmium uptake by *Sesbania drummondii* (Rydb.) (Ruley et al. 2006) and sunflower (*Helianthus annuus* (L.)) (Chen and Cutright 2001; Shen et al. 2002), and was more effective than NTA in *Boehmeria nivea* (L.) Gaudich (Yin et al. 2015) and *Ricinus communis* (L.) (Chhajro et al. 2016). However, EDTA was less effective than EDDS (S,S-ethylenediaminedisuccinic acid) (Luo et al. 2005; Luo et al. 2014), which was not studied in this research project. Based on my results, and the relative cost and availability of the different chelators, it would appear that the most effective way to increase cadmium uptake in radishes would be by utilizing EDTA as a chelator.

The results for zinc solubility and uptake are not as conclusive as the cadmium results. While plants with exogenous DTPA had a 2-fold increase in zinc uptake over EDTA-treated plants and a four-fold increase over HEDTA- and NTA-treated plants, this is contrary to other studies that showed EDTA to be more effective than DTPA in aiding zinc uptake in *Agrostis castellana*, *Corrigiola telephiifolia*, *Vetiveria zizanioides* (Chiu et al. 2005) and *Zea mays* (Pastor et al. 2007). However, my results showed a higher uptake of zinc by plants given DTPA, and even by control plants, than for plants given the EDTA treatment. These inconsistencies may have been due to the studies being done in different conditions (soil vs hydroponics) as well as the different concentrations of chelators used, ranging from 0 to 20 mmol per kilogram of soil. The high affinity

constant of DTPA may have played an important factor in soil studies, binding it tightly to other minerals present in soil, including calcium, and preventing it from being useful as a zinc chelator (Karak et al. 2016). In hydroponics, and according to the results of this study, DTPA would be a good choice in order to increase zinc uptake. A discussion about recommendations in soil will be presented in Section 6.3.

Finally, copper solubility did not differ among any of the chelator treatments that were conducted. Some other studies have shown EDTA to be effective in increasing copper uptake in *Agrostis castellana* and *Corrigiola telephiifolia* (Pastor et al. 2007) and lettuce (*Lactuca sativa*) (Vadas et al. 2007) whereas others have shown DTPA to increase copper uptake in lettuce (*Lactuca sativa*) (Gonzalez and Alvarez 2013), and sunflower (*Helianthus annuus*), Chinese cabbage (*Brassica campestris*), cattails (*Typha latifolia*) and reeds (*Phragmites communis*) (Yeh et al. 2015). In addition, a study of copper uptake in lettuce sprouts found no significant difference between EDTA and DTPA treatments (Inaba and Takenaka 2005). A study comparing the effects of EDTA and NTA treatment on copper uptake in *Brassica juncea* and *Lolium perenne* showed EDTA to be more effective; however, plants treated with NTA showed a high transport of copper from root to shoot, matching its EDTA counterpart (Johnson et al. 2009), which could be a desired effect when using radishes for phytoremediation. It is possible to assume that EDTA and NTA could yield better results for other plants, and with a longer treatment period (> 15 days) one might see an increase in copper uptake.

6.3 Limitations and future work

The main objective of this research project was to determine if a modelling approach could be used to predict metal uptake by radishes grown in hydroponic conditions. Visual MINTEQ 3.1 was used as a speciation and solubility model to predict metal uptake; however, mixed results were found due to several reasons. Some other studies have also shown a poor correlation between predicted metal uptake by using solubility and speciation models (Epstein et al. 1999; Cajuste et al. 2000; Menzies et al. 2007), while others have shown promising results (Parker et al. 1995; Schwab et al. 2008; Wen et al. 2016). It has been determined that the pH value of the surrounding medium is the most important factor that determines ligand exchange and metal complex formation. Some

other factors, such as redox reactions, are of great importance as well for ions that have different oxidation states such as Fe^{2+} and Fe^{3+} (Curie et al. 2009). While Fe^{2+} becomes soluble and is available for plant uptake, Fe^{3+} forms precipitates that are unavailable for plant uptake. Kinetic factors, such as pumping air into the system, may affect the percentage of precipitation of these compounds in the jars. One of the main limitations of this research project is the lack of information about other compounds that may be exuded from the roots. While pH affects directly the stability of complexes formed due to the protonation or deprotonation of molecules, this does not necessarily translate to metal uptake due to the role of other molecules in solution such as peptides, including phytochelatins and metallothioneins, and humic acids (Freisinger 2008).

Without knowing the concentrations of the other molecules that could act as chelators in solution, the accuracy of the model is reduced. However, even with access to all the information from these molecules, it does not guarantee that the model will show a perfect correlation between solubility and metal uptake by any plant, including radishes. This is because chelators inside the plant play a big role on metal uptake and the conditions of pH and redox potential inside the root may determine more accurately what is actually happening with the metals after they cross rhizosphere.

For future work, it may be necessary to keep improving the model by obtaining information about some other organic acids such as butyrate and glutamate, which may also play a role in metal detoxification. This project was focused mostly on the effects of chelators that were exuded to the growth medium; however, the importance of these chelators inside the plant, mostly in the plant sap, may also be of great importance for increasing metal uptake. The pH inside the plant is generally more acidic than in the growth medium and is kept constant, which gives organic acids such as malate and citrate greater capacity to chelate metals with more stability and for longer, increasing their movement towards the vacuoles and reducing damage caused by the presence of metals inside the cytosol.

In order to give a more accurate recommendation for phytoremediation in soil, it may be necessary to conduct an experiment with different ratios of chelator to metal, moving

from zero towards a 2:1 or 3:1 range, and then analyze if these chelators behave differently under soil conditions. It is expected for soil to produce less mobility of metals than hydroponics, therefore metal solubility should remain lower than in the hydroponic experiments. In that scenario, maximizing the amount of chelators applied to soil, while also avoiding chelator toxicity, would be the main objective. It will also be important to assess the frequency at which chelators are applied, especially NTA which is rapidly biodegraded (Nancharaiyah et al. 2006). Leaching of chelators to below the rooting zone will also have to be studied under field conditions; phytoextraction will be enhanced by chelation only if the metals stay in close proximity to the roots.

Although my research project gave different results compared to other studies, the use of modelling is an increasingly popular approach when selecting conditions to improve phytoremediation, mostly in soil but as well in some hydroponic (greenhouse) conditions. With my research results, it is not possible to recommend Visual MINTEQ as the sole source for predicting metal uptake by radishes; however, it is still a good aid when determining which chelators should be utilized to increase metal uptake.

References

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Appendices

Appendix 1: Table of characteristics of chelators. All four chelators used in this project are presented, along with their stability constants (Ueno et al. 1992), biodegradability (Nörtemann 2005), rated from low to high, and the price in \$USD per ton (Alibaba 2017).

Chelator characteristics			
Chelator	Cadmium stability constant	Biodegradability	Price (\$USD per ton)
EDTA	16.46	Low	1500
HEDTA	13.6	Low	2200
NTA	9.54	High	1600
DTPA	19.31	Low	2000

Appendix 2: List of statistical results obtained. All the ANOVA results from this research project, including their F and P values.

Figure 4-1: pH change over time.					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1.692	12	0.141	$F_{(12, 48)} = 3.637$	$P = 0.0007$
Time	1.474	4	0.3686	$F_{(4, 48)} = 9.506$	$P < 0.0001$
Metal	0.6739	3	0.2246	$F_{(3, 12)} = 1.439$	$P = 0.2801$
Subjects (matching)	1.873	12	0.1561	$F_{(12, 48)} = 4.026$	$P = 0.0003$
Residual	1.861	48	0.03877		
Figure 4-2: Plant dry weight.					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1.44	12	0.12	$F_{(12, 56)} = 7.288$	$P < 0.0001$
Metal	6.596	3	2.199	$F_{(3, 56)} = 133.5$	$P < 0.0001$
Days	1.546	4	0.3866	$F_{(4, 56)} = 23.48$	$P < 0.0001$
Residual	0.9221	56	0.01647		
Figure 4-3A: Organic acid exudates (Control).					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	313.9	8	39.24	$F_{(8, 33)} = 1.829$	$P = 0.1066$
Organic acid	2748	2	1374	$F_{(2, 33)} = 64.06$	$P < 0.0001$
Chelator	425.1	4	106.3	$F_{(4, 33)} = 4.956$	$P = 0.0031$
Residual	707.7	33	21.45		
Figure 4-3B: Organic acid exudates (Zinc).					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	75.01	8	9.376	$F_{(8, 42)} = 1.560$	$P = 0.1664$
Organic acid	2815	2	1408	$F_{(2, 42)} = 234.1$	$P < 0.0001$
Chelator	87.6	4	21.9	$F_{(4, 42)} = 3.642$	$P = 0.0123$
Residual	252.5	42	6.012		
Figure 4-3C: Organic acid exudates (Copper).					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	70.88	8	8.86	$F_{(8, 37)} = 2.704$	$P = 0.0189$
Organic acid	1857	2	928.7	$F_{(2, 37)} = 283.4$	$P < 0.0001$
Chelator	11.25	4	2.813	$F_{(4, 37)} = 0.8584$	$P = 0.4978$
Residual	121.2	37	3.277		
Figure 4-3D: Organic acid exudates (Cadmium).					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	33.61	8	4.201	$F_{(8, 36)} = 0.6974$	$P = 0.6914$
Organic acid	2297	2	1149	$F_{(2, 36)} = 190.7$	$P < 0.0001$
Chelator	21.6	4	5.4	$F_{(4, 36)} = 0.8964$	$P = 0.4762$

Residual	216.9	36	6.025		
Figure 4-4A: Concentrations of metals in radish (Control).					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	78.91	12	6.576	F (12, 56) = 1.808	P = 0.0690
Chelator	34.08	4	8.520	F (4, 56) = 2.343	P = 0.0659
Metal	402.4	3	134.1	F (3, 56) = 36.88	P < 0.0001
Residual	203.7	56	3.637		
Figure 4-4B: Concentrations of metals in radish (Zinc).					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	4047	12	337.2	F (12, 56) = 8.872	P < 0.0001
Chelator	2124	4	530.9	F (4, 56) = 13.97	P < 0.0001
Metal	5471	3	1824	F (3, 56) = 47.98	P < 0.0001
Residual	2129	56	38.01		
Figure 4-4C: Concentrations of metals in radish (Copper).					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1162	12	96.86	F (12, 52) = 4.796	P < 0.0001
Chelator	1263	4	315.8	F (4, 52) = 15.64	P < 0.0001
Metal	1247	3	415.7	F (3, 52) = 20.58	P < 0.0001
Residual	1050	52	20.19		
Figure 4-4D: Concentrations of metals in radish (Cadmium).					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	484.5	12	40.37	F (12, 60) = 1.028	P = 0.4362
Chelator	265.7	4	66.44	F (4, 60) = 1.691	P = 0.1639
Metal	26377	3	8792	F (3, 60) = 223.8	P < 0.0001
Residual	2357	60	39.28		
Figure 4-5A: Total metal uptake (Control).					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	78	12	6.5	F (12, 60) = 2.320	P = 0.0164
Chelator	35.75	4	8.936	F (4, 60) = 3.190	P = 0.0193
Metal	307.3	3	102.4	F (3, 60) = 36.56	P < 0.0001
Residual	168.1	60	2.802		
Figure 4-5B: Total metal uptake (Zinc).					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	340.7	12	28.4	F (12, 60) = 4.930	P < 0.0001
Chelator	85.12	4	21.28	F (4, 60) = 3.694	P = 0.0094
Metal	651.4	3	217.1	F (3, 60) = 37.70	P < 0.0001
Residual	345.6	60	5.76		
Figure 4-5C: Total metal uptake (Copper).					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value

Interaction	15.2	12	1.266	$F_{(12, 60)} = 1.252$	$P = 0.2710$
Chelator	13.9	4	3.475	$F_{(4, 60)} = 3.435$	$P = 0.0136$
Metal	24.69	3	8.229	$F_{(3, 60)} = 8.134$	$P = 0.0001$
Residual	60.7	60	1.012		
Figure 4-5D: Total metal uptake (Cadmium).					
ANOVA table	SS	DF	MS	$F_{(DFn, DFd)}$	P value
Interaction	196.7	12	16.39	$F_{(12, 60)} = 3.581$	$P = 0.0005$
Chelator	84.46	4	21.12	$F_{(4, 60)} = 4.614$	$P = 0.0026$
Metal	868.6	3	289.5	$F_{(3, 60)} = 63.26$	$P < 0.0001$
Residual	274.6	60	4.577		
Figure 4-10A: Percentage of metal taken divided by total metal solubility at pH 6.5 according to Visual MINTEQ (Control)					
ANOVA table	SS	DF	MS	$F_{(DFn, DFd)}$	P value
Treatment	103948	2	51974	$F_{(2, 9)} = 9.054$	$P = 0.0070$
Residual	51665	9	5741		
Total	155614	11			
Figure 4-10B: Percentage of metal taken divided by total metal solubility at pH 6.5 according to Visual MINTEQ (Zinc)					
ANOVA table	SS	DF	MS	$F_{(DFn, DFd)}$	P value
Treatment	0.03452	4	0.00863	$F_{(4, 15)} = 1.699$	$P = 0.2024$
Residual	0.07618	15	0.005078		
Total	0.1107	19			
Figure 4-10C: Percentage of metal taken divided by total metal solubility at pH 6.5 according to Visual MINTEQ (Copper)					
ANOVA table	SS	DF	MS	$F_{(DFn, DFd)}$	P value
Treatment	4.988	4	1.247	$F_{(4, 15)} = 11.64$	$P = 0.0002$
Residual	1.608	15	0.1072		
Total	6.595	19			
Figure 4-10D: Percentage of metal taken divided by total metal solubility at pH 6.5 according to Visual MINTEQ (Cadmium)					
ANOVA table	SS	DF	MS	$F_{(DFn, DFd)}$	P value
Treatment	2.196	4	0.5489	$F_{(4, 15)} = 3.804$	$P = 0.0250$
Residual	2.164	15	0.1443		
Total	4.36	19			

Appendix 3: Solubility of iron and amount of finite solids. Solubility was calculated in a solution with NTA at pH 6.0 as modeled by Visual MINTEQ 3.1

Component	% dissolved	% precipitated
Fe+3	0	100
Solid	Equilibrium amount (mol/l)	
Hematite	5	
MnHPO4(s)	2.42	

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