THE HONG KONG
POLYTECHNIC UNIVERSITY
香港理工大學
Pao Yue-kong Library
包玉剛圖書館

Copyright Undertaking

This thesis is protected by copyright, with all rights reserved.

By reading and using the thesis, the reader understands and agrees to the following terms:

1. The reader will abide by the rules and legal ordinances governing copyright regarding the use of the thesis.
2. The reader will use the thesis for the purpose of research or private study only and not for distribution or further reproduction or any other purpose.
3. The reader agrees to indemnify and hold the University harmless from and against any loss, damage, cost, liability or expenses arising from copyright infringement or unauthorized usage.

IMPORTANT

If you have reasons to believe that any materials in this thesis are deemed not suitable to be distributed in this form, or a copyright owner having difficulty with the material being included in our database, please contact lbsys@polyu.edu.hk providing details. The Library will look into your claim and consider taking remedial action upon receipt of the written requests.

Pao Yue-kong Library, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong

http://www.lib.polyu.edu.hk

THE ROLE OF EDDS ON SOIL-PLANT-COPPER INTERACTIONS DURING CHELANT-ASSISTED PHYTOEXTRACTION

ZHAO YANPING

PhD

The Hong Kong Polytechnic University

2018

The Hong Kong Polytechnic University

Department of Civil and Environmental Engineering

The Role of EDDS on Soil-Plant-Copper Interactions during Chelant-assisted Phytoextraction

Zhao Yanping

A thesis submitted in partial fulfilment for the degree of Doctor of Philosophy

January 2018

Certificate of Originality

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

_____(Signed)

Zhao Yanping (Name of Student)

Abstract

Soil pollution with heavy metals is a serious environmental issue around the world. To remediate heavy metals in contaminated soils, chelant-assisted phytoextraction has been paid much attention and investigated widely. However, a thorough understanding is still of lack on the complex interactions among chelants, target metals, soils, and plants. Therefore, the current study aims to investigate the role of the biodegradable chelant on the extraction and transport of a representative metal - Cu in soil and plant processes.

Firstly, the performance of [S,S]-ethylenediaminedisuccinic acid (EDDS) and tetrasodium of N,N-bis(carboxymethyl) glutamic acid (GLDA) on Cu phytoextraction was compared with ryegrass and tall fescue. Results showed that, compared to GLDA, EDDS induced a higher Cu concentration in plants, showed less phytotoxicity, and degraded faster in soils. Therefore, EDDS was selected as a representative biodegradable chelant for the following study.

Secondly, the impact of EDDS on soil processes in phytoextraction was investigated mainly from two aspects. The first aspect concentrated on the chemical interactions of EDDS with soils in rhizosphere of ryegrass. After application into a multi-interlayer rhizobox for 7 d, EDDS transported from non-rhizosphere to rhizosphere of ryegrass. Using synchrotron-based techniques, such as X-ray micro-fluorescence (μ-XRF) and X-ray absorption near edge structure (XANES), EDDS primarily extracted Cu from the adsorbed fraction on goethite instead of clay minerals in tested soils, which was probably associated with the EDDS-promoted dissolution of iron oxides. Transportation of Cu from

non-rhizosphere to rhizosphere was also facilitated in the form of CuEDDS identified by solution speciation modelling. The second aspect focused on the impacts of EDDS to soil nutrients and microbes in the rhizosphere of ryegrass. Results showed that EDDS was beneficial to rhizosphere soil microbes, with the increase of microbial biomass C, microbial biomass N, and urease activities. The benefits of EDDS can be associated with the high concentration of soil nutrients in rhizosphere soils after the application of EDDS.

Finally, the influencing mechanism of EDDS on Cu uptake and transport in ryegrass was studied. EDDS increased the Cu translocation from root to shoot of ryegrass. Cu distribution in roots by μ-XRF of showed that EDDS alleviated the deposition of Cu in meristem of root tip, and in the lateral and primary root conjunction of lateral root zone. Cu speciation by XANES revealed that EDDS formed stable CuEDDS complex, reduced the root sequestration of Cu, and thus improving the transport of Cu within plants. A conceptual model was developed to describe the mechanism of Cu uptake and transport either in the presence or absence of EDDS.

Collectively, this study revealed that EDDS was effective to extract Cu from soils, facilitate Cu transportation to root surface, and improve Cu internal mobility within plants. It unravels the major mechanisms involved in chelant-assisted phytoextraction, which will promote the development and application of this technology in future.

Acknowledgement

Firstly, I would like to express my sincere appreciation to my supervisor, Professor Li Xiangdong, for his patient guidance, continuous encouragement, and kind support during the period of study.

During my PhD. study life, I have received a lot of help from friends and technicians around. Without their help, I could not learn so many skills and solved some tough problems. Firstly, I would like to express a very special gratitude to Dr. Cui Jinli for his continuous assistance from the research design, experiment conduction, and results analysis. Then, I would like to thank Mr. W.S. LAM, Ms. Carman IP, and Ms. Chloe TANG, since they provided warmly help on my experimental work in laboratory. I am grateful to Dr. Jin Ling and Dr. Bing Haijian for sharing their research enthusiasm with me. In addition, thanks must go to Dr. Wang Jianxu, Dr. Yin Runsheng, Dr. Sun Jianteng, Dr. Xiang Nan, Miss. Liang Siyuan, Dr. Ming Lili, and Miss Xie Jiawen for their kind help and support during my study life.

I would like to sincerely appreciate the continuous support from my beloved parents, husband, elder sister, uncle, and other family members.

Finally, I would like to thank the Research Grants Council of Hong Kong for funding this project.

Table of Contents

List of Figures

List of Tables

ABBREVIATIONS

ANOVA	One-way analysis of variance
CCA	Chromate copper arsenate
DOC	Dissolved organic matters
DTPA	Diethylenetriaminepentaacetic acid
EDDS	[S,S]-ethylenediaminedisuccinic acid
EDTA	Ethylenediaminetetraacetic acid
EXAFS	Extended X-ray absorption fine structure
FAO	Food and agriculture organization of the United Nations
GLDA	Tetrasodium of N,N-bis(carboxymethyl) glutamic acid
HPLC	High-performance liquid chromatography
HIDS	3-hydroxy-2,2’-iminodisuccinic acid
PCs	Phytochelatins
PCA	Principal component analysis
PCR-DGGE	PCR-denaturing gradient
PGPB	Plant-growth-promoting microbes
PLFAs	Phospholipid fatty acids
ROS	Reactive oxygen species
ICP-OES	Inductively coupled plasma optical emission
ICP-MS	Inductively coupled plasma mass spectrometry
IDSA	Imminodisuccinic acid
IGOR	Igor Pro 6.0 software
IS	Ionic strength
LMWOAs	Low molecular weight organic acids
LA-ICP-MS	Laser ablation-inductively coupled plasma-mass spectrometry
LCF	Linear combination fitting
MGDA	Methylglycine diacetic acid
MTs	Metallothioneins

NTA	Nitrilotriacetic acid
NSRRC	National Synchrotron Radiation Research Center
SEM-EDX	Scanning electron microscopy combined with energy dispersive X-ray spectroscopy
SEP	Sequential extraction procedure
SSRF	Shanghai Synchrotron Radiation Facility
TT	Target transform
TF	Translocation factor
WHO	World Health Organization
XAS	X-ray absorption spectroscopy
XAFS	X-ray absorption fine structure
XANES	X-ray absorption near edge structure
XRD	X-ray powder diffraction
μ-XANES	Micro-X-ray absorption fine structure
μ-XRF	Micro-X-ray fluorescence

1. Chapter One - Introduction

1.1 Background

Soil is a critical resource for life, as it is the basis of agricultural production, residential use, business affairs, and industrial activities (Chen, 2007). However, soils have been contaminated by heavy metals in Hong Kong, China, and many other places around the world, due to anthropogenic activities including mining, smelting, industrial activities, sewage irrigation, pesticide application, and traffic emissions (Wong et al., 2006; Luo et al., 2011). For instance, a total of 1,500,000 ha (0.016%) of metal-polluted wasteland has been generated in China as a result of mining, and the area keeps enlarging at a rate of 46,700 ha per year (Zhuang et al., 2009). The contaminated soil plays a role as the sink for heavy metals, as well as the source for emission of heavy metals via runoff and flying dusts. The high concentration of heavy metals in contaminated soils pose great risks to the ecosystem, as heavy meals are toxic to plants, animals, and human beings (Li et al., 2014). As a result, the contaminated soil will limit the land use and function, and obstruct land planning and management. Therefore, the appropriate remediation strategies are required to remove the pollutants or mitigate the risks of contaminated sites.

Among the proposed soil remediation strategies, phytoremediation stands out as an environmental friendly technology, which uses green plants for *in situ* treatment of contaminated soils (Salt et al., 1995). As one kind of the distinguished subcategories of phytoremediation, phytoextraction aims to move the non-degradable metals from soils to plant shoots. Because phytoextraction efficiency is critically controlled by the bioavailability of metals, some amendments are often applied to soils to increase the solubility of target metals. Among the amendments, synthetic chelants, such as ethylenediaminetetraacetic acid (EDTA), are the most efficient and widely used in phytoextraction trials, to mobilize metals from soils and increase root-to-shoot translocation of metals in plants (Shahid et al., 2012). However, EDTA is recalcitrant to biodegradation, which leads to leaching risks of metals to subsoil layers or groundwater (Chen et al., 2004). Currently, [S,S]-ethylenediaminedisuccinic acid (EDDS) has been developed as an alternative to EDTA (Grčman et al., 2003), because it is less toxic to plants and microbes, and degrades faster in soils or sludge (Vandevivere et al., 2001; Meers et al., 2008). In view of previous studies, EDDS shows the comparable capacity as EDTA for extracting metals, especially for Cu and Zn, and EDDS limits the leaching risks of metals in field application (Luo et al., 2005; Wang et al., 2012).

Improvement of phytoextraction processes requires a thorough understanding of the

metal complex in the soil-plant continuum. Generally, there are two main processes involved in chelant-assisted phytoextraction of the contaminated site, including soil processes and plant processes (Nowack et al., 2006). On the one hand, soil processes control the metal solublization, speciation, and transport, which determine the metal availability from soils to plant roots. In addition, soil processes also affect the function and composition of indigenous soil microbes, which reflects the soil quality after soil remediation (Epelde et al., 2008; Jelusic et al., 2013). On the other hand, plant processes control the metal uptake, tolerance, and transformation by plant tissues, which ultimately determine the efficiency of phytoextraction. In the past several decades, investigations of chelant-assisted phytoextraction have been extensively conducted primarily from the perspective of environmental engineering, in order to test, evaluate, or enhance the potential use of this technology in field soil remediation (Cao et al., 2007; Evangelou et al., 2007; Bolan et al., 2014). However, studies are still not enough regarding the underlying mechanisms of chelant-assisted phytoextraction (Ali et al., 2013). According to past work, although the enhancement influence of chelant on soil metal solubility has been demonstrated, studies seldom elucidated molecular interactions of metals with chelant and transport processes of metals in rhizosphere (Tandy et al., 2006b). In addition, previous reported results are controversial regarding the impacts of chelants on soil microbes after

chelant-assisted phytoextraction. In spite of that, some pieces of the plant process still remain puzzled on metal uptake and transport by plants in the presence of chelants, although researchers have made some progress (Cestone et al., 2010; Niu et al., 2011). Elucidating the molecular complex of metals from soil to plant can be much helpful for successful phytoextraction.

The understanding on metal spatial distribution and coordination environment in the plant-soil system has been extremely advanced by one of the most developed synchrotron-based technologies, such as micro-X-ray fluorescence (μ-XRF) images and X-ray absorption spectroscopy (XAS) (Gräfe et al., 2014; Castillo-Michel et al., 2016). The use of these synchrotron-based technologies in soil matrix can assist the evaluation of the metal bioavailability, leachability, and partition pattern (Strawn and Baker, 2008). Furthermore, the application of these technologies in plant matrix helps to clarify the metal uptake, transport, and detoxification pathways (Majumdar et al., 2012). Hence, these synchrotron-based technologies may provide a good opportunity to resolve the remaining problems in chelant-assisted phytoextraction.

1.2 Objectives of the present study

The overarching aims of current research are to improve the understanding on the fundamental mechanisms of chelant-assisted phytoextraction. The interactions among chelants, soils, microbes, and plants are thus investigated with particular focus on metal distribution and speciation in the soil-plant continuum. The specific objectives are listed as follows:

1) To select the most effective and safe biodegradable chelants with high-biomass plants, based on a pot experiment using copper mine contaminated soil.

2) To investigate the impact of EDDS on soil chemical interactions, including Cu molecular speciation in soil and soil extracts, and its transport in rhizosphere.

3) To study the effect of EDDS on soil qualities, which are indicated by the variation of soil nutrients, microbial biomass, and enzyme activities.

4) To explore the influence of EDDS on Cu uptake and transport by plants, including Cu distribution pattern in typical root zones and Cu coordination information in plants.

1.3 Organization of the thesis

The thesis is consisted of seven chapters (Fig. 1-1). Chapter One introduces the background, research objectives, and the framework of the study. A comprehensive literature review is delivered in Chapter Two, which describes the history of phytoremediation, concludes the related previous work, and identifies the knowledge gaps. Chapter Three provides a preliminary study to select chelants that used in subsequent mechanism experiments. Interactions among EDDS, Cu, soils, and plants are investigated in the following three chapters. Chapter Four mainly concentrates on the impact of EDDS on Cu interactions in rhizosphere soils. The effects of EDDS on soil nutrients and microbes of rhizosphere are evaluated in Chapter Five. The role of EDDS on Cu distribution and speciation pattern in ryegrass is elucidated in Chapter Six. Finally, Chapter Seven concludes the major findings from current work and provides recommendations for future research.

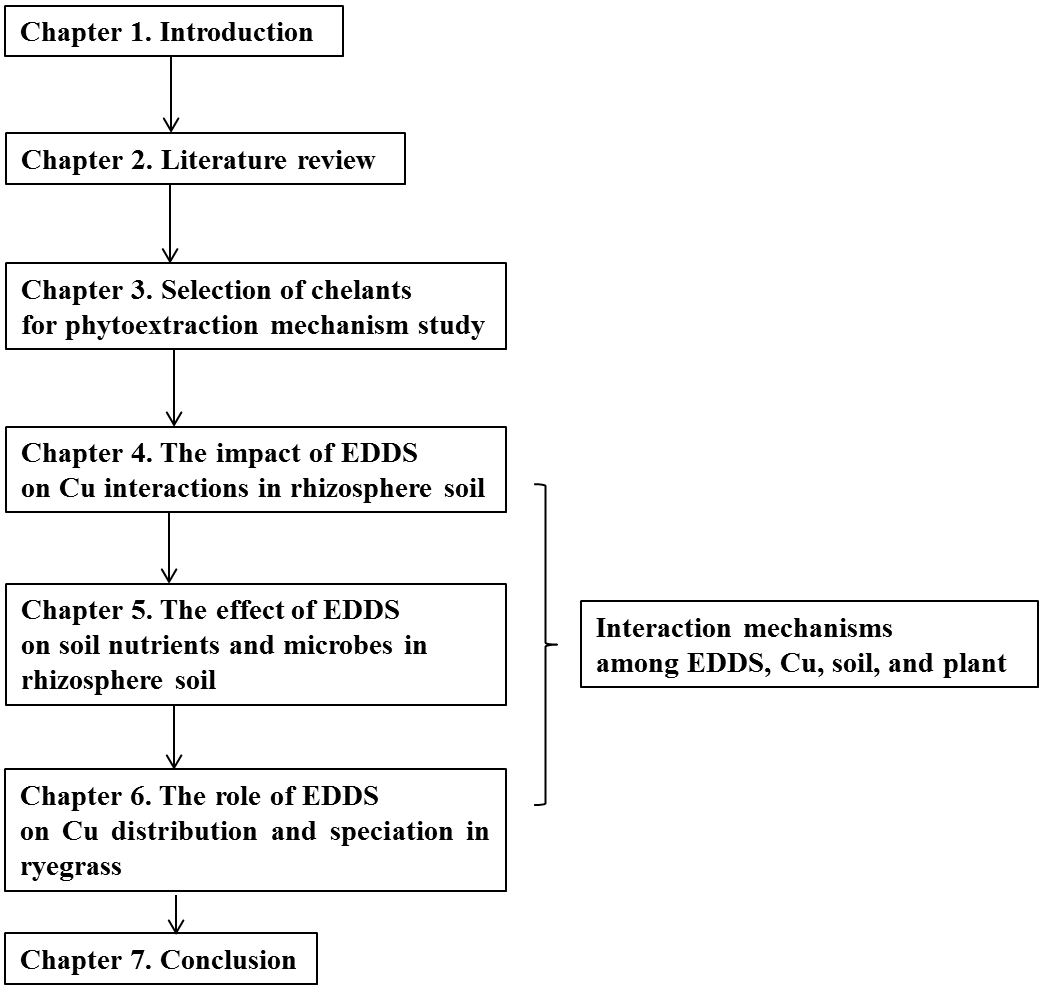

Figure 1-1 Flowchart of the thesis.

2. Chapter Two - Literature Review

This review focuses on the background and development of chelant-assisted phytoextraction of heavy metals from contaminated soil. Firstly, the soil contamination status with heavy metals is introduced, with special introduction of metal speciation and distribution in soil matrix. Secondly, the definition, history, and practice of chelant-assisted phytoextraction for contaminated soil remediation are described. Thirdly, current knowledge on underlying processes in chelant-assisted phytoextraction is reviewed, including soil interactions, rhizosphere dynamics, and plant uptake. Finally, the synchrotron based technology, including μ-XRF and XAS spectra, is introduced to advance the understanding in chelant-assisted phytoextraction process.

2.1 Soil contamination

2.1.1 Current status

With the rapid industrialization and urbanization, soil pollution with heavy metals raises as an important environmental problem in many parts of the world (Li and Thornton, 1993; Micó et al., 2006; Kachenko and Singh, 2006; Wong et al., 2006). According to a current survey (2005-2013) in Chinese land, which collected surface

soil samples from 70% area of China and analyzed thirteen inorganic contaminants and three organic pollutants, 16.1% of soil samples are classified as being polluted compared with the standard set by the Ministry of Environmental Protection of P.R. China (Zhao et al., 2015). Among the polluted soil samples, 82.8% are derived from contamination of inorganic pollutants (heavy metals and metalloids) (Ministry of Environmental Protection and Ministry of Land and Resources of P.R. China, 2014).

The pollution sources of heavy metals are dominant by anthropogenic activities, including traffic emission, fuel combustion, sewage irrigation, mining and smelting, and waste disposal (Wong et al., 2006). In China, the production of metals/metalloids is one of the key foundations for economic development, but at a cost of serious damage to environment (Li et al., 2014). Heavy metals, such as Cu, Pb, Zn, Cd, Ni, Cr, Hg, and As, are usually accompanied with the mineral resources and subsequently emitted to the surrounding environment during mining activities. The level of heavy metals has been reported to be extremely high in cities (*e.g.* Changsha and Jinchang) where large-scale activities of mining and smelting are prosperous (Chen et al., 1999; Wu et al., 2011; Luo et al., 2011). Li et al. (2014) reported that the mean values of As, Cd, Cr, Cu, Ni, Pb, Zn, and Hg in soils collected from 72 mining areas from China are greater than the Grade II environmental quality standard for soils in China (GB15618-1995) by about 6.5, 36.5, 0.4, 2.1, 2.1, 2.1, 4.7, and 7.6

folds, respectively.

Heavy metals in contaminated soils can adversely affect the growth of plants, and the health of animals and human beings (Briat and Lebrun, 1998; Tchounwou et al., 2012). Excessive metals (*e.g.* Pb, Cd, Cu, and Zn) can induce the production of reactive oxygen species (ROS), which cause oxidative stress to cells and inhibit the growth of plants (Sharma and Dietz, 2009). Heavy metals from soils can be further transferred to animals and human bodies through food chain. As heavy metals cannot be degraded, the concentration of heavy metals can keep increase by bioaccumulation in animals and human beings, leaving health danger in long-term view.

2.1.2 Copper pollution in soils

Copper is an important metal resource for the development of human society, which has a long history of use for at least 10,000 years (Alloway, 2013). In ancient times, Cu is mainly utilized for coinage and weapons. In modern times, Cu is primarily applied in electricity (65%), constructions (25%), and other uses (*e.g.* sculptures, musical instruments, and cookware) (7%) (European copper institute, 2010; Alloway, 2013). Due to the long-time and widespread use of Cu, a large quantity of Cu has been emitted into the environment.

The anthropogenic sources of soil Cu varied in different sites depending on the location and history of land use. Mining is one of the most important point sources for soil Cu pollution (Li et al., 2014). Smelting of Cu is another anthropogenic source through emission of Cu-containing dusts, sewage, and solid wastes (Li et al., 2006). Even after the stopping of mining and smelting activities for many years, the contamination is still present in soils. The high concentration of soil Cu (up to 1500 mg kg^{-1}) has been found in the vicinity of abandoned mining and smelting areas (Qin et al., 2012). In spite of that, some agronomic practices, including use of fungicides, pesticides, sewage sludge, and manures, are the major sources for agricultural soils (Brun et al., 1998; Legros et al., 2010). The wood preservation activity using copper chromate arsenate (CCA) can seriously polluted soils (Almaroai et al., 2013). In addition, the recycling of electrical and electronic waste (e-waste) also resulted in additional local input for soil Cu pollution (Cui et al., 2017).

Although Cu is an essential micronutrient for organisms at low amounts, it is toxic to organisms at elevated concentrations. In comparison with many other heavy metals (*e.g.* Zn, Mn, Hg, and Cd), the excessive Cu showed higher toxicity to plants and less to animals and human beings (Adrees et al., 2015). The high concentration of Cu can adversely affect the plant growth and the production of food crops. The fresh weight of wheat and maize has been reported to decrease in response to excess Cu (Azooz et

al. 2012; Dresler et al. 2014). In addition, the yield of rice grain can be reduced by 10-90% when soil Cu ranged from 100-1000 mg kg^{-1} (Xu et al., 2006). However, to our knowledge, soil Cu pollution has received much less attention compared to many other heavy metals (*e.g.* Hg, Pb, and Cd).

2.1.3 Characterization of heavy metals in soils

A thorough understanding of the metal characters in polluted soil is required prior to conducting soil remediation, like metal speciation, distribution, and transformation. Soil is a complex matrix, consisting of organic matters, carbonate minerals, iron oxides, manganese oxides, aluminum oxides, and clay minerals. These components are important sinks for heavy metals. The chemical forms of heavy metals in soil can be related to the pollution sources, soil nature, and aging process. For instance, Cu is primarily adsorbed on soil organic matters in organic, agriculture and vineyard soils, via inner-sphere complexation to amino, carboxyl, or carbonyl functional groups (Karlsson et al., 2006; Jacobson et al., 2007; Strawn and Baker, 2008; Strawn and Baker, 2009). However, iron oxides are reported to play the principle role in binding Cu by inner-sphere complexation in e-waste soils (Cui et al., 2017) and mining soils (Yang et al., 2014). Although the mobility of heavy metals may be controlled by the *in situ* binding to soil constituents, leaching risk is still present in varying soil

conditions, when pH decrease, soil organic matters dissolve, or iron oxides are subject to reductive dissolution in flooded soils (Alloway, 2013).

The remediation approaches for metals are based on either mobilize or immobilize target metals from soil components (Bolan et al., 2014). Mobilization techniques aim to release target metals from soil phase to soil solution, and subsequently remove them from soil components (Begum et al., 2012; Neugschwandtner et al., 2012). Immobilization techniques intend to retain metals in soil phase, and reduce their concentration in soil solution to lower their bioavailability to organisms (Malviya and Chaudhary, 2006; Lee et al., 2011). Therefore, the knowledge on metal speciation, distribution, and transformation, during the process of soil remediation, will assist the formulation of appropriate countermeasures for controlling the metal risks in short- and long-term.

2.2 Chelant-assisted phytoextraction

2.2.1 Origin, definition, and history

Phytoremediation uses plants and associated soil microbes to remove, convert, or sequester the contaminant from the environments (Greipsson, 2011; Samardjieva et al., 2011). Phytoremediation is an *in situ* technology that more appealing to the

public due to the nature of low cost and environmental friendliness, compared with many other remediation approaches including excavation, soil washing, soil incineration, solidification. This technology has gained much interest from scientific communities and environmental engineers in past several decades, especially for treatment of large-scale sites that polluted by metals on soil surfaces (Salt et al., 1995; Reeves and Baker, 2000; Weis and Weis, 2004; Gerhardt et al., 2009; Zhao et al., 2015). It is classified into several categories: phytoextraction, phytodegradation, phytostabilization, phytovotilization, and phytofiltration.

Phytoextraction is a type of phytoremediation technologies that removes heavy metals from soils through plant uptake (Kumar et al., 1995). Since the efficiency of metal uptake by plants is usually limited by the low bioavailability of metals in soils, chelants are applied to soils to solubilize metals and increase metal uptake by plants (Nowack et al., 2006). This is because the chelant is a chemical compound that can form stable and water-soluble complex with metals. Researchers use the terms "chelant-assisted phytoextraction," "chelant-enhanced phytoextraction," or "chelant-induced phytoextraction" to describe the application of chelant in phytoextraction of contaminated soils (Kos and Leštan, 2004; Komárek et al., 2007; Saifullah et al., 2009). "Chelating agents" or "chelators" are synonyms of "chelants" in literature (Wuana and Okieimen, 2010; Lambrechts et al., 2011). The

metal-chelant complex, formed after chelation between metals and chelants, can be named as “chelate.”

The research on chelant-assisted phytoextraction has already lasted for several decades. The idea of applying chelants with plants is initially inspired by studies on plant nutrition dating back to 1950s, when botanists find that the plant deficiency of nutrient metals (*e.g.* Fe) is alleviated after supplying nutrient metals to plants together with chelants (Wallace et al., 1955). In 1990s, Huang and Cunningham (1996) revealed that the use of EDTA (2 g kg^{-1}) in contaminated soils (Pb 2500 mg kg^{-1}) substantially enhanced Pb concentration in corn leaves to 10600 mg kg^{-1}. Later, extensive lab work demonstrate that chelants can increase the accumulation of metals in plants in hydroponic, pot, and field experiments (Vassil et al., 1998; Tandy et al., 2006b; Wang et al., 2012). In recent years, it is recommended to combine new economic opportunities with phytoextraction, such as the production of bioenergy, biochar, and biofortified crops, which would make the phytoextraction or more attractive in practice (Conesa et al., 2012).

2.2.2 Chelants used in phytoextraction

The chelant is a type of compound with high affinity to metals, and it is widely used in different industries, such as production of cosmetics, cleaning detergents,

pharmaceuticals, and scale and corrosion inhibitors, waste or effluents treatment, metal electroplating and other surface treatments (Pinto et al., 2014). Chelants normally used in phytoextraction are natural and synthetic organic ligands, including amino acids (histidine acid), low molecular weight organic acids (citric acid), humic substance (humic acid), and amino polycarboxylates (EDTA) (Najeeb et al., 2009; Karczewska et al., 2011; Shahid et al., 2012). The effective chelants for phytoextraction are required not only able to mobilize metals from soils but also improve metal transport from plant root to shoot.

Among various chelants, the type of amino polycarboxylates is the most efficient and widely studied in chelant-assisted phytoextraction, such as EDTA, EDDS, diethylenetriaminepentaacetic acid (DTPA), nitrilotriacetic acid (NTA), methylglycine diacetic acid (MGDA) and tetrasodium of N,N-bis(carboxymethyl) glutamic acid (GLDA). The structural formulas of these representative amino polycarboxylates are shown in Fig. 2-1, which are featured by one or more amines and two or more carboxylic acid groups. These chelants form strong complex with metal ions through coordinating metals with amine and carboxylic ligands (Fig. 2-2).

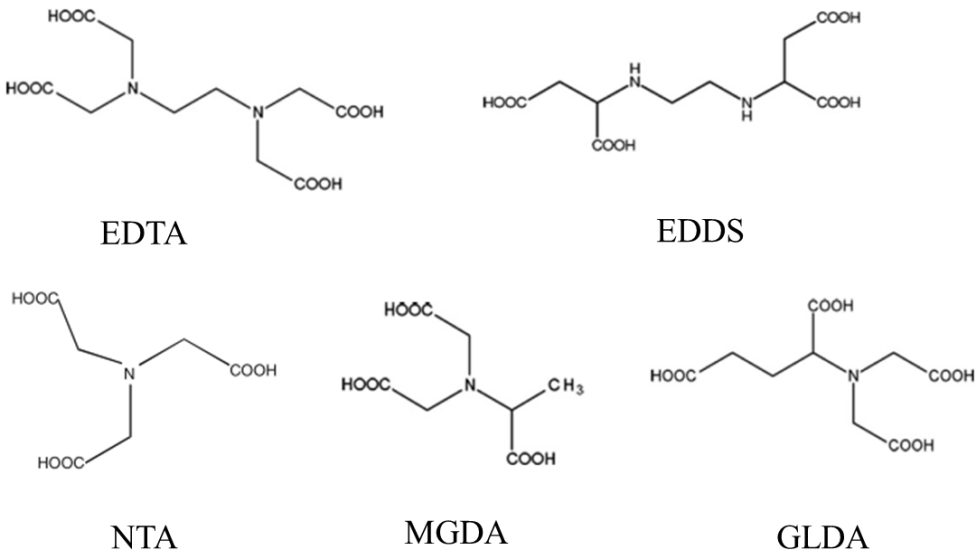

Figure 2-1 The structural formulas of the aminopolycarboxylic acids: EDTA, EDDS, NTA, MGDA, and GLDA (Pinto et al., 2014).

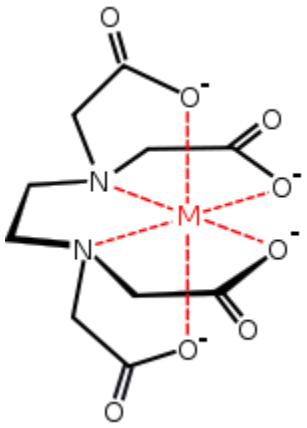

Figure 2-2 The representative structure of EDTA binding to a metal (Rekab et al., 2015).

EDTA is initially regarded as the most efficient on metal solubilizing, especially for Pb, Cu, and Zn, and it is widely used in phytoextraction and many other practices for a long time (Farid et al., 2013). However, EDTA is recalcitrant to degradation in soils or water and it is detected at a high concentration in the river water after widespread

use (Nowack et al., 2002). When EDTA is used in phytoextraction, the persistence of EDTA raises a great concern on the problem of metal leaching to the deep soil layers or shallow groundwater (Sun et al., 2001; Neugschwandtner et al., 2012). In recent years, the biodegradable chelants, such as NTA, EDDS, and MGDA, are recommended as alternatives to EDTA (Saifullah et al., 2009; Wang et al., 2012; Pinto et al., 2014).

2.2.3 Plant species used in phytoextraction

The selection of appropriate plant species in phytoextraction is of great importance, as the success of phytoextraction is dependent on the survival of plants, the concentration of heavy metals in plant aboveground parts, and the plant biomass. The characteristics of suitable plants species for phytoextraction include 1) high tolerance to heavy metals, 2) fast growth rate and high biomass, 3) extensive root systems, 4) high translocation factor, 5) high bioaccumulation factor, and 6) easy agronomic management (Vamerali et al., 2010; Wuana and Okieimen, 2010).

Phytoextraction often uses hyperaccumulator plants, as hyperaccumulator plants can accumulate much more heavy metals in their shoots or leaves than non-accumulating plants. Hyperaccumulators are named for plant species which are capable to accumulate more than 100 mg kg^{-1} Cd, higher than 1000 mg kg^{-1} Cu, Co, Cr, Ni, Pb,

U, and As, and greater than 10000 mg kg^{-1} Zn and Mn in their dry aboveground tissues grown in contaminated soils (Reeves and Baker, 2000). However, a lot of hyperaccumulators, such as *Thlaspi rotundifolium* (hyperaccumulating Pb), have been found to grow slowly with small biomass. In addition, it is difficult to culture hyperaccumulators in fields, due to the scarcity of seeds and the lack of knowledge on agronomic management (Vamerali et al., 2010). These characteristics make hyperaccumulators difficult to be used in phytoextraction projects.

Plant species of high biomass, in combination with the use of chelant, have received much attention from researchers. Chelants have been reported to induce accumulation of metals (especially for Pb and Cu) to hyperaccumulation level in many non-accumulating plants with high biomass, such as maize, Indian mustard, sunflowers, and grasses (Vassil et al., 1998; Tandy et al., 2006c; Johnson et al., 2009; Wang et al., 2012). Therefore, the use of chelant broadens the choices in selection of plants species, and thus facilitating the feasibility of practical application of phytoextraction in different regions with distinguished climate and soil conditions (Vassil et al., 1998). The use of high-yielding plants, which accumulate an enhanced level of metals with assistance from chelants, may be more efficient than hyperaccumulators with low biomass.

2.2.4 Suitable type of contaminated soils for chelant-assisted phytoextraction

The success of chelant-assisted phytoextraction in fields is also determined by the adequate selection of suitable contaminated lands. Considering the characteristics of chelant-assisted phytoextraction, this technology is not applicable for all kinds of polluted areas. As shown below, we conclude several typical features of contaminated lands where chelant-assisted phytoextraction is promising to be used practically.

1) This technology is applicable to sites in which the metal contamination level is low to moderate. This is because that the heavily polluted sites cannot sustain the growth of plants (Ali et al., 2013). Instead, the heavily polluted sites should be more efficiently remediated by other technologies, including soil capping, soil washing with acids or chelating agents, and soil stabilization with lime or other amendments (Saifullah et al., 2009).

2) This technology is suitable for very large areas of contaminated soils. It is not economically feasible to remediate a large quantity of soils using conventional technologies. Compared to conventional clean-up methods, phytoextraction spends much less on the establishment and maintenance (Zhao et al., 2015). Generally, the cost of phytoremediation can be 5% of other conventional technologies (Prasad,

2003).

3) This technology is suitable for sites in which metal pollution is concentrated primarily on the shallow surface layers. The clean-up depth of phytoextraction is restricted by the root zone in polluted soils, as this method mainly use plant roots to directly extract metals from surrounding soils (Kumar et al., 1995). Plant roots typically reach the depth of 30-90 cm underground in fields, depending on climatic conditions, plant species, soil types, and pollution levels (Mahar et al., 2016).

4) This technology is acceptable for sites that are not required to be used in a short period of time. Phytoextraction needs to occupy the polluted lands for a long duration (several years or decades) to lower the concentration of target metals to acceptable levels (Van Nevel et al., 2007; Robinson et al., 2015).

5) This technology is beneficial for soils that to be reused for planting (like gardening, agriculture, and forestry) in future. Phytoextraction is a “gentle remediation option” that being utilized *in situ* for contaminated soils, which preserves soil functions or structure (Gerhardt et al., 2016). With the penetration of plant roots into compacted polluted soils, the soil quality will be improved by the increased soil porosity and aeration (Gerhardt et al., 2016). Additionally, plant roots also exude a variety of organic matters that fertilize the polluted soils. In contrast,

many other quick clean-up technologies often damage the soil components and physical structures, which make the soil not sustainable for plant growth after remediation (Jelusic et al., 2014).

2.3 Mechanisms of chelant-assisted phytoextraction

A large body of study on chelant-assisted phytoextraction has been published in the past twenty years mostly from the point of view of environmental engineering, of which the overarching aims are to test, evaluate, and enhance the potential of applying this technology in field. Although a few studies have intended to explore the mechanisms involved in chelant-assisted phytoextraction, direct evidence is still of lack to clarify the key processes. There are two major processes involved in mechanisms of chelant-assisted phytoextraction, including soil processes and plant processes. Soil processes impact the mobility, speciation, and transport of metals for plant root absorption, and they also affect soil microbes and nutrients which are important for plant growth. Plant processes include the uptake, transformation, and translocation of solubilized metals.

2.3.1 Soil processes

2.3.1.1 The effects of chelants on soil metals

Metal extraction by chelants

Chelants can extract heavy metals from soils, and the efficiency of metal extraction is directly affected by the dosage of chelants to soils. It has been found that the increasing dosage of chelant can solubilize more metals from soil to soil solution, which results in more metals being translocated from roots to shoots (Niu et al., 2011a). However, a high dosage of chelants is usually detrimental for plant growth, leading to plant necrosis, dehydration or death (Grčman et al., 2003; Luo et al., 2005; Cao et al., 2007). Therefore, the dosage of synthetic chelant is mostly controlled at the range of 3-10 mM kg^{-1} in past phytoextraction studies (Cao et al., 2007; Quartacci et al., 2007; Wu et al., 2007; Liu et al., 2008). Moreover, the application of chelant in several smaller dosages is also recommended to reduce the toxicity of chelants to plants (Hadi et al., 2010).

The speciation of target metal in original soil also plays a crucial role in metal extraction process by chelants. The metal speciation in soils can be examined by the traditionally sequential extraction method, and metal fractionations are operationally defined into five phases, including exchangeable, carbonate bound, Fe/Mn oxides

bound, organic matter bound, and residual fraction (Tessier et al., 1979; Li et al., 1995). The former four fractions are able to be extracted by chelants, while the residual fraction is difficult to be released by chelants (Tandy et al., 2003; Guo et al., 2010). Generally, the weakly bound metals (from exchangeable and carbonate bound fraction) is extracted by chelants at a higher rate than the strongly bound metals (from Fe/Mn oxides and organic matter bound fraction) (Wasay et al., 2001; Yip et al., 2009). In other words, chelants facilitate the mobilization of weakly bound metals at a rapid rate, while extract strongly bound metals slowly. In residual fraction, metals are involved in the crystalline lattices, and are not accessible for chelants to form complex, This is supported by Kirpichtchikova et al. (2006), who used extended X-ray absorption fine structure (EXAFS) spectroscopy and found that Zn phosphate component was entirely or selectively solubilized by EDTA and EDDS, while Zn phyllosilicate component was less dissolved.

In spite of heavy metals, chelants can also solubilize mineral cations through dissolution of soil hydroxides. The dissolution of Fe, Al, Ca, and Mn in different soils has been observed in studies of chelant-assisted soil washing and chelant-assisted phytoextraction (Tsang et al., 2007; Komárek et al., 2010; Zhang et al., 2010). The rate and extent of chelant-promoted dissolution of minerals are affected by pH, chelant concentration, and mineral forms in soils (Yip et al., 2010;

Yang et al., 2012). For instance, EDDS dissolves amorphous Fe hydroxide (ferrihydrite) faster than crystalline Fe (goethite) (Komárek et al., 2009). Moreover, these major cations released from mineral dissolution can compete for complexation with chelants, which may reduce the available chelant for extracting target metals. By modelling the effects of chelants on Ca and Pb extraction, Nowack et al. (2006) found that the extraction of Pb by EDTA was greatly reduced around neutral pH in the presence of exchangeable Ca or calcite, while the extraction of Pb by EDDS was almost not affected. In addition, the dissolved Fe and Al have been observed as the competitors for complexation with EDDS in some soils (Komárek et al., 2007; Koopmans et al., 2008; Komárek et al., 2010). However, Yip et al. (2009) found limited competition from major cations for complexation with EDDS, as most dissolved Al was present as colloidal $Al(OH)_3$, Al-DOM, and hydrolyzed species, while Fe, Mn, and Ca dissolution was negligible in soils. In consideration of the previous controvesial results, it still deserves more studies on chelant-promoted dissolution of minerals and the effects of mineral cations on heavy metal extraction in different soils.

Metal and chelant speciation in solution

The knowledge of the speciation of metals and chelants in soil solution is critical for understanding the interactions among the chelant, metals, and plant roots during

phytoextraction process. The *in situ* measurement of the speciation in soil solution is difficult, so most studies performed prediction using programs, such as ECOSAT, Visual MINTEQ, or PHREEQC-2 programs (Tandy et al., 2006; Komárek et al., 2007; Tsang et al., 2009). NICA-Donnan model is involved to predict the binding to humic and fulvic substances. The calculation is based on the stability constants of metal complex, and the results are controlled by the competition between metals for binding with chelant.

In natural plant soil system, the free metal concentration is considered to determine plant uptake of metals from solutions (Parker and Pedler, 1997). The addition of chelants increases the total concentration of dissolved metals, but the free metal amount does not necessarily enhance. Koopmans et al. (2008) found that the addition of EDDS decreased the concentration of free metals in soil extracts by a factor between 1.4 and 1.9 for Cd, 3.4–216 for Cu, 1.3–186 for Ni, and 1.3–3.3 for Zn, by measuring the free metals with Donnan membrane technique (DMT) and comparing to the result from ECOSAT. In chelant treated soils, the solubilized Pb, Cu, and Zn are primarily reported to complex with EDDS and dissolved organic matters (DOM) in soil extracts, and the results are dependent on the stability of metal chelate complex, chelant concentration, and solution pH (Yip et al., 2009; Yang et al., 2012).

The speciation of chelant is controlled by the availability of metals and the binding strength of metal chelate complex. EDDS has been known to primarily chelate with Cu and Ni, due to the high stability of CuEDDS and NiEDDS, and also to Zn and Pb present in soil surface, but not to Cd (Tandy et al., 2006; Koopmans et al., 2008). The kinetic studies of interactions between EDDS and soil revealed that EDDS firstly chelate the available Cu, Zn and Pb from soil surface, and the newly formed PbEDDS and ZnEDDS may be substituted by less available Cu from soil with longer reaction time through metal exchange, especially in deficiency of EDDS (Yip et al., 2009; Tsang et al., 2009). As mentioned above, major cations (*e.g.* Fe, Ca, and Al) dissolved in soil solution would compete for binding with chelants as well (Komárek et al., 2010). Komárek et al. (2007) showed that Cu together with Fe and Zn controlled the speciation of EDDS in contaminated soil from mining and smelting area.

Metal transport in rhizosphere soil

To be taken up by plants, metals or metal complexes in soil solution should be transported to plant root surface. Diffusion and mass flow are two main pathways involved in transport of solutes to roots. Because trace metals are often present at low concentration in natural soil solution, their transport by mass flow is expected to be small. Whiting et al. (2003) reported that Zn was primarily supplied from soils by

diffusion to hyperaccumulator *Thlaspi caerulescens*. However, the relative importance of metal transport pathways may be determined by the particular plant species, metal, and soil properties. The addition of synthetic chelants is expected to considerably enhance metal solubility, increase the transport through mass flow or diffusion, and uptake of metals by plants (Mcgrath et al., 2001; Nowack et al., 2006). Nevertheless, few experimental results are available regarding the effects of chelants on metal transport pathways in rhizosphere soils. A fully understanding on the approaches of metal supply from soil is critical for appropriate soil management in phytoextraction technologies.

2.3.1.2 The effects of chelants on soil microbes

To ensure a successful phytoextraction, it is essential to keep the soil quality both during and after phytoextraction. On the one hand, the maintenance of soil quality is required for supporting plant growth in phytoextraction, as the phytoextraction often need several rounds of planting and harvesting (Yang et al., 2013). On the other hand, the soil quality of remediated soils is important for their reuse in vegetation and agriculture activities. Recent studies revealed that the EDTA-assisted soil washing seriously damage the soil functions, which resulted in the great decrease of the biomass of food crops grown in remediated soils (Jelusic and Lestan, 2014; Jelusic et

al., 2014).

The activities of soil microbes play an important role on plant growth and sensitively reflect the dynamic variation of soil qualities (Fang et al., 2017). Controversial results can be observed from previous studies regarding the effects of different chelants on soil microbes. EDTA was toxic to soil microbes from most studies, inhibiting enzyme activities, decreasing the population of soil microbes, and net N mineralization (Epelde et al., 2008; Mühlbachová, 2009; Usman et al., 2013; Lee and Sung, 2014). However, EDTA showed beneficial impacts on soil microbes in some cases (Chander and Joergensen, 2008). EDDS appeared to exert less toxicity than EDTA on soil microbes in comparative studies (Epelde et al., 2008; Lee and Sung, 2014). Furthermore, biodegradable EDDS and MGDA even allow the proliferation of bacterial population in rhizosphere soil, by providing available carbon and nitrogen sources (Kos and Leštan, 2003; Cao et al., 2007). Conversely, some researchers also found that EDDS inhibits microbial biomass, enzyme activities, and affects the microbial community composition in trials (Mühlbachová, 2011; Yang et al., 2013). In addition, the effects of EDDS on soil microbes may disappear due to its degradation with time in soil (Mühlbachová, 2011; Yang et al., 2013). The varied effects of applied chelants to soil microbes can be related to the composition of indigenous microbial community, the type and dosage of chelants, and the grown

plant species.

2.3.2 Plant processes

The uptake, transport, and tolerance of metals in plant system are critical steps that determine the success of phytoextraction. In fact, these processes are controlled by the metal speciation, plant physiological structures, and plant constitutive tolerance strategies.

2.3.2.1 Plant root structure

Plant roots are responsible for the uptake and transfer of water and nutrients to aerial tissues, and restrict the transfer of hazards from roots to aerial tissues. The physiological structure of a plant root is schematically illustrated in Fig. 2-3. From the longitudinal view (Fig. 2-3A), a plant root includes four parts: 1) root cap, 2) meristematic zone, 3) elongation zone, and 4) mature zone (Nobel, 2009). The apical root cap physically protects the meristem and lubricates roots to help penetrate into the surrounding soils. Cells divide rapidly in the meristematic zone, which gives rise to undifferentiated cells (Dello Ioio et al., 2008). Meristematic cells are small, closely packed, and filled completely with protoplasm, and they contain extremely small vacuoles and no differentiated plastids. Cells elongate rapidly in the elongation zone, resulting in the increase of cell volumes and gradually enlarging vacuoles (Verbelen

et al., 2006). Cells cease elongations in the mature zone, begin to differentiate, and form vessels that function in ion transport (Dolan et al., 1993). From the tranverse view of a mature root zone (Fig. 2-3A), it is mainly consisted of three parts including 1) epidermis, 2) cortex, and 3) root stele (Pugnaire and Valladares, 2007). The epidermis is the outermost root tissue consisting of a single layer of cells. In root tips, root hairs develop from epidermis cells and play an important role in absorption of water and nutrients (Jungk, 2001). The cortex lies between the epidermis and the root stele, and it consists of parenchyma cells. There is a single cell layer called endodermis that defines the interier edge of the cortex. The endodermis has the function to restrict transportation of water and solutes between the cortex and stele, due to the formation of Casparian strips and suberin lamellae (Geldner, 2013; Doblas et al., 2017). Casparian strip is formed by the hydropobic lignin deposits in radial and traverse cell walls in endodermis, blocking the apoplastic space. In addition, there are some specialized endodermal cells called passage cells that are not coated by ligin or subrin deposits. The root stele (vascular cylinder) refers to the inner center part of root, consisting of pericycle, xylem, and phelom. The xylem in root stele is continous from root to leaves, and it is responsible for the transportation of water and nurtients upwards. The phelom transports solube organic compounds made from photosynthesis in multiple direction to plant organs where they are needed (Pugnaire

and Valladares, 2007).

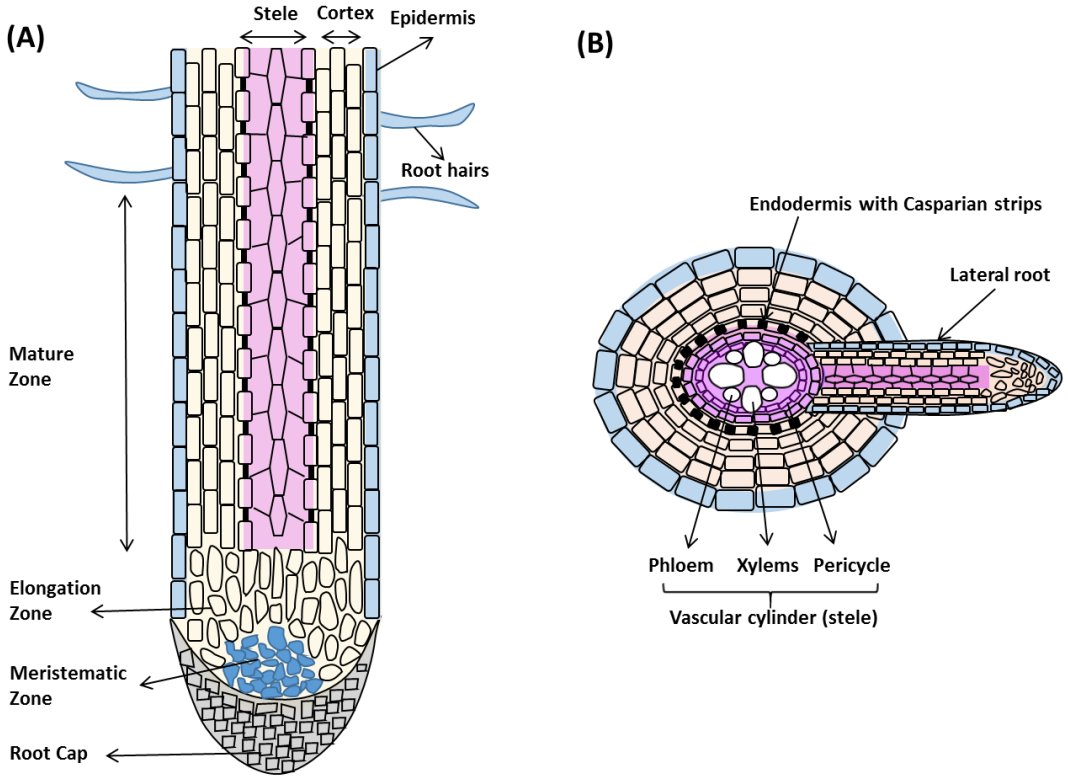

Figure 2-3 Schematic diagrams showing longitudinal section view of primary root (A) and transverse section view of lateral root zone (B) (not to scale) (Johnson, 2012).

With plant maturation, lateral roots will develop from primariy roots and they are important in determining the three dimentional root architecture. Lateral roots arise from root primordia in the pericycle and pass through cortex and epidermis with the elongation of root primordia (Fig. 2-3B) (Péret et al., 2009). At the base of root primodia, the vascular cylinder is differentiated and connected with the vascular system of the primary root. During the development of lateral roots, many cortial

cells are crushed and the Casparian band on endodermis cells is discrupted (Pugnaire and Valladares, 2007). The growth of lateral roots increases the root surface area and allows for the flow of water and nutrients without the control by the Casparian strip (Xie and Yu, 2003; Hodge, 2004).

2.3.2.2 Metal uptake and transport

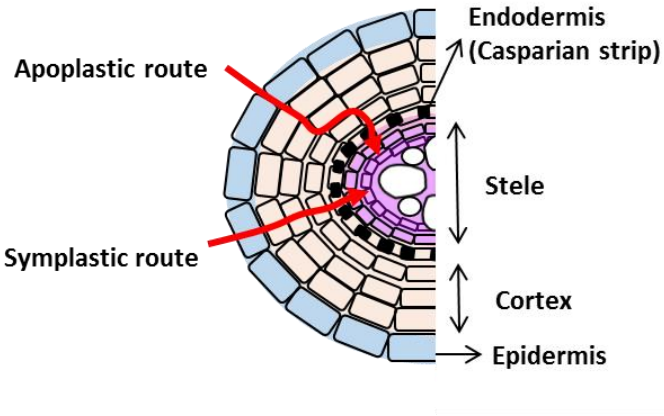

Figure 2-4 Schematic diagrams showing symplastic and apoplastic routes (Johnson, 2012).

Metals can be absorbed and transported radially across the root through symplastic pathway and apoplastic pathway, or the combination of both pathways (Fig. 2-4). The symplastic pathway is selective requiring solutes to pass through cell membranes. The apoplastic pathway is non-selevtive referring to the transport of solutes within the extracelular spaces. For nutrient metals (*e.g.* Fe, Cu, Zn, etc.), there are particular proteins, such as YSL, ZIP, COPT family, emebedded on cell membranes that

mediate metal transport to cytoplasm (Colangelo and Guerinot, 2006). Metal complexes formed with synthetic chelants, such as PbEDTA and CuEDDS, are unlikely to cross cell membranes due to the large molecular size and lack of transporters on cell membranes. It is widely accepted that these metal complexes are primarily transported through the apoplastic pathways (Vassil et al., 1998; Luo et al., 2005; Tandy et al., 2006b). However, Johnson and Singhal (2013) argued that the presence of EDTA and DTPA could enhance the symplastic uptake of Cu, with the observation of increased cell membrane permeability after application of chelants.

To be transported from below- to aboveground organs of plants, metals must be transported through the root cortex and enter into the stele for subsequent flow to aerial tissues (Nowack et al., 2006). The apoplastic flow into the stele can be effectively blocked by the hydrophobic Casparin strip on enderdomis. In order to cross the endodermis cells, solutes should be exchanged from the apoplastic to symplastic pathway and thus enter into cytoplasms of endodermis (Johnson, 2012). To a certain degree, this process allows a plant to select and control the uptake of metals in solutes. Therefore, the apopalsitc flow of metal complexes may be largely restricted by the Casparian strip. However, metal complexes are considered to be mainly transported into the stele from the root apex where the Casparian strip is not fully developed (Tanton and Crowdy, 1972), or the lateral root zone where the

Casparian strip is damaged (Fig. 2-3) (Niu et al., 2011b). Furthermore, Niu et al. (2011b) found that the high concentration of CuEDDS (3000 μM) resulted in damage or death of passage cells on endodermis of maize, which created additional channels for the passage of apoplastic flow of CuEDDS. After the successful transport across the endodermis, metals will be loaded to xylem sap, translocated into the aerial vascular vessels, and unloaded to the plant leaves.

At the early stage, researchers did not believe that the intact metal complex can be absorbed, because it was thought that only the free metal released from the metal complex was able to be absorbed by plant roots (Chaney et al., 1972). Then the discovery of intact metal complex PbEDTA in xylem sap of *Brassica juncea* supports that the intact metal complex is able to be absorbed and transported in long distance by some plant species (Vassil et al., 1998). Up to now, there is still controversy on whether a metal complex is directly absorbed or dissociated prior to or during the uptake and transport, which can be related to the investigated types and dosage of metal complexes, plant species, and experimental conditions (Cestone et al., 2010; Niu et al., 2011a; Tian et al., 2011).

2.3.2.3 Metal sequestration and storgae

The tolerance of plant to heavy metals is important in phytoextraction, as excessive

metal ions are toxic to plant cells and inhibit plant growth (Briat and Lebrun, 1998). Heavy metals may cause oxidative stress to plants by stimulating the formation of free radicals and reactive oxygen species (ROS) (Sharma and Dietz, 2009). The oxidative stress to plants leads to lipid peroxidation, biological macromolecule deterioration, membrane destabilization, ion leakage, and DNA-strand cleavage (Gupta et al., 2013). In addition, metals may inhibit the activity or disrupt the structure of proteins, via binding to thioyl-, histidyl- and carboxyl-groups, or replacing essential elements in proteins (Kasprzak, 2002). Hence, plants adopt an array of mechanisms to maintain the concentration of heavy metals ions within non-toxic ranges in cytoplasm and minimize their cellular toxicity. Cellular detoxification mechanisms include 1) cell wall compartmentalization, 2) vacuolar storage, 3) metal efflux, and 4) metal chelation (Hall, 2002; Yruela, 2009) (Fig. 2-5).

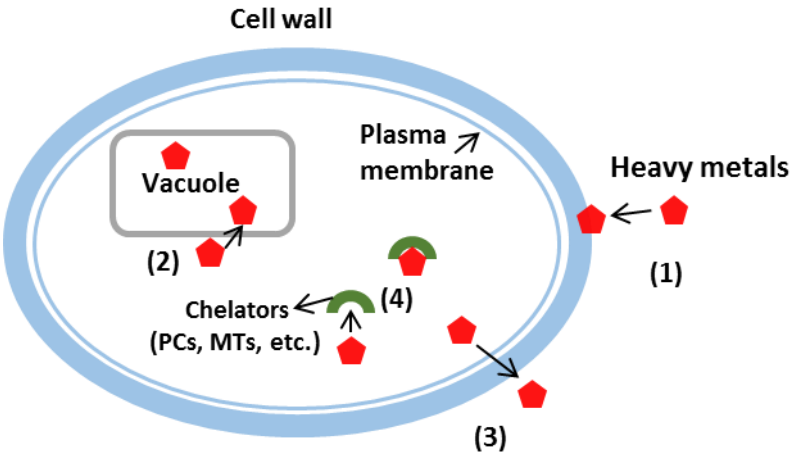

Figure 2-5 Cellular metal tolerance mechanisms in plants. (1) Cell wall compartmentalization, (2) vacuolar storage, (3) metal efflux, and (4) metal

chelation (Hall, 2002).

Plant cell wall are consisted of polysaccharide (cellulose, hemicellulose, and pectin) (up to 90% of dry weight), glycoprotein (2-10%) and phenolic easters (< 2%) (Rose, 2003). The cell wall polysaccharides contain functional groups such as –COOH, –OH and –SH that are able to bind with divalent and trivalent metal ions effectively (Brune et al., 1994; Davis et al., 2003; Krzesłowska, 2011). Compartmentalization of heavy metals ions on cell walls is a common strategy employed by higher plants to reduce the toxicity from free metals (*e.g.* Pb^{2+}, Cd^{+} and Cu^{2+} etc.) (Carrier et al., 2003; Tian et al., 2010; Lu et al., 2017). Vacuole is a safe compartment for storage of a high concentration of heavy metals, which helps to reduce the damage of metals to sensitive organelles in cytoplasm (Liu and Kottke, 2004). Some vacuolar transporters, such as *YCF1* and *TgMTP1* have been characterized to be responsible for pumping metals into vacuole (Tong et al., 2004). In spite of compartmentalization, the selective efflux of metals from cytosol is another way for plant to maintain cellular metal homeostasis. Several classes of heavy metal transporters played an important role in this step, including CPx-ATPases, the Nramps (natural resistance-associated macrophage proteins), the CDF (cation diffusion facilitator) family, and the ZIP family (Hall, 2002; Hall and Williams, 2003; Haney et al., 2005). Chelation with metals in cytosol is a strategy to mask metals and prevent the production of free

radicals that lead to oxidative stress. Two important metal chelators known in plants are phytochelatins (PCs) and metallothioneins (MTs), which are generally cysteine-rich peptides and bind with toxic metals through sulfhydryl groups (Wójcik and Tukendorf, 1999; Jan and Ahmad Parray, 2016). Carboxylic acids (*e.g.* citrate, malate, and oxalate), amino acids (histidine and nicotianamine) and phosphate derivatives can also form stable complex with heavy metals and transform them into non-toxic or less toxic forms (Briat and Lebrun, 1998; Jan and Ahmad Parray, 2016).

2.4 Synchrotron based μ-XRF and XAS spectra

To elucidate metal interactions in plant-soil systems during chelant-assisted phytoextraction, it is vital to acquire accurate, qualitative, and quantitative analysis on the localization and speciation of metals in soil and plant matrices. Fortunately, the current development of synchrotron based techniques, such as synchrotron-based μ-XRF and XAS, provided a good opportunity for the *in situ* characterization of metal distribution and speciation in different environment media.

2.4.1 X-ray fluorescence (μ-XRF)

To image the elemental distribution in soil, sediments, and biological specimens, light microscopy, scanning electron microscopy (SEM), and transmission electron

microscopy (TEM) coupled with energy-dispersive X-ray (EDX) spectroscopy have often been used. However, these techniques demand elaborate sample preparation steps, such as chemical fixation and dehydration process. For example, in order to obtain appropriate contrasting by light microscopy for histochemical investigation, rubeanic acid ($C_2H_4N_2S_2$) was used to produce dark green precipitation with Cu^{2+} in maize tissues (Niu et al., 2011b). The pretreatment approaches may give a chance for metal redistribution and introduce some artifacts into ultrastructure of biological specimens. Laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) is another useful method, which does not require the complex sample preparation, but the laser beam ablates the surface of samples during analysis (Becker et al., 2010; Lu et al., 2013). Therefore, it is essential to utilize the *in-situ* and non-destructive method to image the metal distribution in environmental samples.

The synchrotron-based μ-XRF analysis utilized the synchrotron X-ray to excite emission of secondary X-ray protons from a microscopically small area on the sample surface (Adams et al., 1998). The use of brilliant synchrotron radiation allows a high spatial resolution to nano-microscale levels, and an improvement on elemental sensitivity (Bleuet et al., 2008). In comparison with other imaging techniques, the advantages of μ-XRF arise from the multi-elemental analysis, high sensitivity, high

spatial resolution, simple sample preparation, great penetration depth, and non-destructive approaches (Majumdar et al., 2012). The μ-XRF has been successfully applied in deciphering metal distribution pattern in different matrixes. For instance, Yang et al. (2014) used the μ-XRF to map elemental distribution in a Cu contaminated soil and found that Cu was primarily associated with Fe oxides instead of clay or carbonates. Wang et al. (2013) examined the spatial distribution of different metals (*e.g.* Zn, Ni, Mn, Cu, Hg, Se, As) in root apex, and showed the varied distribution of different metal in cortex, endodermis, pericycle and stele. Therefore, μ-XRF is a useful tool to characterize metal distribution pattern or metal associations, which will help to comprehend metal mobility in soil or plant matrixes.

2.4.2 X-ray absorption spectroscopy (XAS)

Many analytical techniques have been used to assess the speciation of metals in plant and soil. However, most of them are only intended to quantify restricted types of metal species, and the indispensable pretreatment of some samples may lead to transformation of metal species (Gräfe et al., 2014). For instance, the high performance liquid chromatography (HPLC) coupled to ultraviolet (UV) has been used for analysis of CuEDDS in plant and soil, and it requires the sample to be extracted in a liquid form (Niu and Shen, 2009; Cestone et al., 2010). Although

CuEDDS is a relatively stable metal complex (log K=20.46) (Orama et al., 2002), the dissociation and transformation of a similar complex CuEDTA have been reported by algae cell membranes (Walsh et al., 2015). Hence, there is still some concern on the potential transformation of this complex during the extraction step from solid samples. In addition, the extraction efficiency is another problem which dictates the proportion of analyte to be detected. Moreover, metal is generally present as a mixture of many different species in environmental samples; therefore, it is difficult to analyze all potential compounds simultaneously with traditional methods. In consideration of these limitations of traditional analytical methods, the incorporation of more advanced techniques is required in metal speciation analysis.

XAS refers to use a series of high energy X-ray to scan the sample in order to obtain the X-ray absorption spectrum (Lombi and Susini, 2009). The XAS spectrum is divided into two energy regions, including near edge X-ray absorption fine structure (XANES) and extended X-ray absorption fine structure (EXAFS). Interpretation of the spectrum provides information on the oxidation state and local geometry of the element. XAS is an element specific technology that illustrates chemistry of elements directly from soil and plant matrixes (Gräfe et al., 2014). For instance, Cui et al. (2017) examined the exact species of Cu (originally as 65% Cu-ferrihydrite, 15% $Cu(OH)_2$ and 13% $CuCO_3$) and Zn (originally as 43% Zn-ferrihydrite, 38% Zn_2

$(OH)_2CO_3$ and 18% ZnS) in an e-waste soil with XANES, and found the variation of metal speciation after long term leaching with rainwater. Ryan et al. (2013) identified that Cu was primarily present as Cu(I)-glutathione, Cu(I)-cysteine, and Cu-histidine in tomato and oat with XANES, and the supplied Fe amount may affect the Cu speciation in plants. Therefore, XAS can provide qualitative and quantitative information of metal speciation, and elucidate the interactions that affect the association and speciation of metals in soil-plant system.

2.5 Summary and outlook

The soil contamination by heavy metals has raised great concerns in China and many other countries across the world. To clean up the contaminated soils, chelant-assisted phytoextraction has been proposed as a green technology and has received much attention for several decades. However, in recent years, there are controversial opinions towards the development and full-scale application of this technology (Robinson et al., 2015; Mahar et al., 2016). This is because the performance of chelant-assisted phytoextraction is limited by the lack of precise knowledge of many fundamental soil-plant processes and chelant-metal interactions. A better understanding of the key mechanism of chelant-assisted phytoextraction may assist to guide the future direction to develop this technology. However, as indicated above,

large knowledge gaps await to be addressed in further research.

In chelant-assisted phytoextraction, biodegradable chelants, such as EDDS and GLDA, are recommended in application. Because biodegradable chelants are less toxic to plants and microbes compared to recalcitrant EDTA (Meers et al., 2005), and they are also able to reduce the metal leaching risks in fields (Wang et al, 2012). On the other hand, regenerating grass species are convenient to be used in phytoextraction practice, as phytoextraction usually requires repeated harvests to reduce the soil metal burden to acceptable levels. Hence, more trials are needed to use the combination of biodegradable chelants and regenerating species in realistic contaminated soils.

Copper pollution in soils has been widely reported but received less attention compared to other heavy metals (*e.g.* Pb, Cd, Hg, and Zn). Copper is a redox-sensitive metal, which can catalyze the production of ROS, cause oxidative stress, and damage biomolecules in plants (Yruela, 2009). More information is required on the fate and biochemical transformation of Cu in soil-plant systems. In addition, the removal of Cu from polluted soils is important for agricultural production, food safety, and human health (Adrees et al., 2015). As biodegradable EDDS has a high affinity to Cu, it is promising to use EDDS in phytoextraction for

Cu polluted soils. Therefore, it is representative to select the combination of EDDS with Cu polluted soils to investigate interaction mechanisms in chelant-assisted phytoextraction.

The main processes involved in chelant-assisted phytoextraction include soil process and plant process (Nowack et al., 2006). In soil process, the chelant play a role on metal extraction from soils, transportation to roots, and speciation transformation in solution. These steps determined the bioavailability of metals to plant roots. Although interactions of chelants with soils have achieved some progress in soil washing studies (Fabbricino et al., 2016), more study is still required to be conducted in phytoextraction experiments. This is because plant may influence the interactions between chelant and soils in rhizosphere. In addition, more attention should be paid to the soil microbes and nutrients, which are import parameters for soil quality and affect plant growth during phytoextraction. In plant process, the chelant may alter the pathways of metal uptake, transport, and detoxification in the complex plant system. In view of the controversial results from previous studies, more in-depth studies are needed to resolve the exact mechanisms regarding plant metal absorption in the presence of chelants.

A growing body of study has been utilizing and developing μ-XRF and XAS

methods in deciphering different environmental issues over the past decades (Gardea-Torresdey et al., 2005; Lombi et al., 2011). The knowledge, regarding the metal binding characteristics in soils, the essential metal homeostasis in plants, as well as the detoxification strategies of toxic elements in plants, has increased greatly through the use of these synchrotron based techniques in plant-soil system (Gräfe et al., 2014a). Therefore, the application of these techniques into the chelant-assisted phytoextraction will help us to understand the dynamics of metal transport and transformation at molecular scale.

3. Chapter Three - Selection of Chelants for Phytoextraction Mechanism Study

3.1 Introduction

At the starting stage of phytoextraction studies, hyperaccumulators are widely used which naturally accumulate a high concentration of metals in plant leaves (Robinson et al., 1998; Zhao et al., 2003). Then the application of hyperaccumulators is found to be limited in fields, due to the slow growth rate and low biomass production of hyperaccumulators. Later, research interests are shifted to use high biomass species in combination with chelants to clean up the contaminated sites (Sarret et al., 2001).

Because EDTA can result in the leaching risks of metals to subsoil and groundwater (Sun et al., 2001; Chen et al., 2004), various chelants are recommended to be used in soil phytoremediation process, such as EDDS, MGDA, NTA, and GLDA. EDDS has been reported to degrade rapidly in soils with a half-life of 2-8 d (Meers et al., 2005), and it controls the leaching risks of metals in field experiments (Wang et al., 2012). Many researchers support that EDDS can be an substitute for EDTA, since biodegradable EDDS can form complex with metals effectively and shows less toxicity to soil microbes (Grčman et al., 2003; Luo et al., 2005; Meers et al., 2005;

Epelde et al., 2008; Mühlbachová, 2011). GLDA is a new biodegradable chelant that recently launched on the market, of which the production process is green with readily available corn sugar as fermentation source (Bisinger Jr, 2009). GLDA has showed a good performance in extracting metals from soils and sewage sludge (Begum et al., 2012; Tsang and Hartley, 2013; Wu et al., 2015). Therefore, it can be another environmentally friendly alternative for EDTA and deserves more studies in phytoextraction.

The plant species used in contaminated soil are crucial for the success of phytoextraction. The selected plant should be easily propagated, fast growing, deep-rooted, of high biomass production, tolerant to heavy metals, and accumulate target metals (Robinson et al., 2000; Vamerali et al., 2010). Grass species are commonly used in revegetation and rehabilitation of metal contaminated sites (Pierzynski et al., 2002; Arienzo et al., 2004). As grasses can be harvested for multiple times in a single growth period, they are suitable for long term phytoextraction projects to reduce the frequency of sowing after harvesting aboveground parts. Ryegrass is often used in phytoremediation of contaminated sites as a tolerant species to environment stress (Duquène et al., 2009; Gunawardana et al., 2011; Lou et al., 2013). Tall fescue, as a cool-season turfgrass, has been reported to be effective on enhancing Pb accumulation in shoot (2000-3000 mg/kg) during

EDTA assisted phytoextraction for Pb contaminated soil (Begonia et al., 2005).

The interactions of chelants with soils in rhizosphere, which control the metal solubility and speciation, play an important role in phytoextraction efficiency. Rhizosphere is a specific zone of soils around a plant root that is influenced by root respiration, root exudates, microbial activities, and root-microbe interactions (Bais et al., 2006). Metal mobility has been reported to enhance in rhizosphere, due to the rhizospheric acidification, or chelation by organic ligands emitted from roots or microbes (Cieśliński et al., 1998; Jones, 1998; Blossfeld et al., 2009). However, it is not known whether the rhizospheric environment affects the interaction between chelants and soils, as few studies focus on this area previously.

The objectives of this study were 1) to investigate impact of biodegradable EDDS and GLDA on the growth of and Cu contents in ryegrass and tall fescue in comparison with the recalcitrant EDTA; 2) to assess the biodegradation of chelants and leaching risks of Cu; and 3) to test whether there is difference of chemical interactions between rhizosphere and bulk soil in pot experiment during chelant-assisted phytoextraction.

3.2 Materials and methods

3.2.1 Soil pretreatment and characterization

The soil samples used in the pots were collected from a farmland 0-20 cm soil in the vicinity of a past copper mine in Tangshan Town of Nanjing, East China (Wang et al., 2012). The samples were air dried, homogenized, and sieved through a 2 mm mesh. The soil pH was determined with 0.01 M $CaCl_2$ at 1:10 ratio (w/v) using a pH meter. The electrical conductivity (EC) was measured on a soil extract using deionized water at a 1:2.5 ratio (w/v) with a conductivity meter. The soil texture was examined using the hydrometer method (Cater and Gregorich, 2006). The total organic carbon (TOC) of soil was analyzed by a TOC auto-analyzer (Shimadzu) after reaction with HCl to eliminate the inorganic carbon (carbonates). The total metal concentrations were determined by ICP-OES (Agilent Technologies 700 series) after strong acid digestion with concentrated HNO_3 and $HClO_4$ at 1:4 ratios (v/v). X ray diffraction (XRD) (Rigaku Smart Lab) was used to investigate the soil mineralogy with Cu-Kα radiation. XRD analysis was carried out from 5 to 100^o 2θ with a step scan of 0.02^o 2θ. The soil morphology was investigated by scanning Electron Microscope (JEOL Model JSM-6490) with an energy dispersive X-ray spectroscopy (EDX) detector.

3.2.2 Experimental set-up

Seeds of ryegrass (*Lolium multiflorum* Lam. cv. Tetragold) and tall fescue (*Festuca arundinacea* cv. Barlexas) were sterilized with 95% ethanol and germinated on wet filter papers for 7-10 d. About fifty seedlings were then transplanted to pots. Prior to the treatment of chelants solutions, ryegrass was grown for 20 d and tall fescue was grown for 55 d in a plant incubator with conditions of 14 h/8 h light and dark, temperature of 25 ^{o}C/20 ^{o}C day and night, 15000 lux light intensity and 60-70% humidity. The pot was periodically watered every 2-3 d with deionized water to keep the soil humidity around 50% WHC.

The pot (12.5 cm diameter ×15 cm height) was separated by a rhizobag, which was made of a 25 μm nylon cloth (8 cm diameter ×8 cm height), to restrict the root penetration and allow water and nutrients to flow between rhizosphere and bulk soil (Fig. 3-1). The rhizobag was used to study the characteristic of rhizosphere soil in comparison with bulk soil. Seven hundred grams of soils were filled in the pots with half in the rhizobag and the other half outside the rhizobag. Thirty five ml solutions of EDTA, EDDS, and GLDA (50 mM) in the form of sodium salts were added to the rhizosphere and the bulk soil separately from the top of soil surface. The equivalent

dose of chelants was 5 mmol kg^{-1}, which was adopted according to the previous study in phytoextraction (Luo et al., 2005).

The plants were harvested 7 d after the application of chelants (Luo et al., 2005), and were separated into shoots and roots. Plants were cleaned with deionized water and oven dried at 70 ^{o}C for 36 h to obtain constant weight. The soil samples were sampled separately at 7 d, 14 d, 21 d, and 28 d after application of chelants to study the metal leaching risks in rhizosphere soil and bulk soil. The soil was homogenized thoroughly each time before sampling.

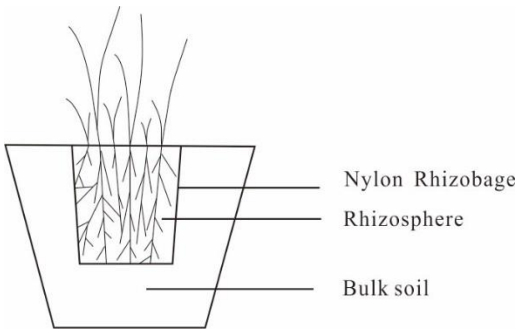

Figure 3-1 The pot with rhizobag used in experiment.

3.2.3 Soil and plant analysis

Soil chemical analysis: Fresh soils were extracted with 0.5 M K_2SO_4 at a 1:4 ratio (w/v) for 30 min, and the soil extracts were filtered. The DOC was analyzed using a TOC auto-analyzer (Shimadzu) in the filtrates. Following extracting 2 g air dried soil

with 20 mL $CaCl_2$ for 1 h, the available fraction of Cu was measured in the extracts using ICP-OES (Agilent Technologies 700 series) (Houba et al., 2000).

Cu distribution with sequential extraction: The distribution of Cu in soil was determined by sequential extraction procedures according to Li et al. (1995). The method classified Cu into groups including exchangeable fraction, carbonate/specifically adsorbed fraction, Fe/Mn oxide fraction, organic/sulphide fraction and residual fraction. After extraction with different chemical agents, Cu concentration was determined by ICP-OES (Agilent Technologies 700 series).

Plant metal analysis: To quantify metals in soil and plants, the soil samples were air dried and plants were oven-dried at 70 ^{o}C and were then grounded with agate mill. 0.25 g soil samples, 0.2 g plant shoots and 0.15 g plant roots were digested with $HNO_3/HClO_4$ (4:1), following the procedures established by Li et al. (2001) and Luo et al. (2005). Certified standard reference materials of NIST 1515 and NIST 2709 were used in the analysis to ensure accuracy of the analysis results. Regent blanks and analytical duplicates were included as part of the protocol. The digestion liquids were stored in 4 ^{o}C before ICP-OES analysis.

3.2.4 Statistical analysis

Sample means and standard errors were calculated by Microsoft EXCEL. The statistical significance of differences ($P < 0.05$) in the means or among different treatment groups were examined by Duncan's multiple range tests using SPSS 19.0 statistical package.

3.3 Results and discussion

3.3.1 Soil characterization

The physiochemical properties of soil are summarized in Table 3-1. The total Cu concentration was 805 mg kg^{-1}, which was over the legislative limits (100 mg kg^{-1}) in agricultural soil according to the second grade of Standards for Soil Environmental Quality of China (GB15618-1995). The total concentration of Zn, Pb, and Ni was below the legislative limit. The major minerals found in this soil sample included quartz, feldspars, calcite, and goethite through XRD analysis (Fig. 3-2). With SEM images and EDX analysis, quartz surface was found to be coated by clay minerals (flake shape) and iron oxides (Fig. 3-3).

Table 3-1 Soil characteristics

pH ($CaCl_2$)	7.72 ± 0.06
Conductivity (μS cm^{-1})	401 ± 12.8
TOC (%)	1.86
Water holding capacity (%)	58.8
Sand/silt/clay (%)	32/47/21
Soil texture	loam
Total heavy metals (mg kg^{-1})	
Cu	805 ± 22.0
Zn	230 ± 1.5
Pb	67.2 ± 1.3
Ni	19.9 ± 0.5
Major metals (g kg^{-1})	
Fe	40.8 ± 2.20
Mn	0.33 ± 0.01
Ca	18.6 ± 1.10
Mg	4.10 ± 0.06
Al	39.1 ± 0.92

Value are mean ± S.D. (n=3)

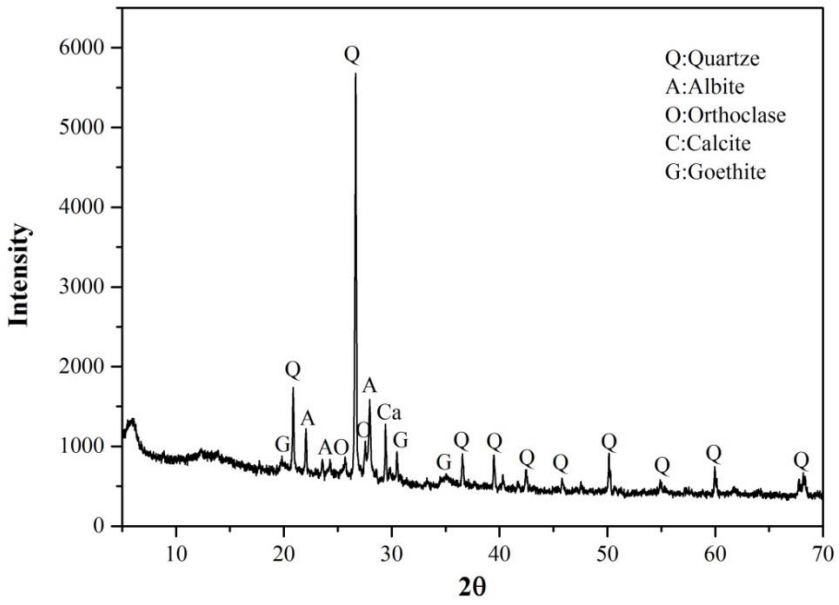

Figure 3-2 X ray diffraction of soil sample used in pot experiment.

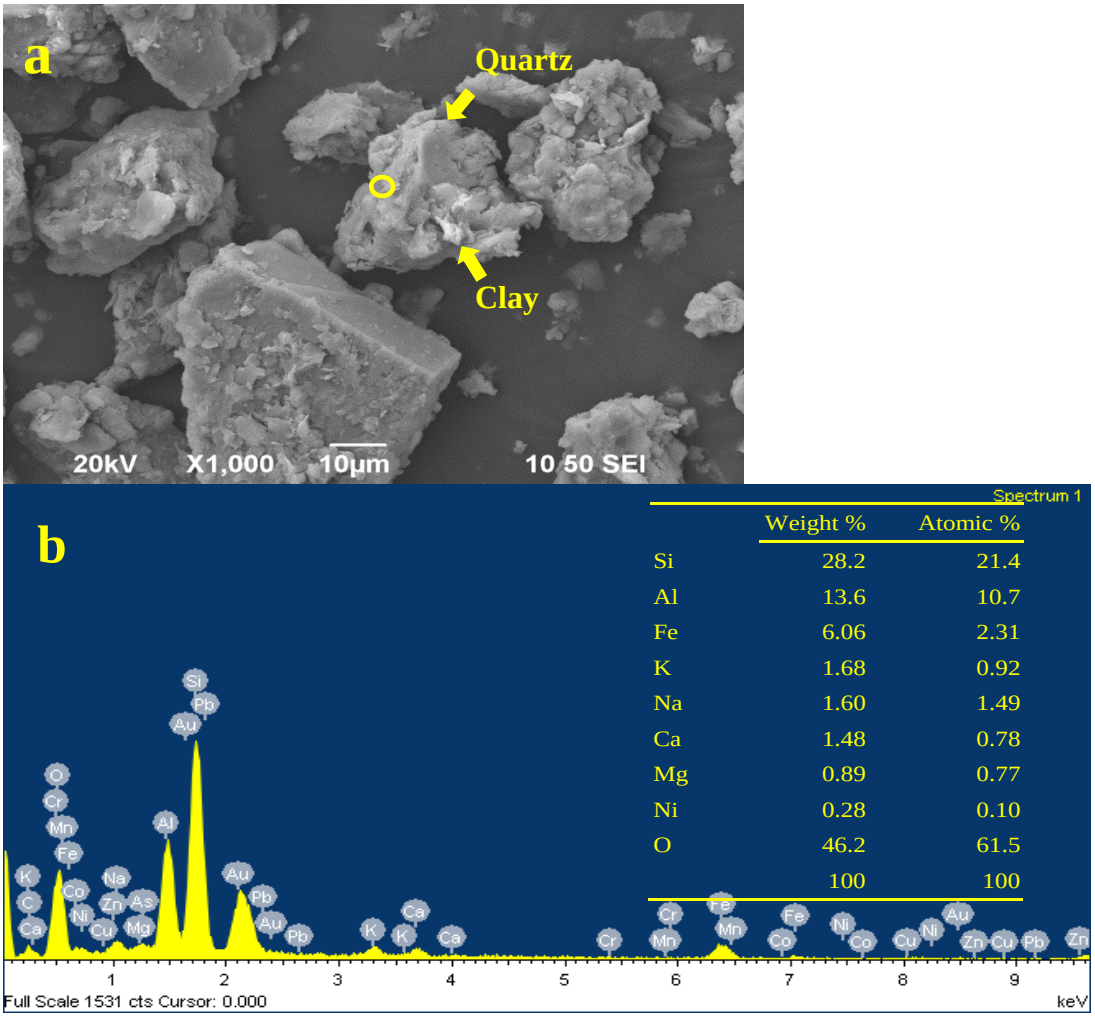

Figure 3-3 SEM photograph (a) and EDX analysis (b) of soil sample. Yellow Circle indicates area where the energy dispersive X-ray spectra displayed was recorded.

3.3.2 Effects of chelants on plant growth

In the present experiment, the application of chelants reduced the biomass of ryegrass and tall fescue (Fig. 3-4), but not resulted in visible toxicity symptoms (*e.g.* necrosis and dehydration) on plants. Compared to the control group, there was a decrease in the shoot biomass by 25.4%, 12.7% and 23.9% for ryegrass and 47.2%, 33.7% and 32.9% for tall fescue in the EDTA-, EDDS-, and GLDA-treated group, respectively (Fig. 3-4). The root biomass was reduced by 16.1%, 17.6%, and 27.1% for ryegrass, respectively, and 49.0%, 41.4%, and 43.2%, respectively, for tall fescue compared with control group after application of EDTA, EDDS, and GLDA (Fig. 3-4). Generally, EDDS appeared to be less toxic than EDTA and GLDA. The inhibitive effects of chelants on the growth of ryegrass were less significant than that of tall fescue.

Grčman et al. (2003) supported that EDTA exhibited a higher phytotoxicity than EDDS to red clover in Pb contaminated soil. However, EDDS showed greater phytotoxicity than EDTA to cardoon, corn and bean (Luo et al., 2005; Epelde et al., 2008). This is because the effects of chelants on plant growth are supposed to correlate to soil pollution levels, plant species, applied chelant dosage, and chelant type (Kos and Leštan, 2004; Niu et al., 2011a). Therefore, the pilot study on the selection of chelants and application dosage should be done with the field contaminated soil before a large scale phytoremediation practice, as the mechanisms of chelant induced toxicity have not been well understood (Johnson and Singhal, 2013).

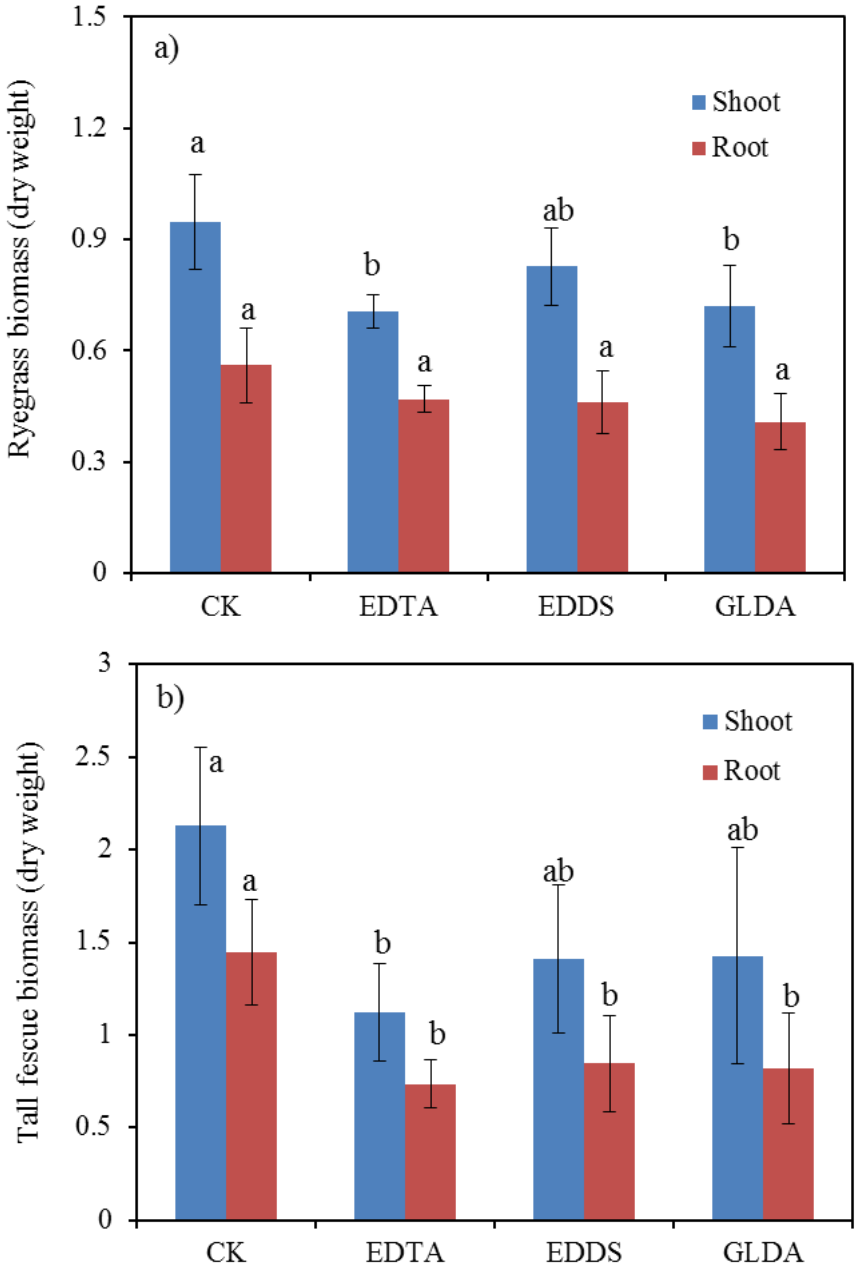

Figure 3-4 Effects of EDTA, EDDS and GLDA on the biomass production of ryegrass (a) and tall fescue (b).Values are means ± S.D. (n = 3) and small letters represent statistical difference at the *P* < 0.05 level.

3.3.3 Effects of chelants on plant Cu concentration

The application of chelants effectively increased Cu contents in shoot of ryegrass and tall fescue (Fig. 3-5). For ryegrass, EDDS was the most effective chelant and increased shoot Cu concentration by 3.8 times over the control treatment. The addition of EDTA and GLDA increased shoot Cu by 2.1- and 0.9- times, respectively, which was slightly less effective compared to EDDS. For tall fescue, the performance of chelant on enhancing shoot Cu concentration followed the sequence: EDTA > EDDS > GLDA. Compared to control, the shoot Cu concentration was enhanced by EDTA, EDDS, and GLDA for 15.0-, 9.0-, and 3.2- folds, respectively. Cu concentration in tall fescue shoot was slightly less than that in ryegrass, either with or without chelant treatments.

Cu distribution in plant roots and shoots can be affected by the application of chelants. Cu in root of ryegrass and tall fescue was significantly higher than shoot in control group. Chelants may increase the shoot to root ratio of Cu in these two grass species, as chelant can form soluble complex with Cu and reduced the sorption of Cu by plant root, which has been reported in many plant species (Luo et al., 2005; Evangelou et al., 2007; Epelde et al., 2008; He et al., 2013). In our study, EDTA and EDDS were effective on stimulating Cu translocation form root to shoot of both grass species in comparison to the control group. GLDA, a newly launched green chelator, exhibited inferior effects than EDTA and EDDS on inducing Cu translocation from root to shoot in ryegrass and tall fescue in this study.

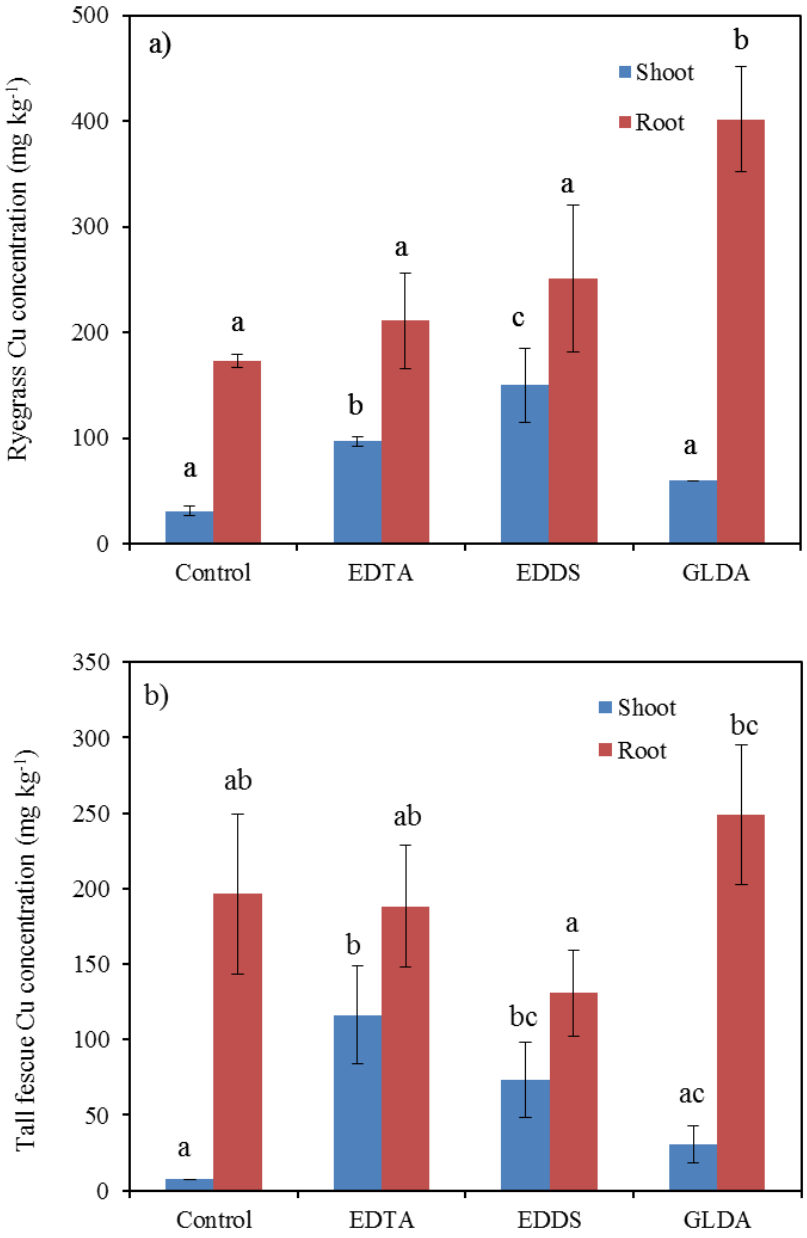

Figure 3-5 Effects of EDTA, EDDS and GLDA on Cu concentration in root and shoot of ryegrass (a) and tall fescue (b).Values are means ± S.D. (n = 3) and small letters represent statistical difference at the *P* < 0.05 level.

3.3.4 Chelant degradation

Chelants were highly soluble and primarily composed of nitrogen and carbon, so the degradation rate of chelants can be indicated by tracking the change of soil DOC (Meers et al., 2008). After treated with chelants for 7 d, the concentration of DOC in rhizosphere was much higher than non-rhizosphere (Fig. 3-6). From 7 d to 14 d after application of chelant, the degradation of EDDS was quite rapid with reduction of DOC for 62-70% in rhizosphere, and 21-42% in non-rhizosphere. EDTA showed less degradation with decrease of DOC for 31-56 % in rhizosphere and 7-11% in non-rhizosphere from 7 d to 14 d, respectively. In addition, GLDA also showed less degradation with the decrease of DOC for 26-45% in rhizosphere and 3-11% in non-rhizosphere from 7 d to 14 d. The grass species grown in pot had no significant effects on the degradation rate of chelants. By the way, the higher percentage of DOC reduction in rhizosphere can be partially resulted from the degradation of root exudates. The root exudate often contains low-molecular-weight organic acids, amino acids, and sugars, which can be readily metabolized by soil microbes (Xu et al., 2007; Dakora and Phillips, 2002).

After 28 d, the remained DOC in EDDS-treated rhizosphere and non-rhizosphere soils was only slightly higher than control by 0.22-0.28 and 0.25-0.35 folds, respectively. However, the remained DOC in EDTA-treated rhizosphere and non-rhizosphere soils was higher than control by 0.78-1.12 and 1.44-1.47 folds, respectively. GLDA treated soil also contained 0.65-1.18 folds and 1.08-1.31 folds higher DOC in rhizosphere and non-rhizosphere, respectively, compared to control. Taken together, these results suggested that the degradation rate of these chelant followed the sequence that EDDS > EDTA ≈ GLDA. EDDS has been reported to degrade with half-lives varied from 3.4 d

to 7.9 d after a lag phase of 7-32 d (Tandy et al., 2006; Meers et al., 2008). Meers et al. (2008) observed distinguished degradation pattern of EDDS in three soil samples with different metal pollution degree and soil texture. Collectively, the degradation rate of EDDS or metal chelate complex was associated with the applied dose, soil type, the degree of pollution and the type of metal involved (Philippe C Vandevivere et al., 2001).

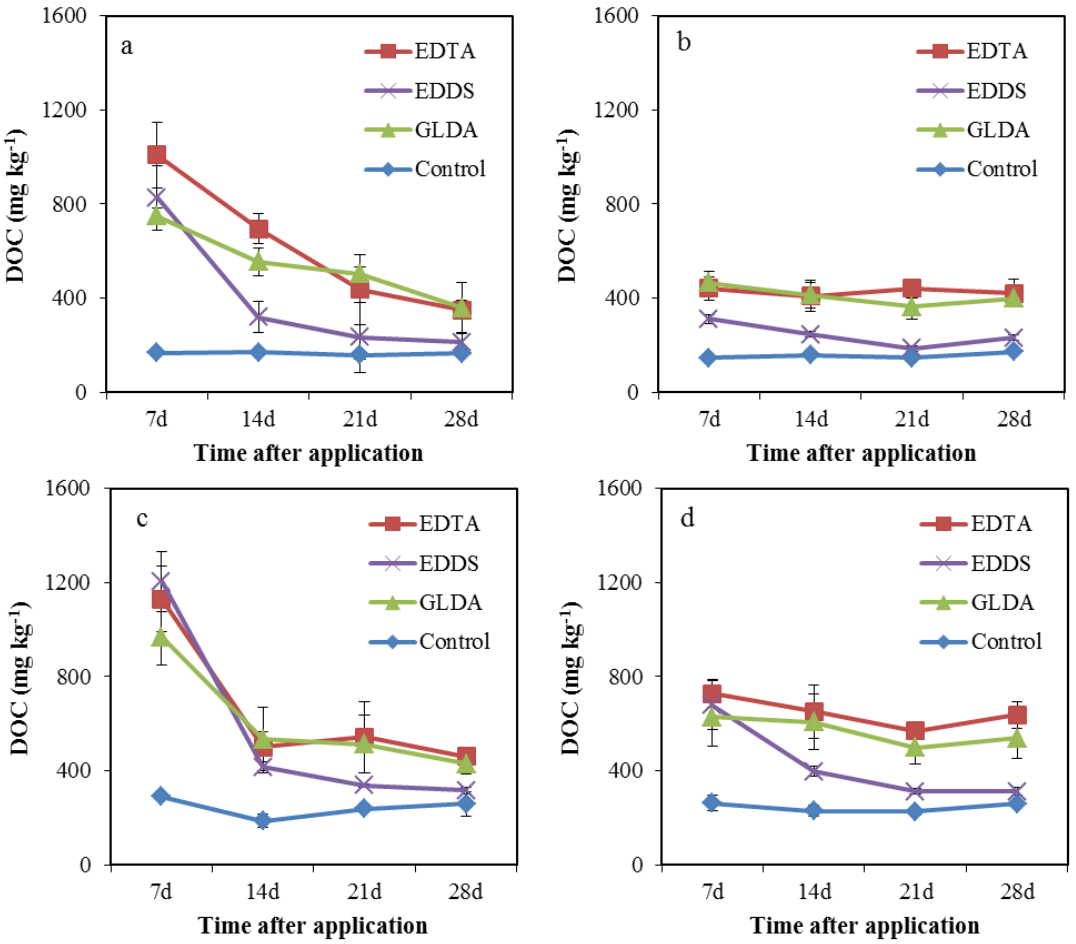

Figure 3-6 The change of soil DOC in ryegrass rhizosphere (a) and non-rhizosphere (b) and tall fescue rhizosphere (c) and non-rhizosphere (d) from 7 - 28 d after application of EDTA, EDDS, and GLDA. Values are means ± S.D. (n = 3).

3.3.5 Copper leaching risks

The $CaCl_2$ extractable Cu from soils is shown in Fig. 3-7, which suggested the Cu mobility in soils. Compared to control, chelants substantially increased the $CaCl_2$ extractable Cu from rhizosphere and non-rhizosphere after application of chelants for 7 d. EDDS resulted in the highest $CaCl_2$ extractable Cu in both rhizosphere and non-rhizosphere, compared to EDTA and GLDA. The extraction efficiency of chelants in this study was in agreement with their affinity to Cu, as the stability constant of Cu-EDDS (log K = 20.46) (Tandy et al., 2006a) is higher than Cu-EDTA (log K = 18.8) and Cu-GLDA (log K = 13.0) (Pinto et al., 2014).

The Cu leaching risks were assessed by the change of $CaCl_2$ extractable Cu in soils from 7 d to 28 d after application of chelants (Fig. 3-7). In EDDS treated group, the $CaCl_2$ extractable Cu rapidly decreased from 7 d to 28 d in accordance with soil DOC (Fig. 3-6). After 28 d, the $CaCl_2$ extractable Cu was largely reduced by 98% in both rhizosphere and non-rhizosphere of both grass species with degradation of EDDS. At 28 d, the $CaCl_2$ extractable Cu in EDDS treated soils was only slightly higher than control by 2.64-11.0 folds. However, EDTA and GLDA still maintained a high concentration of $CaCl_2$ extractable Cu, reaching 48.8-312 and 19.5-153 folds higher than control after application for 28 d. Taken together, these results suggested that EDDS reduced the leaching risks of Cu with degradation, which was safer to environment in field application than EDTA and GLDA.

In consistence with our study, EDTA has been reported to keep soluble metals for several months in deep soil which would cause substantial pollution to groundwater (Neugschwandtner et al., 2008). With OECD 301B Ready Biodegradation tests (Organization for Economic Cooperation and Development, 1992), GLDA is initially

regarded to be not ready biodegradable using inoculum from a U.S. wastewater treatment plant (Itrich et al., 2015). Van Ginkel et al. (2005) demonstrated that GLDA was extensively degraded in activated sludge following a short assimilation period for 11 d. However, the degradation of GLDA in soils has not been investigated. In our study, 52-56% GLDA was degraded at 28 d after application into soil, and the fully degradation time for GLDA still requires further study.

It was noticed that $CaCl_2$ extractable Cu in rhizosphere was much higher than non-rhizosphere, regardless of the treatment of EDTA, EDDS, or GLDA. The results suggested that plant growth affected the interaction of chelants and soils; however, the underlying reason was not known and will be further investigated.

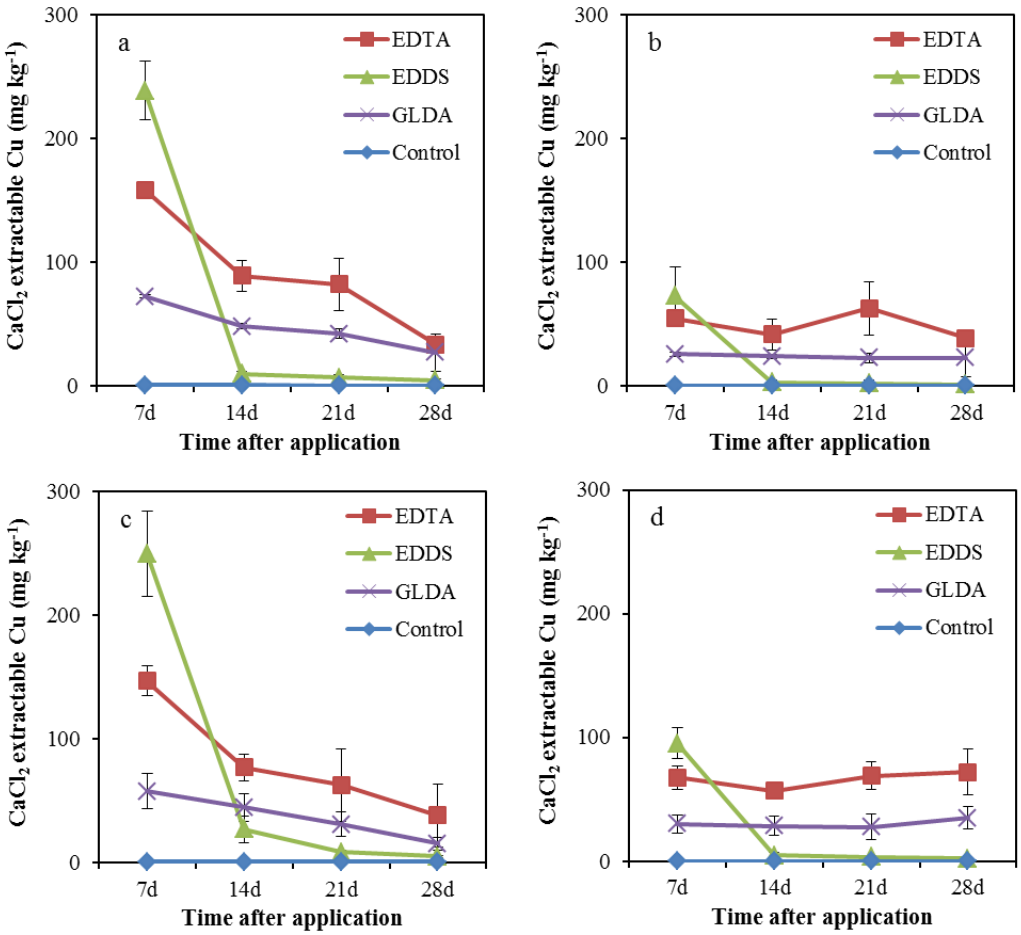

Figure 3-7 The change of $CaCl_2$ extractable Cu in ryegrass rhizosphere (a) and non-rhizosphere (b), and tall fescue rhizosphere (c) and non-rhizosphere (d) from 7 - 28 d after application of EDTA, EDDS, and GLDA. Values are means ± S.D. (n = 3).

3.3.6 Copper fractionation change

The analysis of Cu fractionation in soil is critical for understanding the effects of chelant on chemical transition of Cu in rhizosphere soil system, which sheds light on the underlying process during assisted phytoextraction and Cu mobility with chelant degradation. The traditional sequential extraction was employed in this study to explore the Cu distribution in different soil components (Fig. 3-8). The fraction obtained from sequential extraction is operationally defined, and this method suffers from a lack of specificity and resorption of mobilized metals by soil during extraction. However, it is still a routine tool that widely used for evaluation metal speciation in soils.

In rhizosphere soil of ryegrass without chelants, Cu was primarily associated with iron/manganese oxides (39.4%) and residual fraction (35.4%) in control group. Due to the strong complexation ability of chelants to Cu, Cu distribution in soil was changed dramatically (Fig. 3-8). Exchangeable Cu was substantially enhanced from 0.18% to 18.6%, 28.8% and 11.5% of total Cu by EDTA, EDDS and GLDA respectively, as a result of formation of water soluble Cu-chelate complex that weakly adsorbed on soil. EDTA and EDDS slightly reduced Cu associated with carbonates, but GLDA has no effect on this proportion. A considerable amount of Cu adsorbed on Fe/Mn oxides was reduced from 39.4% to 24.5% (by EDTA), 19.6% (by EDDS) and 30.4% (by GLDA), respectively. Because Fe/Mn oxides are expected to be partially dissolved by EDTA and EDDS via surface complexation, the considerable amount of Cu adsorbed on this fraction can be thus decreased (Zhang et al., 2014). The amount of Cu complexed with soil organic matters was slightly decreased by 14.7%, 22.2%, and 2.2%, respectively, after usage of EDTA, EDDS, and GLDA for 7 d. The difficulty in

extracting Cu from organic matter fraction indicated that Cu-humus in this soil has a strong bonding strength. At last, three chelants were not able to extract Cu from the residual pool, as this fraction of Cu was mainly incorporated in crystalline lattice involved in structure of silicates that hardly to be mobilized. Conclusively, soil Cu was found to be primarily mobilized from Fe/Mn oxides fraction to exchangeable fraction after application of EDTA, EDDS, and GLDA. There was no significant difference on rhizosphere Cu fractionation between pots planted with ryegrass and tall fescue. Our results were in accordance with Sun et al., (2001), who reported that the reduction of Cu in different fraction followed the order that Fe/Mn oxide > organic matter > carbonate > residue after EDTA leaching. However, Tsang and Hartley (2013) found that chelating agents predominantly extracted Cu from the carbonate fraction in a chromate copper arsenate (CCA) contaminated soil, underlining the importance of Cu pollution source on the impact of Cu fractionation change during reaction with chelating agents.

In EDTA treated rhizosphere soils, the enhanced exchangeable Cu was reduced by 45.3-80.0% from 7 d to 28 d, while the other Cu fractionation remained unchanged ($P < 0.05$). This result suggested that a proportion of EDTA-mobilized Cu was leached away from the bottom of pots from 7 d to 28 d after usage of EDTA. However, in EDDS treated group, the exchangeable Cu was reduced substantially by 95.2-95.3%, and Cu associated with Fe/Mn oxides, organic matter, and carbonate increased accordingly to the level similar to control group. This result is mainly due to the rapid degradation rate of EDDS in soils, which resulted in the resorption of mobilized Cu from ryegrass and tall fescue rhizosphere to soil components. In GLDA-treated group, the exchangeable Cu was reduced slightly by 29.0-51.6%, and the other Cu fractionation remained unchanged ($P < 0.05$) from 7 d to 28 d. The slight decrease of

exchangeable Cu is likely due to the metal leaching similar to EDTA-treated group. These results concluded that, compared to EDTA and GLDA treatment, EDDS induced Cu fractionation change in soils was temporary, so that the leaching risks of Cu induced by EDDS can be well controlled with degradation.

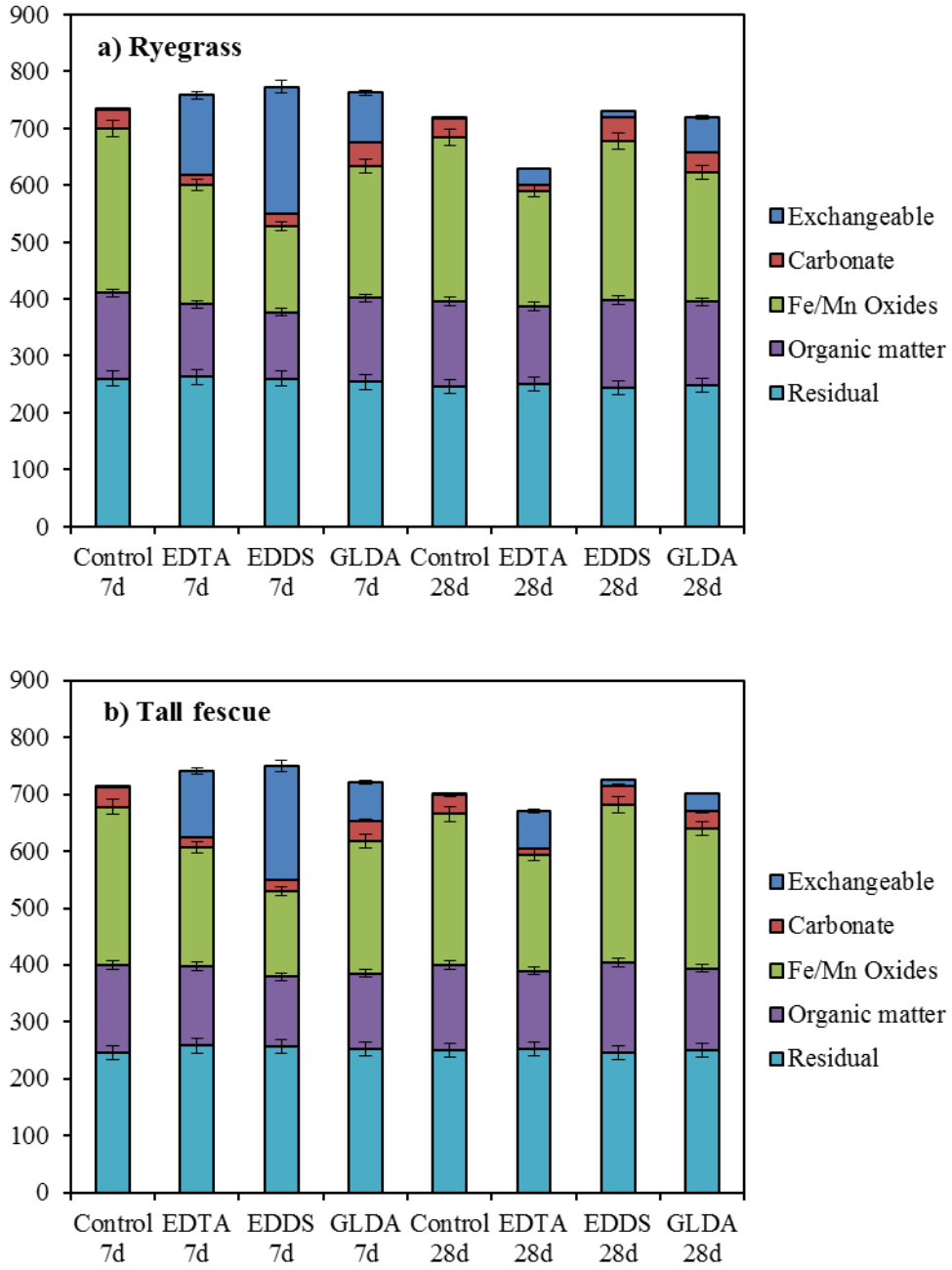

Figure 3-8 Cu fractionation in rhizosphere soil at 7 d and 28 d after application of chelants to pot grown ryegrass (a) and tall fescue (b).

3.4 Summary

According to the current pot experiment, EDDS was more suitable to be used with ryegrass and tall fescue compared to EDTA and GLDA from the viewpoint of phytoextraction. The main results can be summarized as follows:

1) EDDS have less phytotoxicity to ryegrass and tall fescue, and performed better on enhancing Cu contents in shoot of ryegrass and tall fescue in comparison with GLDA.

2) In comparison to tall fescue, ryegrass showed higher tolerance to EDTA, EDDS and GLDA, and could accumulate more Cu in shoots either with or without application of chelants.

3) The leaching risks can be immediately controlled with the rapid degradation of EDDS, with soil DOC and $CaCl_2$ extracted Cu substantially decreased by 76% and 96%, respectively, from 7 d to 28 d after addition to soil. Although GLDA was regarded as a new green chelant, its degradation in this soil was not efficient, which was comparable to EDTA in our study.

4) After application of EDTA, EDDS, and GLDA for 7 d, the exchangeable Cu in rhizosphere soil increased from 0.2% to 11.5-28.8%. Cu was primarily mobilized from Fe/Mn oxides fractions by these chelants. With the effective degradation of EDDS after application for 28 d, Cu was resorbed to soil components.

5) Soil DOC and $CaCl_2$ extracted Cu was higher in rhizosphere compared to bulk soil after application of EDTA, EDDS, and GLDA for 7 d in pots. However, the underlying reason still requires further study.

Collectively, these results suggest that EDDS can be used as a representative biodegradable chelant for investigation the impacts of chelant on phytoextraction mechanisms. Moreover, the interaction of EDDS with soils varied in rhizosphere and non-rhizosphere, which requires a more specific experiment designs (like using rhizobox) to explore the underlying processes.

4. Chapter Four – The Impact of EDDS on Cu Interactions in Rhizosphere Soil

4.1 Introduction

When EDDS was applied, the phytoextraction process is critically determined by the interaction of EDDS with rhizosphere soils. This is because EDDS alters the speciation and concentration of target metals and subsequently affects the uptake and translocation of metals by plants (Nowack et al., 2006). Specifically, EDDS may completely or partially chelate with metals in soil solution, which turn the primary root absorption pathways from that for free metals to metal-EDDS complexes (Koopmans et al., 2008). Furthermore, the concentration of metal-EDDS complex in soil solution is critical for metal translocation in plants. A high concentration of PbEDDS or CuEDDS has been found indispensable to damage the Casparian strip in roots, and facilitate metal translocation to shoots (Mohtadi et al., 2013; Niu et al., 2011).

Many factors have been reported to control the interactions between EDDS and metals within soil. The metal extraction effectiveness by EDDS has been reported to correlate to EDDS concentration, solution pH, reaction duration, liquid-soil ratio, and other soil properties (Yip et al., 2009; Yan and Lo, 2011; Fabbricino et al., 2016). In addition, mineral cations (*e.g.* Fe, Ca, Al, and Mn), of which the dissolution is promoted by EDDS, may compete for complexation with EDDS, alter the speciation of EDDS and extraction effectiveness of target metals (Komárek et al., 2009; Tsang et al., 2009; Yang et al., 2012). Although recent studies have achieved some progress, most of the knowledge is obtained from soil washing experiments or artificially

contaminated soils. More work is required to be conducted in soil-plant system with field contaminated soils, in order to elucidate the realistic interaction for phytoextraction.

Plant growth may influence the interaction of EDDS with metals in rhizosphere soils; however, few studies paid attention to this aspect. Plant transpiration is known to create a water potential gradient in soils near roots, which continuously drives a water flow to surfaces of plant roots (Jackson et al., 2000; Carminati, 2012). In this case, the water flow may bring the applied soluble EDDS from far- to near-root regions, which increase the metal extraction from soils near roots. On the other hand, the metal transport from soils to roots is naturally controlled by diffusion and convection with the water flow (Barber, 1962). EDDS can enhance the convective transport of metals, ascribed to the formation of metal-EDDS complexes as well as the increased metal solubility in soils (Nowack et al., 2006). The multi-interlayer rhizobox has been successfully used to characterize the biochemical changes in rhizosphere soils that caused by plant root activities (Tao et al., 2003; Kim et al., 2010). Hence, this kind of rhizobox can be employed to investigate the potential effects of plants on metal extraction and transport processes in EDDS-assisted phytoextraction.

The mobility and chemical speciation of soil metals is of great concern for EDDS extraction (Kirpichtchikova et al., 2006). EDDS can directly complex with weakly-bound metals, and also indirectly release strongly-bound metals at a relatively slow rate through EDDS-promoted soil dissolution (Zhang et al., 2010). In field-contaminated soils, metals tend to bind strongly to soil components as a function of soil aging, which makes them difficult to be extracted (Tsang et al., 2007). Therefore, the study of soil metal speciation is required with or without use of EDDS,

which can provide mechanistic insights into the metal extraction processes. The application of advanced synchrotron-based spectroscopy, such as micro X-ray fluorescence (μ-XRF) and X-ray absorption near-edge structure (XANES), may provide reliable information on the distribution and speciation of target metals in soil matrix (Kirpichtchikova et al., 2006; Strawn and Baker, 2008; Yang et al., 2014).

The current study aimed 1) to characterize the effects of EDDS on Cu extraction, speciation, and transport in rhizosphere soils, particularly under the interference of plant growth; and 2) to improve the understanding of Cu extraction mechanisms by EDDS from field-contaminated soils.

4.2 Materials and methods

4.2.1 Soil collection

Soil samples of 0-20 cm were collected from a farmland, in the vicinity of an abandoned copper mine (N32^{o}03', 118^{o}47'), from Tangshan Town of Nanjing, Jiangsu, China. Copper pollution in this area was derived from fume dust and wastewater irrigation from smelting plant (Qin et al., 2012), which pose a high health risk of Cu transfer to human being through food chain (Wu et al., 2011). Soils were air dried, mixed thoroughly, and sieved through a plastic mesh of 2 mm for further use. Basic physico-chemical characteristics of the soils were determined by standard analytical methods (Li et al., 2001; Cater and Gregorich, 2006) (Table 3-1).

4.2.2 Experiment set up

A multi-interlayer rhizobox (Fig. 4-1) was made with slight modification according to Wang et al. (2002). The dimension of the rhizobox was 80 mm × 130 mm × 130 mm (length × width × height). Six compartments were divided in the rhizobox by nylon mesh (< 25 μm) at a defined distance. The leftmost compartment grown with ryegrass was marked as rhizosphere, and adjacent compartments next to rhizosphere were named according to the distance from rhizosphere, such as non-rhizosphere (0-1 cm). Such rhizobox restricted plant roots in the rhizosphere compartment by nylon mesh, while the nylon mesh allowed soil pore water, root exudates, and soil microfauna to transfer through each compartment (Xie et al., 2012). Each rhizobox was packed with a total weight of 960 g soil.

Ryegrass seeds were sterilized with 95% ethanol for 15 min, washed thoroughly with deionized water, and then germinated on moist filter paper for 7 d. About 80 seedlings

were transferred to each rhizobox, and the soil humidity was kept at 60% maximum water holding capacity. Deionized water was added to rhizobox every day to keep the soil humidity. Rhizoboxes were placed in a climate growth chamber at 60% humidity, 25 ^{o}C/20 ^{o}C day/night, and 16 h photoperiod per day (325 μmol photons m^{-2} s^{-1}). Each rhizobox was wrapped with aluminum foils to prevent the growth of algae. After growth of ryegrass for three weeks, a total of 40 mL 120 mM EDDS was evenly added from the top surface to compartments in rhizobox, which equaled to a dosage of 5 mM EDDS kg^{-1} soil that frequently used in EDDS-assisted phytoextraction (Luo et al., 2005; Duquène et al., 2009; Almaroai et al., 2013). One set of rhizoboxes was un-planted and applied with EDDS at the same dosage as a control. One set of rhizoboxes was planted without EDDS as a control. Three replicates were employed for each treatment. Following the use of EDDS for 7 d, ryegrass was harvested, separated into roots and shoots, and washed with deionized water. The soil from each compartment was collected, homogenized, and stored at 4 ^{o}C or -20 ^{o}C until further analysis.

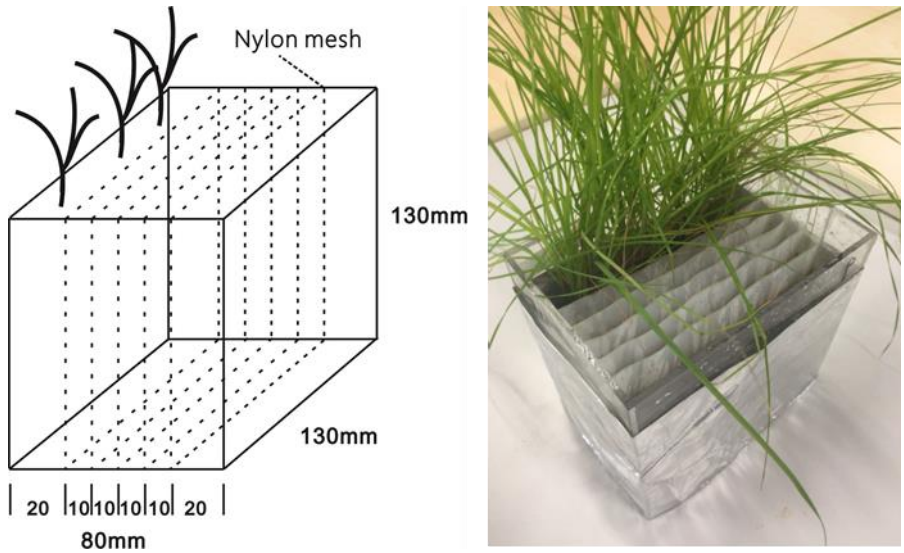

Figure 4-1 A modified multi-interlayer rhizobox. Nylon mesh (< 25 μm) was used to separate soil into different compartment.

4.2.3 Analytical methods

4.2.3.1 Soil solution extraction and analysis

Chemical analysis was carried out on soil extracts to mimic the conditions in soil solution. Fresh soil (2.5 g) was extracted with deionized water in a 1:10 (w: v) ratio on a dry weight soil basis on an end-over-end shaker at 50 rpm for 2 h (Séguin et al., 2004). The suspensions were centrifuged for 10 min at 8000 rpm, and filtered through 0.45 μm cellulosic membranes. Chemical analysis included pH, electrical conductivity (EC), anions (Cl^-, F^-, NO_2^-, NO_3^-, SO_4^{2-}, and PO_4^{3-}) by an ion chromatography, and total dissolved organic carbon (DOC) by a Shimadzu TOC analyzer. Copper, Al, Ca, Fe, Mg, Mn, K, and Na were analyzed with ICP-AES (Agilent 700 series) and ICP-MS (Agilent 7700 series) after digestion of soil extracts with conc. HNO_3 (Cui et al., 2017). Ionic strength (IS) in soil extracts was calculated from EC with the empirical relationship IS＝0.13 EC (Vulkan et al., 2000). EDDS derivation and analysis in soil extracts were performed according to Katata et al. (2006). Generally, EDDS was converted into FeEDDS, separated using a reversed-phase Inertsil ODS-3 C18 (5 μm 4.6*259 mm), and detected at 254 nm wavelength by Waters HPLC 2487. The mobile phase consisted of 10% methanol and 90% tetrabutylammoniumbromide (0.02 M) eluent (with pH adjusted to 4.0 using formic acid). The flow rate was set at 1 mL min^{-1}. The “natural” DOC was calculated from the difference between total DOC and EDDS-contributed DOC.

4.2.3.2 Modeling of metal and EDDS speciation

The software Visual MINTEQ version 3.1 was used to calculate the EDDS and metal speciation in soil extracts from different compartments of rhizobox. Input data included the concentration of EDDS, dissolved metals (Cu, Zn, Pb, Ni, Fe, Mn, Ca,

Mg, and Al), anions (Cl^-, F^-, NO_2^-, NO_3^-, SO_4^{2-} and PO_4^{3-}), and dissolved organic matter (DOM). Parameters such as solution pH and ionic strength were also considered. The stability constants of most metal-EDDS complexes metals were obtained from Tandy et al. (2006), and that of AlEDDS was acquired from Koopmans et al. (2008). The DOM concentration was set as twice of the concentration of "natural" DOC, and the composition of DOM was assumed to be 50% fluvic acid and 50% humic acid (Yip et al., 2009b). The binding of metals to DOM was modeled using the NICA-Donnan model with generic parameters (Milne et al., 2003). Iron and aluminum were allowed to precipitate when exceeding the solubility of $Fe(OH)_3$ (log Ksp = 2.69, 25 ℃) and $Al(OH)_3$ (log Ksp = 8.29, 25 ℃) (Sjöstedt et al., 2010).

4.2.3.3 Metal fractionation in soil by sequential extraction

The speciation of trace metals in the soil was determined by a classic sequential extraction procedure (SEP) (Li et al., 1995). The concentration of trace metals (Cu, Zn, and Pb) in solution was determined by the ICP-OES or ICP-MS. According to this sequential extraction procedure, five binding forms of trace metals were obtained including exchangeable, carbonate bound, iron and manganese oxide bound, organic matter bound, and residual metals.

4.2.3.4 Metal distribution and speciation in soil by μ-XRF, μ-XANES and bulk-XANES

The μ-XRF and μ-XANES experiments were conducted in ambient conditions at beamline 15U in Shanghai Synchrotron Radiation Facility (SSRF), China. Freeze dried soil was grounded to less than 50 μm size fraction and dispersed on tape before microprobe analysis. A selected sample area (1500 × 1500 μm) was scanned in step size of 50 μm. The fluorescence signals of Cu, Fe, Si, Al, Mn, Ca, and K were

selectively acquired with the dwell time of 1.5 s using a one-element Si drift detector. Elemental images from the XRF data were produced using Igor Pro 6.0 software (IGOR). Two hot spots of Cu identified from the XRF image were selected for collecting Cu K-edge μ-XANES spectra (Cui et al., 2013).

Acquisition of the Cu K-edge bulk-XANES data for soil and standards was done on beamline 01C1 at National Synchrotron Radiation Research Center (NSRRC), Taiwan. CuO, CuS, CuCl, $CuSO_4$, $CuCO_3$, $Cu_3(PO_4)_2$, $Cu(OH)_2$ and $Cu(CH_3COO)_2$ standards were obtained from Sigma-Aldrich, and measured in transmission mode (Fig. 4-13). Cu-goethite, Cu-ferrihydrite, Cu-humic acid, and Cu-clay (montmorillonite) were prepared according to Strawn and Baker (2008), and CuEDDS was prepared according to Shi et al. (2008). These standards and soil samples were measured in fluorescence mode (Fig. 4-12 and 4-13). In addition, Fe K-edge bulk-XANES spectra of soils and standards were collected at beamline 16A1 at NSRRC. The XANES sample preparation and data processing were described in our previous study (Cui et al., 2017a; Cui et al., 2017b).

Cu K-edge (8979 eV) XANES spectra were recorded from –200 to 300 eV. Each scan was completed within 15 min, and repeated scan was not derived from the same point. A Cu foil was used to calibrate the inflection point of Cu K-edge at 8,979 eV. The Athena software packages (December 0.9.25) were used for XANES data normalization and linear combination fitting. Principal component analysis (PCA) of sample spectra and target transformation (TT) of standards were performed using Six-Pack. A maximum of three standards were allowed for linear combination fitting (LCF) procedures, according to the minimum value of IND (Table 4-1) (Manceau et al., 2002). The SPOIL values of standards are shown in Table 4-2. LCF analysis of

sample spectra was conducted in the range of -20 to 40 keV. During LCF, the fit standard was incrementally added to improve the linear fit, and one standard was only included when fit residual (normalized sum square) reduced at least by 20%. Additionally, the standard included must accounted for at least 10% of the measured spectra (Sarret et al., 2007; Punshon et al., 2013).

Fe K-edge (7112 eV) XANES spectra were recorded with an energy range of -200 to 400 eV in transmission mode. Fe standards for soil samples included siderite ($FeCO_3$), pyrite (FeS_2), arsenopyrite (FeAsS), goethite, ferrihydrite, lepidocrocite, hematite (Fe_2O_3), and magnetite (Fe_3O_4) (Fig. 4-11). Normalization and LCF procedures of Fe K-edge spectra were conducted as described for Cu, with results shown in Table 4-3.

Table 4-1 Results from the principal component analysis performed on the Cu K-edge XANES spectra.

Component	Eigenvalue	Variance	Cumulative variance	IND
1	77.764	0.927	0.927	0.02542
2	2.897	0.034	0.962	0.01781
3	1.285	0.015	0.977	0.01613
4	0.647	0.007	0.985	0.01961
5	0.416	0.004	0.990	0.03010
6	0.313	0.003	0.994	0.06167
7	0.257	0.003	0.997	0.23568
8	0.235	0.002	1	NA

Table 4-2 Spoil values of Cu references obtained by target transformation.

Standards	Spoil	R	Chi
CuCl	1.2393	0.00809	7.34988
CuO	0.9903	0.00211	1.88339
CuS	1.0671	0.00561	5.25965
$CuSO_4$	3.0277	0.00101	0.89534
$CuCO_3$	1.2305	0.00138	1.23054
$Cu(OH)_2$	1.6692	0.00116	1.03664
$Cu_2(CH_3COO)_4$	1.2292	0.00263	2.34492
$Cu_3(PO_4)_2$	1.8590	0.00182	1.56724
Cu-goethite	2.5400	0.00153	1.39496
Cu-ferrihydrite	2.4734	0.00050	0.46928
Cu-humic acid	2.2993	0.00062	0.56810
Cu-clay	2.4849	0.00033	0.29496
CuEDDS	0.6777	0.00058	0.55000

4.3 Results and discussion

4.3.1 Effects of plants on EDDS distribution in rhizobox

The distribution of EDDS in soil extracts from rhizobox is shown in Fig. 4-2. The concentration of EDDS showed no difference ($P < 0.05$) in soil extracts from different compartments of unplanted rhizobox (Fig. 4-2). Compared to the initial application dosage (5 mM kg^{-1}), a total of 18% EDDS was lost in soil extracts from rhizobox. The loss of EDDS should not be resulted from the adsorption of EDDS on the surface of contaminated soil, since the adsorption is negligible in pH around 8 (Fig. 4-3) (Koopmans et al., 2008; Yip et al., 2009b). Instead, the decrease can be due to EDDS degradation after application to soils for 7 d. Although EDDS degradation has been reported to start in soils after an initial lag phase ranging from 7 to 32 d, the length of lag phase can vary with soil types and the degree of pollution (Tandy et al., 2006a; Meers et al., 2008).

The total amount of EDDS (3.92 ± 0.10 mM) in soil extracts in planted rhizobox was consistent with unplanted rhizobox (4.06 ± 0.12 mM) summarized from Fig. 4-2, suggesting that the degradation rate of EDDS was not affected by ryegrass growth. However, the distribution of EDDS in rhizobox was greatly altered by planting ryegrass (Fig. 4-2). The concentration of EDDS increased substantially in rhizosphere, and decreased in non-rhizosphere after planting ryegrass. In addition, the increment of EDDS in rhizosphere (1.43 ± 0.13 mmol) generally corresponded to the loss in non-rhizosphere (1.30 ± 0.10 mmol). The result indicated that EDDS was transported from non-rhizosphere to rhizosphere of ryegrass, probably driven by the continuous plant-transpiration-induced water flow towards root surface. Similarly, in view of

previous study on soil nutrients, soluble nutrients (*e.g.* Ca, Mg, and NO_3^-) were also observed to transport to root surface through the transpiration stream induced by plants (Moritsuka et al., 2000). Although ryegrass can absorb some EDDS through plant transpiration stream, the uptake amount by plants is usually marginal (accounting for about 3‰ of total EDDS in soils, Tandy et al., 2006b). Therefore, EDDS was gradually accumulated in rhizosphere compartment.

The interaction of EDDS with soils has been known as a kinetic process. In view of previous work for soil washing, most of heavy metals can be extracted by EDDS within 24 h, while the process reached equilibrium after 48-72 h (Yip et al., 2009; Yang et al., 2012). However, the kinetic results, obtained in well agitated condition from EDDS-assisted soil washing, should be different from EDDS-assisted phytoextraction. In phytoextraction, the interaction may require more time for EDDS to penetrate into soil particles or aggregates to extract metals. Therefore, in our study, it is expected that the plant-transpiration-induced water flow towards roots should contain EDDS in both free and complexed forms. Furthermore, the transportation of uncomplexed EDDS to rhizosphere may intensify the chemical interactions of EDDS with rhizosphere soils (which will be discussed later).

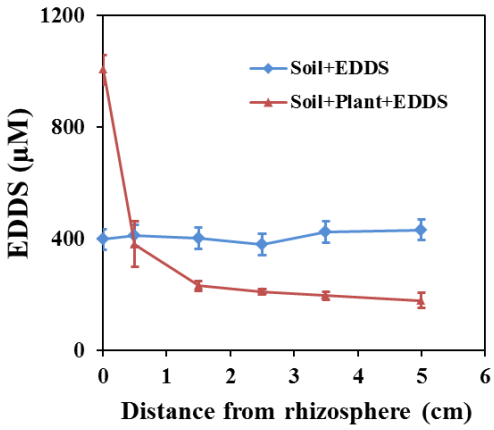

Figure 4-2 Variations of EDDS concentration in soil extracts from the serial compartment of rhizobox.

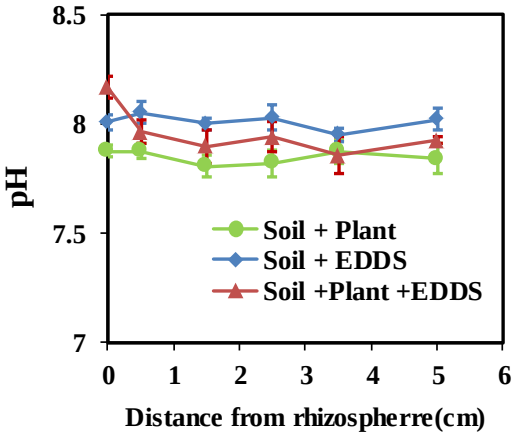

Figure 4-3 Variations of pH in soil extracts from the serial compartments of rhizobox.

4.3.2 Copper extraction and distribution in rhizobox

Cu was negligible in soil extracts of planted rhizobox (Fig. 4-4a), and there was no great difference of soluble Cu between rhizosphere and non-rhizosphere. The result suggests that, during the short period of cultivation (21 d), ryegrass cannot mobilize Cu significantly from soils naturally via root exudates. With use of EDDS, the concentration of Cu in rhizobox increased remarkably by 285–690 folds (Fig. 4-4a). In addition, Zn and Pb concentration increased as well in soil extracts after use of EDDS, but to a much less extent (by 5.97-70.0 and 0.79-66.1 folds, respectively) than Cu (Fig. 4-5). The greater solublization of Cu than Zn and Pb by EDDS was also reported elsewhere (Quartacci et al., 2007; Koopmans et al., 2008), due to the higher affinity of Cu to EDDS (Log K CuEDDS = 20.46, log K ZnEDDS = 15.34 and log K PbEDDS = 14.46) (Orama et al., 2002; Tandy et al., 2006a), and the lower concentration of Zn and Pb in this soil (Table 3-1) (Yan et al., 2010). In spite of that, Cu concentration in soil extracts from rhizosphere was higher than non-rhizosphere by 2.07-3.49 folds.

SEP results supported that the distribution of Cu in original soils followed the sequence of Fe/Mn oxides fraction (37.8%) > residual fraction (35.9%) > organic matter fraction (21.5%) > carbonate fraction (4.8%) > exchangeable fraction (0.08%) (Fig. 4-4b). The result is similar to previous investigation from the sampling region (Wu et al., 2011). The growth of ryegrass alone did not obviously affect the fractionation of Cu in rhizobox at short period, since ryegrass only slightly modified the Cu fractionation in e-waste soils in almost 3 years (Cui et al., 2017).

After application of EDDS into planted rhizobox, Cu fractionation in soil was greatly shifted (Fig. 4-4c), while no significant influence of EDDS was observed for Zn and

Pb (Fig. 4-5), that is consistent with the affinity properties. Generally, EDDS reduced Cu from carbonate (F2), Fe/Mn oxides bound (F3), and organic matter bound fractions (F4), and accordingly increased exchangeable Cu fraction (F1). The fractionation of Cu in rhizosphere and non-rhizosphere was differently influenced by the application of EDDS to rhizobox for 7 d. On the one hand, the quantity of EDDS-extracted Cu from F2, F3, and F4 increased with the decrease of distance from rhizosphere. This result is related to the transport of uncomplexed EDDS from non-rhizosphere to rhizosphere as mentioned above, because the metal extraction efficiency usually increases with the increasing concentration of free chelant (Yan et al., 2010). On the other hand, comparing with the decrease of F2, F3, and F4, the increment of exchangeable Cu was higher in rhizosphere soil and lower in non-rhizosphere, respectively. After mass balance calculation, the excess of exchangeable Cu in rhizosphere (33 ± 4 mg) equaled to the loss in non-rhizosphere (37 ± 5 mg), indicating that abudant exchangeable Cu was transported from non-rhizosphere to rhizosphere after use of EDDS, probably via plant transpiration stream. The absorption of exchangeable Cu by ryegrass (0.58 mg) in the rhizosphere contributed slightly decrease of Cu (0.7%) to the exchangeable pool.

Collectively, SEP results revealed that the higher concentration of Cu in soil extracts from rhizosphere compared to non-rhizosphere was ascribed to two reasons, including the intensified Cu extraction in rhizosphere soils by transported EDDS, and the transportation of EDDS-enhanced exchangeable Cu from non-rhizosphere soils to rhizosphere.

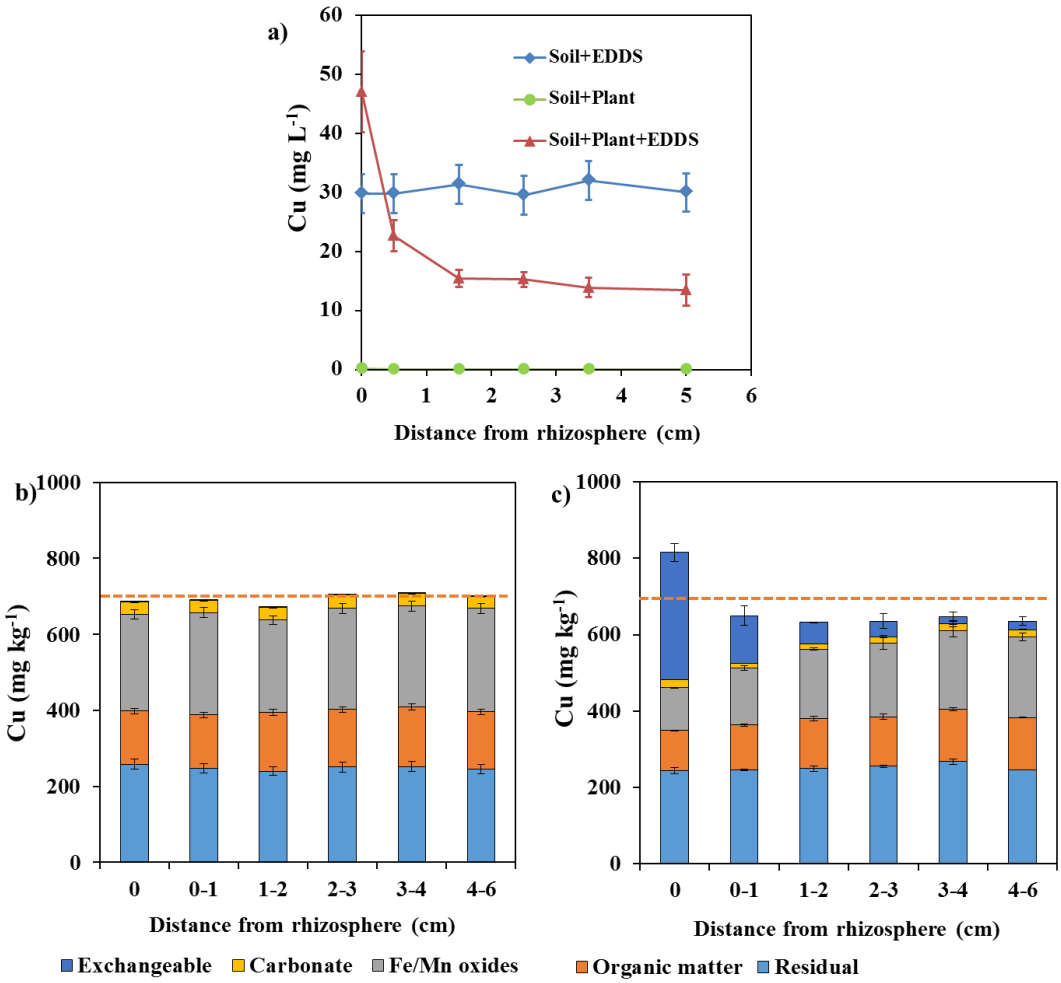

Figure 4-4 Concentrations of Cu in soil extracts (a), sequential extraction fractionations of Cu in soils without (b) or with (c) EDDS treatments from the serial compartments of planted rhizobox. Orange dashed lines in b and c represent the total concentration Cu in original soil by SEP.

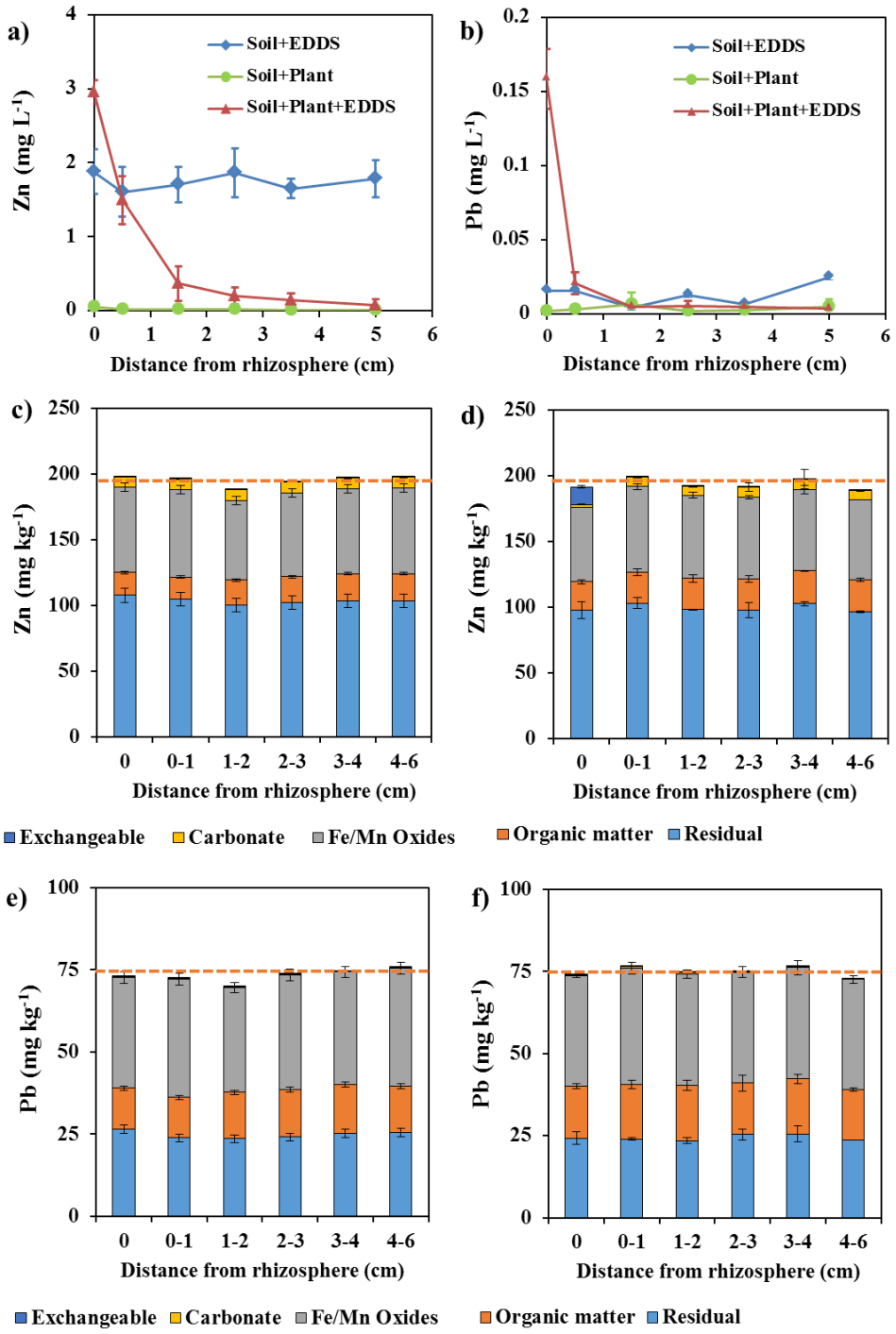

Figure 4-5 Concentrations of Zn (a) and Pb (b) in soil extracts from the serial compartments of rhizobox; the sequential fractionations of Zn and Pb in soil from different compartments of planted rhizobox without (c for Zn and e for Pb, respectively) and with EDDS treatment (d for Zn and f for Pb, respectively). Orange dashed lines in c-f represent the total Zn or Pb concentration in original soil by SEP.

4.3.3 Mineral and organic matter dissolution

Chelants not only extract heavy metals from soils, but also promote the dissolution of minerals (*e.g.* Fe, Mn, Ca, and Al oxides) through the destabilization of metal-oxygen bonds (Nowack et al., 2002). The dissolved cations from minerals often bind with chelant, and thus reduce the extraction efficiency of heavy metals (Tandy et al., 2004; Manouchehri et al., 2006; Komárek et al., 2009; Yip et al., 2009). Our results showed that EDDS increased the dissolution of Fe (by 3.47-60.2 folds) obviously and Al (by 2.43-5.31 folds) slightly comparing with the control soil without EDDS (Fig. 4-6a and b). The potential competition, from dissolved Fe and Al for complexation with EDDS, will be assessed with the subsequent speciation simulation.

Since organic matters often adsorb on Fe/Al oxides in soil aggregates, EDDS may cause the dissolution of organic matters together with Fe/Al oxides (Tsang et al., 2007). This is corroborated by the increase of "natural" DOC (by 2.00-4.44 folds) in EDDS treated rhizobox from current study (Fig. 4-6d). EDDS has been reported to mobilize soil organic matter (SOM) to soil solution in different soils (Hauser et al., 2005; Koopmans et al., 2008; Yang et al., 2012).

Similar to Cu distribution in soil extracts (Fig. 4-4a), the concentration of mineral cations (except Al) and "natural" DOC in rhizosphere soil extract was higher than non-rhizosphere by a factor of 3.03 (Fe), 1.13 (Ca), and 2.34 ("natural" DOC), respectively (Fig. 4-6). The results can be also associated with the plant-transpiration-induced water flow. On the one hand, the transport of extra EDDS towards roots may intensify the dissolution of rhizosphere soils, and increase the dissolution of Fe and organic matters (Yan et al., 2010). Moreover, the high concentration of EDDS may enhance dissolution of Ca in rhizosphere, while the low

concentration of EDDS may even decrease soluble Ca in non-rhizosphere (Fig. 4-6c). It was supported by Luo et al., 2005 that chelants at different dosage exhibited contrary effect on soluble Ca in soils. On the other hand, plant transpiration stream may also bring dissolved Fe and natural "DOC" from non-rhizosphere towards rhizosphere. However, the extraction of minerals from soil is not only controlled by available quantity of EDDS, but also by the availability of mineral cations. Al in this soil can be mostly present as aluminosilicates forms, which is involved in the lattices of AlO_4 tetrahedron and difficult for EDDS extraction. Therefore, EDDS-solubilized Al was low and invariant in rhizosphere and non-rhizosphere (Fig. 4-6b).

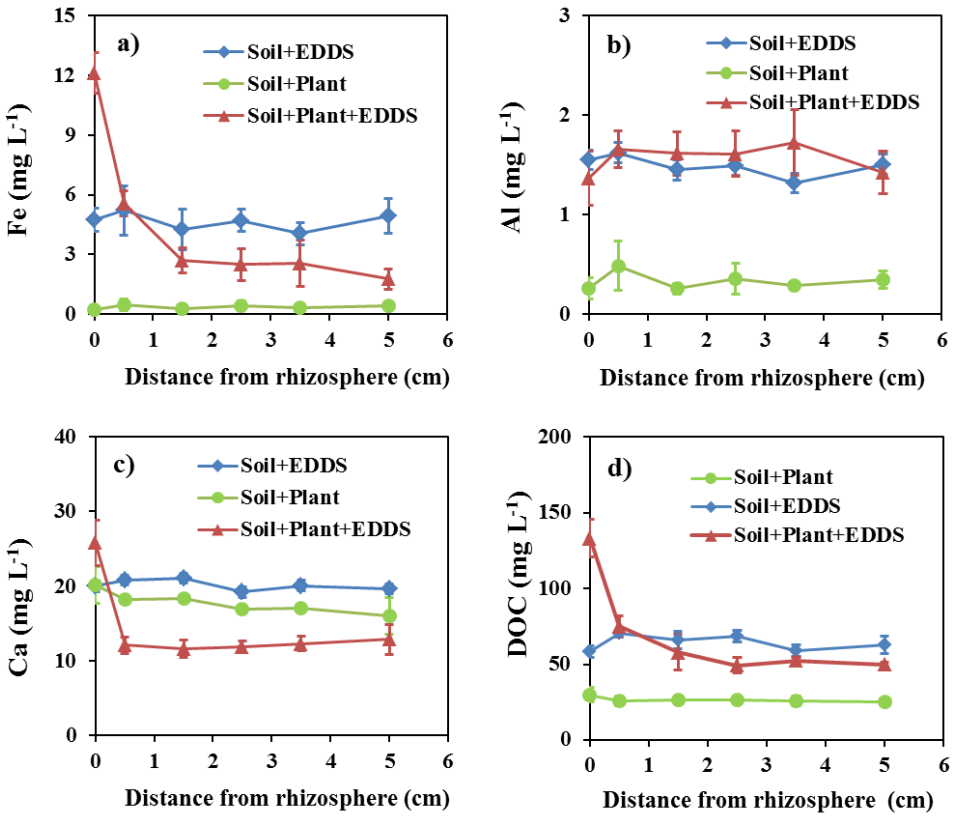

Figure 4-6 Concentrations of Fe (a), Al (b), Ca, (c) and "natural" DOC (d) in soil extracts from the serial compartments of rhizobox. The "natural" DOC was derived from the total detected DOC with subtraction of the DOC involved in EDDS.

4.3.4 Modelling of EDDS and metal speciation in soil extracts

The speciation of EDDS and metals in soil solution may reveal the potential process regarding metal competition in solution (Yip et al., 2009b). In our study, EDDS and Cu showed a high affinity to each other, although the species of EDDS and Cu varied with the relative distance from rhizosphere (Fig. 4-7). In rhizosphere and non-rhizosphere (0-1 cm) (EDDS/Cu >1), EDDS was primarily present as CuEDDS (85.8-93.7%), with a minor proportion as ZnEDDS (5.3-5.8%), free EDDS (0-4.0%), and other metal-EDDS complexes (*e.g.* CaEDDS and FeEDDS, 0-4.4%). Cu was completely chelated with EDDS in rhizosphere and non-rhizosphere (0-1 cm). In non-rhizosphere (1-6 cm, EDDS/Cu < 1), EDDS was almost entirely complexed with Cu. For Cu, a part of Cu (6-16%) was associated with DOM besides CuEDDS, consistent with previous study at low concentration of EDDS (Tandy et al., 2006a).

Our results indicate that the competition from mineral cations (*e.g.* Fe, Ca, and Al) for EDDS poses little influence on Cu extraction in this study. This is because that CuEDDS is a strong complex with a large ionic potential and compact quinquedentate structure, and it is difficult to dissociate or exchanged by other metals (Tsang et al., 2009). Our results showed that Fe was primarily present as Fe-DOM and $Fe(OH)_3$ colloidal, although the concentration of dissolved Fe was high accounting for 20% of total EDDS (molar ratio) in soil extracts (Fig. 4-8) (Yip et al., 2009). In addition, Ca was mostly present as free ions or metal complex with DOM, and Al was mostly present as $Al(OH)_3$ colloidal in all compartments (Fig. 4-8).

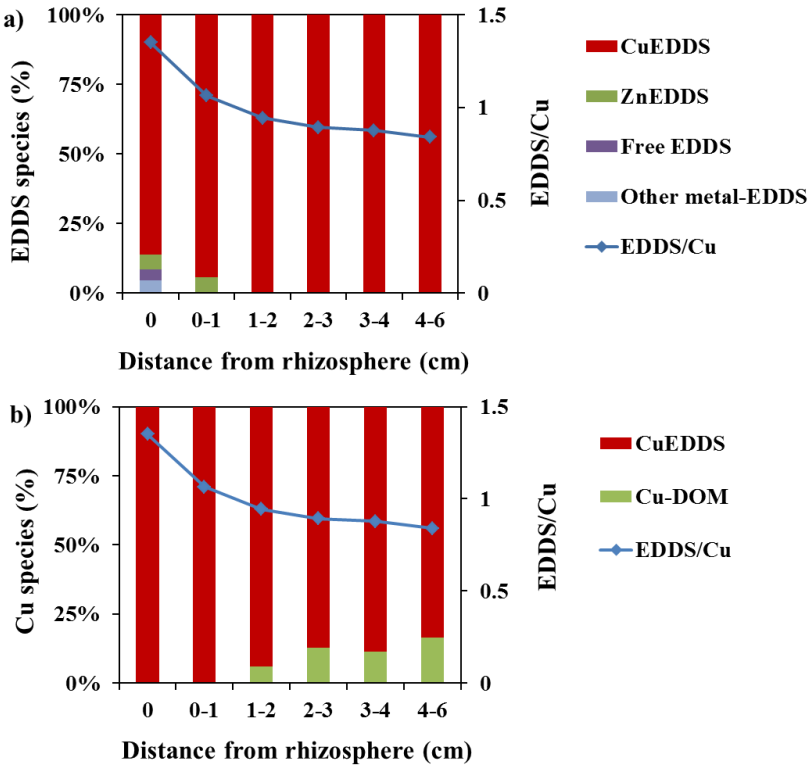

Figure 4-7 Calculated speciation of EDDS (a) and Cu (b) in soil extracts using Visual MINTEQ from different soil compartments and the trend (solid line) of the molar ratio of EDDS/Cu in extracts.

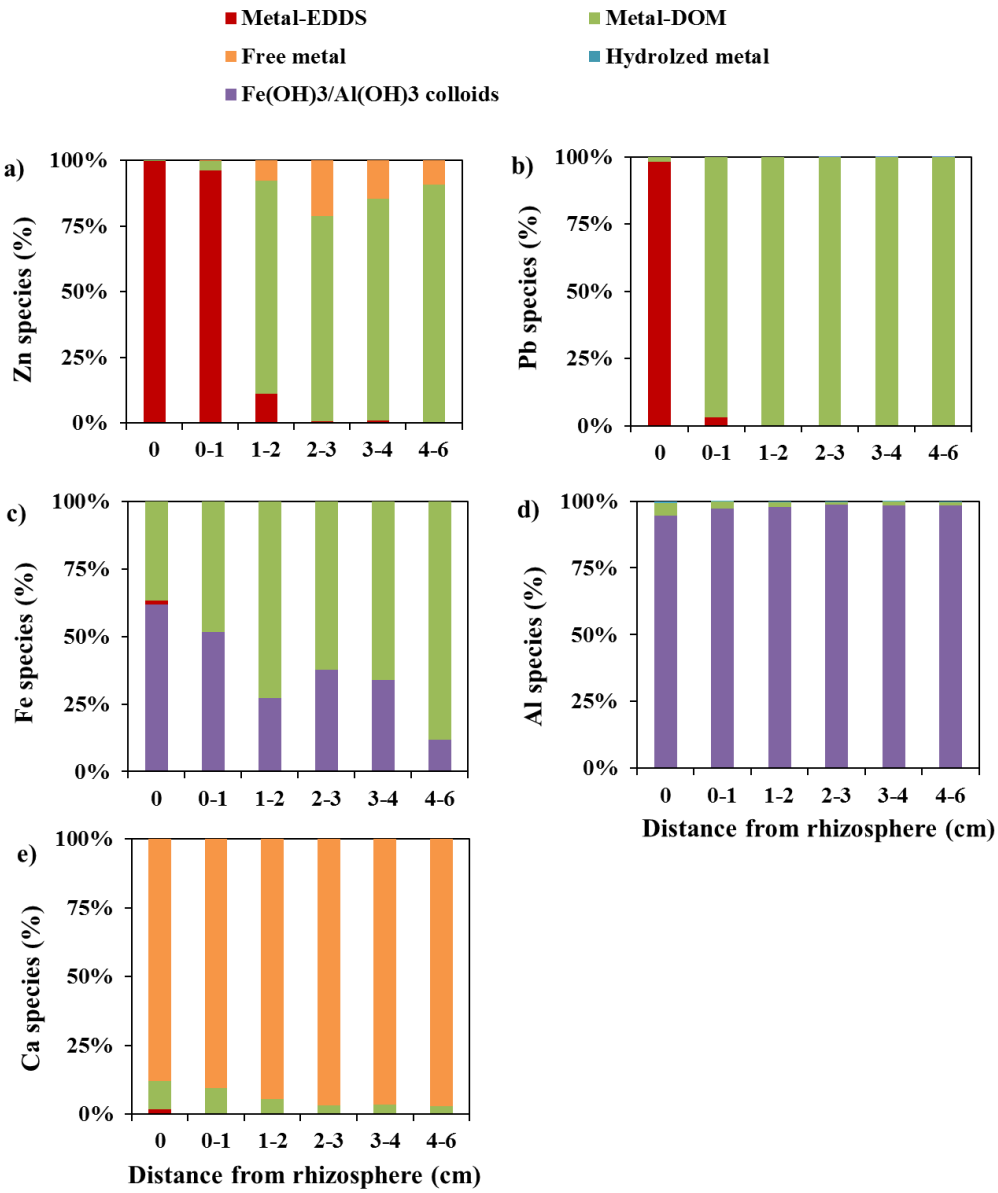

Figure 4-8 Calculated speciation of metals in soil extracts using Visual MINTEQ from different soil compartments of rhizobox.

4.3.5 Copper distribution and speciation in soils by μ-XRF, μ-XANES, and bulk-XANES

The distribution and speciation of metals in soil phase play a vital role the efficacy of EDDS for metal extraction. As mentioned from previous studies, weakly-bound metals (*e.g.* exchangeable and carbonate fractions) are the first to be released, following with the strongly-bound metals (*e.g.* Fe/Mn oxides and organic matter bound fractions), and residual (silicate bound) metals are not extractable by chelants (Guo et al., 2010; Fabbricino et al., 2016). Although SEP has been widely used in soil analysis of metal fractionations, the result suffers from many apparent limitations. The metal fractionation is operationally defined by chemical extraction, and the accuracy is doubted due to non-specific dissolution by extraction agents, incomplete dissolution of target phase, and resorption of solubilized metals (Calmano et al., 2001; Kirpichtchikova et al., 2006). Instead, the non-destructive synchrotron based techniques, such as μ-XRF, μ-XANES, and bulk-XANES, can provide more reliably information on the distribution and speciation of metals, because of their high sensitivity and selectivity to target elements (Majumdar et al., 2012; Cui et al., 2018).

4.3.5.1 Copper distribution in soils by μ-XRF

The μ-XRF analysis of the rhizosphere soil with or without EDDS (Fig. 4-9 and 4-10) showed the distribution pattern of Cu and other elements, which helps to identify the hosting phases of Cu in soils. The results showed that Cu was heterogeneously distributed in soil matrix with the occurrence of hotspots in scanned area.

In rhizosphere soil without EDDS, the position of Cu hotspot overlapped with Fe hotspot, and the correlation analysis revealed that the intensity of Cu and Fe was highly correlated ($R = 0.795$, $P < 0.01$) (Fig. 4-9A). Fe K-edge XANES pointed out

that Fe was present as crystalline goethite (67%) and amorphous ferrihydrite (33%) (Fig. 4-11 and Table 4-3), and iron hydroxides have been known to be important sink for Cu (Peacock and Sherman, 2004; Yang et al., 2014).

Cu also showed a moderate correlation with Si (R = 0.423, $P < 0.01$) and Al (R = 0.355, $P < 0.01$), although the X-ray fluorescence intensities of Si and Al were low (Fig. 4-9A). Si and Al were mainly derived from soil aluminosilicates including quartz, feldspars, and clay minerals. Actually, iron oxides may often precipitate on surface of clay minerals (Goldberg, 1989), which was supported by our SEM-EDX analysis on original soils (Fig. 3-3). Therefore, Cu adsorbed on iron hydroxides can be also spatially correlated with Si and Al from clay minerals. Moreover, clay minerals are also capable to directly sorb Cu (Martínez-Villegas and Martínez, 2008). Nevertheless, correlation coefficients of Cu with Si and Al were much lower than Fe, due to the presence of phases containing Si and Al with little association to Cu, such as quartz and feldspars. In spite of that, Cu hotspot was separated from K, Ca, and Mn hotspots (Fig. 4-10A), indicating that little Cu was associated with K-feldspars, carbonates, and Mn oxides (Yang et al., 2014).

The application of EDDS to rhizobox did not greatly affect the association relationships between Cu and other elements in rhizosphere soils (Fig. 4-9B and Fig. 4-10B). Correlation coefficients of Cu with Fe, Si, and Al were lower compared to that without EDDS treatment, which may be either resulted from the mobilization of Cu by EDDS from hotspots or the heterogeneity of soil samples (Kirpichtchikova et al., 2006).

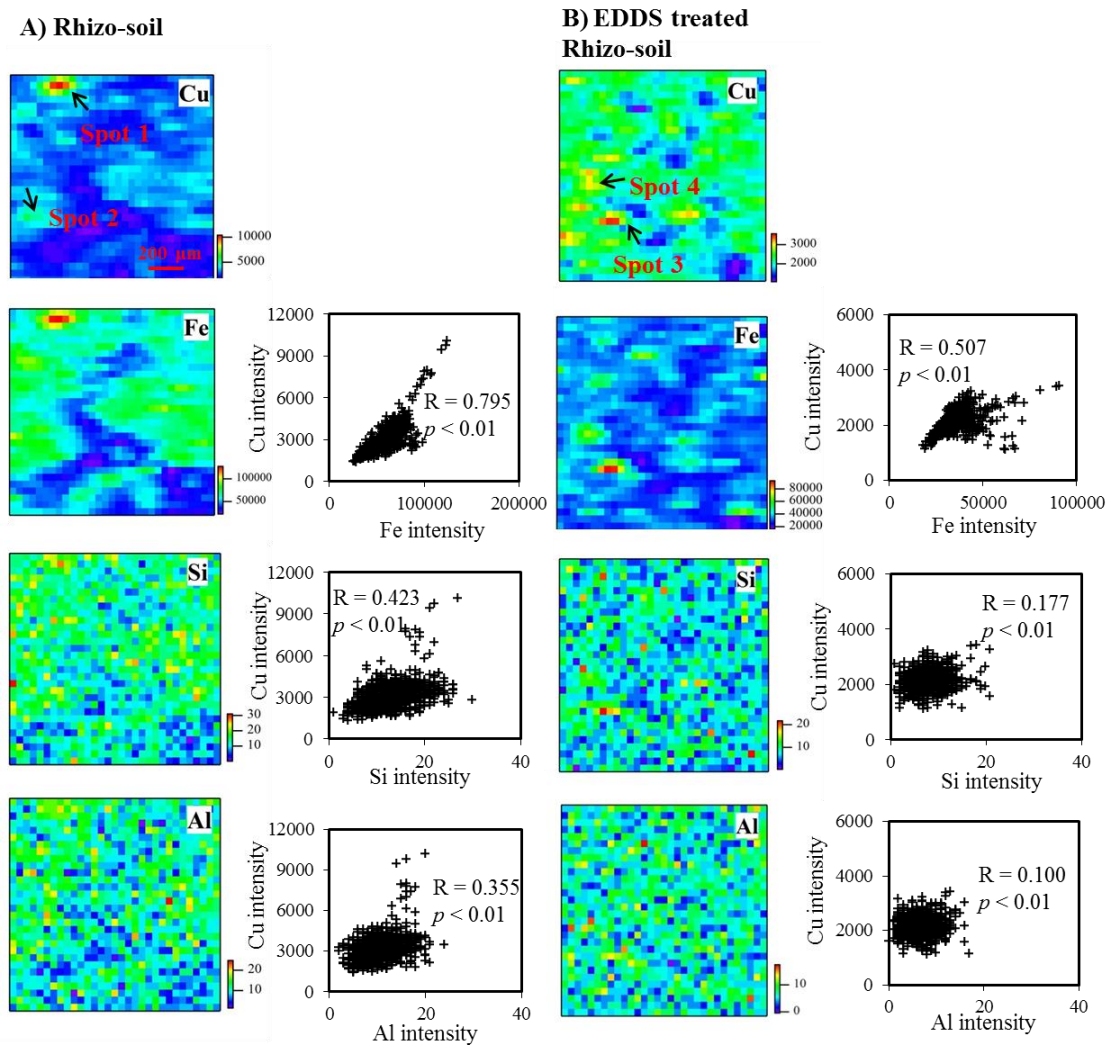

Figure 4-9 Elements (Cu, Fe, Si, and Al) distribution and correlation in an area of 1.5 mm × 1.5 mm from rhizosphere soil without (A) and with (B) treatment of EDDS by μ-XRF. Four hotspots marked with numbers were selected for μ-XANES analysis.

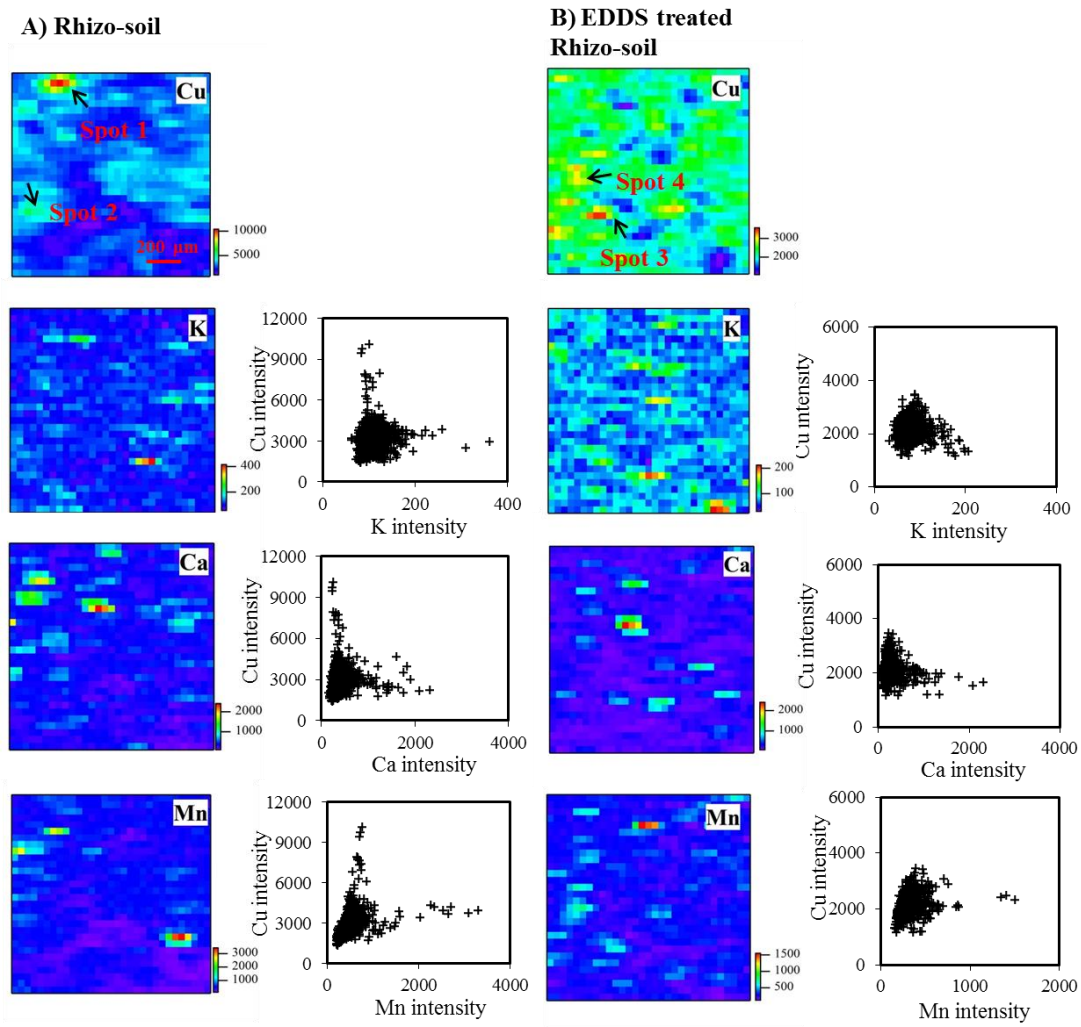

Figure 4-10 Elements (Cu, K, Ca, and Mn) distribution and correlation in an area of 1.5 mm × 1.5 mm from rhizosphere soil without (A) and with (B) treatment of EDDS by μ-XRF. Four hotspots marked with numbers were selected for μ-XANES analysis.

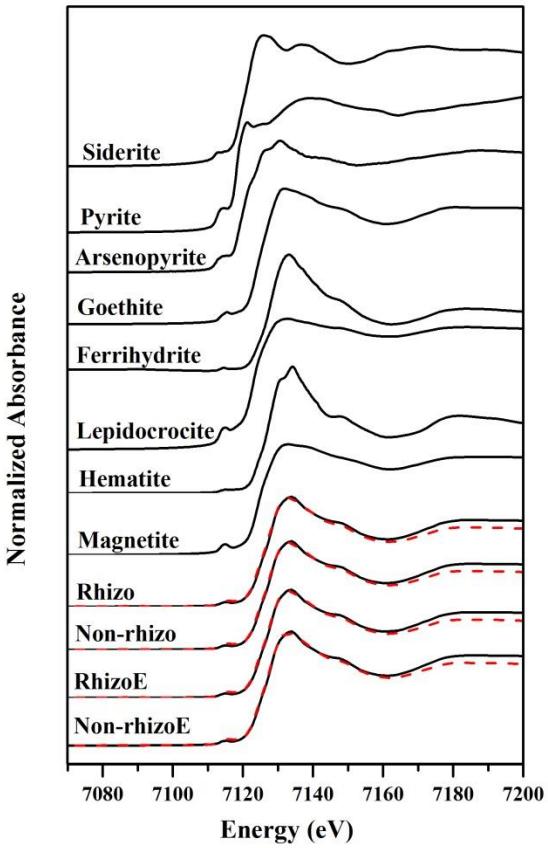

Figure 4-11 Fe K-edge spectra of standards and soil samples treated with or without EDDS.

Table 4-3 Best fit of Fe speciation by linear combination fitting (LCF) of Fe K-edge XANES spectra for rhizobox soil without and with EDDS treatment.

	Goethite (%)	Ferrihydrite (%)	R	Reduced Chi-square
Original soil				
Rhizosphere	65	35	0.0022	0.0001
Non-rhizosphere	67	33	0.0015	0.0001
EDDS treated soil				
Rhizosphere	67	33	0.0015	0.0001
Non-rhizosphere	67	33	0.0012	0.0001

4.3.5.2 Copper speciation in soils by μ-XANES and bulk-XANES

The XANES technique was employed to indicate the exact species of Cu in soils, via characterization the valance and bonding environment of Cu. In original planted rhizobox without EDDS treatment, the LCF analysis of Cu K-edge μ-XANES and bulk-XANES showed that Cu was primarily adsorbed on clay (49-65%), followed by goethite (35-51%) in both rhizosphere and non-rhizosphere soils (Fig. 4-12 and Table 4-4). The results were in consistence with the correlation analysis of Cu to Fe, Si, and Al from XRF (Fig. 4-9A). Clays can capture Cu to surface, interlayers or mineral lattices during weathering or pedogenesis process (Tenginkai et al., 1991; Minkina et al., 2016). Moreover, goethite are effective soil components to bind with Cu by forming innersphere complex (Grossl et al., 1994; Shimizu et al., 2011). The importance of SOM on Cu complexation was often revealed in organic soils (Jacobson et al., 2007; Strawn and Baker, 2008; Strawn and Baker, 2009). However, Cu standards representing Cu associated with SOM (*e.g.* Cu-humic acid and Cu acetate) (Fig. 4-13) contributed little to Cu species in this soil, which was primarily due to the low content of soil organic matters from mining sites (Table 3-1) (Yang et al., 2014). In addition, Cu standards representing readily labile fraction for extraction (*e.g.* $CuSO_4$, $Cu(OH)_2$, and $CuCO_3$) were not present in this soil, which was in agreement with the low percentage (4.8%) of Cu in exchangeable and carbonate fractions by SEP (Cui et al., 2017). Therefore, it can be predicted that Cu extraction by EDDS should be difficult in this long-term contaminated soils from mining sites, where Cu was strongly bound to goethite and clay minerals.

In EDDS-treated rhizobox, μ-XANES and bulk-XANES indicated the formation of CuEDDS (22-38%), the reduction of Cu-goethite, and the unchanged percentage Cu-clay species in the rhizosphere soil (Fig. 4-12 and Table 4-4). However, the

non-rhizosphere bulk-XANES showed no significant change after EDDS addition (Fig. 4-12 and Table 4-4). It can be interpreted by the transport of CuEDDS from non-rhizosphere to rhizosphere, as well as the limited extraction of Cu from non-rhizosphere soils by low concentration of EDDS. The XANES analysis normally cannot detect the Cu species lower than 10%.

The results indicated that EDDS-extracted Cu from rhizosphere was primarily from Cu-goethite instead of Cu-clay. Similarly, SEP analysis also showed that the reduction of Cu associated with Fe/Mn oxides accounted for 73% of the EDDS-mobilized Cu from rhizosphere soils (Fig. 4-4). There are two mechanisms potentially responsible for Cu extraction by EDDS. Firstly, EDDS can directly complex with readily available Cu from soils according to thermodynamic favorability (Yip et al., 2009a). Secondly, strongly-bound metals tend to be released during the disintegration of soil structures that caused by EDDS-promoted dissolution of soil minerals and organic matters (Tsang et al., 2007; Zhang et al., 2010). In this field contaminated soils with little readily available Cu, the second pathway should be more important for the mobilization of Cu that strongly bound to Fe oxides and clay minerals (Fig. 4-12 and Table 4-4). In view of the limited dissolution of clay minerals, as supported by the little soluble Al in soil extracts (Fig. 4-6b), EDDS was not able to extract the proportion of Cu adsorbed on clays. Instead, EDDS was expected to mobilize the proportion of Cu bound with goethite, as the EDDS-promoted dissolution of Fe was substantial (Fig. 4-6a). On the one hand, the dissolution may disintegrate the structure of goethite (Yip et al., 2010a), resulting in the exposure of internal Cu to solution EDDS for direct complexation. Similarly, surfactant TX100 was found to assist dispersion of clayed sediments, and thus to expose Zn for EDTA extraction (Yuan et al., 2010). On the other hand, the newly formed FeEDDS during Fe dissolution is able

to further extract Cu from soil surface and transformed to CuEDDS through metal exchange. The Fe displacement from FeEDDS by Cu was corroborated by the previous observation of substantial formed CuEDDS after application of FeEDDS to soils for 1 d (Ylivainio, 2010). The displaced Fe may be resorbed to soils, or form Fe-DOM and $Fe(OH)_3$ colloids in alkaline soil solutions (Fig. 4-8c). Conclusively, our results suggested that EDDS-promoted dissolution of iron oxides in this soil should facilitate the extraction of Cu in this soil.

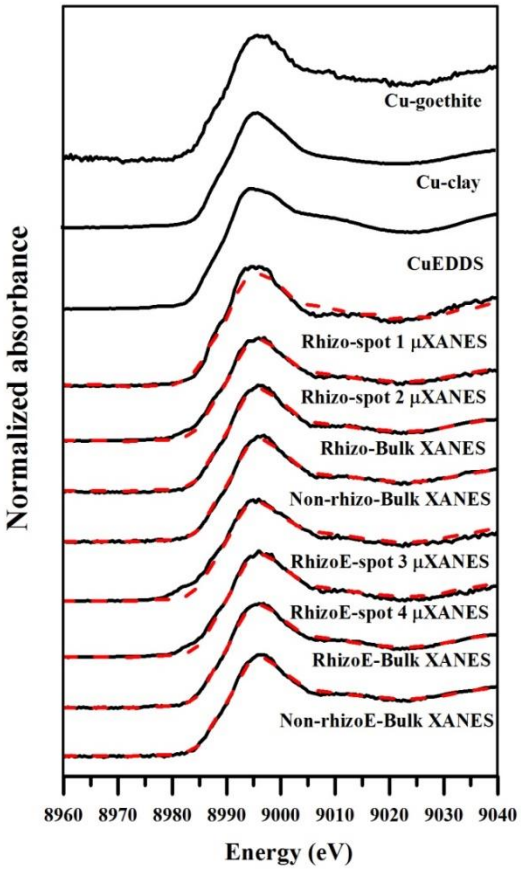

Figure 4-12 Cu K-edge XANES spectra of standards and rhizobox soil samples with or without EDDS treatment, with red dashed lines as the linear combination fitting results. The LCF fitting results are reported in Table 4-4. "Rhizo" refers to rhizosphere soil, "Non-rhizo" refers to non-rhizosphere soil, "RhizoE" refers to rhizosphere soil treated by EDDS, and "Non-rhizoE" refers to non-rhizosphere soil treated by EDDS.

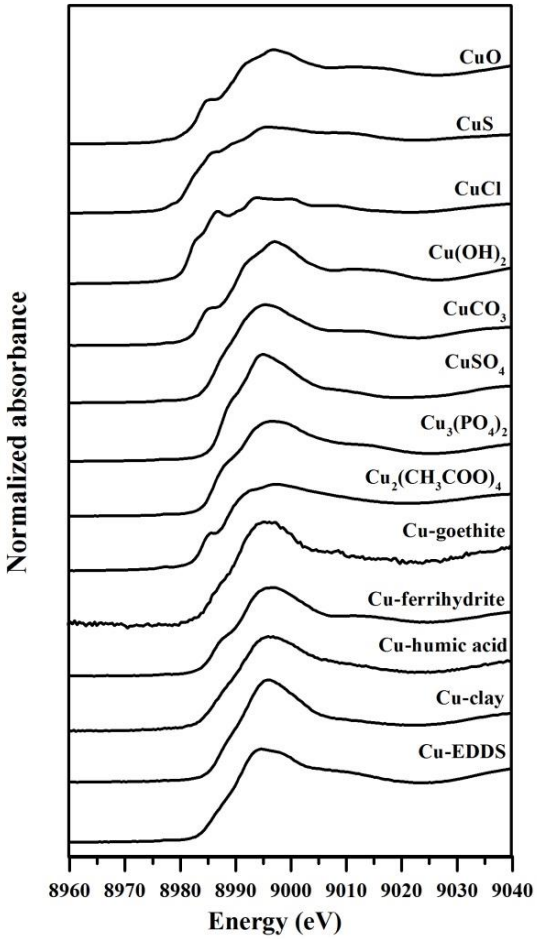

Figure 4-13 Cu K-edge spectra of all standards.

Table 4-4 Copper species (%) in the original and EDDS treated soil from rhizosphere and non-rhizosphere soil using μ-XANES and bulk-XANES analysis by linear combination fit.

Samples	Cu-Goethite	Cu-Clay	CuEDDS	R-factor	Reduced chi-square
Original soil					
Spot 1 Rhizo	35	65		0.0117	0.0024
Spot 2 Rhizo	51	49		0.0059	0.0011
Bulk Rhizo	42	58		0.0039	0.0008
Bulk Non-rhizo	41	59		0.0025	0.0005
EDDS treated soil					
Spot 3 Rhizo		62	38	0.0256	0.0050
Spot 4 Rhizo	14	54	31	0.0057	0.0012
Bulk Rhizo	25	52	22	0.0018	0.0004
Bulk Non-rhizo	48	52		0.0060	0.0012

4.3.6 Implication for chelant-assisted phytoextraction

A plant root is typically able to absorb weakly-bound but not strongly-bound Cu (Wenzel and Jockwer, 1999). In long-term field contaminated soils, it is difficult for plants to extract Cu, since the majority of Cu binds tightly with soil oxides, organic matters, or clay minerals. EDDS can form soluble complex with the strongly-bound metals and release more accessible Cu to roots, of which the process is facilitated by EDDS-promoted dissolution of minerals or organic matters. Soil dissolution may release nutrients (*e.g.* Fe) to benefit plants and soil microbes. However, soil dissolution may partially destruct soil structure and destabilize soil aggregates (Tsang et al., 2007). More attention should be paid on the physical and hydraulic properties of soils after repeated application of EDDS in phytoextraction practice.

Our results suggested that the phytoextraction efficiency is not limited by the soluble Cu in EDDS-treated soils, but by the absorption capacity of plant roots to CuEDDS in rhizosphere soil solution. EDDS is effective to supply soluble Cu (as CuEDDS) to plant roots, through extracting Cu from rhizosphere soils and facilitating Cu transport from non-rhizosphere to rhizosphere. However, the amount of Cu accumulated by ryegrass only accounted for 0.5% of total soluble Cu (as CuEDDS) in rhizosphere. In light of this, further improvement methods, such as using plant growth regulators (*e.g.* plant hormones) or plant growth promoting bacteria (PGPB), can be employed to assist plant growth, enhance the plant transpiration rate, and thus to increase plant absorption of CuEDDS via the plant-transpiration induced water flow. In addition, more studies are required on the uptake mechanisms of CuEDDS by plant roots.

4.4 Summary

This study explored the interaction characteristics and mechanisms of EDDS with Cu-polluted soils in rhizosphere of ryegrass using a multi-interlayer rhizobox. The main results are listed below:

1) After evenly surface application of EDDS to planted rhizobox, EDDS was found transported from non-rhizosphere to rhizosphere after 7 d, probably via plant transpiration-induced water flow.

2) Transported EDDS increased the solublization of soil Cu in rhizosphere compared to non-rhizosphere, and facilitated Cu transportation from non-rhizosphere to rhizosphere.

3) EDDS also caused the dissolution of Fe, Al, and soil organic matters. Cu and EDDS were both mainly present as CuEDDS complex in rhizosphere and non-rhizosphere soil extracts, and mineral cations Fe, Al, and Ca did not compete with Cu for complexation with EDDS.

4) EDDS primarily extracted Cu that adsorbed on goethite instead of clay minerals from rhizosphere soils, probably due to EDDS-promoted dissolution of iron oxides. This study provides an in-depth insight into the interactions of EDDS with soil Cu in rhizosphere.

Taken together, the chemical interactions between EDDS and soil Cu in rhizosphere has been clarified. Nevertheless, the success of phytoextraction is not only determined by the high efficiency of metal extraction from soils but also the maintenance of soil quality during remediation. Therefore, the impacts of EDDS on rhizosphere soil microbes and nutrients still need further analysis, which can be related to the spatial

distribution of EDDS around plant roots.

5. Chapter Five - The Effect of EDDS on Soil Nutrients and Microbes in Rhizosphere Soil

5.1 Introduction

Phytoremediation is often preferred by the public due to the less negative impacts on soil quality in comparison with some destructive strategies (*e.g.* soil washing or soil excavation) (Gupta et al., 1996). The soil qualities and fertility should be maintained during or after soil remediation, in order to support plant growth continuously (Epelde et al., 2008). The soil nutrients and microbial indexes are sensitive indicators to soil qualities, which should be paid great attention.

The impact of chelant on the indigenous soil microbes has been investigated during phytoextraction; however, there are still controversies in different study. The application of EDDS is effective to solubilize heavy metals from contaminated soil, and the enhanced metals may stress the soil microbial community. EDDS shows inhibitory effects on soil enzyme activities (*e.g.* dehydrogenase and arylsulphatase), soil basal respiratory and microbial biomass C (Epelde et al., 2008; Mühlbachová, 2011). Similarly, Mühlbachová (2011) reported that EDDS decreased the microbial biomass C. However, EDDS is readily degradable in soil and the decomposition of EDDS may produce available carbon and nitrogen sources to soil microbes. EDDS exhibited beneficial effects on the metabolic activities of soil microbes for substrate utilization (Venecio Jr et al., 2005; Epelde et al., 2008). In addition, the composition

of soil microbes is not affected by EDDS by analysis of phospholipid fatty acids (PLFAs) and PCR-denaturing gradient (PCR-DGGE) (Yang et al., 2013).

Rhizosphere is a specific region that adjacent to plant roots with different physical, chemical and biological characteristics compared to the non-rhizosphere soil (Bowen and Rovira, 1999). As reported in Chapter Four, plant transpiration resulted in the transport of EDDS from non-rhizosphere to rhizosphere. However, it is not known whether and how this process affects the soil microbes in rhizosphere and non-rhizosphere.

The objectives of this study were to 1) characterize the soil nutrients including soluble cations, dissolve organic carbon, dissolved nitrogen, mineralized nitrogen (NH_4^+ and NO_3^-), and available phosphorus in rhizobox; 2) monitor the microbial indexes including microbial biomass C and N and several enzyme actives with or without the application of EDDS in rhizobox; 3) investigate the toxicity of soil extracts from rhizosphere and non-rhizosphere with microtoxicity assay.

5.2 Materials and methods

All samples to be analyzed were obtained directly from the experiment (Section 4.2) conducted in Chapter Four using a rhizobox (Fig. 4-1).

5.2.1 Chemical properties of soil

Fresh soil from rhizobox (Fig. 4-1) was extracted with deionized water in a 1:10 (w: v)

ratio on a dry weight soil basis and shake on end over end shaker at 50 rpm for 3 h. The suspension was centrifuged for 10 min at 8000 rpm, and filtered through 0.45 μm cellulosic membranes. Soil extracts were digested with conc. HNO_3 and analyzed on K, Ca, Na Mg, Fe, and Mn using inductively coupled plasma atomic emission spectrometry (ICP-AES).

Soil ammonia and nitrate were extracted by 2 M KCl with fresh soil from rhizobox at 1:10 ratio for 30 min, and then filtered before analysis by a portable colorimeter (DR890, Hach) (Cater and Gregorich, 2006). The concentration of ammonia in filtrates was determined based on indophenol formation with sodium salicylate, and nitrate concentration was determined based on cadmium reduction. Soil available P was analyzed according to Margesin and Schinner (2005). Weight of 2 g air-dried soil from rhizobox was mixed with 40 mL 0.5 M $NaHCO_3$, and shaked exactly for 30 min at 20 ^oC before filtration. The concentration P in the filtrates was measured by the absorbance at 880 nm after reaction with mixed regents containing H_2SO_4, ammonium molybdate solution, ascorbic acid solution, and antimony potassium tartrate solution.

5.2.2 Microbial biomass C and N

In the rhizobox experiment, soil microbial biomass carbon and nitrogen were measured by chloroform fumigation extraction method (Vance et al., 1987). Each moist soil sample was divided into two subsamples of 5 g. One subsample was

fumigated with ethanol-free chloroform for 24 h at room temperature. The fumigated and non-fumigated soil was extracted with 20 mL 0.5 M K_2SO_4, shaken for 30 min and filtered. Biomass C (BC) was calculated from BC＝EC/0.45 where EC ＝ (C extracted from fumigated soil) - (C extracted from non-fumigated soil), and biomass N ＝ EN/0.45 where EN ＝ (N extracted from fumigated soil) - (N extracted from non-fumigated soil). The dissolved organic carbon (DOC) and nitrogen (DN) in the extracts were determined by an automated TOC/TN Analyzer (Shimazu). The K_2SO_4 extractable C and N in non-fumigated soil was considered to be soil DOC and DN (Jones and Willett, 2006).

5.2.3 Soil enzymes analysis

Soil urease activities were determined according to Guan (1986). 1 mL toluene was added to 5 g fresh soil. After 15 min, soil was mixed with 10 mL 10% urea and 20 mL citric acid buffer (pH 6.7) and incubated at 37 ^{o}C for 24 h. After incubation, the soil mixture was filtrated through Whatman No. 42. 3 mL of filtrate was mixed with 4 mL 1.35 mol/L sodium phenate and 3 mL 0.9% sodium hypochlorite. After 20 min, the absorbance value of the samples was read at 578 nm. Urease activities were expressed as mg NH_4–N released kg^{-1} dry soil.

The determination of saccharase in the soil was based on glucose formation after incubation with sucrose as substrate (Guan 1986). Five grams of fresh soils were placed in a 50 mL conical flask with addition of 15 mL 8% sucrose, 5 mL phosphate buffer (pH 5.5) and 5 drops of toluene. The mixture was incubated at 37 ^{o}C for 24 h

and then filtered. One mL of filtrate was reacted with 3 mL DNS regent for 5 min in boiling water bath. After cooling for 3 min using tap water, the obtained solutions was measured at 508 nm.

Acid phosphatase was determined according the method from Eivazi and Tabatabai (1977) and Dick (2011). One gram fresh soil was mixed with 0.2 ml toluene, 4 ml modified universal buffer (MUB) solution (pH 6.5) and 1 mL 0.05 M p-nitrophenyl phosphate solution and incubated 37 ^{o}C for 1 h. One ml of 0.5 M $CaCl_2$ and 4 ml of 0.5 M NaOH were then added to the solutions, mixed thoroughly, and filtered through Whatman No. 42. The filtrate was measured at 400 nm to determine the released p-nitrophenol.

For each soil sample, one control was used with substrate addition after incubation. This control value was subtracted from the sample value. In addition, one control with no substrate and no sample was incorporated in a batch experiment to subtract the contribution from regents.

5.2.4 Microtoxicity

To assess the acute microtoxicity of soil extracts, Microtox® (AZUR Environmental Microtox® Model 500) was employed. 1 g freeze dried soil was extracted by 10 mL deionized water, filtered and adjusted to pH 6-8 using 0.1 N NaOH and HNO_3. The 81.9% Basic Test recommended by instrument manual was used to determine the toxicity of soil extracts. The NaCl solution (2%) was used as a control, and the salinity of the soil extracts was adjusted to 2% with adjustment solution. Soil extracts

were diluted with 2% NaCl solution to a series of nine concentrations, and the maximum exposure concentrations of samples were 81.9%. The marine bacterium (Vibrio fisheri) were reconstructed and mixed with soil extracts at 15 ^{o}C. Light emission after 30 min of exposure was recorded using a photometer designed to the Microtox®test (Microtox® Model 500, Azur Environmental). The inhibitory effect of soil extracts was calculated based on the reduction in bioluminescence of a marine bacterium (Vibrio fisheri) after exposure to soil extracts in comparison with the control. Each soil extracts were replicated twice for toxicity assay.

5.2.5 Statistical analysis

Statistical analysis of the experimental data was performed using SPSS software (ver. 22.0, SPSS, Inc.). Comparison of mean values was made through a one-way ANOVA using Duncan's multiple range test at $P < 0.05$.

5.3 Results and discussion

5.3.1 Soluble nutrient cations

The concentration of nutrient metals in soil extracts from different compartment of rhizobox is showed in Fig. 5-1. Before application of EDDS, the growth of ryegrass in rhizosphere compartment consumes a large quantity of K, which decreased the concentration of K by 38% compared to non-rhizosphere (4-6 cm). In addition, plant growth induced mass flow which slightly increased the concentration of Ca and Mg in

rhizosphere compared to non-rhizosphere (4-6 cm) by 25% and 17%, respectively. Plant cultivation did not affect the amount of Na, Fe, and Mn in soil extracts. After application of EDDS, the concentration of these nutrient metals was affected differently. EDDS enhanced the concentration of soluble Fe the most effectively with the increment of 3.47-60.2 folds, and the concentration of soluble Fe increased with the decreasing distance to rhizosphere. Because EDDS can bind Fe from iron oxides in soil matrix, the higher concentration of EDDS present in rhizosphere (Fig. 4-2) would solubilize more Fe than non-rhizosphere. EDDS enhanced the concentration of soluble Mn by 2.2-219 folds, and soluble Mn also increased with decreasing distance to rhizosphere. Soluble K was almost not affected by EDDS, because EDDS cannot complex with monovalent metal from soil matrix. The concentration of Na in soil extracts was largely enhanced by 26.3-53.7 folds, due to the input of EDDS to soil as the form of Na_3EDDS. The concentration of Ca and Mg in rhizosphere increased slightly by 28% and 157%, respectively, but decreased in non-rhizosphere by about 30% and 22% after the dosage of EDDS in rhizobox. Generally, EDDS leaded to the increase of soluble nutrient metals, especially Fe, in rhizosphere soil, which may be beneficial for soil microbial community. Compared to non-rhizosphere, the higher concentration of metal cations in rhizosphere can be solubilized by the higher concentration of EDDS (Fig. 4-2).

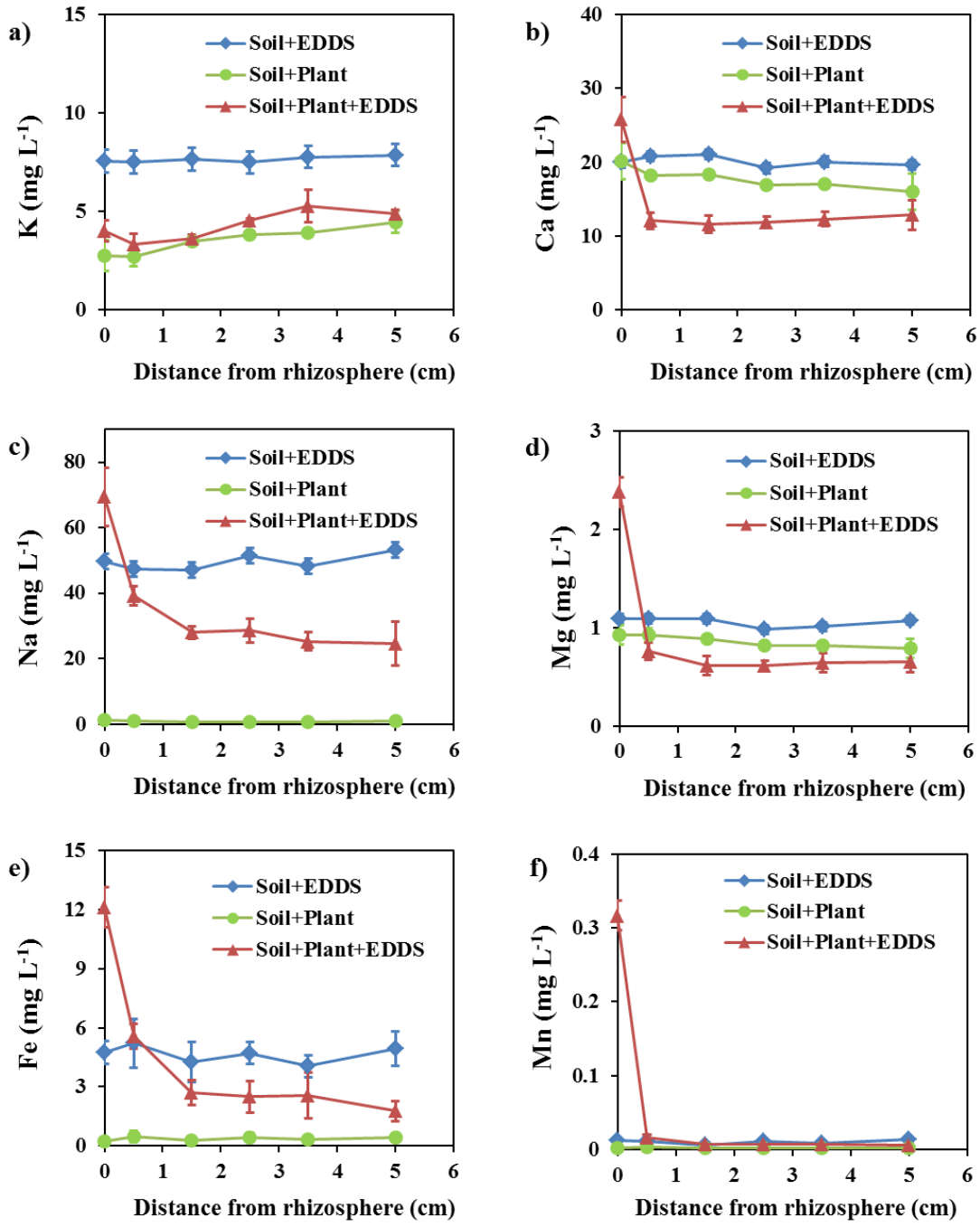

Figure 5-1 The concentration of nutrient metals in soil extracts from different compartment of rhizobox.

5.3.2 Soil DOC, DN, ammonium, nitrate, and available P

Plant growth in soil is considered to enhance soil DOC and DN, as root exudates

release diverse carbon and nitrogen sources including organic acids, amino acids, sugars and phenolic (Dakora and Phillips, 2002; Kim et al., 2010). In this study, ryegrass in rhizosphere did not affect soil DOC and DN (Table 5-1) ($P < 0.05$), due to the short cultivation period of plants. The concentration of soil DOC and DN increased sharply after the treatment with EDDS by 4.62-16.1 and 5.36-20.7 folds, respectively (Table 5-1). EDDS contains soluble carbon and nitrogen, which can be involved in the enhanced soil DOC and DN. In addition, as mentioned in Chapter Four, EDDS destructed soil structure by solubilizing Fe minerals which may lead to the mobilization of soil organic matters that containing organic carbon and nitrogen into soluble fraction. Kim et al. (2010) also showed that soil DOC increased greatly in EDTA- or EDDS- treated soil. Meers et al. (2008) found that soil DOC decreased gradually with the degradation of EDDS in soil, and the half-life of EDDS varied from 3.4-7.9 d in different soil after a lag phase lasting for 10-32 d.

Available nutrients including NH_4^+, NO_3^-, and P in soil are shown in Table 5-2. The growth of ryegrass alone did not affect soil NH_4^+ and NO_3^-, but reduced available P in rhizosphere compared to non-rhizosphere (1-6 cm). Application of EDDS substantially increased soil NH_4^+ and NO_3^-, and the concentration of soil NH_4^+ increased with the decreasing distance to rhizosphere compartment. Actually, soil NH_4^+ in rhizosphere was increased by 1.3 folds after application of EDDS. However, soil available P was not affected ($P < 0.05$) after application of EDDS. In consistence to our results, Yang et al. (2013) showed that the same dosage of EDDS as our study increased soil NH_4^+ grown with corn and bean by 1.4 and 1.0 folds, respectively.

Moreover, the concentration of soil NH_4^+ increased with the increasing dosage of EDDS from 3 to 5 mM kg^{-1}. Therefore, compared to non-rhizosphere, the higher concentration of NH_4^+ in rhizosphere can be due to the presence of higher concentration of EDDS (Fig. 4-2). Moreover, Fang et al., (2017) recorded an increase of NO_3^- in soil solution by 23.5 factors compared to control after 2 d of application EDDS. Collectively, the increase of soil NH_4^+ and NO_3^- after use of EDDS can be due to the microbial degradation of EDDS. It was known that one carbon-nitrogen lyase can catalyze the degradation of EDDS through breaking down the C-N cleavage between ethylenediamine and succinyl part of the molecule (Witschel and Egli, 1997). One of the formed product N-(2-aminoethyl) aspartate can be mineralized by soil microbes to release NH_4^+ and NO_3^-. However, the nitrogen cycle in soils is quite complex and varied in different soils, which controls the nitrification or denitrification processes and thus influence the ratio of NH_4^+ and NO_3^- in soils (Haynes, 2012). Taken together, the increase of soil NH_4^+ and NO_3^- in our study suggested that EDDS was a readily available nitrogen sources for soil microbes. Moreover, the degradation of EDDS may enhance the soil fertility, and be beneficial for soil microbes and plant growth (Fang et al., 2017).

Table 5-1 The amount of soil DOC and DN from rhizobox.

	Rhizo	Non-rhizo (0-1 cm)	Non-rhizo (1-2 cm)	Non-rhizo (2-3 cm)	Non-rhizo (3-4 cm)	Non-rhizo (4-6 cm)
DOC (mg kg^{-1})						
Soil+EDDS	922 ±133 cd	859 ±78cd	985 ±55 d	1055 ±93 d	1134 ±90 d	1053 ±103 d
Soil+Plant	110 ±17 a	94 ±16 a	92 ±24 a	102 ±17 a	94 ±15 a	104 ±20 a
Soil+Plant+EDDS	1772 ±219 e	754 ±101 c	510 ±73 b	473 ±55 b	484 ±44 b	480 ±15 b
DN (mg kg^{-1})						
Soil+EDDS	141 ±10 d	146 ±5 d	156 ±24 d	150 ±12 d	166 ±23 d	163 ±15 d
Soil+Plant	11 ±2 a	9 ±2 a	11 ±1 a	10 ±2 a	12 ±1 a	12 ±1 a
Soil+Plant+EDDS	217 ±1 e	107 ±7 c	74 ±11 b	73 ±10 b	75 ±7 b	67 ±8 b

Table 5-2 The concentration of ammonium, nitrate and available P in soil from rhizobox.

	Rhizo	Non-rhizo (0-1 cm)	Non-rhizo (1-2 cm)	Non-rhizo (2-3 cm)	Non-rhizo (3-4 cm)	Non-rhizo (4-6 cm)
NH_4^+ (mg kg^{-1})						
Soil+EDDS	20.5 ±2.1 d	18.6 ±5.8 cd	21.7 ±3.7 d	18.5 ±0.4 cd	18.5 ±0.2 bcd	17.6 ±1.1 b
Soil+Plant	9.8 ±1.6 a	9.6 ±1.5 a	9.8 ±1.4 a	9.3 ±1.0 a	10.5 ±1.6 a	9.7 ±1.9 a
Soil+Plant+EDDS	22.6 ±3.5 d	22.6 ±3.5 d	15.3 ±2.7 abc	13.5 ±1.2 abc	13.7 ±2.2 abc	11.5 ±4.6 ab
NO_3^- (mg kg^{-1})						
Soil+EDDS	71.6 ±3.4 bc	82.3 ±8.2 c	76.0 ±12.2 bc	77.8 ±1.9 bc	82.9 ±7.0 c	73.1 ±0.9 bc
Soil+Plant	47.7 ±4.2 a	49.5 ±0.1 a	42.7 ±1.6 a	44.0 ±1.8 a	48.5 ±1.4 a	48.3 ±3.6 a
Soil+Plant+EDDS	70.6 ±6.8 bc	74.3 ±4.0 bc	74.1 ±10.2 bc	70.5 ±7.6 bc	83.2 ±6.6 c	76.6 ±10.6 bc
Available P (mg kg^{-1})						
Soil+EDDS	18.6 ±0.40 f	18.6 ±0.37 f	17.6 ±0.74 def	18.3 ±0.39 def	18.5 ±0.23 ef	17.6 ±1.14 def
Soil+Plant	15.0 ±0.56 a	16.3 ±0.80 b	17.3 ±0.91 cd	17.5 ±0.14 de	17.5 ±0.22 de	17.9 ±0.31 def
Soil+Plant+EDDS	15.1 ±0.48 a	15.1 ±0.54 a	16.4 ±0.54 bc	17.3 ±0.33 cd	17.6 ±0.26 de	17.6 ±0.50 def

5.3.3 Microbial biomass C and N

Soil microbial biomass carbon and nitrogen are effective indexes that reflect the population of indigenous microbial community and the soil quality. In this study, the growth of ryegrass alone did not affect soil microbial biomass C and N ($P < 0.05$, Fig. 5-2). The application of EDDS remarkably increased the microbial biomass C and N in rhizosphere by 0.43 and 2.90 fold, respectively (Fig. 5-2). But EDDS did not affect the values of microbial biomass C and N in non-rhizosphere ($P < 0.05$, Fig. 5-2). The different impact of EDDS to microbial biomass C and N in rhizosphere and non-rhizosphere can be related to the distribution of EDDS and dissolved nutrients in rhizobox. In our study, EDDS was transported from non-rhizosphere to rhizosphere (Fig. 4-2), which resulted in more nutrient metals (Fe, Mn, Na, Ca, and Mg), DOC, DN and NH_4^+ (Fig. 5-1 and Table 5-1, 5-2) in rhizosphere. Therefore, the higher concentration of nutrients in rhizosphere after usage of EDDS may facilitate the growth of microbes, while the lower concentration of nutrients in non-rhizosphere can be not enough to promote microbial growth. In contrast to our results, Mühlbachová (2011) reported that EDDS (6.8 mM kg^{-1}) caused the decrease of microbial biomass C from 0 to 10 d after application. The toxicity of EDDS observed from that study can be correlated with the high concentration of Pb (1086-1138 mg kg^{-1}) from tested soils. EDDS can substantially increase the available Pb through solublization from soils and the available Pb is highly toxic to microbial growth. Conclusively, the impact of chelants on soil microbes may vary with different dosage, soil nutrients, and metal pollution level.

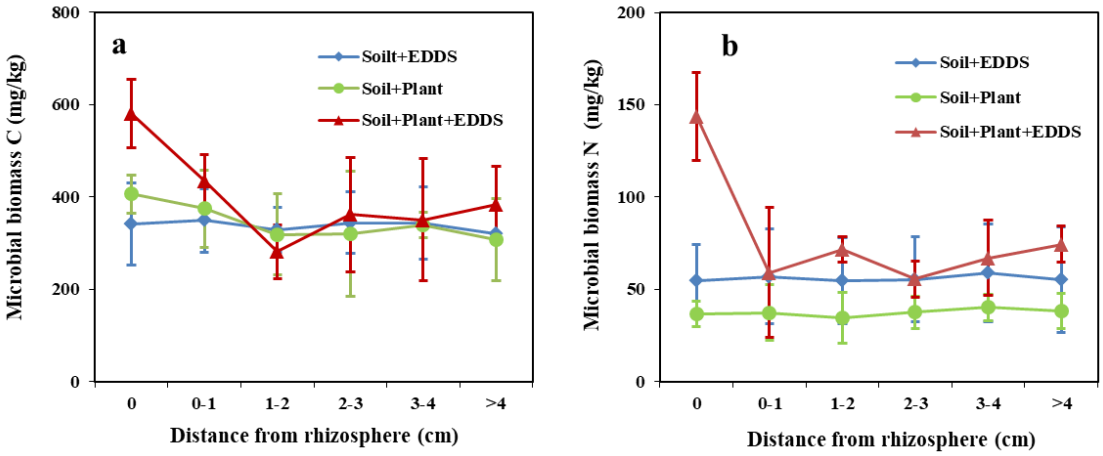

Figure 5-2 Soil microbial biomass carbon and nitrogen in different compartments from rhizobox.

5.3.4 Enzymes activity

Soil enzymes are produced by soil microbes which catalyze the soil transformation process to decompose organic matters and generate nutrients for both microbes and plants. Urease, phosphatase, and saccharase are enzymes that involved in N, P, and C cycling in soils. The growth of ryegrass increased the acid phosphatase, but did not affect the level of urease and saccharase ($P < 0.05$, Fig. 5-3). The application of EDDS resulted in the increase of urease in rhizosphere by 30%, but did not affect the value in non-rhizosphere. Similarly, Beiyuan et al. (2017) reported that urease activity in an e-waste soil enhanced after washing with EDDS (3.34 mM kg^{-1}). The different impact of EDDS to urease activities in rhizosphere and non-rhizosphere can be also interpreted by the distribution of nutrients. There was no difference on acid phosphatase in soil between the rhizobox treated by EDDS and that without amendment ($P < 0.05$), which is consistent with the results from previous study (Epelde et al., 2008; Yang et al., 2013). In addition, after the dosage of EDDS, the saccharase in rhizosphere was not affected. But in non-rhizosphere the saccharase increased with the distance of compartments from rhizosphere. Collectively, EDDS resulted in varied impact on different kind of soil enzymes in rhizosphere and non-rhizosphere.

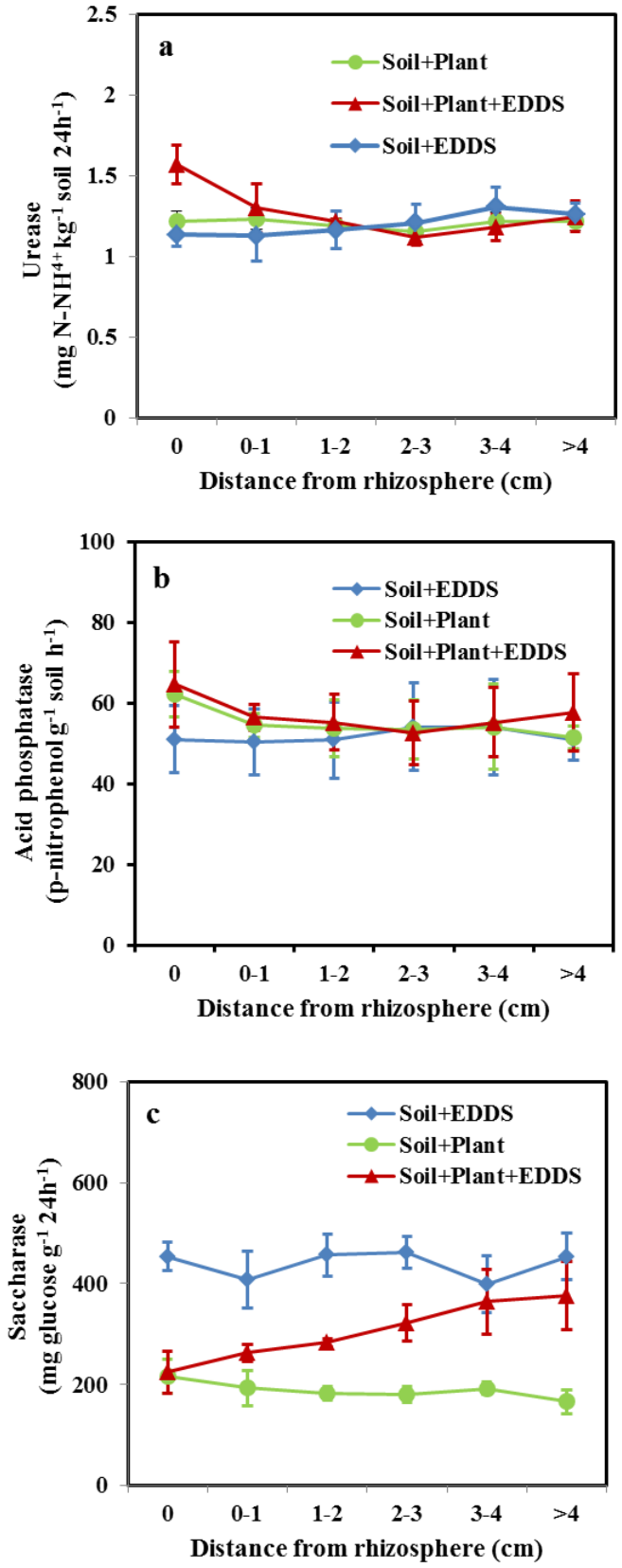

Figure 5-3 The variation of urease and acid phosphatase in soil from rhizobox.

5.3.5 Microtoxicity

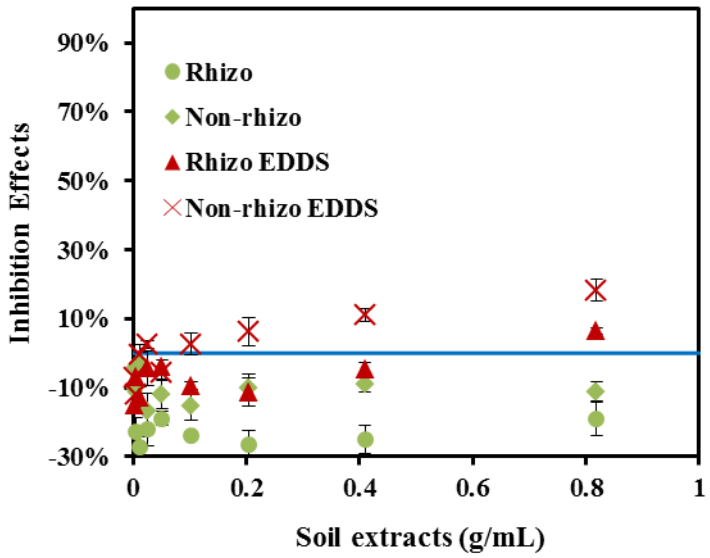

Figure 5-4 The inhibition effects of soil extracts to microbes from rhizosphere and non-rhizosphere with the application of EDDS by Microtoxicity analysis. The blue line indicates no inhibition effects.

The inhibition effect of soil extracts from rhizosphere and non-rhizosphere on the luminescent bacteria (*Vibrio fischer*) is shown in Fig. 5-4. Before the treatment of EDDS, original soil extracts either from rhizosphere or non-rhizosphere did not exhibit toxicity in microtoxicity assay. Similar results were also reported in some aged soils contaminated with heavy metals, in which bioaccessible heavy metals were not enough to induce toxicity to the luminescent bacteria (Smolders et al., 2004; Beiyuan et al., 2017). In addition, the low concentration of toxin may also result in hormesis, a stimulary to microbe growth (Stebbing, 1982). After the application of EDDS, soil extracts from rhizosphere and non-rhizosphere at 0.89 mg L^{-1} showed a slight inhibitory effect (6.5-18.3%) to the luminescent bacteria. The result can be caused by the increase of available Cu after usage of EDDS in soil extracts. The available Cu in soil extracts (1:10, solid-solution ratio) before treatment of EDDS was 0.09 μg L^{-1} in rhizosphere and 0.06 μg L^{-1} in non-rhizosphere. After the treatment with EDDS, the

available Cu increased to 0.06 mg L^{-1} in rhizosphere and 0.02 mg L^{-1} in non-rhizosphere. Although the rhizosphere soil extracts contained two fold higher concentration of Cu than non-rhizosphere, rhizosphere soil extracts showed lower toxicity to the bacteria than non-rhizosphere. The less toxicity of rhizosphere soil extracts treated by EDDS can be related to the higher concentration of soluble nutrients (Fig. 5-1 and Table 5-2) in rhizosphere compared to non-rhizosphere. On the one hand, nutrient cations of Ca, Mg, Na, Fe, and Mn may alleviate Cu toxicity through competition with Cu for the absorption or binding sites by microbial cells (De Schamphelaere and Janssen, 2002; Kahru et al., 2005). On the other hand, the high concentration of nutrient cations and available NH_4^+ (Table 5-2) can promote the growth of microbes which counteracted the toxicity from Cu in rhizosphere soil extracts (Kamilova et al., 2006).

5.4 Summary

The application of EDDS in rhizobox altered the chemical and microbial properties of rhizosphere soil and non-rhizosphere differently. Collectively, the treatment of EDDS in rhizobox showed beneficial effects on soil microbes particularly in rhizosphere compartment, which can be correlated with the elevated concentration of soil nutrients caused by the high concentration of EDDS in rhizosphere. The details of findings are listed below:

1) After the treatment of EDDS for 7 d in rhizobox, there is an obvious increase of soluble nutrient cations (*e.g.* Na, Fe, and Mn), DOC, DN, NH_4^+ and NO_3^- in rhizosphere soil and non-rhizosphere. The concentration of Na, Fe, Mn, DOC, DN, and NH_4^+ parameters is higher in rhizosphere than non-rhizosphere. Ca and Mg increased in rhizosphere but not in non-rhizosphere soil after EDDS treatment. However, the soluble K and available P were not affected by EDDS.

2) The soil qualities indicators, including the microbial biomass C and N, and urease activities, increased in rhizosphere compartment but did not change in non-rhizosphere compartment.

3) The microtoxicity test revealed that soil extracts showed less toxicity in rhizosphere than non-rhizosphere after treatment with EDDS.

Chapter Four and Chapter Five have made a thorough study on the chemical and microbial interactions of EDDS with soils in rhizosphere process. The results clarified the rhizosphere processes of chelant-assisted phytoextraction, which is important for identifying the limiting step of metal extraction in phytoextraction process. Nevertheless, the plant process on Cu uptake, transport, and transformation in the

presence of EDDS is not clear, which will be investigated comprehensively in the following chapter.

6. Chapter Six – The Role of EDDS on Cu Distribution and Speciation in Ryegrass

6.1 Introduction

There are two main pathways that proposed for the uptake of metal-EDDS or metal-EDTA complexes by plants. On the one hand, many researchers believe that plants are able to absorb intact complexes through the apoplastic flow, since metal-complexes (*e.g.* CuEDDS, PbEDTA, and CdEDTA) have been detected in some plants (Schaider et al., 2006). The complexes are unlikely to cross the cell membranes due to its large molecular size and lack of transporters (Leštan et al., 2008; Niu et al., 2011a). In addition, metal-complexes can be transported across the root cortex to stele, particularly in root apex or lateral root zone, where Casparian strip has not been developed or has been damaged (Clarkson, 1991; Niu et al. 2011b; Tao et al., 2016). On the other hand, some researchers maintain that plants mainly absorb free metals dissociated from metal-complexes (Sarret et al., 2001; Tian et al., 2011). Actually, FeEDTA, PbEDTA and ZnEDTA were found to dissociate either prior to or during absorption by plant roots (Chaney et al., 1972; Sarret et al., 2001). Intriguingly, both mechanisms were suggested on the uptake of CuEDDS by different plants according to recent studies (Cestone et al., 2010; Niu et al., 2011b; Johnson and Singhal, 2013), and the relative importance of two absorption pathways can be related to the supplied concentration of CuEDDS. Therefore, direct evidence is still required to verify the exact pathways.

The molecular localization and speciation of Cu in plants can provide valuable insights on the uptake and transport pathways of Cu. Current advanced

synchrotron-based microscopic X-ray fluorescence (μ-XRF) and X-ray absorption near-edge structure spectroscopy (XANES) provide a viable opportunity to acquire the information (Majumdar et al., 2012). To our knowledge, the effect of EDDS on the coordination environment of redox-active Cu in high-biomass grasses has not been studied yet. Because Cu can be reduced during plant absorption and translocation (Jouvin et al., 2012; Ryan et al., 2013), it is still not known whether the reduction process occurs and poses an influence on the dissociation of CuEDDS.

The aim of the present study is to establish a comprehensive understanding of the Cu uptake and transport mechanisms with EDDS in ryegrass (*Lolium multiflorum*). Hydroponic culture was conducted in the current study, due to the convenience in controlling the supplied amount of free Cu or CuEDDS and keeping stable conditions for plant growth. The molecular distribution and speciation of Cu in plants with or without EDDS was investigated using μ-XRF and XANES, respectively.

6.2 Materials and methods

6.2.1 Hydroponic cultivation

Seeds of ryegrass (*Lolium multiflorum* Lam. cv. Tetragold) were germinated on moist filter papers for 10 d after sterilization with 95% ethanol and water soaking. Plant seedlings were cultured with modified Hoagland's nutrient solution (HNS). The compositions of HNS include 1 mM KH_2PO_4, 5 mM KNO_3, 5 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 0.02 mM $FeSO_4$, 0.05 mM HBO_3, 0.01 mM $MnSO_4$, 0.77 μM $ZnSO_4$, 0.32 μM $CuSO_4$, and 0.02 μM $(NH_4)_6Mo_7O_{24}$. The nutrient solution was adjusted to pH 6.0 before use and replaced weekly. Plant seedlings were cultivated in controlled environment by a climate chamber with the relative humidity of 60%, 16 h day (25 ^oC)

and 8 h night (20 ^{o}C) cycle, and light illumination of 325 µmol photons m^{-2} s^{-1}.

After two weeks, plant seedlings were transferred to 100 mL beakers for treatment with free Cu or CuEDDS. Beakers were wrapped with aluminum foils to exclude lights and avoid algae growth in solutions. Each beaker contained about 80 mL HNS with free Cu (0, 50, 150, and 150 µM $CuSO_4$) or CuEDDS (0, 50, 150, 250, 500, 1500, and 3000 µM). Each treatment had three replicates in separate hydroponic containers. K_2HPO_4 was omitted from HNS to avoid precipitation of copper phosphate (Mohtadi et al., 2013). Aliquots of concentrated $CuSO_4$ or CuEDDS solution were added to HNS to create the designed exposure range. CuEDDS was prepared in solution by dissolving equivalent concentration of $CuSO_4$ and EDDS-Na_3 ($C_{10}H_{13}N_2Na_3O_8$, Sigma Aldrich). Specifically, the concentration range of free Cu was designed based on the reported level of Cu (1 to 300 µM) in soil solution from contaminated fields (Zhang et al. 2001; Song et al. 2004; Forsberg et al. 2009). The higher concentration range for CuEDDS was designed since values as high as 3000 µM were recorded in EDDS-assisted phytoextraction sites (Meers et al., 2005).

Plant seedlings were harvested 3 d after treatment with free Cu or CuEDDS (Cestone et al., 2012b). Shoots and roots of ryegrass were washed with deionized water prior to harvest, after which they were separated, oven-dried, weighed, and ground. Plant Cu was subsequently determined using ICP-OES (Agilent 700 series) after acid digestion with HNO_3/$HClO_4$ (4:1) (Luo et al., 2005). A standard reference SRM 1515 (apple leaves) was used for quality control, and the Cu recovery rate reached 92 ±6%. Mean values and standard deviations were obtained based on three replicates. Statistical analysis was performed with one-way analysis of variance (ANOVA) using SPSS software version 22.0. Differences between treatment means were tested using the

Duncan Test at a significance level of 0.05.

6.2.2 Micro-distribution assay of copper in root by μ-XRF

The 2-week-old seedlings were exposed to nutrient solutions either containing free Cu (150 μM) or CuEDDS (150 and 1500 μM) as described above. After incubation for 3 d, roots of ryegrass were rinsed with deionized water. Root segments, including root tips (0-1 cm) and mature root segments (1-3 cm, 3-5 cm, and > 5 cm), were cut off (Fig. 6-5), quickly frozen in liquid N_2, and lyophilized. The total Cu concentration in root segments was measured by ICP-OES after acid digestion. Root tips (0-1 cm) and mature root segments (> 5 cm) were selected for μ-XRF.

Lyophilized root segments were mounted on 3M tapes and analyzed by μ-XRF at beamline 4W1B of the Beijing Synchrotron Radiation Facility (BSRF), Institute of High Energy Physics, Chinese Academy of Sciences. The storage ring was operated at energy of 2.5 GeV with a current intensity ranging from 200-300 mA. Typical root zones, including the root apex from root tips (0-1 cm) and the lateral root zone from mature root segments (> 5 cm), were selected for scanning (Fig. 6-5). The samples were mounted on a XYZ translation stage and the sample platform was moved by a 2D stepping motor along the X direction for 70 μm and Z direction for 100 μm per step. The fluorescence spectra of each point were collected with a dwell time of 15 s by a PGY Si (Li) solid detector. The μ-XRF data reduction and process were performed using the PyMCA package (Solé et al., 2007). The peak intensities of elements were normalized to the current intensity of synchrotron radiation (I_0) in the ionization chamber to correct for the beam flux variation (Zhao et al., 2013).

6.2.3 Copper speciation analysis using Cu K-Edge XANES

Copper molecular speciation was analyzed on root tips (0-1 cm), root segments (> 5 cm), and shoots of ryegrass that were exposed to free Cu (150 μM) or CuEDDS (150 and 1500 μM) for 3 d. Freeze-dried ryegrass samples were ground and stored at -20 ℃ prior to XANES analysis. The Cu K-edge (8979 eV) XANES spectra of plant samples and reference Cu compounds were acquired on beamline 01C1 at the National Synchrotron Radiation Research Center (NSRRC), Taiwan. A Cu foil internal reference was used to calibrate the energy at 8979 eV. Standard Cu species included CuO (solid), CuCl (solid), $Cu(OH)_2$ (solid), $CuCO_3$ (solid), CuS (solid), $CuSO_4$ (solid), $Cu_2(PO_4)_3$ (solid), Cu-acetate (solid), Cu-oxalate (solution), Cu-histidine (solution), Cu(I)-glutathione (solution), Cu-alginate (solution), and CuEDDS (solution). The complexes of Cu-oxalate, Cu-histidine, Cu(I)-glutathione, CuEDDS and Cu-alginate were prepared according to Shi et al. (2008). Spectra of solid standards were acquired in transmission mode, while plant samples and solution standards were acquired in fluorescence mode with a Lytle detector. The obtained spectra were analyzed, using the Athena program in the IFEFFIT computer package for energy calibration, averaging of multiple scans, background subtraction, normalization, and linear combination fitting (LCF) (Ravel and Newville 2005; Cui et al., 2015; Cui et al., 2017).

Cu K-edge (8979 eV) XANES spectra were recorded from –200 to 300 eV. Each scan was completed within 15 min, and repeated scan was not derived from the same point. Principal component analysis (PCA) of sample spectra and target transformation (TT) of standards were performed using Six-Pack. The minimum value of IND was used to determine the number of statistically significant principal components that required to

reconstruct the spectra set (Table 6-1) (Manceau et al., 2002). Hence, a maximum of three standards were allowed for linear combination fitting (LCF) procedures. Standards with SPOIL value over 4.5 were not included in LCF procedures (Table 6-2). The Athena package program was used to conduct LCF analysis of sample spectra (-20 to 40 keV). During LCF, the fit standard was incrementally added to improve the linear fit, and one standard was only included when fit residual (normalized sum square) reduced at least by 20%. Additionally, the standard included must account for at least 10% of the measured spectra (Sarret et al., 2007; Punshon et al., 2013).

Table 6-1 Results from the principal component analysis performed on the Cu K-edge XANES spectra.

Component	Eigenvalue	Variance	Cumulative variance	IND
1	75.064	0.939	0.939	0.01371
2	1.991	0.024	0.964	0.01142
3	1.302	0.016	0.980	0.00798
4	0.481	0.006	0.986	0.00919
5	0.342	0.004	0.991	0.01196
6	0.278	0.003	0.994	0.01688
7	0.208	0.002	0.997	0.02852
8	0.138	0.001	0.998	0.08323
9	0.083	0.001	1	NA

Table 6-2 Spoil values of Cu references obtained by target transformation. Standards with SPOIL value < 1.5 are considered excellent, 1.5-3 are good, 3-4.5 are fair, 4.5-6 are acceptable, and > 6 are unacceptable (Manceau et al., 2002).

	SPOIL	R	Chi square
CuCl	3.4345	0.00255	1.60339
CuO	3.4571	0.00086	0.52062
CuS	2.5006	0.00112	0.70182
$CuSO_4$	8.6235	0.00199	1.21783
$Cu_3(PO_4)_2$	5.515	0.00092	0.56782
$CuCO_3$	11.384	0.00124	0.75692
$Cu(OH)_2$	2.8853	0.00070	0.42359
Cu-acetate	4.6531	0.00151	0.91117
Cu-oxalate	4.1322	0.00147	0.97108
Cu-alginate	4.0915	0.00096	0.60415
Cu-glutathione	1.0936	0.00380	4.00348
Cu-histidine	3.9305	0.00030	0.18730
CuEDDS	1.1346	0.00016	0.10155

6.3 Results

6.3.1 Cu concentration and plant growth of ryegrass

To assess the effects of EDDS on ryegrass Cu concentration, a comparison was made on ryegrass under similar concentration of Cu and CuEDDS (50-250 μM, Fig. 6-1). In the absence of EDDS, root Cu concentration is much higher than shoot Cu, with a translocation factor (TF) ranging from 0.06 to 0.11 (TF = shoot Cu concentration/ root Cu concentration) (Fig. 6-1c). The addition of EDDS substantially decreased the Cu concentration in root by 4.77-9.78 folds, and slightly increased the Cu concentration in shoot by 0.12-0.80 folds. Consequently, the translocation of Cu from root to shoot was greatly enhanced in the presence of EDDS, with the TF values range of 0.60-0.94 under exposure to 50-250 μM CuEDDS (Fig. 6-1c).

As the CuEDDS concentration increased from 50 to 1500 μM, shoot Cu concentration linearly increased and exhibited a saturation trend from 1500 to 3000 μM (Fig. 6-2a). Root Cu concentration continuously increased with supplied CuEDDS from 50-500 μM and displayed a saturation curve afterwards (Fig. 6-2a). The TF values increased with the increasing level of CuEDDS in solution, reaching 4.42 at concentration of 1500 μM (Fig. 6-2b).

During the short period of exposure to Cu or CuEDDS for 3 d, ryegrass did not show obvious toxicity symptoms (*e.g.* necrosis or dehydration) (Fig. 6-3). The dry weight of ryegrass shoot and root at harvest was not greatly affected with free Cu (50-250 μM) and CuEDDS (50-500 μM) ($P < 0.05$, Table 6-3). However, compared to control, the high dosage of CuEDDS (1500 μM) slightly reduced the shoot biomass by 10%, and CuEDDS (3000 μM) decreased the shoot and root biomass by 13% and 17%,

respectively. Because of the minor change of plant biomass by Cu or CuEDDS, the variation of the total Cu content was consistent with the Cu concentration in ryegrass (Fig. 6-4).

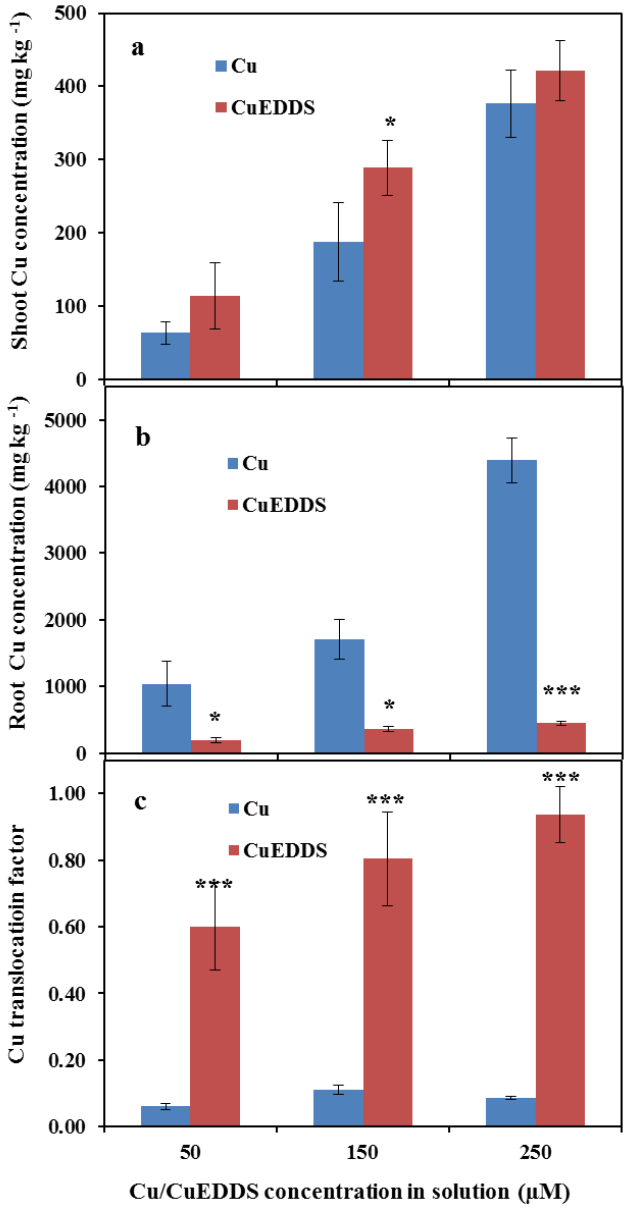

Figure 6-1 Copper concentration in ryegrass shoot (a) and root (b), and Cu translocation factor (c) exposed to Cu or CuEDDS (50, 150, and 250 µM). Significant differences compared to Cu-only treatment were evaluated by student's t test (*$P < 0.05$, ***$P < 0.005$).

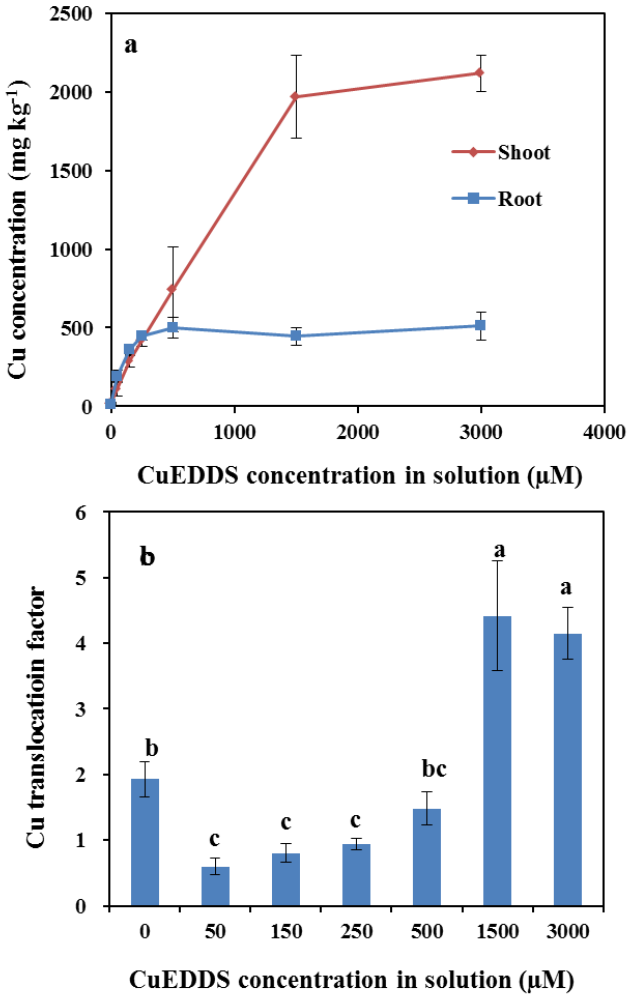

Figure 6-2 Copper concentration (a) and translocation factor (b) of ryegrass exposed to CuEDDS (0, 50, 150, 250, 500, 1500 and 3000 µM). Means with the same letter are not significantly different according to Duncan's Multiple Range test at 5% level.

Figure 6-3 The photograph of ryegrass at harvest under different treatments for 3 d. HNS refers to culture solution without additional Cu and EDDS. Cu50, Cu150, and Cu250 (a) refer to culture solution containing 50, 150, and 250 μM $CuSO_4$, respectively. CuE50, CuE150, CuE250, CuE500, CuE1500 and CuE3000 (b and c) refer to culture solution applied with 50, 150, 250, 500, 1500, and 3000 μM CuEDDS, respectively.

Table 6-3 The dry weight of ryegrass after treatments with Cu or CuEDDS for 3 d. Significant differences compared to control were evaluated by student's t test ($^{*}P < 0.05$).

Treatment	Shoot	Root
Control	1.35 ±0.06	0.48 ±0.04
Cu 50 μM	1.37 ±0.09	0.46 ±0.03
Cu 150 μM	1.32 ±0.12	0.51 ±0.05
Cu 250 μM	1.25 ±0.08	0.43 ±0.06
CuEDDS 50 μM	1.43 ±0.06	0.56 ±0.05
CuEDDS 150 μM	1.49 ±0.15	0.52 ±0.04
CuEDDS 250 μM	1.31 ±0.06	0.46 ±0.04
CuEDDS 500 μM	1.45 ±0.08	0.50 ±0.05
CuEDDS 1500 μM	1.22 ±0.06*	0.43 ±0.05
CuEDDS 3000 μM	1.18 ±0.03*	0.40 ±0.02*

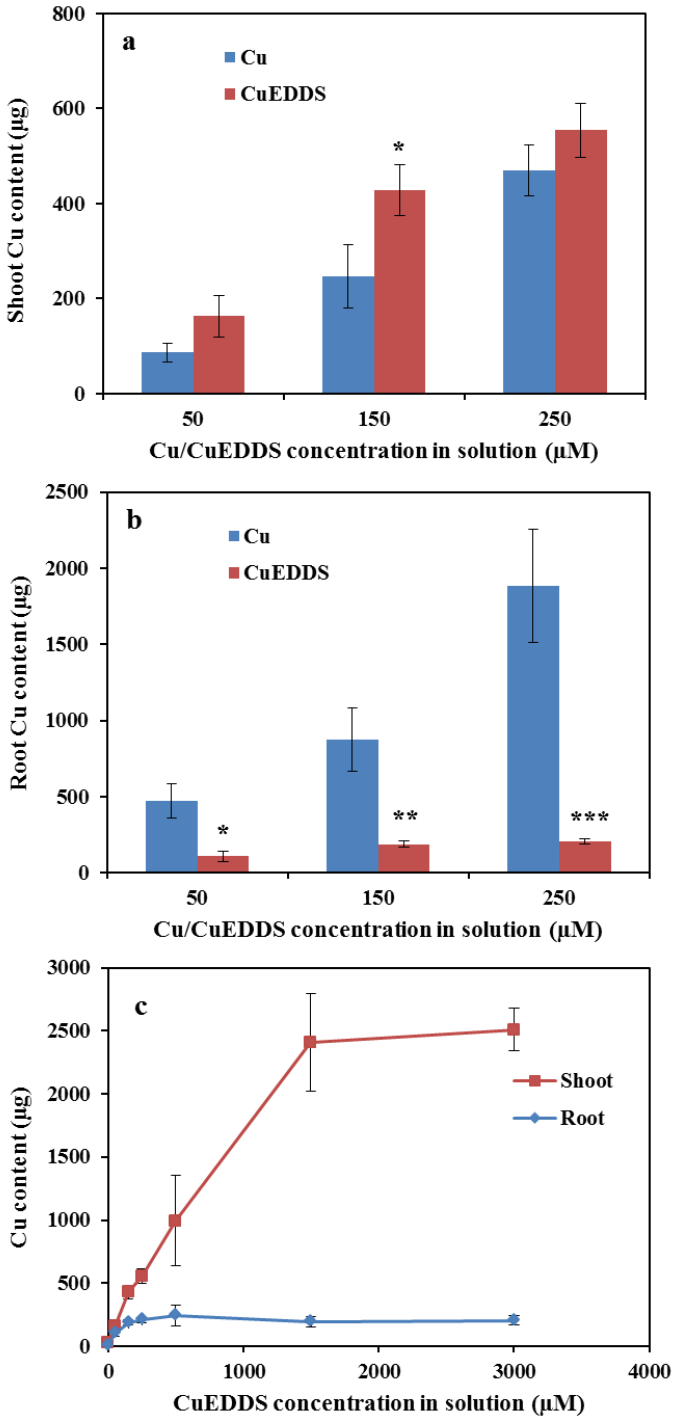

Figure 6-4 Copper content in ryegrass shoot (a) and root (b) exposed to Cu/CuEDDS (50-250 µM), and Cu content in ryegrass exposed to CuEDDS (0-3000 µM) (c). Significant differences compared to Cu-only treatment were evaluated by student's t test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$).

6.3.2 Copper distribution in ryegrass root

The concentration of Cu in different root segments of ryegrass exposed to free Cu (150 μM) and CuEDDS (150 μM and 1500 μM) is shown in Fig. 6-5. For ryegrass exposed to free Cu (150 μM), the Cu content was higher in mature root segments (> 1 cm) (1610-1800 mg kg^{-1}) than in root tips (0-1 cm) (1310 mg kg^{-1}). Consistently, under treatment with CuEDDS (150 μM), mature root segments (> 1 cm) also accumulated more Cu (367-417 mg kg^{-1}) than root tips (0-1 cm) (213 mg kg^{-1}). With the treatment of a higher CuEDDS (1500 μM), root Cu distribution followed the same trend to that treated by CuEDDS (150 μM).

The spatial distribution of Cu identified by μ-XRF in the root apex from root tips (0-1 cm) is shown in Fig. 6-6. Roots treated by 150 μM free Cu showed higher Cu in the meristem zone than the following elongation zone, indicating that Cu was largely localized in the meristem zone (Fig. 6-6a). In contrast, roots treated by CuEDDS (150 μM and 1500 μM) (Fig. 6-6b, c) showed high levels of Cu in both the root meristem and the root stele of elongation zone.

In the lateral root zone from mature root segments (> 5 cm), the spatial mapping of Cu with μ-XRF is shown in Fig. 6-7. Generally, Cu was absorbed by lateral root and further transported from the lateral root stele to the primary root stele (Fig.6-7b and Fig.6-8), either exposed to free Cu (150 μM) or CuEDDS (150 μM and 1500 μM). For free Cu (150 μM) treatment, Cu was primarily accumulated at the junction where the lateral and primary root stele was connected (Fig. 6-7a). Moreover, the higher intensities of Cu at the lateral root stele relative to that of primary root was observed in 150 μM CuEDDS treated root (Fig. 6-7b). Furthermore, Cu in lateral root stele seemed to be transported into primary root effectively when the root was treated by

1500 µM CuEDDS, leading to the higher Cu in the primary root stele than lateral root stele (Fig. 6-7c).

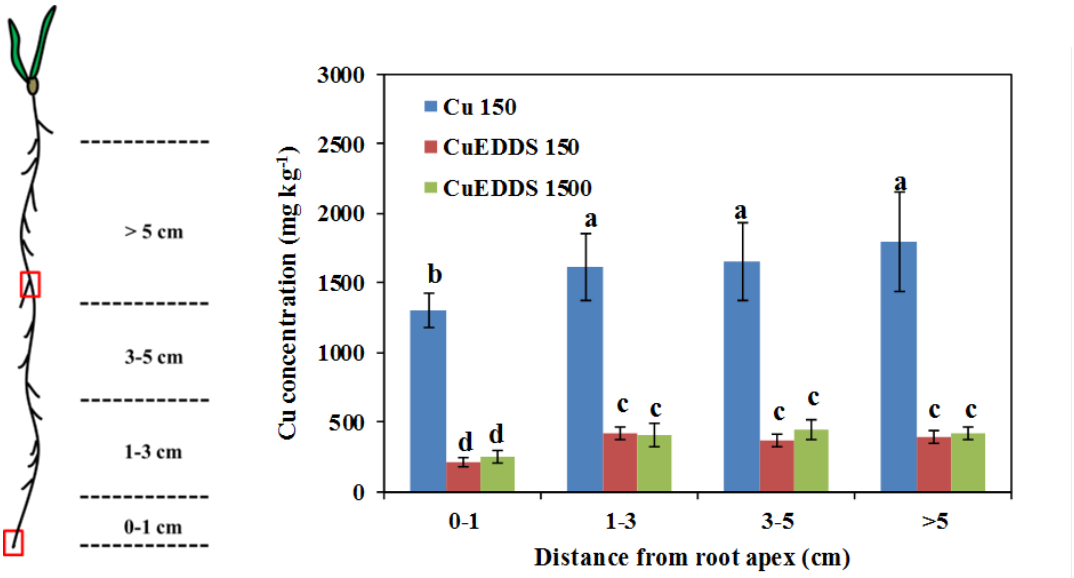

Figure 6-5 Copper concentration in root segments (0-1, 1-3, 3-5 and >5 cm) exposed to free Cu (150 µM) and CuEDDS (150 and 1500 µM). The two red boxes indicated the areas selected for µ-XRF scanning. Means with the same letter are not significantly different according to Duncan's Multiple Range test at 5% level.

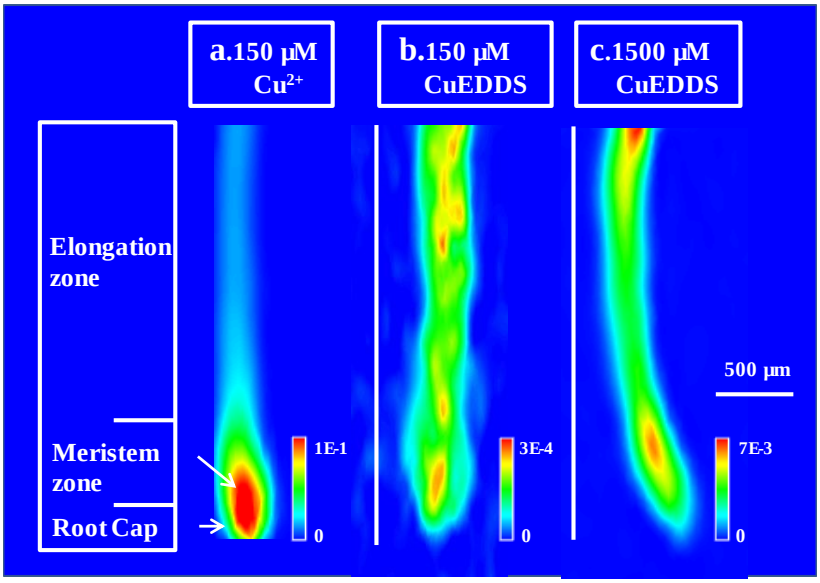

Figure 6-6 The µ-XRF elemental maps for Cu in ryegrass root apex treated with 150 µM free Cu (a), 150 µM CuEDDS (b), and 1500 µM CuEDDS (c) for 3 d. The scanned area was indicated by the red box from root tip (0-1 cm) shown in Fig. 6-5.

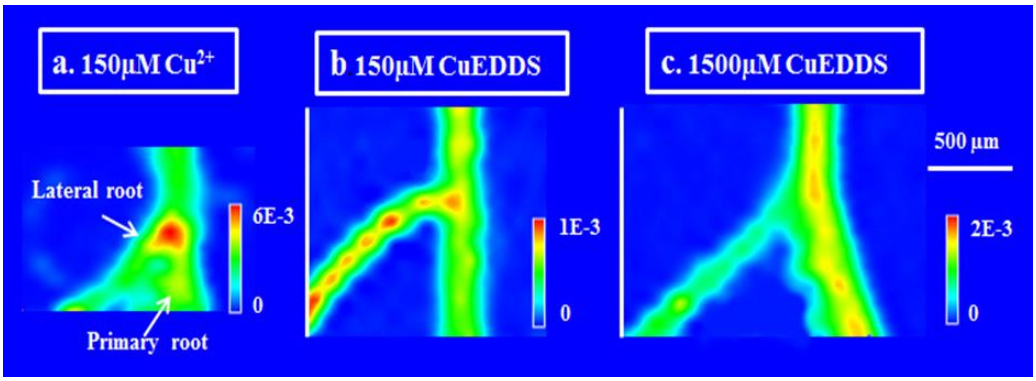

Figure 6-7 The µ-XRF elemental maps for Cu in ryegrass lateral root zone treated with 150 µM free Cu (a), 150 µM CuEDDS (b), and 1500 µM CuEDDS (c) for 3 d. The scanned area is indicated by the red box from mature root segment (>5 cm) shown in Fig. 6-5.

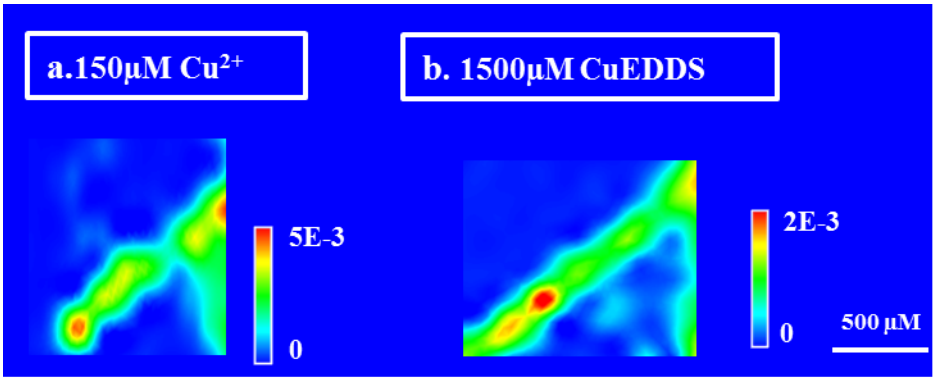

Figure 6-8 The supplementary μ-XRF map for ryegrass lateral root treated with 150 μM free Cu is shown in Fig. 6-7a, and more smaller focused zone in c is shown in Fig. 6-7b.

6.3.3 Copper speciation in ryegrass root and shoot using XAS spectroscopy

Raw and first derivative Cu K-edge XANES spectra of plant samples and standard references are presented in Fig. 6-9. The Cu complex showed different coordination geometries in plant samples under free Cu and CuEDDS treatments (Fig. 6-9). To quantitatively identify the probable Cu species in plant samples treated with free Cu or CuEDDS, LCF was performed (Fig. 6-9a). The fitting results were optimized by gradually increasing fitting component numbers, and minimizing fitting residues. Best fitting results were obtained with Cu-alginate, Cu-histidine, Cu-glutathione, and CuEDDS as model standards (Table 6-4). When plants were exposed to free Cu (150 μM), Cu species were dominated by Cu-histidine (66-80%) in roots and shoots, with a proportion of other forms including Cu-alginate (21-34%) and Cu(I)-glutathione (0-20%). After exposure to CuEDDS (150 and 1500 μM), Cu was mainly present as CuEDDS (49-67%) in roots and shoots, with a proportion of Cu-histidine (21-41%) and Cu-glutathione (0-24%). In addition, shoots contained higher percentage of CuEDDS than roots. Cu species in ryegrass showed no great difference between treatment with 150 and 1500 μM CuEDDS. Based on a previous study (Niu et al.,

2011b), the lateral root zone of maize was reported to be the main sites for absorption of CuEDDS, while the root apex should absorb more dissociated Cu from CuEDDS. However in our study, no obvious difference on Cu speciation was found between root tips (0-1 cm) and mature root segments (> 5 cm) of ryegrass. As the uncertainty in Cu species appointment accounted for 10% of the total amount of Cu, the minor difference (< 10%) may not be deciphered in tested samples.

The rising energy edge of Cu(I) was represented by Cu(I)-glutathione, and Cu(II) was represented by other Cu complex standards (Shi et al., 2008). In view of these spectra, the energy edge of Cu(I) (8982 eV) was lower than that of Cu(II) (8994-8996 eV) (Fig. 6-9a). After transformation of the raw data (Fig. 6-9a) to their first derivatives (Fig. 6-9b), the feature difference between sample spectra became distinguishing, thus the Cu(I) peak was highlighted at ~8980 eV. In all plant samples, Cu was present as a mixture of Cu(I) and Cu(II), with varying Cu(I) percentage from 0 to 24% (Table 6-4).

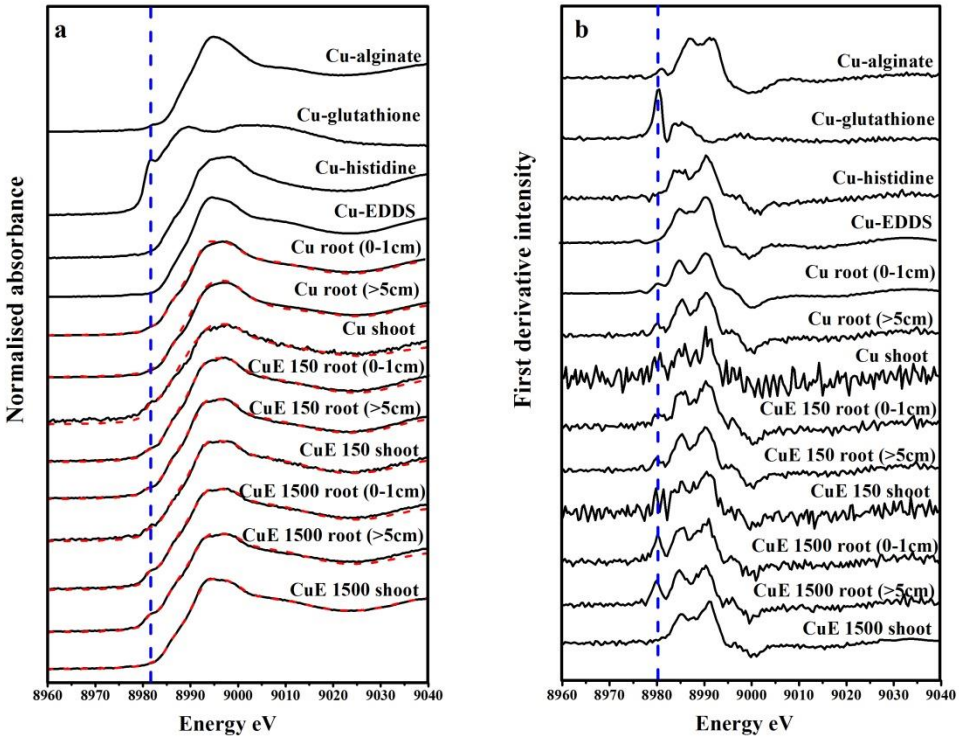

Figure 6-9 Copper K-edge XAFS spectra (solid lines) for reference materials and plant samples exposed to free Cu (150 µM) and CuEDDS (150 µM and 1500 µM) for 3d with red dotted lines as the linear combination fitting results. Both normalized spectra (a) and first derivate (b) of normalized data are presented to highlight spectra features. The vertical blue dotted lines indicate the location of the Cu(I) valent state.

Table 6-4 Species of Cu (%) characterized by Cu K-edge XANES spectra using LCF for plant tissues treated by free Cu (150 µM) and CuEDDS (150 µM and 1500 µM) for 3 d. The inherent error in LCF analysis is +/- 5-10% (Gräfe et al., 2014).

Samples	Cu-alginate	Cu-glutathione	Cu-histidine	CuEDDS	R	Reduced chi-square
Cu 150 µM						
Root (0-1 cm)	21		79		0.0062	0.0009
Root (>5 cm)	34		66		0.0041	0.0007
Shoot		20	80		0.0082	0.0012
CuEDDS 150 µM						
Root (0-1 cm)		13	36	51	0.0014	0.0002
Root (>5 cm)		10	41	49	0.0014	0.0002
Shoot		15	21	64	0.0020	0.0003
CuEDDS 1500 µM						
Root (0-1 cm)		24	26	50	0.0015	0.0002
Root (>5 cm)		24	27	49	0.0022	0.0003
Shoot			33	67	0.0006	0.0001

6.4 Discussion

6.4.1 Effects of EDDS on plant Cu and biomass

It is a typical pattern for non-accumulating plants to restrict heavy metals underground, in order to reduce the toxicity for plant growth (Baker, 1981; Ma et al., 2015). However, the ultimate goal of phytoextraction is to remove heavy metals from soil into plant aerial biomasses. Therefore, methods to enhance metal uptake and translocation are both required to assist phytoextraction. Indeed, the application of chelants has been considered as a feasible method to desorb metals from the soil matrix, and facilitate metals transport from root to shoot (Blaylock et al., 1997).

The enhancement effects of strong chelants (EDTA, DTPA, EDDS, and IDSA) on the shoot-to-root ratio of Pb, Zn, Cu and Cd have been widely reported for ryegrass, maize, and other plants (Luo et al., 2005; Zhao et al., 2010; Niu et al., 2011a; Mohtadi et al., 2013; Johnson and Singhal, 2013). Our study found that in the absence of EDDS, ryegrass root accumulated one magnitude of Cu higher than shoot (Fig. 6-1). Expectedly, the presence of EDDS prominently enhanced the TF values of Cu by 6-9 folds (Fig. 6-1c). Nevertheless, the observed increment (0.1-0.8 fold) of Cu in shoot with EDDS compared to that without EDDS from this study was much lower than that reported by Gunawardana et al. (2009). This study showed a 26 fold Cu increase in shoots of *Lolium perenne* when exposed to 156 μM CuEDDS for 30 d. The lesser enhancing extent of Cu in shoots by EDDS found in our study can be ascribed to the

shorter exposure period of 3 d (Johnson and Singhal, 2013a) and the different plant species used. Furthermore, the translocation of Cu increased with the increasing concentration of CuEDDS particularly over 1500 μM, which can be due to the potential damage to root cells (explained in next section).

In spite of the improvement on metal translocation factor, EDDS has been widely documented to enhance the metal concentration in plant aboveground parts that grown in contaminated soils (Luo et al., 2005; Duquène et al., 2009). However, EDDS did not remarkably alter the Cu level in ryegrass shoot in our hydroponic study (Fig. 6-1). The inconsistent effects of EDDS are mainly derived from the different culture conditions. In contaminated soils, free metal activity is usually low due to the strong binding of metal to soil components, while applied EDDS chelated with metal, greatly enhance metal concentration in soil solution, and thus increase the uptake of metal-EDDS complex by plants (Meers et al., 2005). In contrast, solution culture set the concentration of free metal and metal-EDDS at the same level, and EDDS may substantially decrease the free metal activity for plant absorption. If the uptake and translocation of metal-EDDS cannot compensate for the diminished absorption of free metal, the concentration of metal in plant aboveground parts may not increase even at the presence of EDDS (Tandy et al., 2006; Koopmans et al., 2008). Similarly, Tian et al. (2011) reported that EDTA even reduced the concentration of Pb in xylem and leaves of accumulator *Sedum alfredii* in hydroponic culture.

In view of previous studies, application of EDDS and EDTA appears to increase the

metal uptake at the cost of plant damage or death with the exposure time from 7 d to 67 d (Neugschwandtner et al., 2008; Hadi et al., 2010; Mohtadi et al., 2013). In this study with a short exposure period (3 d), CuEDDS (50-500 μM) did not affect the biomass of *Lolium multiflorum*, while a high dosage of CuEDDS (1500 and 3000 μM) slightly decreased plant biomass (Table 6-3). Up to now, the mechanism of plant toxicity induced by strong chelants is still not clear. On the one hand, in hydroponic studies, the low concentration range of metal-chelant complex (10-200 μM) is non-toxic, and the chelation of free metals by EDDS or EDTA can alleviate metal toxicity and even benefit plant growth (Seth et al., 2011; Cestone et al., 2012a; Johnson and Singhal, 2013; Tan et al., 2014). On the other hand, in hydroponic and soil culture, the high dosage of strong chelants results in plant membrane damage, biomass reduction, or plant death (Vassil et al., 1998; Niu et al., 2011a; Mohtadi et al., 2013). Therefore, the influence of strong chelants on plant growth can be affected by many factors, including the dosage of chelants, exposure time, plant species, plant age, and polluted metal type. In practice, the high dosage of chelants is recommended to apply close to the time of harvest (Vamerali et al., 2010).

6.4.2 Effects of EDDS on Cu localization in roots

EDDS not only decreased total Cu retained in ryegrass roots, but also altered the spatial distribution pattern in roots. In the root apex from current study, a much higher Cu was deposited within the root meristem than in the subsequent elongation zone when exposed to free Cu (Fig. 6-6a). The distribution pattern of Cu in ryegrass roots

was in line with that of dayflower (treated by 100 μM Cu^{2+}) (Shi et al., 2011), cowpea (treated by 1.5 μM Cu^{2+}) (Kopittke et al., 2011; Wang et al., 2013), cucumber (treated by 100 μM Cu^{2+}) (Song et al., 2013), and rice (treated by 50 μM Cu^{2+}) (Lu et al., 2017). The results suggested that free Cu was largely trapped in meristem zone in the ryegrass root apex, thus limiting the transportation into the above root stele. In contrast, under CuEDDS treatment (150 and 1500 μM), Cu was both high in the meristem zone and the root stele of elongation zone (Fig. 6-6b and c), indicating that partial Cu was readily transported upwards into the root stele in the presence of EDDS (Tanton and Crowdy, 1972). Moreover, Cu observed in meristem under CuEDDS treatment is likely resulted from absorption of dissociated Cu, as cells in meristem are tightly packed with limited apoplastic space for absorption of the intact CuEDDS (Niu et al., 2011b; Song et al., 2013). The partial dissociation of CuEDDS was supported by the Cu speciation results which will be discussed later.

In our study, the lateral roots of ryegrass emerged above 1 cm from the root apex, and the contribution of lateral roots on Cu uptake was supported by the higher concentration of Cu detected in mature root segments (> 1cm) than in root tips (1 cm) under all treatments (Fig. 6-5). Free Cu (150 μM) was mainly deposited at the junction between lateral and primary roots (Fig. 6-7a), suggesting that free Cu was largely sequestered by root cells in the junction, leaving a relatively small quantity to enter into xylem sap. In contrast, the presence of EDDS (150 μM) alleviated the deposition of Cu at the junction, inferring that EDDS facilitated Cu transport from lateral root stele to primary root stele (Fig. 6-7b). Due to the development of lateral

roots, the apoplastic flow was not restricted by Casparian strip that was destructed by the penetration of lateral roots on endodermis. Thus, the xylem-loading efficiency of Cu from the primary root stele to xylem was the limiting step for upwards translocation in lateral root zone. The xylem-loading efficiency seemed raised at the exposure to high concentration of CuEDDS (1500 μM), since the majority of Cu located in the primary root stele instead of the lateral root stele (Fig. 6-7c). In addition, a sharply increase of Cu TF value was also noticed at exposure to CuEDDS over 1500 μM (Fig. 6-2). These results indicated that CuEDDS (1500 μM) may injury or kill pericycle and xylem cells, thus permitting more apoplastic bypass of CuEDDS into the xylem. Actually, Niu et al. (2011b) also indicated that CuEDDS (3000 μM) could injury passage cells in the endodermis of maize for additional channels to enter into root stele. Other metal complexes such as PbEDDS (800 and 990 μM) and PbEDTA (1000 μM) also showed toxic lesions to plant roots for subsequent translocation to plant shoots (Schaider et al., 2006; Mohtadi et al., 2013).

6.4.3 Effects of EDDS on the transformation and transport of Cu

In the absence of EDDS, free Cu can be sequestered by abundant ligands in plants during absorption and transport. Polysaccharides in root cell walls have been regarded as an important component for metal stabilization, due to the high affinity of Cu to carboxyl and hydroxyl groups (Krzesłowska, 2011). Alginic acid is a type of natural polysaccharide; therefore, it was used as a model cell-wall-like ligands in our work (Song et al., 2013). Moreover, amino acid residues have been identified to participate

in binding Cu in root cell walls from different plant species, based on recent study using synchrotron techniques (Manceau et al., 2013; Collin et al., 2014; Guigues et al., 2016). Histidine is used to represent amino acids to bond with Cu in this study due to the high affinity to Cu (Log K = 18.4) (Kruck and Sarkar, 1973), although various ligands including nicotianamine and proline can also be used (Irtelli et al., 2009). The sequestration of Cu by root cell walls with these ligands was supported by our results, showing that 21-34% of Cu was bound to alginic acid and 66-79% to histidine in ryegrass roots (Fig. 6-9 and Table 6-4). Immobilization of Cu by cell wall components is also known as an important detoxification strategy employed by plants (Hall, 2002; Sharma et al., 2016). The presence of Cu-histidine and Cu(I)-glutathione may suggest that free Cu was mainly translocated as these complexes to ryegrass shoots, however, there is still doubt that whether the Cu(I)-glutathione was newly produced in shoots (Collin et al., 2014).

In the presence of EDDS, CuEDDS was the major component of Cu in plant roots (49-51%) and shoots (64-67%) (Fig. 6-9 and Table 6-4). The higher percentage of CuEDDS in shoots than roots suggested that CuEDDS was responsible for Cu long distance transport from underground parts to aerial tissues. Our XANES evidence for Cu uptake and transport as CuEDDS was also supported by Niu et al. (2011a), which study used HPLC-UV and showed that CuEDDS accounted for 86.9%–87.7% of total Cu in xylem sap of maize that amended with EDDS (0.5-6.0 mmol kg^{-1}) in soil for 14 d. Additionally, high percentages of other metal complex, including 99% PbEDTA and 89% CdEDTA, were also identified in xylem sap of Indian mustards exposed to

400 μM PbEDTA and CdEDTA (Schaider et al., 2006). Accordingly, strong chelants should form complexes with metals to prevent the root sequestration of metals by carboxyl, hydroxyl, or amine groups, thus promoting the translocation of metals from roots to shoots as complexed forms.

The translocation efficiency of metal by plant was critically affected by the stability of metal-chelate complex within plant systems. For example, weak chelants like citric acids usually fail in enhanced-phytoextraction, due to the readily dissociation property of their metal complex before or after uptake by plants (Luo et al., 2005; Johnson and Singhal, 2013). Although strong chelant EDDS formed a much stable complex with Cu during plant absorption, other Cu complexes like Cu(I)-glutathione (0-24%) and Cu-histidine (21-41%), were also found in ryegrass tissues under exposure to CuEDDS (150 and 1500 μM) (Fig. 6-9 and Table 6-4), indicating the partial dissociation of CuEDDS. Similarly, total dissociation of ZnEDTA and partial dissociation of PbEDTA have also been observed in the roots/shoots of *Phaseolus vulgaris*, with the formation of Zn phosphate dehydrate and unknown Pb species in plants (Sarret et al, 2001).

Since chemical speciation modeling (Visual MINTEQ v3.1) (Gustafsson, 2014) of CuEDDS treatment (150 and 1500 μM) showed that > 99.99% of Cu was present as CuEDDS in the hydroponic solution, so that CuEDDS may dissociate on root surface or within plant tissues under the specific physiology and biochemistry conditions (Cestone et al., 2010). However, the dissociation mechanism of CuEDDS during plant

uptake has not been well understood according to previous studies. On the one hand, the dissociation rate of CuEDDS may be enhanced under a sink effect of plant uptake (Degryse et al., 2012). Since free Cu can be absorbed by plant roots at a much higher rate than CuEDDS (Niu et al., 2011a), the depletion of dissociated Cu from CuEDDS on ryegrass root surface may facilitate the equilibrium of CuEDDS towards dissociation. On the other hand, Cestone et al. (2010) speculated that the dissociation of CuEDDS may be similar to Fe(III)EDTA, as both of them contained redox-sensitive metal element. Reduction based dissociation of Fe(III)EDTA via cell surface reductase has been reported prior to uptake by strategy I plants, with the following steps including weakening chelate bonds on cell surface, reduction of Fe(III) to Fe(II) by Fe reductase, and releasing free Fe(II) and EDTA (Chaney et al., 1972; Römheld and Marschner, 1983; Welch et al., 1993). Nevertheless, more study is deserved on the dissociation rate, extent, and mechanisms of metal-chelate complexes in different plant systems in future.

6.4.4 Conceptual model of Cu uptake and translocation

Based on the observation from current study, a conceptual model is present in Fig. 6-10 showing Cu uptake and translocation by plants either in the absence or presence of EDDS. The major conclusions are listed below:

1) Ryegrass roots have a high affinity to uptake free Cu from solutions, as supported by the high root Cu concentration (Fig. 6-1). The absorbed free Cu was predominantly

sequestered by cell wall components of ryegrass, such as amino acid residues and polysaccharides, as evidenced by Cu species analyzed by XANES spectra (Fig. 6-9 and Table 6-4). Free Cu is largely immobilized in root apoplast, which leads to a relatively low quantity of Cu transportation to root stele, in consistence with the observation of Cu deposition in meristem of root tip, and in the lateral and primary root conjunction (Fig. 6-6a and Fig. 6-7a). Furthermore, Cu-histidine like species should be responsible for Cu transport to xylem (Table 6-4), especially from root tip and lateral root zone where Casparian strip on endodermis is inherently incomplete.

2) Chelation of Cu with EDDS substantially reduces the free Cu activity, and thus diminishes the Cu absorption by ryegrass roots (Fig. 6-1). A proportion of dissociated Cu from CuEDDS can be absorbed as well, as supported from the partial deposition of Cu on meristem of root tip (Fig. 6-6b and c) and Cu species results (Fig. 6-9 and Table 6-4), while the dissociation rate and mechanisms of CuEDDS has not been understood well. Most of CuEDDS remains in ryegrass roots (Fig.6 and Table 2), keeps the mobility of Cu in root apoplast, and readily transports to xylem, with the observation of Cu in root stele from root tip and lateral root zone (Fig. 6-6 and Fig. 6-7). The high concentration of CuEDDS in solution, which can be obtained in polluted fields with high dosage of EDDS (Meers et al., 2005; Fang et al., 2017), further increases the apoplastic loading of CuEDDS. To be noticed, the high CuEDDS is speculated to impair passage cells on endodermis (Fig.6 and Table 2) or pericycle cells, and thus creating additional channels for transportation of CuEDDS to xylem (Fig. 6-7c) (Niu et al., 2011b; Li et al., 2017; Barberon, 2017). Therefore, Cu

hyperaccumulation in ryegrass is only observed at high dosage of CuEDDS (Fig. 6-2).

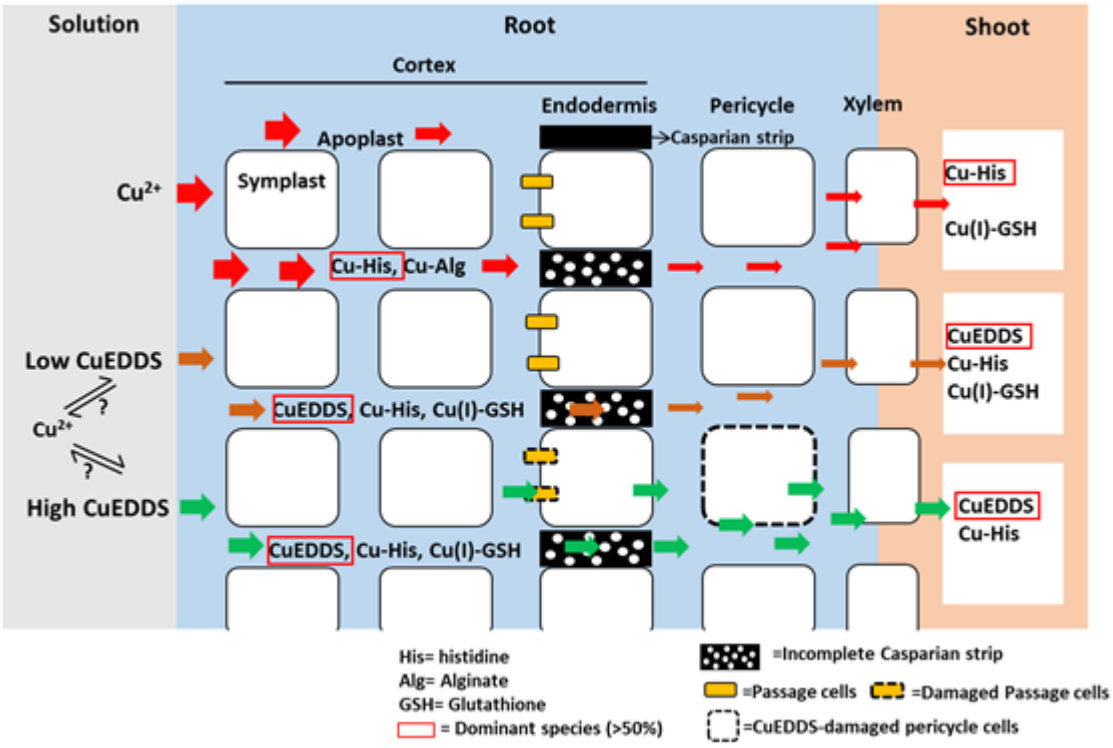

Figure 6-10 A conceptual model of the Cu uptake and transport mechanisms in ryegrass in the absence and presence of EDDS. The width of arrow suggests the relative quantity of Cu influx.

6.5 Summary

According to this hydroponic study, the gathered insights into the speciation, behavior, and fate of Cu in plants elucidated the role of EDDS on Cu uptake and transport process by plants. The main results are shown below:

1) EDDS substantially enhanced the translocation of Cu from roots to shoots of ryegrass. In addition, the high concentration of CuEDDS (1500-3000 μM) further enhanced the root-to-shoot translocation of Cu, which can be related to its damage effects to root cells.

2) Spatial imaging of Cu in root apex and lateral root zone suggested that Cu was more preferentially transported to root stele in the presence of EDDS.

3) The enhancement on Cu mobility by EDDS was interpreted by the molecular speciation of Cu within plants, which supported that EDDS decreased the root sequestration of Cu and facilitated the root-to-shoot translocation of Cu as CuEDDS.

4) Moreover, the partial dissociation of CuEDDS in plants was also noticed, and the dissociation processes still deserve further investigation.

Conclusively, Chapter Four, Chapter Five together with Chapter Six shed light on the full picture of the interactions of EDDS with Cu from underground soil to aboveground plant, which advances our understanding of EDDS-assisted phytoextraction and the biochemistry of Cu in soil-plant systems.

7. Chapter Seven - Conclusion and Recommendations

7.1 Conclusions

This thesis was an attempt to investigate the underlying mechanisms involved in phytoextraction for metal polluted soil with the assistance of biodegradable chelants. Several chelants (EDTA, EDDS, and GLDA) with high biomass species (ryegrass and tall fescue) were used to remediate a Cu contaminated farm soil near an abandoned Cu mine from Nanjing. Then EDDS was selected as a representative biodegradable chelant, in order to study the interaction mechanisms of chelants with soil, plant, and Cu in chelant-assisted phytoextraction. The investigation of the complex interactions was conducted separately from three aspects. Firstly, we studied the impact of EDDS on soil Cu extraction and transport in rhizosphere of ryegrass. Secondly, we examined the effects of EDDS on soil quality (nutrients and microbes). Thirdly, the role of EDDS on Cu uptake and transport mechanisms in ryegrass was explored in a hydroponic experiment. With the advanced synchrotron based techniques, including μ-XRF and XAS, the distribution and speciation of Cu in soil and plant samples provided powerful evidences for understanding the fate of Cu in soil-plant system. There are significant improvements on understanding the impact of EDDS on processes of Cu in rhizosphere soil and ryegrass, which helps the development of phytoextraction and biogeochemical research. The major findings from this study are as following:

1) EDDS have less phytotoxicity and performed better efficiency on enhancing Cu contents in shoot of ryegrass and tall fescue in comparison with GLDA. In addition, the leaching risks can be immediately controlled with the rapid degradation of EDDS, with soil DOC and $CaCl_2$ extracted Cu substantially decreased from 7 d to 28 d after addition to soil. Although GLDA was regarded as a new green chelant, its degradation in this soil was not efficient and comparable to EDTA during our experimental conditions. With the degradation of EDDS, 95% of solubilized Cu was resorbed back to soil components after 28 d.

2) The chemical interaction of EDDS and soil Cu in rhizosphere of ryegrass was investigated in a multi-interlayer rhizobox. The results showed that evenly applied EDDS was accumulated in rhizosphere after 7 d, suggesting the transport of EDDS from non-rhizosphere to rhizosphere probably via transpiration stream. Sequential extraction analysis of rhizobox soils showed that the higher amount of EDDS in rhizosphere solubilized more Cu than non-rhizosphere, and EDDS facilitated Cu transportation from non-rhizosphere to rhizosphere. Solution speciation modelling supported that Cu in soil extracts was preferentially present as CuEDDS with no competition from dissolved Fe, Al, and Ca. Solid speciation analysis of Cu, by X-ray fluoresce and X-ray absorption spectroscopy, revealed that EDDS mainly chelated with Cu that adsorbed on goethite instead of clay, probably due to the EDDS-promoted dissolution of Fe.

3) The impact of EDDS on soil nutrients and microbes was analyzed in the

multi-interlayer rhizobox. The treatment of EDDS in rhizobox remarkably increased soluble nutrient cations (*e.g.* Na, Fe, and Mn), DOC, DN, and NH_4^+ in rhizosphere soil and non-rhizosphere compared to soil without EDDS after 7 d. Moreover, the concentrations of these nutrients parameters were higher in rhizosphere than non-rhizosphere. After application of EDDS in rhizobox, microbial biomass C and N increased in rhizosphere compartment but not changed in non-rhizosphere compartment. Similarly, EDDS enhanced urease activities in rhizosphere but not in non-rhizosphere. Furthermore, the microtoxicity test also revealed that soil extracts showed less toxicity from rhizosphere than non-rhizosphere after treatment with EDDS. Collectively, the treatment of EDDS in rhizobox showed beneficial effects on soil microbes particularly in rhizosphere, which can be correlated with the elevated concentration of soil nutrients in rhizosphere caused by EDDS.

4) To elucidate the influence of EDDS on Cu uptake and transport by ryegrass, a hydroponic experiment is conducted. The results showed that EDDS increased the Cu translocation factor from root to shoot by 6-9 folds under CuEDDS in comparison with free Cu (50-250 μM). Miro-XRF mapping revealed that EDDS alleviated Cu deposition in the root meristem of root apex and the junction of lateral root zone, and facilitated Cu transport to root stele for subsequent translocation upwards. XANES analysis found that free Cu was sequestered in plants as a mixture of Cu-organic ligands. In the EDDS treatment, Cu was primarily present as CuEDDS (49-67%) in plants with partial chemical transformation to Cu-histidine (21-36%) and Cu(I)-glutathione (0-24%). These results suggest that EDDS improves internal Cu

mobility through forming CuEDDS, thus decreasing the root sequestration of Cu, and ultimately facilitating Cu transport to plant shoots.

7.2 Limitations of current study

According to the results produced from current study, EDDS-induced changes on the dynamics of Cu in soil-plant systems were clarified. However, many findings regarding the key processes involved in phytoextraction can be limited by our experiment settings. The detailed limitations are listed below:

1) EDDS substantially enhanced the convective flow of Cu to root surface in our small scale rhizobox study; however, the impact of EDDS on Cu transport in soils should be more complex in filed conditions. This is because that the Cu transport is three-dimensional in field, which is not only controlled by plant transpiration but also vertical leaching. Additionally, the soil hydraulic properties directly controlling metal transport vary with different sites, which are affected by soil vertical layering, soil particle size distribution, agronomic irrigation, and planting density. Moreover, the field metal contamination is typically heterogeneous, which is different with the homogeneous condition used in lab pot experiments and may affect the efficiency of EDDS application.

2) The effects of EDDS on biological activities of soil microbes have been examined in current studies, but the effects on the composition and structure of soil microbes in rhizosphere and non-rhizosphere are not investigated. Moreover, current study

did not involve the study regarding the microbial impact on CuEDDS degradation or dissociation during uptake by plant roots.

3) The impacts of EDDS on Cu uptake and transport by plants were mainly clarified at the biochemical level; however, the process can be also related to the physiological variation of plants. The measurements on physiological properties of plants, such as root architecture, root anatomical structure, cell membrane integrity, plant transpiration rate, are lacking in current study.

4) The transport pathways of CuEDDS were established in hydroponic grown *Lolium multiflorum* in our work; however, the transportation of other metal complexes (PbEDDS, CdEDDS, HgEDDS, and ZnEDDS) in different plants is not known. Although other metal-chelant complex may share many similar properties with CuEDDS, the dissociation and transport of metal complex can be related to their stability , metal oxidative/reducible capacity, and the chelating strength of competing ligands from different plants.

7.3 Recommendations for future work

Given the current progress on the mechanisms of EDDS-assisted phytoextraction, the following research areas could be recommended for future work:

1) Our results suggested that EDDS is effective to extract Cu from soils, facilitates Cu transport to root surface, and improves Cu mobility from root to shoot;

however, the limited uptake rate of CuEDDS by plants from rhizosphere soil solution restricted Cu extraction efficiency from soils. More studies are required on the improvement of plant uptake on CuEDDS from soil solution. For example, the application of plant hormones (*e.g.* indole-3-acetic acid) can be beneficial for plant growth, enhance plant transpiration rate, and improve the uptake of CuEDDS through apoplastic pathways.

2) More studies are recommended to use microbes in combination with chelants for assisted phytoremediation. Some specific rhizobacteria can not only improve the metal availability but also help the plants to establish and grow in contaminated soils.

3) More pilot experiments in fields are needed to promote this technology to market. The experiment results should include cost data and mass balance calculation, in order to provide realistic information on the feasibility of this technology.

4) The phytoremediation technologies face challenges in widespread use, because it is a time-consuming method. To make phytoextraction more commercial attractive, new profitable strategies are recommended to be exploited, such as the production of biochar, the generation of bioenergy, and the recovery of precious metals from biomass.

5) The knowledge obtained from chelant-assisted phytoextraction can be applied not only in soil remediation but also in other areas such as biofortification.

Biofortification aims to increase the concentration of essential trace elements in crops. More studies can be inspired by using chelants to enhance the uptake and accumulation of essential trace elements in crop species.

8. References

Adams, F., Janssens, K., Snigirev, A., 1998. Microscopic X-ray fluorescence analysis and related methods with laboratory and synchrotron radiation sources Plenary Lecture. J. Anal. At. Spectrom. 13, 319–31.

Adrees, M., Ali, S., Rizwan, M., Ibrahim, M., Abbas, F., Farid, M., Zia-ur-Rehman, M., Irshad, M.K., Bharwana, S.A., 2015. The effect of excess copper on growth and physiology of important food crops: a review. Environ. Sci. Pollut. Res. 22, 8148–8162.

Ali, H., Khan, E., Sajad, M.A., 2013. Phytoremediation of heavy metals-concepts and applications. Chemosphere 91, 869–81.

Alloway, B., 2013. Heavy metals in soils: trace metals and metalloids in soils and their bioavailability. UK:Springer.

Almaroai, Y. A., Usman, A.R. A., Ahmad, M., Kim, K. R., Vithanage, M., Sik Ok, Y., 2013. Role of chelating agents on release kinetics of metals and their uptake by maize from chromated copper arsenate-contaminated soil. Environ. Technol. 34, 747–755.

Arienzo, M., Adamo, P., Cozzolino, V., 2004. The potential of Lolium perenne for revegetation of contaminated soil from a metallurgical site. Sci. Total Environ. 319, 13–25.

AzoozMM, Abou-ElhamdMF, Al-FredanMA, 2012. Biphasic effect of copper on growth, proline, lipid peroxidation and antioxidant enzyme activities of wheat (Triticum aestivum'cv. Hasaawi) at early growing stage. Aust J Crop Sci 6,688–694.

Bais, H.P., Weir, T.L., Perry, L.G., Gilroy, S., Vivanco, J.M., 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. Annu. Rev. Plant Biol. 57, 233–66.

Baker, A.J.M., 1981. Accumulators and excluders-strategies in the response of plants to heavy metals. J. Plant Nutr. 3, 643–654.

Barber, S.A., 1962. A diffusion and mass-flow concept of soil nutrient availability. Soil Sci.

Barberon, M., 2017. The endodermis as a checkpoint for nutrients. New Phytol. 4, 1604-1610.

Becker, J.S., Niehren, S., Matusch, A., Wu, B., Hsieh, H.F., Kumtabtim, U., Hamester,

M., Plaschke-Schlütter, A., Salber, D., 2010. Scaling down the bioimaging of metals by laser microdissection inductively coupled plasma mass spectrometry (LMD-ICP-MS). Int. J. Mass Spectrom. 294, 1–6.

Begonia, M.T., Begonia, G.B., Ighoavodha, M., Gilliard, D., 2005. Lead accumulation by tall fescue (Festuca arundinacea schreb.) grown on a lead-contaminated soil. Int. J. Environ. Res. Public Health 2, 228–233.

Begum, Z. a., Rahman, I.M.M., Tate, Y., Sawai, H., Maki, T., Hasegawa, H., 2012. Remediation of toxic metal contaminated soil by washing with biodegradable aminopolycarboxylate chelants. Chemosphere 87, 1161–1170.

Beiyuan, J., Tsang, D.C.W., Valix, M., Zhang, W., Yang, X., Ok, Y.S., Li, X.D., 2017. Selective dissolution followed by EDDS washing of an e-waste contaminated soil: Extraction efficiency, fate of residual metals, and impact on soil environment. Chemosphere 166, 489–496.

Bisinger Jr, E., 2009. A new, biodegradable chelating agent with an excellent safety profile., in: 13th Annual Green Chemistry & Engineering Conference, 23–25 June 2009. College Park.

Blaylock, M.J., Salt, D.E., Dushenkob, S., Zakharova, O., Gussman, C., Kapulnik, Y., Ensley, B.D., Raskin, I., 1997. Enhanced accumulation of Pb in Indian Mustard by soil-applied chelating agents. Environ. Sci. Technol. 31, 860–865.

Bleuet, P., Simionovici, A., Lemelle, L., Ferroir, T., Cloetens, P., Tucoulou, Ŕ., Susini, J., 2008. Hard X-rays nanoscale fluorescence imaging of Earth and Planetary science samples. Appl. Phys. Lett. 92, 90–93.

Blossfeld, S., Perriguey, J., Sterckeman, T., Morel, J.-L., Lösch, R., 2009. Rhizosphere pH dynamics in trace-metal-contaminated soils, monitored with planar pH optodes. Plant Soil 330, 173–184.

Bolan, N., Kunhikrishnan, A., Thangarajan, R., Kumpiene, J., Park, J., Makino, T., Kirkham, M.B., Scheckel, K., 2014. Remediation of heavy metal(loid)s contaminated soils-to mobilize or to immobilize? J. Hazard. Mater. 266, 141–66.

Bowen, G.D., Rovira, A.D., 1999. The rhizosphere and its management to improve plant growth. Adv. Agron. 66, 1–102.

Briat, J.F., Lebrun, M., 1998. Plant responses to metal toxicity. C. R. Acad. Sci. III. 322, 43–54.

Brun, L.A., Maillet, J., Richarte, J., Herrmann, P., Remy, J.C., 1998. Relationships

between extractable copper, soil properties and copper uptake by wild plants in vineyard soils. Environ. Pollut. 102, 151–161.

Brune, A., Urbach, W., Dietz, K, J., 1994. Compartmentation and transport of zinc in barley primary leaves as basic mechanisms involved in zinc tolerance. Plant. Cell Environ. 17, 153–162.

Calmano, W., Mangold, S., Welter, E., 2001. An XAFS investigation of the artefacts caused by sequential extraction analyses of Pb-contaminated soils. Anal. Bioanal. Chem. 371, 823–830.

Cao, A., Carucci, A., Lai, T., La Colla, P., Tamburini, E., 2007. Effect of biodegradable chelating agents on heavy metals phytoextraction with *Mirabilis jalapa* and on its associated bacteria. Eur. J. Soil Biol. 43, 200–206.

Carminati, A., 2012. A Model of Root Water uptake coupled with rhizosphere dynamics. Vadose Zo. J. 11, 0.

Carrier, P., Baryla, A., Havaux, M., 2003. Cadmium distribution and microlocalization in oilseed rape (*Brassica napus*) after long-term growth on cadmium-contaminated soil. Planta 216, 939–950.

Castillo-Michel, H.A., Larue, C., Pradas del Real, A.E., Cotte, M., Sarret, G., 2016. Practical review on the use of synchrotron based micro- and nano- X-ray fluorescence mapping and X-ray absorption spectroscopy to investigate the interactions between plants and engineered nanomaterials. Plant Physiol. Biochem. 110, 13–32.

Cater, M.R., Gregorich, E.G., 2006. Soil sampling and methods of analysis. CRC press.

Cestone, B., Cuypers, A., Vangronsveld, J., Sgherri, C., Navari-Izzo, F., 2012a. The influence of EDDS on the metabolic and transcriptional responses induced by copper in hydroponically grown *Brassica carinata* seedlings. Plant Physiol. Biochem. 55, 43–51.

Cestone, B., Quartacci, M.F., Navari-Izzo, F., 2010. Uptake and translocation of CuEDDS complexes by *Brassica carinata*. Environ. Sci. Technol. 44, 6403–8.

Cestone, B., Vogel-Mikuš, K., Quartacci, M.F., Rascio, N., Pongrac, P., Pelicon, P., Vavpetič, P., Grlj, N., Jeromel, L., Kump, P., Nečemer, M., Regvar, M., Navari-Izzo, F., 2012b. Use of micro-PIXE to determine spatial distributions of copper in *Brassica carinata* plants exposed to $CuSO_4$ or CuEDDS. Sci. Total Environ. 427–428, 339–46.

Chander, K., Joergensen, R.G., 2008. Decomposition of Zn-rich *Arabidopsis halleri*

litter in low and high metal soil in the presence and absence of EDTA. Water. Air. Soil Pollut. 188, 195–204.

Chaney, R.L., Brown, J.C., Tiffin, L.O., 1972. Obligatory reduction of ferric chelates in iron uptake by soybeans. Plant Physiol. 50, 208–213.

Chen, H., Zheng, C., Cong, T., Zhu, Y., 1999. Heavy metal pollution in soils in China : status and countermeasures. Ambio 28, 130–134.

Chen, J., 2007. Rapid urbanization in China: A real challenge to soil protection and food security. Catena 69, 1–15.

Chen, Y.H., Li, X.D., Shen, Z.G., 2004. Leaching and uptake of heavy metals by ten different species of plants during an EDTA-assisted phytoextraction process. Chemosphere 57, 187–196.

Cieśliński, G., Van Rees, K.C.J., Szmigielska, A.M., Krishnamurti, G.S.R., Huang, P.M., 1998. Low-molecular-weight organic acids in rhizosphere soils of durum wheat and their effect on cadmium bioaccumulation. Plant Soil 203, 109–117.

Clarkson, D.T., 1991. Root structure and sites of ion uptake. Plant roots hidden half 417–453.

Colangelo, E.P., Guerinot, M. Lou, 2006. Put the metal to the petal: metal uptake and transport throughout plants. Curr. Opin. Plant Biol. 9, 322–330.

Collin, B., Doelsch, E., Keller, C., Cazevieille, P., Tella, M., Chaurand, P., Panfili, F., Hazemann, J.L., Meunier, J.D., 2014. Evidence of sulfur-bound reduced copper in bamboo exposed to high silicon and copper concentrations. Environ. Pollut. 187, 22–30.

Conesa, H.M., Evangelou, M.W.H., Robinson, B.H., Schulin, R., 2012. A critical view of current state of phytotechnologies to remediate soils: still a promising tool? Scientific World Journal. 2012, 173-829.

Cui, J.L., Jing, C.Y., Che, D.S., Zhang, J.F., Duan, S.X., 2015. Groundwater arsenic removal by coagulation using ferric(III) sulfate and polyferric sulfate: A comparative and mechanistic study. J. Environ. Sci. 32, 42–53.

Cui, J.L., Luo, C.L., Tang, C.W.Y., Chan, T.S., Li, X.D., 2017a. Speciation and leaching of trace metal contaminants from e-waste contaminated soils. J. Hazard. Mater. 329, 150–158.

Cui, J.L., Shi, J.B., Jiang, G. Bin, Jing, C.Y., 2013. Arsenic levels and speciation from

ingestion exposures to biomarkers in Shanxi, China: Implications for human health. Environ. Sci. Technol. 47, 5419–5424.

Cui, J.L., Zhao, Y.P., Li, J.S., Beiyuan, J.Z., Tsang, D.C.W., Poon, C., Chan, T.S., Wang, W.X., Li, X.D., 2017b. Speciation, mobilization, and bioaccessibility of arsenic in geogenic soil profile from Hong Kong. Environ. Pollut. 1–10.

Dakora, F., Phillips, D., 2002. Root exudates as mediators of mineral acquisition in low-nutrient environments. Plant Soil 245, 35–47.

Davis, T.A., Volesky, B., Mucci, A., 2003. A review of the biochemistry of heavy metal biosorption by brown algae. Water Res. 37, 4311–4330.

De Schamphelaere, K.A.C., Janssen, C.R., 2002. A biotic ligand model predicting acute copper toxicity for Daphnia magna: the effects of calcium, magnesium, sodium, potassium, and pH. Environ. Sci. Technol. 36, 48–54.

Degryse, F., Shahbazi, A., Verheyen, L., Smolders, E., 2012. Diffusion limitations in root uptake of cadmium and zinc, but not nickel, and resulting bias in the Michaelis constant. Plant Physiol. 160, 1097–1109.

Dello Ioio, R., Nakamura, K., Moubayindin, L., Perilli, S., Taniguchi, M., T. Morita, M., Aoyama, T., Costantino, P., Sabatini, S., 2008. A genetic framework for genetic control of cell division and differentiation in the root meristem. Science (80). 1380, 1543–1547.

Dick, R.P., 2011. Methods of soil enzymology. Soil Science Society of America, Madison.

Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K., Scheres, B., 1993. Cellular organisation of the *Arabidopsis thaliana* root. Development 119, 71–84.

Doblas, V.G., Geldner, N., Barberon, M., 2017. The endodermis, a tightly controlled barrier for nutrients. Curr. Opin. Plant Biol. 39, 136-143.

Dresler S, Hanaka A, Bednarek W, Maksymiec W, 2014. Accumulation of low-molecular-weight organic acids in roots and leaf segments of *Zea mays* plants treated with cadmium and copper. Acta Physiol Plant. 36, 1565–1575.

Duquène, L., Vandenhove, H., Tack, F., Meers, E., Baeten, J., Wannijn, J., 2009. Enhanced phytoextraction of uranium and selected heavy metals by Indian mustard and ryegrass using biodegradable soil amendments. Sci. Total Environ. 407, 1496–1505.

Eivazi, F., Tabatabai, M.A., 1977. Phosphatase in soils. Soil Biol. Biochem. 9, 167–172.

Epelde, L., Hernández-Allica, J., Becerril, J.M., Blanco, F., Garbisu, C., 2008. Effects of chelates on plants and soil microbial community: comparison of EDTA and EDDS for lead phytoextraction. Sci. Total Environ. 401, 21–8.

European copper institute, 2010. http://www.eurocopper.org/copper/copper-information.html

Evangelou, M.W.H., Bauer, U., Ebel, M., Schaeffer, A., 2007. The influence of EDDS and EDTA on the uptake of heavy metals of Cd and Cu from soil with tobacco *Nicotiana tabacum*. Chemosphere 68, 345–53.

Fabbricino, A., Fabbricino, M., Hullebusch, ERic, D. va., Esposit, G., Francesco, P., 2016. Effect of soil/contamination characteristics and process operational conditions on aminopolycarboxylates enhanced soil washing for heavy metals removal: a review. Rev. Environ. Sci. Bio/Technology 15, 111–145.

Fang, L.C., Wang, M.K., Cai, L., Cang, L., 2017. Deciphering biodegradable chelant-enhanced phytoremediation through microbes and nitrogen transformation in contaminated soils. Environ. Sci. Pollut. Res. 24, 14627–14636.

Farid, M., Ali, S., Shakoor, M.B., Bharwana, S.A., Rizvi, H., Ehsan, S., Tauqeer, H.M., Iftikhar, U., Hannan, F., 2013. EDTA assisted phytoremediation of cadmium, lead and zinc. Int. J. Agron. Plant Prod. 4, 2833–2846.

Forsberg, L.S., Kleja, D.B., Greger, M., Ledin, S., 2009. Effects of sewage sludge on solution chemistry and plant uptake of Cu in sulphide mine tailings at different weathering stages. Appl. Geochemistry 24, 475–482.

Gardea-Torresdey, J.L., Peralta-Videa, J.R., De La Rosa, G., Parsons, J.G., 2005. Phytoremediation of heavy metals and study of the metal coordination by X-ray absorption spectroscopy. Coord. Chem. Rev. 249, 1797–1810.

Gerhardt, K.E., Gerwing, P.D., Greenberg, B.M., 2016. Opinion: Taking phytoremediation from proven technology to accepted practice. Plant Sci. 256, 170–185.

Gerhardt, K.E., Huang, X.D., Glick, B.R., Greenberg, B.M., 2009. Phytoremediation and rhizoremediation of organic soil contaminants: Potential and challenges. Plant Sci. 176, 20–30.

Geldner, N., 2013. The Endodermis. Annu. Rev. Plant Biol. 64, 531–558.

Goldberg, S., 1989. Interaction of aluminum and iron oxides and clay minerals and their effect on soil physical properties: A review. Commun. Soil Sci. Plant Anal. 20, 1181–1207.

Gräfe, M., Donner, E., Collins, R.N., Lombi, E., 2014. Speciation of metal(loid)s in environmental samples by X-ray absorption spectroscopy: A critical review. Anal. Chim. Acta 822, 1–22.

Grčman, H., Vodnik, D., Velikonja-Bolta, Š., Leštan, D., 2003. Ethylenediaminedissuccinate as a new chelate for environmentally safe enhanced lead phytoextraction. J. Environ. Qual. 32, 500.

Greipsson, S., 2011. Phytoremediation. Nat. Educ. Knowl. 3, 7.

Grossl, P.R., Sparks, D.L., Ainsworth, C.C., 1994. Rapid kinetics of Cu(II) adsorption/desorption on goethite. Environ. Sci. Technol. 28, 1422–1429.

Guan, S. ying, 1986. Soil enzymes and research methods. China Agricultural Press, Beijing.

Guigues, S., Bravin, M.N., Garnier, C., Masion, A., Chevassus-Rosset, C., Cazevieille, P., Doelsch, E., 2016. Involvement of nitrogen functional groups in high-affinity copper binding in tomato and wheat root apoplasts: spectroscopic and thermodynamic evidence. Metallomics 8, 366–376.

Gunawardana, B., Singhal, N., Johnson, A., 2009. Amendments and their combined application for enhanced copper, cadmium, lead uptake by Lolium perenne. Plant Soil 329, 283–294.

Gunawardana, B., Singhal, N., Johnson, A., 2011. Effects of amendments on copper, cadmium, and lead phytoextraction by *Lolium Perenne* from Multiple-Metal Contaminated Solution. Int. J. Phytoremediation 13, 215–232.

Guo, H.Y., Wang, W., Sun, Y.Y., Li, H., Ai, F.X., Xie, L., Wang, X.R., 2010. Ethyl lactate enhances ethylenediaminedisuccinic acid solution removal of copper from contaminated soils. J. Hazard. Mater. 174, 59–63.

Gupta, D.K., Corpas, F.J., Palma, J.M., 2013. Heavy metal stress in plants, Plant stress biology. Berlin, DE: Springer.

Gupta, S.K., Vollmer, M.K., Krebs, R., 1996. The importance of mobile, mobilisable and pseudo total heavy metal fractions in soil for three-level risk assessment and risk management. Sci. Total Environ. 178, 11–20.

Hadi, F., Bano, A., Fuller, M.P., 2010. The improved phytoextraction of lead (Pb) and the growth of maize (*Zea mays* L.): the role of plant growth regulators (GA3 and IAA) and EDTA alone and in combinations. Chemosphere 80, 457–62.

Hall, J.L., 2002. Cellular mechanisms for heavy metal detoxification and tolerance. J. Exp. Bot. 53, 1–11.

Hall, J.L., Williams, L.E., 2003. Transition metal transporters in plants. J. Exp. Bot. 54, 2601–2613.

Haney, C.J., Grass, G., Franke, S., Rensing, C., 2005. New developments in the understanding of the cation diffusion facilitator family. J. Ind. Microbiol. Biotechnol. 32, 215–226.

Hauser, L., Tandy, S., Schulin, R., Nowack, B., 2005. Column extraction of heavy metals from soils using the biodegradable chelating agent EDDS. Environ. Sci. Technol. 39, 6819–6824.

Haynes, R.J., 2012. Mineral Nitrogen in the Plant-Soil System, Mineral Nitrogen in the Plant-Soil System. Elsevier.

He, S., Wu, Q., He, Z., 2013. Effect of DA-6 and EDTA alone or in combination on uptake, subcellular distribution and chemical form of Pb in *Lolium perenne*. Chemosphere 93, 2782–8.

Hodge, A., 2004. The plastic plant: root responses to heterogeneous supplies of nutrients. New Phytol. 162, 9–24.

Houba, V.J.G., Temminghoff, E.J.M., Gaikhorst, G. a., van Vark, W., 2000. Soil analysis procedures using 0.01 M calcium chloride as extraction reagent. Commun. Soil Sci. Plant Anal. 31, 1299–1396.

Huang, J.W., Cunningham, S.D., 1996. Lead phytoextraction: species variation in lead uptake and translocation. New Phytol. 134, 75–84.

Irtelli, B., Petrucci, W.A., Navari-Izzo, F., 2009. Nicotianamine and histidine/proline are, respectively, the most important copper chelators in xylem sap of Brassica carinata under conditions of copper deficiency and excess. J. Exp. Bot. 60, 269–277.

Itrich, N.R., McDonough, K.M., van Ginkel, C.G., Bisinger, E.C., LePage, J.N., Schaefer, E.C., Menzies, J.Z., Casteel, K.D., Federle, T.W., 2015. Widespread Microbial Adaptation to L-Glutamate-*N*,*N*-diacetate (L-GLDA) following its market introduction in a consumer cleaning product. Environ. Sci. Technol. 49, 13314–13321.

Jackson, R.B., Sperry, J.S., Dawson, T.E., 2000. Root water uptake and transport: Using physiological processes in global predictions. Trends Plant Sci. 5, 482–488.

Jacobson, A.R., Dousset, S., Andreux, F., Baveye, P.C., 2007. Electron microprobe and synchrotron X-ray fluorescence mapping of the heterogeneous distribution of copper in high-copper vineyard soils. Environ. Sci. Technol. 41, 6343–6349.

Jan, S., Ahmad Parray, J., 2016. Approaches to Heavy Metal Tolerance in Plants.

Japenga, J., Koopmans, G.F., Song, J., Römkens, P.F. a M., 2007. A feasibility test to estimate the duration of phytoextraction of heavy metals from polluted soils. Int. J. Phytoremediation 9, 115–132.

Jelusic, M., Grcman, H., Vodnik, D., Suhadolc, M., Lestan, D., 2013. Functioning of metal contaminated garden soil after remediation. Environ. Pollut. 174, 63–70.

Jelusic, M., Lestan, D., 2014. Effect of EDTA washing of metal polluted garden soils. Part I: Toxicity hazards and impact on soil properties. Sci. Total Environ. 475, 132–141.

Jelusic, M., Vodnik, D., Macek, I., Lestan, D., 2014. Effect of EDTA washing of metal polluted garden soils. Part II: Can remediated soil be used as a plant substrate. Sci. Total Environ. 475, 142–152.

Johnson, A., Gunawardana, B., Singhal, N., 2009. Amendments for enhancing copper uptake by Brassica Juncea and *Lolium Perenne* from solution. Int. J. Phytoremediation 11, 215–234.

Johnson A. Modelling chelate-enhanced phytoextraction mechanisms. ResearchSpace@ Auckland, 2012.

Johnson, A.C., Singhal, N., 2013. Influence of chelation on Cu distribution and barriers to translocation in Lolium perenne. Environ. Sci. Technol. 47, 7688–95.

Jones, D.L., 1998. Organic acids in the rhizosphere–a critical review. Plant Soil 205, 25–44.

Jones, D.L., Willett, V.B., 2006. Experimental evaluation of methods to quantify dissolved organic nitrogen (DON) and dissolved organic carbon (DOC) in soil. Soil Biol. Biochem. 38, 991–999.

Jouvin, D., Weiss, D.J., Mason, T.F.M., Bravin, M.N., Louvat, P., Zhao, F., Ferec, F., Hinsinger, P., Benedetti, M.F., 2012. Stable isotopes of Cu and Zn in higher plants: evidence for Cu reduction at the root surface and two conceptual models for isotopic

fractionation processes. Environ. Sci. Technol. 46, 2652–60.

Jungk, A., 2001. Root hairs and the acquisition of plant nutrients from soil. J. Plant Nutr. Soil Sci. 164, 121–129.

Kachenko, A.G., Singh, B., 2006. Heavy metals contamination in vegetables grown in urban and metal smelter contaminated sites in Australia. Water. Air. Soil Pollut. 169, 101–123.

Kahru, A., Ivask, A., Kasemets, K., Pollumaa, L., Kurvet, I., Francois, M., Dubourguier，Henri-Charles, 2005. Biotests and biosensors in ecotoxicological risk assessment of field soils polluted with zinc, lead, and cadmium. Environ. Toxicol. Chem. 24, 2973–2982.

Kamilova, F., Kravchenko, L. V, Shaposhnikov, A.I., Azarova, T., Makarova, N., Lugtenberg, B., 2006. Organic acids, sugars, and L-tryptophane in exudates of vegetables growing on stonewool and their effects on activities of rhizosphere bacteria. Mol. Plant-Microbe Interact. 19, 250–256.

Karczewska, A., Orlow, K., Kabala, C., Szopka, K., Galka, B., 2011. Effects of chelating compounds on mobilization and phytoextraction of copper and lead in contaminated soils. Commun. Soil Sci. Plant Anal. 42, 1379–1389.

Karlsson, T., Persson, P., Skyllberg, U., 2006. Complexation of copper(ll) in organic soils and in dissolved organic matter-EXAFS evidence for chelate ring structures. Environ. Sci. Technol. 40, 2623–2628.

Kasprzak, K.S., 2002. Oxidative DNA and protein damage in metal-induced toxicity and carcinogenesis 1,3. Free Radic. Biol. Med. 32, 958–967.

Katata, L., Nagaraju, V., Crouch, A.M., 2006. Determination of ethylenediaminetetraacetic acid, ethylenediaminedisuccinic acid and iminodisuccinic acid in cosmetic products by capillary electrophoresis and high performance liquid chromatography. Anal. Chim. Acta 579, 177–184.Kim, K.-R., Owens, G., Kwon, S., 2010. Influence of Indian mustard (*Brassica juncea*) on rhizosphere soil solution chemistry in long-term contaminated soils: A rhizobox study. J. Environ. Sci. 22, 98–105.

Kim, K.R., Owens, G., Kwon, S.L., 2010. Influence of Indian mustard (*Brassica juncea*) on rhizosphere soil solution chemistry in long-term contaminated soils: A rhizobox study. J. Environ. Sci. 22, 98–105.

Kirpichtchikova, T. a., Manceau, A., Spadini, L., Panfili, F., Marcus, M. a., Jacquet, T., 2006. Speciation and solubility of heavy metals in contaminated soil using X-ray

microfluorescence, EXAFS spectroscopy, chemical extraction, and thermodynamic modeling. Geochim. Cosmochim. Acta 70, 2163–2190.

Komárek, M., Tlustoš, P., Száková, J., Chrastný, V., Ettler, V., 2007. The use of maize and poplar in chelant-enhanced phytoextraction of lead from contaminated agricultural soils. Chemosphere 67, 640–651.

Komárek, M., Vanek, A., Mrnka, L., Sudová, R., Száková, J., Tejnecký, V., Chrastný, V., 2010. Potential and drawbacks of EDDS-enhanced phytoextraction of copper from contaminated soils. Environ. Pollut. 158, 2428–38.

Komárek, M., Vaněk, A., Száková, J., Balík, J., Chrastný, V., 2009. Interactions of EDDS with Fe- and Al-(hydr)oxides. Chemosphere 77, 87–93.

Koopmans, G.F., Schenkeveld, W.D.C., Song, J., Luo, Y., Japenga, J., Temminghoff, E.J.M., 2008. Influence of EDDS on metal speciation in soil extracts: measurement and mechanistic multicomponent modeling. Environ. Sci. Technol. 42, 1123–30.

Kopittke, P.M., Menzies, N.W., de Jonge, M.D., McKenna, B. a, Donner, E., Webb, R.I., Paterson, D.J., Howard, D.L., Ryan, C.G., Glover, C.J., Scheckel, K.G., Lombi, E., 2011. In situ distribution and speciation of toxic copper, nickel, and zinc in hydrated roots of cowpea. Plant Physiol. 156, 663–673.

Kos, B., Leštan, D., 2004. Chelator induced phytoextraction and in situ soil washing of Cu. Environ. Pollut. 132, 333–339.

Kos, B., Leštan, D., 2003. Influence of a biodegradable ([S,S]-EDDS) and nondegradable (EDTA) chelate and hydrogel modified soil water sorption capacity on Pb phytoextraction and leaching. Plant Soil 253, 403–411.

Kruck, T.P.A., Sarkar, B., 1973. Equilibria of the simultaneously existing multiple species in the copper(II)– L -histidine system. Can. J. Chem. 51, 3549–3554.

Krzesłowska, M., 2011. The cell wall in plant cell response to trace metals: Polysaccharide remodeling and its role in defense strategy. Acta Physiol. Plant. 33, 35–51.

Kumar, P.B.A.N., Dushenkov, V., Motto, H., Rakin, I., 1995. Phytoextraction: The use of plants to remove heavy metals from soils. Environ. Sci. Technol. 29, 1232–1238.

Lambrechts, T., Gustot, Q., Couder, E., Houben, D., Iserentant, A., Lutts, S., 2011. Comparison of EDTA-enhanced phytoextraction and phytostabilisation strategies with *Lolium perenne* on a heavy metal contaminated soil. Chemosphere 85, 1290–1298.

Lee, J., Sung, K., 2014. Effects of chelates on soil microbial properties, plant growth and heavy metal accumulation in plants. Ecol. Eng. 73, 386–394.

Lee, S. H., Kim, E.Y., Park, H., Yun, J., Kim, J. G., 2011. In situ stabilization of arsenic and metal-contaminated agricultural soil using industrial by-products. Geoderma 161, 1–7.

Leštan, D., Luo, C.L., Li, X.D., 2008. The use of chelating agents in the remediation of metal-contaminated soils: A review. Environ. Pollut. 153, 3–13.

Li, B., Kamiya, T., Kalmbach, L., Yamagami, M., Yamaguchi, K., Shigenobu, S., Sawa, S., Danku, J.M.C., Salt, D.E., Geldner, N., Fujiwara, T., 2017. Role of LOTR1 in Nutrient Transport through Organization of Spatial Distribution of Root Endodermal Barriers. Curr. Biol. 27, 758–765.

Li, X.D., Thornton, I., 1993. Multi-element contamination in soil and plant in the old mining area, UK. Appl. Geochemistry 8, 51–56.

Li, X.D., Coles, B.J., Ramsey, M.H., Thornton, I., 1995. Sequential extraction of soils for multielement analysis by ICP-AES. Chem. Geol. 124, 109–123.

Li, X.D., Poon, C.S., Liu, P.S., 2001. Heavy metal contamination of urban soils and street dusts in Hong Kong 16, 1361–1368.

Li, Y., Wang, Y. Bin, Gou, X., Su, Y.B., Wang, G., 2006. Risk assessment of heavy metals in soils and vegetables around non-ferrous metals mining and smelting sites, Baiyin, China. J. Environ. Sci. (China) 18, 1124–1134.

Li, Z.Y., Ma, Z.W., Kuijp, van der, Jan, T., Yuan, Z.W., Huang, L., 2014. A review of soil heavy metal pollution from mines in China: Pollution and health risk assessment. Sci. Total Environ. 468–469, 843–853.

Liu, D., Islam, E., Li, T., Yang, X., Jin, X., Mahmood, Q., 2008. Comparison of synthetic chelators and low molecular weight organic acids in enhancing phytoextraction of heavy metals by two ecotypes of *Sedum alfredii* Hance. J. Hazard. Mater. 153, 114–122.

Liu, D.H., Kottke, I., 2004. Subcellular localization of copper in the root cells of Allium sativum by electron energy loss spectroscopy (EELS). Bioresour. Technol. 94, 153–158.

Lombi, E., Scheckel, K.G., Kempson, I.M., 2011. In situ analysis of metal(loid)s in plants: State of the art and artefacts. Environ. Exp. Bot. 72, 3–17.

Lombi, E., Susini, J., 2009. Synchrotron-based techniques for plant and soil science: Opportunities, challenges and future perspectives. Plant Soil 320, 1–35.

Lou, Y.H., Luo, H.J., Hu, T., Li, H.Y., Fu, J.M., 2013. Toxic effects, uptake, and translocation of Cd and Pb in perennial ryegrass. Ecotoxicology 22, 207–14.

Lu, L.L, Tian, S.K., Zhang, J., Yang, X. E, Labavitch, J.M., Webb, S.M., Latimer, M., Brown, P.H., 2013. Efficient xylem transport and phloem remobilization of Zn in the hyperaccumulator plant species *Sedum alfredii*. New Phytol. 198, 721–731.

Lu, L.L., Xie, R.H., Liu, T., Wang, H.X., Hou, D. Di, Du, Y.H., He, Z.L., Yang, X.E., Sun, H., Tian, S.K., 2017. Spatial imaging and speciation of Cu in rice (*Oryza sativa L.*) roots using synchrotron-based X-ray microfluorescence and X-ray absorption spectroscopy. Chemosphere 175, 356–364.

Luo, C.L., Shen, Z.G., Li, X.D., 2005. Enhanced phytoextraction of Cu, Pb, Zn and Cd with EDTA and EDDS. Chemosphere 59, 1–11.

Luo, X.S., Yu, S., Zhu, Y.G., Li, X.D., 2011. Trace metal contamination in urban soils of China. Sci. Total Environ. 421–422, 17–30.

Ma, C., White, J.C., Dhankher, O.P., Xing, B., 2015. Metal-based nanotoxicity and detoxification pathways in higher plants. Environ. Sci. Technol.

Mahar, A., Wang, P., Ali, A., Awasthi, M.K., Lahori, A.H., Wang, Q., Li, R., Zhang, Z., 2016. Challenges and opportunities in the phytoremediation of heavy metals contaminated soils: A review. Ecotoxicol. Environ. Saf. 126, 111–121.

Majumdar, S., Peralta-Videa, J.R., Castillo-Michel, H., Hong, J., Rico, C.M., Gardea-Torresdey, J.L., 2012. Applications of synchrotron μ-XRF to study the distribution of biologically important elements in different environmental matrices: A review. Anal. Chim. Acta 755, 1–16.

Malviya, R., Chaudhary, R., 2006. Factors affecting hazardous waste solidification/stabilization: A review. J. Hazard. Mater. 137, 267–276.

Manceau, A., Marcus, M.A., Tamura, N., 2002. Quantitative speciation of heavy metals in soils and sediments by synchrotron X-ray techniques. Rev. Mineral. Geochemistry 49, 341–428.

Manceau, A., Simionovici, A., Lanson, M., Perrin, J., Tucoulou, R., Bohic, S., Fakra, S.C., Marcus, M.A., Bedell, J. P., Nagy, K.L., 2013. *Thlaspi arvense* binds Cu(II) as a bis-(L-histidinato) complex on root cell walls in an urban ecosystem. Metallomics 5, 1674–1684.

Manouchehri, N., Besancon, S., Bermond, A., 2006. Major and trace metal extraction from soil by EDTA : Equilibrium and kinetic studies. Anal. Chim. Acta 559, 105–112.

Margesin, R., Schinner, F., 2005. Manual for soil analysis - monitoring and assessing soil bioremediation. Springer Science & Business Media, Berlin, Heidelberg.

Mart ínez-Villegas, N., Mart ínez, C.E., 2008. Solid- and solution-phase organics dictate copper distribution and speciation in multicomponent systems containing ferrihydrite, organic matter, and montmorillonite. Environ. Sci. Technol. 42, 2833–2838.

Mcgrath, S.P., Zhao, F.J., Lombi, E., 2001. Plant and rhizosphere processes involved in phytoremediation of metal-contaminated soils 207–214.

Meers, E., Ruttens, A., Hopgood, M.J., Samson, D., Tack, F.M.G., 2005. Comparison of EDTA and EDDS as potential soil amendments for enhanced phytoextraction of heavy metals. Chemosphere 58, 1011–22.

Meers, E., Tack, F.M.G., Verloo, M.G., 2008. Degradability of ethylenediaminedisuccinic acid (EDDS) in metal contaminated soils: implications for its use soil remediation. Chemosphere 70, 358–63.

Mic ó, C., Recatal á, L., Peris, M., S ánchez, J., 2006. Assessing heavy metal sources in agricultural soils of an European Mediterranean area by multivariate analysis. Chemosphere 65, 863–872.

Milne, C.J., Kinniburgh, D.G., van Riemsdijk, W.H., Tipping, E., 2003. generic NICA-Donnan model parameters for metal-ion binding by humic substances. Environ. Sci. Technol. 37, 958–971.

Minkina, T.M., Nevidomskaya, D.G., Shuvaeva, V.A., Soldatov, A. V., Tsitsuashvili, V.S., Zubavichus, Y. V., Rajput, V.D., Burachevskaya, M. V., 2016. Studying the transformation of Cu^{2+} ions in soils and mineral phases by the XRD, XANES, and sequential fractionation methods. J. Geochemical Explor.

Ministry of Environmental Protection and Ministry of Land and Resources of P. R. China, 2014. Reports on China's Soil Pollution Survey; http://www.zhb.gov.cn/gkml/hbb/qt/201404/W020140417558995804588.pdf [in Chinese].

Mohtadi, A., Ghaderian, S.M., Schat, H., 2013. The effect of EDDS and citrate on the uptake of lead in hydroponically grown *Matthiola flavida*. Chemosphere 93, 986–9.

Moritsuka, N., Yanai, J., Kosaki, T., 2000. Effect of plant growth on the distribution and forms of soil nutrients in the rhizosphere. Soil Sci. Plant Nutr. 46, 439–447.

Mühlbachová, G., 2009. Microbial biomass dynamics after addition of EDTA into heavy metal contaminated soils. Plant Soil Environ. 55, 544–550.

Mühlbachová, G., 2011. Soil microbial activities and heavy metal mobility in long-term contaminated soils after addition of EDTA and EDDS. Ecol. Eng. 37, 1064–1071.

Mugica-Alvarez, V., Cortés-Jiménez, V., Vaca-Mier, M., Domínguez-Soria, V., 2015. Phytoremediation of mine tailings Using *Lolium Multiflorum*. Int. J. Environ. Sci. Dev. 6, 246.

Najeeb, U., Xu, L., Ali, S., Jilani, G., Gong, H.J., Shen, W.Q., Zhou, W.J., 2009. Citric acid enhances the phytoextraction of manganese and plant growth by alleviating the ultrastructural damages in Juncus effusus L. J. Hazard. Mater. 170, 1156–63. https://doi.org/10.1016/j.jhazmat.2009.05.084

Neugschwandtner, R.W., Tlustoš, P., Komárek, M., Száková, J., 2008. Phytoextraction of Pb and Cd from a contaminated agricultural soil using different EDTA application regimes: Laboratory versus field scale measures of efficiency. Geoderma 144, 446–454.

Neugschwandtner, R.W., Tlustoš, P., Komárek, M., Száková, J., Jakoubková, L., 2012. Chemically enhanced phytoextraction of risk elements from a contaminated agricultural soil using *Zea Mays* and *Triticum Aestivum*: performance and metal mobilization over a three year period. Int. J. Phytoremediation 14, 754–771.

Niu, L.Y., Shen, Z.G., 2009. Ion chromatographic analysis and mass spectrometric identification of complexes of metal and ethylenediamin edisuccinic acid in plants and soils. Chinese J. Chromatogr. 27, 829–834.

Niu, L.Y., Shen, Z.G., Luo, C.L., Deng, Y.E., Wang, C.C., 2011a. Accumulation mechanisms and subcellular distribution of Cu in maize grown on soil treated with [S, S]-ethylenediamine disuccinic acid. Plant Soil 351, 237–247.

Niu, L.Y., Shen, Z.G., Wang, C.C., 2011b. Sites, pathways, and mechanism of absorption of Cu-EDDS complex in primary roots of maize (*Zea Mays* L.): anatomical, chemical and histochemical analysis. Plant Soil 343, 303–312.

Nobel, P.S., 2009. Physicochemical and Environmental Plant Physiology, Academic Press.

Nowack, B., Federal, S., Zu, T., 2002. Critical review environmental chemistry of aminopolycarboxylate chelating agents. Environ. Sci. Technol. 36, 4009–4016.

Nowack, B., Schulin, R., Robinson, B.H., 2006. Critical assessment of chelant-enhanced metal phytoextraction. Environ. Sci. Technol. 40, 5225–5232.

Organization for Economic Cooperation and Development, 1992. *OECD Guidelines for the Testing of Chemicals. OECD TG 301 A-F: Ready Biodegradability*. Paris.

Orama, M., Hyvonen, H., Saarinen, H., Aksela, R., 2002. Complexation of [S,S] and mixed stereoisomers of N,N'-ethylenediaminedisuccinic acid (EDDS) with Fe(iii), Cu(ii), Zn(ii) and Mn(ii) ions in aqueous solution. J. Chem. Soc. Dalt. Trans. 4644–4648.

Parker, D.R., Pedler, J.F., 1997. Reevaluating the free-ion activity model of trace metal availability to higher plants, in: Plant and Soil. pp. 223–228.

Peacock, C.L., Sherman, D.M., 2004. Copper(II) sorption onto goethite, hematite and lepidocrocite: A surface complexation model based on ab initio molecular geometries and EXAFS spectroscopy. Geochim. Cosmochim. Acta 68, 2623–2637.

Péret, B., De Rybel, B., Casimiro, I., Benková, E., Swarup, R., Laplaze, L., Beeckman, T., Bennett, M.J., 2009. Arabidopsis lateral root development: an emerging story. Trends Plant Sci. 14, 399–408.

Pierzynski, G.M., Lambert, M., Hetrick, B.A.D., Sweeney, D.W., Erickson, L.E., 2002. Phytostabilization of metal mine tailings using tall fescue. Pract. Period. Hazardous, Toxic, Radioact. Waste Manag. 6, 212–217.

Pinto, I.S.S., Neto, I.F.F., Soares, H.M.V.M., 2014. Biodegradable chelating agents for industrial, domestic, and agricultural applications-a review. Environ. Sci. Pollut. Res. Int.

Prasad, M.N.V., 2003. Phytoremediation of Metal-Polluted Ecosystems: Hype for Commercialization. Russ. J. Plant Physiol. 50, 764–780.

Pugnaire, F.I., Valladares, F., 2007. Functional plant ecology, Environment.

Punshon, T., Tappero, R., Ricachenevsky, F.K., Hirschi, K., Nakata, P.A., 2013. Contrasting calcium localization and speciation in leaves of the *Medicago truncatula* mutant cod5 analyzed via synchrotron X-ray techniques. Plant J. 76, 627–633.

Qin, C., Luo, C.L., Chen, Y.H., Shen, Z.G., 2012. Spatial-based assessment of metal contamination in agricultural soils near an abandoned copper mine of Eastern China. Bull. Environ. Contam. Toxicol. 89, 113–118.

Quartacci, M.F., Irtelli, B., Baker, A.J.M., Navari-Izzo, F., 2007. The use of NTA and

EDDS for enhanced phytoextraction of metals from a multiply contaminated soil by *Brassica carinata*. Chemosphere 68, 1920–8.

Ravel, B., Newville, M., 2005. ATHENA, ARTEMIS, HEPHAESTUS: data analysis for X-ray absorption spectroscopy using IFEFFIT. J. Synchrotron Radiat. 12, 537–541.

Reeves, R.D., Baker, A.J.M., 2000. Phytoremediation of toxic metals. Using Plants to Clean Up Environ. John Wiley Sons, New York 193–229.

Reeves, R.D., Baker, A.J.M., 2000. Metal Accumulating Plants, in: Phytoremediation of Toxic Metals: Using Plants to Clean up the Environment. pp. 193–230.

Rekab, K., Lepeytre, C., Goettmann, F., Dunand, M., Guillard, C., Herrmann, J.M., 2015. Degradation of a cobalt(II)–EDTA complex by photocatalysis and H_2O_2/UV-C. Application to nuclear wastes containing ^{60}Co. J. Radioanal. Nucl. Chem. 303, 131–137.

Robinson, B.H., Leblanc, M., Petit, D., Brooks, R.R., Kirkman, J.H., Gregg, P.E.H., 1998. The potential of *Thlaspi caerulescens* for phytoremediation of contaminated soils. Plant Soil 203, 47–56.

Robinson, B.H., Mills, T.M., Petit, D., Fung, L.E., Green, S.R., Clothier, B.E., 2000. Natural and induced cadmium-accumulation in poplar and willow: Implications for phytoremediation. Plant Soil 227, 301–306.

Robinson, B.H., Anderson, C.W.N., Dickinson, N.M., 2015. Phytoextraction: Where's the action? J. Geochemical Explor. 151, 34–40.

Römheld, V., Marschner, H., 1983. Mechanism of iron uptake by plants. Plant Physiol. 71, 949–954.

Rose, J.K.C., 2003. The plant cell wall, Annual Plant Reviews, Volume 8.

Ryan, B.M., Kirby, J.K., Degryse, F., Harris, H., Mclaughlin, M.J., Scheiderich, K., 2013. Copper speciation and isotopic fractionation in plants: uptake and translocation mechanisms. New Phytol. 199, 367–378.

Saifullah, Meers, E., Qadir, M., De Caritat, P., Tack, F.M.G., Du Laing, G., Zia, M.H., 2009. EDTA-assisted Pb phytoextraction. Chemosphere 74, 1279–1291.

Salt, D.E., Blaylock, M., Kumar, N.P., Dushenkov, V., Ensley, B.D., Chet, I., Raskin, I., 1995. Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. Nat. Biotechnol. 13, 468–474.

Samardjieva, K. a, Pissarra, J., Castro, P.M.L., Tavares, F., 2011. Insights into phytoremediation solutions for environmental recovery. Recent Pat. Biotechnol. 5, 25–39.

Sarret, G., Isaure, M., Marcus, M.A., Harada, E., Choi, Y., Pairis, S., Fakra, S., Manceau, A., 2007. Chemical forms of calcium in Ca, Zn- and Ca, Cd- containing grains excreted by tobacco trichomes. Can. J. Chem. 746, 738–746.

Sarret, G., Vangronsveld, J., Manceau, A., Musso, M., D'Haen, J., Menthonnex, J.-J., Hazemann, J. L., 2001. Accumulation forms of Zn and Pb in *Phaseolus vulgaris* in the presence and absence of EDTA. Environ. Sci. Technol. 35, 2854–9.

Schaider, L.A., Parker, D.R., Sedlak, D.L., 2006. Uptake of EDTA-complexed Pb, Cd and Fe by solution- and sand-cultured *Brassica juncea*. Plant Soil 286, 377–391.

S éguin, V., Gagnon, C., Courchesne, F., 2004. Changes in water extractable metals, pH and organic carbon concentrations at the soil-root interface of forested soils. Plant Soil 260, 1–17.

Seth, C.S., Misra, V., Singh, R.R., Zolla, L., 2011. EDTA-enhanced lead phytoremediation in sunflower (*Helianthus annuus* L.) hydroponic culture. Plant Soil 347, 231–242.

Shahid, M., Pinelli, E., Dumat, C., 2012. Review of Pb availability and toxicity to plants in relation with metal speciation; role of synthetic and natural organic ligands. J. Hazard. Mater. 219–220, 1–12.

Sharma, S.S., Dietz, K. J., 2009. The relationship between metal toxicity and cellular redox imbalance. Trends Plant Sci. 14, 43–50.

Sharma, S.S., Dietz, K.J., Mimura, T., 2016. Vacuolar compartmentalization as indispensable component of heavy metal detoxification in plants. Plant, Cell Environ. 39, 1112–1126.

Shi, J.Y., Wu, B., Yuan, X.F., YY, C., Chen, X.C., Chen, Y.X., Hu, T.D., 2008. An X-ray absorption spectroscopy investigation of speciation and biotransformation of copper in *Elsholtzia splendens*. Plant Soil 302, 163–174.

Shi, J.Y., Yuan, X.F., Chen, X.C., Wu, B., Huang, Y.Y., Chen, Y.X., 2011. Copper uptake and its effect on metal distribution in root growth zones of *Commelina communis* revealed by SRXRF. Biol. Trace Elem. Res. 141, 294–304.

Shimizu, M., Arai, Y., Sparks, D.L., 2011. Multiscale assessment of methylarsenic reactivity in soil. 2. Distribution and speciation in soil. Environ. Sci. Technol. 45,

4300–4306.

Sjöstedt, C.S., Gustafsson, J.P., Köhler, S.J., 2010. Chemical equilibrium modeling of organic acids, pH, aluminum, and iron in Swedish surface waters. Environ. Sci. Technol. 44, 8587–8593.

Smolders, E., Buekers, J., Oliver, I., McLaughlin, M.J., 2004. Soil properties affecting toxicity of zinc to soil microbial properties in laboratory-spiked and field-contaminated soils. Environ. Toxicol. Chem. 23, 2633–2640.

Solé, V.A., Papillon, E., Cotte, M., Walter, P., Susini, J., 2007. A multiplatform code for the analysis of energy-dispersive X-ray fluorescence spectra. Spectrochim. Acta Part B At. Spectrosc. 62, 63–68.

Song, J., Yang, Y.Q., Zhu, S.H., Chen, G.C., Yuan, X.F., Liu, T.T., Yu, X.H., Shi, J.Y., 2013. Spatial distribution and speciation of copper in root tips of cucumber revealed by μ-XRF and μ-XANES. Biol. Plant. 57, 581–586.

Song, J., Zhao, F.J., Luo, Y.M., McGrath, S.P., Zhang, H., 2004. Copper uptake by *Elsholtzia splendens* and *Silene vulgaris* and assessment of copper phytoavailability in contaminated soils. Environ. Pollut. 128, 307–315.

Stebbing, A.R.D., 1982. Hormesis - The stimulation of growth by low levels of inhibitors. Sci. Total Environ. 22, 213–234.

Strawn, D.G., Baker, L.L., 2009. Molecular characterization of copper in soils using X-ray absorption spectroscopy. Environ. Pollut. 157, 2813–2821.

Strawn, D.G., Baker, L.L., 2008. Speciation of Cu in a contaminated agricultural soil measured by XAFS, μ-XAFS, and μ-XRF. Environ. Sci. Technol. 42, 37–42.

Sun, B., Zhao, F.J., Lombi, E., McGrath, S.P., 2001. Leaching of heavy metals from contaminated soils using EDTA. Environ. Pollut. 113, 111–120.

Tan, J., He, S., Yan, S., Li, Y., Li, H., Zhang, H., Zhao, L., Li, L., 2014. Exogenous EDDS modifies copper-induced various toxic responses in rice. Protoplasma 251, 1213–1221.

Tandy, S., Ammann, A., Schulin, R., Nowack, B., 2006a. Biodegradation and speciation of residual SS-ethylenediaminedisuccinic acid (EDDS) in soil solution left after soil washing. Environ. Pollut. 142, 191–199.

Tandy, S., Bossart, K., Mueller, R., Ritschel, J., Hauser, L., Schulin, R., Nowack, B., 2004. Extraction of heavy metals from soils using biodegradable chelating agents.

Environ. Sci. Technol. 38, 937–944.

Tandy, S., Schulin, R., Nowack, B., 2006b. Uptake of metals during chelant-assisted phytoextraction with EDDS related to the solubilized metal concentration. Environ. Sci. Technol. 40, 2753–2758.

Tandy, S., Schulin, R., Nowack, B., 2006c. The influence of EDDS on the uptake of heavy metals in hydroponically grown sunflowers. Chemosphere 62, 1454–63.

Tanton, T.W., Crowdy, S.H., 1972. Water pathways in higher plants. II. water pathways in roots. J. Exp. Bot. 23, 600–618.

Tao, Q., Jupa, R., Luo, J.P., Lux, A., Kováč, J., Wen, Y., Zhou, Y.M., Jan, J., Liang, Y.C., Li, T.Q., 2016. The apoplasmic pathway via the root apex and lateral roots contributes to Cd hyperaccumulation in the hyperaccumulator *Sedum alfredii*. J. Exp. Bot. 68, 739–751.

Tao, S., Chen, Y.J., Xu, F.L., Cao, J., Li, B.G., 2003. Changes of copper speciation in maize rhizosphere soil. Environ. Pollut. 122, 447–454.

Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., Sutton, D.J., 2012. Heavy metals toxicity and the environment, Molecular, clinical and environmental toxicology. Springer, Basel.

Tenginkai, S.G., Ugarkar, A.G., Koti, M. V., Mookherjee, A., 1991. Copper-bearing clay minerals of the oxidized zone of the Rakha-Chapri Block, Singhbhum Copper Belt, India. Proc. Indian Acad. Sci. - Earth Planet. Sci. 100, 13–29.

Tessier, A., Campbell, P.G.C., Bisson, M., 1979. Sequential extraction procedure for the speciation of particulate trace metals. Anal. Chem. 51, 844–851.

Tian, S., Lu, L., Yang, X., Webb, S.M., Du, Y., Brown, P.H., 2010. Spatial imaging and speciation of lead in the accumulator plant *Sedum alfredii* by microscopically focused synchrotron X-ray investigation. Environ. Sci. Technol. 44, 5920–5926.

Tian, S.K., Lu, L.L., Yang, X.E., Huang, H.G., Brown, P., Labavitch, J., Liao, H.B., He, Z.L., 2011. The impact of EDTA on lead distribution and speciation in the accumulator *Sedum alfredii* by synchrotron X-ray investigation. Environ. Pollut. 159, 782–788.

Tong, Y.P., Kneer, R., Zhu, Y.G., 2004. Vacuolar compartmentalization: A second-generation approach to engineering plants for phytoremediation. Trends Plant Sci. 9, 7–9.

Tsang, D.C.W., Hartley, N.R., 2013. Metal distribution and spectroscopic analysis after soil washing with chelating agents and humic substances. Environ. Sci. Pollut. Res. Int.

Tsang, D.C.W., Yip, T.C.M., Lo, I.M.C., 2009. Kinetic interactions of EDDS with soils. 2. metal−EDDS complexes in uncontaminated and metal-contaminated soils. Environ. Sci. Technol. 43, 837–842.

Tsang, D.C.W., Zhang, W., Lo, I.M.C., 2007. Copper extraction effectiveness and soil dissolution issues of EDTA-flushing of artificially contaminated soils. Chemosphere 68, 234–243.

Usman, A.R. a, Almaroai, Y. a, Ahmad, M., Vithanage, M., Ok, Y.S., 2013. Toxicity of synthetic chelators and metal availability in poultry manure amended Cd, Pb and As contaminated agricultural soil. J. Hazard. Mater. 262, 1022–30.

Vamerali, T., Bandiera, M., Mosca, G., 2010. Field crops for phytoremediation of metal-contaminated land. A review. Environ. Chem. Lett. 8, 1–17.

Van Ginkel C G, Geerts R, Nguyen P D., 2005. Biodegradation of L-glutamatediacetate by mixed cultures and an isolate. ACS Symp. Ser. 910, 183–194.

Vance, E.D., Brookes, P.C., Jenkinson, D.S., 1987. An extraction method for measuring soil microbial biomass C. Soil Biol. Biochem. 19, 703–707.

Van Nevel, L., Mertens, J., Oorts, K., Verheyen, K., 2007. Phytoextraction of metals from soils: How far from practice? Environ. Pollut. 150, 34–40.

Vandevivere, P.C., Saveyn, H., Verstraete, W., Feijtel, T.C.J., Schowanek, D.R., 2001. Biodegradation of metal-[S,S]-EDDS complexes. Environ. Sci. Technol. 35, 1765–1770.

Vandevivere, P.C., Saveyn, H., Verstraete, W., Feijtel, T.C.J., Schowanek, D.R., 2001. Biodegradation of Metal−[S,S]-EDDS Complexes. Environ. Sci. Technol. 35, 1765–1770.

Vassil, A., Kapulnik, Y., Raskin, I., Salt, D., 1998. The role of EDTA in lead transport and accumulation by Indian mustard. Plant Physiol. 117, 447–53.

Venecio Jr, U., Yano, a, Iwasaki, K., Tanaka, S., Kang, Y., Sakurai, K., 2005. Influence of chelating agent addition on copper distribution and microbial activity in soil and copper uptake by brown mustard (*Brassica juncea*). Soil Sci. Plant Nutr. 51, 193–202.

Verbelen, J.-P., De Cnodder, T., Le, J., Vissenberg, K., Baluska, F., 2006. The root apex of *Arabidopsis thaliana* consists of four distinct zones of growth activities: meristematic zone, transition zone, fast elongation zone and growth terminating zone. Plant Signal. Behav. 1, 296–304.

Vulkan, R., Zhao, F.J., Barbosa-Jefferson, V., Preston, S., Paton, G.I., Tipping, E., McGrath, S.P., 2000. Copper speciation and impacts on bacterial biosensors in the pore water of copper-contaminated soils. Environ. Sci. Technol. 34, 5115–5121.

Wallace, A., Mueller, R.T., Lunt, O.R., Ashcroft, R.T., Shannon, L.M., 1955. Comparision of five chelating agents in soils, in nutrient solutions, and in plant responses. Soil Sci. 80, 101–108.

Walsh, M.J., Goodnow, S.D., Vezeau, G.E., Richter, L. V., Ahner, B. a., 2015. Cysteine Enhances Bioavailability of Copper to Marine Phytoplankton. Environ. Sci. Technol. 49, 12145–12152.

Wang, A.G., Luo, C.L., Yang, R.X., Chen, Y.H., Shen, Z.G., Li, X.D., 2012. Metal leaching along soil profiles after the EDDS application–A field study. Environ. Pollut. 164, 204–210.

Wang, P., Menzies, N.W., Lombi, E., McKenna, B.A., de Jonge, M.D., Donner, E., Blamey, F.P.C., Ryan, C.G., Paterson, D.J., Howard, D.L., James, S.A., Kopittke, P.M., 2013. Quantitative determination of metal and metalloid spatial distribution in hydrated and fresh roots of cowpea using synchrotron-based X-ray fluorescence microscopy. Sci. Total Environ. 463–464, 131–139.

Wang, Z.W., Shan, X.Q., Zhang, S.Z., 2002. Comparison between fractionation and bioavailability of trace elements in rhizosphere and bulk soils. Chemosphere 46, 1163–1171.

Wasay, S. a, Parker, W.J., Van Geel, P.J., 2001. Contamination of a calcareous soil by battery industry wastes. I. characterization. Can. J. Civ. Eng. 28, 341–348.

Weis, J.S., Weis, P., 2004. Metal uptake, transport and release by wetland plants: Implications for phytoremediation and restoration. Environ. Int. 30, 685–700.

Welch, R.M., Norvell, W.A., Schaefer, S.C., Shaff, J.E., Kochian, L. V., 1993. Induction of iron(III) and copper(II) reduction in pea (*Pisum sativum* L.) roots by Fe and Cu status: Does the root-cell plasmalemma Fe(III)-chelate reductase perform a general role in regulating cation uptake? Planta 190, 555–561.

Wenzel, W.W., Jockwer, F., 1999. Accumulation of heavy metals in plants grown on mineralised soils of the Austrian Alps. Environ. Pollut. 104, 145–155.

Whiting, S.N., Broadley, M.R., White, P.J., 2003. Applying a solute transfer model to phytoextraction: Zinc acquisition by *Thlaspi caerulescens*. Plant Soil 249, 45–56.

Witschel, M., Egli, T., 1997. Purification and characterization of a lyase from the EDTA-degrading bacterial strain DSM 9103 that catalyzes the splitting of [S,S]-ethylenediaminedisuccinate, a structural isomer of EDTA. Biodegradation 8, 419–428.

Wójcik, M., Tukendorf, A., 1999. Cd-tolerance of maize, rye and wheat seedlings. Acta Physiol. Plant. 21, 99–107.

Wong, C.S.C., Li, X.D., Thornton, I., 2006. Urban environmental geochemistry of trace metals. Environ. Pollut. 142, 1–16.

Wuana, R.A., Okieimen, F.E., 2010. Phytoremediation potential of maize (*Zea mays L.*). A Review 6, 275–287.

Wu, F., Liu, Y.L., Xia, Y., Shen, Z.G., Chen, Y.H., 2011. Copper contamination of soils and vegetables in the vicinity of Jiuhuashan copper mine, China. Environ. Earth Sci. 64, 761–769.

Wu, J., Hsu, F.C., Cunningham, S.D., 1999. Chelate-assisted Pb phytoextraction: Pb availability, uptake and translocation constraints. Environ. Sci. Technol. 33, 1898–1904.

Wu, L.H., Sun, X.F., Luo, Y.M., Xing, X.R., Christie, P., 2007. Influence of [S, S]-EDDS on phytoextraction of copper and zinc by *Elsholtzia splendens* from metal-contaminated soil. Int. J. Phytoremediation 9, 227–41.

Wu, Q., Cui, Y., Li, Q., Sun, J., 2015. Effective removal of heavy metals from industrial sludge with the aid of a biodegradable chelating ligand GLDA. J. Hazard. Mater. 283, 748–754.

Xie, X.M., Liao, M., Fang, S., Peng, Y., Yang, J., Chai, J.J., 2012. Spatial characteristics of pyrene degradation and soil microbial activity with the distance from the ryegrass (Lolium perenne L.) root surface in a multi-interlayer rhizobox. J. Hazard. Mater. 213–214, 156–160.

Xie, Y., Yu, D., 2003. The significance of lateral roots in phosphorus (P) acquisition of water hyacinth (*Eichhornia crassipes*). Aquat. Bot. 75, 311–321.

Xu J.K., Yang L.X., Wang Z.Q., Dong G.C., Huang J.Y., Wang Y.L., 2006. Toxicity of copper on rice growth and accumulation of copper in rice grain in copper contaminated soil. Chemosphere. 62, 602–607.

Xu, W. H., Liu, H., Ma, Q. F., Xiong, Z. T., 2007. Root exudates, rhizosphere Zn fractions, and Zn accumulation of ryegrass at different soil Zn levels. Pedosphere 17, 389–396.

Yan, D.Y.S., Lo, I.M.C., 2011. Enhanced multi-metal extraction with EDDS of deficient and excess dosages under the influence of dissolved and soil organic matter. Environ. Pollut. 159, 78–83.

Yan, D.Y.S., Yip, T.C.M., Yui, M.M.T., Tsang, D.C.W., Lo, I.M.C., 2010. Influence of EDDS-to-metal molar ratio, solution pH, and soil-to-solution ratio on metal extraction under EDDS deficiency. J. Hazard. Mater. 178, 890–894.

Yang, J., Liu, J., Dynes, J.J., Peak, D., Regier, T., Wang, J., Zhu, S., Shi, J., Tse, J.S., 2014. Speciation and distribution of copper in a mining soil using multiple synchrotron-based bulk and microscopic techniques. Environ. Sci. Pollut. Res. 21, 2943–2954.

Yang, L., Wang, G., Cheng, Z., Liu, Y., Shen, Z.G., Luo, C.L., 2013. Influence of the application of chelant EDDS on soil enzymatic activity and microbial community structure. J. Hazard. Mater. 262, 561–570.

Yang, R.X., Luo, C.L., Zhang, G., Li, X.D., Shen, Z.G., 2012. Extraction of heavy metals from e-waste contaminated soils using EDDS. J. Environ. Sci. 24, 1985–1994.

Yip, T.C.M., Tsang, D.C.W., Lo, I.M.C., 2010a. Interactions of chelating agents with Pb-goethite at the solid–liquid interface: Pb extraction and re-adsorption. Chemosphere 81, 415–421.

Yip, T.C.M., Tsang, D.C.W., Ng, K.T.W., Lo, I.M.C., 2009. Kinetic Interactions of EDDS with Soils. 1. Metal Resorption and Competition under EDDS Deficiency. Environ. Sci. Technol. 43, 831–836.

Yip, T.C.M., Yan, D.Y.S., Yui, M.M.T., Tsang, D.C.W., Lo, I.M.C., 2010b. Heavy metal extraction from an artificially contaminated sandy soil under EDDS deficiency: Significance of humic acid and chelant mixture. Chemosphere 80, 416–421.

Ylivainio, K., 2010. Effects of iron(III)chelates on the solubility of heavy metals in calcareous soils. Environ. Pollut. 158, 3194–3200.

Yruela, I., 2009. Copper in plants: Acquisition, transport and interactions. Funct. Plant Biol. 36, 409–430.

Yuan, S.H., Wu, X.F., Wan, J.Z., Long, H.Y., Lu, X.H., Wu, X.H., Chen, J., 2010.

Enhanced washing of HCB and Zn from aged sediments by TX-100 and EDTA mixed solutions. Geoderma 156, 119–125.

Zhang, H., Zhao, F.J., Sun, B., Davison, W., Mcgrath, S.P., 2001. A new method to measure effective soil solution concentration predicts copper availability to plants. Environ. Sci. Technol. 35, 2602–2607.

Zhang, S.C., Yang, H., Singh, L., 2014. The remobilization of metals from iron oxides and sediments by metal-EDTA complexes. CEUR Workshop Proc. 1225, 41–42.

Zhang, W., Huang, Z., He, L., Sheng, X., 2012. Assessment of bacterial communities and characterization of lead-resistant bacteria in the rhizosphere soils of metal-tolerant *Chenopodium ambrosioides* grown on lead-zinc mine tailings. Chemosphere 87, 1171–8.

Zhang, W.H., Huang, H., Tan, F.F., Wang, H., Qiu, R.L., 2010. Influence of EDTA washing on the species and mobility of heavy metals residual in soils. J. Hazard. Mater. 173, 369–376.

Zhao, F.J., Lombi, E., McGrath, S.P., 2003. Assessing the potential for zinc and cadmium phytoremediation with the hyperaccumulator *Thlaspi caerulescens*. Plant Soil 249, 37–43.

Zhao, F.J., Ma, Y.B., Zhu, Y.G., Tang, Z., McGrath, S.P., 2015. Soil Contamination in China: Current Status and Mitigation Strategies. Environ. Sci. Technol. 49, 750–759.

Zhao, J.T., Yi, H., Yuxi, G., Li, Y.F., Li, B., Dong, Y.X., Chai, Z.F., 2013. Mercury modulates selenium activity via altering its accumulation and speciation in garlic (*Allium sativum*). Metallomic 5, 896–903.

Zhao, Z.Q., Xi, M.Z., Jiang, G.Y., Liu, X.N., Bai, Z.K., Huang, Y.Z., 2010. Effects of IDSA, EDDS and EDTA on heavy metals accumulation in hydroponically grown maize (*Zea mays, L.*). J. Hazard. Mater. 181, 455–9.

Zhuang, P., McBride, M.B., Xia, H., Li, N., Li, Z., 2009. Health risk from heavy metals via consumption of food crops in the vicinity of Dabaoshan mine, South China. Sci. Total Environ. 407, 1551–1561.

9. Publication from the Research Project

Journal articles

Zhao, Y.P., Cui, J.L., Chan, T.S., Dong. J.C., Dong, J.C., Chen, D.L., Li, X.D., 2018. Role of chelant on Cu distribution and speciation in *Lolium multiflorum* by synchrotron techniques. Sci. Total Environ. 621, 772-781

Zhao, Y.P., Cui, J.L., Chen, Y.H., Li, X.D., 2018. Interaction mechanisms of EDDS and a copper polluted soil in rhizosphere. (to be submitted)

Cui, J.L., **Zhao, Y.P.**, Li, J.S., Beiyuan, J.Z., Tsang, D.C.W., Poon, C., Chan, T.S., Wang, W.X., Li, X.D., 2018. Speciation, mobilization, and bioaccessibility of arsenic in geogenic soil profile from Hong Kong. Environ. Pollut. 232, 375–384.

Conference presentation

Zhao, Y.P., Cui, J.L., Li, X.D., 2016, September 26-29. Bioaccumulation and biotransformation of CuEDDS by *Lolium multiflorum* using synchrotron-based μ-XRF and XANES techniques. The 13th international phytotechnologies conference plant-based solutions for environmental problems from lab to field. Hangzhou, China.

Li, X.D., **Zhao, Y.P.**, Cui, J.L., 2016, November 20-27. Interactions between EDDS and Cu in ryegrass rhizosphere investigated by μ-XRF and μ-XANES. The 3rd international conference on contaminated land, ecological assessment and remediation. Taipei, Taiwan.