Biochelators as an alternative to EDTA and other synthetic chelators for the phytoextraction of heavy metals (Cu, Cd, Pb) from soil

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der Rheinisch-Westfälischen Technischen Hochschule Aachen zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften genehmigte Dissertation

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Doktorarbeit

...nach mehreren Jahren harter Arbeit hast du ein Buch in deinen Händen, welches deine Eltern nicht verstehen und den Rest der Welt nicht interessiert

PhD-thesis

...after several years of hard work you have a book in your hands, which your parents do not understand and the rest of the world does not care about

Michael W.H. Evangelou (1976-)

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Declaration / Erklärung

Herewith I declare that I have written this doctorate thesis myself, using only the referenced literature.

Hiermit versichere ich, dass ich die vorliegende Doktorarbeit selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe.

Aachen, Januar 2007

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Abbreviations

AAS	atomic absorption spectrometer
APCA	aminopolycarboxylic acid
Cd	Cadmium
CBA	calcium binding protein
CEC	cation exchange capacity
CyD	Cyclodextrins
Cu	Copper
DTPA	diethylene triamine pentaacetic acid
ED3A	ethylene diamine triacetate
EDDS	ethylene diamine disuccinate
EDDA	ethylene diamine diacetate
EDDHA	ethylenediamine-di(*o*-hydroxyphenylacetic acid)
EDMA	ethylene diamine monoacetate
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylenebis-(oxyethylenenitrilo)-tetraacetic acid
HEDTA	N-(2-hydroxyethyl) ethylenediaminetriacetic acid
HPLC	high performance liquid chromatography
LMWOA	low molecular weight organic acids
MALDI	matrix assisted laser desorption/ionisation
MS	mass spectrometry
NLMWOA	natural low molecular weight organic acids
NTA	nitrilo triacetic acid
Pb	Lead
TEA	Triethanolamine
TOF	time of flight

Summary

Heavy metal pollution of soil is a global problem. Diffuse contamination of large areas causes particular difficulties, since most classical engineering technology directed at soil decontamination is associated with a loss of land, and is usually costly. For arable land, in which low to medium level contamination threatens soil fertility and human health in the long term, gentle remediation techniques are required which not only reduce toxicological risks, but also restore soil fertility.

In order to overcome these problems, the use of plants to extract metals from soil (Phytoextraction) has repeatedly been suggested as a novel clean-up technology. Phytoextraction using hyperaccumulator plants is usually limited by low biomass, whereas metal uptake by high biomass plants usually suffers from low phytoavailability of the metal. One strategy to overcome these limitations is to enhance metal phytoavailability by the application of chelates. EDTA has been the most commonly used chelating agent for phytoextraction. Its disadvantage, though, is its persistence in the environment. This leads to a high risk of metal leaching into the groundwater. Furthermore, there have been signs of high toxicity towards plants and microorganisms.

Various naturally occurring chelating agents, such as natural low molecular weight organic acids (NLMWOA), cyclodextrins (CyD), (S,S)-N,N'-ethylenediamine disuccinic acid (EDDS) and wool in hydrolysed form were selected for use. All these chelating agents have a common denominator, they are synthesised by natural processes, such as microorganisms, plants or animals and are thus biodegradable.

The ability of the above mentioned chelating agents to mobilise heavy metals was assessed in slurry and column experiments. Phytotoxicity experiments evaluating the toxic effect of the chelates on plants were succeeded by phytoextraction pot experiments in the greenhouse. The degree of biodegradability of the chelates was evaluated with oxitop and pot experiments. Attention was given on the influence of the chelating agents on the bioavailability of heavy metals during the degradation process. Microorganisms which degrade the applied chelating agents were characterised in the case of NLMWOA.

The studied NLMWOA, citric acid, oxalic acid and tartaric acid, displayed low toxicity to plants and high Cu mobilisation effectiveness. The high effectiveness was not confirmed in the phytoextraction experiment, where EDTA, considering the amounts applied was more effective. The reason for this lies in the high degree of biodegradability of the NLMWOA. All three investigated NLMWOA were degraded by the same microorganisms and displayed a

significant pH increase in the soil during the degradation. This pH increase was accompanied by a decrease in the bioavailability of the heavy metals.

The CyD revealed a high toxicity towards tobacco and a very low capacity mobilising heavy metals. Additionally a high degree of biodegradability was observed.

EDDS, compared to EDTA, displayed a higher toxicity to tobacco. Both showed the same effectiveness in mobilising Cd and Cu in a column experiment. In the phytoextraction experiment EDDS increased the uptake of Cu but not of Cd, and both chelates failed to increase the root to shoot translocation rate. EDDS displayed a degree of biodegradability lower than often currently stated. A replanting of the pots revealed a prolonged toxicity effect of the remaining EDDS in the soil on the seedlings revealing new challenges for the field of chelate assisted phytoextraction.

Wool hydrolysate displayed a high efficiency mobilising Cu in soil. Depending on the degree of hydrolysation for the applied wool, it increased the uptake by up to 850%. It revealed a very high degree of biodegradability, which, moreover was not accompanied by side effects, such as the increase of soil pH, and the accompanied decrease of the bioavailability of the heavy metals.

The results from this study demonstrate that hyrolysed wool may be applied as a perfect substitute of the commonly used EDTA for enhancement of phytoextraction. The other tested biochelators, however, are not suitable to replace EDTA.

Zusammenfassung

Diffuse Verunreinigungen größerer Flächen mit geringer bis mäßiger Schwermetall-Kontamination ist ein ernst zu nehmendes globales Umweltproblem, da die meisten heute zur Verfügung stehenden Technologien zur Reinigung von Böden mit der Zerstörung der Bodenstruktur verbunden und zudem sehr kostenintensiv sind. Für Landwirtschaftsflächen mit einer Schwermetallbelastung im Prüfwertbereich werden sanfte Sanierungsmethoden benötigt, welche nicht nur die toxikologischen Risiken minimieren, sondern auch die Bodenfruchtbarkeit weitgehend wiederherstellen. In den letzten Jahren wurde daher der Einsatz metallakkumulierender Pflanzen (Phytoextraktion) vorgeschlagen, die als kostengünstige *in situ*-Methode die Nachteile klassischer Ingenieurverfahren beseitigen sollte.

Phytoextraktion mit hyperakkumulierenden Pflanzen ist normalerweise durch die geringe Biomasse der Pflanzen limitiert, während die Metallaufnahme durch Pflanzen mit hoher Biomasse von der oftmals geringen Bioverfügbarkeit der Metalle im Boden bestimmt wird. Eine Strategie, diese Einschränkung zu umgehen, ist es die Metallverfügbarkeit durch Zugabe von Komplexbildnern zu erhöhen. EDTA ist der am häufigsten für diese Anwendung eingesetzte Komplexbildner. Sein Nachteil ist seine Persistenz in der Umwelt, welche zum Risiko des Auswaschens der Metalle ins Grundwasser führt. Weiterhin zeigt EDTA auch eine hohe Toxizität zu Pflanzen und Mikroorganismen.

Verschiedene natürlich vorkommende Chelatoren wie z.B. natürliche niedermolekulare organische Säuren (NLMWOA), Cyclodextrine (CyD), Ethylendiamindibernsteinsäure (EDDS) und Wolle in einer hydrolysierten Form, die durch Mikroorganismen, Pflanzen oder Tiere gebildet werden und dadurch biologisch abbaubar sind, wurden in der vorliegenden Arbeit untersucht.

Die Fähigkeit der oben genannten Chelatoren, Schwermetalle zu mobilisieren, wurde in Schüttel- und Säulenversuchen bewertet. Nach Phytotoxizitätsversuchen, um die toxische Wirkung der Chelatoren auf Pflanzen einzuschätzen, wurden Phytoextraktionsversuche im Gewächshaus durchgeführt. In Oxitop- und Topfexperimenten wurde die Bioabbaubarkeit der Chelatoren untersucht. Besonderes Augenmerk wurde auf den Einfluss der Chelatoren auf die Bioverfügbarkeit der Schwermetalle während des Abbauprozesses gelegt. Im Fall der NLMWOA wurden Mikroorganismen, die beim Abbau beteiligt sind, charakterisiert.

Die untersuchten NLMWOA, Zitronen-, Oxal- und Weinsäure, wiesen eine geringe Toxizität gegenüber Pflanzen auf. Weiterhin zeigten sie ein hohes Potential, Cu zu mobilisieren; entsprechende Schüttelversuche konnten aber nicht im Phytoextraktionsversuch

bestätigt werden. Die Ursache dafür lag in ihrer Abbaubarkeit. Alle drei untersuchten NLMWOA wurden durch dieselben Mikroorganismen abgebaut. Während des Abbaus stieg der pH des Bodens signifikant, so dass die Bioverfügbakeit der Schwermetalle abnahm.

Die CyD offenbarten eine hohe Toxizität gegenüber Tabak und eine geringe Fähigkeit Schwermetalle zu mobilisieren. Zusätzlich wurde ein rascher Abbau beobachtet.

EDDS wies eine höhere Toxizität gegenüber Tabak als EDTA aus. EDDS und EDTA zeigten die gleiche Effektivität, Cu und Cd in einem Säulenversuch zu mobilisieren. Im Phytoextraktionsversuch steigerte EDDS die Pflanzenaufnahme von Cu, jedoch nicht von Cd. EDDS wies eine geringere Abbaurate auf als in der Literatur öfters angegeben. Eine erneute Bepflanzung der Böden aus früheren Versuchen zeigte, dass noch im Boden vorliegender EDDS phytotoxische wirkte.

Wollhydrolysat wies eine hohe Effektivität in der Mobilisierung von Cu im Boden auf. Abhängig von dem Hydrolysierungsgrad der applizierten Wolle stieg die Metallaufnahme um bis zu 850% an. Des Weiteren traten keine Nebeneffekte wie ein pH-Wertanstieg im Boden auf. Auf Grund dieser Tatsachen könnte hydrolysierte Wolle in Zukunft eine sehr gute und billige Alternative zu den bisher eingesetzten synthetischen Chelatoren, wie z.B. EDTA, sein.

Die Resultate dieser Studie zeigen, dass keiner der untersuchten Chelatoren außer Wollhydrolysat geeignet ist, EDTA als Chelator bei der Phytoextraktion zu ersetzen.

I INTRODUCTION

Historically, pollution of soil has been of limited concern. Over the centuries, human activities have contaminated large areas in both developed and developing countries. In the Sudbury region of Canada, for example, a combination of metal mine waste disposal and smelting emissions commencing in the late 19th century, led to widespread devastation of land, even up to 30 km from the sources of pollution (Winterhalder, 1996). More than 50,000 ha of land incapable of supporting other than very limited vegetation cover and an ongoing programme of soil and water remediation over more than 40 years, were the result. More recently, Mulligan et al. (2001) have estimated that 22,000 Mt of Cd and 1,372,000 Mt of Zn were deposited globally on soils during the 1980s. In recent years there have been numerous examples of tailing dam failure in Europe, (e.g. Aznalcollar mine, Spain as reported by Lopez-Pamo et al. (1999) leading to widespread pollution of floodplain soils.

In the EU, an estimated 52 million hectares of soil, representing more than 16% of the total land area, are affected by some kind of deterioration (when soil loses its capacity to carry out its functions). The European Environment Agency has estimated the total costs for the clean-up of contaminated sites in Europe to be between EUR 59 and 109 billion (Commission of the European Communities, 2002). In the United States, 1200 sites are on the National Priority List (NPL) for the treatment of contaminated soils, indicating the extensiveness of the problem. Approximately 63% of the sites on the NPL include contamination from toxic heavy metals (Hazardous Waste Consultant, 1996). At present not even highly industrialised countries can afford to clean up contaminated sites. In Germany, for instance, only 30% of the soils from contaminated areas are cleaned up in soil remediation facilities (Umweltgutachten, 2004), while the rest is stored untreated in waste disposal facilities.

Whereas water and air have been subject to environmental regulation since the 19th century, equivalent measures for soil, or, indeed even the concept of polluted soils, were not considered until the later decades of the 20th century. Early efforts at environmental regulation focused on the protection of human health (Scullion, 2006), but the link between soil pollution and human health was less obvious than that for air and water. The earliest measures to regulate soil pollution, relating to sewage disposal on agricultural land (Sewage Sludge Directive 86/227/EC), were linked to food production. This was followed by regulations aiming to protect water resources from pollutants leaching from soils. Only more recently have we recognised that there are multiple pathways by which polluted soils pose risks to humans and the broader environmental system. In consequence a few industrial countries, such as Germany (Bachmann, 1991), have implemented soil protection policies, but the

necessity of treating soil as an important resource has only been more widely recognised at the end of the last century (Scullion, 2006).

I.1 Heavy metals

I.1.1 General Information

'Heavy metals' is a general collective term applying to the group of metals and metalloids with a density greater than 6 g cm^{-3}. Although it is only a loosely defined term, it is widely recognised and usually applied to the elements such as cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), lead (Pb), and zinc (Zn), which are commonly associated with environmental pollution and toxicity problems. An alternative, but barely used name for this group of elements is 'trace metals' (Alloway and Ayrea, 1997). Heavy metals are natural constituents of the Earth's crust and are present in varying concentrations in all ecosystems. Both natural and anthropogenic processes and sources emit metals into the air and water. Since heavy metals cannot be degraded or destroyed, they remain in the environment as stable and persistent contaminants. Thus they tend to accumulate in the soils, seawater, freshwater, and sediments.

I.1.2 Biochemical properties of heavy metals

For normal healthy growth most living organisms require some heavy metals in small but critical concentrations (referred to as 'micronutrients' or 'essential trace elements'). Excess concentrations, though, cause toxic symptoms. These metals, which are unequivocally essential, and whose deficiency causes disease under normal living conditions include Cu, manganese (Mn), iron (Fe), and zinc (Zn) for both plants and animals, cobalt (Co), chromium (Cr), and the half-metal selenium (Se) for animals, boron (B) and molybdenum (Mo) for plants. Most of the micronutrients owe their essentiality to being constituents of enzymes and other important proteins involved in key metabolic pathways. A deficient supply of the micronutrient will result hence in a shortage of the enzymes, which lead to metabolite dysfunction causing disease (Alloway and Ayrea, 1997).

Heavy metals, which have no known essential biochemical function are called 'non-essential elements', and are sometimes incorrectly referred to as 'toxic' elements. These elements, which include arsenic (As), Cd, Hg, Pb, plutonium (Pu), antimony (Sb), thallium

(Tl) and uranium (U), cause toxicity at concentrations which exceed the tolerance of the organism, but do not cause deficiency disorders at low concentrations such as micronutrients (Alloway and Ayrea, 1997). These toxic symptoms are clearly shown by the typical dose-response curves in Figure 1-1, where the concentration ranges of the observed effects vary within the individual metals.

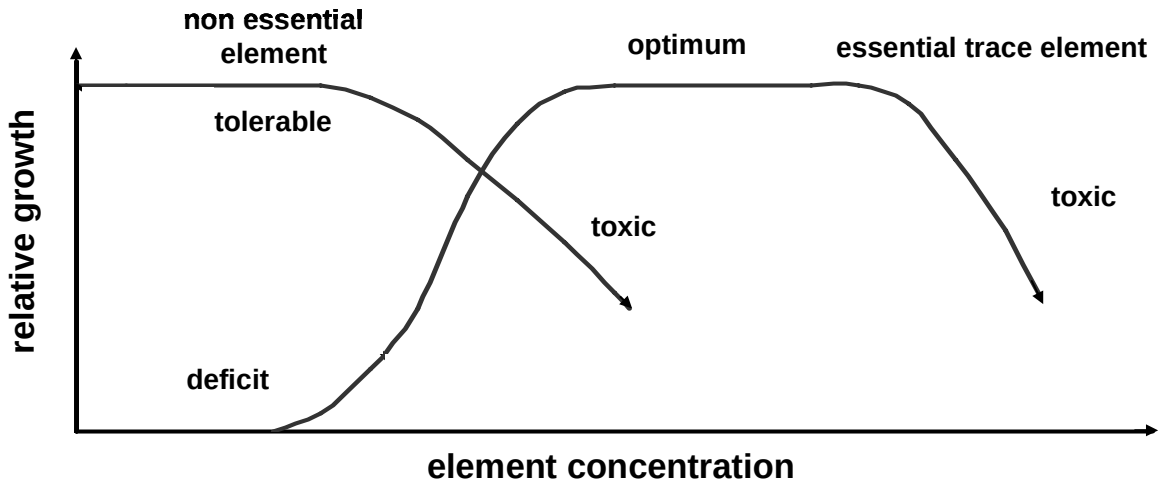


Figure 1-1: Dose-response curve of non essential and essential elements.

I.1.3 Heavy metal sources

Heavy metals are used widely in electronics, machines and the artefacts of everyday life as well as in "high-tech" applications. Consequently, they tend to reach the environment from a vast array of anthropogenic sources as well as through natural geochemical processes. The following are significant sources of metals to the environment (Alloway and Ayrea, 1997).

- *Metalliferous mining*: The metals utilised in manufacturing are obtained from either the mining of ore bodies in the rocks of the earth's crust, or the recycling of scrap metal originally derived from geological sources.
- *Agricultural materials*: The main sources are components (e.g. Cu containing fungicides) or impurities in fertilisers, pesticides, wood preservatives, sewage sludge, composts and manures and corrosion of metal objects.
- *Fossil fuel combustion*: A wide range of heavy metals are found in fossil fuels. They are either emitted into the environment as particles during combustion, or accumulate in ash, which may itself be transported in air and contaminate soil or waters from which they may be leached in situ.

- *Metallurgical industries*: Both the manufacture and disposal or recycling of the alloys in scrap metal can lead to environmental pollution by a wide range of metals.
- *Other sources* are batteries, pigments and paints, catalysts, polymer stabilisers, additives in fuels and lubricants etc.

I.1.4 Heavy metal behaviour in the environment

After their release in the environment, the metal pollutants can behave in various ways.

- *Atmospheric aerosol particles*: While suspended in the air, metal aerosol particles may be inhaled by humans and animals and subsequently absorbed into the bloodstream through the alveoli of the lungs. Particles reaching the earth's surface may enter plant tissues or the soil and be ingested through food.
- *Aqueous and marine environments*: Aerosols deposited into water, either directly or washed off surfaces into water streams, react with the constituents of the water or settle to the bottom where they may bind or react with the sediments. Subsequently, the metals can be accumulated in marine invertebrates or deposited in the drinking water.
- *In soils*: The most long lasting effects of heavy metal pollution can be seen in soil owing to the strong adsorption of many metals to the humic and clay colloids in soils. Unlike organic pollutants, which can be decomposed, metal atoms will remain unchanged, (Alloway and Ayrea, 1997) but can change their bondage type which subsequently may influence their availability and toxicity.

I.1.5 Specific heavy metals

In this thesis the elements Cd, Cu and Pb have been selected for thorough investigation. These three metals were chosen as they differ in their transfer properties from soil to plants. Cd is very mobile and toxic, Pb is immobile and toxic, while Cu has a medium mobility, tends to form complexes, is essential (Bliefert, 2002) but highly toxic to aquatic organisms and microorganisms.

I.1.5.1 Cadmium (Cd)

Cd was discovered by Strohmeyer in 1817 in the course of investigating zinc carbonate. Strohmeyer recognised that the yellow colour of a sample of zinc oxide produced by roasting

was due to the presence of an unknown metal oxide. As this new element also occurred in the zinc ore calamine, a name derived from the Latin word “cadmia,” he named the new element cadmium (Ullmann, 2002). Unlike some other heavy metals, such as Pb or Hg, which have been used since ancient times, Cd has been refined and utilised only relatively recently. Since the 1940's production and consumption have risen distinctly, thus achieving a high abundance in the environment (Ullmann, 2002).

Cd is a relatively volatile element, not essential for plants, animals and human beings. It occurs naturally, in absence of obvious pollution and geochemical mobility in the Earth’s crust (~ 0.11 mg kg^{-1}) and seawater (~ 0.01 – 0.1 µg kg^{-1}). Cd can occur in minerals such as monteponite (CdO), greenockite (hexagonal CdS), hawleyite (cubic CdS), $CdSO_4$, and $CdCl_2$ which, except for CdS and CdO, are soluble in water (Merian, 1991). Cd minerals are scarce, but, as a result of its similarity to Zn, Cd occurs by isomorphous replacement in almost all Zn ores (Merian et al., 2004).

CdS is used in the semiconductor industry, in high-temperature and high pressure tolerant colours, for the coating of metals (e.g. Aluminium) to provide a combination of corrosion resistance and low coefficient of friction. It is also used as a stabiliser in polyvinylchloride (PVC) to retard degradation by heat and ultraviolet light and in small amounts in alloys to improve their physical, mechanical or electrochemical properties. Additionally, before the advent of lithium batteries Cd was used in the production of Nickel (Ni)-Cd batteries. The recycling rate of Ni-Cd batteries rose from 2% in 1993 to 15% in 1995, with a value of 70% in 2001 (Merian et al., 2004).

Pollution

The anthropogenic enrichment factor for the total global emission of Cd is 89%. The remainder 11% is from natural sources, such as volcanoes (Merian et al., 2004). Cd soil contamination is very high in industrial areas exceeding the Cd soil concentration of agricultural soils on average 7-fold (Alloway and Ayrea, 1997). Moreover, dredged material of western European rivers is also often polluted with Cd and other heavy metals, making it unsuitable for soil improvement in agriculture (Mertens et al., 2001)

Effects on humans, animals and plants.

Cd(II) is toxic to many animals, because of its ability to combine with sulfhydryl groups (SH, thiols), thereby preventing normal functions of SH-group enzymes including the formation of disulfide bridges and consequent conformational changes in the proteins. Cd may also affect organisms by inducing deficiencies of essential elements through competition of active sites in biologically important molecules (antagonism). Calcium (Ca) resorption is influenced by the Cd content in food as both Ca and Cd bind to the "calcium binding protein" (CBP). The best-known incident in connection with Cd poisoning is the "itai-itai" disease in Toyama Prefecture, Japan in 1950. The symptoms are massive osteoporosis and bone deformation in combination with pancreatic diarrhoea. The cause of these symptoms is the blocking of the CBP as well as the blocking of the vitamin D_3-production pathway, both being essential for the stability of the bones (Marquadt and Schaeffer, 1994).

Since plants generally have no influence where they germinate and grow, they must adapt to unfavourable environmental conditions to survive. In order to tolerate Cd-rich soils, plants have four general strategies: a) accumulation, by binding metal to the cell wall, b) sequestration, by reducing transport across cell membrane, c) compartmentalization, d) chelation by Cd-binding complexes (phytochelatins) and their stabilization by sulphide ions and subsequent damage- rescue by heat shock proteins and phytochelatin constituting organics (Prasad, 1995). Most plants utilise one or more of these strategies to avoid Cd toxicity. Excess Cd causes a number of toxic symptoms in plants, e.g. growth retardation, inhibition of photosynthesis, induction and inhibition of enzymes, altered stomatal action, water relations, efflux of cations and generation of free radicals (Prasad, 1995). At great concentrations plants show symptoms of toxicity through chlorosis and subsequent necrosis of the leaves, reduction of biomass and necrosis of the root zone.

I.1.5.2 Copper (Cu)

Cu has been known since the dawn of human civilization, some 10,000 years ago. The first metals found by Neolithic man were gold (Au) and Cu, later silver (Ag) and meteoric iron (Fe). The earliest findings of Cu are presumed to be nearly nine millennia old and came from the region near Konya in southern Anatolia (Turkey). Until recently the six-millennia-old Cu implements from Iran (Tepe Sialk) were presumed to be the oldest. The origin of the name comes from the Mediterranean island of Cyprus, formerly *Kypros* (Greek), where Cu

was exploited millennia before Christ. Indeed, the Romans first named cyprium and later called cuprum, which became the origin of the word *"copper*" in most Roman and Germanic languages, e.g., cobre (Spanish and Portuguese), cuivre (French), Kupfer (German), koper (Dutch), and koppar (Swedish) (Ullmann, 2004).

Cu is a heavy metal which is distributed ubiquitously in the Earth's crust (~ 20 mg kg^{-1}) and seawater (~ 3 μg kg^{-1}). It readily forms complexes with various anions. As it is neither a soft nor a hard metal, it does not have a preference for a donor with certain electronegativity (Martell and Hancock, 1996). Cu is involved in numerous metabolic processes of the living organisms, especially those which involve the redox potential of Cu(I)/Cu(II). It occurs naturally in many minerals such as cuprite (Cu_2O), malachite (Cu_2CO_3 $Cu(OH_2)$), azurite ($2Cu_2CO_3$ $Cu(OH_2)$), chalcopyrite ($CuFeS_2$), chalcocite (Cu_2S), and bornite (Cu_3FeS_4) (Merian et al., 2004).

Worldwide, the largest use of Cu is in electrical wire, cable and other electronic applications. Cu is used in several alloys including those with Zn (brass), Sn (bronze) and nickel (Ni) (money metal). Cu sulphate is used as an algaecide and molluscicide in water and with lime as a plant fungicide. Cu oxide has been used as a component of paint for ship bottoms, while Cu chromates are pigments, catalysts for liquid phase hydrogenation, and potato fungicides (Merian et al., 2004).

Pollution

Cu soil pollution is widespread in urban areas. In studies from countries as varied as Naples, Italy (Imperato et al., 2003), Hong Kong, China (Chen et al., 1997) and Warsaw, Poland (Pichtel et al., 1997), Cu levels have greatly exceeded the requirements set by the corresponding Ministry of Environment for soils of public, residential and private areas. From the end of the eighteenth century, Cu, has been used to protect vines against fungus disease (Lafforgue, 1928), and in consequence studies have shown very high amounts of the metal in the soil of vineyards, (Ripolzi et al., 2002), reaching concentrations 500 mg kg^{-1} (Brun et al., 1998). The emission from Cu-Ni smelters is also a universal problem, and levels of pollution have been shown to be very high even several km away from the smelter (Uhlig et al., 2001).

Effects on humans, animals and plants

Cu is one of the several heavy metals which are essential to life, despite being as inherently toxic as nonessential heavy metals (e.g., Cd, Pb, or Hg). Toxic effects on humans and animals are seldom. Toxicity of Cu may arise if excess Cu provokes the following adverse reactions: (a) structural impairment of essential metal binding sites by displacement of metals, (b) functional impairment by binding of Cu to crucial sites in macromolecules as DNA or enzymes, and (c) cellular injury due to the production of oxyradicals (Merian et al., 2004). Ruminants are the only animals which are susceptible to Cu. Significant, and even lethal Cu toxicity, can occur by the addition of dietary supplements.

Cu toxicity on plants is low because of various protective mechanisms: (a) Cu ions thermodynamic activity is reduced by utilising the metal only as a prosthetic element tightly bound to specific Cu proteins; and (b) by an interaction between Zn and Cu. In general, visible symptoms of Cu toxicity are small, consisting of chlorotic leaves and early leaf fall, growth is stunted, and the initiation of roots and the development of root laterals is poor. Chlorotic spots may be due to cellular injury by the production of oxyradicals (Merian et al., 2004)

I.1.5.3 Lead (Pb)

Pb is one of the oldest metals known to man. The knowledge and use of Pb goes back as far as 5000 B.C. to the ancient Egyptians. The metal was used extensively by the Phoenicians, Romans, Indians, and Chinese. The first Pb mines in Spain were operated around 2000 B.C. The uses of the metal included weight standards, coinage (ultimately replaced by silver), statuary, sheathing and lining, trinkets, anchoring of iron rods, and the making of seals. The Greeks operated Pb mines at Laurium in the fifth century B.C. and the Romans mined in the Rio Tinto region of Spain in 300 B.C. The Romans used Pb extensively for water piping, and, indeed the Latin “plumbum” for Pb is derived from the word for a water spout.

Pb is a non-essential element; it is a neurotoxin and a good example for a multimedia pollutant. The most common Pb ore is galena (PbS), followed by anglesite ($PbSO_4$) and cerussite ($PbCO_3$). The two latter minerals originate from galena by natural weathering.

Pb´s high density makes it particularly applicable as a shield against radiation in the nuclear industry and against X-rays in medicine. The most important use of Pb today, however, is in Pb-acid storage batteries, which provide power in numerous situations, such as

in vehicles. Pb is also used in paint pigments, glass, ammunition and cable-covering material. Today, in the western world, more Pb is produced by recycling than by mining (Ullmann, 2004).

Pollution

In 1989, Nriagu estimated that globally in the late 1980s, anthropogenic emissions of Pb were more than 20 times greater than natural emissions, an enrichment factor far greater than in any other trace metal. Pb was used in organolead compounds such as tetramethyl lead and tetraethyl led as fuel additive because of their anti-knocking properties. Pb contaminated soil is, therefore, widespread. Although the introduction of "Pb-free" gasoline substantially reduced Pb emissions worldwide concern about toxicity and risks of future groundwater contamination remain, as Pb has a long residence time in soils (Miller and Friedland, 1994; Jonhson et al., 1995). In Shenyang, China, for instance, the Pb concentration of soil exceeds the China Environment Protection Agency guideline to protect human from health risk, in many cases to levels of 350 mg kg^{-1} (Ren et al., 2006). Pb has not just contaminated soils in cities, but also in many remote areas, such as Greenlands snow (Rosman et al., 1993), numerous lakes in Sweden (Renberg et al., 2002), remote areas of Swiss National Parks (Nowack et al., 2001) and woodlands in Ontario, Canada (Watmough and Hutchinson, 2004).

Effects on humans, animals and plants

Pb is one of the seven metals used in antiquity by the Eurasian civilization. Its uses were various. Physicians prescribed various forms of Pb to heal ailments ranging from constipation to highly infectious diseases, such as the plague. The symptoms of Pb poisoning have been recognised for centuries. Hippocrates described Pb colic in 370 B.C. and Pliny reported poisoning among Pb workers in about 50 A.D (Ullmann, 2004). Pb causes damages to the nervous system, kidney damage, inhibits several enzymes of heme synthesis and has shown to have a potential for carcinogenicity.

Pb is absorbed and accumulated, but not to any great extent by plants from the soil. Pb is either unavailable to plants or it is fixed in the roots, with only small amounts being translocated to the above-ground portions. Pb interferes with several processes in the development of the plant, including growth, maintenance, and photosynthesis (Merian et al., 2004) resulting in toxic symptoms such as chlorosis and necrosis.

I.1.6 Bioavailability of heavy metals

Total metal concentration refers to the fraction of soil metal which is removed by strong extractants such as aqua regia and perchloric acids. This includes not only the readily exchangeable ions but also those metals more strongly bound within solid phases of the soil and not available for uptake by soil organisms. Receptors such as plants are exposed to heavy metals via the soil solution. Importantly, total metal concentrations do not necessarily reflect levels of metals in the soil solution (Rieuwerts et al., 1998). The degree of availability for uptake i.e. the phytoavailability of metals is affected by numerous soil processes, although clear distinctions cannot always be made between processes, such as cation exchange capacity (Moore et al., 1995), specific adsorption, precipitation and complexation (Rieuwerts et al., 1998), and factors affecting these soil processes, such as pH (Hornburg and Brümmer, 1993; Reddy et al., 1995, Schmidt, 2003), organic matter content (Bliefert, 1994; Li and Shuman, 1996), redox-potential, clay content, iron-manganese oxides content, presence of cations and anions in soil solution, and miscellaneous factors (Rieuwerts et al., 1998). In addition, the speciation of the metal, which is correlated to the factors mentioned above (Reddy et al., 1995), and the metal species (Atanassova, 1999) itself, play an important role in the bioavailability of metals in soil.

I.1.6.1 Soil processes involved in the solid solution partition of metal

Charged surfaces of soils

Soil particles, particularly those found within the clay fraction (< 2 μm), are commonly electrically charged, and ions in the soil solution are attracted to their surfaces. These electrical charges are contributed by both mineral (permanent charges) and humic constituents (variable charges) and arise from different mechanisms of charge generation (Evans, 1989).

The permanent structural charge which exists in phyllosilicates is generated by charge imbalances in their structures brought about by isomorphous substitution or by nonideal occupancy in octahedral sheets. The substitution of Si^{4+} by $A1^{3+}$ generates a negative charge within a tetrahedral sheet; similarly, the substitution of $A1^{3+}$ by Mg^{2+}, or Mg^{2+} by Li^{+}, will generate a negative charge within the octahedral sheet. Positive charges can also be generated in octahedral sheets by substituting Ti^{4+} for $A1^{3+}$, or $A1^{3+}$ for Mg^{2+} (Evans, 1989).

The variable charge in soils usually arises on the edges of lattice clay minerals, and on the surfaces of sesquioxides, amorphous materials such as allophone, imogolite and organic matter. The reactive groups responsible for variable charge are similar in all the inorganic colloids and different in organic matter.

The total intrinsic charge, on soil particles is made up of the charge density of permanent structural charge plus the charge density of net proton, or variable charge.

The surface charge is created by the adsorption of potential determining ions (H^+ and OH^-) onto the surface [≡ **S** -] with the developing electrical charge the net proton charge being determined by that ion sorbed in excess (Evans, 1989; Naidu et al., 1997). Thus, these charges differ, compared to the permanent structural charges in clay minerals as the magnitude and sign of the charge depend on the pH of the soil.

A summarised more simple display of the proton dissociation reactions at the surfaces or edges of minerals can be represented by the two surface protolysis reactions:

$$\equiv S\text{-}OH_2^+ \leftrightarrow \equiv S\text{-}OH^o + H^+$$

$$\equiv S\text{-}OH^o \leftrightarrow \equiv S\text{-}O^- + H^+$$

Variable charge sites in humic materials arise from the ionization of carboxylic acid, functional groups within the humic and fulvic acid structures (Evans, 1989).

$$\equiv R\text{-}COOH \leftrightarrow \equiv R\text{-}COO^- + H^+$$

Cation exchange reactions

In order to maintain electroneutrality the negatively charged edges and surfaces of soil particles attract positively charged cations in solution to an equal quantity, by either electrostatic or coulombic forces. If the cations do not form covalent bonds with the surface, thereby retaining their water of hydration to form only outer sphere complexes, only a weak association is formed between the adsorbed ion and the soil particle. Owing to this weak association, the adsorbed ions are easily exchanged by other cations which similarly form only outer sphere complexes with the surface. Such bonding is reversible and is referred to as non-specific adsorption or, more commonly, as cation exchange (Evans, 1989; Rieuwerts et al., 1998).

Cation exchange is a particularly important mechanism of retention for many of the alkali and alkaline earth metals such as Na^+, Ca^{2+}, and Mg^{2+}, and, to a lesser extent, for ion pairs such as $CaC1^+$ and $MgCl^+$.

Usually, there is a selectivity for cations at the charged surface. This selectivity is attributable primarily to differences in the valencies and hydrated radii of cations at the charged surface. The preference of the adsorbing solid for one cation over another is proportional to the valency and proportional to the unhydrated radii (Evans, 1989; Elliot et al. 1986). Metals with small ionic radii have larger hydrated radii because of the greater attraction of the 0-H dipoles in water molecules to the charged ion, reflecting an increase in the polarizing power of the cation (Evans, 1989).

Specific adsorption

Another adsorption process, specific adsorption of inner sphere complexes, involves the exchange of metal cations with surface ligands to form partly covalent bonds with charged mineral surfaces, so the adsorbed species are not readily displaced (Evans, 1989; Alloway, 1995).

The mechanism of adsorption involves the replacement of surface-OH or $–OH_2^+$ groups on variable charge surfaces by the adsorbing ligand L^-. The process of ligand exchange for those anions that form monodentate complexes with a variable-charge surface can be described by:

$$\equiv S{-}OH_2^+ + L^- \leftrightarrow \equiv S{-}L + H_2O$$

(Evans, 1989)

Specific adsorption of metallic ions occurs most readily for metals which hydrolyze in water.

$$Me^{2+} + H_2O \leftrightarrow MeOH^+ + H^+$$

(Naidu et al., 1997)

Such metals include most of the transition elements and the rare earths, in addition to other ions, such as Hg^{2+} and Pb^{2+}. The adsorption reaction generally involves the formation of an inner sphere complex between the hydroxo-metal complex and the negatively charged

deprotonated surface of oxides, hydroxides, and oxyhydroxides of Al, Mn, and Fe. The oxides and oxyhydroxides of Mn, in particular, are very effective scavengers of metallic ions, particularly Co^{2+}. The reactions of divalent hydrolyzed metals at variable-charged surfaces can be described by:

$$\equiv S{-}O^{-} + MeOH^{+} \leftrightarrow \equiv S{-}O{-}Me{-}OH$$

Specific adsorption, which is strongly pH dependent, involves both organic and inorganic colloids and often occurs when concentrations of metals are low. The maximum amount of adsorption generally occurs at a pH somewhat below the pK of the hydrolysis reaction of the metal in water. According to Alloway, 1995, metals are specifically adsorbed in the preferential order Cd < Zn < Cu < Pb. Highly selective adsorption sites specifically adsorb metal cations which have affinities for particular sites. Therefore, adsorption on metals may not always be affected by competition from other cations. McLaren et al. 1981 made the observation that, while major cations such as Ca^{2+} may be present in soil solution in greater concentrations than Cu(I)/(II) ions, Cu adsorption may not be greatly affected because of specific adsorption of Cu ions. Nevertheless, an increasing metal concentration saturates the surface sites and subsequently the affinity of adsorbing surfaces for particular metals decreases (Rieuwerts et al., 1998).

Precipitation

Besides adsorption, metals may dissipate from the soil solution via precipitation. They can be precipitated as oxides, oxyhydroxides, hydroxides (removing OH^- ions from solution), and carbonates; phosphates and silicates probably are of lesser importance. In addition to these secondary precipitates, certain sulphide minerals may also form through the interaction of metals with H_2S, HS^-, which itself is formed by the biological reduction of SO_4^- ions (Evans, 1989; Rieuwerts et al., 1998).

Metals which might be expected to occur as hydroxides under some soil conditions are Fe^{3+}, Al^{3+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, and Cd^{2+}. The hydroxo-complexes of some metals, especially Al and Fe, are extremely important for controlling the behaviour of these metals in soils. The formation of the $Al(OH)_4^-$ and, to a lesser extent, $Fe(OH)_3^-$ anions at neutral and alkali pH's, for example, increases the solubility of these metal hydroxides at higher pH values. The presence of such hydroxo-complexes sharply increases the total concentration of metal in

solution. Metals which might be expected to precipitate as carbonates in soil include Ca^{2+}, Sr^{2+}, Ba^{2+}, Fe^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+}. Metals which might be expected to occur as sulfides under reducing conditions include Ag^{+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, and Fe^{3+} (Evans, 1989).

Natural soil-water interactions are very complex, the complexity arises from numerous components and factors, such as the various ligands, humic substances and microbiota, which are present in soil solutions.

Complexation

Metal complexation involves a centrally located metal ion in solution being surrounded by one or more organic or inorganic ligands. The most important inorganic ligands with regard to metal binding in solution appear to be the hydroxide and chloride ions. Complexation of metal cations has important consequences for metal binding, depending on the affinity of the complexing ligand for the soil surface. For example, the hydroxide has a high affinity for both the adsorbent (e.g. the soil surface) and the metal ion at relatively low pH levels and, therefore, increases metal sorption. By contrast, the chloride ion may reduce Cd sorption on soil surfaces due to its high affinity for the adsorbent (Rieuwerts et al., 1998).

Complexant organic ligands include citric, oxalic or gallic acids, or more structurally complex acids such as those contained in the soluble fulvic and humic fractions. For chelation to occur, the ligand must contain donor atoms capable of bonding to the same metal ion and positioned within the ligand so that the formation of a ring is sterically possible. Donor atoms are generally the more electronegative nonmetallic elements, such as O, N, and S**.** These elements are usually contained within basic groups, such as $-NH_2$ (amino), =O (carbonyl), -OH (alcohol) and -S- (thioether); or within acidic groups, such as -COOH (carboxyl), -OH (enolic or phenolic), and -SH (thiol) (Stevenson, 1994). S-containing groups are particularly strong, soft Lewis bases which can form strong complexes with soft Lewis acids such as Hg^{2+} and Cd^{2+}. Humic substances contain a highly complex mixture of functional groups whose metal-complexing abilities may be expected to vary considerably. Both the abundance and the abilities of these functional groups are constrained and controlled by the composition and structure of the humic materials present in soil. Humic substances may thus be expected to behave as a heterogeneous mixture of polymeric molecules (Evans, 1989).

I.1.6.2 Factors influencing the solid solution partition of metal

pH

pH is generally acknowledged to be the principal factor governing concentrations of soluble and plant available metals. Metal solubility of Cd, Cu and Pb tends to increase at lower pH and decrease at higher pH values (Rieuwerts et al., 1998). Al and Fe display the lowest solubility near the neutral pH, while the solubility increases as the pH decreases or increases (Bliefert, 1997).

The association between adsorption and pH is partly due to competition of H^+ (and Al^{3+}) ions of adsorption sites at low pH resulting in decreased metal adsorption. Another important mechanism may be metal hydrolysis at higher pH levels followed by strong, preferential adsorption of the resulting metal hydroxo complexes. Beyond a threshold pH (6 for Pb, 7 for Cd) virtually all metal ion is removed from solution, presumably as adsorbed hydroxo species. Acid catalysed dissolution of oxides, and their adsorption sites, may be another factor, causing decreased adsorption at low pH (Elliott et al., 1986).

The effect of pH on metal solubility can also be described in terms of its effect on precipitation-dissolution reactions. At neutral and higher pH levels various metal-phosphates and metal-carbonates may be precipitated. Complexation of metals also appears to decrease in increasingly acidic conditions. The organic ligands presumably complex H^+ ions and dissolve Al^{3+} and Fe^{3+} in preference to heavy metals (Rieuwerts et al., 1998).

Redox potential

Redox reactions in soils are controlled by the aqueous free electron activity, which can also be expressed by the redox potential. High redox potentials are typically recorded in dry, well aerated soils, whereas soils prone to waterlogging and rich in organic matter tend to have low redox potentials (Evans, 1989). The redox potential can have increasing or decreasing influence on the metals solubility depending on the metal, the soil characteristics and the time frame.

Solubility of heavy metals increases when redox potential decreases. It is possible that, due to the dissolution of Fe-Mn oxyhydroxides under reducing conditions, adsorbed metals are released. In contrast the solubility can decrease due to the formation of metal sulphide

(MeS). Under reducing conditions, metal sulphates are normally reduced by bacteria to relatively insoluble sulphides.

Clay content

Clays are thought to absorb metal ions through both ion exchange and specific adsorption. Metals may be bound to the surface hydroxyl groups along edges of clay particles as well as being directly bound to the clay surface. In soils where clay minerals with permanent negative surface charges are dominant, a large proportion of the adsorption capacity is due to permanent, pH-independent charge.

The influence of clay on the solubility of metals depends on the metal species. The influence of clay on the migration of Pb and Zn is very high, while on Cu and Cd it is very low. For the latter metals the influence of organic matter and hydrous oxide is to a greater extent much more important.

Organic matter content

Soil organic matter is comprised of humic substances or humus, and non-humic substances. Humic substances result from the decomposition of plant and animal residues (MacCarthy, 2001). They are comprised of humic acids, fulvic acids, and humin. Fulvic acids have a lower molecular weight, a higher oxygen and lower carbon content compared to humic acids. They are soluble at all pH values, while humin is not soluble and humic acids are soluble only under alkaline conditions in aqueous media.

The mechanisms involved in the retention of metals by organic matter include both complexation and adsorption (Evans, 1989). The ability of the humic substances to form complexes with trace elements is due to their high content of the functional groups (Stevenson, 1994). The active components in metal binding are carboxyl and phenolic OH groups, alcoholic OH and carbonyl (quinonoid and ketonic C=O) groups (Hofrichter and Steinbuechel, 2001) as well as amino groups (Rieuwerts et al., 1998).

Humic substances interact with metal ions to form both water soluble and water insoluble complexes (Schnitzer, 1978, Stevenson, 1994), which can either increase or decrease the metal solubility. This apparent contradiction lies in the fact that metals interact with organic components in both the solid and solution phases of soil. Fulvic acids are readily soluble and the formation of soluble complexes results in solubilisation of metals.

Iron and manganese oxides

Hydrous Fe and Mn oxides occur in clays as coatings on phyllosilicates and as free gels and crystals. They may reduce the concentration of metals in soil solution by both precipitation and specific adsorption reactions. Mn oxides have a stronger affinity for several metals, but as the Fe oxides are more abundant their influence is balanced (Rieuwerts et al., 1998).

Presence of cations and anions in soil solution

Base cations, particularly Ca^{2+} (constitutes > 90% of the total cation concentration, in many soils) influence the quantities of soluble trace elements. The presence of Ca affects the adsorption of Cd greatly, while Pb is influenced less strongly. Additionally, heavy metals themselves compete for the same adsorption sites and therefore influence the bioavailability of themselves and of the other present contaminants. Anions, inorganic and organic, can affect the solubility of metals, by forming complexes with metal ions (Rieuwerts et al., 1998).

Miscellaneous factors

There are a number of other factors which may affect the solubility of metals in soils. Microbial activity, for example, influences the solubility of metals. Microorganisms may immobilise soil metals by aiding the precipitation of sulphides and hydrated ferric oxides. The presence of surface organic functional groups on bacterial cell walls plays a role in the adsorption of metals from soil solutions (Rieuwerts et al., 1998).

Roots exude acid materials, such as H_2CO_3 which lower the pH of the rhizosphere and increase the absorption by plants. Furthermore, symbiotic fungi may increase or decrease the plant available metal fraction in soil (Leyval et al., 1997).

Temperature is positively correlated with plant uptake of metals, probably due to higher transpiration of the plant. Additionally, physical soil properties such as fracturing and permeability, presence of earthworms, and calcium carbonate content have an influence on metal fixation.

Natural contaminated soil and heavy metal spiked soil

The processes which influence the bioavailability of heavy metals in the soil such as adsorption, complexation and precipitation are processes which are interminable. The processes establish equilibrium between the bioavailable, water soluble heavy metal fraction and the bound heavy metal fraction which is not available to plants and soil organisms. The bioavailability of heavy metals in natural contaminated soil is lower than the heavy metal bioavailability in a heavy metal spiked soil. In phytoextraction experiments, the spiked soil passes a 3 week wet-dry cycles to enable the added heavy metals to reach a steady state (Blaylock et al., 1997). Nevertheless, the bioavailability of heavy metals is still higher than in comparable natural contaminated soil.

I.2 Soil remediation

Over the years several remediation techniques have been proposed and applied on contaminated sites. Contaminants can be isolated and contained to prevent further movement. Vertical barriers are applied to reduce the movement of contaminated groundwater or uncontaminated groundwater through a contaminated area. Solidification/stabilization technologies are very common as they contain the contaminants and not the contaminated area as physical barriers. The contaminants are encapsulated, either physically (solidification) or chemically (stabilization). This technique is usually applied ex situ, because mixing in situ is difficult to evaluate (Mulligan et al., 2001). Vitrification is also a solidification/stabilization process, which requires thermal energy. During this technique the contaminated soil area is solidified. During vitrification toxic gases can be produced and leaching of contaminants is possible. In the mechanical separation process, larger cleaner particles are removed from the smaller more polluted ones. To accomplish this, several processes are used. They include: hydrocyclones, fluidised bed separation, gravimetric settling and floating. Pyrometallurgical processes use high temperature furnaces to volatilise metals in contaminated soils. After volatilization metals are then recovered or immobilised. This treatment is performed off site because of the lack of mobile units (Mulligan et al., 2001). Chemical treatment by reductive as well as oxidative mechanisms may be used to detoxify or decrease the mobility of metal contaminants (Evanko and Dzombak, 1997). It can be performed in situ by injection into the groundwater but potentially it can introduce further contamination. Electrokinetic processes involve passing a low intensity electric current between a cathode and an anode imbedded in the contaminated soil. The process can be used in situ (Mulligan et al., 2001) but in order to

be effective, the pH of the soil has to be maintained at a low level. Heavy metals can be removed from soils by washing, using various agents added to the soil. These agents are inorganic acids, organic acids and chelating agents (USEPA, 1991). After treatment the soil can be returned to the original site. The use of biosurfactants to remove heavy metals from soil is a method which is gaining more interest. The biosurfactants can be produced by bacteria or yeast and have the ability to remove heavy metals in water (Mulligan et al., 1999). This technique, though, is very new and needs further development.

A summary of the various remediation techniques is shown in Table 1-1. Physical containment is the least expensive approach, but it does not remove the contaminants. Solidification/stabilization and vitrification can be performed in situ, and this reduces handling costs. Long term stability of the solidification/stabilization matrix is the major unknown factor. Vitrification is very expensive for an in situ process, and the produced toxic gases could pose a further risk. Ex-situ treatments are very cost intensive and require a long regeneration time for the remediated soil. Electrokinetics have been used in few sites, and the greatest disadvantages is the very low pH of the soil.

The above mentioned techniques do not offer a satisfactory solution for many sites, especially for agricultural sites or those with low or moderate contamination. In consequence, phytoremediation, a technique which is cost effective, and moreover, accepted by the public because it is aesthetically pleasing and causes minimal environmental disturbance as it does not alter the soil matrix, could offer a solution for sites with moderate and slightly elevated contaminant concentration.

Table 1-1: Summary of remedial technologies (adapted from Mulligan et al. (2001) and Glass (1999))

Technology	Description	Applicability	Costs ($US/ton)
Containment			
Physical	Prevent movement by preventing fluid flow	Landfill covers and slurry walls	10-90
Encapsulation	Creation of an inert waste	Injection of solidifying chemicals	60-290
Vitrification	Application of electrical energy to vitrify contaminant		400-870
Ex-situ treatment			
Physical separation	Includes, froth flotation, gravity separation, screening, etc.	For high metal concentration	60-245
Soil washing	Addition of surfactants and other additives to solubilise	For water soluble contaminants	25-300
Pyrometallurgical	Elevated temperature extraction and processing for metal removal	Highly-contaminated soils (5-20%)	200-1000
In situ			
Electrokinetic	Application of electrical current	Applicable for saturated soils with low groundwater flow	No info
Phytoremediation	Use of plants for contaminant removal (see I.2.1)	Shallow soil and water	25-100 ≈ (50,000-200,000/acre)

I.2.1 Phytoremediation

Phytoremediation is defined as the use of green plants to remove pollutants from the environment or to render them harmless (Raskin et al., 1997). Targets for phytoremediation are both toxic heavy metals and organic pollutants. Currently, the following areas are summarised under the term phytoremediation (Salt et al., 1998):

- **Phytoextraction:** the use of pollutant-accumulating plants to remove metals or organic pollutants from soil by concentrating them in harvestable parts;
- **Phytodegradation:** the use of plants and associated micro-organisms to degrade organic pollutants;
- **Rhizofiltration:** the use of plant roots to absorb pollutants, mainly metals, from water and aqueous waste streams;
- **Phytostabilization:** the use of plants to reduce the bioavailability of pollutants in the environment;
- **Phytovolatilization:** the use of plants to volatilise pollutants; also the use of plants to remove pollutants from air. (Salt et al., 1998)

This work focuses on phytoextraction, which, as proposed by a number of authors (Banuelos and Schrale, 1989; Raskin et al., 1994; Baker et al., 1995; Kumar et al., 1995; McGrath, 1998), is a 'gentle' in situ decontamination technique making use of the natural ability of plants to extract heavy metals from soil. Phytoextraction is primarily used to remove heavy metals from soil but it has also been found useful in the removal of radionuclides, such as Caesium, and is occasionally used to extract organic pollutants. Phytoextraction causes a minimal environmental disturbance, as it does not alter the soil matrix compared to the conventional remediation techniques, with the result that after a successful phytoextraction a soil can be used for agricultural purposes.

I.2.2 Phytoextraction

The basic idea that plants can accumulate extraordinarily high metal levels is very old and cannot be traced to any particular source. Miners in the 19th century found ore deposits by looking for fields covered with the Cu-tolerant mustard plant. In 1855, Baumann identified plants capable of accumulating uncommonly high Zn levels. In 1935, Byers documented the accumulation of selenium in *Astragalus spp*., which could accumulate up to 0.6% selenium (Se) in dry shoot biomass. Thirteen years later, in 1948, a study was conducted by the Italian

scientists Minguzzi and Vergnano, who noticed dense accumulations of Ni in the Alyssum, a low herb, and identified plants capable of hyperaccumulating up to 1% Ni in shoots. By definition, a hyperaccumulator must accumulate at least 100 mg kg^{-1} (0.01% dry wt.) Cd, As and some other trace metals, 1000 mg kg^{-1} (0.1% dry wt.) Co, Cu, Cr, Ni and Pb and 10,000 mg kg^{-1} (1% dry wt.) Mn and Ni (Reeves and Baker, 2000; Wantanabe, 1997). Metal hyperaccumulation is an ecophysiological adaptation of plants to metalliferous soils (Maywald and Weigel, 1997). Its function is not yet known, but experiments have been performed, which support the hypothesis that metal-hyperaccumulation works as a defence mechanism against plant pathogens (Boyd et al., 1994), and also prevents predation (Sagner et al., 1998). *Streptanthus polygaloides* (Brassicaceae), a Ni-hyperaccumulating plant, was more slowly infected by a powdery mildew (*Erysiphe polygoni*) than low-Ni plants (Boyd et al., 1994). A repellent effect of the plant sap was observed on the fruit fly Drosophila melanogaster indicating that in hyperaccumulating plants nickel functions as an agent to prevent predation (Sagner et al., 1998).

Scientific phytoremediation studies have continued to focus on hyperaccumulating species such as *Thlaspi caerulescens* (Baker and Walker, 1990; Brown et al., 1994; Brown et al., 1995), *Thlaspi rotondifolium* (Reeves and Brooks, 1983) and *Alyssum lesbiacum* (Kramer, 1996). To date over 400 plant species have been identified as natural metal hyperaccumulators, representing <0.2% of all angiosperms (McGrath and Zhao, 2003).

For the phytoextraction technique to be feasible, sufficient amounts of heavy metals have to be extracted. Plants have thus to be highly efficient in metal uptake and translocation into their aboveground parts. Hyperaccumulators have the above mentioned characteristics but, unfortunately, they are combined with slow growth and limited biomass production. As total metal extraction is the product of biomass and tissue concentration, the speed of metal removal, in hyperaccumulators, is accordingly limited (Cunningham et al., 1995; Comis 1996; Ebbs et al. 1997). Calculations showed that, in the case of Pb, this technology can only be feasible if systems can be developed to employ high biomass plants, which are capable of accumulating more than 1% Pb in shoots and produce more than 20 t of biomass ha^{-1} $year^{-1}$ (Huang et al., 1997). Hyperaccumulating species so far cannot meet such requirements.

I.2.3 Strategies to cope with the limitations of phytoextraction

To overcome the limitations of phytoextraction techniques several approaches were taken. Efforts have been made to increase growth of hyperaccumulators by crossbreeding them with

related plants which produce more biomass (Cunningham and Ow, 1996). Others have selected clones of high biomass crop plants for their ability to accumulate increased levels of heavy metals (Ow, 1993). In addition, transgenic plants containing animal or plant genes encoding for different metallothioneins were tested for their ability as phytoextraction plants (Elmayan and Tepfar, 1994; Hattori et al., 1994; Pan et al., 1994; Daghan, 2004). To understand the mechanisms involved in heavy metal tolerance more deeply, molecular mechanisms and genes leading to hyperaccumulation in tolerant species were investigated (Brown et al., 1995 a, b). Additionally, attempts were made to improve metal uptake capabilities of high biomass plants by somaclonal variation and selection techniques (Guadagnini, 2000). The results were positive demonstrating a significant increase in metal tolerance. However, owing to restrictive laws in several countries, such as the countries of the EU (Directive 2001/18/EC, 2001), an application of genetic modified plants in the near future is not likely.

As suggested by Robinson et al. (2000), a different approach lies in the use of plants which are fast growing, deep-rooted, easily propagated and accumulate the target metal, combined with an increase of the phytoavailability of the metals in soil (Felix, 1997). Research has focused, therefore, on crops such as maize (*Zea mays*), tobacco (*Nicotiana tabacum*), Indian mustard (*Brassica juncea*), oat (*Avena sativa*), barley (*Hordeum vulgare*), pea (*Pisum sativa*), poplar (*Populus spec*.) and sunflower (*Helianthus annus*) (Mench et al., 1989; Salt et. al., 1995; Huang and Cunningham, 1996; Blaylock et al., 1997; Huang et al., 1997; Salt et al., 1997; Ebbs and Kochian, 1998; Huang et al., 1998; Wu et al., 1999; Chen and Cutright, 2001; Wenger et al., 2002; Liphadzi, 2003; Dickinson and Pulford, 2005).

The ability to cultivate a high biomass plant with a high content of toxic metals in a contaminated soil will be the determining factor in the success of phytoremediation. Enhancing metal accumulation in existing high yielding crop plants without diminishing their yield is the most feasible strategy in the development of phytoremediation. To improve the uptake speed of non hyperaccumulating plants, the addition of chelating agents has been implemented.

The concept behind chelate-assisted/induced phytoextraction (Figure 1-2) is as follows: hyperaccumulating plants continuously extract heavy metals from soil (Figure 1-2). A non hyperaccumulating high biomass plant can only extract very little heavy metal by nature. The addition of chelating agents enables the plant to extract high amounts of the heavy metals in the soil.

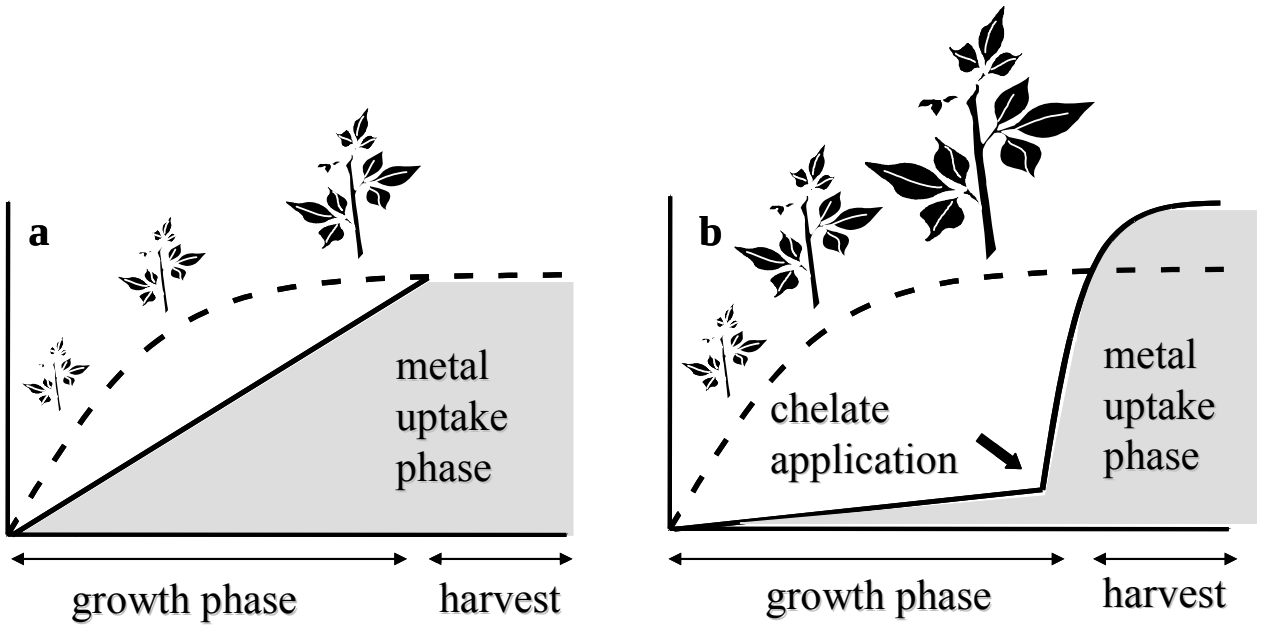


Figure 1-2: Schematic representation of continuous phytoextraction (a) and chelate-assisted phytoextraction (b). Solid line represents metal concentration in shoot biomass; dashed line represents shoot biomass. (adapted from Salt et al., 1998).

I.2.4 Chelate assisted phytoextraction

Chemically/chelate-enhanced, -induced, -assisted phytoextraction is based on the fact that the application of chemicals/chelating agents to the soil significantly enhances metal accumulation by plants (Garbisu and Alkorta, 2001). A chelate is a chemical compound composed of a metal ion and a chelating agent. A chelating agent is a substance whose molecules can form several bonds to a single metal ion. In other words, a chelating agent is a multidentate ligand (Ullmann, 2006), which forms strong, water soluble metal complexes with di- and trivalent cations. For more than 50 years chelating agents have been used to supply plants with micronutrients both in the soil and in hydroponic solutions (Salt et al., 1995). The formation of chelates prevents precipitation and sorption of the metals, thereby maintaining their availability for plant uptake (Salt et al., 1995). The addition of chelating agents to the soil can also bring metals into solution through desorption of sorbed species, dissolution of Fe and Mn oxides, and dissolution of precipitated compounds (Norwell, 1984). These complexes can greatly alter the reactivity of the metal ion. They can alter the oxidation – reduction properties of transition-metal ions, such as iron (Fe) and manganese (Mn), and therefore increase or decrease the reactivity of these systems (Ullmann, 2006). Chelating agents also influence the toxicity of metals. They can increase the toxicity, have very low or

no influence on the toxicity, or reduce noticeably the toxicity of metals. The inhibitory effect on metal toxicity is explained to be due to binding of the heavy metal ions and thus preventing their normal action (Sillanpää and Oikari, 1996).

The uptake mechanism depends on the characteristics of the chelating agent such as its lipophilicity, stability constant to the metal, pKa and complex size. In general though solute transport from the external parts of the root to the central root xylem, takes place through two major pathways: the apoplastic (cell wall space between cell membranes) and the symplastic (crossing many cell membranes along the pathway). In the apoplastic pathway, the presence of the lipophilic Casparian strip at the root endodermis disrupts the apoplastic water flow and directs it to cross cell plasma membranes at least twice, where selective transport as well as passive permeation of solutes occur (Figure 1-3). In order to enhance heavy metal uptake and translocation within the plants by the use of organic ligands physiological barriers have to be overcome.

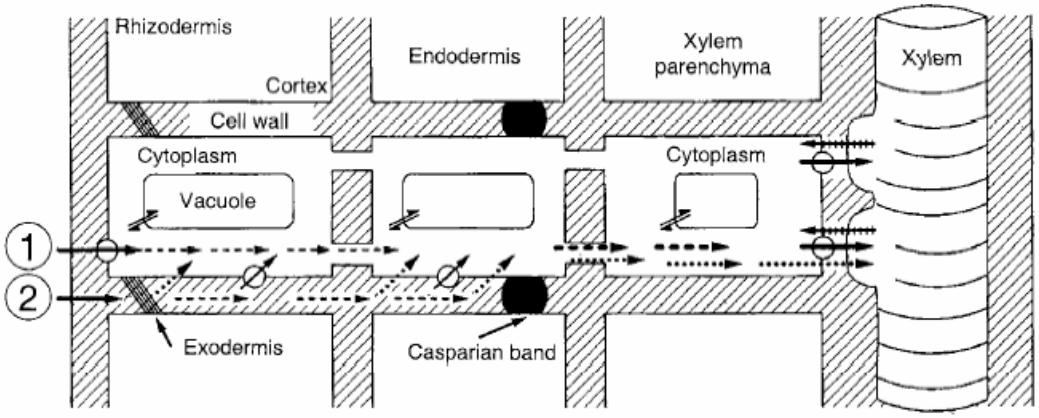


Figure 1-3: Model for symplastic (1) and apoplastic (2) pathways of radial transport of ions and solutes across the root into the xylem. Key: ⊖▶ , active transport; ◀ resorption. (modified from Lauchli, 1976, from Wenger, 2000).

Independent of the chelating agents used for enhanced phytoextraction, the effectiveness depends largely on the plants and heavy metals used in the study. Members of the *Brassicacae* have an ability to take up heavy metals from contaminated soils and transport them to the shoots (Kumar et al., 1995). In a study with cabbage (*Brassica rapa*), mung bean (*Vigna radiata*) and wheat (*Triticum aestivum*) Shen et al. (2002) demonstrated the different translocation effectiveness of these plants. Chen and Cutright (2001) reported an effective root to shoot translocation for Cd and Ni after the addition of EDTA, whereas for Cr no translocation could be observed. Additionally the stability constant is not a reliable

measurement scale for the effectiveness of a chelating agent. EDTA for instance has a stability constant value of $\log K_s = 18.0$ for Pb, whereas DTPA has a value of $\log K_s = 18.8$ for the same metal (Martel and Smith, 1974), but as stated by Blaylock et al. (1997) EDTA was significantly more effective than DTPA for the phytoextraction of Pb. According to their chemical affinity for Pb a similar or higher effectiveness by DTPA would have been expected.

In the last decade the use of persistent aminopolycarboxylic acids (APCA) such as ethylene diamine tetraacetic acid (EDTA), N-(2-hydroxyethyl) ethylenediaminetriacetic acid) (HEDTA), diethylene-triamine-pentaacetic acid (DTPA), ethylenebis-(oxyethylenenitrilo)-tetraacetic acid (EGTA), ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDHA) and others have been tested for their usability in phytoextraction (Huang et. al., 1997). Recently the biodegradable APCA, ethylene diamine disuccinate (EDDS) (Luo et al., 2005; Meers et al., 2005) and nitrilo triacetic acid (NTA) (Kayser et al., 2000; Wenger et al., 2003) have been proposed as an alternative to EDTA and other persistent APCA. Some experiments using low molecular weight organic acids (LMWOA) (Gramss et al., 2004) and EDDS (Kos and Lestan, 2004; Meers et al., 2005) have also been performed.

I.2.5 Chelating agents

I.2.5.1 Synthetic chelating agents

Ethylene diamine tetraacetic acid (EDTA)

In the late 1980´s and early 1990´s EDTA was suggested as a chelating agent for the assistance of phytoextraction processes. In the many pot experiments described in the literature the influence of EDTA varied largely. While some authors report that the use of synthetic chelating substances, such as EDTA increased metal uptake by plants (Jørgensen, 1993; Blaylock et al., 1997; Huang et al., 1997; Grčman et al., 2001), others did not observe an enhancement (Athalye et al., 1995), but rather a reduction of heavy metal uptake by plants (Robinson, 1997).

Although EDTA has been shown in several publications to be effective in enhancing phytoextraction, EDTA and EDTA-heavy metal complexes are toxic to soil microorganisms (Grčman et al., 2001) and to plants by severely decreasing shoot biomass (Epstein et al., 1999; Chen and Cutright, 2001). Owing to its low biodegradability (Bucheli-Witschel and Egli, 2001) EDTA may remain adsorbed to soil particles, even after soil cleaning (Wasay et al.,

1998). Its prolonged presence in the soil and its non selective nature dramatically increase the leaching risk of heavy metals (Grčman et al., 2003) and alkaline earth metals such as Ca and Mg (Barona et al., 2001). It has been suggested that groundwater pollution by EDTA application could be prevented by proper management of irrigation. In climates displaying a positive water balance difficulties will arise in controlling seepage, particularly in sandy substrates, which are representative in many polluted areas (Wenzel et al., 2003). As a consequence, EDTA occurred at higher concentration in river water than any other identified organic compound (Nowack, 2002).

However, APCA in general and EDTA, in particular, do slowly disintegrate in the environment because of abiotic processes. Photodegradation and chemical degradation are substantially affected by the chelated metal ion. Complexes of Fe(III)EDTA and Cu(III)EDTA are rapidly photodegraded, while most other complexes are not or only slightly photosensitive. The rapid photodegradation of Fe(III)EDTA results in a mean half-life of EDTA in river water of a few hours during summer and several days in winter (Nowack, 2002). In the case of Fe(III)EDTA a stepwise degradation leads under successive decarboxylation to ethylenediaminetriacetate (ED3A), ethylenediaminediacetate (EDDA), and ethylenediaminemonoacetate (EDMA) (Nowack, 2002). The disintegration of EDTA in aqueous environment seems to be rapid, but in the soil it is a more time intensive process. In a soil solution experiment of Meers et al. (2005) the amount of mobilised metals (Zn, Cu, Cd, and Ni) after EDTA application did not or only very slightly decreased in 40 days. This underlines the high environmental persistence of EDTA in the soil. The low biodegradability of EDTA combined with the several above mentioned environmental drawbacks, forced scientist to search for new biodegradable chelating agents.

I.2.5.2 Natural chelating agents

Ethylene diamine disuccinate (EDDS)

EDDS is an aminopolycarboxylic acids (APCA), which is produced naturally by a number of microorganisms (Nishikiori et al., 1984; Goodfellow et al., 1997). It can also be synthesized from ethylene diamine and maleic anhydride (Schowanek et al, 1997). In the last ten years several phytoextraction experiments have been performed with the addition of EDDS, which have proven to be very effective in enhancing the uptake of several metals.

Schowanek et al. (1997) described a good degree of biodegradability for [S,S]-EDDS, with observed half lives ranging from 2.5 days in a soil experiment to 4.6 days in an unacclimated Sturm test, while [R,R]-EDDS remained undegraded. Interestingly, the [S,S]-isomer of EDDS was also reported to be produced naturally by a number of microorganisms (Nishikiori et al., 1984), such as *Amycolatopsis japonicum sp. nov.* (Goodfellow et al., 1997). Vandevivere et al. (2001) verified that uncomplexed [S,S]-EDDS is rapidly degraded, but added that the degradation of metal-[S,S]-EDDS complexes depended on the metal type. Ca-, Cr(III)-, Fe(III)-, Pb-, Al-, Cd-, Mg-, Na-, or ZnEDDS (the latter only after extensive lag phase) were readily biodegraded. On the other hand, the Cu-, Ni-, Co-, and Hg-complexes remained essentially undegraded. Only in the case of HgEDDS was the lack of biodegradation due to metal toxicity. Metsärinne et al. (2001) reported the total disappearance of Na_4-EDDS from lake water exposed to sunlight in a time period of two weeks. Since Na_4-EDDS does not absorb light within natural UV radiation range, it is thought that it was converted to a Fe(III) complex. This is similar to the case of EDTA, where Fe(III)-EDTA is the only environmentally relevant EDTA species that undergoes direct photolysis (Kari et al., 1995). Indirect photolysis is also relevant. Such a process enables energy to be transferred from e.g. humic acid triplet states to chemicals or generated oxidants to react with the chemical (Mill and Mabey, 1986).

Natural low molecular weight organic acids (NLMWOA)

It is known that exudation of organic compounds by roots may influence the solubility of essential and toxic ions both indirectly and directly; indirectly, through their effects on microbial activity, rhizosphere physical properties and root growth dynamics and, directly, through acidification, chelation, precipitation and oxidation-reduction reactions in the rhizosphere (Uren and Reisenhauer, 1988; Marschner et al., 1995). Their nature, amount, source and persistence vary both longitudinally and radially away from the root tip. The release is enhanced when the plants are exposed to iron stress, low concentrations of calcium and phosphorous and a host of other factors (Uren and Reisenauer, 1988). Of these compounds, natural low molecular weight organic acids (NLMWOA), such as citric acid, oxalic acid, or malic acid are of particular importance, because of their complexing properties. They play a significant role in heavy metal solubility, (Mench and Martin, 1991; Krishnamurti et al., 1997; Nigam et al., 2000) and the mobilisation of mineral nutrients (Zhang et al., 1989; Jones et al., 1996). It has been maintained that NLMWOA are even more important for the

uptake than the pH of the soil (Huang et al., 1998). In addition, as earlier studies suggested, NLMWOA have also the capability to detoxify intracellular heavy metals via binding (Lee et al., 1977), thus increasing the plants heavy metal capacity. Many of these organic acids are released from the root in greater quantities in time of stress (Dinkelaker et al., 1989; Delhaize et al., 1993; Jones and Darah, 1995), either to protect the plant from the metal or to increase the uptake of metal nutrients. Over 99% of the organic acids lost by the root were predicted to remain within 1 mm of the root, showing that they rapidly biodegraded. This, though, varied according to the root growth-rate, soil and efflux rates (Jones et al., 1996).

Huang et al. (1998) stated that an addition of 20 mmol kg^{-1} of citric acid increased the concentration of U (uranium) in the studied plants (*B. Juncea, B. Chinensis, B. narinosa* and amarath) 1000 fold in comparison to the control. The authors also stated that citric acid is advantageous in the use of chelate assisted phytoextraction, because it is biodegradable, rapidly degrading to carbon dioxide and water (Dodge and Francis, 1994; Yan et al., 1996; Huang et al., 1998b). Experiments by Meers et al. (2005) showed that soils treated with 55 mmol kg^{-1} or 220 mmol kg^{-1} NH_4-citrate contained heavy metal concentrations in the soil solution at levels comparable to the control 2 weeks after harvesting.

Cyclodextrins (CyD)

Cyclodextrins (CyD) are cyclic oligomers of α-D-glucose formed by the action of certain enzymes on starch. Three cyclodextrins are readily available: α -CyD, having six glucose units (and also named cyclohexaamylose or cyclomaltohexaose); *β*-CyD (seven units, cycloheptaamylose, cyclomaltoheptaose); and *γ*-CyD (eight units, cyclooctaamylose, cyclomaltoheptaose) (Connors, 1997). The glucose units are connected through glycosidic α-1,4 bonds. The first reference to a cyclodextrin was published by Villiers, in 1891, when he digested starch with *Bacillus amylobacter* (Szejtli, 1998). These lampshade-shaped molecules have a hydrophobic, nonpolar interior and a hydrophilic, polar exterior. Relatively nonpolar organic contaminants partition to the interior of the molecule (i.e., an inclusion complex is formed), while the highly polar exterior provides the molecule with a large aqueous solubility (e.g., approximately 50% by mass) (McCray and Brusseau, 1998).

By controlling (increase or decrease) the solubility of organic molecules, by reducing volatilisation, by directing chemical reaction, by aiding processes, by controlling fluorescence, by stabilising reaction compounds and by masking effects of guest molecules (Hedges, 1998), CyD have a wide industrial application.

In general CyD have shown a poor ability to increase the bioavailability or mobilisation of heavy metals in soil. In Khodadoust et al. (2004) CyD were found to be ineffective for the removal of nickel. A removal efficiency of less than 8% was observed. On the other hand Brusseau et al. (1997) showed that a 0.5% carboxymethyl-β-cyclodextrin + 0.5% hydroxypropyl-β-cyclodextrin solution mobilised approximately 90% of the Cd in soil. To the present time it is unknown if a phytoremediation application is possible, as the toxicity of these substances regarding plants has not yet been tested.

Wool

Wool, like other types of fine and coarse animal hair and also silks, is composed of natural animal fibers. Although the term "wool" can be used in conjunction with the names of various animals, e.g., "angora wool", it is here understood to include only the hair of the various breeds of domesticated sheep (*Ovis aries*) (Zahn, 2001).

The production of wool and woollen textiles and the trade in these commodities have played an important role in political and economic history. The first country to process and trade in wool was Babylon (Babylonia = land of wool). Various found clay panels prove that corn, native oil and wool were the most important products of the country (Zahn et al., 1991). The oldest known wool fabrics, dating from the second half of the second millennium b.c., were found in Danish tree coffins. For centuries wool has also been used for isolation and floor cloth in housing (Zahn et al., 1997)

Unlike cotton, which because of its plant origin consists of cellulose, wool consists to a large extent of proteins. The protein fiber wool consists of carbon, hydrogen, oxygen, nitrogen, and sulfur. The elemental analysis of wool (free of water) is as follows:

Carbon	50.5 wt %
Hydrogen	6.8 wt %
Oxygen	22.0 wt %
Nitrogen	16.5 wt %
Sulfur	3.7 wt %
Ash	0.5 wt %

With the exception of the sulfur content this composition is typical for all proteins. The high sulfur content is due to the high content of cystine, a double amino acid containing two sulfur atoms in a disulfide bond: $HOOCCH(NH_2)CH_2S–SCH_2CH(NH_2)COOH$.

Water free wool consists mainly (approximately 97 %) of wool proteins, the remainder being made up of approximately 2 % structural lipids, approximately 1 % mineral salts, nucleic acids and carbohydrates (Zahn, 2001). Proteins and free amino acids have the ability to complex heavy metals (Martell and Calvin, 1958). For example, various wool dyes on the market are metal complex dyes with Cr(III) and Co(II) compounds (Bruckinshaw, 1992). Some of the dyes bind to the wool proteins over a coordinative bond of the metal ion to the carboxylic groups.

Table 1 shows the amino acid composition and concentrations analysis for a total acid hydrolysate of the wool used in this thesis. It is a typical amino acid composition for wool. Asparagine and glutamine are not measured owing to their change to aspartic acid and glutamic acid during a total acid hydrolysis. The sum of the protein content is 82.9, for wool, because wool is hygroscopic and thus it was not water free when it was hydrolysed.

Table 1: Amino acid analysis of wool

Name of amino acids	Wool (g 100 g^{-1})
Cysteine	0.27
Aspartic acid	5.40
Threonine	4.90
Serine	7.01
Glutamic acid	11.53
Proline	4.03
Glycine	3.50
Alanine	2.82
Valine	3.97
Cystine	8.68
Methionine	0.35
Isoleucine	2.77
Leucine	6.88
Tyrosine	4.76
Phenylalanine	3.34
Ornithine	0.08
Lysine	3.01
Histidine	1.03
Arginine	8.58
Glutamine	n.m.
Asparagine	n.m.
Sum of protein content (g 100 g^{-1})	82.9

Like the other organic natural fibers, cotton and silk, wool is an environmentally friendly, renewable, textile raw material. Woollen materials are reusable. Wool wastes are biodegraded by bacteria, fungi, and insects (moths, carpet beetles) and can be used as long-acting nitrogen fertilisers (Leeder, 1984).

Amino acids and various proteins as for example haemoglobin or calmodulin have been shown to have the ability to complex heavy metals. The application of wool or wool hydrolysates as chelating agent for the purpose of heavy metal phytoextraction however has

been hitherto ignored and was not tested before. Its usefulness in enhancing phytoextraction will thus be examined in this work for the first time.

I.2.6 Commercial future of chelate assisted-phytoextraction

Phytoremediation is already practised commercially in the U.S.A., Canada, Europe and several other countries. The current markets outside the U.S., however, are believed to be small. In 1999 Canadian phytoremediation revenues were estimated to be at U.S. $1-2 million, and European revenues at $2-5 million, whereas the U.S. revenues in 1999 amounted to $30-49 million (D. Glass Associates, Inc., 1999). Calculations for the U.S. phytoremediation market have been put between $100-150 million.

Despite more than 15 years of intensive research on the subject, and positive market prognostics for the *in situ* remediation of large areas, few commercial phytoextraction operations have been carried out. It has been suggested that this technology should be combined with a profit making operation, which is unaffected by any elevated plant metal loadings, such as forestry and bioenergy production (Robinson et al., 2003). Suitable crops, such as poplar and willow trees, could be either incinerated or used for bio-fuel production. As the heavy metals in the remaining ash are tightly bound, the ash would not pose a threat for the environment. Such ash containing heavy metals has been used for years as road base material, and no leaching has occurred (EPA, 2005). A subset of phytoextraction is phytomining, which aims to produce a commercial ´bio-ore´ by planting a hyperaccumulator crop over a low grade-ore body or mineralised soil (McGrath and Zhao, 2003). High biomass Ni hyperaccumulators are particularly suitable for this purpose. Commercially viable phytoremediation/phytomining technologies employing *Alyssum* Ni-hyperaccumulator species to extract quantitatively Ni from soils have already been developed (Broadhurst, 2004). Companies initiated commercial contracts for the production of *Alyssum murale* on serpentine soils in several locations (Chaney, 2003). Although phytomining appears promising, it is momentarily restricted to Ni metal ore extraction, as only the Ni-hyperaccumulators *Alyssum murale* and *Alyssum corsicum* are suitable. Several companies in the field of phytoextraction, mainly in the USA, have emerged (D. Glass Associates, Inc., 2000). In addition, NATO Advanced Study Institute has shown much interest in the phytoremediation of metal-contaminated sites (NATO, 2002).

I.2.7 Subject in dispute

Field studies are necessary to observe the uptake efficiency of heavy metals by the application of chelating agents under natural conditions. The literature is controversial, the results being partially concurrent, and partially different to the results in the greenhouse. Kayser et al. (2000), for instance, performed a field study and a preliminary greenhouse study. Their findings showed much higher tissue concentrations of the extracted metals (Cd, Zn and Cu) in the greenhouse studies than under natural conditions. Other studies dealing with the phytoextraction of gold with corn (*Zea mays*) and Indian mustard (*Brassica juncea*) by Anderson et al. (2005) give hope as to the effectiveness of phytoextraction, the results in the field correlating with those in greenhouse experiments. In contradiction to the above mentioned results, Clemente et al. (2005) observed low uptakes of Zn, Cu and Pb in field studies with Indian mustard (*Brassica juncea*). Their findings indicate the problems involved in employing phytoextraction for the clean-up of pluri-contaminated sites. The results from the study of Jiang et al. (2004) show that shoot Cu concentrations of Haichow elsholtzia herb (*Elsholtzia splendens*) were only up to 10 mg kg^{-1} grown under glasshouse conditions in the pot, but as high as 250 mg kg^{-1} grown under field conditions. The ability of the whole plant for extracting Cu from the polluted soil under field conditions was greater than under glasshouse conditions. An evaluation of the reasons for the differences in the results is very difficult, as the condition in the greenhouse and the field are different, and all plants do not react similarly to the conditions.

The application of chelating agents on field experiments has not been widely performed. Zhuang et al. (2005) conducted field trials with *Viola baoshanensis, Vertiveria zizanioides,* and *Rumex K-1 (R. patientia X R. timschmicus)* and the application of EDTA. Pb phytoextraction rates of *V. baoshanensis, V. zizanioides* and *Rumex K-1* were improved by 19-, 2-, and 13-fold, respectively, compared with the control treatment. Liphazi et al. (2003) performed experiments with sunflower (*Helianthus annuus*) and EDTA. The EDTA application of 1.0 g kg^{-1} rate resulted in 3-fold enhanced uptake of the three non-essential heavy metals (Cd, Ni, Pb) compared to the controls.

As more and more field trials are conducted, the question of heavy metal leaching arises. In experiments with and without barley *(Hordeum vulgare L.)* planted soil columns, Madrid et al. (2003), reported that, even though roots retarded the movement of Cd, Fe, Mn, Ni, Pb, and Zn through the EDTA-treated (0.5 g kg^{-1}) soil from 1 d (Cd) to 5 d (Fe), the drainage water from columns with EDTA had concentrations of Cd, Fe, Mn, and Pb exceeding drinking water standards by 1.3, 500, 620, and 8.6 times, respectively. Similar results were observed

for EDTA by Grčman et al. (2001), Jiang et al. (2003) and Lombi et al. (2001), for EDDS by Meers et al. (2005) and for EDGA by Römkens et al. (2002). The work of Chiu et al. (2005) has also indicated the potential environmental risk of metal mobility caused by NTA and HEIDA. Cooper et al. (1999) also expressed concern about the leaching probability of CDTA and DTPA.

Strategies to control leaching have already been suggested. Kayser et al. (2000) and Shen et al. (2002) proposed splitting the application in separate dosages. Although this division could reduce leaching, it could simultaneously retard the effectiveness. Grčman et al. (2001) reported that the same amount of EDTA applied in different dosages was less effective in enhancing the uptake of heavy metals than a single dose. Irrigation control has also been proposed. Kos and Leštan (2004) successfully tested permeable barriers with the reactive materials, nutrient enriched vermiculite, peat or agricultural hydrogel and apatite. Madrid et al. (2003) proposed that the metal-enriched drainage water should be collected using a dual-pipe subirrigation-drainage system, and should be recycled for further phytoremediation. Kirkham and Horton (1993) proposed this type of irrigation system as means of reducing evaporation and conserving nutrients. These techniques, though, increase the price of phytoremediation and do not solve the problem of leaching from heavy rainfall.

Wenzel et al. (2003) indicated that chelate-assisted phytoextraction is limited by the risk of groundwater pollution arising from metal mobilisation. Robinson et al. (2003) likewise stress that the use of chelating agents significantly increases the risk of contaminant leaching and will do little to enhance the ability of phytoextraction to meet the demands of current environmental legislation. As a concequence chealting agents are needed, which have a high uptake efficiency but at the same time owing to theit rapid biodegradation limit metal leaching into the groundwater.

I.3 Aims and scope

EDTA is toxic to soil microorganisms and to plants, it has a low biodegradability and may remain adsorbed to soil particles, even after soil cleaning, thus dramatically increasing the leaching risk of heavy metals. It is, therefore, desirable to find other biodegradable chelators, which do not exert adverse effects on soil, groundwater and their inherent biota, whilst still offering a high complexation potential for toxic heavy metals. Since nature itself offers such compounds in the form of NLMWOA, cyclodextrins, EDDS and wool, their applicability for enhancing phytoextraction from soil shall be tested. Wool will be applied in the form of wool hydrolysate, which is gained after an enzymatic hydrolysis.

Current research has neglected to determine the influence of the biodegradation process of the chelating agents on the bioavailability of heavy metals and the phytotoxicity of the chelates themselves. The biodegradation of the chelating agents has a direct effect on follow up treatments in the phytoextraction process. Therefore a focus will be laid on the biodegradation process of the chelating agents and an assessment will be made regarding its influence on follow up treatments.

In particular the following questions were addressed:

- What is the efficiency of the above mentioned biochelating agents to metal solubilization in soils and metal uptake and removal by plants?
- At which concentration do the applied chelating agents reveal phytotoxicity?
- What is their degradation rate and what influence do they have on the plant- and bioavailability of heavy metals during the degradation process?
- Is a successive application of the NLMWOA citric acid, oxalic acid and tartaric acid efficient or do the same microorganisms degrade all applied NLMWOA making a successive application useless?
- Which is the effective fraction of the wool hydrolysate?

For studying the efficiency of the chelating agents in enhancing phytoextractions, the main focus will be on greenhouse pot experiments, since metal concentrations and their distribution in the soil can be controlled. Tobacco (*Nicotiana tabacum* SR-1) plants in particular will be used as model plants as they are easily distributed, and have a high growth rate and biomass.

Based on the results, the potential of NLMWOA, cyclodextrins, EDDS and wool hydrolysate to replace compounds, such as EDTA or other synthetic chelators as enhancing agents for the phytoremediation of heavy metal contaminated soils, shall be assessed.

II MATERIALS AND METHODS

II.1 Material

II.1.1 Equipment

AAS, 1100B	Perkin Elmer (California, Fremont, USA)
AAS-lamp, Cd 527423	Helmut Saur Laborbedarf (Reutlingen, Germany)
AAS-lamp, Cu 527446	Helmut Saur Laborbedarf
AAS-lamp, Pb 528136	Helmut Saur Laborbedarf
Analytical balance, SBA31, (Precision 0.0001g)	ScalTec (Göttingen, Germany)
Analytical balance, Kern Kb (Precision 0.01 g)	Kern & Sohn GmbH (Balingen, Germany)
Balance, P1200N	Mettler-Toledo GmbH (Giessen, Germany)
CHN-Analyser, Vario EL III	Elementar Analysensysteme GmbH (Hanau, Germany)
Centrifuge, 2K15, with Rotor 11192, Bucket 13097	Sigma (Osterode, Germany)
Centrifuge, 4404	Heraeus Christ (Hanau, Germany)
Centrifuge, RC-5B Refrigerated super speed centrifuge, with Rotor SLA-3000	Sorvall (Minnesota, USA)
Centrifuge bottles, 50ml polypropylene copolymer, screw closure	Nalgene (Rochester, USA)
Column-oven, STH 585	Dionex (Sunnyvale, USA)
Conductivity-electrode, HI 991300	HannaInstruments (Woonsocket, USA)
Diaphragm vacuum pump, MZ 2C	Vacuubrand (Wertheim, Germany)
HiTrap IMAC HP Columns	GE Heathcare (München, Germany)
Lyophilisator, alpha 1-2, Braun biotech	Heraeus Christ
Lyophilisator, Gamma IA	Heraeus Christ
MALDI-TOF, BRUKER BIFLEX^{TM} III MALDI Flugzeitmassenspektrometer	(Bruker-Franzen Analytik GmbH, Bremen)
Microwave, Start 1500	MLS GmbH für Mikrowellen-Laborsysteme (Leutkirch im Allgäu, Germany)

Oven	
pH-Electrode SE 103	Knick (Berlin, Germany)
Rotoring evaporator, R-114	Büchi (Essen, Germany)
Water-system Milli-Q	Millipore (Schwalbach, Germany)

II.1.2 HPLC-System

Autosampler, 232 XL Samplin Injector	Abimed/Gilson (Langernfeld, Germany)
HPLC, Fa. Chromeleon Software (6.40, Build 681)	Dionex
HPLC pump, P 580 Pump	Dionex
HPLC-Column, Shodex Carbonhydrate Pb^{2+}	ERC-GmbH (Riemeling, Germany)
HPLC-Column, Carbonhydrate-Resin	CS-Chromtographie (Langerwehe, Germany)
Spezialsäule Carbohydrate Pb^{2+}	
UV-detector, UVD 340S	Dionex

II.1.3 Chemicals

AAS standard Cd	Fluka (Taufkirchen, Germany)
AAS standard Cu	Fluka
AAS standard Pb	Fluka
Agar	Applichem GmbH (Darmstadt, Germany)
$BaCl_2\,2{\cdot}H_2O$	Merck KGaA (Darmstadt, Germany)
β-cyclodextrins, min. 98%,	Applichem
$CaCl_2$	Merck KGaA
$CaNO_3\,4{\cdot}H_2O$, min. 99%	Acros Organics (Geel, Belgium)
$3{\cdot}CdSO_4\,8{\cdot}H_2O$, min 98%	Sigma-Aldrich Chemie GmbH (Hannover, Germany)
Citric acid H_2O, min. 99%	Fluka
$CuCl_2$, min. 99%	Fluka
DNeasy Tissue Kit (250)	Qiagen (Hilden, Germany)
DTPA, 98%	Acros Organics
EDDS-trisodium salt, 30%, density 1.274	Fluka
EDTA-Fe(III)-sodium salt, ca. 14%	Fluka

EDTA-disodium salt, min. 99%	Fluka
Ethidiumbromide, 1% in H_2O	Merck KGA
HCl, 37%, density 1.19	Merck KGA
HNO_3, 65% density 1.40, Merck KGaA	Merck KGA
HCl, 32%, density 1.16	Fluka
Imidazole, min. 99%	Roth GmbH (Karlsruge, Germany)
KCl	Merck KgaA
KH_2PO_4	Merck KGaA
$NaHCO_3$, min 99%	Merck KGaA
NaOH, pellets GR for analysis	Merck KGaA
$NaPO_4$, min. 99%	Roth GmbH
NH_4NO_3, min. 99%	Merck KGaA
Oxalic acid $2{\cdot}H_2O$, min. 99%	Fluka
$Pb(NO_3)_2$, min. 99%	Sigma-Aldrich Chemie GmbH
Tartaric acid, 99%	Fluka
TEA, 99%, density 1.124	Acros Organics

II.1.4 General consumables

Filters, 589/3, 110 mm, blue ribbon	Schleicher & Schuell (Dossel, Germany)
Filters, 595, 110 mm	Schleicher & Schuell
Filter unit, 0.45 µm	Schleicher & Schuell
Filter unit, 0.2 µm	CS Chromatographie (Langerwehe, Germany)
Syringe, 2 mL	Braun (Melsungen, Germany)

II.1.5 Tobacco seeds germination medium

Seeds of tobacco plants (*Nicotiana tabacum* SR-1) were germinated in a peat and sand mixture. After 2-3 weeks (depending on the temperature and the greenhouse conditions), the seedlings were transferred into the pots.

II.1.6 Soil characterisation

Depending on the chelates applied in the experiments in this thesis, two different types of soil were used. Both soils were collected from 0-30 cm surface layer of the Melaten field in Aachen, Germany. Soil (a) a silty-loamy sand was used in the experiments with NLMWOA and cyclodextrins, whereas the experiments with wool and EDDS were performed on soil (b) a silty-loamy agricultural soil USDA (Soil Survey Manual, 1951). The soil was air-dried at room temperature, sieved through a 2-mm sieve and characterised as follows. The sand, clay and silt fractions of the samples were determined by the hydrometer methods (Bouyoucous, 1952), and the organic matter content was determined by the Walkley-Black method (Nelson and Sommers, 1996). The pH was measured by the $CaCl_2$-method (Lewandowski et al., 1997). The initial total Cu, Cd and Pb content of the soil, as determined by the aqua regia method according to DIN 38414 Teil 7, are listed in Table 2-1. Cu, Cd and Pb analysis in the filtrate was performed by flame AAS (Perkin-Elmer 1100B). Standards for the AAS calibration were prepared in the extraction solution by the addition of appropriate quantities of Cu and Pb respectively. The soil properties are listed in Table 2-1.

Table 2-1: Properties of the soils used in the experiments.

Character		Content	
		(a)	(b)
Texture[1]	Clay (%)	8.49	18.6
	Silt (%)	42.10	56.4
	Sand (%)	49.41	25.0
pH ($CaCl_2$)[2]		6.74	6.59
Organic matter content (%)[3]		3.54	4.45
$CaCO_3$ (%)[4]		3.2	0.8
CEC (cmol kg^{-1})[5]		18.06	14.16
Maximum water holding capacity (%)[6]		41	47
Electric conductivity (µS cm^{-1})[7]		177.3	128.3
C/N-ratio (%)[8]		10.4	16.75
Total Cd (mg kg^{-1})[9]		1.62	2.51
Total Cu (mg kg^{-1})[9]		21.8	89.9
Total Pb (mg kg^{-1})[9]		35.7	259.9

[1]DIN ISO 11277

[2]DIN ISO 10390

[3]DIN ISO 19684-3 (über Glühverlust)

[4]DIN ISO 10693 (Variation of Scheibler-Apparatus according to Passon)

[5]DIN ISO 11260

[6]Schinner et al. (1993), with alterations

[7]DIN ISO 11265

[8]CHN-Analyser

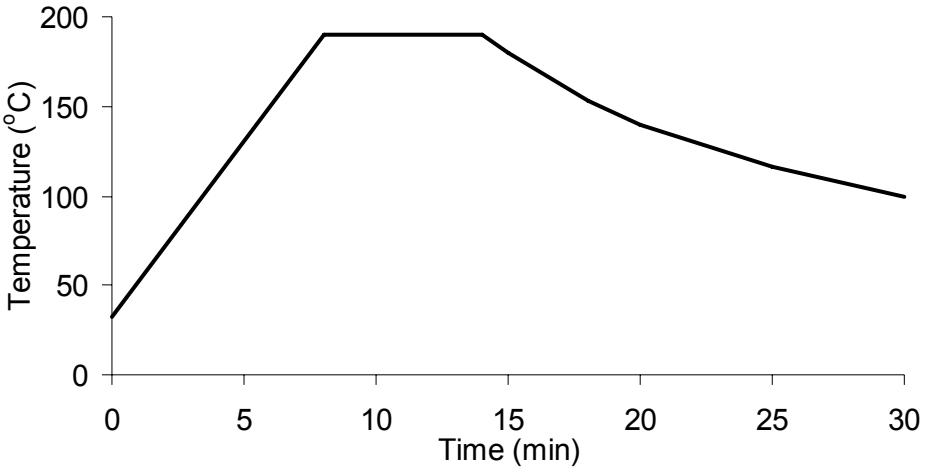
[9]Aqua regia extraction (Kopp, 1999)

Maximum water holding capacity

The maximum water holding capacity was determined according to Schinner et al. (1993). The method was adjusted to the possibilities of the laboratory equipment. 20 g of air dried soil were filled into glass cylinders (24 mm inner diameter and 140 mm in length). One side of these was covered with gaze, and these were subsequently put in a water filled plate for 40 h. Thereafter, the soil was saturated with water. Subsequently, the cylinders were covered on one side with filter papers and placed on a plate with sand for 24 h. During this time they were placed in a 4 ^{o}C thermo constant chamber. The upper side of the columns was covered with parafilm to avoid evaporation. After 24 h, the samples were weighed and subsequently dried in 105 ^{o}C chamber for 18 h at which a constant weight was achieved. The dry samples were then weighed again. The weight difference before and after drying determined the maximum water holding capacity of the soil in per cent of the soils dry weight.

Aqua regia-extraction

With the aid of the aqua regia-method it is possible to determine the total amount of Cd and other metals in the soil (DIN ISO 11047). The method was adjusted to microwave combustion according to Kapp et al. 1999. Three g of air dried soil were boiled with aqua regia (3 mL 32% HCl, 10 mL 63% HNO_3, 5 mL H_2O) in the microwave. Figure 2-1 depicts the time and temperature of the extraction. The extraction was performed in triplicates.



Step 1: 8 min, 190 °C; Step 2: 6 min, 190 °C; Step 3: 20 min ventilation

Figure 2-1: Time and temperature-conditions of the microwave extraction.

Subsequently the samples were transferred to a graduated cylinder with a pipette and diluted with Millipore water to a volume of 25 mL. The Cu, Cu and Pb content was then measured via AAS.

II.2 Methods

II.2.1 Mobilisation experiments

II.2.1.1 Slurry experiment

For the slurry experiment 1 g of the agricultural soil was weighed into a 50 mL polypropylene copolymer centrifuge tube. The centrifuge tubes were pre-washed with dilute HNO_3 to eliminate any adsorbed metals. The soil was suspended with 15 mL of a 62.5 mM solution containing, either, citric acid, oxalic acid, tartaric acid, malic acid, or 0.125 mM EDTA. Each suspension was performed at three different pH values, 9, 7, and 5 adjusted with 1 M NaOH. The tubes were then shaken for 18 h at 70 rpm, and were subsequently centrifuged at 14.000 g for 10 min. Each sample was then filtered through a quantitative filter paper (589/3, Schleicher & Schuell Filters). In order to keep the metals in the samples in solution 100 µL of 65% HNO_3 was added to each of them. After maximum 2 d the Cu and Pb content in the solution was determined by AAS. Each treatment was performed in triplicates.

II.2.1.2 Column experiment

Glass columns (24 mm inner diameter and 140 mm in length) were pre-washed with diluted nitric acid to eliminate any adsorbed metals. 25 g of metal spiked soil was packed into the column. The soil was spiked 3 months before the column experiment, with either 450 mg kg^{-1} of Cu applied as $CuCl_2$, 600 mg kg^{-1} of Pb applied as $Pb(NO_3)_2$, or 100 mg kg^{-1} of Cd applied as 3 $CdSO_4$ 8 H_2O. For this purpose large volumes of dissolved $CuCl_2$ and $Pb(NO_3)_2$ or 3 $CdSO_4$ 8 H_2O were added at low concentrations in order to achieve an equal distribution. The pore volume of the column, which was determined according to DIN 19683-13 was 56%, which equals 14 mL. The chelating agents solutions, described in Table 2-2, were continuously poured into the columns in order to maintain a constant solution level of approximately 6 cm above the soil surface during the leaching. The effluent flow rate was kept at an average of 1 pore volume h^{-1} (14 ± 1 mL h^{-1}). Each column was eluted with 60 - 100 mL of the chelating agents with the concentrations shown in Table 2-2. The solution level varied slightly depending on the leachate flow. At the bottom of the columns, the leachates were collected in 14 mL aliquots and measured at the same day. To avoid pH dependent effects chelating agents' solutions were adjusted to a pH value of 6.8 with 1 M NaOH, which equals the soil's pH value. As a control a 10 mM $CaCl_2$ solution was used, since it has a comparable ionic strength to the natural soil solutions (Shuman, 1990). Each solution-column experiment was performed in five replicates. The Cu and Pb content was determined by AAS.

Table 2-2: Chelating agents and their corresponding concentrations used in column experiments.

Chelating agent	Concentration
NLMWOA	
Citric acid	62.5 mM
Tartaric acid	62.5 mM
Oxalic acid	62.5 mM
EDTA	0.125 mM
β-cyclodextrins	4.4 mM
EDDS	1.5 mM
Wool hydrolysate	6.6 g L^{-1}

II.2.2 Pot experiments

II.2.2.1 Soil preparation

The pot experiments were conducted in a greenhouse. For the NLMWOA experiments 400 g of air dried and sieved soil was used, whereas in the cyclodextrins, wool and EDDS experiments 450 g were used. The soil was filled in 0.5 L plastic pots with 6 small holes at the bottom. The 0.5 L plastic pots with 6 small holes at the bottom were pre-washed with diluted nitric acid to eliminate any adsorbed metals. The soil was filled into the pots and a pot-plate was placed under each pot. The following amounts of fertiliser were applied to each pot: 674.4 mg $Ca(NO_3)_2\,4H_2O$, 175.6 mg KH_2PO_4, resulting in a concentration of 200 mg kg^{-1} and 100 mg kg^{-1} respectively. Fe(III)EDTA was deliberately not added to avoid the influence of EDTA on the phytoextraction experiments.

II.2.2.2 Cultivar source, seedling preparation and plant growth

Tobacco plants (*Nicotiana tabacum* SR-1) were used for the pot experiments. This selection was based on the plant's ability to produce a great biomass in a very short time. Seeds of tobacco plants were germinated in a peat/sand mixture. After an incubation of 4 weeks, seedlings with similar biomass were transferred into the pots with the metal spiked soil. One seedling was planted into each pot.

The work of Walch-Liu et al. (2000) was used as a reference for the growth of the tobacco plants with conditions adapted to the technical capabilities provided in our laboratory. All tobacco plants were grown under controlled environmental conditions with a 16 h light period (light intensity of 120 µmol m^{-2} s^{-1}), a 25/20°C light/dark temperature regime, and 60% relative humidity. The plants were harvested after 4 weeks of growth on heavy metal spiked soil.

II.2.2.3 Toxicity pot experiment

Toxicity experiments were performed with NLMWOA, EDTA, EDDS and cyclodextrins. The experiment included the control treatment (no additional chelating agents), and treatments with NLMWOA, EDTA and cyclodextrins according to the concentrations in Table 2-3.

In the experiments with NLMWOA, EDTA and cyclodextrins the chelating agents were applied in solid state. The application of the solid state was necessary because cyclodextrins have very low water solubility and the amount of NLMWOA was too high to be applied in a dissolved state. After thorough mixing of the soil, seedlings with similar biomass were transferred into the pots containing chelating agents spiked soil. One seedling was planted into each pot. The experiment was performed in triplicates.

In the toxicity experiment with EDDS and EDTA, the chelating agents were applied to the pots 2 weeks before harvesting in dissolved form, while the plants were already planted for 14 d. The chelating agents' concentrations in soil were according to Table 2-3. The toxicity effects were based on observation of the dry mass, and of chlorosis and necrosis symptoms.

Table 2-3: Chelating agents and their corresponding concentrations used in phytotoxicity experiments.

Chelating agent		Concentration (mmol kg^{-1})
NLMWOA		
	Citric acid	62.5, 125, 250
	Tartaric acid	62.5, 125, 250
	Oxalic acid	62.5, 125, 250
EDTA		0.125, 0.25, 1.25
β-cyclodextrins		3.92, 7.83, 11.75
EDDS		1.5, 3.125, 6.25, 12.5, 25, and 50
EDTA		1.5, 3.125, 6.25, 12.5, 25, and 50

II.2.2.4 Phytoextraction pot experiments

The experiments included the control treatments (no added heavy metals), and treatments with Cu applied as $CuCl_2$, Pb applied as $Pb(NO_3)_2$, and Cd applied as 3 $CdSO_4$ 8 H_2O. Each treatment was performed in triplicates. In order to achieve an equal distribution of the heavy metals large volumes (50 mL) of dissolved $CuCl_2$, $Pb(NO_3)_2$ or 3 $CdSO_4$ 8 H_2O were applied at low concentrations. The heavy metal soil concentrations are displayed in Tab.

In the phytoextraction pot experiments with NLMWOA or EDTA only Cu or Pb were added. One day after the application of Cu or Pb, the fertilisers, as well as NLMWOA (citric, oxalic, and tartaric acid) or EDTA were applied to the soil. Subsequently one seedling was

planted into each pot, the experiment was initiated thereafter. All seedlings used had similar biomass.

In the experiment with EDDS and wool as chelating agents, the soil was spiked 3 weeks before the pot experiment. During these 3 weeks the soil passed 3 wet-dry cycles to enable the added heavy metals to reach a steady state (Blaylock et al., 1997). Three weeks after the application of Cu and Cd (for EDDS-experiment), one seedling was placed in each pot. Two weeks before harvesting the chelating agents EDDS or EDTA were applied, but in the case of wool the addition was made just 2 d before harvesting.

In order to avoid the leaching of heavy metals from the soil the distilled water, used for watering the plants, was never added directly to the soil surface, but into the pot-plate. The chelating agents' concentrations in soil are displayed in Table 2-4.

Table 2-4: Chelating agents, heavy metals and their corresponding concentrations in soil used in phytoextraction experiments.

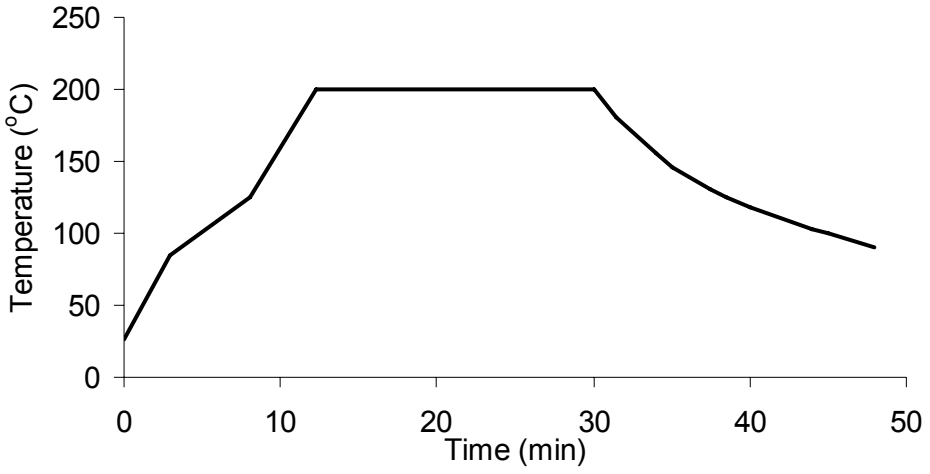
Chelating agent-Experiment	Heavy metal	Concentration (mg kg^{-1})
NLMWOA		
Citric acid	Cu	225, 450
	Pb	300, 600
Tartaric acid	Cu	225, 450
	Pb	300, 600
Oxalic acid	Cu	225, 450
	Pb	300, 600
EDTA	Cu	225, 450
	Pb	300, 600
EDDS	Cu	150, 300, 450
		5, 10, 15
EDTA	Cd	150, 300, 450
		5, 10, 15
Wool-hydrolysate	Cu	200, 400, 600
	Cd	10, 20, 30

II.2.2.5 Plant harvest and analysis

Plants used in the pot experiments were harvested after approximately 3 weeks of growth. During harvesting, plants were cut short above ground, and separated into stems and leaves. The subsequent steps were performed according to Jones and Case (1990). Plant samples (stem and leaves) were rinsed briefly in deionised water, dried between Kleenex tissues to remove surface contamination, and oven dried at 70°C to a constant weight. The dry weight was determined and the samples were homogenised in particle size by grinding in a ball mill.

The heavy metals from the plant samples of the NLMWOA and EDTA experiment were extracted via dry digestion. After milling, 200 ± 5 mg of dried plant tissue were weighed into a 15 mL high form porcelain crucible. The plant tissue was incinerated at 500 °C for 5 h in a muffle furnace and cooled down. At 60 °C, 2 mL of 15% HCl were added and evaporated. At room temperature, an additional 2 mL of 15% HCl was added. The ash was suspended with the assistance of a glass stick, and the suspension subsequently filtered through a quantitative filter paper (595, Schleicher & Schuell Filters, pore size 4 µm). The filtrate was adjusted to 20 mL with deionised water and analysed for Cu and Pb by AAS.

The plant samples of the EDDS, EDTA and wool experiment were extracted via wet digestion. After milling, plant samples of approximately 500 mg were digested via microwave in 4 mL HNO_3 (65%), 3 mL H_2O_2 (30%), and 5 mL H_2O for metal analysis (Kopp, 2000). The digested samples were diluted to 15 mL in Millipore water and analysed for Cu and Cd by AAS. The temperature and duration of the extraction is shown in Figure 2-2.



Step 1: 3 min, 85 °C; Step 2: 5 min, 125 °C; Step 3: 4:30 min, 200 °C, Step 4: 17:30 min, 200 °C; Step 5: 18 min, ventilation

Figure 2-2: Time and temperature-conditions of the microwave extraction.

II.2.3 Biodegradation of chelating agents

II.2.3.1 Influence of the biodegradation on Cu bioavailability

In the same pots as the one used for the toxicity experiment 450 g of spiked soil containing 450 g kg^{-1} Cu were added. The chelating agents NLMWOA (citric, tartaric, or oxalic acid) or cyclodextrins were added to the soil, resulting in a soil concentration of 62.5 mmol kg^{-1} and 3.92 mmol kg^{-1} respectively. Throughout the experiment, the soil was kept at a constant dampness of 25% of the maximum water holding capacity. Samples for pH measurement and Cu bioavailability were taken after 0, 24, 48, 96, and 144 h. The bioavailable Cu was extracted by the DTPA method (Risser and Baker, 1990). The Cu concentration was determined thereafter via AAS.

II.2.3.2 Biodegradation in oxitop systems

The Oxitop system consists of an individual number of reactors consisting of glass bottles (500 mL nominal volume) with a carbon dioxide trap (potassium hydroxide) in the headspace. Forty-five mL of soil were filled into the bottles. The soil was adjusted to 50% maximum water holding capacity, and either glucose, EDDS, EDTA, NLMWOA, cyclodextrins, or wool was added (Table 2-5). A control, containing only the soil at 50% maximum water holding capacity, was run separately. The experiment was performed in triplicates. The bottles were sealed with a cap containing an electronic pressure indicator, quantifying the CO_2 production by metabolism of the added substrate. An incubator chamber was used to maintain the respirometer units at constant temperature (20 ^{o}C) during the test run. The decrease in headspace pressure in the closed test vessel was continuously recorded and the biochemical oxygen demand (BOD) was calculated according to the following equation:

$$BOD = \frac{M(O_2)}{RT}\left(\frac{V_{Total} - V_{sample}}{V_{sample}} + \alpha\frac{T_{20}}{T_0}\right)\Delta p(O_2)$$

$M(O_2)$ is the molecular weight of oxygen (32000 mg/mol), R is the gas constant (83.144 mbar/(mol K)), T_0 is the temperature at 0 ^{o}C (273.15 K), T_{20} is the incubation temperature (293.15 K = 20 ^{o}C), V_{Total} is the total volume of the test vessel, V_{sample} is the sample volume in the test vessel, α is the Bunsen absorption coefficient (0.03103) and $\Delta p(O_2)$ is the difference of the partial pressure of oxygen (mbar).

Table 2-5: Chelating agents and their corresponding concentrations used in oxitop biodegradation experiments.

Chelating agent		Concentration (mmol kg^{-1})
NLMWOA		
	Citric acid	62.5
	Tartaric acid	62.5
	Oxalic acid	62.5
Cyclodextrins		3.92
EDDS		0.05, 0.005
EDTA		0.05, 0.005
Glucose		0.05
Wool		6.6 g kg^{-1}

II.2.3.3 Influence of the biodegradation on the phytotoxicity

Additionally, 20 d after the chelating agent application (6 d after harvest) new tobacco seedlings were planted in the already used pots without spiked Cd and Cu, but with the applied EDDS and EDTA. Only the control pots were used to avoid toxic effects from the mobilised heavy metals, Cd and Cu, in the other pots.

II.2.4 Identification of NLMWOA degrading microorganisms

II.2.4.1 Isolation of microorganisms on minimal medium agar plates

One gram of the air dried agricultural soil was weighed into a 50 mL polypropylene copolymer centrifuge tube. To the soil, NLMWOA (citric acid, tartaric acid or oxalic acid) resulting in a concentration of 62.5 mmol kg^{-1}, were added. The soil was adjusted to a 50% maximum water holding capacity and stored for 24 h at 18 ^{o}C, 28 ^{o}C and 37 ^{o}C. The temperatures were chosen in order to isolate bacteria and as fungi. Each treatment was performed in duplicates.

After 24 h, 10 mL of a 0.9% NaCl-solution was added to the soil. The solution was shaken for 1 min and then left for sedimentation. After 2 hours 10 µL of the solution were added to corresponding minimal medium agar plates. The composition of the agar plates are listed in

Table 2-6. The concentration of agar was 15 g L^{-1}. Amounts of 6.3 g citric acid, 4.5 g tartaric acid, or 3.78 g oxalic acid, resulting in a NLMWOA concentration of 0.03 mol L^{-1}, were added as the sole C-source. The pH of the agar was adjusted to 5.5 with NaOH, which was the pH of the soil at the beginning of the phytoextraction experiment. The agar plates were stored in 18 ^{o}C, 28 ^{o}C and 37 ^{o}C until the growth of microorganisms was visible. Each treatment was performed in triplicates.

Table 2-6: Composition of minimal medium for agar plates. Contains no C-source

Component	Concentration in medium (mM)
Stock A	
Na_2HPO_4	10
KH_2PO_4	10
Stock B	
$(NH_4)_2SO_4$	2.5
Stock C	
$MgSO_4$	0.4
Stock D	
$CaCl_2$ $2H_2O$	0.05
Stock E	
EDTA	0.0086
$FeSO_4$ $7H_2O$	0.0100
$ZnSO_4$ $7H_2O$	0.0040
$MnSO_4$ H_2O	0.0100
$CuSO_4$ $5H_2O$	0.0015
$Co(NO_3)_2$ $6H_2O$	0.0008
$(NH_4)_6$ Mo_7 O_{24} 4 H_2O	0.0001

II.2.4.2 DNA extraction of the microorganisms on the minimal medium agar plates

Samples of the microorganisms were taken from the agar plates and put in 2 mL eppendorf tubes. The samples were frozen with liquid nitrogen and grinded. DNA extraction was performed with Quiagen´s DNAeasy Plant MiniKit. The extracted samples were

subsequently verified via agarose-gel-electrophorese. Agarose in the concentration of 1% (w/v) was dissolved in 1 x TAE-buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) in microwave. After cooling down 3 µL of ethidiumbromide were added to the solution. To the gel 10 µL DNA + 3 µL 6x loading dye and 10 µL ladder were added. The duration was 40 min at a constant current of 120 mA.

II.2.4.3 Polymerase Chain Reaction (PCR)

PCR is as procedure for rapid in vitro enzymatic replication of a specific segment of DNA (Saiki et al., 1985).The PCR was used to amplify parts of the extracted DNA strand, through the application of oligonucleotides as a starter molecule (Primer) for thermostable DNA-polymerase. The PCR consisted of 3 steps. 1. The DNA is denaturated, 2. the primers attach themselves to the DNA strands, 3. the DNA-polymerase copies the DNA. The three steps are repeated several times, until a sufficient amount of the amplified DNA-segment is reached.

The amplification of the 16S rDNA (bacteria) was achieved with the application of the primers complementary to the positions 968 (primer U968GC) and 1401 (primer 1401) of the 16S rDNA of *Escherichia coli.* The result was approximately 450 bp long samples of the 16S rDNA, amplified via PCR, which contain the variable regions V6 to V8 (Bulawa, 2004). The used primers and the PCR composition and conditions are depicted in Table 2-7.

Table 2-7: Applied primers and composition and condition of 16 S rDNA PCR reaction.

Applied primers:

U968GC: 5´CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC 3´

L1401: 5´CGG TGT GTA CAA GAC CC 3´

Components	Volume per sample (µL)
Goldstar Ready-Mix (Eurogentec)	25
$MgCl_2$	3
L1401	1
U968GC	1
H_2O	19
DNA-extract	1

Cycle number	Step definition	Time/Temperature
	Activation of DNA-polymerase	10 min at 95 ^oC
1-35 cycles		
	Denaturation	1 min at 94 ^oC
	Annealing	1 min at 54 ^oC
	Extension	1 min at 72 ^oC
36. cycle	Final extension	10 min at 72 ^oC

For the amplification of the fungi 18S rDNA, the forward primer Ns1 complementary to the positions 20-38 of *Saccharomyces cerevisiae* and the reverse primer GCFung complementary to the positions 351-368 of *S. cerevisiae* were used. The result was approximately 350 bp long samples of the 18S rDNA, amplified via PCR (Bulawa, 2004). The used primers and the PCR composition and conditions are depicted in Table 2-8.

Table 2-8: Applied primers and composition and condition of the 18S rDNA PCR reaction.

Applied primers:

NS1: 5´GTA GTC ATA TGC TTG TCT C3´

GCFung: 5´CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CAT TCC CCG TTA CCC GTT G3´

Components	Volume per sample (µL)
Goldstar Ready-Mix (Eurogentec)	25
$MgCl_2$	3
Ns1	1
GCFung	1
H_2O	19
DNA-extract	1

Cycle number	Step definition	Time/Temperature
	Activation of DNA-polymerase	10 min at 95 °C
1-35 cycles		
	Denaturation	30 sec at 95 °C
	Annealing	1 min at 50 °C
	Extension	1 min at 72 °C
36. cycle	Final extension	10 min at 72 °C

The PCR products were subsequently verified via agarose-gel-electrophorese. Agarose in the concentration of 1% (w/v) was dissolved in 1 x TAE-buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) in microwave. After cooling down 3 µL of ethidiumbromide were added to the solution. To the gel 10 µL DNA + 3 µL 6x loading dye and 10 µL ladder were added. The duration was 40 min at a constant current of 120 mA.

II.2.4.4 Sequencing

The PCR products were purified with the QIAquick PCR Purification Kit according to the specifications of the manufacturer. The sequencing was performed by the Fraunhofer, IME, Aachen. The received sequences were subsequently compared with 16S rDNA, for bacteria, and 18S rDNA, for fungi, sequences of reference organisms. The comparison of the sequence

data with public reference data was conducted via the sequence-database NCBI (National Center for Biotechnology Information) program BLAST (http://www.ncbi.nlm.nih.gov./BLAST/; Altschul et al., 1997).

II.2.4.5 HPLC detection in NLMWOA batch experiments

20 mL of minimal medium (Table 2-6) were filled into 100 mL Erlenmeyer flasks. The sole carbon source was 2 g L^{-1} citric acid, 2 g L^{-1} tartaric acid, and 2 g L^{-1} oxalic acid. Additionally a similar batch experiment was prepared with 2 g L^{-1} oxalic acid as the sole carbon source. The flasks were inoculated with samples of the isolated microorganisms on the agar plates. The flasks were shaken for one week on rotary shaker at 18 ^{o}C, 28 ^{o}C and 37 ^{o}C.

After one week 1.5 mL of each flask were sterilised and filtered through a 0.2 µm HPLC filter. HPLC-standards were prepared by dissolving 0.02, 0.08, 0.1, 0.2, 0.8, 1.2, 2.0 g L^{-1} of citric acid, tartaric acid and oxalic acid in 1 mM H_2SO_4. A Shodex Carbonhydrate Pb^{2+} (ERC-GmbH) and Carbonhydrate-Resin Spezialsäule Carbohydrate Pb^{2+} (CS-Chromtographie) column was used. The flow rate of the eluent (1 mM H_2SO_4) was 0.8 mL min^{-1} and the temperature was 60 ^{o}C. The NLMWOA were detected with a UV-detector at 254 nm.

II.2.5 Characterisation of wool hydrolysate

II.2.5.1 Wool hydrolysis

The experiments were carried out on wool fibres originating from Merino sheep. A commercial available thermo stable and alkali-resistant serine protease Esperase 8.0 L (8 KNPU/g) by Novo Nordisk A/S, Bagsvaerd Denmark, was used. Esperase 8.0 L was used because Elling and Zahn, 1988 revealed that the enzyme has a high proteolytic activity for wool. In a 500 mL round bottom flask 10 g of wool were treated with 250 mL 0.01 M $NaHCO_3$, pH of 8.1 and 2 mL of the enzyme. The temperature of treatment was 50 ^{o}C and the time of treatment was 7 d for the 3 g (6.6 g kg^{-1}soil) treatments and 14 d for the 6 g (12.2 g kg^{-1} soil) treatment. During this process the solution was agitated every 12 h.

II.2.5.2 Molar mass determination by MALDI-TOF MS

Part of the 14 d hydrolysed wool was centrifuged and the supernatant was collected and analysed for its molar mass by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS).

In MALDI-TOF MS a co-precipitate of an UV-light absorbing matrix and a biomolecule is irradiated by a nanosecond laser pulse. Most of the laser energy is absorbed by the matrix, which prevents unwanted fragmentation of the biomolecule. The ionised biomolecules are accelerated in an electric field and enter the flight tube. During the flight in this tube, different molecules are separated according to their mass to charge ratio and reach the detector at different times. In this way each molecule yields a distinct signal.

The samples were lyophilised for the determination of the molecular weight of the individual peptides in the hydrolysed wool solution. The residue was solved in 0.5% (v/v) TFA (trifluoroacetic acid) and applied on the sample carrier. After the evaporation of the solvent, the matrix sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) solved in a solution of 0.1% TFA and H_2O:Acetonitril (2:1) was applied, in a 1:1 sample to matrix ratio, on the sample carrier.

The MALDI-TOF massspectra were measured at the Deutschen Wollforschungs Institut (DWI) of the RWTH-Aachen University. A BRUKER BIFLEX^{TM} III MALDI was used, which was equipped with a SCOUT-Ionsource, an ion mirror (reflector) and standard-microchannel-plate-detectors. The stimulation of the matrix was performed by a nitrogen laser at 337 nm a pulse duration of 3 ns. The acceleration voltage was 20 kV. The recorded mass spectra show the generated ion signal from a summary of approximately 100 laser pulses. The standards used for calibration were sinapinic acid, substance P (neuropeptide) and the adrenocorticotropic hormone (ACTH).

II.2.5.3 Amino acid analysis

The amino acid composition and concentrations were determined using ion-exchange chromatography (Moore et al., 1958). The method is known as the Speckman, Stein, and Moore method. The hydrolysate was applied on the sulfon polystyrol matrix of the chromatographic column in a lithiumcitrate buffer with a pH of 2.2. The separation of the hydrolysate is achieved by a buffer alternation of pH 3.53 over 4.34 and 5.25 to 10.75. The

amino acids are quantified through a ninhydrin post column derivatisation and a photometric measurement at 570 and 440 nm.

The amino acid composition and concentrations analysis was performed for a total acid hydrolysate of the wool and of the enzymatic hydrolysate. Additionally, an analysis was performed with the free amino acids in the enzymatic hydrolysate. The total acid hydrolysis, which can be performed in concentration ranging from 0.1 to 10 mg mL^{-1}, is performed as follows: 0.2 mg of sample in 0.5 mL of 5.7 N HCl for 24 h at 110 °C according to Spitz (1973). Methionine and cystine were determined using the performic acid oxidation method as described by Moore (1963). The measurement was performed by the Deutsche Wollforschungs Institut (DWI) in Aachen.

II.2.5.4 Immobilised metal ion affinity chromatography (IMAC)

IMAC is based on the specific interaction between immobilised metal ions and certain amino acid side chains exposed on the surface of proteins. Although, widely implemented as a separation method for purifying a broad range of proteins and peptides, it was used in this case to isolate peptides which have a high affinity to Cu^{2+}.

HiTrap IMAC HP 1 ml columns were prepacked with uncharged IMAC Sepharose High Performance, containing a covalently immobilised chelating group (NTA).

The medium was subsequently charged with Cu^{2+} using a 0.1 M $CuSO_4$ solution according to the manual delivered with the HiTrap IMAC HP 1. The binding buffer consisted of a 20 mM sodium phosphate, 0.5 M NaCl, solution adjusted to pH 7.4, while the elution buffer consisted of a 20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, adjusted to pH 7.4.

The column was prepared according to the manual. A blank run and a purification step were included to decrease the possible leakage of Cu^{2+}. The flow rate was installed at 1 mL in^{-1} with a peristaltic pump. The samples (hydrolysed wool) were solved 1:10 in binding buffer to adjust the sample to the conditions of the column. On one column 2 mL of the hydrolysed wool were poured.

After the elution of the peptides an analysis for Cu was performed via AAS to detect any Cu which could have leached from the IMAC-columns. Cu is harmful for the chromatographic column used in the amino acid analysis. The Cu content of the samples was too high, and thus an uncharged IMAC-column was used to purify the samples. The samples were poured into the uncharged IMAC-column and were subsequently eluted with the elution

buffer, in order to achieve a removal of the Cu from the peptide solution. A subsequent AAS measurement revealed no Cu in the samples. An amino acid analysis and a MALDI-TOF MS were then performed.

III RESULTS

III.1 Natural low molecular weight organic acids

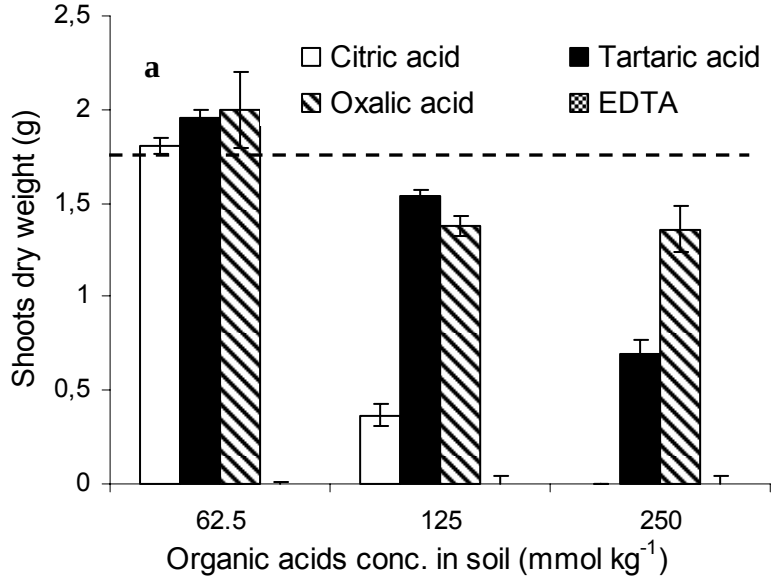
The three natural low molecular weight organic acids (NLMWOA), citric acid, oxalic acid and tartaric acid, were used in slurry, column, phytotoxicity, phytoextraction and biodegradation experiments to investigate their potential as an alternative to synthetic chelators. The same experiments were performed with the synthetic chelator EDTA, in order to achieve a direct comparison with the NLMWOA. The phytotoxicity experiment revealed a low phytotoxic potential for NLMWOA and a very high phytotoxic potential for EDTA. At a NLMWOA concentration of 62.5 mmol kg^{-1} of soil no adverse effects were observed on the biomass production, whereas in the case of EDTA no adverse effects were observed at an EDTA concentration of 0.125 mmol kg^{-1} of soil. The slurry experiment showed a higher Cu and Pb mobilisation potential by the NLMWOA than by EDTA. The column experiment confirmed these results. The Cu amount mobilised by the NLMOWA was 5-fold higher than the mobilised Pb amount. The phytoextraction experiment revealed a significant increase in Cu uptake only in the citric acid treatment (67 mg kg^{-1}) in comparison to the EDTA treatment (42 mg kg^{-1}). The NLMWOA could not enhance the uptake of Pb. The biodegradation of the NLMWOA in the soil decreased the bioavailability of Cu, owing to an increase in the soil pH. In order to cope with the short effective time period of the NLMWOA, owing to a rapid biodegradation rate, the question of a successive application of the three NLMWOA was followed. Isolated NLMWOA degrading microorganisms revealed that the same microorganism could degrade the three tested NLMWOA thus making a successive application not feasible.

III.1.1 Phytotoxicity pot experiment

In this experiment the effect of the added NLMWOA and EDTA on the dry weight of shoots was investigated after a 3 week growth period. Dry matter yields of the shoots (sum of leaves and stem) are shown in Figure 3-1. The application of NLMWOA to the soil did not adversely affect dry matter production of the plants at a concentration of 62.5 mmol kg^{-1} of soil. Toxicity symptoms, such as lower dry weight and chlorosis, were visible in higher concentrations, depending on the chelating agents. Citric acid showed significant ($P < 0.05$) toxicity effects at a concentration of 125 mmol kg^{-1}, whereas tartaric acid and oxalic acid showed only slight toxicity at this concentration. Regarding the concentration of 250 mmol

kg^{-1}, all tested NLMWOA exhibit toxicity symptoms, such as chlorosis and necrosis. The plants on the 250, 125 and 62.5 mmol kg^{-1} EDTA treated soil died shortly after the exposure to EDTA, therefore their dry weight in Figure 3-1a is not visible. EDTA did not show any adverse effects at a concentration below 0.125 mmol kg^{-1}. At 1.25 mmol kg^{-1} of EDTA (Figure 3-1b) the leaves showed necrotic lesions and the dry weight was negligible. At a concentration of 0.25 mmol kg^{-1} EDTA, the dry weight is only slightly lower than the 0.125 mmol kg^{-1} application, but the standard deviation is higher.

The phytotoxicity experiment revealed the highest no effect concentration of NLMWOA and EDTA to tobacco, therefore NLMWOA and EDTA can be applied in the phytoextraction experiment according to these results without causing additional stress to the plants. Potential toxicity symptoms appearing on the plants in the phytoextraction experiment can thus not derive from the chelating agent's application.



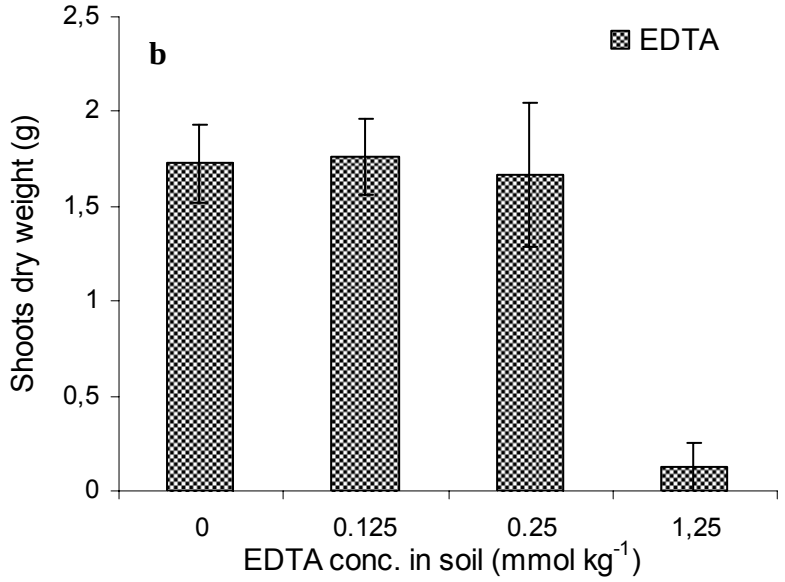


Figure 3-1: Dry weight of tobacco (*Nicotiana tabacum* SR-1) grown for 3 weeks in soil containing NLMWOA and EDTA (a) and EDTA (b). Error bars represent SD of triplicates. ---- symbolises shoots dry weight of control plants.

III.1.2 Slurry experiment

In this experiment the mobilisation of Cu and Pb in natural contaminated soil was analysed. The concentrations of NLMWOA and EDTA used in this experiment were based on the phytotoxicity pot experiment. Figure 3-2 shows the Cu and Pb mobilisation in proportion to the natural heavy metal content, (21.8 and 48.7 mg kg^{-1} respectively) in percent of the total amount of Cu and Pb in soil, concerning different NLMWOA and EDTA. The amounts mobilised by Millipore water (control) i.e., approximately 1% Cu and 0.5% Pb, have been subtracted. The amount of Cu mobilised by the different NLMWOA was significantly ($P < 0.05$) higher in comparison to that of EDTA, irrespective of the NLMWOA used. However, higher amounts of the NLMWOA were applied resulting in 500-fold higher soil concentration compared to EDTA. The heavy metal amounts mobilised by the different NLMWOA were in the same order of magnitude. The NLMWOA mobilised approximately 20-25% of Cu irrespective of the pH (Figure 3-2a) and approximately 8% of Pb (Figure 3-2b) at the initial concentrations. The capability NLMWOA and EDTA to mobilised Cu differed significantly, whereas the mobilisation of Pb by NLMWOA and EDTA was of the same order of magnitude, with the exception of citric acid. At pH 5, citric acid was able to mobilise approximately 36% of the Pb, whereas the other NLMWOA were only able to mobilise approximately 8% of the Pb.

The slurry experiment revealed a high Cu mobilisation potential for the NLMWOA in an application amount according to the phytotoxicity experiment. The results provide the opportunity to make estimations about the outcome of the phytoextraction experiment. A significant enhanced Cu uptake would be expected by the application of NLMWOA.

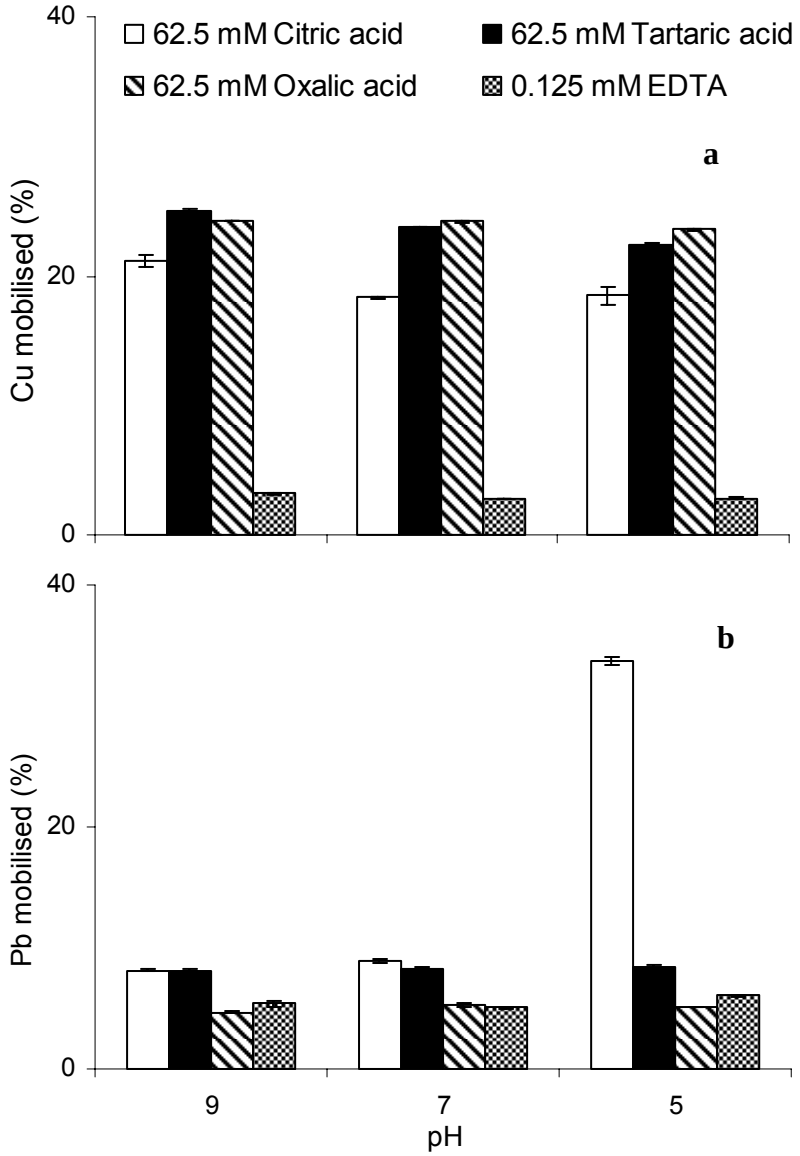


Figure 3-2: Mobilisation of Cu (a) and Pb (b) by NLMWOA and EDTA from soil at various pH of soil solution. Natural, aged metal concentrations were 21.8 mg Cu kg^{-1} soil and 48.7 mg Pb kg^{-1} soil, respectively. NLMWOA and EDTA were applied at the highest no effect concentrations (see chapter III.1.1). Error bars represent SD of triplicates.

III.1.3 Column experiment

In a column experiment the mobilisation capability of heavy metals by chelating agents was investigated, thus the tendency of a chelating agent to move through soil can be assessed. Figure 3-3 depicts the amounts of Cu and Pb mobilised from a soil spiked with 450 Cu and 600 mg kg^{-1} Pb respectively, compared to the total Cu and Pb content of the soil. The amount of Cu mobilised by NLMWOA was to a significant degree ($P < 0.05$) higher than that mobilised by EDTA. The latter mobilised approximately 0.4% more than the control with $CaCl_2$ but both average out at the same order of magnitude, mobilising negligible amounts of Cu compared to the NLMWOA. Regarding the different NLMWOA, citric acid mobilised approximately 25% of the Cu in the first fraction, thereafter mobilising less Cu in each following fraction. Oxalic and tartaric acid in contrast reached their maximum at the fourth pore volume, only mobilising approximately 9% and 3% respectively. Regarding the amount of Pb mobilised by the tested chelating agents, these ranged at the same order or magnitude, between 0.5 and 2%. Oxalic and tartaric acid, showed a similar curve progression as in the extraction of Cu, whereas the extraction curve of citric acid differed. Compared to Cu, the latter reached its maximum of extraction in the second pore volume.

A column experiment is a more realistic approach to the heavy metal mobilisation potential of NLMWOA and EDTA, but it is not as simple as a slurry experiment, which is optimal for a first estimation of the potential of a chelating agent. The column experiment revealed a high Cu mobilisation potential and a low Pb mobilisation potential for NLMWOA. Nevertheless the mobilisation is significantly higher than the mobilisation by EDTA, thus an uptake in a phytoextraction experiment should also be significantly higher.

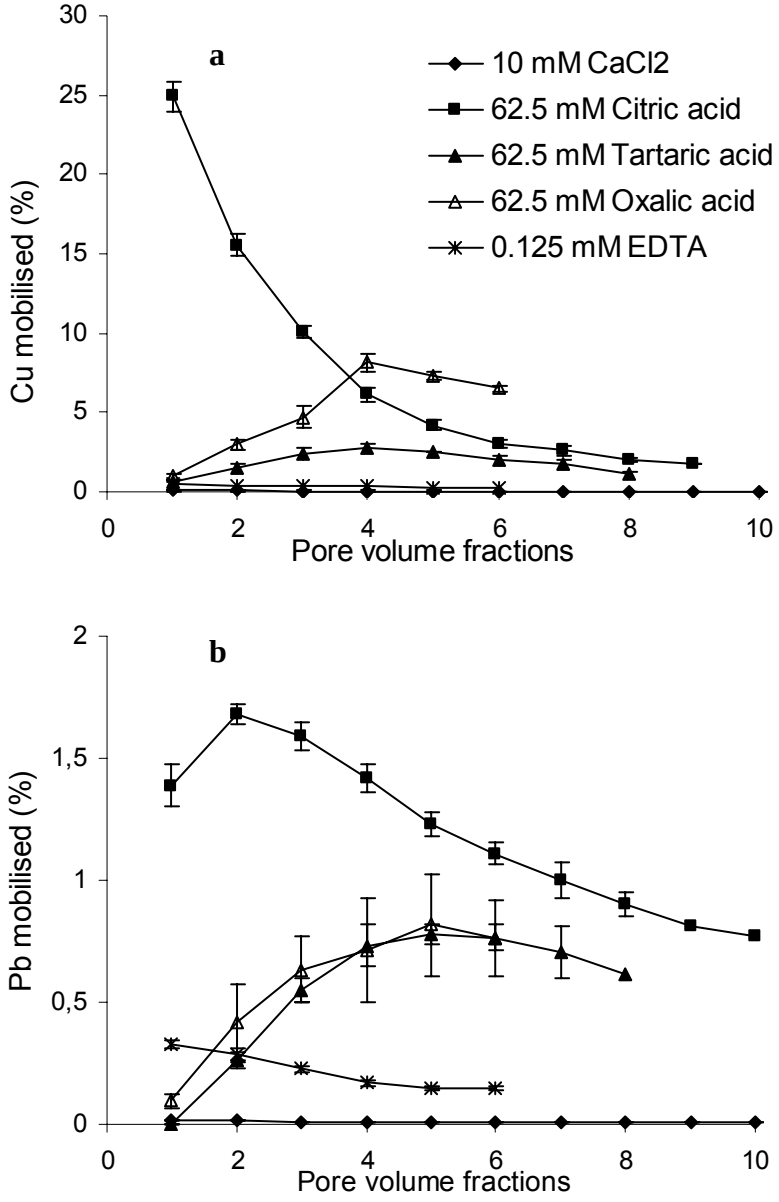


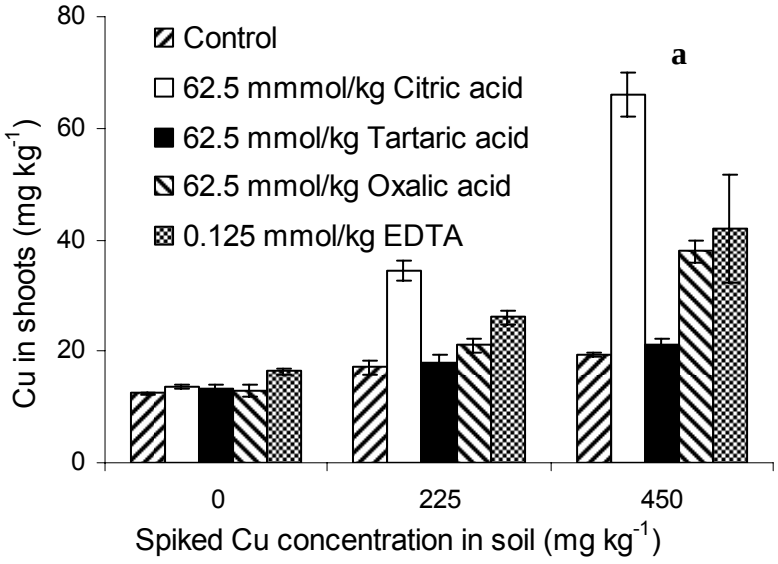
Figure 3-3: Leaching of Cu (a) and Pb (b) in soil after the addition of NLMWOA or EDTA and $CaCl_2$ as a control in column experiments. Cu and Pb were spiked to the soil at 450 and 600 mg kg^{-1}, respectively. NLMWOA and EDTA were applied at the highest no effect concentration (see chapter III.1.1). Error bars represent SD of five parallels.

III.1.4 Phytoextraction pot experiment

The enhanced uptake of heavy metals in plants under the effect of chelating agents was investigated. Figure 3-4 shows that Cu and Pb concentrations in the shoots increased with increasing Cu and Pb concentrations in the soil. Concerning Cu, uptake into the shoots was particularly enhanced after the application of citric acid. A significantly ($P < 0.05$) higher

concentration of Cu in the shoots compared to the control, the other NLMWOA and EDTA applications was caused by the treatment of 62.5 mmol kg^{-1} citric acid, the concentrations in the shoots reaching 67 mg kg^{-1}. The effect of oxalic and EDTA additions on Cu plant uptake was less prominent, but nevertheless showed an increase in comparison to both the control and tartaric acid, which showed no enhancing effect on Cu uptake by the plant. Regarding Pb, the application of the NLMWOA had no significant ($P < 0.05$) effect, on its uptake, which averaged to the same order of magnitude as the control. EDTA, in contrast, showed an enhancing effect on the Pb uptake into the shoots, the latter containing approximately 63 mg kg^{-1} of Pb at a spiked soil concentration of 600 mg kg^{-1}.

The phytoextraction experiment showed that the uptake was not as enhanced by the application of the NLMWOA as expected by the impression given from the previous experiments. The cause for this inefficiency could be a rapid biodegradation of the NLMWOA.



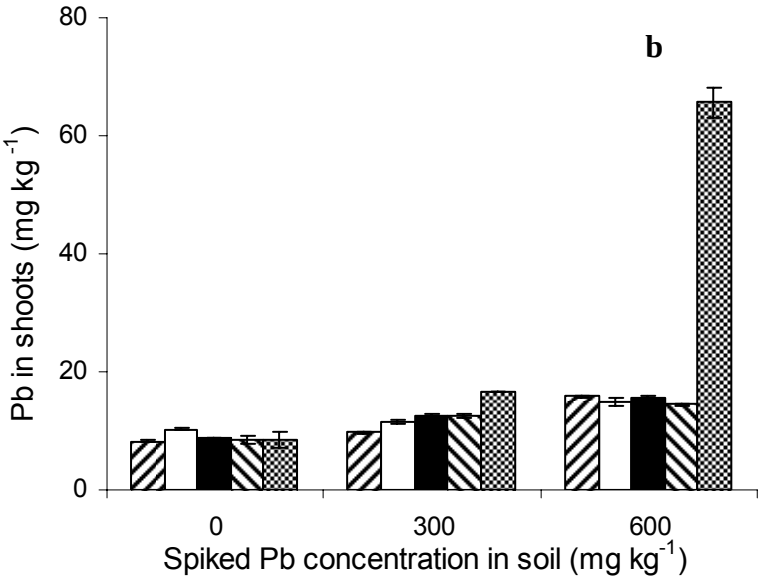


Figure 3-4: Cu (a) and Pb (b) concentrations in shoots of tobacco plants (*Nicotiana tabacum* SR-1) grown for 3 weeks in soil spiked with Cu and Pb, containing citric, oxalic, tartaric acid or EDTA. Error bars represent SD of triplicates.

III.1.4.1 pH change

The pH-change measured in this experiment is an indication of the biodegradation of the added chelating agents. The pH-values at the beginning and at the end of the phytoextraction experiments are shown in Table 3-1. At the beginning of the experiment the pH value of the control soil was 0.3 lower than its native pH 6.8, probably owing to the application of fertilisers. By the end of the experiment, the control soil reached its native pH. The pH was furthermore decreased by the application of the acidic Cu- and Pb-solutions. The application of EDTA barely changed the pH of the soil, whereas the addition of NLMWOA lowered the pH to an average value of 5.6 at the beginning of the experiment. Concerning the latter, the pH changed exceedingly during the experiment reaching values of around 7.7 after 4 weeks, a level 0.9 higher than the native pH of the soil.

The rise of the pH supports the potential biodegradation of the NLMWOA.

Table 3-1: The soil pH before and after the growth of tobacco plants (*Nicotiana tabacum* SR-1) for 3 weeks in soil spiked with Cu and Pb and treated with NLMWOA (62.5 mmol kg^{-1} soil) and EDTA (0.125 mmol kg^{-1} soil).

Application	Control		Cu 225 mg kg^{-1}		Cu 450 mg kg^{-1}	
	pH start	pH end	pH start	pH end	pH start	pH end
Control	6.46±0.09	6.79±0.08	6.23±0.02	6.67±0.02	6.18±0.01	6.64±0.14
Citric acid	5.65±0.07	7.45±0.06	5.54±0.04	7.44±0.08	5.48±0.11	7.45±0.04
Oxalic acid	5.66±0.05	7.64±0.06	5.42±0.01	7.73±0.11	5.42±0.01	7.73±0.11
Tartaric acid	5.72±0.03	7.45±0.10	5.62±0.03	7.71±0.01	5.53±0.03	7.76±0.09
Na_2EDTA	6.40±0.09	6.83±0.10	6.36±0.03	6.67±0.03	6.36±0.03	6.67±0.03

Application	Control		Pb 300 mg kg^{-1}		Pb 600 mg kg^{-1}	
	pH start	pH end	pH start	pH end	pH start	pH end
Control	6.46±0.09	6.79±0.08	6.41±0.03	6.73±0.03	6.19±0.01	6.91±0.07
Citric acid	5.65±0.07	7.45±0.06	5.48±0.03	7.41±0.00	5.34±0.04	6.91±0.07
Oxalic acid	5.66±0.05	7.64±0.06	5.55±0.08	7.59±0.08	5,54±0.01	7.68±0.12
Tartaric acid	5.72±0.03	7.45±0.10	5.65±0.04	7.80±0.07	5.65±0.04	7.71±0.10
Na_2EDTA	6.40±0.09	6.83±0.10	6.38±0.14	6.90±0.01	6.40±0.01	6.78±0.15

III.1.5 Biodegradation

III.1.5.1 Influence of biodegradation of NLMWOA on bioavailability of Cu

In this experiment the influence of the biodegradation of NLMWOA on the bioavailability of Cu was investigated. Cu was spiked to the soil at 450 mg kg^{-1}. Figure 3-5a, depicts the influence of the biodegradation of NLMWOA on the potentially bioavailable (DTPA-extractable) Cu concentration in a Cu spiked soil (450 mg kg^{-1}). Time 0 d is the addition of 62.5 mmol kg^{-1} of citric acid, oxalic acid, or tartaric acid. The application of the NLMWOA significantly increased the bioavailability of Cu by 15 mg kg^{-1} to approximately 190 mg kg^{-1}, compared to the bioavailable Cu of the control samples. After 96 h the Cu bioavailability of the soils treated with citric acid, tartaric acid or oxalic acid decreased significantly to 142 mg kg^{-1} 143 mg kg^{-1} and 138 mg kg^{-1} respectively. Simultaneously the pH of the soil increased from approximately 5.5 to approximately 7.7 (Figure 3-5b). Regarding the control, the pH

stayed constant during the observed time period. After 96 h, the pH as well as the potentially bioavailable Cu remained constant.

The increase of the pH deriving from the biodegradation of NLMWOA results in the decrease of the bioavailability of Cu, thus making an application of NLMWOA to increase the uptake and the mobility of heavy metals inefficient.

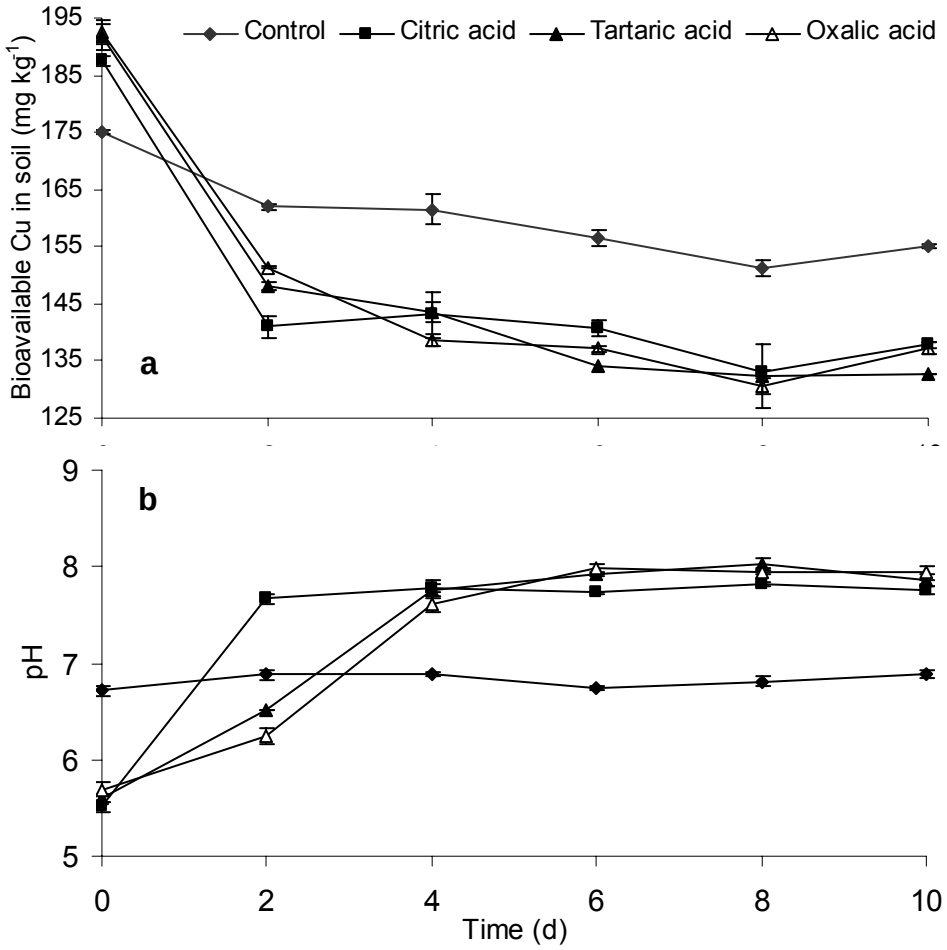


Figure 3-5: Influence of biodegradation of NLMWOA (62.5 mmol kg^{-1} soil) on (a) potential bioavailable Cu in soil and (b) on the pH of soil. Error bars represent SD of triplicates.

III.1.5.2 Oxitop-experiment

In the oxitop experiment the biodegradation of a compound through the consumption of oxygen can be observed. The oxitop-system is an air sealed system, thus the amount of oxygen is limited. The basal respiration of a soil is the respiration deriving from soil microorganisms under normal (without the application of additional carbon source) conditions. During the experimental time period the oxygen is consumed and exchanged for

carbon dioxide which is bound to the KOH solution in the system, thus the pressure in the system drops. Therefore, the graph of the control rises, as the amount of oxygen decreases in the system, but the rate remains constant. The results from the Oxitop degradation experiment are shown in Figure 3-6. The addition of 62.5 mmol kg^{-1} NLMWOA displayed a significant increase of respiration in comparison to the control (basal respiration), reaching a plateau after approximately 240 min (4 h). Tartaric acid and citric acid behaved similarly, both reaching a plateau at approximately 668 and 710 mg O_2 L^{-1} respectively. Oxalic acid in contrast showed only a slight increase in respiration, reaching a plateau of approximately 350 mg O_2 L^{-1} after approximately 120 min (2 h). At 3360 min (56 h) the respiration reached a value lower than the basal respiration, resulting in an oxygen consumption of 380 mg O_2 L^{-1}.

The oxitop-experiment revealed a very high biodegradation rate for the NLMWOA and also revealed that NLMWOA are not toxic for soil microorganisms.

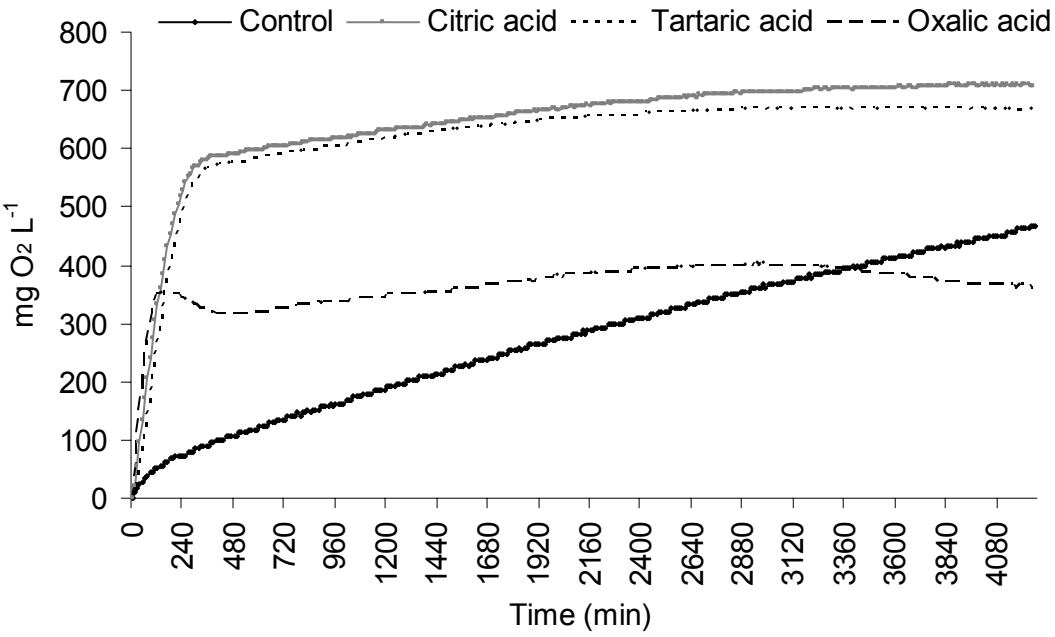


Figure 3-6: Influence of NLMWOA on the oxygen consumption during their degradation in the Oxitop system. NLMWOA were applied at 62.5 mmol kg^{-1} soil.

III.1.5.3 Identification of microorganisms

The results of the previous experiments indicate a biodegradation of the NLMWOA which could be due to the biodegradation through microorganisms. Soil was incubated for 24 h with 62.5 mmol kg^{-1} of citric acid, oxalic acid or tartaric acid. A volume of 50 µL from the NLMWOA incubated soil suspension was transferred on minimal medium agar plates with the sole carbon source one of the NLMWOA (37.5 mmol kg^{-1}) tested. The NLMWOA was

decreased in the medium of the agar plates to decrease the salt payload resulting from the pH adjustment. The pH was adjusted to the same pH as determined in the biodegradation experiment in soil (see chapter III.1.5.1). As a control soil suspension (without additional carbon source) grown on minimal medium agar plates (without carbon source) was used. The incubation time was between 5-20 d, depending on the carbon source and the incubation temperature. Not all different colonies, but only the most dominant ones were isolated. The isolation of the microorganisms from the agar plates resulted in 7 different microorganisms; two bacteria, 4 fungi and one unknown microorganism. Table 3-2 depicts the microorganisms and the corresponding NCBI database reference. *Cordyceps sp., Paecilomyces sp*. and *Burkholderia sp*. grew on all three plates; whereas *Ralstonia sp*., *Saccharomyces sp*. and *Basidiomyces sp*. grew only on the plates that contained citric acid or tartaric acid as a carbon source. The control displayed only few very small colonies which were not sequenced.

Table 3-2: Name and NCBI database reference of the isolated microorganisms from the NLMWOA biodegradation experiment.

Micro-organism	NCBI database reference
Bacteria	
Burkholderia sp.	gi|110589882|gb|DQ777739.1|
Burkholderia sp.	gi|110589882|gb|DQ777739.1|
Ralstonia sp.	gi|114224468|gb|DQ785822.1|
Ralstonia sp.	gi|114224468|gb|DQ785822.1|
Fungi	
Saccharomyces sp.	gi|55416147|gb|AY790536.1|
Cordyceps sp.	gi|27807830|dbj|AB099942.1|
Basidiomyces sp.	gi|46405650|gb|AY520246.1|
Paecilomyces sp.	gi|116174501|dbj|AB277860.1|
Paecilomyces sp.	gi|45861408|gb|AY526475.2|

III.1.5.4 Detection of biodegradation of NLMWOA via HPLC

Table 3-3 shows the NLMWOA consumption by the isolated bacteria and fungi in batch experiments. The batch experiment consisted of 100 mL flasks containing 20 mL minimal medium with NLMWOA (2 g L^{-1}) as the sole carbon source and isolated microorganisms. The pH was adjusted to 5.5 (see chapter III.1.5.1). It was not possible to apply the same number of

microorganisms into each flask; therefore the results give an idea of which microorganisms have the ability to degrade all three NLMWOA rather than their effectiveness to degrade NLMWOA. In the first batch experiment, all three simultaneously added NLMWOA were the sole carbon source (citric acid, tartaric acid and oxalic acid) (Table 3-3), whereas in the second batch, oxalic acid was the sole carbon source (Table 3-3). Experimental medium without microorganism were used as a control. The incubation time was 3 weeks.

The measurements were performed via HPLC. The decrease of the NLMWOA concentration owing to degradation by the microorganisms was measured.

In the first batch experiment all of the isolated microorganisms were able to degrade citric acid as well as tartaric acid. With the exception of *Saccharomyces sp*., citric acid (1.902 g L^{-1}) was more effectively degraded than tartaric acid (1.343 g L^{-1}) by the microorganisms. Oxalic acid, by contrast, was not degraded by any of the microorganisms when citric acid and tartaric acid were present as a carbon source. The concentration of oxalic acid remained above 2 g L^{-1} in the first batch experiment. In the second experiment oxalic acid was used as the sole source. It is of interest that the fungi, *Cordyceps sp.*, *Paecilomyces sp.* and the bacteria *Burkholderia sp.* were able to degrade oxalic acid, when this was the only carbon source, whereas *Basidiomyces sp., Saccharomyces sp*. and *Ralstonia sp.* could not.

The identified microorganisms *Cordyceps sp.*, *Paecilomyces sp*. and *Burkholderia sp*. were able to degrade the three NLMWOA (citric acid, oxalic acid and tartaric acid), thus making a successive application of the three NLMWOA inefficient. A successive application of the NLMWOA could have the potential to overcome the rapid biodegradation rate of the NLMWOA, but it would have only been feasible if the microorganism were not the same. In that case with each application of a different NLMWOA new microorganisms would be in the lag (adaptation) phase of growth, but as the microorganisms are the same the microorganisms remain in the log (exponential) growth phase.

Table 3-3: NLMWOA (2 g L^{-1}) consumption by isolated microorganisms after 3 weeks incubation in batch experiments.

	All three NLMWOA added as carbon source (batch exp. 1)			Only oxalic acid added as carbon source (batch exp. 2)	
	Citric acid (g L^{-1})	Tartaric acid (g L^{-1})	Oxalic acid (g L^{-1})		Oxalic acid (g L^{-1})
Fungi				Fungi	
Cordyceps	0.535	1.121	2.169	*Cordyceps*	1.117
Paecilomyces	0.309	1.162	2.149	*Paecilomyces*	1.105
Basidiomyces	0.374	1.268	2.279	*Basidiomyces*	2.092
Paecilomyces	0.433	1.114	2.238	*Paecilomyces*	1.619
Saccharomyces	1.902	1.343	2.178	*Saccharomyces*	2.142
Bacteria				Bacteria	
Burkholderia	0.073	1.096	2.184	*Burkholderia*	0.675
Ralstonia	0.811	1.107	2.219	*Ralstonia*	2.158
Control	2.131	2.115	2.117	Control	2.078

III.2 Cyclodextrins (CyD)

β-CyD were used in phytotoxicity, column, and biodegradation experiments to investigate their potential as an alternative to synthetic chelators. β-CyD showed a relatively high toxicity to tobacco (*Nicotiana tabacum* SR-1) (3.92 mmol kg^{-1} of soil) and a very low ability to mobilise Cd and Cu. The biodegradation of β-CyD caused an increase of the soil pH, thus reducing the bioavailability of Cu significantly. No phytoextraction experiments were performed owing to the low heavy metal mobilisation potential and the high biodegradation rate.

III.2.1 Phytotoxicity pot experiment

Dry matter yields of the shoots (sum of leaves and stem) are shown in Figure 3-6. The application of β-cyclodextrins (β-CyD) to the soil adversely affected dry matter production of

the plants at a concentration of 3.92 mmol kg^{-1}, but no toxicity symptoms such as chlorosis or necrosis were visible. The dry weight of the tobacco shoots at a β-CyD concentration of 3.92 mmol kg^{-1} was significantly lower ($P > 0.05$), than that of the control reaching 3.3 and 4.4 g respectively. At a concentration of 7.83 mmol kg^{-1} β-CyD the dry weight is only slightly lower, but the standard deviation is higher in comparison to the 3.92 mmol kg^{-1} application. At a concentration of 11.75 mmol kg^{-1} β-CyD toxicity effects such as chlorosis were visible.

The phytotoxicity experiment revealed the highest no effect concentration of β-CyD; therefore β-CyD can be applied in the phytoextraction experiment according to these results without causing additional stress to the plants. Potential toxicity symptoms appearing on the plants in the phytoextraction experiment can thus not derive from the chelating agent's application.

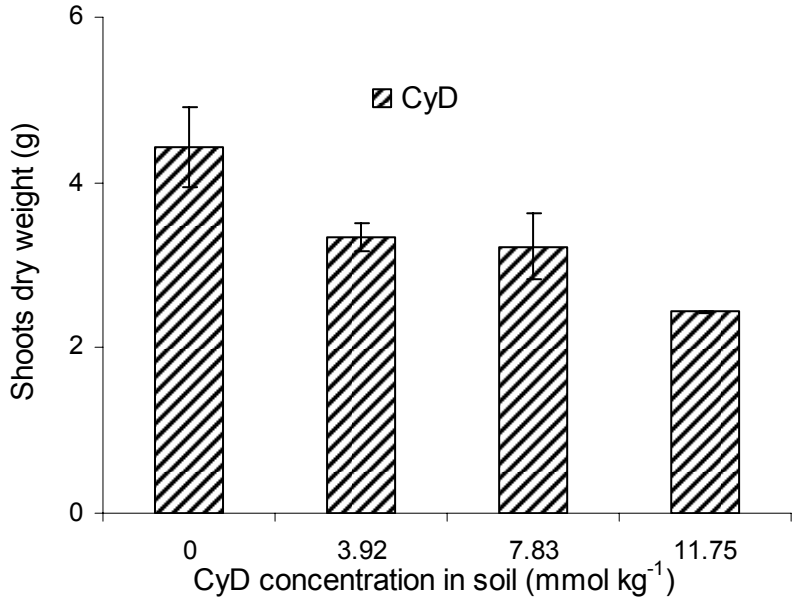


Figure 3-6. Dry weight of tobacco (*Nicotiana tabacum* SR-1) grown for 3 weeks in soil containing β-CyD. Error bars represent SD of triplicates.

III.2.2 Column experiment

In this experiment the potential of β-CyD to mobilise Cu and Cd were assessed. Figure 3-7 depicts the amount of Cu and Cd mobilised from a soil spiked with 450 Cu and 100 mg kg^{-1} Cd respectively, as a percentage of the total amount of Cu and Cd in the soil. The amount of Cu mobilised by β-CyD, was significantly higher compared to the amount mobilised by

$CaCl_2$. The difference was approximately 1.4% higher. Cd on the other hand was significantly less mobilised by β-CyD than by the control. In general though, both β-CyD and $CaCl_2$ average out to the same order of magnitude.

A column experiment is a more realistic approach to the heavy metal mobilisation potential of β-CyD, in comparison to a slurry experiment. In a slurry experiment the soil is shaken with a solution, thus all soil particles are in constant contact with the solution; whereas in a column experiment the solution has to pass through soil, as it has to in a pot experiment. The column experiment revealed a low Cu and Cd mobilisation potential β-CyD, thus making it a very weak chelating agent for these two metals.

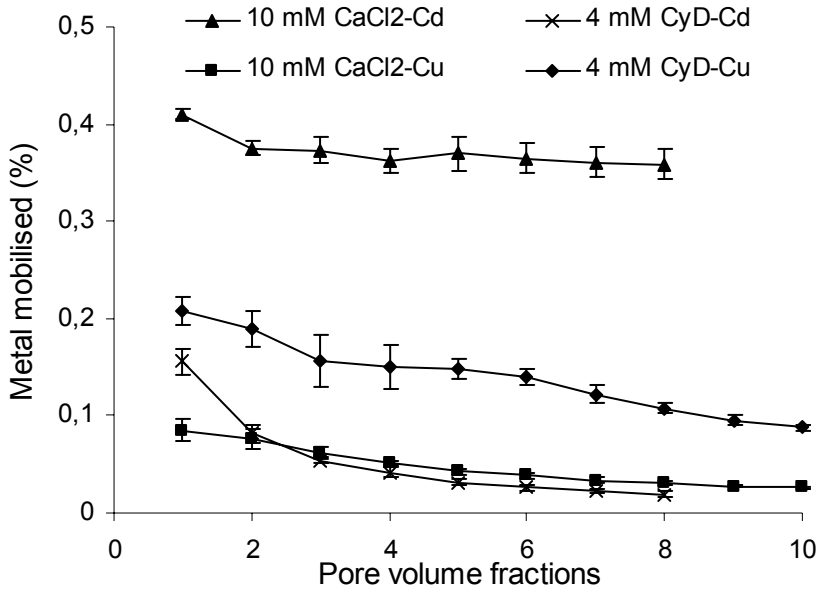


Figure 3-7: Leaching of Cu and Cd with β-CyD in column experiments. Error bars represent SD of five parallels.

III.2.3 Biodegradation

The object of this study was to investigate the use of β-CyD as an alternative to synthetic chelators. Column-, phytotoxicity- and biodegradation experiments were performed. β-CyD showed a relative high toxicity to plants (3.92 mmol kg^{-1} of soil) and a very low ability to mobilise cadmium and copper. The biodegradation of β-CyD caused the increase of the soil pH, thus reducing significantly the bioavailability of copper.

III.2.3.1 Metal-bioavailability

Figure 3-8, depicts the influence of the biodegradation of the β-CyD added to the soil on the potentially bioavailable (DTPA-extraction) Cu concentration in soil. At 0 d 3.92 mmol kg^{-1} of the β-CyD were added to the soil. This did not affect the potentially bioavailable (DTPA-extraction) Cu concentration in the soil significantly. The potentially bioavailable Cu of samples treated with the β-CyD amounted to 162 mg kg^{-1} Cu, in comparison to the control's levels of 166 mg kg^{-1}. After 24 h the Cu bioavailability of the soils treated with β-CyD decreased significantly to 148 mg kg^{-1} remaining constantly at that level until the termination of the experiment (6 d).

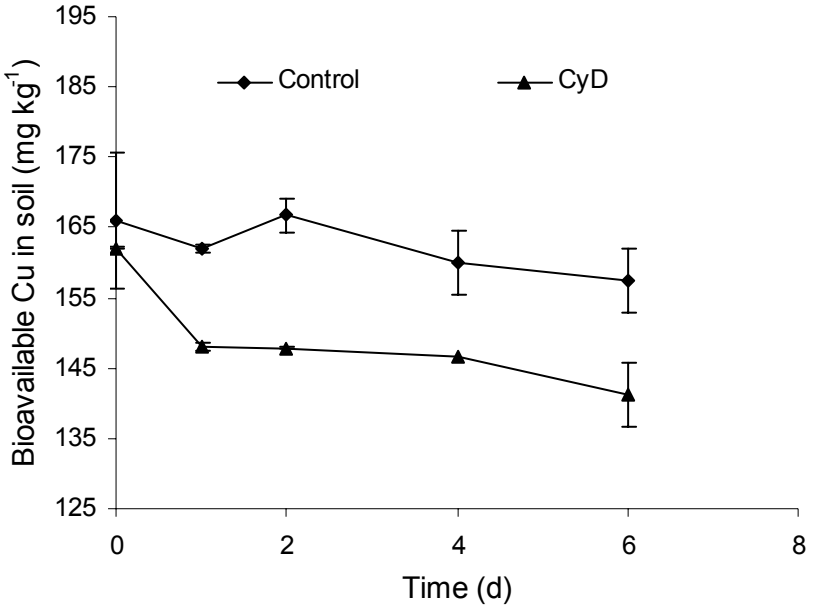


Figure 3-8: Potential bioavailable Cu in soil after an application of β-CyD (3.92 mmol kg^{-1} of soil). Error bars represent SD of triplicates.

III.2.3.2 pH-change

Table 3-4 shows the pH-values at the beginning and at the end of the degradation experiment. The pH of the control soil and the soil containing β-CyD were equal at the beginning of the experiment, whereas the pH of the β-CyD treated soil increased by approximately 0.5 to 7.3, the pH of the control staying constant. At the beginning of the experiment the pH value of the control soil and the soil containing β-CyD are equal. At the end of the experiment the pH reached values in average around 7.3, which is approximately 0.45 higher than the control soil.

The increase of the pH deriving from the biodegradation of β-CyD results in the decrease of the bioavailability of Cu (see chapter III.2.3.1), thus making an application of β-CyD to increase the uptake and the mobility of heavy metals inefficient.

Table 3-4: The soil pH at the beginning and at the end of the Cu bioavailability experiment.

Application	pH-value start	pH-value end
Control	6.78±0.03	6.85±0.04
CyD	6.80±0.02	7.28±0.01

III.2.3.3 Oxitop

The oxitop-system is an air sealed system, thus the amount of oxygen is limited. The basal respiration of a soil is the respiration deriving from soil microorganisms under normal (without the application of additional carbon source) conditions. During the experimental time period the oxygen is consumed and exchanged for carbon dioxide which is bound to the KOH solution in the system, thus the pressure in the system drops. Therefore, the graph of the control rises because the amount of oxygen decreases in the system, but the rate remains constant. The results of the degradation experiment are shown in Figure 3-9. The addition of β-CyD displayed a significant increase of respiration in comparison to the control (basal respiration). The respiration increase in the 0.039 mol kg^{-1} β-CyD treated samples was visible after approximately 14 h, afterwards the degradation was very rapid.

The respiration increase in the oxitop experiment supports the results of the extraction of the potential bioavailable heavy metal fraction (see chapter III.2.3.2). The rapid biodegradation rate combined with low heavy metal mobilisation potential make β-CyD inefficient for the enhancement of phytoextraction.

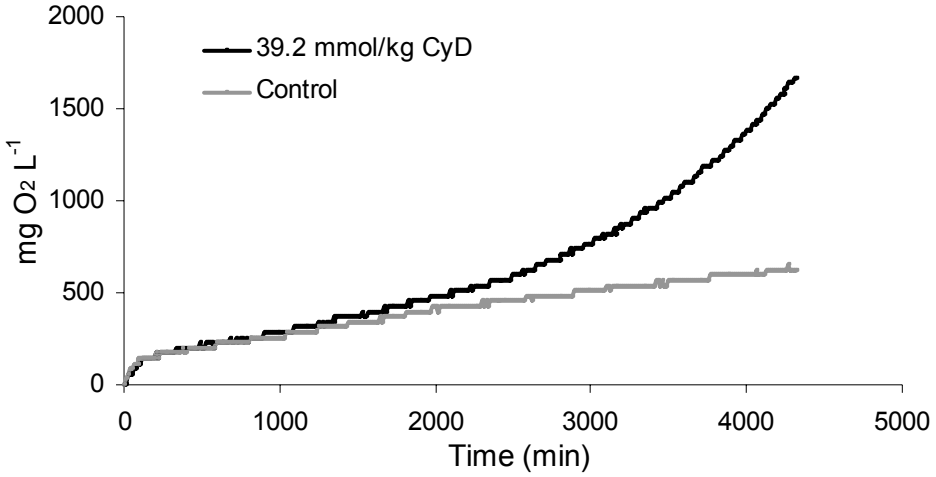


Figure 3-9: Influence of CyD (39.2 mmol kg^{-1}) on the oxygen consumption during their degradation in the Oxitop system.

III.3 Ethylene diamine disuccinate (EDDS)

The biodegradable chelator, ethylene diamine disuccinate (EDDS), was used in column, phytotoxicity, phytoextraction and biodegradation experiments to investigate its potential as an alternative to synthetic chelators. The same experiments were performed with the synthetic chelator EDTA, in order to achieve a direct comparison to EDDS. EDDS revealed a higher toxicity to tobacco (*Nicotiana tabacum* SR-1*)* in comparison to EDTA. At an EDDS concentration of 1.5 mmol kg^{-1} of soil no adverse effects were observed on the biomass production, whereas in the case of EDTA no adverse effects were observed at an EDTA concentration of 6.25 mmol kg^{-1} of soil. The concentration of 1.5 mmol kg^{-1} was chosen in order to have a direct comparison of the effectiveness of the two chelating agents without adverse effect on the plants. The column experiment revealed a higher Cu mobilisation potential for EDDS compared to EDTA and an equal mobilisation potential in the case of Cd. The Cd amount mobilised by the EDDS and EDTA was approximately 3-fold and 8-fold higher respectively than the mobilised Cu amount. The phytoextraction experiment revealed a significant increase in Cu uptake by the EDDS application but it had no effect on the Cd uptake, whereas EDTA could enhance the uptake of Cu and Cd. The biodegradation experiment revealed a slow biodegradation rate of EDDS. EDDS remained in the soil even 4 weeks after the application, causing toxicity symptoms to new seedlings planted on the soil.

III.3.1 Phytotoxicity pot experiment

A preliminary phytotoxicity experiment was performed. Dry matter yields of preliminary phytotoxicity experiments are shown in Figure 3-10. The application of EDDS at a concentration of 3.125 mmol kg^{-1} did not affect dry matter production of the plants, although toxicity symptoms, such as chlorosis and necrosis, were visible at concentrations ≥ 3.125 mmol kg^{-1}. This correlated with a decreasing biomass at concentrations ≥ 3.125 mmol kg^{-1}. By contrast, the application of up to 6.25 mmol kg^{-1} EDTA did not result in a reduction of the biomass or in toxicity symptoms. Moreover, toxicity symptoms such as necrotic lesions and a reduction of the dry weight were observed at concentrations ≥ 12.5 mmol kg^{-1}.

The phytotoxicity experiment revealed the highest no effect concentration of EDDS and EDTA to tobacco. Therefore, EDDS and EDTA can be applied in the phytoextraction experiment according to these results without causing additional stress to the plants. Potential toxicity symptoms appearing on the plants in the phytoextraction experiment can thus not derive from the chelating agent's application. The concentration of 1.5 mmol kg^{-1} was chosen for both chelating agents in order to have a direct comparison of their effectiveness to mobilise heavy metals. The dry weight of the plants after EDTA treatment is higher than the control because of higher amounts of mobilised nutrients made available to the plants. The highest no effect concentration for EDTA is higher in this experiment than in the experiments with NLMWOA (see chapter III.1.1). In this experiment EDTA was applied while the seedlings were already growing in the soil for a time period of 2 weeks, whereas in chapter III.1.1 EDTA was applied shortly before the seedlings were planted.

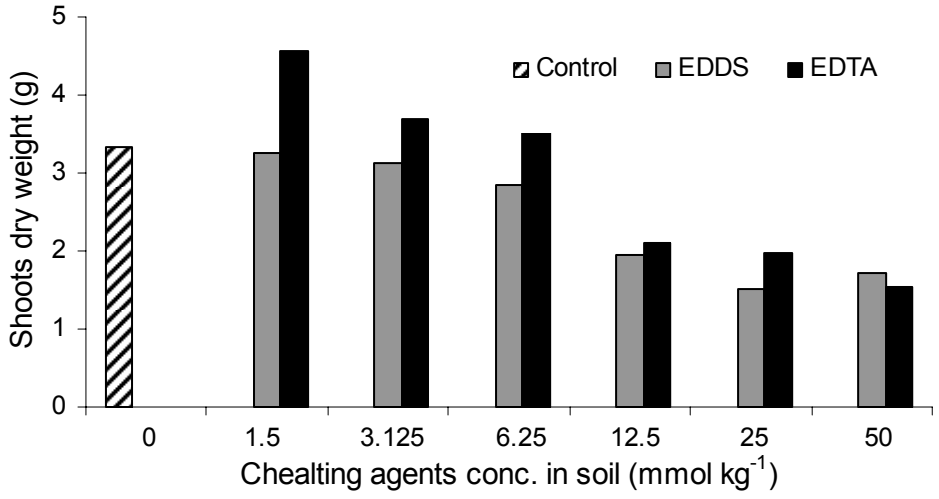


Figure 3-10: Dry weight of tobacco (*Nicotiana tabacum* SR-1) grown for 2 weeks in soil containing either EDDS or EDTA.

III.3.2 Column experiment

In a column experiment the mobilisation capability of heavy metals by chelating agents is investigated. The tendency of a chelating agent to move through soil can be assessed. Figure 3-11 depicts the amounts of Cu and Cd mobilised from a soil spiked with 450 mg kg^{-1} Cu and 100 mg kg^{-1} Cd respectively, compared to the total Cu and Cd content of the soil. The amount of Cu mobilised by EDDS (30%) was significantly ($P < 0.05$) higher than that mobilised by EDTA. The latter mobilised approximately 14% more than the control with $CaCl_2$. Regarding the amount of Cd mobilised by the tested chelating agents, these ranged at the same order or magnitude, between 74-77%. In comparison to the amounts mobilised by EDDS and EDTA, the amounts mobilised by $CaCl_2$ are significantly ($P < 0.001$) lower.

The heavy metal mobilisation potential determined by column experiment was higher for EDDS in the case of Cu and equal for EDDS and EDTA in the case of Cd. Therefore, the uptake in a phytoextraction experiment is expected to be higher for Cu by the application of EDDS and equal for Cd by the application of EDTA and EDDS.

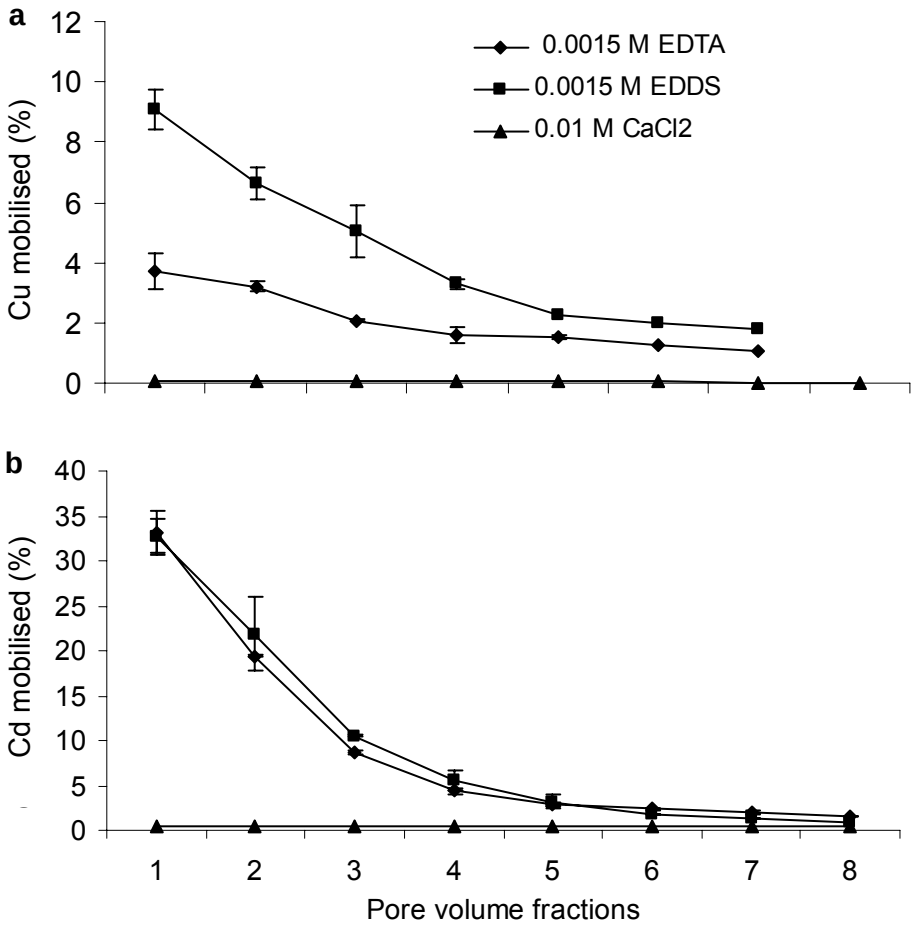


Figure 3-11: Leaching of Cu (a) and Cd (b) with EDDS or EDTA and $CaCl_2$ as a control in column experiments. Error bars represent SD of five parallels.

III.3.3 Phytoextraction pot experiment

Dry matter yields of the shoots (sum of leaves and stem) are shown in Figure 3-12. The application of Cd and Cu, combined with and EDDS or EDTA to the soil did not affect the dry matter production of the plants adversely. The dry weight of the shoots ranging from 2.5-3 g. Likewise no symptoms of chlorosis and necrosis were observed. The chelating agents EDTA and EDDS were applied 2 weeks after planting of tobacco (*Nicotiana tabacum* SR-1) and the incubation time was 2 weeks, therefore the total growth time period was 4 weeks.

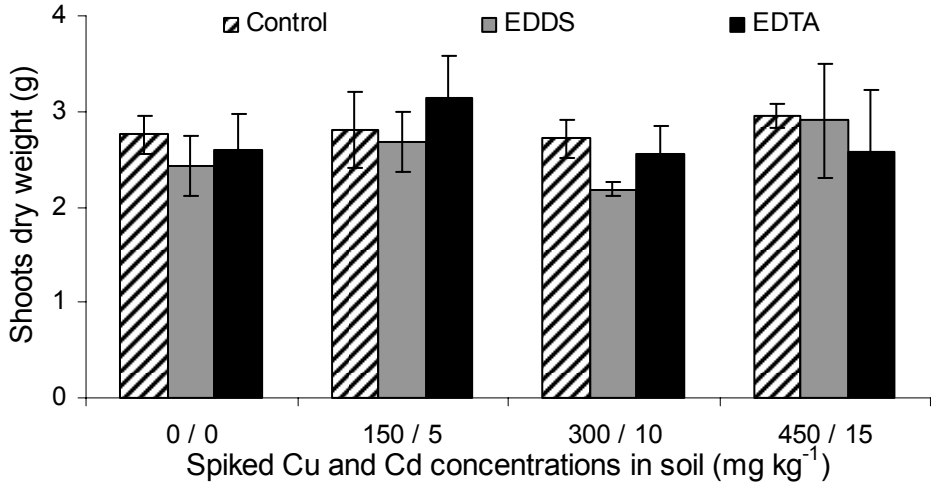


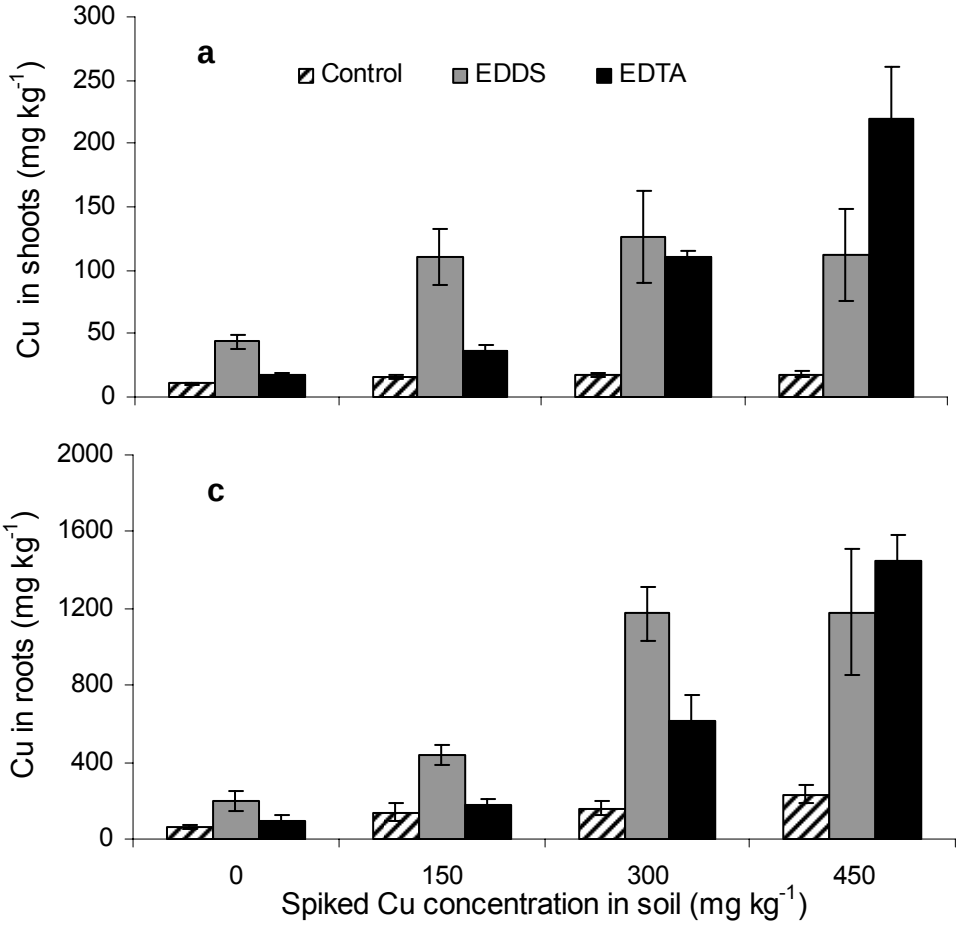
Figure 3-12: Effect of the application of Cu/Cd and either EDDS or EDTA (1.5 mmol kg^{-1}) to soil on shoot dry weight of tobacco (*Nicotiana tabacum* SR-1) grown for 2 weeks. Error bars represent ± SE of triplicates (n = 3).

As shown in Figure 3-13a and 3-13b increasing Cu and Cd concentrations of the soil resulted in increasing Cu and Cd concentrations in the shoots of the plants. The uptake of Cu into the shoots was particularly enhanced by the application of EDDS and EDTA. Compared to the control the treatment with 1.5 mmol kg^{-1} EDTA and EDDS caused a significantly ($P < 0.05$) higher concentration of Cu in the shoots. In the case of EDTA application, Cu concentrations in the shoots reached 219 mg kg^{-1}, whereas with EDDS Cu concentrations in the shoots rose to 200 mg kg^{-1}. Compared to EDTA, the effect of EDDS on Cu plant uptake was more prominent in the lower soil Cu concentrations (0, 150, 300 mg kg^{-1}), whereas at a Cu concentration of 450 mg kg^{-1} and greater, the addition of EDDS and EDTA had approximately the same enhancing effect on the uptake. Regarding Cd, the application of

EDTA and EDDS showed no significant ($P < 0.05$) effect on the uptake, which averaged to the same order of magnitude as the control.

Metal concentrations in the roots (Figure 3-13c, 3-13d) showed a similar picture as those in the shoots. The treatment with 1.5 mmol kg^{-1} EDTA and EDDS caused a significantly ($P < 0.05$) higher concentration of Cu in the roots reaching 1440 mg kg^{-1} and 1200 mg kg^{-1} respectively. With respect to Cd the case is somewhat different. Whilst the application of EDTA showed a significant effect ($P < 0.01$) in comparison to the control, that of EDDS had no influence.

The phytoextraction experiment showed that the uptake of Cd was not as enhanced by the application of EDDS as expected by the impression given from the column experiment, whereas EDTA increased the uptake significantly. Additionally, the application of EDDS enhanced the Cu uptake but remained in the same order of magnitude contrary to the results from the column experiment. The heavy metal concentration in the roots is higher than the concentration in the shoots, indicating a low root to shoot translocation. This low heavy metal translocation is a disadvantage for the chelating agents EDTA and EDDS as the largest amount of the extracted heavy metals is located in non harvestable parts of the plant.



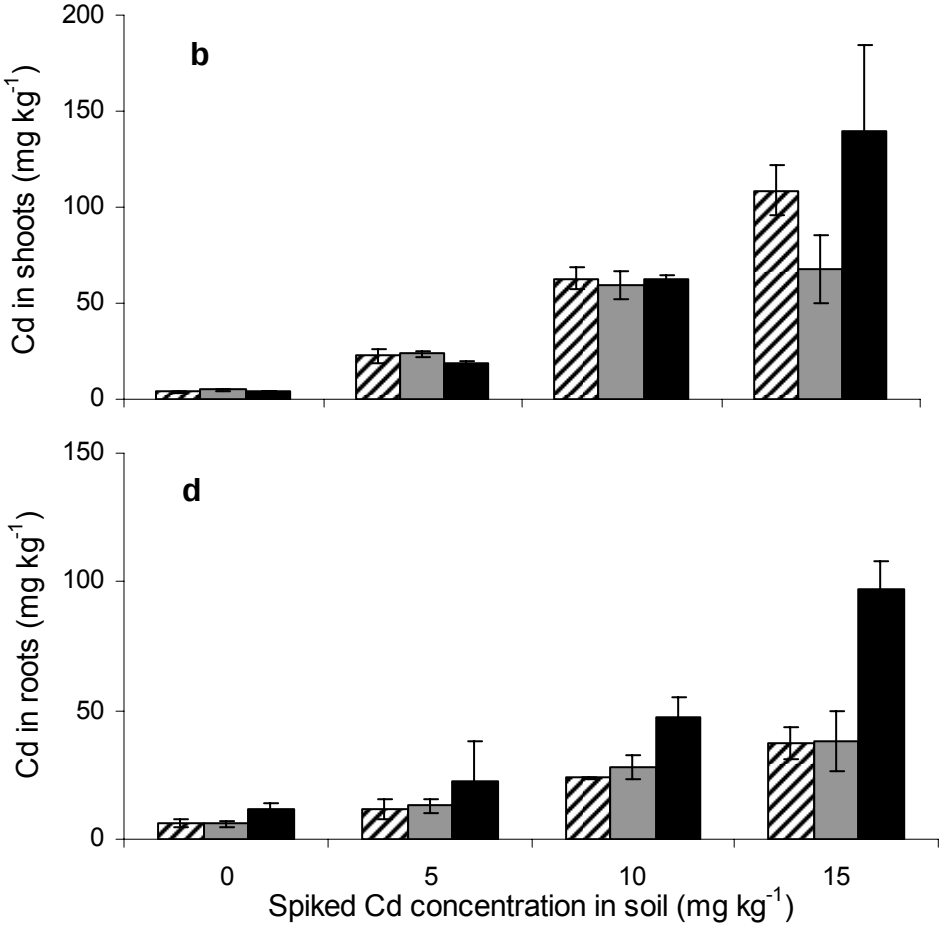


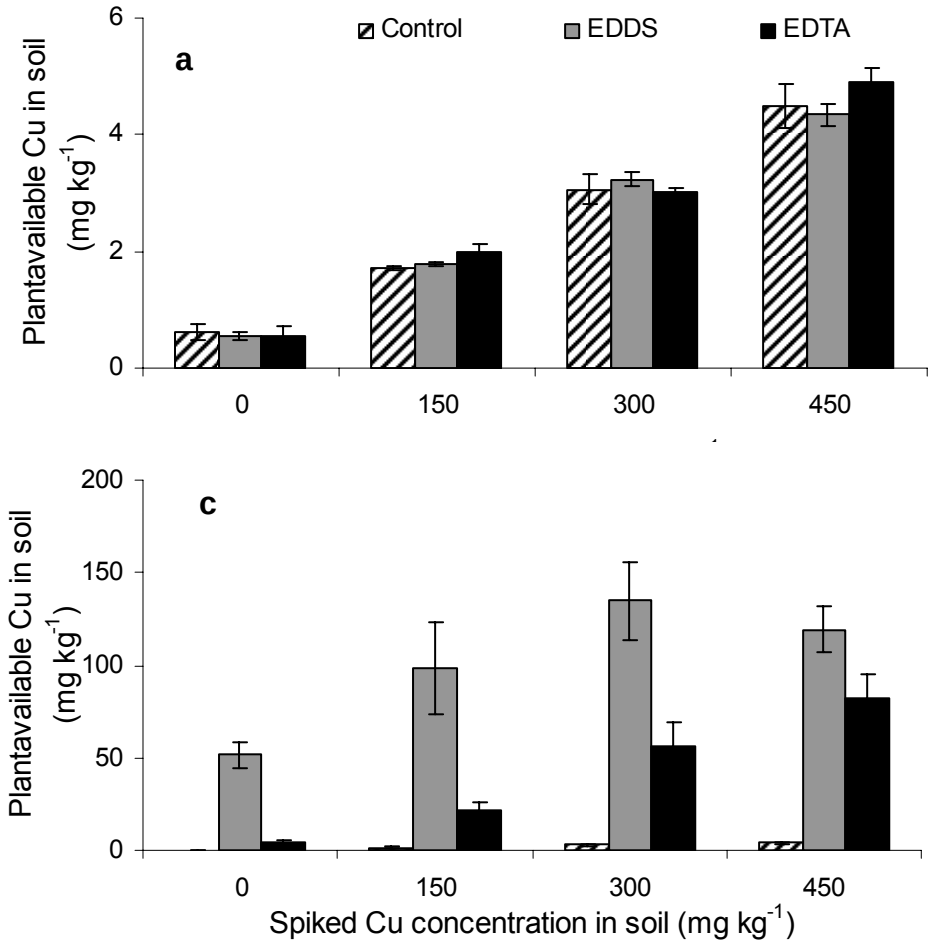
Figure 3-13: Cu and Cd concentration in shoots (a, b) of tobacco (*Nicotiana tabacum* SR-1) and roots (c, d) grown for 2 weeks in soil after the application of EDTA of EDDS (1.5 mmol kg^{-1}). Error bars represent ± SE of triplicates (n = 3).

III.3.4 Soil analysis of phytoextraction pot experiment

The plant available Cd and Cu before and after the phytoextraction experiment was determined. At the beginning of the experiment the pH value of the control soil was 0.3 lower than its native pH, owing to the application of fertilisers and the acidic heavy metal solutions. At the end of the experiment the control soil reached approximately its original native pH levels. The plant available Cd and Cu concentrations in the soil, as determined by NH_4NO_3 extraction, are shown in Figures 3-14a, 3-14b. The plant available Cu mobilised by EDTA and EDDS was significantly ($P < 0.01$) higher in comparison to the control. EDDS application mobilised significantly ($P < 0.01$) more Cu than EDTA at Cu soil concentrations of 0, 150, and 300 mg kg^{-1}. At 450 mg kg^{-1} the margin between the plant available Cu mobilised by EDTA and EDDS diminished. The plant available Cu of the control was reduced on average

by 0.2 mg kg^{-1} of soil, indicating an uptake of this heavy metal fraction by the plant. Figures 3-14c, 3-14d display the plant available Cd. Irrespective of the EDDS added to the soil before planting and after harvesting, the plant available Cd concentration in the soil was not enhanced in comparison to the control. The addition of EDTA, however, increased the plant available Cd significantly ($P < 0.05$). The plant available Cd was reduced in the controls and in the EDDS treated soils on average by 0.09 mg kg^{-1} after harvest.

Soil analysis revealed an increase of plant available Cu after the application of EDTA and EDDS. The plant available Cu is significantly higher than that of the control, even after an incubation time of 2 weeks, indicating the biodegradation rate of EDDS is low. Furthermore, owing to the high amounts of plant available Cu, it is shown that the plants could not extract the total amount of plant available Cu, thus causing a leaching risk. In the case of Cd only EDTA increased the amount of the plant available Cd fraction. It supports the results from the shoot and root analysis (see chapter III.3.3), where only EDTA could enhance the Cd uptake, whereas EDDS had no influence on the uptake.



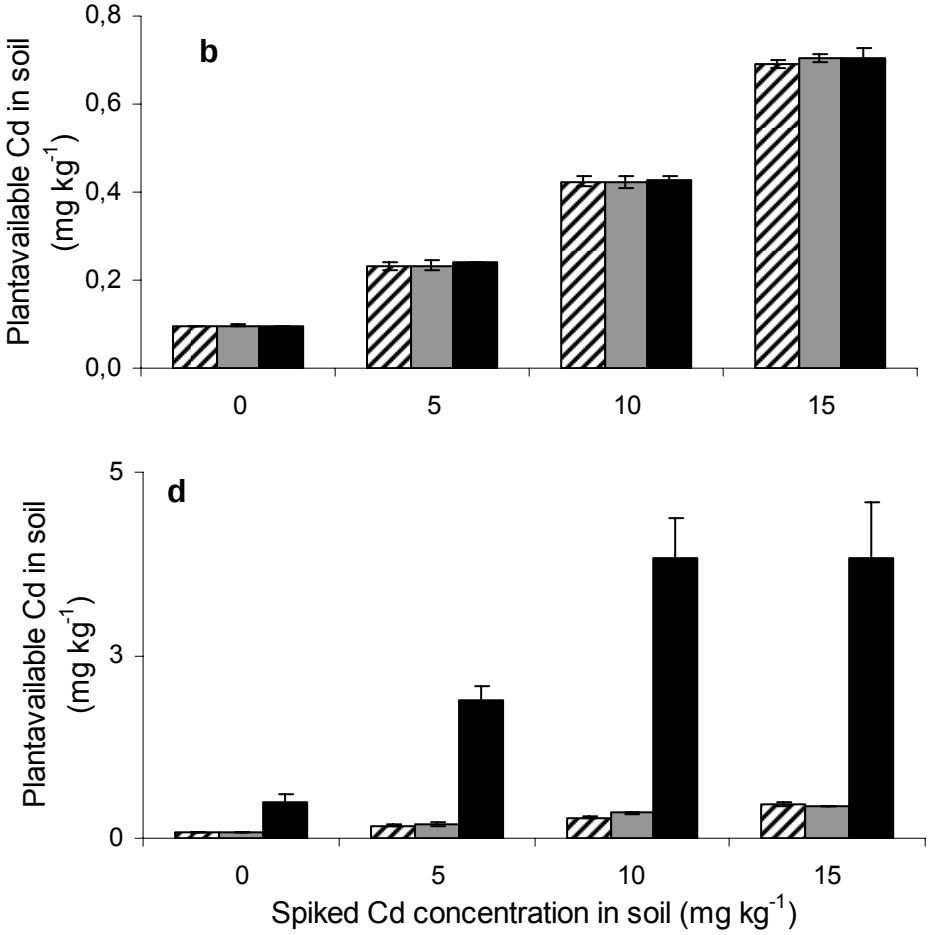


Figure 3-14: Plant available Cu and Cd (a, b) before and (c, d) after the application of EDTA and EDDS. Soil samples were taken before (a, b) and (c, d) after harvesting. The growth time period of tobacco (*Nicotiana tabacum* SR-1) was 2 weeks after the application of EDTA and EDDS but the total growth time period was 4 weeks. Error bars represent ± SE of triplicates (n = 3).

III.3.5 Biodegradation of EDDS and EDTA

New tobacco seedlings were planted 1 week after harvesting of the phytoextraction experiment on the same soil. The new seedlings were planted on the EDDS and EDTA treated pots, but only in the pots without spiked Cu and Cd, in order to avoid any heavy metal related toxicity symptoms. Dry weights of the new planted seedlings in the control pots are depicted in Figure 3-16. The seedlings in the control and EDTA treated pots did not show any chlorosis or necrosis. In contrast the seedlings in EDDS treated pots showed severe necrotic and chlorotic toxicity effects. 5 d after planting more than 50% of the leaves were completely

necrotic, the remaining leaves being partially necrotic. After 10 d no new symptoms of toxicity arose and the plants subsequently produced healthy leaves. The biomass during the first 10 d of the experiment was visibly lower for the EDDS treated plants, thereafter diminishing. Nevertheless, compared to the control and EDTA treated pots, the biomass stayed lower.

The replanting of the pots from the phytoextraction experiment revealed that EDDS is not readily degradable. Beside the high heavy metal leaching probability (see chapter III.3.4) it causes toxicity symptoms to the plants that are planted in a follow up phytoextraction treatment, thus making an application of EDDS not suitable for chelate assisted phytoextraction.

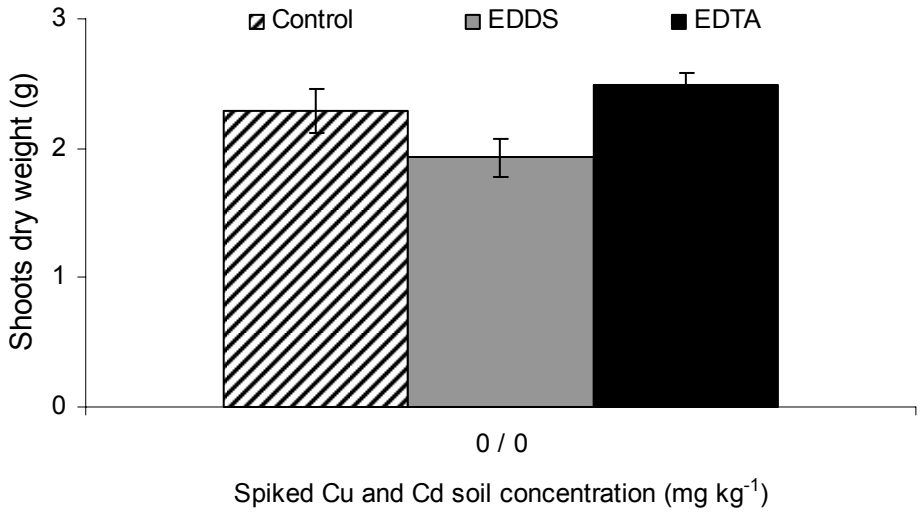


Figure 3-16: Effect of the application of EDDS or EDTA to soil on shoot dry weight of replanted tobacco (*Nicotiana tabacum* SR-1) grown for 3 weeks. Error bars represent ± SE of triplicates (n = 3).

III.4 Hydrolysed wool

Wool was enzymatically hydrolysed and used in column, phytoextraction and biodegradation experiments to investigate its potential as an alternative to synthetic chelators. Furthermore, a characterisation of the wool hydrolysate was performed to determine the effective parts of the hydrolysate. The wool was hydrolysed in two different time periods; the first was a period of 7 d and the second was a period of 14 d in order to observe the influence of the hydrolysation time on the heavy metal mobilisation and phytoextraction efficiency. The column experiment revealed a higher Cu and Cd mobilisation potential for the 14 d wool hydrolysate. However, the Cd amount mobilised by the 14 d wool hydrolysate was only 2-

fold higher than that of the control, whereas the Cu amount mobilised was approximately 25-fold higher than that by the control. The phytoextraction experiment with tobacco (*Nicotiana tabacum* SR-1*)* and the addition of a 7 d hydrolysate revealed a non significant enhanced uptake of Cd. Phytoextraction experiments with Cu were performed under the application of 7 d hydrolysate (6.6 g kg^{-1} of soil) and 14 d hydrolysate (12.2 g kg^{-1}). The 14 d hydrolysate application achieved an 850% increase in the uptake of Cu. Soil analysis showed that the time period of heavy metal mobilisation, owing to the application of hydrolysed wool is short, thus a leaching probability is low. The biodegradation experiment confirmed the results of the soil analysis by displaying a rapid biodegradation rate of hydrolysed wool. The characterisation of the wool hydrolysate revealed that the most efficient amino acids involved in the mobilisation of Cu were cysteine and histidine.

III.4.1 Column experiment

The capability of wool hydrolysate to mobilise heavy metals was investigated in a column experiment. Figure 3-17a, 3-17b depicts Cu and Cd mobilised from a soil spiked with 450 mg kg^{-1} Cu and 100 mg kg^{-1} Cd respectively, in a column experiment, in percentage of the total amount of Cu and Cd in the soil. The Cu mobilised by hydrolysed wool (14 d) was to a significant ($P < 0.05$) extent higher than that by hydrolysed wool (7 d). In comparison to the 7 d or 14 d wool hydrolysate, the control with $CaCl_2$ mobilised negligible amounts of Cu. Hydrolysed wool (14 d) mobilised approximately 13% in the first fraction. The mobilised amounts declined with the number of pore volumes. The total Cd amount mobilised by both applied hydrolysed wool grades and $CaCl_2$ ranged at the same order or magnitude, between 3 and 5.5%.

The heavy metal mobilisation potential determined by column experiment was higher for the 14 d wool hydrolysate in the case of Cu and Cd. The wool hydrolysates show a low mobilisation potential for Cd in comparison to the control. The amounts of Cd mobilised by the application of the wool hydrolysates is much lower compared to the control, than in the case of Cu. Therefore, the Cu uptake in a phytoextraction experiment is expected to be enhanced significantly by the application of the wool hydrolysates, whereas the uptake of Cd is not expected to be enhanced.

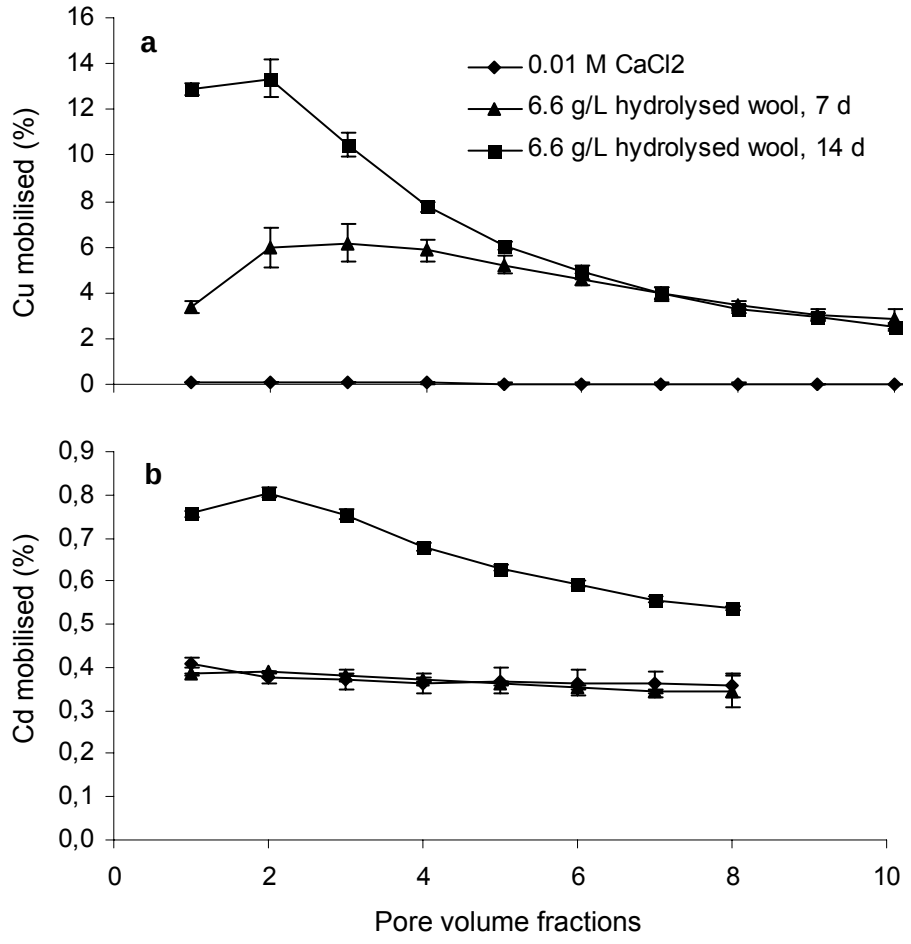


Figure 3-17: Leaching of Cu (a) and Cd (b) with hydrolysed wool and $CaCl_2$ as a control in column experiments. Error bars represent SD of five parallels.

III.4.2 Phytoextraction pot experiment

The enhanced heavy metal uptake effect by the addition of chelating agents was investigated in phytoextraction pot experiments. The wool hydrolysate was added 2 d before harvesting. In this application the first experimental setup was made with the addition of 6.6 g kg^{-1} wool (7 d-hydrolysate), while the second was with the addition of 12.2 g kg^{-1} wool (14 d-hydrolysate).

III.4.2.1 Phytoextraction of Cd

Dry matter yields of the shoots (sum of leaves and stem) are shown in Figure 3-18. The amount of 6.6 g kg^{-1} of 7 d wool hydrolysate was applied to the soil. Neither the application of the wool hydrolysate to the soil, nor the extracted Cd adversely affected the dry matter production of the plants. Toxicity symptoms, such as chlorosis and necrosis, were not visible in any spiked Cd concentrations in soil.

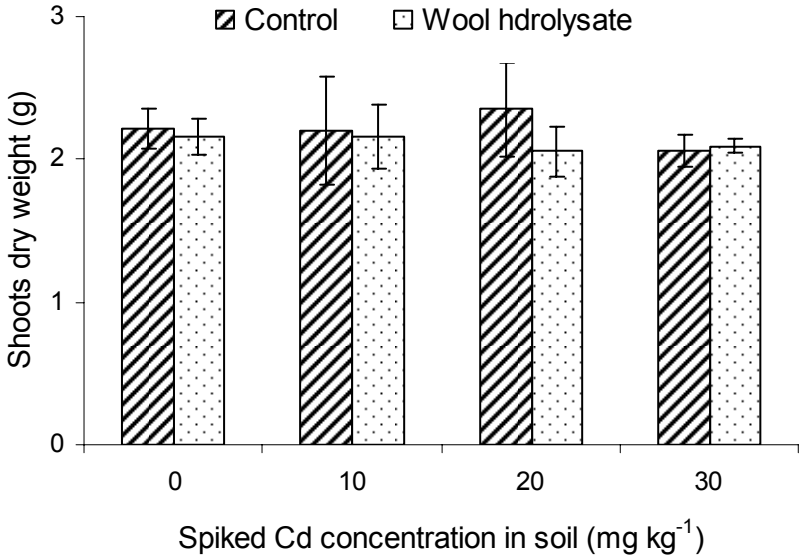


Figure 3-18: Dry weight of tobacco (*Nicotiana tabacum* SR-1) grown for 3 weeks on a soil treated with a 7 d wool hydrolysate (6.6 g kg^{-1}). Error bars represent SD of triplicates.

Figure 3-19a shows that Cd concentrations in the shoots, increased with increasing Cd concentrations in the soil. The Cd uptake into the shoots was slightly enhanced by the application of wool hydrolysate. Compared to the control the treatment with 6.6 g kg^{-1} soil 7 d wool hydrolysate caused a significantly ($P < 0.05$) higher concentration of Cd in the shoots. In the case of the wool hydrolysate application, Cd concentrations in the shoots reached 56.9 mg kg^{-1}, while the control reached Cd concentrations of 43.6 mg kg^{-1} in the shoots. Figure 3-19b depicts the total amount of Cd in the shoots. The picture is similar to Figure 3-19a as the dry weights are similar. A significantly ($P < 0.05$) higher total amount of Cd in the shoots compared to the control is visible at the 30 mg kg^{-1} Cd soil concentration. The total Cd amount of the 7 d wool hydrolysate treated plants and the control plants in the shoots reached 119.3 and 89.3 mg kg^{-1} respectively.

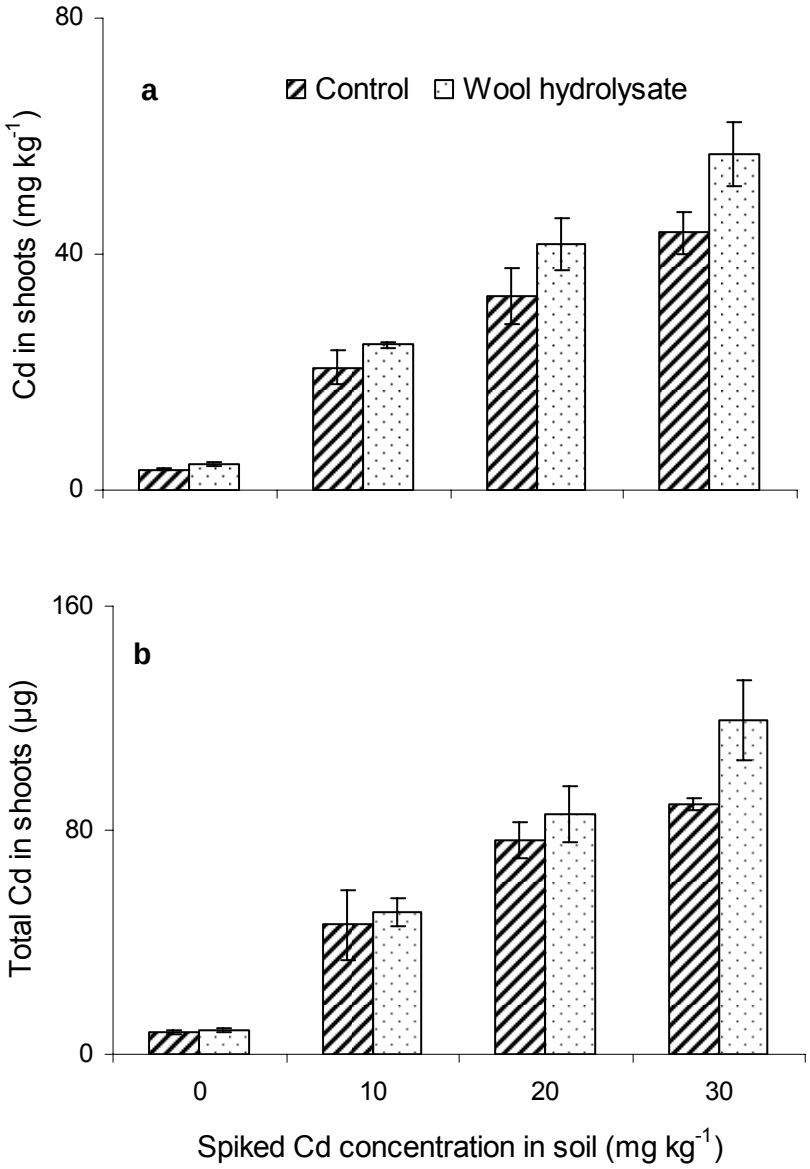


Figure 3-19: Cd (a) concentration and (b) total amount in tobacco (*Nicotiana tabacum* SR-1) grown for 3 weeks on a soil treated with a 7 d wool hydrolysate (6.6 g kg^{-1}). Error bars represent ± SE of triplicates (n = 3).

The phytoextraction experiment revealed that the 7 d wool hydrolysate applied to the soil enhanced the uptake of Cd contrary to the expected results, based on the column experiment (see chapter III.4.1). It is possible that the column experiments display a higher mobilisation of heavy metals by the control ($CaCl_2$-solution) than the corresponding uptake of heavy metals by the control plants. Therefore, the difference between the uptake by control and the chelating agent application in the pot experiment is greater than one might expect from the results of the column experiment.

III.4.2.2 Phytoextraction of Cu (6.6 g kg^{-1}, 7 d wool hydrolysate)

Dry matter yields of the shoots (sum of leaves and stem) are shown in Figure 3-20. The amount of 6.6 g kg^{-1} of a 7 d wool hydrolysate was applied to the soil. The application of Cu, combined with wool hydrolysate to the soil did not affect the dry matter production of the plants adversely. Toxicity symptoms, such as chlorosis, or necrosis were not observed.

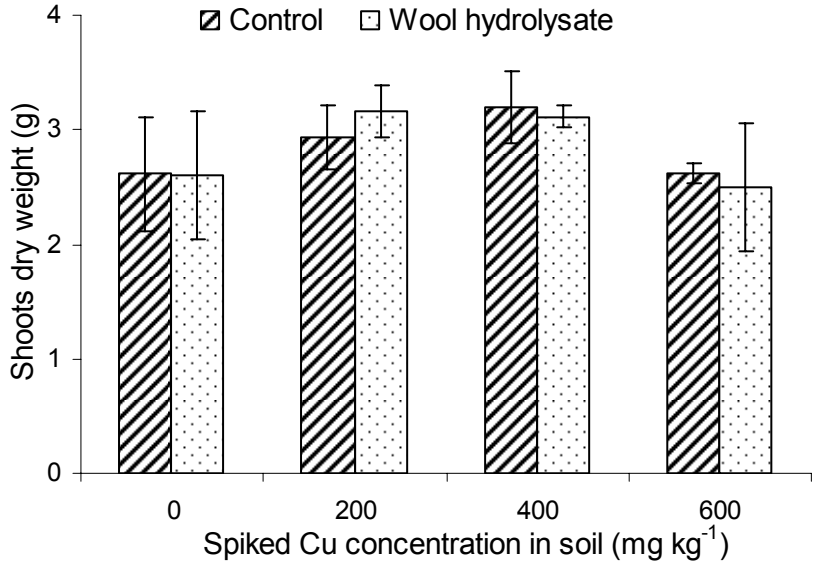


Figure 3-20: Dry weight of tobacco (*Nicotiana tabacum* SR-1) grown for 3 weeks on a soil treated with a 7 d wool hydrolysate (6.6 g kg^{-1}). Error bars represent SD of triplicates.

As shown in Figure 3-21a, increasing Cu concentration of the soil resulted in increasing Cu concentration in the shoots of the plants. The addition of 7 d wool hydrolysate enhanced the uptake of Cu into the shoots, resulting in a significantly ($P < 0.05$) higher Cu concentration in the shoots in comparison to the control. The plants treated with 7 d wool hydrolysate (6.6 g kg^{-1}) reached Cu concentrations of 33.7 mg kg^{-1}, as compared to the control which reached Cu concentrations of 19.8 mg kg^{-1} in the shoots. The total amount of Cu in the shoots was also significantly ($P < 0.05$) enhanced in comparison to the control (Figure 3-21b). The total Cu amount of the wool hydrolysate treated plants and the control plants in the shoots reached 80.9 and 61.3 mg kg^{-1} respectively.

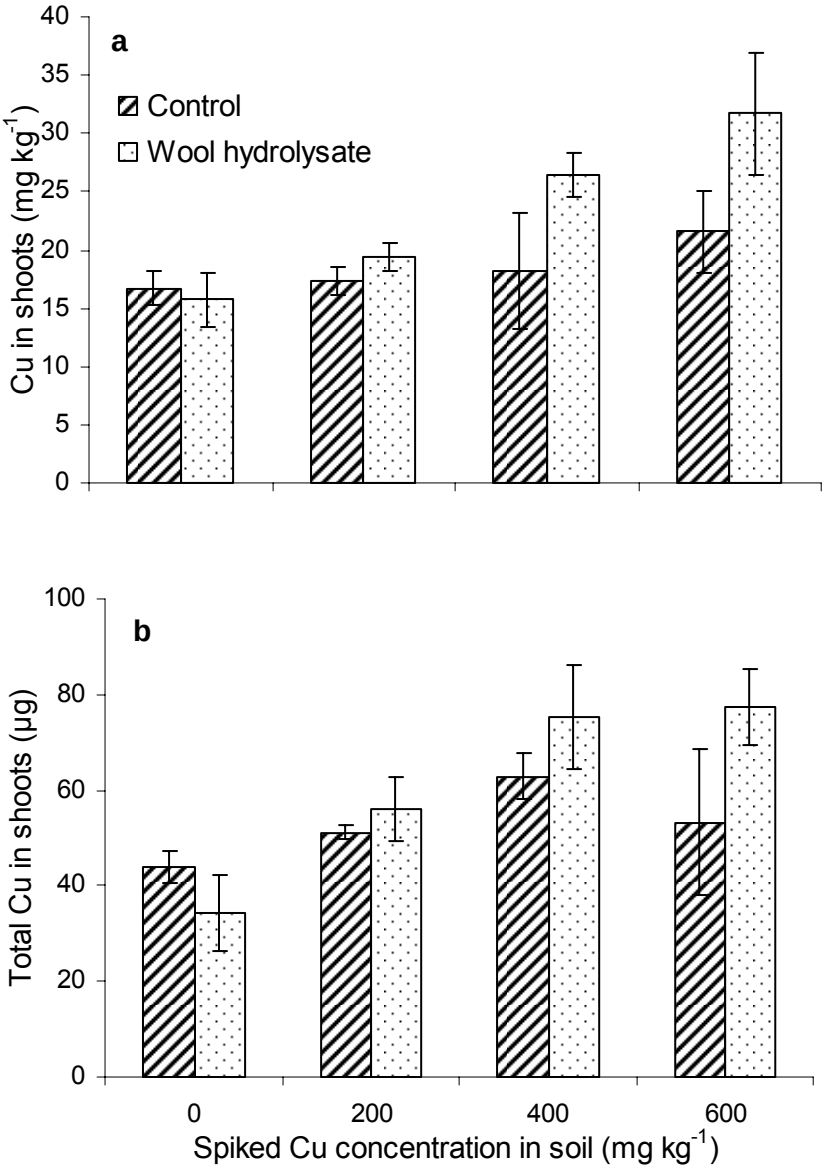


Figure 3-21: Cu (a) concentration and (b) total amount in tobacco (*Nicotiana tabacum* SR-1) grown for 3 weeks on a soil treated with a 7 d wool hydrolysate (6.6 g kg^{-1}). Error bars represent ± SE of triplicates (n = 3).

The Cu uptake was enhanced significantly after a 7 d wool hydrolysate application to the soil. The increase in uptake was not as high as expected from the results of the column experiment (see chapter III.4.1). The 7 d wool hydrolysate could probably not fulfil the expectations of the column experiment owing to the biodegradation of wool. The number of effective molecules was probably too rapidly degraded to display an enhancement according to the column experiment.

III.4.2.3 Phytoextraction of Cu (12.2 g kg^{-1}, 14 d wool hydrolysate)

Dry matter yields of the shoots (sum of leaves and stem) are shown in Figure 3-22. In this case the amount of 12.2 g kg^{-1} of a 14 d wool hydrolysate was applied to the soil. The application of hydrolysed wool to the soil did not adversely affect dry matter production of the plants. However, the leaves showed necrotic lesions 2 d after the application of the hydrolysed wool.

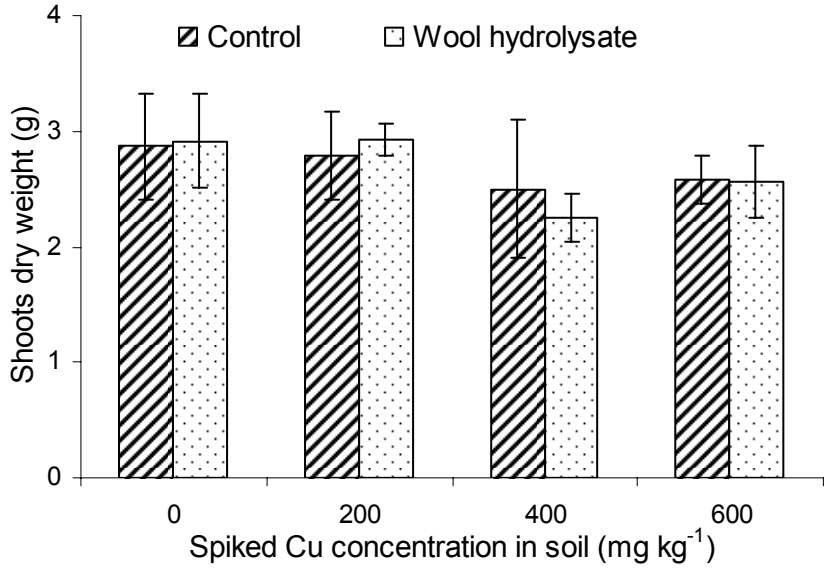


Figure 3-22: Dry weight of tobacco (*Nicotiana tabacum* SR-1) grown for 3 weeks on a soil treated with a 14 d wool hydrolysate (12.2 g kg^{-1}). Error bars represent SD of triplicates.

Figure 3-23a shows that Cu concentrations in the shoots increased with increasing Cu concentrations in the soil. Concerning Cu uptake into the shoots it was particularly enhanced after the application of the 14 d wool hydrolysate. A significantly ($P < 0.001$) higher concentration of Cu in the shoots compared to the control was caused by the treatment with hydrolysed wool. In the case of the 14 d wool hydrolysate application, Cu concentration in the shoots reached 207.7 mg kg^{-1}, whereas the Cu concentration in the shoots of the control reached 24.1 mg kg^{-1}. Figure 3-23b depicts the total amount of Cu in the shoots. Similar to Figure 3-23a significantly ($P < 0.001$) higher total amounts of Cu were extracted by the plants through the application of 14 d wool hydrolysate as compared to the control. The total Cu amount in the shoots for the plants treated with 14 d wool hydrolysate was 530.4 mg kg^{-1}, compared to 61.9 mg kg^{-1} for the control plants.

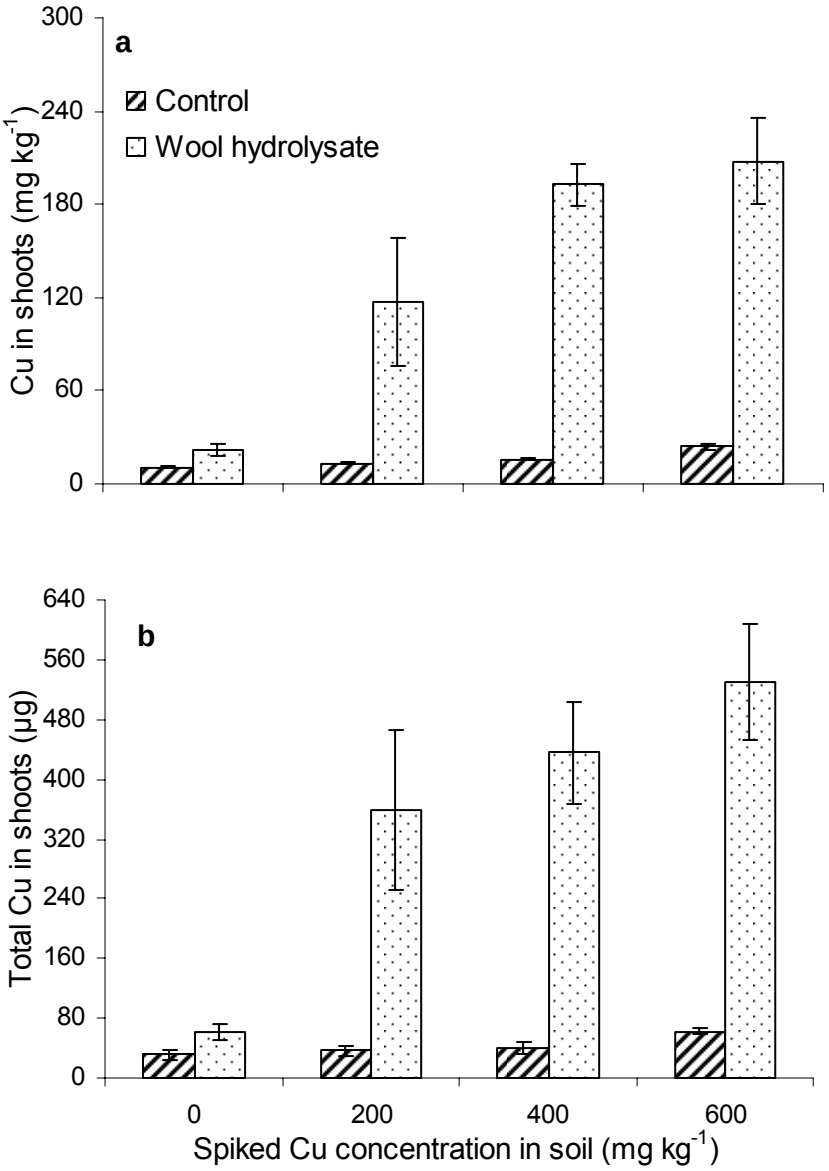


Figure 3-23: Cu (a) concentration and (b) total amount in tobacco (*Nicotiana tabacum* SR-1) grown for 3 weeks on a soil treated with a 14 d wool hydrolysate (12.2 g kg^{-1}). Error bars represent ± SE of triplicates (n = 3).

The Cu uptake was enhanced significantly after a 14 d wool hydrolysate application to the soil. The uptake increase was as high as expected from the results of the column experiment (see chapter III.4.1). A higher hydrolysation time period increases the effectiveness of the wool hydrolysate as shown in the column and the phytoextraction experiment.

III.4.3 Soil analysis of phytoextraction pot experiment

The pH, as well as the DTPA-extractable (potentially bioavailable) and the NH_3NO_4-extractable (plant available) heavy metal fraction of the soil was analysed after the phytoextraction experiments. The potentially bioavailable fraction is the heavy metal fraction in soil which can become bioavailable in time under the influence of weathering, whereas the plant available fraction is the heavy metal fraction in soil which becomes available to plants when they release their root exudates.

III.4.3.1 Phytoextraction of Cd (6.6 g kg^{-1}, 7 d wool hydrolysate)

pH of the soil

The soil pH of the control and that of the pots treated with 7 d wool hydrolysate were measured at the end of the phytoextraction experiment. The pH-values of the Cd phytoextraction experiment are shown in Figure 3-24. The pH of the control pots is on average approximately 0.2 lower than that of the hydrolysed wool treated pots. The pH was decreased by the application of fertilisers and Cd. The pH of the pots treated with 7 d wool hydrolysate was higher than that of the control and exceeded the native pH 6.59 of the soil by maximum 0.1.

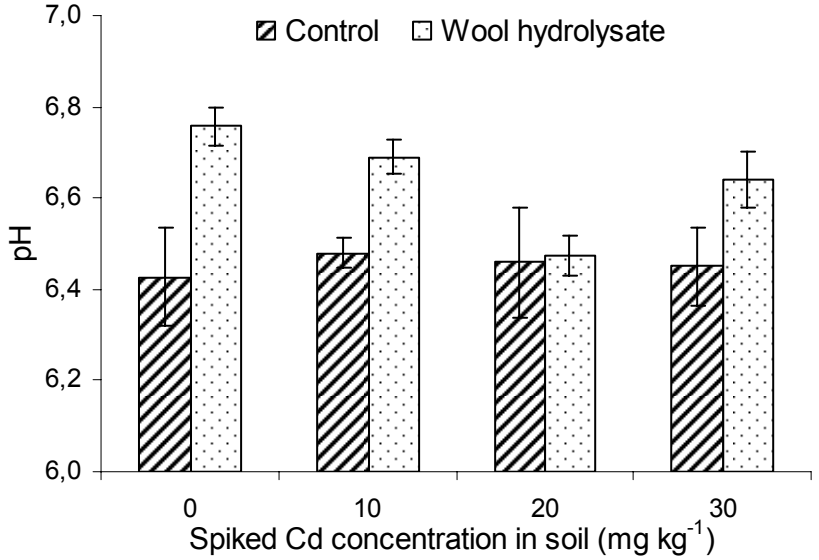


Figure 3-24: pH values of the soil after the application of 7 d hydrolysed wool. Error bars represent ± SE of triplicates (n = 3).

DTPA-extractable Cd fraction in the soil

The DTPA-extractable Cd fraction was measured for the control pots and the 7 d wool hydrolysate treated pots after the phytoextraction experiment. The DTPA-extractable (potentially bioavailable) Cd concentrations in the soil are shown in Figures 3-25. The potentially bioavailable Cd fraction in the control pots and the chelate assisted pots was not significantly ($P < 0.005$) different.

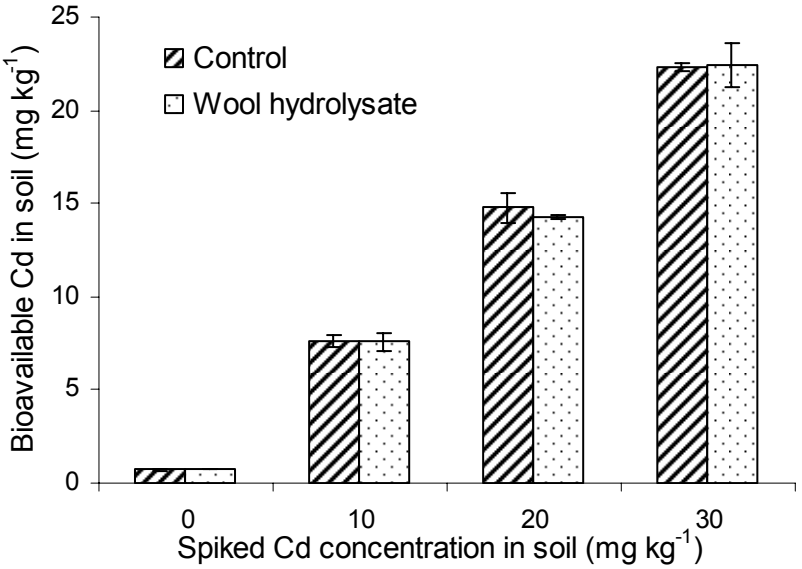


Figure 3-25: Potentially bioavailable Cd after the application of 7 d hydrolysed wool. Error bars represent ± SE of triplicates (n = 3).

NH_4NO_3 -extractable Cd fraction in the soil

The plant available Cd concentrations in the soil, as determined by NH_4NO_3 extraction, are shown in Figure 3-26. The NH_4NO_3 -extractable Cd fraction was measured for the control pots and the 7 d wool hydrolysate treated pots following the phytoextraction process. Irrespective of the 7 d wool hydrolysate added to the soil before harvesting, the plant available Cd concentration in the soil was not enhanced significantly ($P < 0.005$), with the exception of the 30 mg kg^{-1} Cd treatment, in comparison to the control.

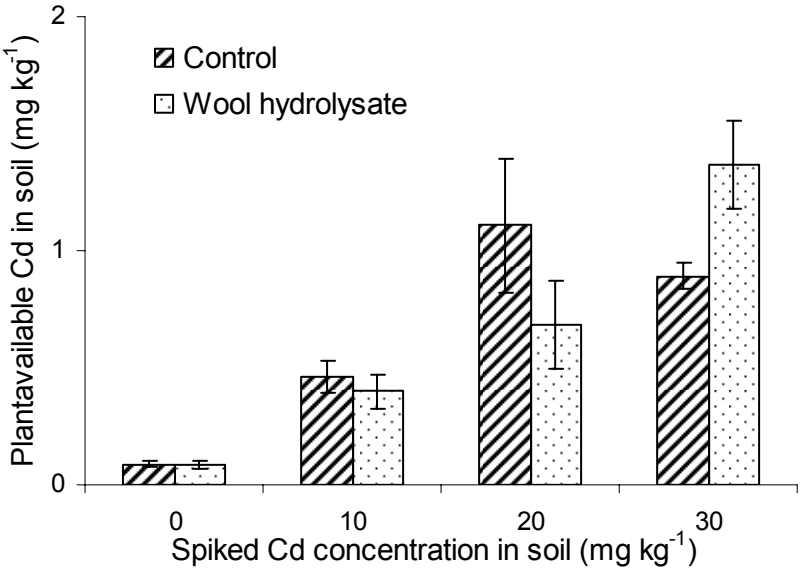


Figure 3-26: Plant available Cd after the application of 7 d hydrolysed wool. Error bars represent ± SE of triplicates (n = 3).

The results show that after a 7 d wool hydrolysate application the enhanced Cd mobilisation diminishes quickly thus reducing the leaching probability, in comparison to other chelating agents, such as EDDS and EDTA, which have a high heavy metal leaching probability.

III.4.3.2 Phytoextraction of Cu (6.6 g kg^{-1}, 7 d wool hydrolysate)

pH of the soil

The pH-values of the soil measured following the Cu phytoextraction experiment are shown in Figure 3-27. The pH was measured for the control as well as that of the pots treated with 7 d wool hydrolysate. The pH of the 7 d wool hydrolysate treated pots is in average approximately 0.3 higher than that of the control pots. The pH of the control pots was probably decreased by the application of fertilisers and Cu. As the Cu concentration in the soil increased the pH of the control pots decreased accordingly. The pH of the pots treated with 7 d wool hydrolysate was higher than that of the control, and exceeded the native pH 6.59 of the soil only at the 600 mg kg^{-1} spiked Cu concentration in the soil.

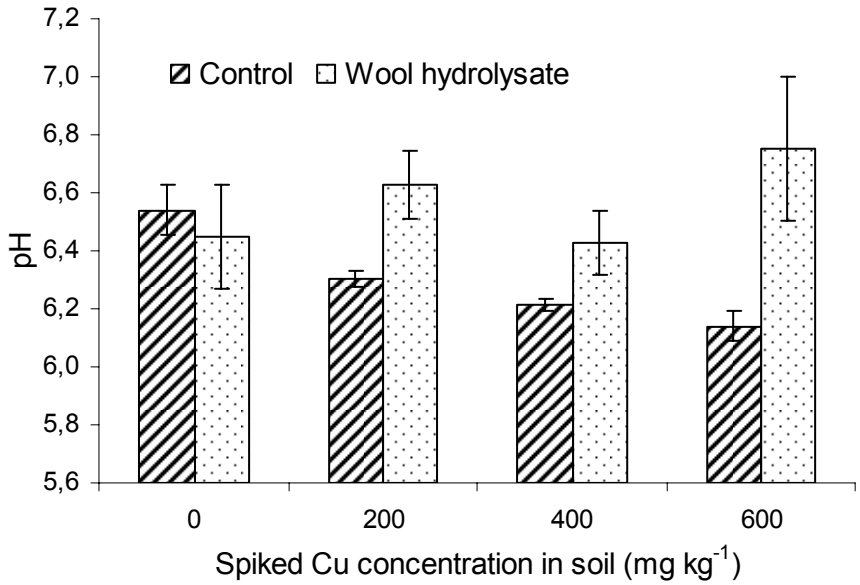


Figure 3-27: pH values of the soil after the application of 7 d hydrolysed wool. Error bars represent ± SE of triplicates (n = 3).

DTPA-extractable Cu fraction in the soil

The DTPA-extractable (potentially bioavailable) Cu concentrations in the soil are shown in Figure 3-28. The DTPA-extractable Cu fraction was measured for the control pots and the 7 d wool hydrolysate treated pots following the phytoextraction experiment. The potentially bioavailable Cu fraction in the 7 d wool hydrolysate treated pots was never significantly higher than that of the control. At the 600 mg kg^{-1} spiked Cu concentration in the soil the DTPA-extractable Cu was significantly ($P < 0.005$) lower in the chelate treated pots in comparison to the control.

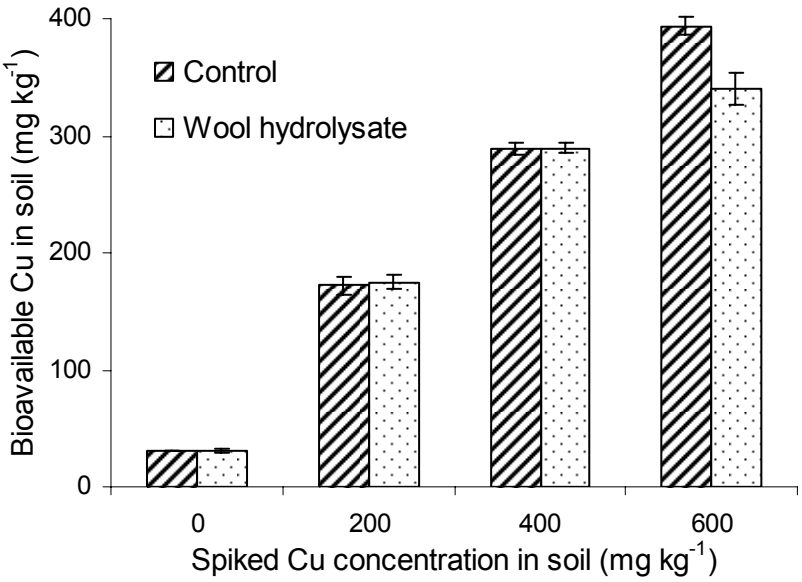


Figure 3-28: Potentially bioavailable Cu after the application of 7 d hydrolysed wool. Error bars represent ± SE of triplicates (n = 3).

NH_4NO_3-extracrable Cu fraction in the soil

The NH_4NO_3 -extractable Cu fraction was measured for the control pots and the 7 d wool hydrolysate treated pots following the phytoextraction experiment. The plant available Cu concentrations in the soil, as determined by NH_4NO_3 extraction, are shown in Figure 3-29. Irrespective of the 7 d wool hydrolysate added to the soil before harvesting, the plant available Cu concentration in the soil was not enhanced significantly ($P > 0.1$) in comparison to the control, at any spiked Cd concentration in the soil.

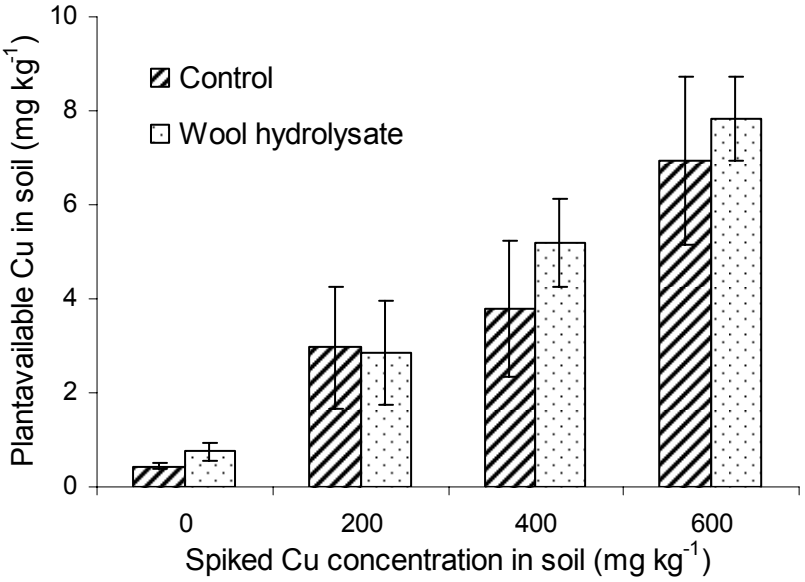


Figure 3-29: Plant available Cu after the application of 7 d hydrolysed wool. Error bars represent ± SE of triplicates (n = 3).

The results show that after application of a 7 d wool hydrolysate the enhanced Cu mobilisation diminishes quickly thus reducing the leaching probability.

III.4.3.3 Phytoextraction of Cu (12.2 g kg^{-1}, 14 d wool hydrolysate)

pH of the soil

The pH was measured for the control as well as that of the pots treated with 14 d wool hydrolysate. The pH-values of the soil following the Cu phytoextraction experiment are shown in Figure 3-30. The pH of the 14 d wool hydrolysate treated pots was significantly ($P < 0.005$) higher than that of the control pots. On average, it was approximately 0.45 higher. The decrease of the pH in the control pots was probably due to the application of fertilisers and Cu. As the Cu concentration in the soil increased the pH of the control pots decreased accordingly. The pH of the pots treated with 14 d wool hydrolysate was higher than that of the control, and exceeded the native pH 6.59 of the soil at all spiked Cu concentration in soil.

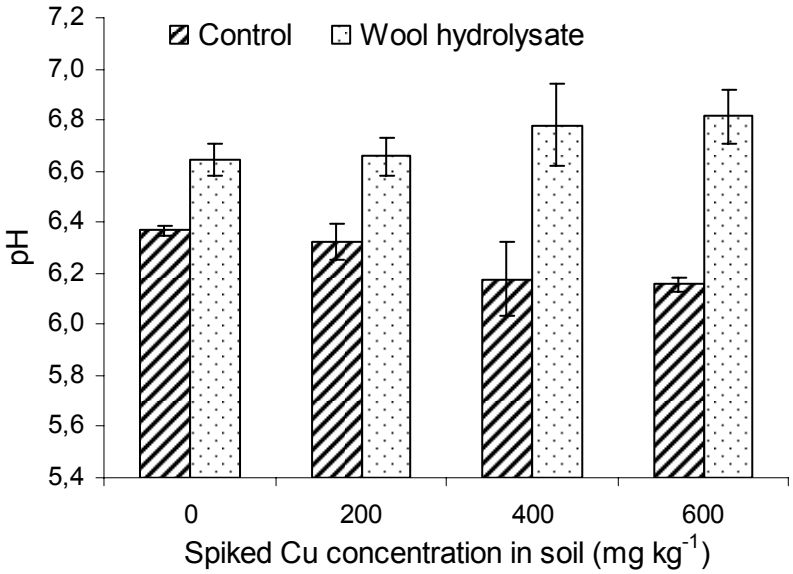


Figure 3-30: pH values of the soil after the application of 7 d hydrolysed wool. Error bars represent ± SE of triplicates (n = 3).

DTPA-extractable Cu fraction in the soil

The DTPA-extractable Cu fraction was measured for the control pots and the 14 d wool hydrolysate treated pots following the phytoextraction experiment. The DTPA-extractable (potentially bioavailable) Cu concentrations in the soil are shown in Figure 3-31. The potentially bioavailable Cu fraction in the 14 d wool hydrolysate treated pots was never significantly ($P < 0.1$) higher than that of the control.

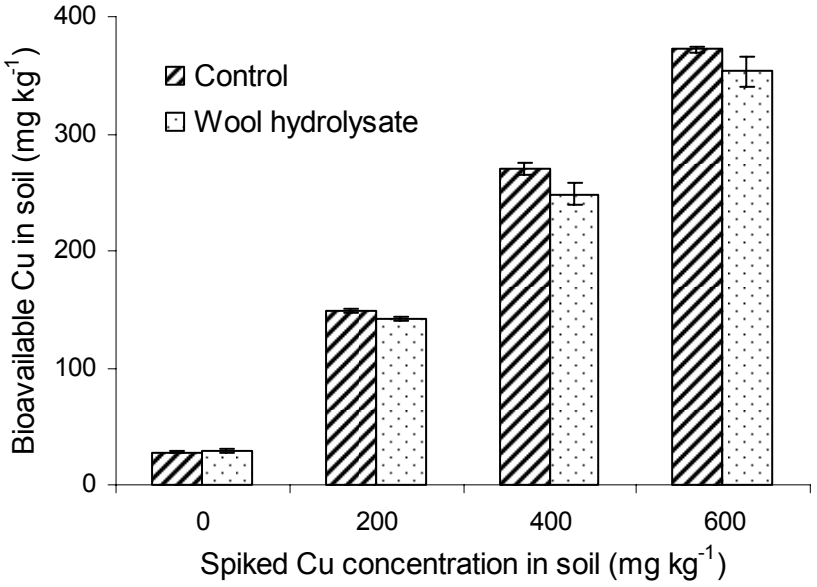


Figure 3-31: Potentially bioavailable Cu after the application of 14 d hydrolysed wool. Error bars represent ± SE of triplicates (n = 3).

NH_4NO_3-extracrable Cu fraction in the soil

The plant available Cu concentrations in the soil, as determined by NH_4NO_3 extraction, are shown in Figure 3-32. The NH_4NO_3 -extractable Cu fraction was measured for the control pots and the 14 d wool hydrolysate treated pots following the phytoextraction experiment. The 14 d wool hydrolysate added to the soil before harvesting, enhanced significantly ($P < 0.01$) the plant available Cu fraction in the soil. Only at the 600 mg kg^{-1} spiked Cu concentration in the soil, the increase in plant available Cu was not significant.

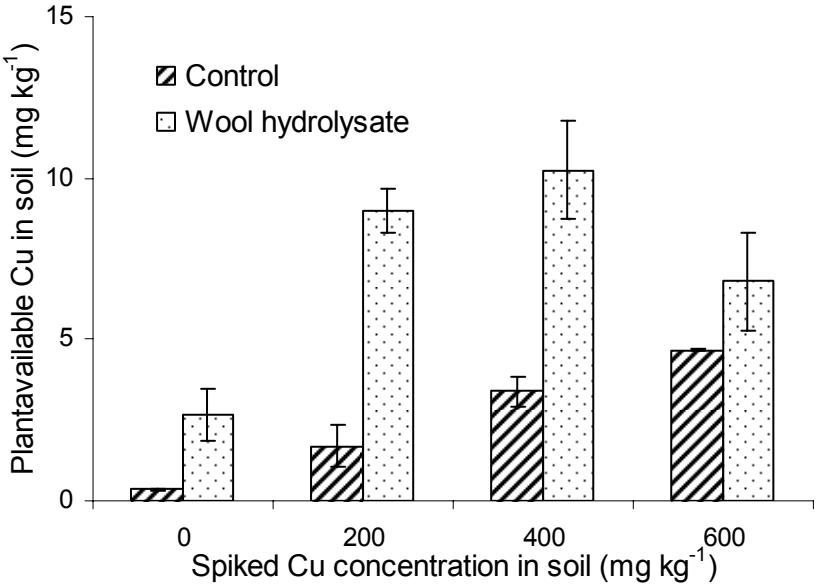


Figure 3-32: Plant available Cu after the application of 14 d hydrolysed wool. Error bars represent ± SE of triplicates (n = 3).

The application of 14 d wool hydrolysate (12.2 g kg^{-1} of soil) to soil enhanced the Cu plant available fraction in soil. Nevertheless the increase is still small, thus Cu having a low leaching probability.

III.4.3.4 Summary of the phytoextraction experiments with hydrolysed wool

The 14 d wool hydrolysate was most efficient in the enhancement of the uptake of Cu by tobacco (*Nicotiana tabacum* SR-1). Additionally it displayed a short lived enhancement effect thus reducing the leaching probability. The 7 d wool hydrolysate increased the uptake of Cu and Cd although only slightly. Judging from the column experiment and the phytoextraction experiment the wool hydrolysate displays a higher efficiency in mobilising Cu than Cd. This difference in efficiency could lie in the higher stability constants of amino acids Cu-complexes.

III.4.4 Biodegradation of the hydrolysed wool

III.4.4.1 Oxitop

The results of the degradation experiment are shown in Figure 3-33. The addition of hydrolysed 7 d wool hydrolysate (6.6 g kg^{-1} of soil) displayed a significant increase of

respiration in comparison to the control (basal respiration). The respiration of the hydrolysed wool treated samples surpassed the basal respiration after approximately 10 h. Afterwards the degradation was very rapid.

The respiration increase in the oxitop experiment shows that the wool hydrolysate is not toxic for the soil microorganisms. The rapid biodegradation rate combined with high heavy metal mobilisation potential makes wool hydrolysate an efficient chelating agent for the enhancement of phytoextraction.

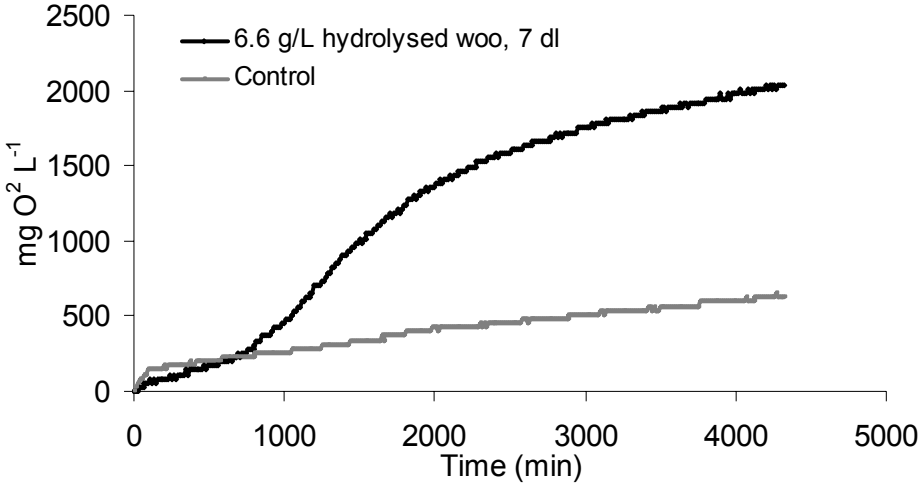


Figure 3-33: Influence of hydrolysed wool on the oxygen consumption during degradation in the Oxitop system.

III.4.5 Characterisation of wool hydrolysate

III.4.5.1 Molar mass analysis by MALDI-TOF MS

The matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) is used for the detection and characterisation of biomolecules, such as proteins, peptides, oligosaccharides and oligonucleotides. The molar mass distribution of the wool after a 14 d hydrolysis is depicted in Figure 3-34. It revealed peptides of varying sizes. No abundance was observed of peptides above a molar mass of 1010. Most of the peptides masses range between 758.9 and 595.1, corresponding to peptides in the range of 8, 7 and/or 6 amino acids. Abundance is also observed above a mass of 900, probable owing to nona-

and/or decapeptides. An intense signal with m/z = 471.5 was observed, depicting probably tri- and/or tetrapeptides.

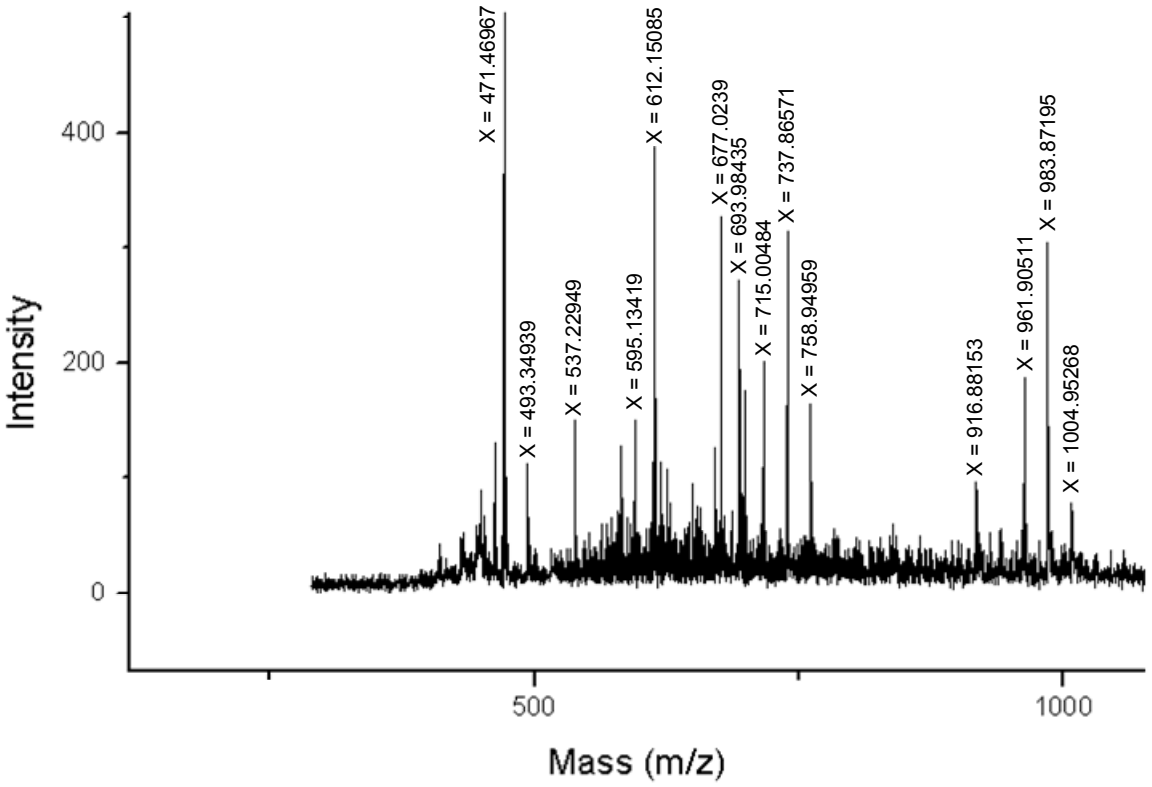


Figure 3-34: MALDI time-of-flight mass spectrum; molar masses of the most abundant mono-isotopic species from the 14 d wool hydrolysate as determined by MALDI-TOF MS analyses are depicted.

IMAC-columns were used to determine which peptides were the most effective in the mobilisation of Cu. IMAC-columns are columns which can be loaded with various metals, thus binding peptides which have a high affinity for a specific metal. The IMAC-columns were loaded with Cu, which was tightly bound on the columns. When the 14 d wool hydrolysate (in dissolved state) passed through the IMAC-columns only peptides with a high affinity for Cu were bound to the column. Therefore, a selection of these peptides was possible which were the most efficient in mobilising Cu in soil. The molar mass distribution of a 14 d wool hydrolysate after passing through the IMAC-columns is depicted in Figure 3-35. It revealed peptides of varying sizes. No abundance was observed of peptides above a molar mass of 917. Most of the peptides masses range between 693.0 and 471.2, consisting

probably of hepta-, and tripeptides. Signals above a mass of 900, might be nona- and/or decapeptides.

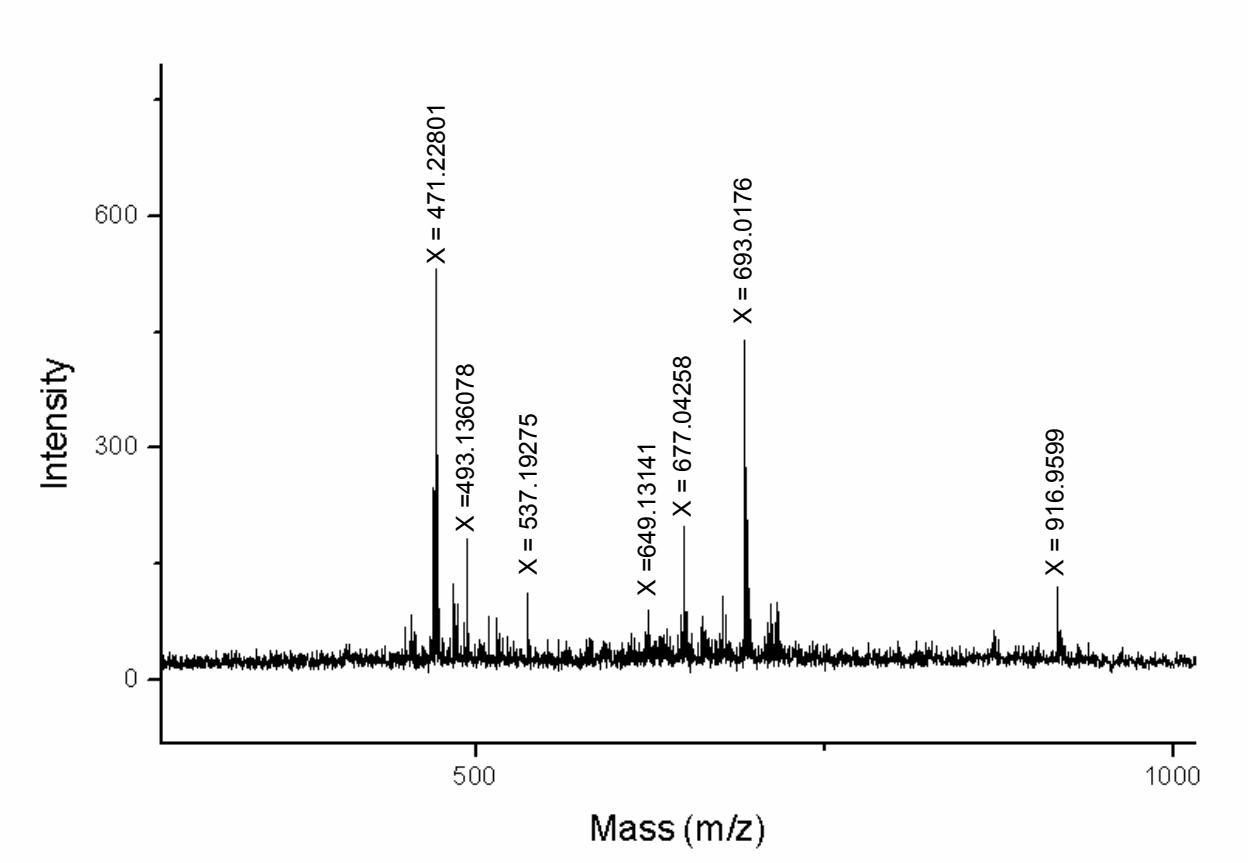


Figure 3-35: MALDI time-of-flight mass spectrum of the 14 d wool hydrolysate after passing a IMAC-column; molar masses of the most abundant mono-isotopic species as determined by MALDI-TOF MS analyses are depicted.

III.4.5.2 Amino acid composition of wool and wool hydrolysate

Table 3-5 depicts the amino acid composition and molar percentage concentration for a total acid hydrolysis of the wool and of the enzymatic hydrolysate before and after the IMAC-columns. Due to total acid hydrolysis, glutamine and asparagine are not measured, as they are transformed to glutamic acid and aspartic acid during the total acid hydrolysis process. In the total acid hydrolysis process cysteine is partially transformed to cysteic acid. The wool hydrolysate has roughly the same composition as the wool before enzymatic hydrolysis. The molar percentage concentration of glycine and leucine increased, whereas that of serine

decreased. The wool hydrolysate before the IMAC-columns contains more ornithine and less arginine than the wool. The enzymatic wool hydrolysation process was not performed under sterile conditions, therefore during the 14 d hydrolysation process microorganisms (such as *Saccharomyces cerevisiae*) may have developed, which have the ability to degrade arginine and transform it to ornithine by the enzyme arginase (Martin et al., 2003), thus increasing the amounts of ornithine compared to arginine. The content of cystine, which is an oxidised dimeric form of cysteine, formed by linking two cysteine residues via a disulfide bond (cys-S-S-cys) between the -SH groups, decreased during the enzymatic hydrolysation process. High amounts of cystine are contained in Keratin associated proteins (KAP). KAP have been classified on the basis of their amino acid composition as high sulfur (16-30 mol % cysteine), ultra-high sulfur (>30 mol % cysteine), and high glycine/tyrosine proteins (Shimonura et al., 2002). In the absence of reducing agents, KAP are nearly insoluble, and thus a decrease of cystine content in the dissolved protein hydrolysate was expected. As a concequence the cystine concentration in the hydrolysate decreases. The cystine concentration is still rather high, posably owing to the fact that the enzymatic hydrolysis was not sterile, and thus microorganisms will have developed that reduced the disulfide bonds by the aid of reductases.

After the treatment with the IMAC-columns a higher amount of ornithine, cysteine (cysteic acid), and histidine was found in the hydrolysate compared to the wool hydrolysate before the IMAC-columns, whereas the amounts of valine, methionine, isoleucine, leucine and tyrosine decreased. Cystine decreased and cysteic acid increased because in the total acid hydrolysis process Cu residues catalysed the oxidation of the disulfide bond to cysteic acid. The Cu originated from the IMAC-column treatments, where Cu leaching was not avoidable.

Table 3-5: Amino acid analysis of wool and hydrolysed wool before and after IMAC-treatment; after a total hydrolysis.

Amino acids	Wool (mol 100 mol^{-1})	Hydrolysed wool (mol 100 mol^{-1})	Hydrolysed wool after IMAC (mol 100 mol^{-1})
Cysteic acid	0.25	0.27	10.01
Aspartic acid	6.52	6.96	6.31
Threonine	6.75	6.22	5.03
Serine	11.20	8.70	11.59
Glutamic acid	12.42	12.31	13.5
Proline	5.78	5.59	6.84
Glycine	8.54	10.46	9.80
Alanine	5.51	5.80	6.48
Valine	5.57	5.94	0.0
Cystine	5.91	3.45	0.0
Methionine	0.37	0.31	0.0
Isoleucine	3.40	3.55	0.0
Leucine	8.46	9.44	4.07
Tyrosine	4.06	4.81	2.21
Phenylalanine	3.16	3.88	3.62
Ornithine	0.10	1.77	5.62
Lysine	3.27	3.71	2.67
Histidine	1.04	1.30	10.28
Arginine	7.64	5.44	3.53
Glutamine	n.m.	n.m.	n.m.
Asparagine	n.m.	n.m.	n.m.

Cysteine and histidine increased significantly compared to the increase of alanine before and after the IMAC-columns indicating that cysteine (cysteic acid) and histidine are the amino acids, which are responsible for the mobilisation and the enhanced uptake of Cu into the plants.

IV DISCUSSION

Phytoextraction, the use of plants to extract inorganic contaminants from the soil, has been so far performed with either hyperaccumulating plants, i.e. plants which can reach a high heavy metal concentration in their shoots, but have a slow growth rate and a low biomass; or with fast growing, deep-rooted, easily propagated plants with a high biomass but which can only accumulate heavy metals in a very low degree. Regardless of the plants used, availability of heavy metals to plant roots is considered the key factor limiting the efficiency of phytoextraction (Felix, 1997). The degree of availability for uptake i.e. the phytoavailability of metals, can be increased by the application of chelating agents (chelators).

Chelators have shown an ability to enhance phytoremediation of heavy metals from contaminated soil, a property which could balance the characteristics of the hyperaccumulating plants. Although synthetic chelators, such as EDTA, have shown positive effects on the enhancement of phytoextraction of metals from soil, their use also has disadvantages: EDTA is non-selective in extracting metals (Barona et al., 2001) and has a poor biodegradability (Wasay et al., 1998), thus causing leaching of toxic and essential metals. It has also the effect of decreasing the plant growth severely (Chen and Cutright, 2001) even at very low concentrations. An alternative to synthetic chelators could be found in natural occurring chelating agents, so called biochelators. These biochelators should have a high heavy metal mobilisation capability in order to enhance the phytoextraction of heavy metals; simultaneously they should be easily biodegradable so that the risk of heavy metal leaching can be reduced. Furthermore, they should display a low phytotoxic potential. Biochelators, such as humic acids, have already been shown to have positive effects in the phytoextraction of heavy metals from soil (Evangelou et al., 2004), but the efficiency was not high enough, thus alternative biochelators have to be found and tested in order to replace the synthetic chelators on the field of chelate assisted phytoextraction. Possible alternatives could be natural low molecular weight organic acids (NLMWOA), cyclodextrins (CyD), ethylene diamine disuccinate (EDDS) and wool in a hydrolysed form.

IV.1 Natural low molecular weight organic acids (NLMWOA)

NLMOWA are exudated by plants into the soil and have an influence on the phytoavailability of metals in soil owing to their acidification, chelation, precipitation and oxidation-reduction reactions in the rhizosphere, but also owing to their effects on microbial

activity. The addition of NLMWOA to the soil could enhance the uptake of heavy metals into the plant.

The objective of this study was to investigate the ability of NLMWOA in enhancing the phytoextraction of Cu and Pb from soil by the use of tobacco plants (*Nicotiana tabacum* SR-1) under laboratory conditions. EDTA was also tested in order to have a direct comparison to the NLMWOA and thus to assess the potential of NLMWOA in replacing compounds such as EDTA or other synthetic chelators as enhancing agents for the phytoremediation of heavy metal contaminated soils. Up to now research focused on the effect of the application of NLMWOA on the uptake of heavy metals, but has neglected to observe the influence of the biodegradation of NLMWOA on the bioavailability of heavy metals and subsequently the influence on follow up NLMWOA applications. Therefore, in this thesis the biodegradation of the NLMWOA was observed and microorganisms participating in the process were identified.

Phytotoxicity experiments were performed to determine the highest no effect concentration of NLMWOA (citric acid, tartaric acid and oxalic acid) on tobacco (*Nicotiana tabacum* SR-1). Based on the results of the phytotoxicity experiment, the Cu and Pb mobilisation potential of NLMWOA and EDTA applied in corresponding amounts, was determined in slurry and column experiments. Phytoextraction experiments were performed applying NLMWOA and EDTA according to the determined highest no effect concentration. The phytoextraction experiment was followed by biodegradation experiments in order to assess a leaching potential by the application of NLMWOA and the efficiency by a successive application of the tested NLMWOA.

IV.1.1 Phytotoxicity pot experiment

The phytotoxicity pot experiments depicted a great difference between NLMWOA and EDTA concerning their toxicity to plants. Consistent with the results of Chen and Cutright (2001), EDTA showed a very high toxicity (Figure 3-1), which lead to a decreased plant growth at the concentration of 1.25 mmol kg^{-1} in the soil. At an EDTA concentration of 0.125 mmol kg^{-1} and 0.25 mmol kg^{-1} in the soil the plants showed no toxicity symptoms. In both applications the dry weight was similar to that of the controls. However, the standard deviation of the triplicates treated with 0.25 mmol kg^{-1} was higher than the standard deviation of the triplicates treated with 0.125 mmol kg^{-1}. The latter concentration was thus used in the phytoextraction experiments (Figure 3-1b). The NLMWOA application of 62.5 mmol kg^{-1} showed no adverse effects on the dry matter production of the shoots, and indeed even a slight increase in shoots yield is visible (Figure 3-1a). As a consequence, this concentration was

chosen for the phytoextraction and mobilisation (slurry and column) experiment. Higher concentrations of NLMWOA resulted in biomass decrease, probably owing to the destruction of the physiological barrier by NLMWOA in roots, which controls the uptake of solutes (Vassil et al., 1998). NLMWOA may have damaged the plasma membrane surrounding root cells which is thought to play a major role in forming this barrier. The plasma membrane is normally stabilised by Ca^{2+} and Zn^{2+} ions (Pasternak, 1987; Kaszuba and Hunt, 1990), therefore NLMWOA may induce metal-chelate uptake and accumulation by removal of stabilizing Zn^{2+} and Ca^{2+} from the plasma membrane. This damage may confer access of random metal complexes of soil-solution to root xylem and to the shoot via the transpiration stream, (Vassil et al., 1998) resulting in plant necrosis. Additionally necrosis can also occur owing to a facilitated access of pathogens into the plant, because of a disrupted plasma membrane.

Prior to the NLMWOA phytotoxicity tests, Cu and Pb toxicity experiments were performed. These showed no apparent harmful effects up to a soil concentration for Cu of 450 mg kg^{-1} and for Pb of 600 mg kg^{-1}. These two heavy metal concentrations were, therefore, applied in the phytoextraction pot experiment and column experiment.

IV.1.2 Mobilisation (slurry and column) experiments

The concentrations of the solution containing NLMWOA and EDTA used in the slurry and in the column experiment were obtained from the phytotoxicity experiment. In the slurry experiment the chelating agent's solutions were adjusted to several pH values, in order to observe the effect of the pH on their mobilisation capability. In the column experiment the pH of the solution was adjusted to the pH of the soil to avoid pH related effects in the mobilisation of the heavy metals Pb and Cu.

In the slurry experiments the addition of 62.5 mM NLMWOA showed an enhanced mobilisation of Cu compared to the addition of 0.125 mM EDTA (Figure 3-2). The mobilisation of Cu by citric acid and tartaric acid decreased significantly ($P < 0.01$) with decreasing pH of the solution, however, no corresponding pH effect was observed for oxalic acid and EDTA. This is explained by the pKa values of the chelates. The pKa_1 of oxalic acid and EDTA is 1.19 and 1.70, whereas that of citric acid and tartaric amounts to 3.06 and 3.02, respectively. The low pKa_1 values in oxalic acid and EDTA lead to a high proportion of fully deprotonated chelating species ranging from alkaline to slightly acidic pH. In contrast, owing to their higher pKa, citric acid and tartaric acid have a higher proportion of protonated

chelating species with a lower complexing potential in slightly acidic pH. Therefore, oxalic acid can complex metals in more acidic conditions compared to citric acid and tartaric acid. Schmidt (2003) stated that the Cu solubility increases with decreasing pH. However, as visible in this experiment, NLMWOA influence the mobility of Cu to a higher degree than the pH of the soil (Huang et al., 1998a). This is the case for Cu (Ullmann, 2004), and other metals, such as Fe, Ni, Zn (Holleman and Wiberg, 1995), which have a greater tendency to complexate. Pb, however, is very tightly bound to the soil (Bliefert, 1995) and does not display the same tendency to complexate as Cu. As such, the addition of NLMWOA, as compared to EDTA, showed very little effect on the mobilisation of Pb (Figure 3-2b). Surprisingly, at pH 5, only citric acid (Figure 3-2b) mobilised significantly ($P < 0.005$) more Pb than EDTA, oxalic and, tartaric acid.

The column experiment with NLMWOA underlined the great differences in the extractability and the complexation capabilities with respect to the two metals Cu and Pb. 0.5% and 0.1% of the initial amount of Cu and Pb in soil were extracted by 10 mM $CaCl_2$ solution, respectively, thus revealing the binding difference of the two metals to the soil. In comparison to $CaCl_2$ and EDTA, NLMWOA mobilised significantly more Cu. The Cu mobilisation by EDTA and $CaCl_2$ was in the same order of magnitude. Cu mobilisation by NLMWOA, especially citric acid, was significantly ($P < 0.05$) higher than the Cu amounts extracted by EDTA (Figure 3-3a). Citric acid has a higher affinity for Cu ($\log K_s = 14.24$) than oxalic acid ($\log K_s = 8.29$) (Martell and Calvin, 1958), which explains the significant difference in mobilisation among the NLMWOA. EDTA has a higher chemical affinity for Cu ($\log K_s = 18.3$) (Martell and Calvin, 1958), than citric acid, but 500-fold more citric acid (62.5 mM) than EDTA (0.125 mM) was applied thus outbidding the higher affinity and effectiveness of EDTA. Pb mobilisation by NLMWOA was very low (Figure 3-3b), as all of them have a low chemical affinity for Pb, e.g., citric acid ($\log K_s = 6.5$) (Luo et al., 2005). In the case of Pb, NLMWOA mobilised more Pb than EDTA with 8% and 1.8% respectively, but the difference was not as great as in the case of Cu, where NLMWOA mobilised approximately 60% whereas EDTA only approximately 2%. EDTA has a much higher affinity for Pb ($\log K_s = 18.2$) (Martell and Calvin, 1958) than the used NLMWOA so that the impact of higher applied NLMWOA amounts, as seen in the case of Cu, did not have such a great influence. Given these results a significantly higher Cu and Pb uptake by the NLMWOA treated plants would be expected in the phytoextraction experiment.

IV.1.3 Phytoextraction pot experiment

The analysis of plant material indicated that citric acid (62.5 mmol kg^{-1}) and EDTA (0.125 mmol kg^{-1}) treated soil significantly ($P < 0.05$) increased the concentrations of Cu in the shoots by 2.3 and 1.1-fold respectively in comparison to the control plants (Figure 3-4). The increase by the addition of EDTA and NLMWOA was not as high as stated by Schmidt (2003) and Gramss et al. (2004) respectively. Although tartaric acid showed, in the column experiment, a mobilisation potential equal to that of oxalic acid (Figure 3-3), it did not increase the Cu concentrations in the shoots (Figure 3-4), whereas oxalic acid increased the Cu concentration in the shoots in the same order of magnitude as EDTA. In the mobilisation experiments, the added NLMWOA were not able to enhance the Cu-uptake as much as expected. In the case of Pb the plant analysis revealed that the NLMWOA did not enhance the uptake, although NLMWOA treatments showed higher mobilisation capabilities in slurry (Figure 3-2b) and column experiments (Figure 3-3b) than EDTA. EDTA addition led to an approximately 3-fold increase of Pb concentration in the shoots (Figure 3-4). This though, is lower than that stated by Grčman et al. (2001). Given that the concentration of NLMWOA in the soil used in the phytoextraction experiment was 500 times higher than that of EDTA, the effect was of the NLMWOA were minimal.

The inefficiency may be owing to the biodegradation of the NLMWOA or the inability of the heavy metal-NLMWOA complexes to access the root and therefore into the shoots of the plant. The cause of the inefficiency owing to the inability of the heavy metal-NLMWOA complexes to access the plant is rejected, because as in the case of EDTA Grčman (2001), Senden et al. (1990) and Guo (1995) detected heavy metal-NLMWOA complexes which accessed the plant from the roots and were translocated via xylem to the shoots (Senden et al., 1990; Guo, 1995). Therefore, it is possible that the unexpected results arise from biodegradation. An indication of the biodegradation of the NLMWOA is reflected by the increase of the soil pH in the NLMWOA treated pots (Table 3-1).

IV.1.4 Biodegradation

IV.1.4.1 Influence of biodegradation on Cu bioavailability

The final pH of the soil in the phytoextraction pot experiment where NLMWOA were applied was approximately 7.7 (Table 3-1). An increase in the pH of the NLMWOA treated

soil is owing to the biodegradation of the NLMWOA. The microbial degradation of carboxylic acids (in this case NLMWOA) consumed H^+ liberated OH^- and CO_2 (Gramss et al., 2004), thus increasing the pH. The degradation of the NLMWOA results in the absence of complexing agents and a pH increase. The bioavailability of metals is among other factors (see chapter I.1.6) associated to the pH (Schmidt, 2003) and to the existence of chelating agents (Huang et al., 1998), thus an increase in pH and an absence of chelating agents could reduce the bioavailability of Cu and Pb. In contrast EDTA is characterised by persistence in the soil, (Meers et al., 2005) resulting in a very low biodegradation, as seen from a non significant increase in the pH of the EDTA treated pots (Table 3-1).

The biodegradation pot experiment revealed a direct influence of the degradation of the NLMWOA on the potential bioavailability of Cu. As the pH of the soil increased, the bioavailability of Cu decreased significantly in the NLMWOA treated pots, in comparison to the control pots (Figure 3-5). After approximately 4 d the biodegradation of the NLMWOA was complete. The pH reached its maximum after 4 d and subsequently remained constant. There were some fluctuations in the potential bioavailable Cu measurements (Figure 3-5), but these were slight and could have been owing to the changes in the humidity of the soil and the temperature in the greenhouse.

The oxitop system revealed a much more rapid degradation of the NLMWOA (Figure 3-6). The curves reached their plateau after 4 h instead of approximately 48-96 h as determined in the biodegradation pot experiment. The reason for this was probably the higher soil humidity of 50% as compared to only 25% in the biodegradation pot experiments. The difference in the humidity was due to practical reasons. The soil was mixed every day and a soil with 50% humidity does not allow an equal mixing. A higher humidity creates more preferable conditions for microbial growth. Moreover, the oxitop system is an air tight system, thus no air or humidity can escape from the system. Therefore, in contrast to the biodegradation pot experiment (water was added every 24 h to reach constant humidity, nevertheless a decrease in the humidity during this 24 h was unavoidable) the humidity of the soil in the oxitop system remained constant. The temperature of the system also remains constant, as it is stored in a thermoconstant chamber. The conditions, therefore, remain constant and the biodegradation is not influenced by changing environmental conditions, such as in a greenhouse.

The conditions in an oxitop system are constant so that the microorganisms do not have to adapt to changing humidity or temperature condition, thus revealing a high degradation rate under such favourable conditions. Similar results have been presented for EDDS, where an

oxitop similar system (sturm test) revealed half lives ranging from 2.5 to 4.6 d in a soil experiment (Schowanek et al., 1997), whereas Hauser et al. (2005), in a pot experiment, revealed half lives of over 7 weeks.

IV.1.4.2 Identification of microorganisms participating in biodegradation

The growth of microorganisms (bacteria, unicellular fungi e.g. yeasts, and mycelial fungi) is divided into different phases. During the first phase, the lag phase, microorganisms adapt themselves to the growth conditions. It is the period where the individual microorganisms are maturing and not yet able to divide, or when the cell division is very slow. During the exponential phase, the number of new microorganisms appearing per time unit is proportional to the present population. This gives rise to the classic exponential growth curve, in which the logarithm of the population density rises linearly with time (Figure 4-1). The actual rate of this growth depends upon the growth conditions, which affect the frequency of cell division events and the probability of both daughter cells surviving. This phase will continue until one or more nutrients become limiting, and/or metabolic by-products accumulate to toxic levels. This may be followed by a stationary phase, during which there is no discernible change in cell concentration or biomass. Finally, a phase of cell death and lysis may be observed, which results in a decrease in cell number and/or biomass (Schlegel, 1992; Antranikian, 2006).

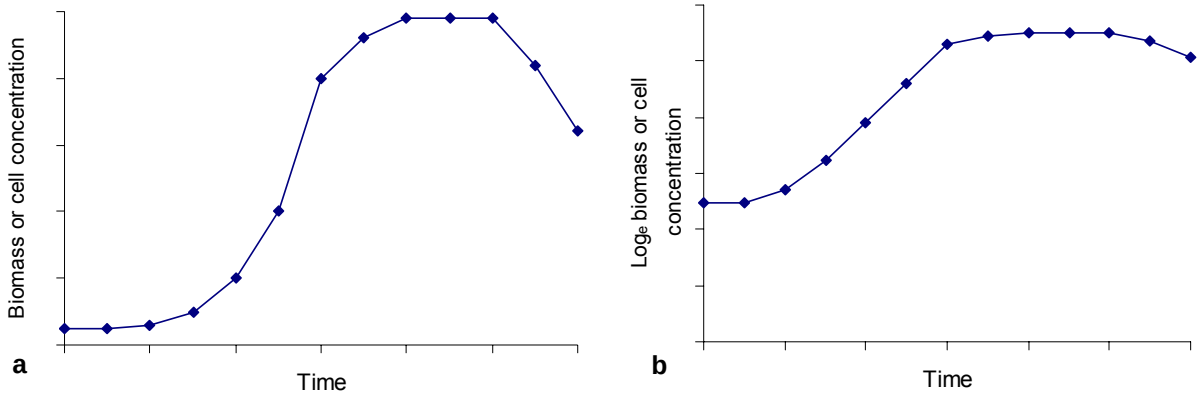


Figure 4-1: Growth kinetics of microorganisms a) characteristic s-shaped growth curve, b) $\log_e$ vs. time (modified from Schlegel, 1992)

The degradation rate of NLMWOA is very rapid and the bioavailability of Cu in NLMWOA treated pots is lower than that of the control pots. Thus the time period where the NLMWOA enhance the uptake of Cu and Pb into the plants is very short. To extend this short time period where NLMWOA enhance the uptake of heavy metals in plants, a successive

application of the three NLMWOA, citric acid, oxalic acid, and tartaric acid could be adopted. The successive application of citric acid, oxalic acid and tartaric acid on to the soil would create a new carbon source with each successive application. The success of the application technique requires the microorganisms which degrade the three applied NLMWOA, to be different. In the case that the microorganisms able to degrade the applied NLMWOA are not the same the degradation of the NLMWOA is slow, whereas if they are the same the degradation is very rapid. In the first case the microorganisms have to adapt to a changing carbon source, thus they are in a lag phase. Additionally, the microorganisms which can degrade the applied carbon source have to assert themselves over the other microorganisms which could degrade the previous carbon source, thus reducing the biodegradation rate. In the case in which the microorganisms which can degrade all three NLMWOA are the same, the degradation rate is very rapid because with each new applied NLMWOA the number microorganisms increases thus accelerating the degradation process.

The identification via agar-plates cultivation and additional sequencing revealed the bacteria *burkholderia sp.* and *ralstonia sp.* (uncultured bacteria) and the fungi *cordyceps sp.*, *paecilomyces sp.*, *basidiomyces sp.*, and *saccharomyces sp.*. Subsequently, the identified microorganisms were cultivated in batch liquid culture containing minimal medium and all three NLMWOA and, in an additional experiment, just oxalic acid. After several days of cultivation, HPLC measurements were performed to quantify the remaining NLMWOA in the medium. The HPLC measurements revealed that each of *cordyceps sp.*, *paecilomyces sp.*, and *burkholderia sp.* was able to degrade all three applied NLMWOA, whereas *ralstonia sp., basidiomyces sp.*, and *saccharomyces sp.* could only degrade citric acid and tartaric acid. A successive application of the three NLMWOA is thus ineffective, because the microorganisms *cordyceps sp.*, *paecilomyces sp.*, and *burkholderia sp.* would remain in the log phase which would result in a very rapid degradation of the three NLMWOA.

IV.1.5 Evaluation of the NLMWOA effectiveness

NLMWOA displayed a low phytotoxicity (Figure 3-1) in comparison to EDTA. This is in accordance with published studies. Huang et al. (1998a) observed no biomass decrease or other adverse effects on Indian mustard (*Brassica juncea*), after an application of 20 mmol kg^{-1} citric acid, in contrast to an application of 10 mmol kg^{-1} EDTA (Epstein et al., 1999). Citric acid, oxalic acid, and tartaric acid showed a high mobilisation capability (Figure 3-3) for Cu; this could not be verified, to this degree, in a phytoextraction experiment (Figure 3-4). This

may in part be owing to their high biodegradation rate, and perhaps also to the experimental plant chosen. Huang et al. (1998) showed that the effects of citrate additions on the uptake of heavy metals vary significantly amongst the plant species. Meers et al. (2005) used sunflower (*Helianthus annuus*) and showed that an addition of citrate (220 mmol kg^{-1}) did not increase the uptake of Zn, Cu, Cd, or Ni in comparison to the control. In experiments with Indian mustard (*Brassica juncea*), the addition of 20 mmol kg^{-1} citric acid induced a 3-fold increase of Cd concentration in the shoots (Quartacci et al. 2005). In the case of Pb, NLMWOA were inefficient in enhancing the uptake.

The enhanced uptake of Cu by the NLMWOA is probably due to the fact that Cu is more mobile in soil than Pb (Bliefert, 1995), as also seen in the column experiment where the 10 mM $CaCl_2$ solution mobilised 5-fold more Cu than Pb, although the soil contained 1.3-fold more Pb than Cu. Additionally, the NLMWOA, especially citric acid have a higher stability constant to Cu ($\log K_s$ = 14.24) (Martell and Calvin, 1958), than to Pb ($\log K_s$ = 6.5) (Luo et al., 2005). Cu was, therefore, extracted to a higher degree from the plants before the NLMWOA were degraded.

The high biodegradation rate of NLMWOA decreased the leaching probability of heavy metals to a minimum (Figure 3-5a). This is also verified by several authors. Gramss et al. (2004) observed a degradation of NLMWOA, accompanied by a concurrent increase of the soil pH by 0.5 to 1.3, similar to the pH increase of the soil in the phytoextraction experiment (Figure 3-5b). Experiments by Meers et al. (2005) showed that soils treated with 55 mmol kg^{-1} or 220 mmol kg^{-1} NH_4-citrate contained heavy metal concentrations in the soil solution at levels comparable to the control, 2 weeks after harvesting. An increase in the pH of the soil delays the remediation process, since the decreased bioavailability leads to a decreased uptake. A successive application of the three NLMWOA could not cope with this issue, since they are degraded by the same microorganisms.

Moreover, the amounts of NLMWOA which had to be applied to the soil before any effect was visible were too high, to make such a treatment feasible. In this aspect EDTA was much more efficient. Additionally, the soil used in the experiment was spiked with Cu and Pb. Therefore, the availability of the applied heavy metals is higher than in a naturally contaminated/aged soil. The process of metal binding on to the soil is a process that takes years to reach equilibrium between plant-/bioavailable and not plant-/bioavailable metals. In order to simulate an aged soil for phytoextraction experiments, the spiked soil passes a normal wet-dry cycle (3-5 weeks) (Blaylock et al., 1997), in order to accelerate the ageing of the soil. Although spiked soil passes a wet-dry cycle it has nevertheless a higher bioavailable heavy

metal fraction than a comparable naturally contaminated/aged soil. It is thus unlikely that the technique will be successful under field conditions. These findings make a continuous application of NLMWOA unsuitable for a clean up over several years.

IV.2 Cyclodextrins (CyD)

Cyclodextrins (CyD) are cyclic oligomers of α-D-glucose formed by the action of certain enzymes on starch. The glucose units are connected through glycosidic α-1,4 bonds. These lampshade-shaped molecules have a hydrophobic, nonpolar interior and a hydrophilic, polar exterior. The addition of β-CyD into the soil could therefore enhance the uptake of heavy metals into the plant.

The objective of this study was to investigate the ability of β-CyD in enhancing the phytoextraction of Cu and Cd from soil by the use of tobacco plants (*Nicotiana tabacum* SR-1) under laboratory conditions. The potential of β-CyD was assessed in replacing compounds such as EDTA or other synthetic chelators as enhancing agents for the phytoremediation of heavy metal contaminated soils. Current research has so far not applied β-CyD on soil in order to enhance the phytoextraction of heavy metals.

Phytotoxicity experiments were performed to determine the highest no effect concentration on tobacco (*Nicotiana tabacum* SR-1). Based on the results of the phytotoxicity experiment, the Cu and Cd mobilisation potential of β-CyD and EDTA applied in corresponding amounts, was determined in column experiments. Phytoextraction experiments were not performed because the Cu and Cd amounts mobilised by β-CyD in the column experiment were too low and the highest no effect concentration of β-CyD determined in the phytotoxic experiment was also very low. Biodegradation experiments were performed in order to assess a leaching potential by the application of β-CyD.

IV.2.1 Phytotoxicity pot experiment

The application of β-CyD to the soil adversely affected dry matter production of the plants at a concentration of 3.92 mmol kg^{-1} (Figure 3-6). At a concentration of 7.83 mmol kg^{-1} β-CyD the dry weight is only slightly lower, but the standard deviation is higher in comparison to the 3.92 mmol kg^{-1} application. The following experiments were adjusted to the concentration of 3.92 mmol kg^{-1}. The phytotoxicity of β-CyD to other plants has not been previously published, thus a comparison to tobacco (*Nicotiana tabacum* SR-1) is not possible.

IV.2.2 Column experiment

The column experiment displayed a low potential of β-CyD to mobilise Cu and Cd. The Cu mobilised by β-CyD, approximately 1.4%, was to a significant extent higher than by $CaCl_2$. Cd, on the other hand, was significantly less mobilised by β-CyD than by the control. Both β-CyD and $CaCl_2$ average out at the same order of magnitude. The amounts of Cu and Cd mobilised by β-CyD were too small to enhance the phytoextraction of these two metals substantially. The reason for the inefficiency of β-CyD to mobilise Cu and Cd is based on the conditions required for the formation of β-CyD-heavy metal complexes. The authors Norkus et al. (2002, 2003, 2005) discovered that β-CyD-Pb(II), -Cd(II), -Cu(II) complexes are formed at a pH > 10. This pH-value exceeds that of the experiment by more than 3.2, and does not allow the growth of plants. A formation of β-CyD-heavy metal complexes only in alkaline conditions is owing to the pKa value of β-CyD. The pKa of β-CyD is 12.2 (Hamai, 1996), thus β-CyD is protonated in acidic and neutral conditions and therefore a β-CyD-heavy metal complex can be formed in alkaline conditions where β-CyD is deprotonated. The natural pH of soil ranges from slightly acidic to neutral, a range where β-CyD cannot from complexes, thus β-CyD is not suitable for chelate enhanced phytoextraction.

IV.2.3 Biodegradation

Irrespective of the β-CyD concentrations added to the soil, the potentially bioavailable Cu concentration in the soil, as determined by DTPA extraction, was not affected significantly by the application of β-CyD (Figure 3-8). During the degradation of the β-CyD the potentially bioavailable Cu decreased significantly. The reason for this decrease could be the increase in pH as the solubility of heavy metals in soils is connected to the pH of the soil (Schmidt, 2003). At the beginning of the experiment the pH value of the control soil and the soil containing β-CyD are equal, but during the experiment the pH of the β-CyD treated soil rose and surpassed the pH of the control by approximately 0.45. The rapid microbial degradation of the β-CyD, presumably in the first 48 h, via carboxylic acid intermediates resulted, therefore, in alkalisation of the soil (Gramss et al., 2003), and thus, in a decrease in the bioavailability of Cu in soil.

IV.2.4 Evaluation of the CyD effectiveness

As the β-CyD have low water solubility, they have to be applied to the soil before planting, because otherwise an equal distribution cannot be guaranteed if β-CyD are applied after planting. β-CyD showed a high toxicity towards plants, and, as a result a planting directly after the application of β-CyD is not suitable. Simultaneously, they also display a high degree of biodegradability, so that their effectiveness decreases rapidly. Additionally, they demonstrated a poor capability of mobilising heavy metals from the soil. This was also demonstrated in the case of Ni by Khodadoust et al. (2004). However, modified CyD such as 0.5% carboxymethyl-*β*-cyclodextrin + 0.5% hydroxypropyl-*β*-cyclodextrin, have shown a high mobilisation capability (Brusseau et al., 1997). These though, have an even higher price of purchase than the β-CyD making them unsuitable for chelate enhanced phytoextraction. These results, in addition to the high purchase costs, show that β-CyD are unsuitable for enhanced phytoextraction of heavy metals from the soil.

IV.3 Ethylene diamine disuccinate (EDDS)

EDDS is a naturally occurring chelator, which is produced naturally by a number of microorganisms. The S,S-stereoisomer is produced naturally and is biodegradable, whereas the technically produced (R,R, R,S, S,R)-stereoisomers are non-biodegradable or only partially degradable. The S,S-stereoisomer was used in this study. EDDS has shown to have I high chemical affinity for Cu and Cd, therefore could the addition of EDDS to the soil enhance the uptake of Cd and Cu into the plant.

The objective of this study was to investigate the ability of EDDS in enhancing the phytoextraction of Cu and Cd from soil by the use of tobacco plants (*Nicotiana tabacum* SR-1) under laboratory conditions. EDTA was also tested in order to have a direct comparison to the EDDS and thus to assess the potential of EDDS in replacing compounds such as EDTA or other synthetic chelators as enhancing agents for the phytoremediation of heavy metal contaminated soils. Current research has neglected to focus on the effects of the applied EDDS after harvesting. Therefore, tobacco plants were planted in a follow up experiment to observe the phytotoxic effects of EDDS on new planted seedlings and the bioavailability of Cu and Cd was determined in order to assess a leaching potential.

Phytotoxicity experiments were performed to determine the highest no effect concentration on tobacco (*Nicotiana tabacum* SR-1). Based on the results of the phytotoxicity

experiment, the Cu and Cd mobilisation potential of EDDS and EDTA applied in corresponding amounts, was determined in column experiments. Phytoextraction experiments were performed applying EDDS and EDTA according to the determined highest no effect concentration. The translocation factor (TF) was calculated in order to assess the effectiveness of the chelating agents EDDS and EDTA to transport Cu and Cd from roots to shoots. The phytoextraction experiment was followed by soil analysis and a biodegradation experiment in order to assess a leaching potential by the application of EDDS. The soil of the phytoextraction experiment was replanted with new tobacco seedlings to examine any possible phytotoxic symptoms from the nondegraded EDTA and EDDS in the soil, thus allowing an evaluation of the possibility to continue the remediation of the soil.

IV.3.1 Phytotoxicity pot experiment

The phytotoxicity experiments depicted a similar effect of EDDS and EDTA with respect to their toxicity to plants. As reported by Chen and Cutright (2001), EDTA showed a very high toxicity (Figure 3-10), and plant growth decreased at a concentration of 12.5 mmol kg^{-1} in the soil. The cause of this toxicity could be an EDTA-induced foliar necrosis due to the presence of free protonated EDTA in the leaves. It has been predicated that uncoordinated EDTA would bind various essential divalent cations, including Fe^{2+}, Zn^{2+}, and Cu^{2+}, disrupting the biochemistry of the leaf cells, and ultimately causing cell death (Vassil et al., 1998). The dry weight of the plants after EDTA treatment is higher than the control because of higher amounts of mobilised nutrients made available to the plants and the absence of phytotoxic effects at these EDTA concentrations.

EDDS showed a similar toxicity towards tobacco (*Nicotiana tabacum* SR-1) with respect to the dry weight, and an even higher toxicity, with respect to visible symptoms, such as necrosis and chlorosis, compared to EDTA. With the exception of 1.5 mmol kg^{-1} EDDS, chlorosis and necrosis were visible at all levels. EDDS displayed a similar degree of toxicity towards sunflower (*Helianthus annuus*) (Meers et al., 2005) and a higher one towards corn (*Zea mays*) and white bean (*Phaseolus vulgaris*) (Luo et al., 2005) compared to tobacco (*Nicotiana tabacum*). Meers et al. (2005) applied 1.77 mmol kg^{-1} EDDS and observed no toxicity symptoms, whereas Luo et al. (2005) applied 5 mmol kg^{-1} EDDS and the dry weight decreased by approximately 55% in comparison to the control. With respect to EDTA, it showed a similar degree of toxicity towards Indian mustard (Brassica juncea) (Epstein et al., 1999; Wu et al., 2004) and sunflower (*Helianthus annuus*) (Meers et al., 2005). As with

EDDS, this was not the case with tobacco (*Nicotiana tabacum*). The degree of toxicity is in concurrence with present literature. In this respect tobacco (*Nicotiana tabacum*) is no more susceptible to these to chelating agents than other plant species.

Although EDTA did not show such symptoms at concentrations of 1.5, 3.125 and 6.25 mmol kg^{-1}, the concentration of 1.5 mmol kg^{-1} was chosen for the phytoextraction and mobilisation (column) experiments, because the highest no effect concentration of EDDS was 1.5 mmol kg^{-1} and a direct comparison of the efficiency of EDDS and EDTA to enhance the phytoextraction of Cu and Cd was desired, without causing toxicity symptoms to the plant due to the application of chelating agents.

IV.3.2 Column experiment

According to Bliefert (1995), Cu is more tightly bound to the soil than Cd. The column experiment supports this classification. The $CaCl_2$ solution could mobilise in total approximately 0.4% Cu and 3% Cd of the initial amount of the 2 metals in the soil (Figure 3-11). Cu mobilisation by EDDS, was significantly higher ($P < 0.05$) than the Cu amounts mobilised by EDTA (Figure 3-11a) although both have an equal chemical affinity for Cu (EDDS-Cu $logK_s$ = 18.45 and EDTA-Cu $logK_s$ = 18.86), and thus an equal Cu mobilisation would be expected (Whitburn et al., 1999). It is probable that other heavy metals in the soil, for instance Pb, for which EDTA has a high chemical affinity, decreased the effectiveness of EDTA. Cd mobilisation by EDTA and EDDS was higher than that of Cu (Figure 3-11b), and equal amounts were mobilised by both chelating agents. As EDTA and EDDS have a different chemical affinity for Cd, $logK_s$ = 16.62 and logKs = 10.8, respectively (Whitburn et al., 1999), a high mobilisation by EDTA would be expected. However, the comparison of stability constants data needs to be considered with caution. Speciation in a metal-chelator system is controlled by the concentration of all metals and chelators, the stability constants of all complexes and by the kinetic of coordination reactions. In addition, other chelator reactions, adsorption in the soil solid phases, mineral dissolution and chelator degradation are substantially affected by the chelated metal ion (Nowack, 2002).

Owing to these results a significantly higher Cu uptake by EDDS treated plants and an equal uptake in the case of Cd might be expected in the phytoextraction pot experiment.

IV.3.3 Phytoextraction pot experiment

The dry weights of the plants in the pot experiment (Figure 3-12) were of the same order of magnitude in all samples, irrespective of both the heavy metals soil content and the chelating agent application. No adverse effects concerning the dry weight of the plants were observed. The absolute uptake of heavy metals is, therefore, in direct proportion to the heavy metal concentration in the plants. The analysis of plant material indicated that soil containing 450 mg kg^{-1} Cu, which was treated with either EDDS or EDTA ($P < 0.05$), increased the concentration of Cu in the shoots by 7 and 12-fold respectively in comparison to the control plants (Figure 3-13a). The increase is higher in comparison to the increase stated by Kos and Lestan (2004) and Meers et al. (2005) who, however, used natural multicontaminated soil. However, the increase of Cu uptake by the addition of EDDS in the control is 4-fold and, thus in the same order of magnitude as the increase stated by Kos and Lestan (2004) and Meers et al. (2005). The control has not been spiked and has a moderate concentration of Cu (Table 2-1) thus is can be used to compare it with studies where natural contaminated soil was used. Luo et al. (2006) and Ultra et al. (2005) also used a spiked soil, but they observed a much lower increase compared to this thesis. A higher uptake would have been expected because the bioavailable metal fraction in spiked soils is higher. Multicontaminated soil contains several different metal species with various chemical affinities for the chelating agent, which compete for a limited number of chemical sites of the chelating agent for complexation. Furthermore, spiked soil usually has a higher metal bioavailability as the binding of metals to the soil is process of long duration.

The Cu concentration in the roots was enhanced by the addition of EDDS and EDTA in comparison to the control, and was higher than the concentration in the shoots. This indicates that the translocation of the heavy metals is very low. The largest part of the heavy metal is therefore not, in the harvestable part of the plant. Consequently, the extraction effectiveness is low.

Cd uptake in the shoots, however, was not enhanced by the application of EDDS and EDTA (Figure 3-13b). This result is at variance with the work of Lai and Chen (2004) and Luo et al. (2005), in which a significantly enhanced uptake was observed in the case of Cd. It is, though, in agreement with Meers et al. (2005) and Tandy et al. (2006), who did not notice an enhanced uptake. In the case of Cd EDTA enhanced the uptake in the roots, but the Cd concentration in the shoots was only slightly higher than in the roots.

Lombi et al. (2001) and Heiss et al. (2003) stressed that in evaluating a chelator´s effectiveness at mobilising the metals to the root zone, the extent of translocation to the shoots

should be determined. If, for instance, the metal concentrations surrounding the roots rose quickly, the plant could exhibit toxic symptoms of damaged roots and growth inhibition. The translocation factor (TF) is defined as the ratio of metal(loid) concentration in shoots to that in roots. It can be used to evaluate the capacity of a plant to transfer metals from roots to shoots (TF is usually >1 (or ≥1) in (hyper)accumulators and <1 in excluders (McGrath and Zhao, 2003). The translocation factors for the heavy metals Cd and Cu and the applied chelating agents EDTA and EDDS are depicted in Table 4-1.

Table 4-1: Effects of the chelates (EDDS, EDTA) on the translocation of Cu and Cd from roots to shoots of tobacco (*Nicotiana tabacum* SR-1) 14 d after treatment. The soil was spiked with 0, 5, 10 and 15 mg kg^{-1} Cd and 0, 150, 300, 450 mg kg^{-1} Cu.

Cd (mg kg^{-1})	TF			Cu (mg kg^{-1})	TF		
	Control	EDDS	EDTA		Control	EDDS	EDTA
0	0.61±0.16	0.83±0.18	0.35±0.09	0	0.17±0.01	0.24±0.05	0.24±0.00
5	2.06±0.39	1.89±0.32	1.41±0.04	150	0.13±0.00	0.21±0.13	0.16±0.09
10	2.64±0.26	2.18±0.57	1.23±0.07	300	0.11±0.02	0.09±0.05	0.07±0.01
15	2.93±0.34	1.86±0.66	1.43±0.37	450	0.08±0.01	0.10±0.03	0.19±0.01

The translocation factors (TF) of the control plants for Cd were always higher (TF: 2.06, 2.64 and 2.93) compared to the TF of the EDDS (1.89, 2.18, 1.86) and the EDTA (1.41, 1.23, 1.43) application. Only in the case of the non Cd spiked soil (TF: 0.61) did the EDDS application increase the TF (0.83), but it was not significant. This shows that the plant could not transport Cd from the roots to the shoots. The uptake into the root was higher than the translocation to the shoots. Tandy et al. (2006) observed that the translocation could be assisted by the application of EDDS. Santos et al. (2006) observed that through the addition of EDDS the Cd concentration in the roots could be reduced in comparison to the control. Both these statements are not in agreement with the findings of this study as the TF was not higher than that of the control and the Cd concentration in the roots was not lower compared to that of the control (Figure 3-13). In the case of Cu the translocation factors increased through the addition of the chelating agents, though not significantly. This is not in agreement with Luo et al. (2005), who claimed that after the application of either EDTA or EDDS, the translocation factors for both Cd and Cu increased substantially.

Tobacco has not previously been used in a phytoextraction experiment with the addition of EDDS as a chelating agent. In the study of Meers et al. (2005) the authors used sunflower (*Helianthus annuus*) and the study is comparable to the control of this study as both soils have approximately the same Cu concentration in the soil and similar amounts of EDDS were applied. The enhancement effect owing to the addition of EDDS was similar but tobacco reached a higher Cu concentration in the shoots. In a study performed by Keller et al. (2003), tobacco displayed a lower Cu and Cd concentrations in the shoots compared to maize (*Zea mays*), and Indian mustard but revealed a higher TF.

IV.3.4 Soil analysis of phytoextraction pot experiment

The soil samples for the NH_4NO_3-extraction (plant available heavy metals) were taken 14 d after the application of EDDS and EDTA. Due to the application of EDDS and EDTA, the plant available Cu (Figure 3-14) was significantly ($P < 0.01$) increased by 25 and 20-fold respectively, as compared to the control plants (Figure 3-14), even after the first step in the remediation process was completed. In the case of Cd only EDTA increased the plant available Cd 8-fold. Thus significantly more heavy metals were plant available, indicating the hazard of leaching, even for the biodegradable EDDS, which is in agreement with Meers et al. (2005). The authors observed a higher leaching probability in the case of Cu, but not for Cd, 20 d after the application of EDDS. With respect to the application of EDTA a higher Cu and Cd bioavailability and thus leaching probability was observed. Kos and Lestan (2004) also observed that more Cu was leached than extracted by cabbage (*Brassica rapa*) after the addition of EDDS. The findings of Grčman et al. (2003) are also in agreement with this study who for the case of Cd observed an increased leaching after the application of EDTA, while no leaching could be detected after a treatment with 5 mmol kg^{-1} EDDS.

Only a limited fraction of the mobilised Cu was effectively absorbed and translocated by the plant: after an EDDS application approximately 0.55% of the mobilised Cu was recovered in the shoots, while for the EDTA treatment it amounts to approximately 1.3%. This is in agreement with Meers et al. (2005) who observed a similar percentage of uptake in comparison to the mobilised Cu in soil. The high leaching probability in this study cannot be explained by a varying pH level, as this remained constant to that of a natural soil.

The observed high plant available heavy metal fraction in the soil after harvesting cannot be explained by a varying pH level, as this remained constant at that of the natural soil. An

explanation could be the low biodegradability of EDDS and EDTA, which kept the heavy metals Cu and Cd mobilised, even after harvesting of the plants.

IV.3.5 Biodegradation

To observe the biodegradation and the phytotoxic effect of the chelating agents, new seedlings were placed into the control pots one week after harvesting. Those seedlings placed in EDDS treated pots displayed chlorosis, necrosis and lower dry weight indicating a lack of biodegradation. The pots did not contain any spiked soil, thus the toxicity could only derive from the EDDS in the soil. By comparison, though, no toxic symptoms were observed in seedlings placed in EDTA treated control pots (Figure 3-16). This probably reflects the different application levels. In the case of EDDS the highest application level at which the plants did not show any toxicity effects, was chosen. EDTA, on the other hand, was applied in a two fold lower concentration than the highest non toxicity symptoms concentrations. This is the first time that the toxicity and biodegradation of chelating agents with a following planting after the phytoextraction experiment was observed. Apart from the increased heavy metal leaching probability, the lack of biodegradation has also a direct effect on the following phytoextraction procedure. If the new plants had been germinated directly in the pots, the plants would probably not have survived. This means that even if the heavy metal contamination is low, toxicity could result directly from the applied chelating agents. Although the time frame between harvesting and replanting is very short, with this follow up experiment the author wants to stress out that it is not enough to just perform one experiment to evaluate the effectiveness or phytotoxicity of a chelating agent. A new difficulty in the area of chelate assisted phytoextraction has therefore to be tackled. Follow up experiments have to be performed to evaluate a possible continuous planting without occurring toxicity owing to the added chelating agents. A reduction of the amounts of chelating agents added would though result in a reduced effectiveness in the enhancement of the phytoextraction of heavy metals.

IV.3.6 Evaluation of the EDDS effectiveness

EDDS displayed a high degree of phytotoxicity, higher than that of EDTA (Figure 3-10). The phytotoxicity depends on the plant studied. In Luo et al. (2005) the application of 5 mmol kg^{-1} EDTA and EDDS caused toxicity symptoms to corn (*Zea mays*) and white bean

(*Phaseolus vulgaris*), but in contrast to this study they were more severe in the case of EDTA. The results of the study showed that the addition of EDDS and EDTA (1.5 mmol kg^{-1}) had a positive effect on Cu bioavailability, and enhanced the Cu uptake 7 and 12- fold respectively. This was higher than the uptake observed by Meers et al. (2005), who observed an increase by 4.1-fold by the application of EDDS and 1.5-fold by EDTA. Meers et al. (2005) used sunflower (*Helianthus annuus*) as a phytoextraction plant, which could be the reason for this vast difference in uptake because, as observed by Huang et al. (1998), not all plants react with the same uptake enhancement by the application of a chelating agent. A reason could also be the use of aged multicontaminated soil by Meers et al. (2005), which has a lower bioavailable metal fraction than spiked soil, as used in this thesis. In contrast to Cu, the Cd uptake was not affected by the addition of EDDS and not increased significantly by the addition of EDTA. The addition of EDDS and EDTA failed to increase the translocation factor (TF) (concentration of metals in shoots divided by concentration of metals in roots). This is contrary to Luo et al. (2005) and Tandy et al. (2006) who observed an increase in the TF by the application of EDDS to the soil. Therefore, the highest metal concentration was in the roots, the not harvestable part of the plant, thus making the addition of EDDS not feasible. Furthermore, the biodegradation was very low, with the result that a high leaching risk was imminent.

In conclusion, therefore, EDTA and EDDS are not suitable as chelating agents for the enhancement of phytoextraction. The reasons are as follows: a) the translocation factor was not enhanced substantially by neither EDTA nor EDDS, b) both EDTA and EDDS displayed a propensity to the leaching of heavy metals, as the significantly elevated bioavailable Cd and Cu levels showed, c) 14 d after the addition of EDDS there was no sign of degradation, which caused toxicity effects to the following plant generation. The variance of these results with literature underlines the importance of assessing each case separately, according to soil, plant and chelating agents.

IV.4 Hydrolysed wool

The natural fiber wool, like other types of fine and coarse animal hair and also silks, is an animal fiber, which consists mainly (ca. 97%) of wool proteins, the remainder being made up of ca. 2% structural lipids, ca. 1% mineral salts, nucleic acids and carbohydrates (Zahn, 2001). Proteins and free amino acids have the ability to complex heavy metals (Martell and Calvin, 1958). The addition of hydrolysed wool into the soil could therefore enhance the uptake of heavy metals into the plant.

The objective of this study was to investigate the ability of hydrolysed wool in enhancing the phytoextraction of Cu and Cd from soil by the use of tobacco plants (*Nicotiana tabacum* SR-1) under laboratory conditions. The wool was hydrolysed in two different time periods (7 d and 14 d), in order to observe the influence of the hydrolysation time on the heavy metal mobilisation and phytoextraction efficiency.

The Cu and Cd mobilisation potential of hydrolysed wool in the two hydrolysation time-periods (7 d and 14 d), was determined in column experiments. Phytoextraction experiments were performed applying hydrolysed wool in the following amounts: in the case of Cu hydrolysed wool 7 d, 6.6 g kg^{-1} and 14 d, 12.2 g kg^{-1} and in the case of Cd 7 d, 6.6 g kg^{-1}. The phytoextraction experiment was followed by soil analysis experiments determining the DTPA-extractable (potentially bioavailable) and NH_4NO_3-extractable (plant available) metal fraction in order to assess a leaching potential by the application of hydrolysed wool. A biodegradation experiment was performed to observe any toxicity to soil microorganisms by the application of hydrolysed wool and to verify the results of the soil analysis, because a fast degradation is connected to a low leaching probability.

IV.4.1 Column experiment

The column experiment depicted a high mobilisation capability of hydrolysed wool for Cu. Both, the 7 d and the 14 d wool hydrolysate were significantly ($P < 0.05$) more effective in mobilising Cu than the control. Additionally, between the 7 d and the 14 d wool hydrolysate a significant difference was visible. The Cu amounts mobilised by the 14 d hydrolysate, were significantly ($P < 0.05$) higher than the Cu amounts extracted 7 d hydrolysate (Figure 3-17a). However, in the case of Cd, only the 14 d wool hydrolysate was significantly ($P < 0.005$) more effective than the $CaCl_2$ solution. The amount of Cd extracted by the 7 d wool hydrolysate and the $CaCl_2$ solution ranged in the same order of magnitude.

The difference in the effectiveness of the 14 d wool hydrolysate to mobilise Cu or Cd probably lies in the different chemical affinity of amino acids towards Cu and Cd. As an example, asparagine has a $\log K_s = 8.05$ (Boraei et al., 1996) for Cu and $\log K_s = 4.11$ (Ahmed et al., 1996) for Cd and glutamic acid has a $\log K_s = 8.50$ (Boraei et al., 1996) for Cu and $\log K_s = 4.39$ for Cd (Martell and Calvin, 1958). The stability constant ($\log K_s$) describes the chemical affinity of a molecule to a metal ion. Owing to its logarithmic scale a small difference in the $\log K_s$ value results in a great difference regarding the chemical affinity. The difference between the stability constants for Cu and Cd differs by approximately a $\log K_s$ of 4. Therefore, the chemical affinity of the amino acids to Cd is much lower compared to the

chemical affinity to Cu, resulting in a significant difference concerning the capability of amino acids to mobilise Cu and Cd.

The difference between the 7 d and the 14 d wool hydrolysate concerning their mobilisation capability of Cu and Cd, may also reflect the different amount of free amino acids, or small peptides in the solutions. The authors Martell and Calvin (1958) showed that the metal stability decreases with increasing length of the peptides. Glycine has a $\log K_s = 8.62$ for Cu, while the tripeptide GlycineGlycineGlycine has a $\log K_s = 5.4$. The 14 d wool hydrolysate contains smaller peptides than the 7 d wool hydrolysate, thus more molecules with a high chemical affinity to a metal are present resulting in a higher metal mobilisation capability. This does not apply to all peptides as it depends on the amino acids, from which they are composed. With varying amino acids, the functional groups and the sterical stability vary, thus influencing the stability constant for a metal.

Given the results of the column experiment, it is possible to make predictions about the uptake in the subsequent pot experiments. The Cu uptake should be enhanced significantly, by the addition of the 14 d wool hydrolysate and to a lesser extent by the 7 d wool hydrolysate.

IV.4.2 Phytoextraction pot experiment

IV.4.2.1 Phytoextraction of Cd (6.6 g kg^{-1}, 7 d wool hydrolysate)

The dry matter yields of the shoots revealed no adverse effects after the application of 6.6 g kg^{-1} 7 d wool hydrolysate on the plants. Additionally, no toxicity symptoms, such as chlorosis and necrosis were visible. The analysis of plant material indicated that the 7 d wool hydrolysate (6.6 g kg^{-1}) treated soil increased significantly ($P < 0.05$) both the concentration and the total amount of Cd in the shoots as compared to the control plants (Figure 3-19) at the highest spiked Cd concentration in the soil (30 mg kg^{-1}). At the other spiked Cd soil concentrations the increase was not significant.

Regarding the potential of the 7 d wool hydrolysate, as shown in the column experiments, the uptake was more enhanced than expected. This may be owing to the fact that the control solution ($CaCl_2$-solution) used in the column experiments display a higher mobilisation of heavy metals than the actual uptake of heavy metals by the control plants, thus overestimating the mobilisation by the control. Therefore, the difference between the uptake by control and the chelating agent application in the pot experiment is greater than one might expect from the results of the column experiment.

IV.4.2.2 Phytoextraction of Cu (6.6 g kg^{-1}, 7 d wool hydrolysate)

In the highest spiked Cu soil concentration (600 mg kg^{-1}), the analysis of plant material revealed an increase of 70% in the 7 d wool hydrolysate (6.6 g kg^{-1}) treated pots in comparison to the control (Figure 3-21). In other spiked Cu soil concentrations the increase was not as prominent. The application of the 6.6 g kg^{-1} 7 d wool hydrolysate revealed no adverse effects on the dry matter yields of the shoots. The enhanced Cu concentration in the shoots is thus proportional to the total amount of Cu taken up.

The potential of the 7 d wool hydrolysate, as shown in the column experiments, was not verified in the phytoextraction pot experiment. A much higher uptake by the plants was expected. This could be due to the rapid degradation of the small peptides and amino acids, which are the effective molecules for the enhanced uptake. Therefore, if the amount of small peptides is increased, the uptake has to be enhanced. Indication for this hypothesis are given in the column experiment where the 14 h wool hydrolysate, which contained more small peptides than the 7 d hydrolysate, mobilised significantly more Cu and Cd than the 7 d wool hydrolysate. Nevertheless, since the plants did not show any toxicity symptoms, recurring application of the extract would be possible.

IV.4.2.3 Phytoextraction of Cu (12.2 g kg^{-1}, 14 d wool hydrolysate)

The application of the 14 d wool hydrolysate (12.2 g kg^{-1}) treated pots revealed a significant ($P < 0.001$) Cu extraction enhancement. The uptake was enhanced approximately 850% in comparison to the control (Figure 3-23). This increase in Cu uptake is higher than the increase achieved by the natural chelating agents NTA (Kayser et al., 2000; Wenger et al., 2002), citric acid (Wu et al., 2004) and the synthetic chelating agent HEIDA (Chiu et al., 2005) who achieved an increase of 2-fold (NTA), no significant increase (citric acid) and 3-fold (HEIDA).

The amounts of wool extract applied as well as the hydrolysis duration of the wool was doubled in comparison to the previous phytoextraction pot experiment with Cu, leading to a remarkably increased uptake. The 14 d wool hydrolysate contained more small peptides than the 7 d hydrolysate, thus increasing its efficiency in enhancing the uptake of Cu. Cu is probably taken up in form of amino acid-Cu ion complexes. These amino acid-Cu ion complexes possibly passed the intact plasma membrane of the roots. Kinraide (1981) as well

as Persson and Näsholm (2001) have proven that intact amino acids are taken up by plant roots and that the root has specific channels for specific amino acids. The enhancement of the uptake is however so high that is likely that the amino acid-Cu ion complexes were to some extent taken up through a damaged plasma membrane caused by the high amounts of hydrolysed wool applied. The plasma membrane surrounding the root cells is thought to play a major role in forming the root barrier. The damage of the plasma membrane resulted in a high Cu uptake by the plant. The plasma membrane is normally stabilised by Ca^{2+} and Zn^{2+} ions (Pasternak, 1987; Kaszuba and Hunt, 1990), therefore the amino acids and peptides similar to the NLMWOA may induce metal-chelate uptake and accumulation by removal of stabilising Zn^{2+} and Ca^{2+} from the plasma membrane. This damage may confer access of random metal complexes of soil-solution to root xylem and to the shoot via the transpiration stream (Vassil et al., 1998), thus causing chlorosis and necrosis to the plant. In this case the chlorotic and necrotic plant symptoms are probably caused by fungi that could pass the damaged membrane which surrounds the root thus. The potential of the 14 d wool hydrolysate, as shown in the column experiments was verified in the phytoextraction pot experiment.

IV.4.3 Soil analysis of phytoextraction pot experiment

The soil samples for the pH measurement, NH_4NO_3-extraction (plant available heavy metals), and DTPA-extraction (potentially bioavailable heavy metals) were taken 6 d after the application of hydrolysed wool. Three d after the application of the hydrolysed wool the plants were harvested, thus the soil samples were taken 3 d after harvesting.

The pH-values of the soil in the hydrolysed wool treated pots exceeded the natural pH of the soil by only 0.1 in all phytoextraction pot experiments. The pH of the control soil was lower than the pH of the native soil, probably owing to the application of fertilisers and heavy metals (Figure 3-27). As shown in Figures 3-27 and 3-30, the soil pH decreases with increasing spiked Cu concentration in soil. If $CuCl_2$ and 3 $CdSO_4$ 8 H_2O are dissolved in an aqueous solution the pH of the solution decreases because these salt react acidic. When applied to the soil, the salts are dissolved in the water contained in the soil, thus reducing the soil pH. The soil pH was not affected by the application of Cd (Figure 3-24), probably because the amounts applied were too low (10-30 mg kg^{-1}), whereas the soil pH decreased by the amounts applied in the case of Cu (200-600 mg kg^{-1}).

The application of hydrolysed wool did not have an effect on the fraction of the DTPA-extractable heavy metals in the soil. DTPA is a very strong complexing agent with a logKs of 21.2 (Martell and Calvin, 1958) for Cu. Owing to this high chemical affinity to Cu, DTPA mobilised Cu fractions in soil, which were not available to the amino acids. Only a small fraction of the heavy metals in soil are water soluble or plant available. When higher amounts of Cu is mobilised by a strong chelating agent such as DTPA, smaller variations in the plant availability are not detectable.

The NH_4NO_3-extractable metals correspond to the plant available metal fraction in soil. This fraction in the soil was not uniformly affected by the application of hydrolysed wool. At a Cd soil concentration of 20 mg kg^{-1} the plant available Cd fraction was decreased, whereas at 30 mg kg^{-1} it was increased. Owing to the degradation of the hydrolysed wool, which was probably not the same in all the pots, the plant available Cd fraction varied. The plant available Cu (Figure 3-29) was not significantly increased in the 7 d hydrolysate (6.6 g kg^{-1}) application, but significantly ($P < 0.01$) enhanced by the 14 d hydrolysate (12.2 g kg^{-1}) application, as compared to the control plants (Figure 3-32). The enhancement by the 14 d wool hydrolysate ranged from 3-fold to insignificant, according to the spiked Cu soil concentration. The maximum 3-fold increase of the plant available Cu fraction is very low compared to the increased uptake of 8.5-fold measured in the phytoextraction experiment (see III.4.2.3). This increase in plant available Cu is also very low compared to the mobilisation of Cu by other chelating agents, which had a similar uptake enhancement, e.g. EDTA increased the plant available Cu fraction by 30-fold (Wu et al., 2004), NTA by 20-fold (Wenger et al., 2002) and HEIDA by 300-fold (Chiu et al., 2005).

The pH-change in soil is an indication of the biodegradation of the added chelating agent. The biodegradation of the wool hydrolysate did not affect the pH level; however, biodegradation did occur, because 6 d after the application of hydrolysed wool the enhanced mobilisation of Cu (seen by the enhanced Cu uptake in tobacco) decreased to a level where no significant enhanced availability of Cu was visible (except for the NH_4NO_3-extractable fraction in the 14 d hydrolysate (12.2 g kg^{-1}) application).

The biodegradation experiment, which was performed in the oxitop apparatus, displayed a rapid degradation of the wool hydrolysate (Figure 3-33). The respiration increased rapidly, it surpassed the basal respiration after 10 h and started reaching a plateau after approximately 40 h. Therefore hydrolysed wool must have been degraded in approximately 40 h. In the phytoextraction experiment the biodegradation rate was probably lower (approximately 4 d) because the conditions in the greenhouse are not constant as in the oxitop system, thus

mimicking shorter half lives. Similar observations have been made in the case of EDDS. Schowanek et al. (1997) revealed half lives ranging from 2.5 to 4.6 d in a soil experiment by using an oxitop similar system (sturm test), whereas Hauser et al. (2005), performed a pot experiment and revealed half lives of over 7 weeks.

IV.4.4 Characterisation of wool hydrolysate

The MALDI-TOF MS analysis of the 14 d wool hydrolysate displayed several different oligopeptides (Figure 3-34). After passing through IMAC-column smaller oligopeptides were present in the eluate compared to the 14 d wool hydrolysate (Figure 3-35). Several peptides with equal masses (471, 493, 537, 649, 677, 693, 916) were visible before and after the IMAC treatment. An identification of the sequence of the oligopeptides was impossible as Esperase 8.0 L is an unspecific enzyme, and thus the possible combinations which could correspond to the masses measured by the MALDI-TOF MS are too high.

The amino acid analysis, before and after the IMAC-column depicted a change in the amino acid composition of the oligopeptides (Table 3-5) with increased proportions of histidine and cysteine (determined as cysteic acid after the total hydrolysis) and loss of valine, leucine and isoleucine. IMAC-columns are columns which can be loaded with various metals, thus binding peptides which have a high affinity for a specific metal. The IMAC-columns were loaded with Cu, which was tightly bound to the columns. The IMAC columns selected amino acids and accordingly peptides which contained a high rate of amino acids with high binding constants to Cu. Therefore, a selection of the peptides and amino acids, which were the most efficient in mobilising Cu in soil, was possible. Histidine and cysteine have a high chemical affinity to Cu, with stability constants of $\log K_s = 10.6$ and $\log K_s = 19.2$ respectively (Furia, 1972). By contrast, amino acids with low chemical affinity to Cu, such as glycine ($\log K_s = 8.2$), alanine ($\log K_s = 5.8$) (Sovago et al., 1993), lysine ($\log K_s = 7.6$), arginine ($\log K_s = 7.9$) (Yamauchi and Odani, 1996), were found in similar or decreased frequency in the solution after the IMAC column. Additionally, methionine ($\log K_s = 7.9$) (Berthon, 1995), valine ($\log K_s = 7.9$) and isoleucine ($\log K_s = 8.3$) (Sovago et al., 1993) were not present in the solution after the IMAC column. Although both isoleucine and leucine have a similar chemical affinity to Cu, $\log K_s = 8.27$ and $\log K_s = 8.26$ (Sovago et al., 1993), respectively, leucine was present in the solution after the IMAC column. This, though, may be explained by the presence of more leucine than isoleucine in the initial solution.

The increase or decrease of amino acids compositions such as arginine, ornithine, cysteic acid and of cystine are related to chemical processes during the enzymatic hydrolysis and the total acid hydrolysis. The wool hydrolysate before the IMAC-columns contains more ornithine and less arginine than the wool. The enzymatic wool hydrolysis process was not performed under sterile conditions, therefore during the 14 d hydrolysation process microorganisms (such as *Saccharomyces cerevisiae*) may have developed, which have the ability to degrade arginine and transform it to ornithine by the enzyme arginase (Martin et al., 2003), thus increasing the amounts of ornithine compared to arginine. Cystine, which is an oxidised dimeric form of cysteine, formed by linking two cysteine residues via a disulfide bond (cys-S-S-cys) between the -SH groups, decreased during the enzymatic hydrolysis process. High amounts of cystine are contained in Keratin associated proteins (KAP). KAP have been classified on the basis of their amino acid composition as high sulfur (16-30 mol % cysteine), ultra-high sulfur (>30 mol % cysteine), and high glycine/tyrosine proteins (Shimonura et al., 2002). The cystine concentration in the wool hydrolysate decreased because the KAP are nearly insoluble, thus only a small portion of the KAP goes into solution. During the total acid hydrolysis the presence of Cu (deriving from the IMAC-columns) catalyses the oxidation of cystine to cysteic acid and leads to decreased cystine value. The Cu originates from the IMAC-column treatments, where Cu leaching was not avoidable.

Other chemical characteristics of the amino acids, such as the hydrophobicity or the van der Waals volume also play a role for their occurrence in the solution after the IMAC treatment. Table 2A displays the chemical characteristics of amino acids with isoleucine, valine, leucine, and methionine as very hydrophobic (Kyte and Doolittle, 1982; Hessa et al., 2005). Hessa et al. (2005) and Kyte and Doolittle (1982) use two different scales. In the scale of Hessa et al. (2005) more negative values reflect greater hydrophobicity, whereas in the scale by Kyte and Doolittle (1982) more positive values stand for greater hydrophobicity. For example isoleucine is a hydrophobic amino acid and according to Hessa et al. (2005) it has a hydrophobicity value of -0.60 whereas according to Kyte and Doolittle (1982) it has a hydrophobicity value of 4.5 (Table 2A). Both Hessa et al. (2005) and Kyte and Doolittle (1982) describe isoleucine as the most hydrophobic amino acid, among the amino acids listed in Table 2A. Due to their hydrophobicity, hydrophobic amino acid could shield other less hydrophobic amino acids in a larger peptide, thereby preventing a binding to the IMAC column. Cysteine is also very hydrophobic but has a very high chemical affinity to Cu, $\log K_s$

= 19.2, compared to isoleucine, valine, leucine, and methionine $logK_s$ = 8.3, $logK_s$ = 7.9, $logK_s$ = 8.26, and $logK_s$ = 7.9, respectively. Its binding was, therefore, possible.

Tyrosine is rather hydrophilic, and has a chemical affinity to Cu similar to the majority of the amino acids, $logK_s$ = 7.9 (Yamauchi and Odani, 1996). It was present though at a lower frequency in the solution after the IMAC treatment. A reason could be its van der Waals volume, which is the volume occupied by the atoms when considered to be hard spheres with van der Waals radii (Connolly, 1985). The van der Waals volume is related to the solvent-excluded volume, which is made up of the van der Waals volume plus the interstitial volume Tyrosine displays a very high van der Waals volume (Nelson and Cox, 2000), which could hinder the tyrosine containing peptide, to bind to the IMAC column.

On the basis of the results of the IMAC column one can conclude that histidine und cysteine (the amino acids with the highest abundancy after the IMAC column) and the peptides containing these amino acids are the effective components of the wool hydrolysate. Tyrosine, owing to its van der Waals radius, leucine and isoleucine owing to their hydrophobicity and alanine, lysine etc. owing to their low chemical affinity for Cu are ineffective in mobilizing Cu.

IV.4.5 Evaluation of the wool hydrolysate effectiveness

Hydrolysed wool displayed a low phytotoxicity as seen in the dry weight of the phytoextraction experiments. The application of the 14 d wool hydrolysate increased the uptake of Cu in tobacco (*Nicotiana tabacum* SR-1) by 850% and the 7 d wool hydrolysate by 60%. In contrast to Cu, the 7 d wool hydrolysate did not increase the uptake of Cd significantly. This result verifies the results from the column experiment, where both, the 14 d and the 7 d wool hydrolysate were not able to increase the mobilisation of Cd substantially. This is owing to the lower chemical affinity of amino acids for Cd than for Cu. Besides the significant increase of Cu uptake by the 14 d wool hydrolysate, the application of hydrolysed wool displayed a very low heavy metal leaching probability. The cause for this low leaching probability lies in the rapid biodegradation rate of hydrolysed wool, which interestingly did not significantly change the pH of the soil. The pH of the soil did probably not change because the wool hydrolysate applied on the soil was a mixture of many amino acids, which can be divided in basic, acidic and neutral, according to their acidity, thus in summary reacting neutral. Additionally, the amino acids themselves have active groups of an amine and a carboxylic acid thus they can be considered both acid and base (though their natural pH is

usually influenced by the R group). Therefore, the amino acids work as a buffer, which keeps the soil pH stable. The pH of the soil influences the mobility of metals in soil and thus by remaining constant during the biodegradation of the wool hydrolysate, the mobility of the metals is not influenced by the degradation of the hydrolysed wool.

The amounts of hydrolysed wool which were applied to the soil were very high, e.g. 12.2 g kg^{-1} soil, approximately 1000 kg of wool hydrolysate ha^{-1} soil. Whilst these amounts would produce practical difficulties (application of high amounts of hydrolysed wool on contaminated sites) in handling the technique, the advantage would be its low cost, as wool is cheap. To cope with the high amounts of hydrolysed wool, which have to be applied in order to achieve a high heavy metal uptake, hydrolysed wool could be applied in the same way as sludge, and owing to its capacity as a nitrogen fertiliser, it would balance the additional costs. The amounts that have to be applied to reach a satisfactory phytoextraction enhancement can probably be reduced, with the help of experiments which would optimise the hydrolysation duration and the application practise of wool hydrolysate.

Hydrolysed wool combines two very important aspects for enhanced phytoextraction: a) it increased the uptake of Cu significantly (by 850%) and b) it showed only a very low heavy metal leaching probability, thus making hydrolysed wool a very capable tool for chelate assisted phytoextraction.

IV.5 Assessment of the investigated chelating agents

Potential chelating agents in the field of enhanced phytoextraction have to meet certain requirements, such as low phytotoxicity, enhancement of heavy metal uptake, increase of the root to shoot translocation, high biodegradation rate, low leaching probability, and low cost of purchase. EDTA has revealed a significant enhancement of heavy metal uptake (Huang et al., 1997) combined with a low cost of purchase, but it has also displayed a high degree of phytotoxicity (Epstein et al., 1999; Chen and Cutright, 2001), low biodegradation rate (Bucheli-Witschel and Egli, 2001), high leaching probability (Barona et al., 2001; Grčman et al., 2003; Jiang et al., 2003), and a varying translocation effectiveness depending on the plant (Shen et al., 2002) and the heavy metal (Chen and Cutright 2001) used. The chelating agents tested in this thesis have displayed positive and negative properties with respect to the above mentioned requirements.

The adverse effects on a plant caused by a chelate limit the amounts which can be applied to the soil in order to enhance phytoextraction, and subsequently have a direct effect on the effectiveness of the technique. NLMOWA displayed a very low degree of phytotoxicity (Figure 3-1) in comparison to EDDS and CyD (Figure 3-10 and Figure 3-6). Higher amounts

of NLMWOA could thus be applied to the soil for the phytoextraction experiments. However, higher amounts of a chelate applied to the soil do not necessarily correspond to a higher effectiveness.

Hydrolysed wool applied as a 7 d hydrolysate at 6.6 g kg^{-1} did not produce any phytotoxic symptoms. The 12.2 g kg^{-1} did cause chlorosis and necrosis to the plant, probably caused by the disruption of the plasma membrane surrounding the root, thus allowing fungi to enter the plant. The NLMWOA increased the Cu uptake by approximately 2.3-fold, EDDS by 7-fold and the application of 14 d wool hydrolysate by 8.5-fold.

Various amounts of the chelating agents were applied in order to achieve the increased uptake. In a discussion of the effectiveness of a chelate not only do the applied amounts of a chelating agents, which determine the cost and the practicability of the application play a role, but also its persistence in the soil. Its persistence in soil has a direct influence on its time of impact, which in turn is associated with the leaching probability of heavy metals. NLMWOA and CyD displayed a rapid degradation rate, which not only kept leaching probability very low, but also decreased the initial bioavailability of heavy metal arising from the pH increase of the soil. EDDS however, displayed a low degradation rate, which maintained the heavy metals in the soil highly bioavailable for weeks, thus increasing the risk of leaching. Hydrolysed wool combined a rapid degradation rate, which did not affect the pH of the soil, thus keeping the bioavailable heavy metal fraction in the same level as previous to the application, with a significantly enhanced Cu uptake.

Hydrolysed wool also displayed positive side effects which would give this chelate an economic advantage over NLMWOA, CyD, and EDDS. It, could also, for instance be used as a nitrogen fertiliser (Table 4-1). Table 4-1 displays the nitrogen content of the soil after harvesting the plants which were treated with hydrolysed wool, EDDS, EDTA and NLMWOA. The control before planting (bp.) is an average of the nitrogen content of all control experimental pots before the experiment. The nitrogen in the soil is so high, because the soil was fertilised before each experiment. The control after harvest (ah.) is an average of the nitrogen content of all control experimental pots after harvesting. The nitrogen content has sunk because the plants used the nitrogen in the soil. The nitrogen content of the soil from the wool application is significantly higher (0.246%) than the nitrogen content of the other chelate applications (EDDS (0.199%), EDTA (0.202%), and NLMWOA (0.199%)); though still lower than the initial nitrogen content.

Table 4-1: Nitrogen content (%) in soil after harvesting (ah.) of the control and the chelates and before planting (bp.) of the control

	Wool	EDDS	EDTA	NLMWOA	Control bp.	Control ah.
N (%)	0.246	0.199	0.202	0.199	0.267	0.202

The practicability of the chelate assisted technique is also an important criterion. Humic acids, for example have displayed positive characteristics (Evangelou et al., 2004), but the amounts which have to be applied are too large in order to achieve a significant enhancement of the uptake. The EDDS amounts applied are very low in comparison to NLMOWA and in particular wool. Hydrolysed wool, though, could be applied in the same way as sludge, and owing to its capacity as a nitrogen fertiliser, would balance the additional costs. The amounts that have to be applied to reach a satisfactory phytoextraction enhancement can probably be reduced with the help of experiments which would optimise the hydrolysation duration and the application practice of wool hydrolysate.

V CONCLUSION

Metal pollution of soils is a widespread problem across the globe, and the clean up is a difficult and economically challenging task. Phytoremediation is a low-cost and simple-technology method of combating this pollution. The results indicate that wool hydrolysate was the only chelate, which may eliminate two of the major limiting factors in heavy metal phytoextraction from contaminated soils: low heavy metal uptake and the leaching risk of heavy metals into the groundwater.

Wool not only increased the uptake of Cu by 850%, but it also showed a very low risk of leaching of the heavy metals because of the readily biodegradability of the chelates, thus having a very short impact period on the bioavailability of heavy metals.

In contrast, (ethylene diamine tetraacetic acid) EDTA and (ethylene diamine disuccinate) EDDS reveal a high leaching potential of heavy metal after application to soil. Contrary to several publications, EDDS showed a relatively high persistence in the soil. The risk of heavy metal leaching into the groundwater was high, and this feature offsets the usefulness of EDTA and EDDS in enhancing the uptake of heavy metals.

Natural low molecular weight organic acids (NLMWOA) increased the uptake of Cu, but were ineffective for the uptake of Pb. Owing to their fast degradation the leaching probability was low, but due to the pH increase during the degradation the bioavailability of heavy metals declined thereby reducing the effectiveness of the technique.

Cyclodextrins (CyD) displayed a high phytotoxicity and a very low mobilisation capability of heavy metals, thus making them unsuitable for chelate assisted phytoextraction..

From the chelates tested only hydrolysed wool has the potential to replace EDTA on the free market, and to reduce the phytoextraction time in an economical way. It combines a high effectiveness for heavy metal uptake and a low risk of heavy metal leaching. Additionally, apart from the low price of purchase its potential use as a nitrogen fertiliser is an advantageous factor, which would further more decrease the cost of application.

VI OUTLOOK

Soil remediation by phytoextraction of metals has become a major interest to many scientists over the last decades. Despite enormous efforts and some striking results, this technique is still in the development stage and is primarily conducted in laboratory or greenhouse experiments.

The results form the phytoextraction experiments show that the use of phytoextraction will probably be limited to the soils which show only a moderate degree of contamination.

The addition of chelating agents to increase the uptake rate bears the risk of heavy metal leaching. The solutions which have been proposed to cope with this problem (i.e. subirrigation-drainage system) are very costly. These additional costs would diminish the high economical efficiency, one of the major advantages of phytoremediation.

It has been suggested that research on chemically-induced phytoextraction should be continued, enabling the discovery of new mobilizing agents, which enhance accumulation of metals and which are environmentally safe. Research on phytoextraction has perhaps reached a turning point, where it should distance itself from adding chelating agents. The supporters of enhanced phytoextraction have always criticised the infeasibility of phytoremediation without chelates, as too much time is required. It should though not be forgotten that these long periods of time required in the remediation of contaminated fields, could be combined with profit making operations, such as the use of the plants as a source for bioenergy.

Nevertheless, hydrolysed wool has shown great potential in increasing the uptake of heavy metals in plants. At present, as the world demand for natural textiles slump, the wool industry searches for alternatives in the use of its product. Filter material and industrial bakeries have provided some outlet. Its function as a nitrogen fertiliser, and, moreover as a chelating agent simultaneously, could be of great interest. Research, however, should focus on the influence of the long-term wool application on the microbial community of soil and its characteristics, in order to avoid a long-term negative impact on the soil biota. Additionally, the effectiveness of wool hydrolysate for other heavy metals should be tested, as well as the efficiency of successive applications of the hydrolysed wool on the phytoextraction of heavy metals.

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VIII APPENDIX

Table 1A: Sequences of identified microorganisms.

Micro-organism	Sequence	Expect value
Burkholderia sp.	gi|110589882|gb|DQ777739.1| GNNNNNNNNNGCTNNNACNNNNNNNNNAGAATAN TCNGNNNNNNNGCNNNNGAGTTGCANAGTGCGATC CGGACTACGATCGGTTTTCTGGGATTGGCTCCCCCT CGCGGGTTGGCGACCCTCTGTTCCGACCATTGTATG ACGTGTGAAGCCCTACCCATAAGGGCCATGAGGAC TTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGG CAGTCTCCCTAGAGTGCTCTTGCGTAGCAACTAGGG ACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACA TCTCACGACACGAGCTGACGACAGCCATGCAGCAC CTGTGTTATGGCTCCCTTTCGGGCACTCCCACCTCTC AGCAGGATTCCATACATGTCAAGGGTAGGTAAGGT TCTTCGCGTTCCCCCCGTGCCCNN	0.0
Ralstonia sp.	gi|114224468|gb|DQ785822.1| GGNNNNNNNNNNNNNTNNNCNNGNTTACTAGCGAN TNNAGNNNNACGTATTCNAGTTGCNNACTACAATC CGGACTACCATGCATTTTCTGGGATTANCTCCACCT CGCGGCTTGGCAACCCTCTGTATGCACCATTGTATG ACGTGTGAAGCCCTACCCATAAGGGCCATGAGGAC TTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGG CAGTCTCTCTAGAGTGCCCTTTCGTAGCAACTAGAG ACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACA TCTCACGACACGAGCTGACGACAGCCATGCAGCAC CTGTGTCCACTTTCTCTTTCGAGCACCTAATGCATCT CTGCTTCGTTAGTGGCATGTCAAGGGTAGGTAAGGT TCTTCGCGTTCCCCCCGTGCC	0.0
Ralstonia sp.	gi|114224468|gb|DQ785822.1| NNNNNNNNNNCNGNNNNNACNNNNNNANANNNNN AACAGNTANATGNATNNNAGTTNNNCACTACAAAA CGGANTACNANGNATTTTCTGGGATTANCTCCACCT CGCGGCTTGGCAACCCTCTGTATGCACCATTGTATG ACGTGTGAAGCCCTACCCATAAGGGCCATGAGGAC TTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGG CAGTCTCTCTAGAGTGCCCTTTCGTAGCAACTAGAG ACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACA TCTCACGACACGAGCTGACGACAGCCATGCAGCAC CTGTGTCCACTTTCTCTTTCGAGCACCTAATGCATCT CTGCTTCGTTAGTGGCATGTCAAGGGTAGGTAAGGT TCTTCGCGTTCCCCCCGTGCN	$5e^{-176}$

Basidiomycete yeast sp.	gi|46405650|gb|AY520246.1| NNNNNNNNNNNNNNNNNNCNNNNANNTNNNANT TNNNCTGNGAACTNNNANGGNCNTTNNNGTCAGTT NNNNTNCNNTTGATNATATCTTGCTACATGGATAAC TGTGGTAATTCCAGAGCTNATACATGCTGAAAAGCC CCGACTTCTGGAAGGGGTGTTTTTATTAAATAAAAA ACCAATNNNCNNNCNNNNNCTTTGGTGATTAATAA TACCTTCTCGAATCGCTTGGNNTTGTGCNGGCGATG CTTCATNCAATTATCTGCCCTATCAACTTTCGATGG NAGGATANAGGCCTACCATGGTATNNAACGGGTAA CGGGAANNAA	$1e^{-33}$
Paecilomyces sp.	gi|45861408|gb|AY526475.2| NNNNNNNNNNNNNNCNAANNNNANNNTTATACG GCGAAACTGCGAATGGCTCATTATATAAGTTATCGT TTATTTGATAGCACCTTACTACTTGGATAACCGTGG TAATTCTAGAGCTAATACATGCTAAAAATCCCGACT TCGGAAGGGATGTATTTATTAAATTAAAAACCAATG CCCTCTGGGCTCTCTGGTGATTCATGATAACTTCTCG AATCNCATGGCCTTGCGCCGGCGATGGTTCATTCAA ATTTCTTCCCTATNAACTTTCGATGTTTGNGTAGTGG CCTAACATGGTTGCAACGGNNAACGNNGAAAAA	$4e^{-145}$
Saccharomyces cerevisiae	gi|55416147|gb|AY790536.1| CCCNNNNNNNNNNNNNNNNNNNANNNTTNANA CNGNGAAANTGNNNTGGCCNNTNAAAGNAGTTANC GTTNCCNNNATANNNGCTTTACTANATGGAATAACT GNGGTANNNCTAGAGCTANTACATGCTAAAAATCT CTNCCCTTTGNANGAGNNGTTTTTATTANGTANAAN CTCAATGTCTTCCGNNNCTNTGANNATTCNTAATNN NNTTTTNAAACGAATGGCCNTNNNNTGNTGATGNN NTNNGCAGTTTCNNTNCNCNNAATNNNTTNTNTGNN NNNANNNTGNCCTTANNNGNTTANANNNNCNTNNN ACNNAANANN	$3e^{-10}$
Cordyceps sp.	gi|27807830|dbj|AB099942.1| NNNNNNNNNNNNNNNNCNNNNNNNGCGTTTNN ACGGCGAAACTGNGAATGGCTCNTTATAGAAGTTA TNGTTTNNNTNATANCANNTTACTACTTGNATAACC GTNNNAATTCNNNATCTAATACATGCNAAAAATCC CNACNCCTTAAGAGATGNATNTATTANANTAAAAA CCCCCGCCCNTTGNCCNCNCTGGTCANACANGATAG NTNCTNNAATCTCATGGCCNNGCGCCGNNNAAAGN CNATNCNAGTTTCTCCNNNATCTTCNTTAGANGTTG GNNTGNNGGCCNTACNCGTTTNCAANTNNNATTAA TAAAANNNT	$5e^{-12}$
Burkholderia sp.	gi|110589882|gb|DQ777739.1| NNNNNNNNNNCCNNNNGNNCNNNNNNNNNNNNN NNNNNNNGNAGANNNGAGNGGGNGAGTGNGANNC NNTNNNNNNNNNNTTTCTGGGATTGGCTCCCCCTC	$4e^{-109}$

	GCGGNNNTNNNNAANCTCTGTTCCGACCATTGTATG ACGTGTGAAGCCCTACCCATAAGGGCCATGAGGAC TTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGG CAGTCTCCCTAGAGTGCTCTTGCGTAGCAACTAGGG ACAAGGGTTGCGCTCGTTGCGGGACTTAACCCANN ATCTCACGANNCGAGNNGACGACAGCCATGCAGCN CCTGTGTTATGGNTCCCTTTCGGGCANNCNNNNNNN NCANNNNNNNANCNTACNTGNCNNNGGTAGNNNA NGTTCTTCNNNNACNNNCNACNNACNTNNNNNN	
Paecilomyces sp.	gi|116174501|dbj|AB277860.1| NNNNNNNNNNNNNNNNNNNNNNNNNNANNNNTT ATACGNGCGNNACTGCGAATGGCTCATTATAGNNG TTAGNGTTNNCCCNNANAACAANTTACNACTTGNAT AACCGNGNNAATTCNNGAGCTAATACATGCCNAAA ATCCCGACTTCGNAAGGGATGNNTTTNTTANATNNN NANNNNANGCCCTCTNNNNNCNNNNNTCNTTCNTG AAANNNNCTCGAANCNCATGGCCATCCGACGNNGA GGGCCCTTNCNAATTTCNCCGNTATCNNCCTTANAC NTAGGCNTNNNGGGCTNACTTNGTTGCANCGGGAT ACGNNTNNNAN	$8e^{-11}$

Table 2A: Chemical characteristics of amino acids.

Name	3-letter symbol	1-letter symbol	Molecular weight	Molecular formula	pKa[1]	pKb[2]	pKx[3]	pI[4]	Hydrophobicity[a]	Hydrophobicity[b]	Van-der Waals Volume[c]
Alanine	Ala	A	89.09	$C_3H_7NO_2$	2.34	9.69	-	6.00	1.8	0.11	67
Arginine	Arg	R	174.20	$C_6H_{14}N_4O_2$	2.17	9.04	12.48	10.76	-4.5	2.58	148
Asparagine	Asn	N	132.12	$C_4H_8N_2O_3$	2.02	8.80	-	5.41	-3.5	2.05	96
Aspartic acid	Asp	D	133.10	$C_4H_7NO_4$	1.88	9.60	3.65	2.77	-3.5	3.49	91
Cysteine	Cys	C	121.15	$C_3H_7NO_2S$	1.96	10.28	8.18	5.07	2.5	-0.13	86
Glutamic acid	Glu	E	147.13	$C_5H_9NO_4$	2.19	4.25	9.67	3.22	-3.5	2.68	109
Glutamine	Gln	Q	146.15	$C_5H_{10}N_2O_3$	2.17	9.13	-	5.65	-3.5	2.36	114
Glycine	Gly	G	75.07	$C_2H_5NO_2$	2.34	9.60	-	5.97	-0.4	0.74	48
Histidine	His	H	155.16	$C_6H_9N_3O_2$	1.82	9.17	6.00	7.59	-3.2	2.06	118
Isoleucine	Ile	I	131.17	$C_6H_{13}NO_2$	2.36	9.60	-	6.02	4.5	-0.60	124
Leucine	Leu	L	131.17	$C_6H_{13}NO_2$	2.36	9.60	-	5.98	3.8	-0.55	124
Lysine	Lys	K	146.19	$C_6H_{14}N_2O_2$	2.18	8.95	10.53	9.74	-3.9	2.71	135
Methionine	Met	M	149.21	$C_5H_{11}NO_2S$	2.28	9.21	-	5.74	1.9	-0.10	124
Phenylalanine	Phe	F	165.19	$C_9H_{11}NO_2$	1.83	9.13	-	5.48	2.8	-0.32	135
Proline	Pro	P	115.13	$C_5H_9NO_2$	1.99	10.60	-	6.30	-1.6	2.23	90
Serine	Ser	S	105.09	$C_3H_7NO_3$	2.21	9.15	-	5.68	-0.8	0.84	73
Threonine	Thr	T	119.12	$C_4H_9NO_3$	2.09	9.10	-	5.60	-0.7	0.52	93
Tryptophan	Trp	W	204.23	$C_{11}H_{12}N_2O_2$	2.83	9.39	-	5.89	-0.9	0.30	163
Tyrosine	Tyr	Y	181.19	$C_9H_{11}NO_3$	2.20	9.11	10.07	5.66	-1.3	0.68	141
Valine	Val	V	117.15	$C_5H_{11}NO_2$	2.32	9.62	-	5.96	4.2	-0.31	105

[1]pKa is the negative of the logarithm of the dissociation constant fort he –COOH group
[2]pKb is the negative of the logarithm of the dissociation constant fort he $–NH_3^+$ group
[3]pKx is the negative of the logarithm of the dissociation constant of any other group in the molecule
[4]pI is the pH at the isoelectric point

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IX CURRICULUM VITAE

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Education and training

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Publications

Journals:

Evangelou, M.W.H., Ebel, M., Schaeffer, A., 2007. Chelate assisted phytoextraction of heavy metals from soil. Effect, mechanism, toxicity, and fate of chelating agents: A review. Chemosphere 68, 989-1003.

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