INVESTIGATION OF THE USE OF RHAMNOLIPID BIOSURFACTANT FOR CADMIUM PHYTOEXTRACTION IN SOILS

By

Jia Wen

Soil Science

School of Agriculture, Food and Wine

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Abstract

Phytoextraction is a technology to remove hazardous metals from soil which has developed rapidly in the last 20 yearsby using metal-tolerant hyperaccumulating plants or using plants with high biomass production and enhancing metal uptake using chelants. Although phytoextraction of metals is not a routine remediation approach, continued efforts have been made to refine this "green" technology in the hope that it could replace traditional remediation measures (e.g. excavation, soil washing, etc.), as soil handling less and costs are typically lower, leaving the soil fertile and able to support subsequent vegetation. Currently phytoextraction has not yet had significant success in field applications, due to difficulties in seeking environmental-friendly and effective chelants and low biomass production of many metal-tolerant plants. Conventional chelants enhance metal solubility in soil, but there are concerns over their use due to persistence in the environment and the potential for metal leaching to ground water. Therefore, there is a need to identify and evaluate chelants with potentially good metal-extraction efficiency and environmental compatibility. Recently, rhamnolipid, a biologically synthesized microbial product, has been used to remove metals from contaminated soils. In particular, rhamnolipid has been used to wash cadmium (Cd) from contaminated soils due to its strong affinity for Cd compared to many other metals. Unlike many conventional chelants, such as ethylenediaminetetraacetic acid (EDTA), rhamnolipid can form uncharged, lipophilic complexes with metal cations. There is some evidence that the uncharged or lipophilic metal rhamnolipid complexes are equally or more available than the free metal ions for plant uptake. Therefore, rhamnolipid could potentially be useful for Cd phytoextraction. This study therefore investigated the possibility of using rhamnolipid as a potential chelant to enhance the phytoextraction of Cd and its co-contaminant zinc (Zn). As well as cost and availability, the three key attributes of chelants that need to be considered in chelant-assisted phytoextraction are (i) persistence of the chelants in soil; (ii) adsorption behaviour of the chelants and its metal complexes; and (iii) toxicity of the chelants to plants.

Chelant biodegradation is of importance to phytoextraction efficiency. Persistence in the environment is not regarded as favourable and too rapid degradation results in poor enhancement of metal availability. Hence rhamnolipid biodegradation in soil was compared with conventional chelants, i.e. EDTA and citric acid, in Chapter 2. Rhamnolipid (applied at low – 2.0 mmol/kg soil and high – 10.0 mmol/kg soil concentrations) was found to be biodegradable in soil, but persisted long enough to enable metal mobilisation during phytoextraction.

Chapter 3 showed that soil sorption of rhamnolipid was concentration dependent, as was its ability to mobilise metal ions. In soil batch adsorption experiments, rhamnolipid was found to adsorb to soil when applied at low concentrations (< 1.7 mM applied concentration, equivalent to 8.5 mmol/kg soil), whereas when the concentration increased to 4.4 mM (22 mmol/kg soil), rhamnolipid adsorption to soil reduced and Cd desorption was significantly increased. This effect may have been due to the formation of larger rhamnolipid aggregates (e.g. vesicles) at the higher rhamnolipid concentrations. Determination of the octanol/water partition coefficients for metal-rhamnolipid complexes showed that rhamnolipid complexes change their morphology and hydrophilicity with concentration, which could be an alternative reason for the reduced rhamnolipid adsorption in soil. A solution concentration of rhamnolipid ranging from 4 – 5 mM was found to be most promising for Cd and Zn phytoextraction because of the low rhamnolipid sorption to soil.

High concentrations of applied rhamnolipid (4 – 5 mM, equivalent to 20 – 25 mmol/kg, as multiple weekly doses or applied as a single dose before harvest) were found to be the most suitable conditions selected for Cd and Zn mobilisation. However, rhamnolipid at those concentrations induced toxicity symptoms in maize (*Zea mays*). Therefore, the use of high concentrations (≥ 4 mM) was not suitable for phytoextraction. In a subsequent experiment, low concentrations of rhamnolipid (≤ 1.4 mmol/kg, equivalent to 1 mM in soil solution) were assessed for their ability to enhance Cd uptake by maize (*Zea mays*) and sunflower

(*Helianthus annuus*). Cadmium uptake was moderately improved ($P \leq 0.001$) following rhamnolipid application at 0.02 and 0.2 mmol/kg/week with no observable phytotoxicity. However, in general, rhamnolipid did not dramatically improve Cd and Zn uptake by the plant shoots.

An aseptic hydroponic experiment (Chapter 4) was undertaken to understand the effect of rhamnolipid on plant uptake of Cd and Zn at low concentrations (4 – 20 μM). At constant total Cd and Zn concentrations in solution and with increasing EDTA concentrations, plant uptake of Cd decreased, conforming to the free ion activity model. Rhamnolipid complexed Cd and Zn to a lesser extent than EDTA, but enabled a relatively constant Cd and Zn uptake in the root as complexation increased and free ion activities declined. This effect may have been due to the absorption of intact metal-rhamnolipid complexes. At normal Ca concentrations (2 mM, commonly found in soil solutions) in the uptake solutions, Cd translocation from roots to shoots was inhibited compared to low Ca concentrations (0.035 mM). This indicates that there will be no beneficial effect of rhamnolipid application on Cd phytoextraction in soil where Ca concentrations are several orders of magnitude higher than Cd.

The body of the work emphasizes the key criteria for selecting suitable chelants for phytoextraction. Rhamnolipid was deemed to be unsuitable for Cd phytoextraction.

Declaration

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Jia Wen **Date**

List of Publications

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Wen, J., McLaughlin, M. J., Stacey, S. P., Kirby, J. K., 2010. Is rhamnolipid biosurfactant useful in cadmium phytoextraction? J. Soils Sed., 10, 1289-1299.

Wen, J., McLaughlin, M. J., Stacey, S. P., Kirby, J. K., 20-23 Sep, 2009. Investigating the use of rhamnolipid for cadmium phytoextraction in soils. 13th Australasian Society for Ecotoxicology Conference: Toxicants in a Changing Environment. Adelaide, SA, Australia.

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Abbreviations

AEAA	N-(2-aminoethyl) aspartic acid
ANOVA	analysis of variance
ASV	anodic stripping voltammetry
ATSDR	Agency for Toxic Substances & Disease Registry
CA	citric acid
CEC	cation exchange capacity
DDC	diethyldithiocarbamate
DI	deionised water
DMT	Donnan membrane technique
DTPA	diethylenetriaminepentaacetic acid
EDDS	ethylenediaminedisuccinic acid
EDGA	ethylenediaminediglutaric acid
EDTA	ethylenediaminetetraacetic acid
EK	electrokinetics
EXAFS	synchrotron-based extended X-ray absorption fine structure
FIAM	free ion activity model
HAs	humic acids
HMWOAs	high molecular weight organic acids
IARC	International Agency for Research on Cancer
ICP-MS	inductive coupled plasma mass spectroscopy
ICP-OES	inductive coupled plasma optical emission spectroscopy
IDSA	iminodisuccinic acid
IUPAC	International Union of Pure and Applied Chemistry
LMWOAs	low molecular weight organic acids
LSC	liquid scintillation counting
MWHC	maximum water holding capacity
NTA	nitrilotriacetic acid
PC	phytochelatins
PIPES	piperazine-N,N'-bis(2-ethane-sulfonic acid)
PS	phytosiderophores
SEM/EDX	scanning electron microscopy with energy dispersive X-rays
SEPs	sequential extraction procedures
XAS	X-ray absorption spectroscopy
XRD	X-ray diffraction
XANES	X-ray absorption near edge spectroscopy

Chapter 1. Introduction and literature review

1.1 Introduction

Cadmium (Cd) contaminated soil is of concern because of the potential adverse effects on soil flora and fauna, and most importantly on human health. Intake of excessive Cd through the food chain can result in humans suffering from diseases such as kidney dysfunction and skeletal problems (Omarova and Phillips, 2007). Rice grown on geogenic Cd-contaminated paddy soils or adjacent to mining precincts increases the risks of dietary Cd intake. Subsistence rice diets (e.g. polished rice grain) that supply marginal or deficient calcium (Ca), iron (Fe), and zinc (Zn) also promote the risk of duodenal Cd absorption by humans (Chaney et al., 2004; Reeves et al., 2005). In Japan and China, soil Cd has been found to cause a high incidence of adverse health effects in farmers who rely on rice for their sustenance. A serious case of Cd toxicity occurred in Toyama Prefecture in Japan in the 1950s, where people lived mainly on rice grown in Cd contaminated soil caused by nearby Pb-Zn mining and smelting and also relied on the metal-polluted water for their drinking water (Alloway and Ayres, 1997). The risks of Cd-induced health effect on humans through uptake from their diets highlight the importance of minimising Cd concentrations in heavily-industrialized or contaminated arable regions. An understanding of Cd contamination in soil is essential to developing and improving methods for Cd remediation.

1.2 Cadmium in the environment

1.2.1 Sources of cadmium contamination in soils

Cadmium is believed to be a "non-essential element" due to its non-essential biochemical function, except for its biological role (at very low inorganic Cd concentrations) in the marine diatom *Thalassiosira weissflogii* under the conditions of Zn limitation (Lane and Morel, 2000; Lee et al., 1995). The potential for Cd transfer through the food chain highlights the importance of Cd decontamination in soil. In the early 1980s, Cd and its compounds were placed on the priority list of pollutants in the United Kingdom (Alloway and Ayres, 1997). Most soils contain < 1 mg Cd/kg, except those naturally high in Cd due to the presence of phosphorites (e.g. Jamaican soils and surface soils from Raine Island, Great Barrier Reef) (Barry and Rayment, 1997; Garrett et al., 2008), contaminated from point sources with Cd concentrations exceeding 100 mg/kg, or developed on parent materials such as black shale with Cd contents ranging from 0.30 to 219 mg/kg (Alloway, 1990).

Cadmium in soils originates from different sources, e.g. atmospheric sources, aquatic sources, and anthropogenic sources. Anthropogenic sources, e.g. metalliferous mining, agricultural inputs, metallurgical industry emissions, manufacturing, and disposal of products, generally contribute most to Cd contamination in soil. For example, manufacturing and processing facilities were responsible for the discharge of an estimated total of 299 tons (2.99×10^5 kg) of Cd to land in the United States in 1996, amounting to 22% of the total environmental release (ATSDR, 1999). It is the recognition of the potential toxicity of Cd and the current situation

of diffuse pollution through industrial and agricultural sources that has resulted in an average reduction in world production, from 20,300 tons (2.03×10^7 kg) in 2000 to 18,700 tons (1.87×10^7 kg) in 2004 (Buckingham, 2006). In the United States, the refinery production of Cd has declined more than a half from 1,470 tons (1.47×10^6 kg) in 2005 to 700 tons (7.0×10^5 kg) in 2009 (Tolcin, 2010). Cadmium emissions from some major point sources of emission (**Figure 1.1**) has declined over the last two decades in Europe (Pacyna et al., 2009). It is estimated that the total emissions of Cd will be reduced by 37% by implementing the Maximal Feasible Technological Reduction in Europe between the years of 2005 and 2010 (Pacyna et al., 2009).

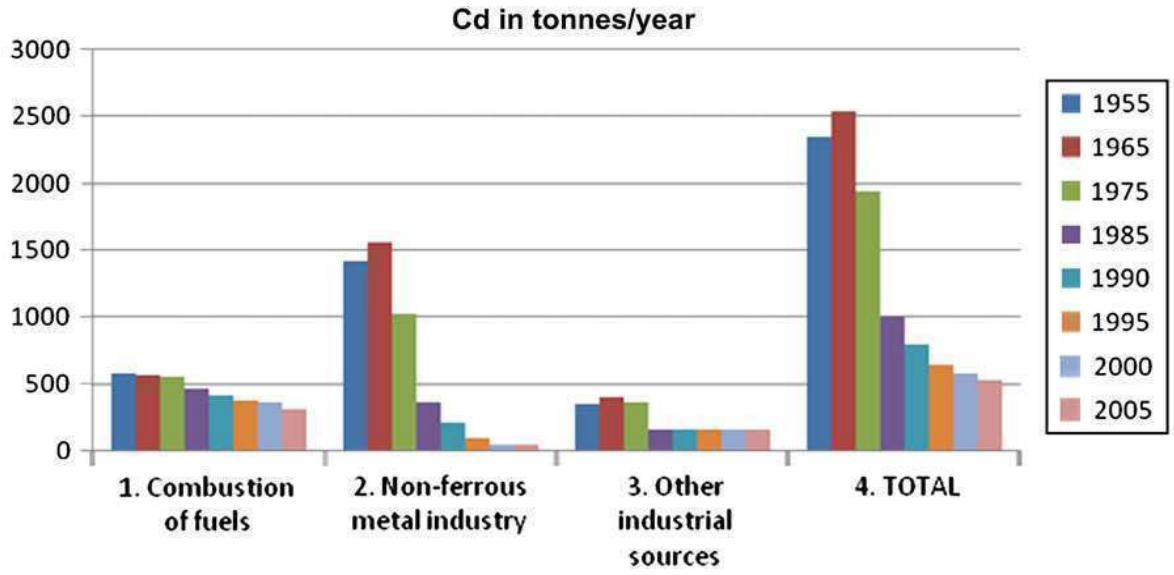


Figure 1.1 Change of atmospheric emissions of Cd in the period from 1955 through 2005 (in t/year). (Reprint with permission from Pacyna, J. M., Pacyna, E. G., Aas, W., 2009. Changes of emissions and atmospheric deposition of mercury, lead, and cadmium. Atmos. Environ. 43(1), 117-127. Copyright Elsevier Publishing.)

In Australia, the total national facility emissions for Cd and its compounds during 2004 and 2009 were relatively steady (from 16,000 to 20,000 kg), based on 1,093 industrial facilities (**Figure 1.2**). Burning/wildfires, paved/unpaved roads and basic non-ferrous metal

manufacturing are the top three atmospheric Cd emission sources in Australia. Emissions to land are most from non-ferrous production sources (NPI, 2010). On a global scale, in the non-ferrous metal production sector, the bulk of Cd being mined is often found in Zn ores e.g. sphalerite (ZnS) because of their chemical similarity. Estimated global Cd extraction in ores, in the annual report of U.S. Geological Survey in 2007 (Peter, 2007), was about 6 million tons (6.0×10^9 kg), based on 1.9 billion tons (1.9×10^{12} kg) identified as Zn ores containing 0.3% Cd. Therefore, Zn production, as well as copper (Cu) and lead (Pb) smelting, can give rise to significant environmental contamination with Cd.

Figure 1.2 Total national facility emissions for the substance: Cadmium and compounds from 2004 to 2009 (NPI, 2010).

Agriculture constitutes one of the most important non-point sources of Cd pollution to soils. Phosphatic fertilizers, composts, manures, and sewage sludge may contain high concentrations of Cd which may cause elevated concentrations of Cd in food crops (McLaughlin et al., 1996; Nicholson et al., 1994; Nicholson et al., 2003). In Australia, the

main source of Cd added to agricultural soils is through the use of phosphatic fertilizers (McLaughlin et al., 1996).

1.2.2 Cadmium chemistry in soils

Elemental Cd is in Group IIB of the Periodic Table and its normal oxidation state in almost all of its compounds is +2. The chemical properties of Cd resemble those of Zn, especially under reducing conditions and in covalent compounds; whereas under oxidizing conditions, it may behave similarly to Ca (Morrow, 2001). Cadmium forms a large number of complex ions with other ligand species (e.g. ammonia, cyanide, and chloride). An understanding of the distribution and speciation of Cd in soils is central to understanding its behavior in soil environments.

1.2.2.1 Speciation and bioavailability of Cd

In soils, Cd can partition into either the solution phase or the solid phase. The soil solution acts as an important link between soil solid phase or other components by which dissolved Cd species are transported to root surfaces, leached to ground waters or transported via surface waters (Ritchie and Sposito, 1995). The dynamics of metals between solution phase and solid phase are influenced by various biological and chemical processes, including dissolution/precipitation, adsorption/desorption, redox reactions, complexation, etc., which change metal partitioning in soil. Cadmium adsorption processes in soil are strongly affected by soil pH and can be broadly divided into two groups: non-specific adsorption and specific adsorption. Non-specific adsorption enables Cd bound by electrostatic forces to balance the

negative charge on surfaces, while specific adsorption is more pH specific and Cd ions are bound much more tightly to the adsorbent and consequently desorbed less effectively (Christensen and Huang, 1999). An increase in soil pH would favour the specific adsorption process either by decreased competition (e.g. H^+ and aluminum (Al)) in solution or by the increase in the negative charge of the soil sorbent surfaces. Change in the stoichiometric ionic strength of the background solutions could also affect Cd partitioning in soil: the adsorption of Cd decreases with an increase in ionic strength in most soils (Naidu et al., 1994; Petruzzelli et al., 1985), due to the competition of other cations for sorption sites. Compared to some other heavy metals such as Cu, nickel (Ni), Pb, and Zn, Cd has a lower affinity to soil because of its lower extent of hydrolysis, higher solubility and its inclination to non-specific adsorption (Appel and Ma, 2002; Asami et al., 1995; Bruemmer et al., 1988).

The speciation of metals is informative and fundamental to understanding metal mobility and bioavailability in soil. The International Union of Pure and Applied Chemistry (IUPAC) in 1993 gave the definition of speciation as "the determination of the exact chemical form or compound in which an element occurs in a sample, and the quantitative distribution of the different chemical forms that may coexist". In the soil solution phase, Cd may exist in the form of free and complexed ions, such as Cd^{2+}, $CdOH^+$, $CdCl^-$, $CdHCO_3^+$, and $CdSO_4^0$ (Alloway, 1990). An analysis of Cd speciation in the soil solution of 64 field-contaminated soils showed that the estimated Cd^{2+} species constituted between 0% and 60% of the dissolved fraction (average ~ 20%) (Sauvé et al., 1999). The solution phase also contains colloidal material such as Cd bound to high molecular weight organic material (humic

substances) or to dispersed metal colloids such as hydroxyl polymers of Al, Fe and other metals (Helmke, 1999). In the solid phase, Cd is present by sorbing to organic matter or mineral surfaces, or forming hydroxide, carbonate, phosphate, sulfate and sulfide precipitates. For instance, cadmium sulfide (CdS) will be produced as a result of Cd^{2+} reacting with sulfur under anoxic conditions (Alloway, 1990).

Chemical speciation, which is controlled by environmental physicochemical conditions, plays a critical role in mediation of metal bioavailability. The term 'bioavailability' refers to the relative proportion of a contaminant that produces a biological response (beneficial or detrimental) or can be taken up by an organism (Sauvé, 2002). For a metal to be bioavailable, it will have to be mobile and be in an accessible form to the plant (Adriano et al., 2004). In the solution phase, it was believed that the free Cd ion forms the major part of Cd in soil solution and correlates best with plant uptake and toxicity (García-Miragaya and Paje, 1978). However, other Cd species may also contribute to the bioavailable pool (Nolan et al., 2005), which indicates biota may take up metal ions from soil solution in forms other than free ions, or that complexation affects the uptake of free ions. For example, increasing chloride (Cl^-) concentration in nutrient solution was found to directly result in an increase of Cd absorption by Swiss chard (Smolders and McLaughlin, 1996b). In soils under saline conditions, Cd bioavailability has been found to be related to the Cl concentration in solutions. Cadmium concentrations of potato tuber (*Solanum tuberosum* L.) were found to be related neither to the total or extractable (chelant or salt extracts) Cd concentrations, nor to the ionic activities of

Cd^{2+} in soil solution, but to the activities of $CdCl_n^{2-n}$ species in solution ($P \leq 0.001$) (McLaughlin et al., 1997). Factors affecting Cd bioavailability are illustrated in Section 1.3.

1.2.2.2 Measurement of Cd species in soils

An assessment of the total concentration and speciation of Cd in soil is essential for understanding its mobility, bioavailability and toxicity. Speciation techniques can be grouped into those examining metal species in soil solutions, and those attempting to measure metal species in the soil solid phase.

Metal speciation in the solution phase can be broadly predicted or measured via the following approaches: computer-based models such as GEOCHEM (exclusively used in soil systems) and MINEQL (applied for water bodies) and some of their updated versions such as SOILCHEM and MINTEQA2 (Jon Peter Gustafsson, KTH, Dept. of Land and Water Resources Engineering, Stockholm, Sweden), anodic stripping voltammetry (ASV) (Andrewes et al., 1996), Donnan dialysis (Cox et al., 1984; Cox and Twardowski, 1980; Nolan et al., 2003; Nolan et al., 2005), resin exchange methods (Holm et al., 1995), and ionic-selective electrodes (Cavallaro and McBride, 1980). The development of these methods was stimulated by the interest in free ion activity as a potential key factor controlling trace metal uptake by organisms (Campbell, 1995; Parker and Pedler, 1997).

Spectroscopic techniques can be used to determine the chemical forms or species of metals in the solid phase of soils and may also provide information on their oxidation states. These techniques include X-ray diffraction (XRD), X-ray absorption spectroscopy (XAS), scanning

electron microscopy with energy dispersive X-rays (SEM/EDX), synchrotron-based extended X-ray absorption fine structure (EXAFS) and X-ray absorption near edge spectroscopy (XANES). These techniques have been applied to some metals in soils such as Cu (Strawn and Baker, 2007), mercury (Hg) (Skyllberg et al., 2006), and Pb (Manceau et al., 1996). However, measuring solid-phase Cd species in soil has been problematic due to the typically low concentrations found in most soils, which normally makes the spectroscopic methods unsuitable (Traina, 1999). These techniques have also been suggested or applied to investigation of Cd speciation in plants (Adriano et al., 2004; Gardea-Torresdey et al., 2005) with findings that the majority of Cd is bound with phytochelatins containing sulfur (S) ligands and in addition with oxygen (O) or nitrogen (N) ligands (Salt et al., 1995; Vogel-Mikuš et al., 2010).

In order to determine the readily plant-available forms of Cd in soil, the amount of extractable Cd can be quantitatively measured by chemical treatments. However, metal extractability does not equal bioavailability: only a part of Cd in soil is actually available to plants, and this part needs to be defined in specific species. Chemical analysis using a single extractant is one method for determining the available fraction of Cd in soil. For water-soluble and exchangeable metal in soil, a single reagent can be used such as $CaCl_2$, $MgCl_2$, NH_4Cl, KNO_3, $Mg(NO_3)_2$, and $Ca(NO_3)_2$ as extractants to displace metal ions on particle surfaces (Krishnamurti et al., 1995). However, single extractants are generally not specific to a single phase and often extract elements bound in other phases. Therefore, they are better described as isolating phases operationally.

A common strategy using multiple reagents to determine the chemical forms of metals in soil, sediments and suspended solids in natural waters are called sequential extraction procedures (SEPs) (Ure, 1991). The two basic protocols used to assess metal fractionation in soil are the Tessier et al. (1979) scheme and the Standards, Measurements and Testing Programme (formerly BCR) of the European Community (Young et al., 2006) (**Table 1.1**). However, the extractants used in SEPs may not accurately determine metal species because of their lack of selectivity, exemplified by BCR scheme in which the 'exchangeable' and 'carbonate' fractions are combined and determined within one stage (Young et al., 2006). Re-distribution and re-adsorption in the course of extraction may also occur due to pH changes and the new release of metals from residues and adsorption sites (Asami et al., 1995; Nirel and Morel, 1990; Young et al., 2006). Moreover, the speciation results may differ markedly in terms of different extractants, extraction times, and soil types.

Table 1.1 Sequential extraction procedures for soils: Tessier and BCR (Young et al., 2006)

Fraction (nominal)	Tessier		BCR	
Salt-extractable 'Exchangeable'	F1	1 M $MgCl_2$, pH 7	F1	0.11 M CH_3COOH
Acid-soluble 'Carbonate-bound'	F2	1 M NaOAc, pH 5		
Reducible 'Fe/Mn oxide-bound'	F3	0.04 M $NH_2OH{\cdot}HCl$ in 25% HOAc, pH 2	F2	0.5 M $NH_2OH{\cdot}HCl$ in 0.01 M HNO_3, pH 2
Oxidizable 'Sulphide/humus-bound'	F4	30% H_2O_2, 0.8 M NH_4OAc, pH 2 HNO_3	F3	30% H_2O_2, 1 M NH_4OAc, pH 2 HNO_3
Acid-digestible 'Residual'	F5	HF, $HClO_4$		Aqua Regia (ISO 11466 protocol)

1.3 Factors affecting cadmium bioavailability to plants

The speciation and bioavailability of Cd in soils are also greatly affected by the chemical and physical characteristics of soils as well as several factors related to plant growth and root metal acquisition.

1.3.1 Soil factors

1.3.1.1 pH

The effect of pH is of great importance to Cd bioavailability. Multiple regression analysis showed that soil pH and total soil Cd concentration were the two most significant variables that influenced the concentration of Cd in wheat grain (Adams et al., 2004). The effect of pH on metal bioavailability should however be examined differently in soil and solution cultures. In solution culture, decreasing pH has been found to give rise to a competitive inhibition of Cd uptake by hydrogen ions (Hatch et al., 1988; Tyler and McBride, 1982). In soil, decreasing pH will increase metal solubility in soil by reducing the partitioning of Cd to the solid phase (i.e. decreasing the negative surface charge on soil particles). Therefore, a negative correlation between soil pH and plant Cd uptake has frequently been reported (Castilho and Chardon, 1995; McBride, 2002; Miller et al., 1976).

Interpretation of the Cd uptake regulated by pH should be based on a function of both solubility and pH in the system. Low soil pH does not always increase Cd accumulation by plants because of the complex interaction of pH with other soil properties: the increased concentration of hydrogen ions in soil solution and the release of other ions during

acidification may compete with Cd for root uptake; there may also be variation in the response of plant species to increased available Cd because of physiological differences (Eriksson, 1989). Plant damage occurring at low pH has also been found to affect Cd uptake (Eriksson, 1989; Wang et al., 2006b). Wang et al. (2006b) studied the effect of pH on the uptake of Cd and Zn by *Thlaspi caerulescens* by adjusting soil pH to six different levels in two (low and high) Cd and Zn co-spiked soils. In both soils, the highest metal uptake was not found at the lowest pH level because of pH-induced Al and manganese (Mn) toxicity, but at the intermediate pH levels.

Reducing pH could also have significant negative impacts on soil microbial activity. Wang et al (2006a) found that soil alkaline phosphatase activity, arylsulphatase activity, nitrification potential and respiration were significantly reduced after acidification of soil. It indicates that although soluble Cd and Zn concentrations in the soil will be enhanced by reducing pH, care must be taken to avoid further degradation of the soil ecosystem.

1.3.1.2 Competitive cations

Metals in soil affect Cd bioavaiability in two ways: by competing with Cd for metal binding sites in the soil solid phase and complexation with natural or added ligands in solution phase; or by competing for uptake by plant roots. In soil, metals competing for binding sites or ligand complexation include Ca, cobalt (Co), chromium (Cr), Cu, Fe, Ni, Pb, Zn (Alloway, 1990; Christensen, 1987). Calcium and Fe are the major competitors of target metals due to their high concentrations found in soils and the relatively high stability constant of their

complexed species (Martell and Calvin, 1952). Below neutral pH, chelants added to soil will be bound to Fe at equilibrium because of the much higher stability of Fe-complexes than other metal complexes; whereas at alkaline pH where Fe compounds are insoluble, Ca becomes the primary competitor of the target metal with a solution ligand (Nowack et al., 2006).

As Cd is always found as a co-contaminant in Zn mining sites and also because of physico-chemical similarities, Zn has a competitive effect on Cd bioavailability and uptake in soil, while there also have been studies showing no effect or a stimulating effect of Zn on Cd uptake (Adiloglu, 2002; Honma and Hirata, 1978; Iwai et al., 1975). The different effects can be shown to be a result of different Zn supply from the growth media. Under Zn-deficient conditions, Cd accumulation in various plant organs including roots and shoots decreases with increasing Zn supply. This is because in Zn deficient soil, root-cell plasma membranes are disrupted in a manner that leads to abnormally high accumulation of Cd. Correcting Zn deficiency lowers the amount of Cd accumulated by plants by restoring the integrity of root-cell plasma membranes (Welch and Norvell, 1999). However, when Zn nutrition is adequate, addition of further Zn usually stimulates Cd accumulation in shoots and leaves (Welch and Norvell, 1999). Adequate or high Zn nutrition could help protect the plant from Cd toxicity, thus resulting in more accumulation of Cd in plant tissues (Köleli et al., 2004).

1.3.1.3 Soil salinity

Increasing soil salinity can significantly mobilise some metals in soil. Formation of chlorocadmium complexes can be shown by the following reaction equation:

$$Cd^{2+} + nCl^{-} \leftrightarrow CdCl_n^{2-n}, \quad (n=1, 2, 3, \text{and } 4) \qquad [1]$$

Increased uptake of Cd in plants was found with an increased Cl^- concentration in soil (Bingham et al., 1983; McLaughlin et al., 1994; McLaughlin et al., 1997; Smolders and McLaughlin, 1996a, 1996b; Weggler et al., 2004). Soil salinity can modify plant functions that are related to metal acquisition and translocation. Khoshgoftarmanesh et al. (2006) found increasing salinity could result in significant increases in shoot Cd concentrations of Zn-deficient genotypes of bread wheat. The enhanced Cd translocation to the leaves of *L. leucocephala* by sodium chloride (NaCl) indicates that transfer of metals through the food chain under saline conditions may be a matter of considerable agroecological significance (Helal et al., 1999). The mechanism involved in the process of stimulated plant uptake of Cd by high concentrations of Cl^- ion may be an enhanced diffusion of Cd to the plant root surface (Degryse et al., 2006; Smolders and McLaughlin, 1996a) and/or that the $CdCl_n^{2-n}$ species (in addition to Cd^{2+}) are phytoavailable (Smolders and McLaughlin, 1996a).

1.3.1.4 Phosphorus nutrition

Cadmium concentration varies among different phosphate fertilizers, and was found to be positively related to plant Cd concentration (Grant et al., 1995; He and Singh, 1994). Low Cd-containing P fertilizers has therefore been suggested as a method to reduce Cd

phytoavailability by the process of P-induced immobilisation (Bolan and Duraisamy, 2003). However, the total Cd concentration in soil is not changed by this remediation technique and the immobilised Cd may become phytoavailable when environmental conditions change, such as natural weathering processes. The effect of low-Cd P fertilizers on Cd uptake in plants may also be marginal. McLaughlin et al. (1995) demonstrated that changing Cd concentrations in a P fertilizer had an insignificant effect on Cd accumulation by potato tubers (*Solanum tuberosum* L), suggesting the natural Cd, or Cd accumulated through previous fertilization, was the major source of Cd taken up by potato crops. Sparrow et al. (1993) also found increased Cd uptake in potatoes fertilized with P; this was found not to be due to an increased Cd supply in the fertilizer but the application of P promoting root activity and enhancing access to soil Cd. Although the introduction of low-Cd P fertilizers in soil may not immediately affect Cd concentration in plants, it will minimise further Cd accumulation in soil.

1.3.1.5 Organic matter

The association of Cd with soil organic matter (SOM) may result in different effects on Cd solubility and availability depending on the phase of OM in soil. Application of organic materials such as animal manure, poultry litter and pig slurries have been found to decrease Cd availability and retain Cd in soil through increased sorption to the solid phase (Adriano et al., 2004; Bolan and Duraisamy, 2003; John et al., 1972; Zhang et al., 1998). However, in the solution phase, the opposing reactions of Cd sorption to the solid phase OM may occur because of the solubilisation of Cd by dissolved OM (DOM), the effects of which vary

according to soil pH. Under alkaline conditions, the complexation of Cd by DOM maintains the total Cd concentration in soil solution greater than that could be predicted from its solubility. For example, Grant et al (1999) found that at pH < 6, addition of SOM could actually decrease Cd concentrations in soil solution, whereas at pH 6 – 8, higher Cd concentrations were measured in solutions of soils with higher organic matter content, owing to complexation of Cd by soluble organic substances. This finding was also supported by Khan et al. (2006), who found that addition of humic acids (HAs) precipitated metals in a low pH soil and reduced metal solubility; under alkaline conditions, addition of HAs resulted in increased Cd and Pb solubility in soils and metal concentrations in plants. However, the interaction of metal, organic matter and pH is complex. It should be noted that at low soil pH, metal sorption on minerals and SOM is suppressed so that high metal concentrations in crops are generally found under these conditions (McBride, 2002). As toxicity is directly related to the concentration of free metal ions (Parker et al., 1988), it should also be recognised that increased OM may in another respect reduce the concentrations of free metal ions by complexation and therefore reduce subsequent metal availability and toxicity (Cabrera et al., 1988; Vaughan et al., 1993).

1.3.1.6 *Redox potential*

The solution Cd concentration varies greatly with changes in soil conditions, in particular, redox potential. Cadmium solubility was found to be reduced with decreased redox potential in sediment (Guo et al., 1997) and after one to three reduction-oxidation cycles in soils (Contin et al., 2007). However, findings are not always consistent. Chuan et al. (1996)

observed an increased Cd solubility with reduction and they attributed this to increased dissolution of Fe–Mn oxyhydroxides under the reduction conditions and the subsequent release of adsorbed metals.

The role of redox reactions on the behavior of Cd can be exemplified by the transformation of Cd in the rice paddies where flooding and drainage are required as irrigation management. Flooding of the paddies during the early growth stage of rice is to keep weeds down (Adriano, 1986). During this time, soil redox potential decreases, and sulfide formation in the submerged soil may allow for the formation of CdS and consequently reduces Cd availability (Bingham et al., 1976). The overall reduction of Cd concentration in rice shoots (at vegetative stage), straw and polished rice at maturity was reported to be 84, 89, and 79% under flooded conditions (Kashem and Singh, 2001). Rice paddies are then drained to facilitate harvesting and become oxic. Drainage increases Cd availability (Iimura and Ito, 1978; Minagawa et al., 1974) either by solubilisation of CdS during oxidation or by reduced competition from Fe and Mn for root absorption sites when Fe and Mn become insoluble (Adriano, 1986).

1.3.2 Plant factors

1.3.2.1 Plants species and genotype

The amount of Cd transported to shoots and edible organs differs between plant species and genotypes. Most of the monocotyledonous cultivars such as maize, oat, rye, and sorghum, and some dicotyledonous cultivars such as sunflower, cardoon and rape were found to be able to contain naturally high metals in their shoots (Hernández-Allica et al., 2008). In the edible

parts of six vegetables (i.e. Chinese cabbage, pakchoi, water spinach, towel gourd, eggplant and cowpea), water spinach had the highest Cd concentration while cowpea had the least (Wang et al., 2006c). One explanation for the various amounts of Cd accumulated by plants may be the effect of different root exudates in the rhizosphere of various plant species (**Table** 1.2). Some plants may also possess phytochelatins (PC), metallothioneins or phytosiderophores (PS) in internal tissues, which can complex metals and improve the plant's tolerance to metal toxicity (Kazuaki et al., 2007; Li et al., 2006; Schat et al., 2002).

The uptake and translocation of Cd in plant can vary greatly among different cultivars of the same species (He et al., 2006; Hocking and McLaughlin, 2000; Maxted et al., 2007; Pongrac et al., 2009). A field experiment conducted in China during 2002 – 2004 compared 38 rice genotypes. There was a large difference of Cd concentrations in straw, brown rice and grain chaff among the genotypes, with the total Cd uptake by brown rice varying between 0.96 and 28.58 μg/plant (He et al., 2006).

Table 1.2 Root exudation of organic acid of wheat, tomato, chickpea and white lupin under phosphorus-sufficient conditions (Neumann and Römheld, 1999)

Species	Exudation (μmol/h/g root fwt)				
	Malic	Citric	Malonic	Fumeric	Oxalic
Wheat	0.25±0.1	ND*	ND	trace	0.009±0.002
Tomato	0.14±0.03	0.13±0.07	ND	0.005±0.001	ND
Chickpea	0.001	0.001	0.014±0.002	trace	0.007±0.002
White lupin	trace	0.006±0.001	ND	trace	trace

***ND — Not detectable**

1.3.2.2 Root architecture

Among the different tissues of a plant, roots are critical organs for their role in absorbing and the translocation of Cd to shoots and reproductive organs, and for their role in nutrient acquisition. In order to transfer a large amount of soil metal from soil to plant shoots in phytoremediation, plant roots must tolerate metal toxicity and be active in contaminated soil zones. It was demonstrated by Mullins et al. (1986) and Li et al. (2005) that the greater the root length, root surface area and root volume of an ecotype, the higher concentrations of metals in leaves and stems. However, at very high root density, the total uptake levels off due to competition between roots for access to metals in soil (Mullins et al., 1986). In order to accumulate more metals from soil, some plants exhibit distinct root architecture, such as *Thlaspi caerulescens* and willows. *Thlaspi caerulescens* has a shallow root system with length per soil volume decreasing rapidly with depth. Both *Thlaspi caerulescens* and willows have a large proportion of fine roots (0 – 100 μm class) in the topsoil (0 – 0.1 m depth) (Keller et al., 2003).

1.3.2.3 Root rhizosphere

The rhizosphere is important for metal acquisition due to the abundance of dissolved organic matter and microbes in this microenvironment (Lorenz et al., 1997). Plant roots are known to release considerable amounts of organic carbon into the rhizosphere, resulting in a high population density of microorganisms. These microorganisms may alter the rhizosphere environment by affecting (1) the growth and morphology of roots; (2) the physiology and

development of plants; (3) the speciation of metals; and (4) root uptake processes (Lasat, 2002; Perry et al., 2007; Tao et al., 2005) (**Figure 1.3**).

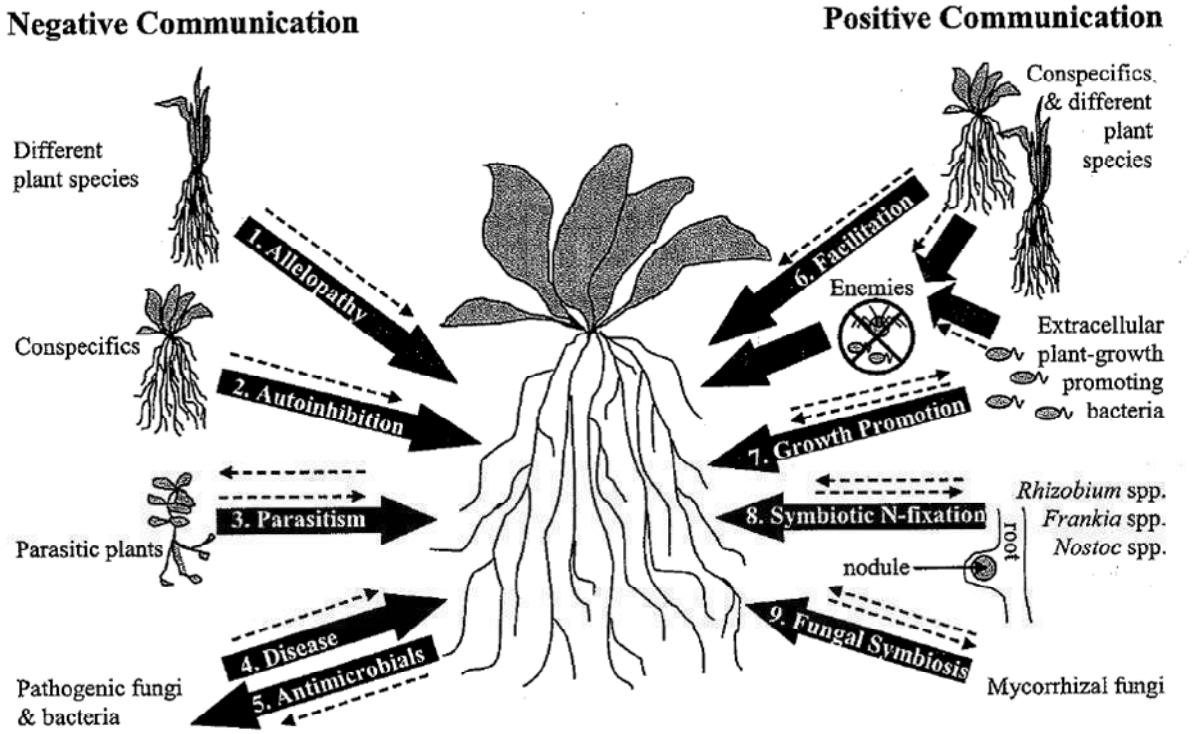


Figure 1.3 Modes of chemical-mediated communication between plant roots and between plant roots and soil microbes in rhizosphere. Block arrows indicate different types of interactions (Perry et al., 2007).

The OM that is released into the rhizosphere by plants and microorganisms includes a variety of high- and low-molecular-weight organic acids (HMWOAs and LMWOAs), PS, mucilages, proteins, amino acids, carbohydrates, and phenolics (Violante et al., 2005). The concentrations of root excreted LMWOAs, such as formic acid, acetic acid, oxalic acid, malic acid and citric acid, may vary markedly depending on the species and nutrient adequacy/deficiency (Jones, 1998; Neumann and Römheld, 1999). The pH in root cell cytosol ranges from 7.1 to 7.4 (Marschner, 1986), therefore the organic acids dissociate in the cytosol before being released to the rhizosphere because of their low pKa, and the species observed in

soil solution are their conjugated bases (Hinsinger et al., 2003). Although the release of citrate was found to account for a root acidification process in a particular case of the clustered roots of white lupins (Dinkelaker et al., 1989), the rates of release of organic anions for most plant species are two or three orders of magnitude lower than the rates of H^+ release that arise from a larger cation than anion uptake (Hinsinger et al., 2003). Thereby the rhizosphere acidification and metal solubilisation by organic acids are limited. Chaney et al. (2007) pointed out that engineering plants to secrete chelating ligands into the rhizosphere and then absorb the metal-ligand complexes into roots in the manner of PCs, metallothioneins or PS, is unlikely to be successful because of its trivial improvement, non-specificity or instability.

1.4 Remediation of Cd-contaminated soils

In order to reduce Cd concentrations in soil, various remediation measures have emerged during the last few decades. These include physicochemical methods that either reduce the soluble but not the total Cd concentrations (e.g. immobilisation), or reduce the Cd availability in soil (e.g. electrokinetics, soil washing), and 'green' methods that are less harmful to the environment (phyto- or bio-remediation).

1.4.1 Physicochemical methods for remediation of Cd-contaminated soil

The *in-situ* immobilisation of Cd in soils through the use of chemical agents or amendments can reduce Cd mobility to ground water and Cd phytoavailability for plant roots. Phosphate compounds are ideal immobilisation agents, because they can either adsorb metals (e.g. Cd,

Cu, nickel(Ni), Pb, and Zn) or co-precipitate with them (Bolan and Duraisamy, 2003). Dheri et al. (2007) found addition of phosphate (as KH_2PO_4) to a Cd-toxic soil could enhance dry matter yield of spinach shoot by immobilising Cd. Other chemical agents have also been proven to markedly decrease Cd mobility in soils and mitigate Cd uptake by plants, such as lime, zeolites, iron oxides/hydroxides, manganese hydroxides and sand-cement-clay mixtures (Contin et al., 2007; Grant et al., 1999; Hong et al., 2009; Shawabkeh, 2005). These immobilisers reduce the Cd partitioning to the solution phase by changing the pH or/and increasing solid surface adsorption (section 1.2.2.1). However, immobilisation does not reduce the total Cd concentrations in soil, and some immobilising agents may not perform consistently because of inactivation of their performance with time.

If the aim of soil remediation is to reduce the total Cd concentration, electrokinetics (EK) can be a promising *in situ* or *ex situ* decontamination process. It involves applying a low direct current or a low potential gradient to electrodes that are inserted in the contaminated soil. Charge (positive/negative) and the direction of the pore water flow dominate the metal's transport towards the anode or cathode electrodes depending on the element of interest. Chen et al. (2006) investigated Cd fractionation in a Cd-spiked soil before and after EK remediation. They observed that the exchangeable (1M sodium acetate, pH 8.2) and soluble Cd easily migrated to the cathode region with an average Cd removal efficiency of 69% of the initial total Cd concentration. However, the soil pH decreased drastically from 6.7 to 1.7 after 60 h. Soil acidification to this extent is an undesirable change in soil quality. Other limitations

of this technique are precipitation of species close to the cathode, electrode corrosion and excess soil heating (Kim et al., 2002).

The remediation practice of soil washing is the injection or infiltration of an aqueous solution into a contaminated soil or groundwater area, followed by down gradient extraction of groundwater, aboveground treatment and discharge or reinjection (Rosario et al., 2004). It is the reverse process of *in situ* immobilisation in that Cd availability and mobility is increased by using flushing agents such as aminopolycarboxylic acids, (bio-)surfactants, natural organic acids and inorganic acids. However, some flushing agents (e.g. acids) may damage soil physicochemical properties and cause the loss of soil nutrients, some others may result in soil recontamination after application.

1.4.2 Phytoremediation of Cd-contaminated soil

Plant-based remediation techniques, termed phytoremediation, offer an alternative soil remediation method with potentially lower cost, little influence on the physical and chemical structure of the soil, and the ability to recycle the extracted metal (Chaney et al., 1997). It is often referred to as bioremediation, botanical-bioremediation, or "green remediation". At present, phytoremediation is only an economically important clean-up technique for slightly contaminated soils. Heavily contaminated soils can only be cropped using highly metal-resistant plants (Ernst, 1996). Phytoremediation is further divided into phytoextraction, phytovolatilisation, phytofiltration and phytostabilisation. For Cd, the most widely employed and applicable phytoremediation method is phytoextraction.

Phytoextraction uses plants to extract metals from soil and has received considerable attention in recent decades (Chaney et al., 1997; Robinson et al., 1999). In a phytoextraction operation, plants are grown in a soil that has been contaminated by one or more metals. The plants used for phytoremediation either accumulate the metals specifically (i.e. hyperaccumulators) to high concentrations, or they are induced to do so by soil amendments. When mature, the crop is harvested and burned, leaving a small volume of ash containing a high concentration of the target metal that can be smelted for recovery. If the metal is of low value, the ash can be stored or immobilised so it does not pose a risk to the environment (Robinson et al., 1999).

Most hyperaccumulators, e.g. *Thlaspi caerulescens* for Cd and Zn, *Spirodela polyrhiza* for Cd and *Salvinia molesta* for Pb (McCutcheon and Schnoor, 2003), do not have a large biomass yield. Therefore, high biomass crops which do not accumulate specific metals have been proposed for use in phytoremediation; these include sunflower (Fässler et al., 2010; Lesage et al., 2005; Meers et al., 2005b; Tandy et al., 2005; Turgut et al., 2004), mustard (Ebbs et al., 1997; Jiang et al., 2003; Römkens et al., 2002; Schaider et al., 2006), maize (Hernández-Allica et al., 2008; Luo et al., 2006; Meers et al., 2004; Zhao et al., 2010), lupin (Collins et al., 2002; Meers et al., 2004) and rice (Murakami et al., 2007). Some woody species have also been investigated for use in phytoextraction, e.g. Dos Santos Utmazian and Wenzel (2007) grew willow and poplar species on polluted soils to examine their efficacy for uptake of Cd and Zn. Generally, the tested species tolerated high metal concentrations in soils and had higher Cd and Zn concentrations in leaves than in roots.

High biomass plants used in phytoextraction are often assisted by addition of chelants, due to the inability of most plant species to accumulate high concentrations of metals in their shoots. Though chelant-assisted phytoextraction has been asserted to be useful for Cd remediation (Table 1.3), the results and conclusions from these studies are inconclusive. A number of phytoextraction studies were conducted in solution culture (Laurie et al., 1991a, 1991b; Tandy et al., 2006a; Wang et al., 2008) or in soils spiked only with Cd (Jiang et al., 2003; Quartacci et al., 2005). Addition of soluble Cd to solution cultures or soils does not reflect the chemistry of Cd in soils in the field. Cadmium is rarely found present in soils on its own, is rarely present in highly soluble species, and is usually accompanied by Zn and other metals at most contaminated sites. Consequently, the assessment of plant uptake from solutions or from soils spiked only with Cd likely overestimates plant extraction efficiencies. In addition, during solution culture studies there is the possibility of the depletion of essential nutrients, which could result in increased Cd uptake owing to less competition from other cations, such as Ca or Zn (Jarvis et al., 1976; Karez et al., 1990), or the stimulative role of phytosiderophores driven by Fe deficiency (Kazuaki et al., 2007). In addition, the Cd concentrations used in some studies (Lehmann and Rebele, 2004; Quartacci et al., 2005) were well above those encountered even in contaminated soils (Lorenz et al., 1997), which makes the phytoextraction efficiencies measured unrealistic.

Table 1.3 Case studies of chelants used in phytoextraction

Chelator	Soil or solution pH	Metal	Phytotoxicity	Dose giving highest metal uptake	Reference
EDTA	5.5 (KCl)	Cd, Cr, and Ni	Stunted growth	0.27 mmol/kg	Turgut et al. (2004)
	5.0 (H_2O)	Zn	Depressed transpiration	3.4 mmol/kg	Collins et al. (2002)
	6.8 ($CaCl_2$)	Pb		10 mmol/kg	Kos and Lestan (2003)
	5.2 (Hydroponics)	Cu, Fe, Mn, and Zn	Chlorotic, inhibited growth	40 μM for Fe and Mn, 24 μM for Cu and Zn	Laurie et al. (1991a)
	7.2 (H_2O)	Cd, Cr, Cu, Ni, Pb, and Zn		2 mmol/kg EDTA for Pb	Meers et al. (2004)
	NA[†] (Hydroponics)	Cd, Cu, Pb, and Zn		500 μM EDTA for Pb	Zhao et al. (2010)
EDDS	6.0 (Hydroponics)	Cu, Pb, and Zn	No toxic effect	500 μM for Pb	Tandy et al. (2006a)
	6.42 ($CaCl_2$)	Cd, Cu, Pb, and Zn	Reduced shoot/root dry mass	10 mmol/kg for Cu and Pb	Tandy et al. (2006b)
	6.8 ($CaCl_2$)	Cd, Pb, and Zn		10 mmol/kg	Crčman et al. (2003)
CA	5.5 (KCl)	Cd, Cr, Ni	Stunted growth	5.2 mmol/kg	Turgut et al. (2004)
	4.2 (KCl)	Cd, Zn	No toxic effect	(1 mmol/kg EDGA[#]+1 mmol/kg CA)*2	Römkens et al. (2002)
IDSA[*]	NA (Hydroponics)	Cd, Cu, Pb, and Zn		500 μM IDSA for Cd and Cu	Zhao et al. (2010)

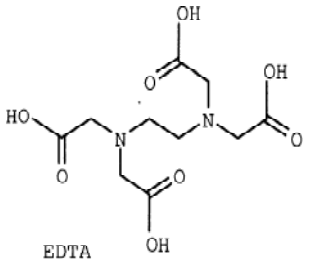
[†]NA — not available

[#]EDGA — ethylenediaminediglutaric acid

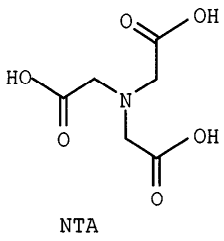
[*]IDSA — iminodisuccinic acid

1.4.2.1 Synthetic chelants

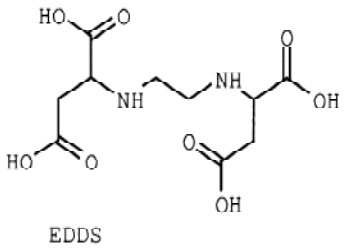
Chelants have been widely used in phytoextraction because they can increase metal solubility in soils and may enhance metal uptake and translocation to shoots (Meers et al., 2005a; Nowack et al., 2008; Quartacci et al., 2005; Tandy et al., 2005; Turgut et al., 2004; van Engelen et al., 2007). Bioavailability of metals may be artificially increased by adding synthetic chelants, such as ethylenediaminetetraacetic acid (EDTA) and nitrilotriacetic acid (NTA) (**Figure 1.4**).



(Ethylenediaminetetraacetic acid)



(Nitrilotriacetic acid)



(Ethylenediaminedisuccinic acid)

Figure 1.4 Structural formulas of EDTA, NTA and EDDS. **(**Reprint with permission from Bucheli-Witschel, M., Egli, T., 2001. Environmental fate and microbial degradation of aminopoly-carboxylic acids. FEMS Microbiol. Rev. 25, 69-106. Copyright John Wiley and Sons Publishing.)

Ethylenediaminetetraacetic acid (EDTA), which contains six donor atoms and acts as a hexadentate ligand, has been widely used in metal phytoextraction (Blaylock et al., 1997; Komárek et al., 2007; Laurie et al., 1991b; Vassil et al., 1998; Wallace et al., 1977). It was

originally believed to be the most efficient for metal phytoextraction with lower doses found to yield the best results (Turgut et al., 2004). Van Engelen et al. (2007) found a 10-fold increase of Cd concentrations in the shoots of *Brassica juncea* after EDTA application (1:1 chelant to metal molar ratio) to a Cd-contaminated soil. However, contradictory results have been reported showing no observable impact of EDTA added as a solution to the soil at a rate of 1.78 mmol/L on plant Cd uptake (Jiang et al., 2003) or an actual decrease in Cd accumulation due to severe growth depression using a rate of 7 mmol EDTA/kg soil (Lesage et al., 2005). Based on its strong metal complexation ability and poor degradability, EDTA also increases environmental risks by potentially leaching the mobilised metals down through soil profile to ground water. Chen et al. (2004) found that 23% of the initial total Cd, 16% of Cu, 4% of Pb, and 13% of Zn in soils were leached from the soil columns in an EDTA-assisted phytoextraction process, and cultivation of sunflower in the system did not change the leaching patterns.

Nitrilotriacetic acid (NTA), a quadridentate chelant with four donor atoms, usually forms 1:1 complexes with metal ions by establishing three chelate rings with four co-ordination sites of the metal (Bucheli-Witschel and Egli, 2001). Quartacci et al. (2005) investigated the utility of NTA for phytoextraction of Cd by Indian mustard *(Brassica juncea* (L.) Czernj, *Brassicaceae)*, and concluded that NTA could be useful in making Cd available at both environmental and phytotoxic concentrations. However, in that study the soil was spiked with only Cd (50 to 200 mg/kg), therefore it did not take into account the possible competition with other metals for chelant binding. Unlike the results of Quartacci et al. (2005), Meers et

al. (2004) did not observe significant effects of NTA amendment (2 mmol/kg, applied prior to sowing) to a dredged contaminated sediment on Cd uptake by maize. They attributed the phenomenon to the rapid mineralisation and the low dose of the NTA used. Indeed, the complexation strength of NTA is lower than that of EDTA (**Table 1.4**), and it is also more biodegradable (Means et al. 1980). The International Agency for Research on Cancer (IARC) has listed NTA as a possible carcinogen to humans (Group 2B)(IARC, 1990) that restricts its use for soil remediation practices.

Table 1.4 Stability constants (log K) of various metal chelates (exampled by EDTA, NTA and citric acid)(Furia, 1972)

Metal (to right) Ligand (below)	Al(Ш)	Ba	Ca	Cu	Co(II)	Fe(II)	Fe(Ш)	Hg	Mg	Mn	Ni	Sr	Zn
EDTA	16.1	7.8	10.7	18.8	16.2	14.3	25.7	21.5[a]	8.7	13.6	18.6	8.6	16.5
NTA	>10	4.8	6.4	12.7	10.6	8.8	15.9		5.4	7.4	11.3	5.0	10.5
Citric acid	11.7[b]	2.3	3.5	6.1	4.4	3.2	11.9	10.9[a]	2.8	3.2	4.8	2.8	4.5

[a] Martell and Smith (1989)

[b] Martin (1994)

1.4.2.2 Natural chelants

Due to the drawbacks of synthetic chelants outlined above, natural chelants have been proposed for use in phytoextraction, mainly due to their biodegradability and low toxicity. One widely studied natural chelant is citric acid (CA)/citrate, which is the dominant low molecular weight organic acid (LMWOA) present in the proteoid root exudates of *Lupinus albus* and is effective in metal and phosphate mobilisation (Jones, 1998). Citric acid added at

a rate of 10 mmol/kg soil was reported to improve Cd solubility in a soil without the risk of leaching, and the net removal of Cd, Cu and Pb by *E. Crusgalli* was as efficient as synthetic chelants, e.g. EDTA (Kim and Lee, 2010). Given that CA has a much lower metal binding strength than EDTA (**Table 1.4**), the observed high efficiency could be due to a transporter-specific active uptake of citrate or its analogue in plants suggested by Bell et al. (2003). However, the metal solubilisation and phytoextraction efficiency of natural chelants is not always observed to be good (do Nascimento, 2006; Meers et al., 2007; Turgut et al., 2004). do Nascimento et al. (2006) compared the performance of synthetic chelants with several LMWOAs in solubilising metals using an artificially spiked soil. They found the removal of Cd from soils was not effective using citrate or oxalate added at a rate of 10 or 20 mmol/kg, compared to good extraction efficiencies for Cd, Cu, Ni, and Zn by EDTA and diethylenetriaminepentaacetic acid (DTPA). In the study of Turgut et al. (2004), CA applied at 1.0 and 3.0 g/kg (equivalent to 5 and 15 mmol/kg) did not enhance the uptake of Cd by *Helianthus annuus*, indeed it caused a severe phytotoxiciy. When the doses were reduced to 0.1 and 0.3 g/kg (equivalent to 0.5 and 1.5 mmol/kg), CA treatment was found not to be different from the control. Natural chelants normally have a lower metal complexation ability compared to synthetic chelants (**Table 1.4**), thus the metal solubilisation is considerably lower accordingly. It addition there is the possibility of re-adsorption and re-precipitation of metals released during CA/citrate degradation that could limits its phytoextraction efficiency.

Ethylenediaminedisuccinic acid (EDDS) (**Figure 1.4**) was the first natural aminopolycarboxylic acid that was isolated from culture filtrates of the actinomycete

Amycolatopsis orientalis (Bucheli-Witschel and Egli, 2001). It has substituted the traditional synthetic chelant EDTA in a number of commercial products (e.g. industrial detergents) due to its similar chemical properties and biodegradability. Ethylenediaminedisuccinic acid is a structural isomer of EDTA, and it exhibits two chiral C-atoms which result in the existence of three different stereoisomers, i.e. [S,S]-, [R,S]- and [R,R]-EDDS (Bucheli-Witschel and Egli, 2001). Uptake of metals was found to be more effective when EDDS was used to assist phytoremediation, compared to EDTA, but the effects were still considered insufficient for remediation purposes (Meers et al., 2005c). Several studies have shown EDDS exhibited no enhanced Cd extraction when compared with other chelants (e.g. EDTA) (Evangelou et al., 2007; Komárek et al., 2007; Luo et al., 2006; Luo et al., 2005). This is likely because EDDS is not Cd-specific (Koopmans et al., 2008; Tandy et al., 2006b). As EDDS is a biodegradable product, Luo et al. (2006) found that metal solubility (Cd, Cu, Pb, and Zn) in a co-spiked soil decreased following EDDS addition as time progressed. The poor phytoextraction efficiency of Cu obtained by Komárek et al. (2010), who examined use of EDDS for phytoremediation of Cu-contaminated vineyard soils under "ideal" conditions (single metal, low contamination, metal tolerant plant species, root colonization by mycorrhizal fungi), indicates that "research focused on EDDS-enhanced phytoextraction of metals from contaminated soils has probably reached a dead end".

1.4.2.3 Biosurfactants

Considering their environmental compatibility and relatively strong metal binding strength, biosurfactants have been used in soil washing (Almeida et al., 2009; Mulligan et al., 2001).

They can increase degradation of hydrophobic organic soil contaminants, enhance removal of organics by biodegradation (Maslin and Maier, 2000; Zhang and Miller, 1992, 1995) or soil washing (Urum et al., 2005), and facilitate removal of metals (e.g. Cd, Ni, Pb, and Zn) (Herman et al., 1995; Wang and Mulligan, 2004a). Rhamnolipid, produced by *Pseudomonas aeruginosa*, is one of the most widely studied biosurfactants (**Figure 1.5**).

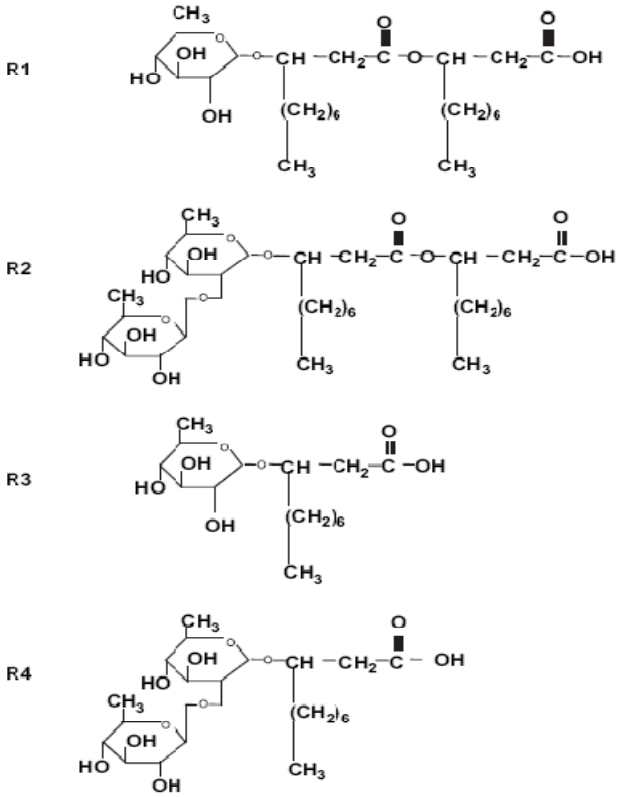


Figure 1.5 Structure of four rhamnolipids produced by *P. aeroginosa*. (Reprint with permission from Mulligan, C. N., 2005. Environmental applications for biosurfactants. Environ. Pollut. 133, 183-198. Copyright Elsevier Publishing.)

Pseudomonas aeruginosa mainly produces two forms of rhamnolipid called monorhamnolipid (R1), consisting of one molecule of rhamnose and two molecules of β-hydroxydecanoic acid, and dirhamnolipid (R2), where two molecules of rhamnose are linked to two molecules of β-hydroxydecanoic acid (Nitschke et al., 2005) (**Figure 1.5**). The morphology of their molecular aggregates changes with pH, from vesicles under acidic

conditions, to lamella, lipid particles, and finally to micelles around pH 6.8 (Ishigami et al., 1987). Rhamnolipid biosurfactants can reduce the surface tension of water from 72 mN/m to values below 30 mN/m (Syldatk et al., 1985). Their hydrophilic carboxyl group is responsible for complexation with a variety of metals.

Some authors have reported the use of rhamnolipid for removal of metals by soil washing (Aşçi et al., 2008; Juwarkar et al., 2007; Mulligan and Wang, 2006; Wang and Mulligan, 2004b). The efficiency of rhamnolipid application in soil will partly depend on its interaction with soil constituents, because any sorption and precipitation of rhamnolipid will decrease its solution-phase concentration. Concentrations of clay, metal oxides and organic matter are considered the three major factors affecting rhamnolipid sorption to soils (Herman et al., 1995; Ochoa-Loza et al., 2007; Torrens et al., 1998). However, these studies only examined acidic or neutral soils. Noordman et al. (2000) demonstrated a linear relationship between rhamnolipid hydrophobicity and sorption to soil, and suggested the adsorption of rhamnolipid to soils involved the formation of surface aggregates. It was considered that R1 and R2 mixtures might sorb less strongly than R1 alone due to the more hydrophilic nature of R2 (Ochoa-Loza et al., 2007), and thus could be more available to mobilise Cd and increase extraction efficiency. Torrens et al. (1998) reported that increasing potassium (K) in solution led to a rapid rise in rhamnolipid sorption by cation bridging, thus confirming the importance of maintaining low K concentration in soil remediation using rhamnolipid.

Studies using surfactants for phytoextraction are limited. Only three research papers have discussed the potential of rhamnolipid in phytoextraction (Gunawardana et al., 2010; Johnson

et al., 2009; Jordan et al., 2002). Jordan et al. (2002) found the application of rhamnolipid at 1 mmol/kg in soil did not increase metal concentrations (Cu, Pb, and Zn) in both *Atriplex* and *Z. mays* (**Figure 1.6**). The authors attributed the absence of any improved metal phytoextraction to the relatively large structure of the rhamnolipid-metal complex. However, in the same study, but using hydroponics, they identified the presence of rhamnolipid (applied at 20 mM) in the plant shoots, whereas this was not seen for EDTA. There was no toxicity data provided in this study, so it is not possible to relate the uptake of rhamnolipid in shoots to a lipophilic uptake or toxicity. Furthermore, the design of the experiment (one or two concentrations only and pre-harvest application only) may not have determined the optimum conditions for metal uptake.

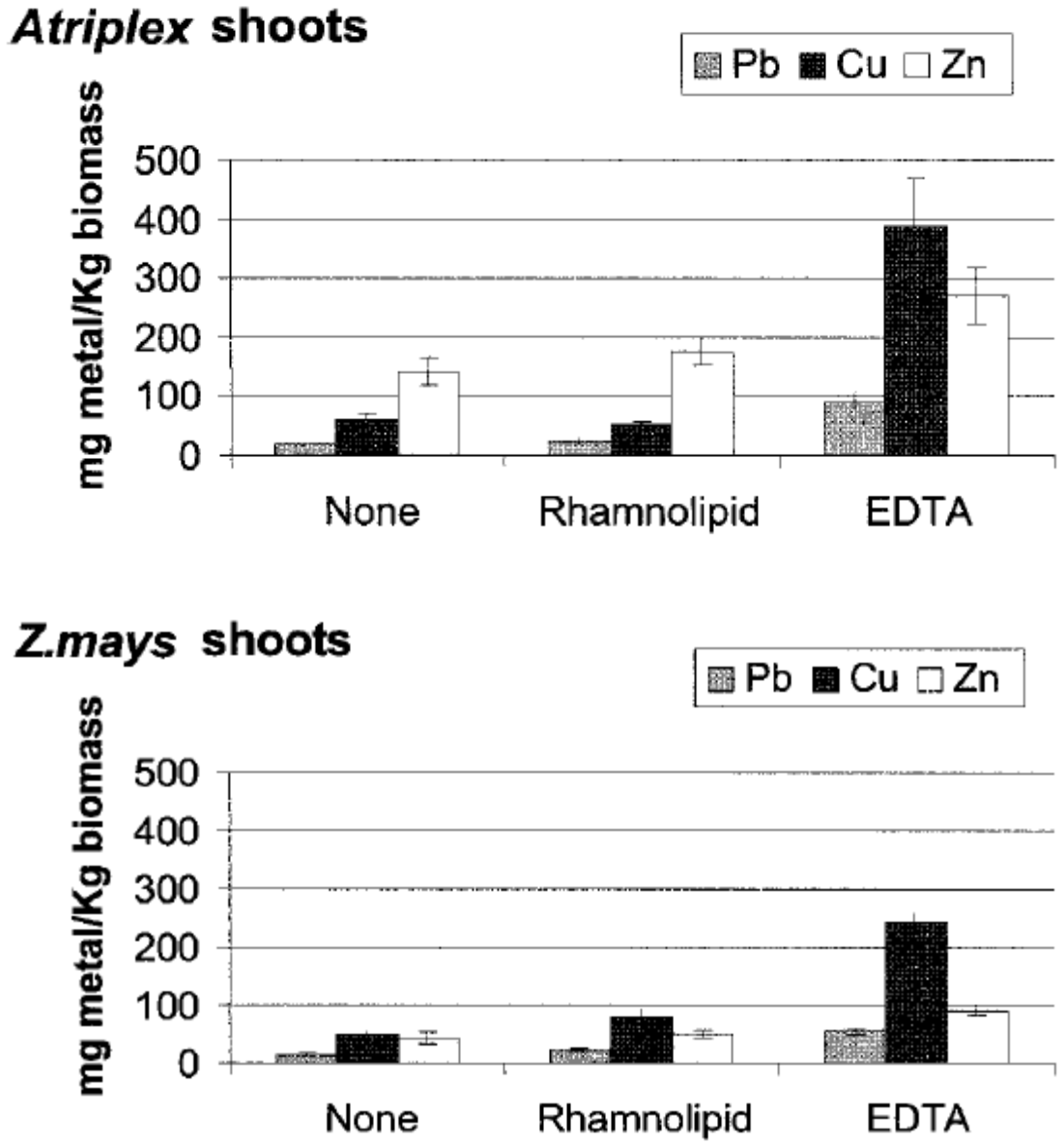


Figure 1.6 Shoot metal (Pb, Cu, and Zn) concentrations per kilogram plant biomass (dry wt) receiving 1 mmol of EDTA, 1 mmol of rhamnolipid, or no chelator treatment over a one-week period prior to harvesting. (Reprint with permission from Jordan, F.L., Robbin-Abbott, M., Maier, R.M., Glenn, E.P., 2002. A comparison of chelator-facilitated metal uptake by a halophyte and a glycophyte. Environ. Toxicol. Chem. 21, 2698-2704. Copyright John Wiley and Sons Publishing)

Gunawardana et al. (2010) and Johnson et al. (2009) examined the effect of rhamnolipid on Cd, Cu and Pb phytoextraction in hydroponic experiments. In the study of Johnson et al. (2009), rhamnolipid applied at low and high concentrations (0.04 mM and 0.35 mM) did not

improve Cu uptake in roots and shoots of Indian mustard and caused a reduction of root biomass yield. The authors suggested there was no increased uptake by roots or shoots because the intact rhamnolipid micelles would not be likely to traverse cell membranes and cortical tissues. Gunawardana et al. (2010) found a rhamnolipid concentration of 1.5 mM moderately increased Cd concentrations in the shoots of *Lolium perenne*, and they believed that this was due to a greater uptake of the rhamnolipid-Cd complex allowed by a rhamnolipid-induced damage to the plants. However, the rhamnolipid complexed form of Cd only constituted 5% of the Cd species in their study which could not explain the improved Cd uptake by shoots. Moreover, the suggestion by the authors of using rhamnolipid to moderately enhance Cd uptake, which was made on the observation of shoot concentration but not accumulation, is in fact weakened by the large variation of shoot biomass yield in this study (Gunawardana et al., 2010).

A recent study of Stacey et al. (2008) showed that rhamnolipid concentrations ≤ 10 µmol/kg applied to a Zn-deficient calcareous soil improved Zn concentrations in durum (*Triticum durum* L. cv. Balcali-2000) and bread wheat (*Triticum aestivum* L. cv. BDME-10) shoots. In addition, Zn was found predominately in the form of intact Zn-rhamnolipid complexes in the roots of the rhamnolipid-treated samples (**Table 1.5**, **Figure 1.7**), compared to a predominant presence of Zn-phytate in roots treated with either $ZnSO_4$ or Zn-EDTA (Stacey et al., 2008). This finding suggests rhamnolipid could actually increase metal uptake by a direct absorption of the intact metal-rhamnolipid complex (Stacey et al., 2008).

Table 1.5 Percentage of Zn species in canola roots at selected Zn hotspots determined by linear combination fitting of k^2-weighted μ-XAS spectra (adapted from Stacey et al. (2008)).

Root treatment	Phytate (%)	Rhamnolipid (%)	Glutamate (%)	Lysine (%)	$ZnSO_4$ (%)
no Zn	70		23.1	6.9	
$ZnSO_4$	87				13
Zn-rhamnolipid A	16.7	55.3			28
Zn-rhamnolipid B	12.4	87.6			

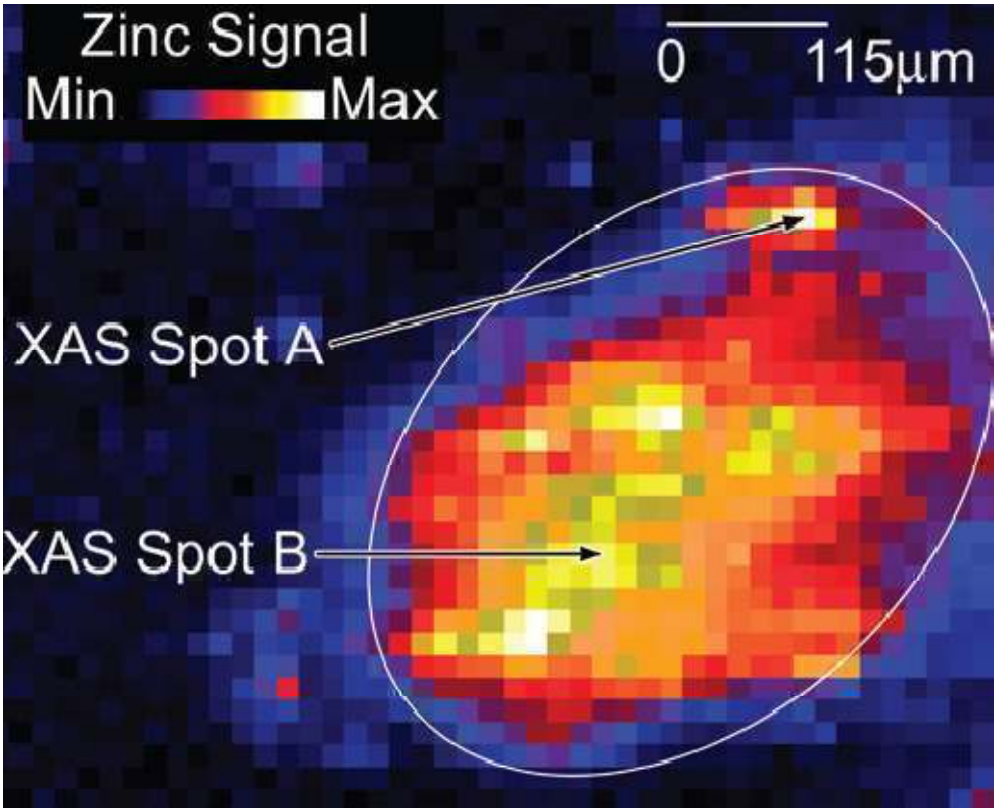


Figure 1.7 Zinc μ-X-ray fluorescence showing the distribution of Zn in a canola root treated with Zn-rhamnolipid. (Reprinted with permission from Stacey, S. P., McLaughlin, M. J., Cakmak, I., Hetitiarachchi, G. M., Scheckel, K. G.,Karkkainen, M., 2008. Root uptake of lipophilic zinc-rhamnolipid complexes. J. Agric. Food Chem. 56(6), 2112-2117. Copyright American Chemical Society Publishing.)

1.4.3 Mechanisms explaining chelant-enhanced phytoextraction

Plant uptake of trace elements is normally the first step in their entry to agriculture food-chains. Changing soil pH or the presence of root exudates in the rhizosphere can alter Cd speciation and change the rate and extent of root Cd uptake (John, 1972; Kim et al., 2010; Luo et al., 2008). Artificially adding chelants to soil can also mobilise Cd. Subsequently, the three basic steps involved in metal phytoextraction are (a) transport of metals across the plasma membrane of root cells; (b) xylem loading and translocation; and (c) detoxification and sequestration of metals at the whole plant and cellular levels (Yang et al., 2005).

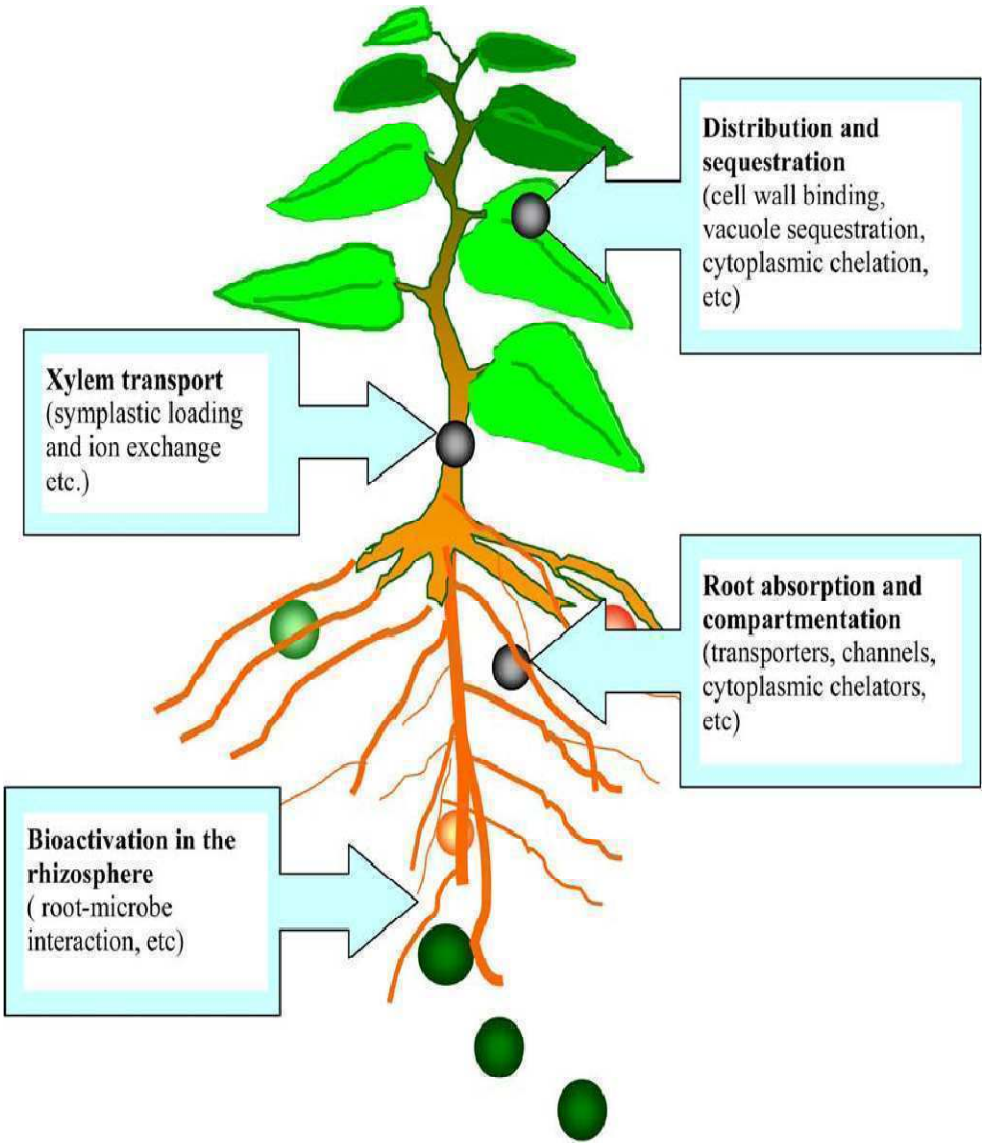


Figure 1.8 Major processes involved in metal phytoextraction. (Reprinted with permission from Yang, X. E., Feng, Y., He, Z. L., Stoffella, P. J., 2005. Molecular mechanisms of heavy metal hyperaccumulation and phytoremediation. J. Trace Elem. Med. Biol. 18, 339-353. Copyright Elsevier Publishing.)

Roots have a strict selectivity for the metal species they prefer to be taken up. In plant roots, two parallel transport pathways for water through the root cortex toward the stele are: 1) a pathway of passive transport by diffusion and convection through the apoplasm, namely cell walls and intercellular spaces, and 2) a pathway of active transport from cell to cell in the symplasm (selective transport across membranes) (Nowack et al., 2006). Essential metals like

Cu and Zn are normally selectively taken up by plants through a symplastic pathway in the form of free ions, whereas they may take an apoplastic uptake pathway in the presence of chelants (Tandy et al., 2006b). The non-essential metal Cd may be metabolically facilitated for plant uptake showing multiple phase absorption (Cataldo et al., 1983) within the normal Cd concentration range of 3 to 200 nM encountered in soil solutions (Helmke, 1999), while at higher concentrations, apoplastic influx of Cd may outweigh its absorption through the symplasm (Cataldo and Wildung, 1978).

There is wide acceptance that free metal ions are best correlated with metal bioavailability and are the metal species most readily absorbed by plant roots (Bingham et al., 1983; Checkai et al., 1987; Pavan and Bingham, 1982). Many studies have also shown that chelants can alter metal speciation and consequently influence metal bioavailability (Collins et al., 2002; Laurie et al., 1991a, 1991b; McLaughlin, 2002; Tandy et al., 2006b). The influence of chelants on Cd uptake can be either positive or negative (Wallace et al., 1977) because of the complexity of the interrelationships of Cd with other trace elements (e.g. Cu, Fe, Mn) (John, 1976), the biodegradability of the chelants, and the direct effect of the chelant on root growth and function. In the rhizosphere, the absorption of metals will be independent of the nature of the complexing agent if the dissociation rate of metal complexes at the plasma membrane is faster than the rate of uptake by the roots. In this case the dissociated metals may bind with intracellular binding groups, such as sulflhydryl functional groups on proteins, after bypassing the cell membrane. Inversely, if dissociation of metal from the complexing agent is slower

than absorption, the uptake of metals will depend on the nature of the complexing agent, in terms of kinetic and thermodynamic properties of the complexes (Laurie et al., 1991b).

The effect of chelant on metal uptake in soil experiments should be distinguished from that in solution cultures. In solution culture, the addition of chelants reduces the free metal ion activity in solution and subsequently reduces metal uptake (DeKock, 1956; DeKock and Mitchell, 1957; Nor and Cheng, 1986). However in soil experiments, increased metal uptake can be found in the presence of the chelant if a diffusion limitation to metal uptake is alleviated (Elgawhary et al., 1970) or if the complexed chelate is taken up through apoplastic pathway following root damage (Epstein et al., 1999; Vassil et al., 1998). Collins et al. (2002) and Luo et al. (2006) confirmed in their studies that a significantly enhanced uptake of metals by the application of chelants might be attributed to root damage and to the further breakdown of a root exclusion mechanism. Indeed, the endodermis of the roots may be disrupted when higher concentrations of chelants are added to soil or if a crop is transplanted into contaminated soil, which facilitates phytoextraction by allowing the free passage of chelated metals into the stele (Nowack et al., 2006). The toxic effect of chelants on plants is a problem that needs to be avoided or managed (through different chelant application strategies) in effective phytoremediation.

Normally, metal complexes with negative charge will not be favoured for root uptake because of the repulsion from the negative charged cell wall and cytoplasm (Bell et al., 2003; Gregory, 2006). Unlike the negatively charged hydrophilic organic metal complexes, uncharged or lipophilic metal complexes are bioavailable through direct assimilation. For instance, the

lipophilic compound diethyldithiocarbamate (DDC) can form neutral complexes with metals. It was shown that substantially more metals were found in the cell of *T. weissflogii* in lipophilic $Cd(DDC)_2^0$ and $Pb(DDC)_2^0$ complexes than that when hydrophilic complexes formed (Phinney and Bruland, 1994). Uncharged inorganic or lipophilic organic Cd complexes have been found to be taken up by plant through direct apoplastic diffusion (Berkelaar and Hale, 2003; McLaughlin et al., 1998a; McLaughlin et al., 1998b; Smolders and McLaughlin, 1996b). Wu et al. (1999) also suggested that designing a metal-binding ligand for phytoremediation of contaminated soil should optimise the metal-chelate's lipophilicity. In a recent study, Stacey et al. (2008) found that rhamnolipid increased Zn uptake by canola roots at ice-cold temperatures, indicating that the lipophilic Zn-rhamnolipid was taken up via a non-metabolically mediated pathway in plant roots. Therefore, rhamnolipid could be an alternative to traditional chelants to improve Cd phytoextraction efficiency.

1.5 Improving cadmium phytoavailability using chelants

There is still a need to develop improved phytoremediation strategies for Cd-contaminated soils. Use of synthetic chelants has not proved successful, and the search for natural chelants to improve phytoextraction continues. The requirements of a successful chelant for Cd phytoextraction are 1) relatively high binding strength with Cd; 2) lack of toxicity to plants; 3) low sorption to soil; 4) degradability, and 5) low cost.

1.5.1 Metal binding strength

The strength of the complexation between organic ligands and metals is usually expressed in terms of their stability constant. The following equilibrium involves the interaction of an ion with a chelant,

$$M + xL \rightarrow ML_x$$
$$K = (ML_x)/(M)(L)_x$$

where M is metal concentration at equilibrium, L is ligand concentration at equilibrium, and K is the conditional stability constant.

The stability of complexes of bivalent metal ions with organic ligands usually follows the order Pd > Cu > Ni > Pb > Co > Zn > Cd > Fe > Mn > Mg, irrespective of the nature of the ligands involved (Irving and Williams, 1948; Martell and Calvin, 1952).

Among the chelants mentioned above, EDTA has the highest stability constant when complexed with Cd, followed by EDDS and NTA (**Table 1.6**). Compared to synthetic chelants, CA has the lowest stability constant. The stability constants for the Cd-rhamnolipid complex is relatively lower than those of Cd-EDTA and Cd-NTA, but higher than the Cd-CA

complex. With respect to potential leaching of complexed metals during phytoremediation, some studies have shown an increased risk of metal leaching after application of chelants with strong metal binding strength, e.g. EDTA. In a soil phytoextraction experiment, the longevity of EDTA effectiveness, reported as a "minimum observed effect half life" (i.e. the decrease of heavy metal mobilisation by soil amendments over time), varied from 36 days to infinity, much longer than the 3.8 – 7.5 days observed for EDDS (Meers et al., 2005c). Although the longevity increases the persistence of aqueous soluble Cd, it also renders a large fraction of soil Cd vulnerable to leaching with potential environmental risks, and potentially increases the loss of micronutrients from soils (Jiang et al., 2003; Wu et al., 2004).

Table 1.6 Equilibrium stability constant (log K) for Cd(II) complexes with natural and synthetic chelants

Ligand	Method	Ionic Strength	T (°C)	Log K_1
EDTA	Polarograph	0.1 M KNO_3	20	16.46[a]
	Glass electrode	0.1 M KCl	20	16.59[b]
CA	Polarograph	various	25	4.2[b]
	Glass electrode	0.1 M $NaClO_4$	20	3.75[b]
NTA	Glass electrode	0.1 M KCl	20	9.54[a]
	Polarograph	0.2 M KCl	20	9.16[a]
	Polarograph	0.1 M KNO_3	20	9.83[a]
[S,S]-EDDS		0.1 M	20	10.8[c]
Rhamnolipid	Cadmium-ion selective electrode			-2.47[d]
	Cation-exchange resin			6.5[e]
	Cation-exchange resin			6.9[f]

[a] Sillén et al (1964), [b] Sillén et al (1971), [c] Bucheli-Witschel and Egli (2001), [d] Tan et al. (1994), [e] Herman et al. (1995) and [f] Ochoa-Loza et al. (2001)

In solution, the affinity of rhamnolipid for various metals followed the order (from strongest to weakest) $Al^{3+} > Cu^{2+} > Pb^{2+} > Cd^{2+} > Zn^{2+} > Fe^{3+} > Hg^{2+} > Ca^{2+} > Co^{2+} > Ni^{2+} > Mn^{2+} > Mg^{2+} > K^{+}$ (Ochoa-Loza et al., 2001). However in soil, desorption of metals by rhamnolipid

may not exactly follow this order as the metal adsorption/desorption process will be affected by numerous other chemical and biological factors (section 1.2.2.1). Juwarkar et al. (2007) found that dirhamnolipid selectively favoured mobilisation of Cd over Pb in soil. They attributed this to the finding that Cd was found to be more present in the exchangeable fraction than Pb in soils (Wasay et al., 1998).

Complexation of Cd by rhamnolipid was investigated by Tan et al. (1994), who found that pH and concentrations of both metal and rhamnolipid influenced complexation. The stability constant for the Cd-rhamnolipid complex was reported to be -2.47 in their study, in which the Cd concentration at equilibrium was measured by adding increasing amounts of Cd solution to a concentration-fixed rhamnolipid solution. The value is different from that in the study of Ochoa-Loza et al. (2001) and Herman et al. (1995), using the resin-exchange method. This marked difference in the reported log K values could be due to the measurement methods used.

1.5.2 Ecotoxicology

When evaluating the potential use of a certain chemical compound in an ecosystem, the potential ecotoxicology is of overriding importance. The impact of a chelants on plant root and shoot growth is perhaps of most importance in phytoremediation, but adverse effects on microbial activity should also be considered.

Normally, the concentration of chelant applied in phytoextraction was suggested to follow a chelant-to-metal ratio of at least 1:1 (Nowack et al., 2006), in order to achieve rapid metal

solubilisation and complete complexation of metals in soil solution. High concentrations of chelants may mobilise the target metal and increase its uptake and translocation via the nonselective uptake pathway. However, these high concentrations can reduce the water potential in the root-zone, increasing concentrations of dissolved salts in soil solution, thereby reducing plant transpiration (Nowack et al., 2006). Once transpiration is reduced, the assimilation process of plants for both nutrients and non-essential metals will be hindered, and reductions in plant growth are likely. For example, EDTA application has been reported to have deleterious effects on plant growth, decreasing both shoot and root yields and causing chlorosis (Jiang et al., 2003; Kos and Lestan, 2003; Laurie et al., 1991a; Meers et al., 2004; Robinson et al., 2000). Stunted growth and diminished uptake rates were found in sunflower (*Helianthus annuus*) with increasing doses of CA in soils (Lesage et al., 2005; Römkens et al., 2002).

Toxicity of chelants may also be observed to soil microorganisms which are capable of chelant biodegradation. This may cause low chelant biodegradation and consequently a greater potential for metal leaching. Kos and Lestan (2003) found in a Pb phytoextraction experiment that while [S,S]-EDDS (≤ 20 mmol/kg) promoted rather than decreased glucose-induced soil respiration, implying no toxic effect, EDTA applied at the same amount decreased soil respiration. Compared to synthetic chelants, rhamnolipid has better environmental compatibility. The use of rhamnolipid (concentration of 0.1%) for the decontamination of Cd and Pb in a contaminated garden soil showed no toxic effects of this chelant on soil microbial populations (Juwarkar et al., 2007).

1.5.3 Sorption

Adsorption of chelants in soil is detrimental for metal remediation as it causes chelant loss and reduces their effectiveness for metal complexation and mobilisation. In order to achieve high mobilisation efficiency, a lower chelant adsorption to soil is required. Although the adsorption of rhamnolipid to soil has been studied (Noordman et al., 2000; Ochoa-Loza et al., 2007; Torrens et al., 1998) with findings showing that rhamnolipid is inclined to adsorb to soils with high OM and clay contents, no study has been conducted on the effect of chelant adsorption on Cd mobilisation in various soil types.

1.5.4 Degradation

Biodegradation rates of chelants are considered to be affected by a number of environmental factors including temperature, pH, salinity, presence of water, oxygen, nutrients and toxins, chelant concentration, nature and concentration of microorganisms, and nature of reaction medium (adsorption, permeability, etc.) (Means et al., 1980). In enrichment culture where all the conditions are well adjusted, biodegradation of the uncomplexed chelants, such as EDTA, NTA and CA is possible and quick, whereas the biodegradation process is inhibited by complexation with metals (e.g. Cd, Co, Cu, and Zn) (Bucheli-Witschel and Egli, 2001; Means et al., 1980; Satroutdinov et al., 2003; White and Knowles, 2000). The degradability of some chelants also depends on their stereospecificity: [R, R]-EDDS is recalcitrant, [R,S]-EDDS can disappear quickly but its transformation leads to a recalcitrant intermediary *N*-(2-aminoethyl)

aspartic acid (AEAA), only [S,S]-EDTA is biodegradable and suggested for phytoremediation (Bucheli-Witschel and Egli, 2001).

Many studies have observed an inverse correlation between chelate biodegradability and stability constant (Klüner et al., 1998; Satroutdinov et al., 2003), whereas other studies claim that it is more the type of metal (Kos and Lestan, 2003), or the formation of the free chelant ions (Bolton et al., 1996; Vandevivere et al., 2001), than the complex stability constant that influences chelate degradation. For example, in the study of the biodegradation of the metal-[S,S]-EDDS complexes, Vandevivere et al. (2001) found that although Pb- and Zn-EDDS have similar stability constants (log K = $10^{12.7}$ and $10^{13.5}$, respectively), Pb-EDDS is degradable while Zn-EDDS is not. Therefore, the use of the thermodynamic stability constant as a predictor for chelate degradation is flawed, other factors such as enzymology which is not crucially affected by stability constant should be considered.

Most of the data on degradability of rhamnolipid are from studies of behaviour in composts or in nutrient solutions. For example, in a study of co-degradation of rhamnolipid with glucose in a nutrient solutions and in compost, 90% of the rhamnolipid added was degraded within 400 hours after addition (Zeng et al., 2007). A biodegradation study of surfactants in a respirometer system showed that rhamnolipid was more degradable than Triton X-100, in that it was degraded under aerobic, anaerobic conditions and nitrate/sulfate reducing conditions (Mohan et al., 2006). However, there is limited information on the persistence/degradation of rhamnolipid in soils, especially in Cd and Zn co-contaminated soils. Although Maslin and Maier (2000) observed an ultimate degradation of 28% to 38% of rhamnolipid (0.1 mM and

1.0 mM) in two soils, the soils were contaminated solely by Cd up to 1777 mg/kg which is well beyond normal contamination levels where plants could normally grow.

1.5.5 Cost

The use of synthetic chelants in phytoextraction has never been a cheap measure for remediation. The basic cost for enhancing Pb accumulation in plants to levels greater than 10 g Pb/kg dry shoot using EDTA would be US $30,000/ha in 2002, and EDDS is much more expensive (Chaney et al., 2007). Rhamnolipid has the potential to be a relatively cheap natural chelant as it can be manufactured using cheap substrates, such as vegetable oil and waste oils (Mukherjee et al., 2006). The major bottlenecks in rhamnolipid production are its low yield and relatively high recovery and purification costs. Optimisation of bioprocesses and recombination with hyper-producing strains may make it commercially competitive in future. Indeed, large-scale production of rhamnolipid has commenced in USA for many applications (see http://www.rhamnolipid.com/), so that production of low-cost material in large quantities is becoming a reality.

1.6 Summary and Research Gaps

Cadmium contamination of soils is of public concern since soil Cd can pose a threat to human health through the intake of Cd-contaminated crops. This problem is particularly severe in large areas of soil in South-East Asia. Decontamination, or the removal of Cd from soils, requires effective and low-cost remediation strategies based on different chemical, physical, and biological techniques. Phytoremediation is one of the few remediation techniques capable of being deployed on large areas of land. Chelants increase metal solubility in soil, which might enhance Cd removal through phytoremediation. The potential risks of synthetic chelants to the environment and the high costs have led researchers to seek alternatives to synthetic compounds that have properties more suited to phytoremediation. The ideal chelant for Cd phytoextraction should meet the following requirements: 1) have strong Cd binding strength; 2) have a low leaching potential; 3) be harmless or have low toxicity to plants and soil organisms; 4) relatively weak adsorption to soil so that complexed Cd is plant available; 5) be degradable, but not so rapid as to compromise efficacy; and 6) have low cost. Rhamnolipid biosurfactant has been used to enhance Cd removal from contaminated soil by washing (Mulligan and Wang, 2006; Mulligan et al., 2001; Torrens et al., 1998) and to increase plant uptake of Zn from fertilizers (Stacey et al., 2008). It potentially possesses many of the attributes for an ideal chelant for phytoextraction. This suggests that rhamnolipid may be a substance worthy of investigation for phytoextraction of Cd from contaminated soils.

There are a number of key gaps in our understanding of rhamnolipid behaviour in soils and its potential use for enhancing Cd phytoextraction. The longevity of rhamnolipid in the soil

environment is not known, particularly in contaminated soils where the chelant is likely to be complexed by metals, and where microbial activity may be less than in uncontaminated systems. While there is some information on the sorption of rhamnolipid to model soil components, sorption in soils is also poorly understood. Degradation and sorption of chelants are both important properties that determine application strategies for chelant-enhanced phytoextraction. Finally, the phytotoxicity of rhamnolipid and its effect on enhancing plant accumulation of Cd is not known, particularly in the most common environmental situation where Zn is a co-contaminant with Cd.

There is a need therefore, to investigate the possible role of rhamnolipid in enhancing Cd phytoextraction and to determine the optimum application strategy for rhamnolipid in Cd phytoextraction. The working hypothesis for this thesis is that rhamnolipid is potentially a useful chelant for phytoextraction of Cd from contaminated soils.

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CHAPTER 2

BIODEGRADATION OF RHAMNOLIPID, EDTA AND CITRIC ACID IN CADMIUM AND ZINC CONTAMINATED SOILS

J. WEN[a], S.P. STACEY[a], M.J. MCLAUGHLIN[ab], J.K. KIRBY[b]

[a] Soil and Land Systems, Department of Earth and Environmental Sciences, The University of Adelaide, PMB 1, Glen Osmond, SA 5064, Australia
[b] CSIRO Land and Water, PMB 2, Glen Osmond, SA 5064, Australia

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WEN, J. (Candidate)

Performed analysis on all samples, interpreted data, wrote manuscript and acted as corresponding author
I hereby certify that the statement of contribution is accurate
Signed

……..*Date*……

STACEY, S.P.

Data interpretation and manuscript evaluation
I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis
Signed

……..*Date*……

MCLAUGHLIN, M.J.

Supervised development of work, helped in data interpretation and manuscript evaluation
I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis
Signed

……..*Date*……

KIRBY, J.K.

Helped in data interpretation and manuscript evaluation
I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis
Signed

……..*Date*……

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Biodegradation of rhamnolipid, EDTA and citric acid in cadmium and zinc contaminated soils

Jia Wen [a,*], Samuel P. Stacey [a], Mike J. McLaughlin [a,b], Jason K. Kirby [b]

[a] Soil and Land Systems, Department of Earth and Environmental Sciences, The University of Adelaide, PMB 1, Glen Osmond, SA 5064, Australia
[b] CSIRO Land and Water, PMB 2, Glen Osmond, SA 5064, Australia

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ABSTRACT

Rhamnolipid, a metal sequestering agent produced by *Pseudomonas* Sp., has been effective in the removal of metals in soil washing technologies. Rhamnolipid has a strong affinity for cadmium (Cd) compared to some other metals (e.g. cobalt (Co), nickel (Ni)) and might also be useful in chelate-assisted phytoextraction. There have been many studies investigating the formation of metal–rhamnolipid complexes and the ability of rhamnolipid to remove metals from soil. However, to date, the longevity of rhamnolipid in soil has not been measured. Therefore, this study investigated the rate of rhamnolipid degradation in soils of varying physicochemical properties and contaminated with varying concentrations of Cd and zinc (Zn). The rate of rhamnolipid degradation was compared with ethylene diamine tetraacetic acid (EDTA) and citric acid. Our results indicate that citric acid was rapidly degraded, with 20% degradation occurring between 1 and 4 d depending on the level of soil contamination and 70% degradation within 20 d. EDTA was more persistent in the soils; only 14% of the EDTA was degraded after 20 d. Rhamnolipid had cumulative degradation between those of citric acid and EDTA. In most contaminated soils, cumulative degradation of the chelates and ligands were lower than in the uncontaminated soils. These results show that rhamnolipid may remain in the soil long enough to enhance metal phytoextraction, but not remain long enough to raise concerns regarding metal transport in the long-term.

1. Introduction

Metal contamination in soil is an important concern to human and environmental health in many industrialized regions of the world. Chelating agents, such as EDTA, nitrilotriacetate acid (NTA), ethylene diaminedissucinnic acid (EDDS) and citric acid, have been widely used to enhance soil washing and phytoextraction of metals from soils. Many studies have shown improved metal removal from soil following the use of chelating agents (Chaney et al., 1997; Collins et al., 2002; Meers et al., 2005, Evangelou et al., 2007a,b; Hernández-Allica et al., 2008). However, many synthetic chelating agents form chemically and microbiologically stable complexes with metals and therefore are recalcitrant in soil (Brynhildsen and Rosswall, 1997; Alkorta et al., 2004). For example, EDTA is poorly photo-, chemo- and biodegradable in soils (Tiedje, 1975; Means et al., 1980; Bucheli-Witschel and Egli, 2001) and many authors have found high concentrations of metals in soil pore waters or leachates following EDTA treatment (Lombi et al., 2001; Kos and Lestan, 2004). Therefore, when EDTA is applied prior to irrigation or rainfall, the losses of mobilised metals due to leaching is unavoidable and has been linked to groundwater contamination (Thayalakumaran et al., 2003; Alkorta et al., 2004; Evangelou et al., 2007a). Alternative chelating agents, such as citric acid, are more readily biodegradable (Jones, 1998; Römkens et al., 2002) but are less effective in the removal of metals (Chen et al., 2003; Quartacci et al., 2005; Evangelou et al., 2008), probably due to their short half-life (1.5–5.7 d) (Meers et al., 2005) in the soil. Hence, it is of paramount importance for environmental health and remediation of metals that the added ligands be readily biodegradable, yet last long enough to enhance metal removal.

Rhamnolipid is a biosurfactant produced by *Pseudomonas* bacteria. It is also a chemical ligand which has a strong affinity for metals such as Cd, Zn and lead (Pb) through its single carboxyl group (Ochoa-Loza et al., 2001), and has been used in soil washing of these metals (Cd, Zn and Pb) over recent years (Herman et al., 1995; Miller, 1995; Singh and Cameotra, 2004; Juwarkar et al., 2007). A number of studies have used rhamnolipid to successfully remove contaminants by soil washing and were fully reviewed by Mulligan (2005). To date, many studies have shown that rhamnolipid can facilitate the degradation of aliphatic and aromatic organic compounds sorbed onto soil constituents by stimulating mass transport (Zhang and Miller, 1992, 1995; Maslin and Maier, 2000;

* Corresponding author. Tel.: +61 8 8303 6519; fax: +61 8 8303 6511.
E-mail address: j.wen@adelaide.edu.au (J. Wen).

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Christofi and Ivshina, 2002; Zeng et al., 2007). However, few studies have assessed the longevity of rhamnolipid in soils. The longevity of rhamnolipid sorbing to soils or remaining in soil pore waters following soil washing should be considered in the ecological risk assessment.

Due to the high affinity of rhamnolipid to the toxic metal Cd, which in soil is mostly found to be accompanied with Zn, the aim of this study was to determine the influence of Cd contamination and Cd + Zn co-contamination on the degradation of rhamnolipid in soils. A comparison was made to the traditional chelating agents EDTA and citric acid. Radiolabelled chelating agents and ligands were used to quantify the mineralisation by $^{14}CO_2$ evolution.

2. Materials and methods

2.1. Soil source characterization and preparation

Two uncontaminated soils from Keith and Booleroo in South Australia and a rice paddy soil from Changsha, China with past contamination from mining were selected for this study (Table 1). The soils were collected in the field to a depth of 20 cm, air-dried and sieved to <2 mm. The two uncontaminated soils were further spiked with 10 mg/kg Cd and 10 mg/kg Cd plus 1000 mg/kg Zn using cadmium nitrate ($Cd(NO_3)_2{\cdot}4H_2O$) and zinc sulphate ($Zn(SO_4)_2{\cdot}7H_2O$). The ratio of Cd to Zn concentration (i.e. 1:100) was chosen to mimic the ratios found in many contaminated soils. The soils were oven-dried, digested in *aqua regia* (3:1 HCl:HNO_3) and analysed by an inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7500ce, USA) to confirm the total Cd and Zn contents of the soils. Soil pH(H_2O) and soil water extracts (soil:water = 1:5) were tested after spiking the soils with either Cd or Cd and Zn. Major soil properties are presented in Table 1.

2.2. ^{14}C-Labelled rhamnolipid production

A ^{14}C-labelled rhamnolipid was synthesised in-lab to examine the biodegradability of rhamnolipid in Cd or/and Zn contaminated soils (Maslin and Maier, 2000). *Pseudomonas aeruginosa* strain IGB83 (ATCC BAA-228) was inoculated into a 500 ml conical flask containing 100 ml culture medium and agitated at 37 °C for 24 h. The culture medium contained 2.6 mmol ammonium phosphate ($NH_4H_2PO_4$), 1.2 mmol potassium phosphate (K_2HPO_4), 0.2% glucose, 0.41 mmol magnesium sulphate ($MgSO_4{\cdot}7H_2O$), 3.0 mmol iron sulphate ($FeSO_4$).

The ^{14}C-labelled rhamnolipid was prepared by adding 1% of the culture medium with 2.22 MBq of uniformly labelled ^{14}C-glucose (GE Healthcare, UK) into a volumetric flask containing 1 l of mineral salt medium (MSM) and fermented on an agitator at 37 °C for 7 d. The MSM was prepared by taking 1 ml of trace element solution containing 5.3 μmol $ZnSO_4{\cdot}7H_2O$, 8.9 μmol manganese sulphate ($MnSO_4{\cdot}H_2O$), 4.8 μmol boric acid (H_3BO_3), 0.63 μmol cobalt chloride ($CoCl_2{\cdot}6H_2O$), 0.6 μmol copper sulphate ($CuSO_4{\cdot}5H_2O$), 0.4 μmol sodium molybdate ($Na_2MoO_4{\cdot}2H_2O$) to a 1 l solution containing 29.4 mmol sodium nitrate ($NaNO_3$), 2.6 mmol magnesium sulphate ($MgSO_4{\cdot}7H_2O$), 13.4 mmol potassium chloride (KCl), 17.1 mmol sodium chloride (NaCl), 0.3 mmol calcium chloride ($CaCl_2{\cdot}2H_2O$), 146.6 mmol phosphoric acid (H_3PO_4) and 2% glucose. The MSM was then adjusted to pH 7.0 by 3.0 M sodium hydroxide (NaOH) and autoclaved at 121 °C for 20 min.

At the end of fermentation period, the suspension was decanted into a 250 ml centrifuge bottle and centrifuged at 16 000 *g* for 20 min. The supernatant was then removed and acidified to pH 2.0 using hydrochloric acid (HCl) and left overnight at 4 °C in order to precipitate rhamnolipid. After this period the mixture was then centrifuged at 16 000 *g* for 20 min and the supernatant was removed. The pellet was then transferred to a separation funnel and extracted 3 times with a 2:1 (v/v) mixture of chloroform and ethanol. The organic chloroform phase was then collected and evaporated using a rotary evaporator (BÜCHI Rotavapor R-200, BÜCHI Heating Bath B 490). The raw ^{14}C-rhamnolipid was then purified by column chromotography, using silica gel 60 (0.040–0.063 mm, Merck, Darmstadt, Germany) and a gradient chloroform/methanol mobile phases of: stage 1–50:3 v/v (1000 ml), stage 2–50:5 v/v (200 ml) and stage 3–50:50 v/v (100 ml) at a flow rate of 1 ml/min (Sim et al., 1997). The rhamnolipid fractions were eluted from the column in stage 2 and 3 and evaporated to dryness. The final specific activity of ^{14}C-rhamnolipid was 0.21 MBq/g, which equated to an 8.9% yield of ^{14}C-rhamnolipid from ^{14}C-glucose. The recovery of ^{14}C-rhamnolipid from ^{14}C-glucose in this study was higher than the recovery in the study of Zhang and Miller (1995) (2.9%) and Schenk et al. (1997) (3.9%). The majority of the ^{14}C either remained in the nutrient solution or was evolved as $^{14}CO_2$ during fermentation.

The rhamnolipid composition was further examined by mass spectrometry using a TSQ Quantum Discovery Max-triple quadruple (Thermo Fisher Scientific). The rhamnolipid fraction was re-suspended in methanol before being directly infused into a mass spectrometer using a syringe pump (Stacey et al., 2008). The instrument was operated in negative ion acquisition mode in the scanning *m*/*z* range of 400–1000. The initial mass spectra of the rhamnolipid fraction identified two major molecular ions occurring at *m*/*z* 503 (monorhamnolipid, R1) and 649 (dirhamnolipid, R2) (Fig. 1). The relative abundance of R1 was found to be higher than R2, with the approximate ratio of 2:1 (Fig. 1).

Table 1
Physical and chemical characteristics of the soils selected in this study.

Soil properties		Keith	Booleroo	Changsha
pH (1:5 soil/water)		5.4	7.3	7.9
MWHC[a] (%)		30.2	40.7	49.4
Moisture (%)		1.3	2.2	1.3
EC[b] (dS/m)		0.07	0.10	0.32
CEC[c] ($cmol^+$/kg)		8.64	13.90	6.70
Organic Carbon (%)		0.6	1.0	1.4
Clay (%)		12	23	13
Silt (%)		2	22	38
Sand (%)		40	56	47
Cd concentration (mg/kg)	Background	0.0	0.3	4.0
	Contaminated	10.6	10.2	
Zn concentration (mg/kg)	Background	4.4	56.5	1591
	Contaminated	1052	1072	

[a] Maxium water holding capacity.
[b] Electrical conductivity.
[c] Cation exchange capacity.

2.3. Rhamnolipid, EDTA, citric acid degradation

The Keith, Booleroo and Changsha soils were incubated at 50 ± 5% of their maximum water holding capacity (MWHC) in the dark at 25 °C for 28 d to stimulate microbial activity. Working solutions of ^{14}C-EDTA and ^{14}C-citric acid were prepared by spiking 0.1 mmol/l solutions of EDTA and citric acid with ^{14}C-EDTA (0.9 mg, 1.85 MBq, Sigma, St. Louis, MO) and ^{14}C-citric acid (1 ml, 1.85 MBq, Amersham Life Science), respectively. The ^{14}C-rhamnolipid was prepared at two concentrations representing low (0.1 mmol/l) and high (0.5 mmol/l) application concentrations.

After incubation, 0.5 ml of each ^{14}C-labelled working solution was added to plastic vials containing 25 g of each soil by pipette, then mixed throughout the soil using a spatula to obtain spiking rates of 2 mmol/kg for EDTA and citric acid, and either 2 or 10 mmol/kg for rhamnolipid. Each vial was placed in a sealed

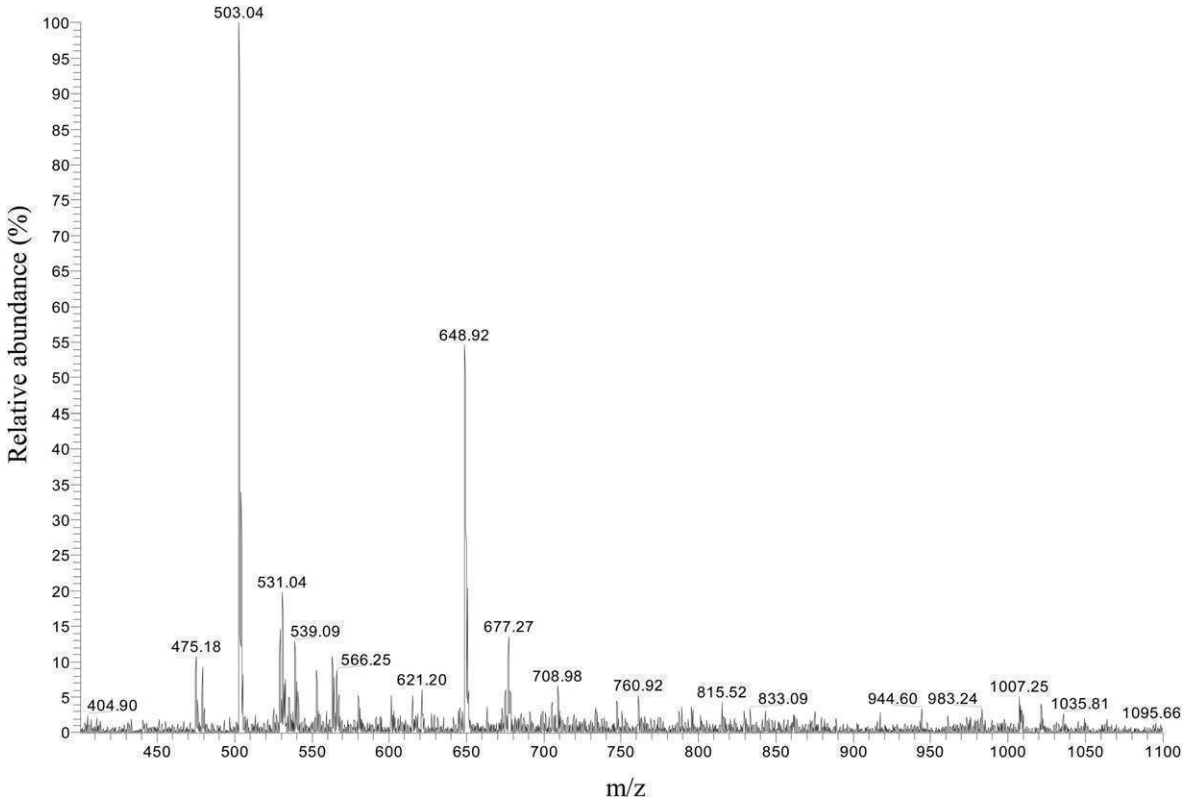

Fig. 1. Relative abundance of ^{14}C-monorhamnolipid (m/z 503.04) and ^{14}C-dirhamnolipid (m/z 648.92).

250 ml polypropylene jar and incubated at 25 °C in the dark for 20 d. All soil samples amended with different treatments were analysed in triplicate. The $^{14}CO_2$ evolved through biodegradation was collected in a 2 ml trap solution of 1 M NaOH placed inside the polypropylene jars. The ^{14}C trapped in NaOH was determined by liquid scintillation counting (Wallac 1414 scintillation counter, Wallac EG&G, Finland) using alkali compatible scintillant (Wallac Optiphase Hisafe 3, Wallac EG&G, Finland).

2.4. Calculations and statistical analysis

The degradation of each amendment between samplings was calculated using formula (1).

$$\text{Degradation}(\%) = \frac{\text{Evolved}^{14}C}{\text{Total}^{14}C} \times 100\% \quad (1)$$

where evolved ^{14}C is the radioactivity of NaOH trapped $^{14}CO_2$ (Bq), Total ^{14}C is the radioactivity of each ligand amended in the soil (Bq).

Exponential decay functions were fitted to the data using formula (2) in GraphPad Prism (5th edition):

$$N = (N_0 - \text{Plateau})\exp^{-KX} + \text{Plateau} \quad (2)$$

where N is the residual organic ^{14}C (Bq); N_0 is the N value when X (time) is zero (Bq); Plateau is the N value at infinite time (Bq); K is the rate constant, expressed in the reciprocal of the time units (time^{-1}).

Rate constant (k) of decline of the residual organic ^{14}C were calculated using formula (3) (Van Veen et al., 1985).

$$k = -\ln\frac{(^{14}C_{\text{resid.}})t_2}{(^{14}C_{\text{resid.}})t_1}/(t_2 - t_1) \quad (3)$$

where $(^{14}C_{\text{resid.}})t_1$ and $(^{14}C_{\text{resid.}})t_2$ are the residual ^{14}C content (% of input) at time t_1 and t_2 respectively. The data collected from sampling on Day 1 and Day 3 were used to calculate k_1 (initial rate constant of degradation) and those from Day 15 and Day 20 were used to calculate k_2 (long-term rate constant of degradation).

In this study, we found the calculated $T_{0.5}$ (50% chelating agent or ligand degradation) exceeded the longest incubation period (20 d). In order to prevent bias caused by extrapolation, we calculated and compared $T_{0.2}$ values (Jones and Edwards, 1998). After calculating N for each soil treatment, the time required for 20% chelating agent/ligand degradation ($T_{0.2}$) was calculated by considering the residual organic ^{14}C equates to 80% of the input to compare the biodegradability of rhamnolipid, EDTA and citric acid.

Treatments were arranged in a completely randomised design with three replications. Cumulative degradation, the degradation rates and $T_{0.2}$ of chelating agents/ligands were analysed for statistical differences using one or two-way analysis of variance (ANOVA) in Genstat (10th edition). When the F value indicated significant differences ($P \leq 0.05$), means were separated by the least significant difference method at $P = 0.05$.

3. Results

3.1. Change of metal solubility in soil with pH

Soil pH was decreased to pH 5.3 and 3.7 in the Cd and Cd + Zn spiked Keith soil, respectively. Similarly in the Cd and Cd + Zn spiked Booleroo soil, pH dropped down to 6.3 and 5.8, respectively. A water extraction experiment showed that 722.5 mg/kg of Zn and 4.6 mg/kg of Cd in the Cd + Zn contaminated Keith soil could be extracted in deionised water, compared to 59.1 mg/kg of Zn and 0.3 mg/kg of Cd in the Cd and Zn contaminated Booleroo soil.

3.2. Rhamnolipid, EDTA and citric acid degradation

There was a significant difference found in the cumulative degradation of rhamnolipid, EDTA and citric acid in the three different soils ($P \leq 0.001$) (Fig. 2). Citric acid was found to degrade rapidly in all soils; on average 69% of the citric acid was degraded after 20 d. Up to 36% of the 2 mmol/kg rhamnolipid treatment was degraded, compared to 29% of the 10 mmol/kg treatment over the same time period. In contrast, only 14% of the EDTA was readily degraded in all three soils (Fig. 2). Despite the contamination level, rhamnolipid degradation showed a continuing increasing degradation after the experimental period (Fig. 2).

Soil contamination was found to affect the cumulative degradation (Table 2), $T_{0.2}$ and degradation rates of the three amendments (Table 3). The $T_{0.2}$ of all three ligands increased with soil contamination, except for rhamnolipid (2 mmol/kg and 10 mmol/kg) in the Keith soil and citric acid in both the Keith and the Booleroo soil where the $T_{0.2}$ did not increase in the presence of Cd (Table 3). The degradation rate constants k_1 of rhamnolipid in all soils were between those of citric acid and EDTA when applied at 2 mmol/kg but were close to those of EDTA when applied at 10 mmol/kg (Table 3). The degradation rate constants for rhamnolipid and EDTA were significantly higher in the Booleroo soil than in the Keith soil, while citric acid degraded faster in the Keith soil (Table 3). Degradation was the slowest in the soils co-contaminated with Cd and Zn (Table 3). The three amendments degraded faster in the Changsha soil than in the Cd and Zn spiked Keith and Booleroo soils (Table 2).

In the Keith and Booleroo soils, rhamnolipid continued to rapidly degrade between Day 3 and Day 20, whereas significant amounts of citric acid had been degraded by Day 2 in all soils (Figs. 3 and 4). Rhamnolipid degradation rate constant k_1, at the application rate of 2 mmol/kg, was not affected by Cd alone in the Keith soil, and $T_{0.2}$ was halved to 3.5 d compared to that in the uncontaminated soil (Table 3). However, the addition of Cd slowed rhamnolipid (2 mmol/kg) degradation rate constant k_1 in the Booleroo soil to $(26 \pm 4.3) \times 10^{-3}$/day and increased $T_{0.2}$ to 8.3 ± 1.6 d. There was a reduction of rhamnolipid degradation (10 mmol/kg) as demonstrated by the lower k_1 in the Cd-contaminated Keith soil in the initial stages, but the effect did not persist as shown by the improved k_2 and unaffected $T_{0.2}$ (Table 3). In the Booleroo soil, the inhibitory effect of Cd contamination was mainly shown in the increased $T_{0.2}$ (from 7.4 ± 0.54 d to 12 ± 1.2 d). In Cd and Zn spiked Keith and Booleroo soils, rhamnolipid degradation at both application rates was found to drop by at most 20% compared with the Cd-contaminated soil (Figs. 3 and 4, Table 3). In both soils, the degradation of 10 mmol/kg rhamnolipid commenced later than 2 mmol/kg rhamnolipid, but maintained higher degradation rate constants (k_2) at the end of the experiment (Table 3). Meanwhile, the long-term degradation rate constants (k_2) of the other three treatments slowed at the end of the experimental period in all three soils (Table 3).

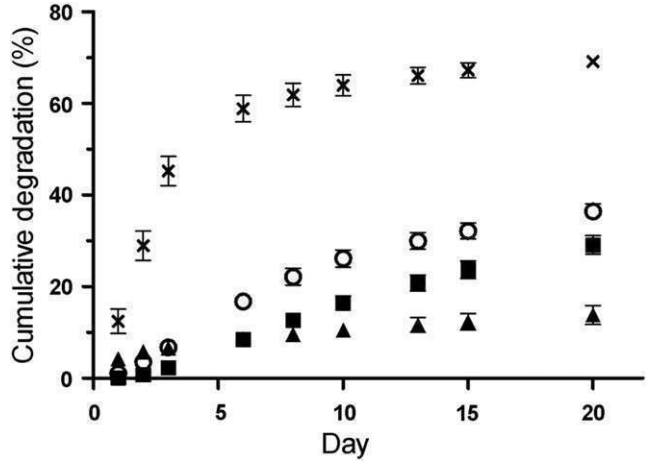

Fig. 2. Cumulative degradation (%) (means ± 1 S.E., $n = 3$) across Keith, Booleroo and Changsha soil (○ Rhamnolipid 2 mmol/kg, ■ Rhamnolipid 10 mmol/kg, ▲ EDTA 2 mmol/kg, × Citric acid 2 mmol/kg).

Table 2
Averaged cumulative degradation (% of added) of chelating agents in different soils.

Contamination	Soil		
	Cumulative degradation (%)		
	Keith	Booleroo	Changsha
Uncontaminated	23.3^{bc*}	30.8^{c}	
Cd-contaminated	25.7^{c}	22.2^{abc}	
Cd + Zn contaminated	17.1^{ab}	15.7^{a}	26.8^{d}

* Letters indicate significant differences ($P \leq 0.05$).

The $T_{0.2}$ for EDTA exceeded the duration of the experiment in most of the soil treatments (Table 3). In the Keith and Changsha soils, approximately 10% of the EDTA degraded during the course of the experiment (Figs. 3 and 5). In the uncontaminated Booleroo soil, 30% of the EDTA degraded after 20 d (Fig. 4). The inhibitory effect of Cd and Zn on EDTA degradation was shown by the high levels of residual ^{14}C (Figs. 3 and 4) and prolonged $T_{0.2}$ (Table 3) in both Keith and Booleroo soils. The degradation rate constants, k_1 and k_2, of EDTA were the lowest among all the amendments (Table 3) and the cumulative degradation reached a plateau after 8 d incubation (Fig. 2).

The degradation rate constants (k_1 and k_2) of citric acid were found to be unaffected by Cd contamination in both the Keith and the Booleroo soils, but were reduced due to the addition of Cd + Zn (Table 3). Nevertheless, cumulative mineralisation was not reduced in the co-contaminated soils after 20 d (Figs. 3 and 4). In the co-contaminated soils, reduced k_1 but increased k_2 indicate the inhibitory effect of metals on citric acid degradation was mainly during the initial stage. The highest degradation rate constant k_1 of citric acid occurred in the uncontaminated Booleroo soil where the value of residual ^{14}C was reduced to below 70% on Day 1 (Fig. 4), indicating a stronger microbial activity in this soil.

4. Discussion

The degradation of EDTA, citric acid and rhamnolipid was found to be inhibited by Cd or/and Zn contamination in all three soils examined. This result supports previous studies that showed decreased mineralisation of organic compounds in organic-metal co-contaminated soils (Cornfield, 1977; Giller et al., 1998; Maslin and Maier, 2000). In addition, various metals added to soils have been shown to exert different effects on degradation of organic materials. Cornfield (1977) studied the carbon release from degradation of native soil organic matter (SOM) in an acidic sandy soil contaminated with twelve different metals at a single dose rate of 100 mg/kg. The degradation of SOM was found to be inhibited by metals in the following order: bismuth < cadmium < cobalt < copper < lead < nickel < thallium < antimony < tin < zinc < mercury < silver. In this study, there was a decrease in $^{14}CO_2$ evolution following Cd and Zn contamination, while single contamination by Cd had a less inhibitory effect, especially in the Keith soil where no decrease in $^{14}CO_2$ evolution was found (Table 3). The spiking concentrations of Zn were ten times higher than that in the study of Cornfield (1977), which is possibly linked to an increased Zn toxicity. Due to the co-existence of Cd in soils, there may have been an increase in metal toxicity caused by Cd + Zn interaction effects. The inhibited ligand degradation in the co-contaminated Keith

Table 3
Degradation rates (k)[a] and $T_{0.2}$ of rhamnolipid, EDTA and citric acid in soils (Mean ± 1 S.E.).

	Rhamnolipid (2 mmol/kg)			Rhamnolipid (10 mmol/kg)			EDTA (2 mmol/kg)			Citric acid (2 mmol/kg)		
	$k_1 \times 10^{-3}$ (/day)	$k_2 \times 10^{-3}$ (/day)	$T_{0.2}$ (days)	$k_1 \times 10^{-3}$ (/day)	$k_2 \times 10^{-3}$ (/day)	$T_{0.2}$ (days)	$k_1 \times 10^{-3}$ (/day)	$k_2 \times 10^{-3}$ (/day)	$T_{0.2}$ (days)	$k_1 \times 10^{-3}$ (/day)	$k_2 \times 10^{-3}$ (/day)	$T_{0.2}$ (days)
Keith	24 ± 1.0[bb]	15 ± 1.5[b]	6.7 ± 1.7[bc]	4.1 ± 0.46[b]	15 ± 1.2[b]	11 ± 0.92[c]	12 ± 1.1[cd]	1.6 ± 0.27[b]	na[c]	395 ± 28.8[c]	7.4 ± 0.058[ab]	0.50 ± 0.058[ab]
Keith Cd	20 ± 2.0[b]	8.5 ± 0.95[a]	3.5 ± 0.17[a]	1.4 ± 0.44[a]	21 ± 1.1[c]	8.9 ± 0.52[bc]	6.7 ± 0.86[b]	1.2 ± 0.38[ab]	na[c]	353 ± 42.3[c]	4.2 ± 0.75[a]	0.53 ± 0.088[ab]
Keith Cd + Zn	8.1 ± 1.8[a]	14 ± 0.6[b]	14 ± 1.6[d]	1.6 ± 0.16[a]	11 ± 0.80[a]	23 ± 1.9[e]	1.2 ± 0.44[a]	0.62 ± 0.073[a]	na[c]	307 ± 14.5[b]	13 ± 3.3[b]	0.77 ± 0.12[bc]
Booleroo	42 ± 0.46[c]	11 ± 2.0[b]	3.2 ± 0.058[a]	14 ± 2.0[cd]	21 ± 1.4[c]	7.4 ± 0.54[ab]	21 ± 1.8[e]	11 ± 2.8[d]	11 ± 0.84	165 ± 9.0[a]	6.7 ± 2.3[ab]	0.93 ± 0.088[c]
Booleroo Cd	26 ± 4.3[b]	18 ± 5.0[b]	8.3 ± 1.6[c]	15 ± 2.4[de]	40 ± 0.93[d]	12 ± 1.2[c]	16 ± 2.7[de]	7.1 ± 1.2[d]	na[c]	168 ± 26.3[a]	7.0 ± 2.1[ab]	1.1 ± 0.21[c]
Booleroo Cd + Zn	27 ± 2.7[b]	14 ± 3.4[b]	7.3 ± 0.57[bc]	8.9 ± 1.1[c]	9.4 ± 1.0[a]	20 ± 1.3[d]	8.8 ± 1.2[bc]	0.61 ± 0.076[a]	na[c]	67 ± 3.6[a]	52 ± 22[c]	4.3 ± 0.75[d]
Changsha	44 ± 6.7[c]	11 ± 1.5[b]	5.2 ± 0.74[ab]	24 ± 3.1[d]	13 ± 0.64[ab]	5.7 ± 0.56[a]	17 ± 0.46[de]	3.3 ± 0.33[c]	na[c]	318 ± 50.1[b]	5.3 ± 1.4[a]	0.30 ± 0.00[a]

[a] k_1 is calculated from data of sampling on Day 1 and Day 3, k_2 is calculated from data of Day 15 and Day 20.
[b] Letters indicate significant differences ($P \leq 0.05$).
[c] Data are not available as the chelating agent was unable to reach 20% degradation within the experimental period.

and Booleroo soils was therefore likely due to either the individual effect of Zn or the interaction of Zn and Cd.

Soil properties were found to play a pivotal role in the degradation rate constants of chelating agents and chemical ligands (Table 3). Among the soils used in the study, rhamnolipid and EDTA

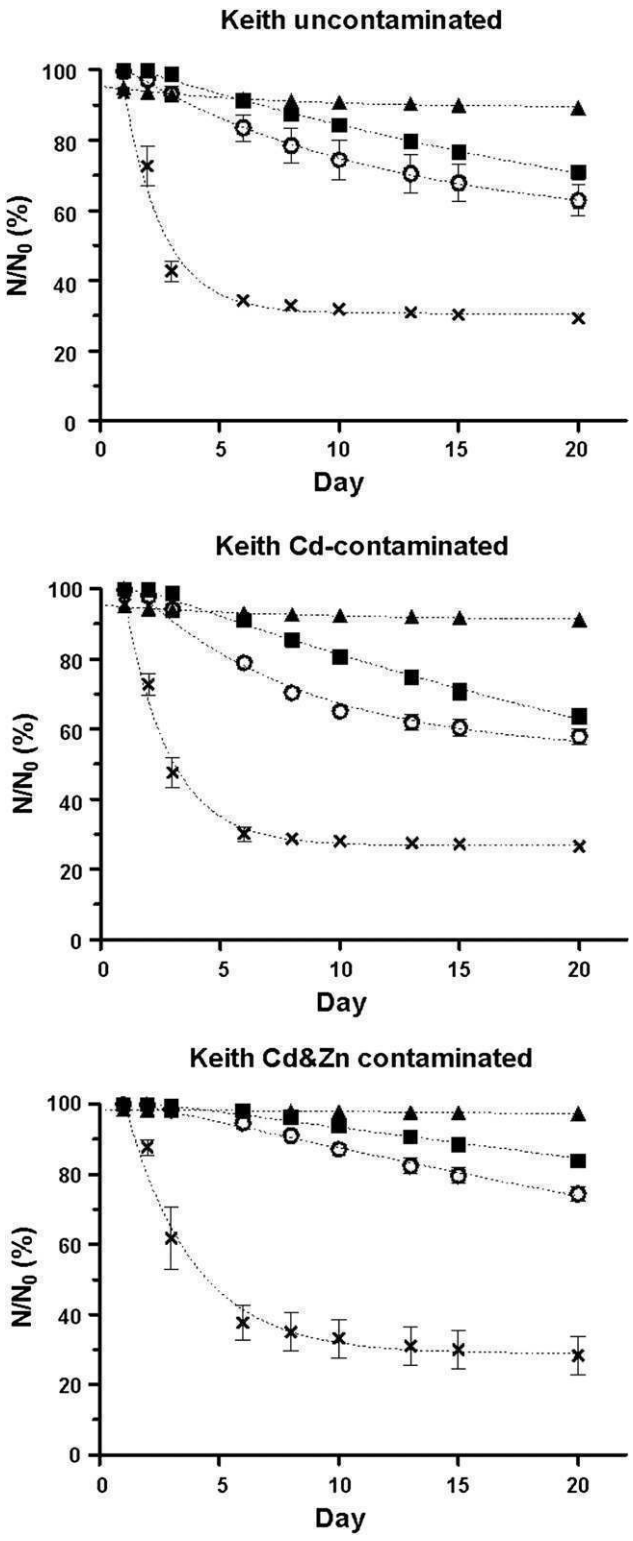

Fig. 3. Exponential decay (means ± 1 S.E., $n = 3$) of chelates in Keith soil (○ Rhamnolipid 2 mmol/kg, ■ Rhamnolipid 10 mmol/kg, ▲ EDTA 2 mmol/kg, × Citric acid 2 mmol/kg).

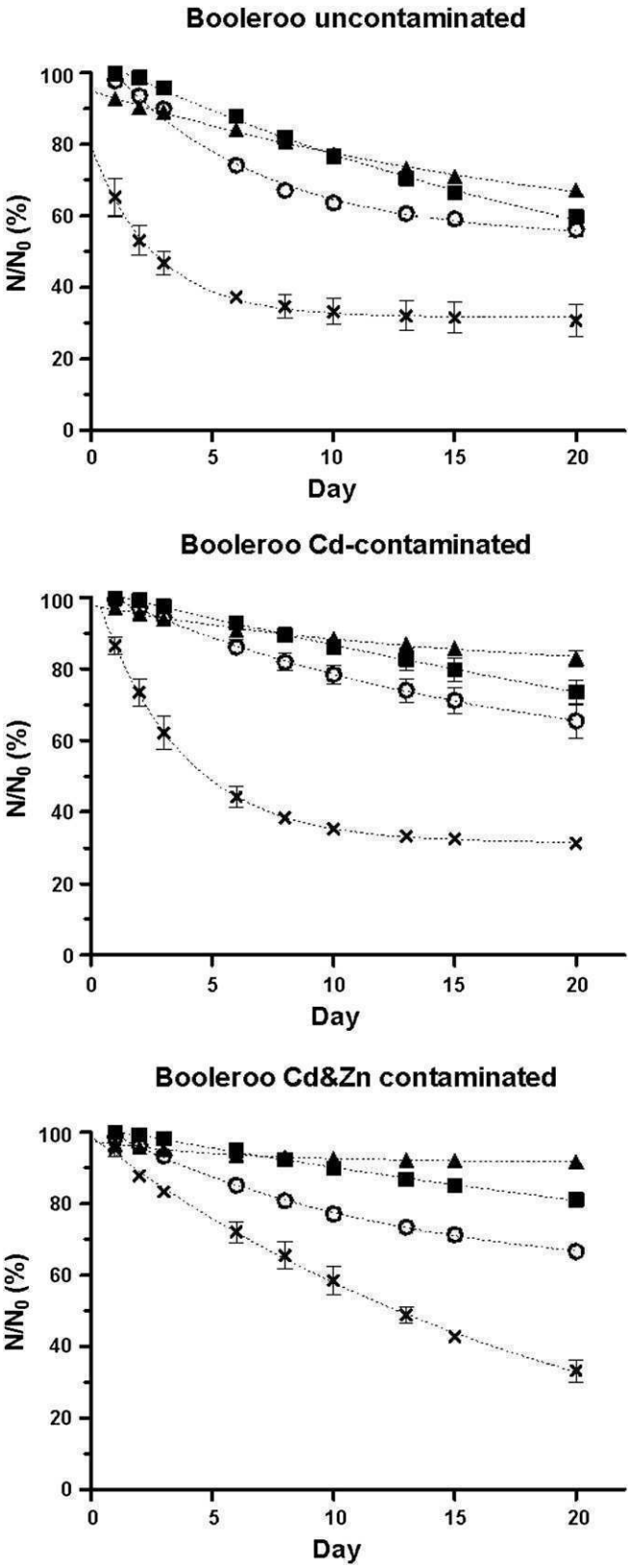

Fig. 4. Exponential decay (means ± 1 S.E., $n = 3$) of chelates in Booleroo soil (○ Rhamnolipid 2 mmol/kg, ■ Rhamnolipid 10 mmol/kg, ▲ EDTA 2 mmol/kg, × Citric acid 2 mmol/kg).

degraded faster in the uncontaminated Booleroo soil (k_1 ranging from $(14 \pm 2.0) \times 10^{-3}$/day to $(42 \pm 0.46) \times 10^{-3}$/day) than in uncontaminated Keith soil (k_1 ranging from $(4.1 \pm 0.46) \times 10^{-3}$/day to $(24 \pm 1.0) \times 10^{-3}$/day), as shown in Table 3. The higher organic matter and cation exchange capacity (CEC) associated with the fertility of the Booleroo soil probably produced a more active microbial biomass than that in the Keith soil. Previous studies have correlated decreased soil pH (range ~ 8.3–3.7) with reduced soil microbial biomass carbon (Stuczynski et al., 2003; Aciego Pietri and Brookes, 2008). In addition, metal solubility in soil increases with decreasing pH. The Keith soil had a more acidic pH than the Booleroo soil before and after spiking, which lead to the higher content of water extracted metals. Therefore, the resulting increase in metal solubility may have decreased microbial activity.

There was a significant difference ($P \leq 0.001$) found in the degradation rate constants among the co-contaminated soils (i.e. Cd and Zn spiked Keith and Booleroo soils and Changsha soil). The average time for 20% degradation of citric acid or rhamnolipid was shorter in the field-contaminated Changsha soil than in the two spiked Keith and Booleroo soils. This difference between the spiked and field soils may be due to the higher pH and organic carbon, which are positively associated with biomass carbon, in the Changsha soil (Aciego Pietri and Brookes, 2008). A study by DiazRavina (1996) measured microbial adaptation to Zn contamination (equivalent to 1040 mg/kg soil) over a four month period and metal adaptation by indigenous soil populations is now well established (Rusk et al., 2004). The study indicated a possible physiological and genetic microbial adaptation to contamination in soils. In a separate study by Frostegård et al. (1996), they found gradual changes in microbial community structure in two Zn contaminated soils with time using phospholipid fatty acid patterns. The Changsha soil has a past history of metal contamination and therefore metal tolerance in the microbial community may have contributed to the higher degradation rates of the amendments.

Rhamnolipid is considered to be a biodegradable surfactant (Maslin and Maier, 2000; Zeng et al., 2007). A study of rhamnolipid biodegradation in a compost matrix found 90% of rhamnolipid was degraded after 400 h (Zeng et al., 2007). Composting material is comprised of organic materials, air and water, which are ideal for

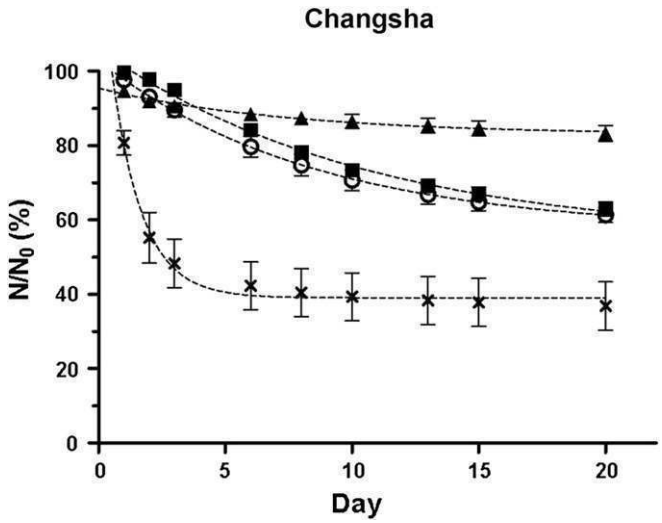

Fig. 5. Exponential decay (means ± 1 S.E., $n = 3$) of chelates in Changsha soil (○ Rhamnolipid 2 mmol/kg, ■ Rhamnolipid 10 mmol/kg, ▲ EDTA 2 mmol/kg, × Citric acid 2 mmol/kg).

the diversity and growth of microorganisms. Rhamnolipid degradation in soil is likely to be influenced by additional factors that have not been comprehensively studied. Maslin and Maier (2000) used rhamnolipid applied at low concentrations (0.1 mM and 1.0 mM) in Cd-contaminated soil (394 mg/kg to 1777 mg/kg), but did not find any effect of Cd on rhamnolipid mineralisation. Earlier work done by Bewley and Stotzky (1983) showed no significant effect of Cd at concentrations of 100 and 1000 mg/kg on organic carbon mineralisation in glucose-supplemented (1%) soils. It is worth noting that the soils were contaminated with Cd at extremely high levels, which, in most cases, is unrealistic for contaminated field soils. In this study, Cd contamination at 10 mg/kg in the Keith soil was found to have no inhibitory influence on the biodegradation of rhamnolipid compared to the uncontaminated soil, with degradation levels reaching 30–40% within 20 d. Furthermore, studies done by Stuczynski et al. (2003) and Liao et al. (2005) have even suggested that Cd could have a stimulatory effect on the soil microbial biomass by potentially increasing dehydrogenase and nitrification activities. However, Cd decreased the rate of rhamnolipid degradation in the Booleroo soil, which indicated that the Booleroo microbial population was more susceptible to Cd toxicity.

The rate constant k_1 of rhamnolipid degradation was found to be slower at the higher rhamnolipid application rate (10 mg/kg) in the first three days (Table 3). However, an increased k_2 was observed using this application rate during the latter stages of the incubation (Table 3). At least 2 mmol/kg of rhamnolipid from the higher application rate could be degraded within the 20 d. By contrast, the time needed for complete degradation of rhamnolipid applied at 2 mmol/kg would be much longer because of the decreasing k. This suggested that the degradation process of rhamnolipid would not be inhibited when applied at higher doses in soil washing techniques, and again verified that concerns related to the residence time in soil may be small for rhamnolipid relative to other chemicals.

A complete mineralisation of citric acid was not measured in this study for all soils (Figs. 3–5). The cumulative degradation of citric acid in our soils were higher than that in a cultivated and a forest soil which had citrate mineralisation of 64% and 51% within a 40-d incubation, respectively (Brynhildsen and Rosswall, 1997). In both studies, the degradation was not 100%. Other authors have hypothesized that this effect may be due to 1) an inhibited decomposition when citric acid was sorbed to ferric hydroxide in soil (Jones and Edwards, 1998); 2) the uptake of some ^{14}C-chelates into microbial cells and subsequent conversion to other compounds incapable of decomposition (Brynhildsen and Rosswall, 1997; Ström et al., 2001); and 3) re-trap of the evolved CO_2 by the increased soil pH resulting from decarboxylation of organic ligands (Yan et al., 1996). The incomplete decomposition of citric acid in our study could have been due to either sorption to these soils or to incomplete recovery of evolved $^{14}CO_2$ due to the abovementioned processes.

5. Conclusion

The degradation of chelating agents and chemical ligands in soil should be considered when chelate-assisted remediation is conducted. The extent of contamination, soil properties, and metal toxicity may all influence the degradation of the amendments. Mineralisation of chelating agents and chemical ligands is not always inhibited in the presence of Cd alone, but may be impeded by co-contamination of Cd and Zn. In light of our results, we conclude that rhamnolipid is more biodegradable than EDTA but more stable in the soil than citric acid. Therefore, concerns related to the resilience time of metal-complexing agents in soil may be smaller for rhamnolipid than for other ligands, such as EDTA. However, further studies may be required to fully evaluate the residual effects of high rhamnolipid rates applied *in situ*.

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CHAPTER 3

IS RHAMNOLIPID BIOSURFACTANT USEFUL IN CADMIUM PHYTOEXTRACTION?

J. WEN[a], M.J. MCLAUGHLIN[ab], S.P. STACEY[a], J.K. KIRBY[b]

[a] Soil and Land Systems, Department of Agriculture, Food and Wine, The University of Adelaide, PMB 1, Glen Osmond, SA 5064, Australia
[b] CSIRO Land and Water, Agricultural Sustainable Flagship, Environmental Biogeochemistry Program, Waite Campus, Urrbrae, SA 5064, Australia

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WEN, J. (Candidate)

Performed analysis on all samples, interpreted data, wrote manuscript and acted as corresponding author
I hereby certify that the statement of contribution is accurate
Signed

……..*Date*……

MCLAUGHLIN, M.J.

Data interpretation and manuscript evaluation
I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis
Signed

……..*Date*……

STACEY, S.P.

Supervised development of work, helped in data interpretation and manuscript evaluation
I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis
Signed

……..*Date*……

KIRBY, J.K.

Helped in data interpretation and manuscript evaluation
I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis
Signed

……..*Date*……

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SOILS, SEC 3 • REMEDIATION AND MANAGEMENT OF CONTAMINATED OR DEGRADED LANDS • RESEARCH ARTICLE

Is rhamnolipid biosurfactant useful in cadmium phytoextraction?

Jia Wen · Mike J. McLaughlin · Samuel P. Stacey · Jason K. Kirby

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Abstract
Purpose Successful chelant-assisted phytoextraction requires application of an eco-friendly metal-complexing agent which enhances metal uptake but does not pose a significant risk of off-site movement of metals. Rhamnolipid biosurfactant has been used to enhance cadmium (Cd) removal from contaminated soil by washing. It has a strong affinity for Cd compared to some other hazardous metals, suggesting that rhamnolipid could be useful in Cd phytoextraction. This study investigated the potential use of rhamnolipid to enhance Cd phytoextraction.
Materials and methods Adsorption patterns of rhamnolipid in soils were investigated by batch adsorption experiments. Hydrophobicity of rhamnolipid–metal complexes were determined by assessing partitioning in an octanol/water system. Phytotoxicity of rhamnolipid to maize (*Zea mays*) and chelant-assisted phytoextraction efficiency of maize and sunflower (*Helianthus annuus*) were determined in pot experiments.
Results and discussion The results showed that rhamnolipid was prone to adsorb strongly to soil at low application rates (0.1–1.7 mM) possibly due to its hydrophobic interactions with soil organic matter, hence reducing its capacity to complex and transport metals to plant roots. Rhamnolipid mobility increased (i.e. decreased soil phase partitioning) at elevated concentrations (~4.4 mM), which increased soil solution Cd concentrations possibly due to its reduced hydrophobic nature. The use of rhamnolipid at concentrations >4.4 mM severely reduced maize biomass yield, reducing the potential for chelant-assisted phytoextraction. At lower concentrations of rhamnolipid (0.02–1.4 mmol/kg), there was insignificant enhancement of Cd accumulation by plant (*Z. mays* and *H. annuus*) shoots, likely through strong retention of the chelant (or Cd-associated rhamnolipid) on soil surfaces.
Conclusions High rates of rhamnolipid addition to soils in this study caused severe phytotoxicity to maize and sunflower. Lower rates of rhamnolpid addition to soils in this study did not improve Cd accumulation by plants. Therefore, the sorption of rhamnolipid (or Cd-associated rhamnolipid) to soils, along with the phytotoxicity and phytoextraction results, suggests that neither low nor high concentrations of rhamnolipid are likely to consistently assist Cd phytoextraction using maize or sunflower.

Keywords Adsorption · Partition coefficient · Phytoextraction · Phytotoxicity · Rhamnolipid

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J. Wen (✉) · M. J. McLaughlin · S. P. Stacey
Soil and Land Systems, School of Agriculture, Food and Wine, The University of Adelaide, PMB 1, Glen Osmond, SA 5064, Australia
e-mail: j.wen@adelaide.edu.au

M. J. McLaughlin · J. K. Kirby
CSIRO Land and Water, Agricultural Sustainable Flagship, Environmental Biogeochemistry Program, Waite Campus, Urrbrae, SA 5064, Australia

1 Introduction

Chelants can be used to increase the phytoextraction of metals from contaminated soil (Chaney et al. 1997; Kos and Lestan 2004; Laurie et al. 1991a, b; Luo et al. 2005). Efficiency of chelants for enhancing phytoextraction of metals depends on a number of factors—complexing capacity, charge, stability in soil, uptake efficiency, plant species, toxicity and cost.

Traditional chelants (e.g. ethylenediaminetetraacetic acid (EDTA) or citric acid) used in soil washing or phytoex-

traction techniques are usually strong anionic chelants and confer a negative, neutral or reduced positive charge on metallic cations once complexed. This dramatically increases the solubility and mobility of the metals, therefore improving metal migration through the soil to the plant root (Elgawhary et al. 1970), which is often the rate-limiting step in metal accumulation by plants (McLaughlin 2002). Being anionic, these chelating anions are also weakly sorbed by soil minerals or organic matter, which improves complexation effectiveness. In addition, at elevated concentrations, the negative charge conferred on the metal can reduce the effectiveness for metal absorption by plant roots (Laurie et al. 1991b), unless normal uptake pathways (through the symplasm) are disrupted and uptake is via an apoplastic pathway or as a result of root leakage (Collins et al. 2002; Nowack et al. 2006). Furthermore, traditional chelants are also compromised for phytoextraction as they are either recalcitrant in soil (e.g. EDTA; Means et al. 1980), creating a potential hazard of groundwater contamination through leaching (Nowack et al. 2006), or are susceptible to rapid microbial degradation (e.g. citric acid; Römkens et al. 2002; Wen et al. 2009), which reduces their potential effectiveness.

Uncharged or lipophilic metal complexes are much more easily accumulated by plant roots (McLaughlin et al. 1998; Stacey et al. 2008), and hence, chelants with the potential to form these complexes offer promise for enhancing phytoextraction. This, combined with the awareness of the need to use eco-friendly and effective chemicals in soil remediation, has resulted in increasing interest in biosurfactants as an alternative to traditional chelating agents. Rhamnolipid, an amphiphilic compound produced by *Pseudomonas aeroginosa*, is a commercially available biosurfactant and is extensively used in remediation of soil and water (Mulligan 2009). It is not recalcitrant to microbial degradation, but is persistent enough to remain in soil for periods useful for phytoextraction (Wen et al. 2009). It has been widely used in soil washing to remove excess toxic metals such as cadmium (Cd), nickel (Ni) and lead (Pb; Herman et al. 1995; Juwarkar et al. 2007; Mulligan 2005; Wang and Mulligan 2004). It was also found to improve the utilisation of zinc (Zn) fertilisers by plant roots in Zn-deficient soils and in solution culture (Stacey et al. 2008). Furthermore, Cd is preferentially bound to rhamnolipid compared to other cations naturally present in soil, such as calcium (Ca), potassium (K), magnesium (Mg) and Zn (Ochoa-Loza et al. 2001), which suggests that it could be useful for enhancing Cd phytoextraction without being affected by the presence of other cations.

However, remediation efficacy of rhamnolipid for Cd-contaminated soils will depend on its sorption by soil, its toxicity to terrestrial plants and its effectiveness in selectively complexing Cd and enhancing plant uptake compared to other metallic cations present in many Cd-contaminated soils (e.g. Ca, iron (Fe), Mg, Zn, etc.). Increased content of clay, soil organic matter and hydrous oxides of aluminium (Al), Fe and Mn increase rhamnolipid adsorption to soils (Ochoa-Loza et al. 2007), which may reduce phytoextraction effectiveness and require higher rates of chelant addition. Considering that rhamnolipid is an amphiphilic compound, the effect of concentration on phytoextraction will depend on the labile (i.e. unadsorbed) portion in the soil solution which will be available to facilitate the mobilisation of soil-bound metals. Thus, it is important to identify a concentration where low adsorption to soil occurs. High concentrations of rhamnolipid (25–80 mM) were previously used for soil washing purposes and achieved high metal removal (Aşçi et al. 2007, 2008). However, use of high concentrations of rhamnolipid could be phytotoxic and would necessitate a different application strategy compared to a non-toxic chelant (Alkorta et al. 2004).

Studies of effectiveness and toxicity of rhamnolipid for phytoextraction of metals are limited. Jordan et al. (2002) examined the chelant-assisted phytoextraction of Cu, Pb and Zn by maize (*Zea mays*) and saltbush (*Atriplex numilaria*) from a soil contaminated by mine tailings and found that whilst rhamnolipid application increased the solubility of these metals (by chelation), it did not increase metal phytoextraction. Only one rate of rhamnolipid application (5 mmol/kg) was tested. Subsequently, Johnson et al. (2009) examined the ability of two concentrations (43 and 347 μM) of rhamnolipid (along with several other chelants) to improve Cu accumulation by Indian mustard (*Brassica juncea*) and ryegrass (*Lolium perenne*) from hydroponic solutions and found little toxicity of rhamnolipid to plant shoot growth, but also little enhancement of metal uptake.

Use of rhamnolipid in phytoextraction therefore requires an evaluation of the optimum dose for metal complexation and mobilisation along with an evaluation of the potential phytotoxicity of this compound at these doses. This will determine the optimum application method for rhamnolipid in phytoextraction schemes. Chelants are currently being applied to soil in two different ways: (1) if the chelant is phytotoxic, as a single dose when optimal growth has been achieved, or (2) in multiple small doses to allow adaptation of the plants to chelant application (Alkorta et al. 2004).

The aims of the study therefore were (1) to identify the optimal concentrations of rhamnolipid to mobilise soil Cd; (2) to investigate possible phytotoxicity of rhamnolipid at these concentrations; and (3) to establish application strategies for the use of rhamnolipid in Cd phytoextraction.

2 Materials and methods

2.1 Overview of study design

Solution experiments, including a batch adsorption experiment and a partition coefficient study, were used to investigate rhamnolipid concentrations potentially suitable for phytoextraction, as well as changes in rhamnolipid lipophilicity as a function of pH, metals and added concentrations. Pot experiments were used in the phytotoxicity and phytoextraction studies. Considering that Cd contamination rarely occurs without co-contamination by Zn, we conducted experiments using soils and solutions spiked with Cd alone or with Cd and Zn together.

2.2 Rhamnolipid batch adsorption

The 25% rhamnolipid liquid extract (Jeneil Biosurfactant Company, $C_{26}H_{48}O_9$, $C_{32}H_{58}O_{13}$, CAS no.147858-26-2) was used throughout this experiment. The critical micelle concentration of the rhamnolipid mixture was found to be 0.1 mM (Shin et al. 2008). One millimolar rhamnolipid solution was prepared by diluting the rhamnolipid extract in deionised water (DI), and the solution pH was tested. An aliquot of the solution was digested in concentrated nitric acid (Mallinckrodt) and analysed by inductively coupled plasma optical emission spectroscopy (ICP-OES, Spectro Acros) or inductively coupled plasma mass spectroscopy (ICP-MS, Agilent 7500ce, USA) for element analysis.

Samples of two uncontaminated soils were collected from Monarto and Booleroo in South Australia, dried at 40°C and sieved to <2 mm for chemical analysis. Monarto and Booleroo soils were selected based on low clay, carbonate and organic matter content. A portion of the Booleroo and Monarto soils were spiked with 1.34 mg/kg Cd in a solution of Cd nitrate ($Cd(NO_3)_2{\cdot}4H_2O$) or 1.34 mg/kg Cd plus 134 mg/kg Zn in a solution of Cd $(NO_3)_2{\cdot}4H_2O$ and Zn sulphate ($ZnSO_4{\cdot}7H_2O$). These spiking rates were chosen because a preliminary seedling emergence experiment (maize, Hycorn 424, Pacific Seeds Company, Australia) had shown that the EC_{25} value of Zn (the effective concentration of Zn in soil which induces 25% inhibitory effect on seedling emergence) in Booleroo soil was 134 mg/kg. Therefore, Cd was added at a concentration based on the Cd-to-Zn ratio of 1:100 found in many contaminated soils (Chaney et al. 1997). Specific soil properties are presented in Table 1.

Five grams of either the Booleroo or Monarto soils was suspended in 50 mL of rhamnolipid solutions (0, 0.1, 1.7, 3.6, 4.4, 7.4, 8.9 and 12.2 mM) in 50-mL polypropylene centrifuge tubes with a drop of toluene added to each sample in order to inhibit microbial activity. Blank samples without soil were included in the experiment in order to detect possible rhamnolipid adsorption during sample preparation (e.g. on vessel walls and membrane filters). After 2 days of end-over-end shaking, the incubation vessels were centrifuged (6,000×*g*, 20 min), supernatants filtered through 0.20-μm membrane filters (Sartorius Stedim, Germany) and the solution pH measured. The total rhamnolipid concentrations added and remaining after adsorption were measured using the 6-dehydroxes assay (Chandrasekaran and BeMiller 1980) by which the rhamnose moiety of rhamnolipid was measured at a wavelength of 400 nm on a UV-1601 spectrophotometer (Shimadzu Corp., Kyoto, Japan). Two millilitres of the extract solution was digested with concentrated nitric acid and analysed for concentrations of Cd and Zn using ICP-OES or ICP-MS. Rhamnolipid adsorption was calculated using Eq. 1:

$$\text{Rhamnolipid}_{adsorbed}(\%) = \frac{\text{Rhamnolipid}_{initial} - \text{Rhamnolipid}_{equilibrium}(\text{mM})}{\text{Rhamnolipid}_{initial}(\text{mM})} \times 100\%. \quad (1)$$

Table 1 Basic physical and chemical properties of the Booleroo and Monarto soils

		Booleroo	Monarto
Soil pH (1:5 H_2O)		7.3	7.2
Moisture content (%)		2.2	0.77
Organic Carbon (%)		1.0	0.7
CEC ($cmol^+/kg$)		13.9	5.4
Clay (%)		23	7.2
Silt (%)		22	5.5
Sand (%)		56	87
Cd (mg/kg)	Background	0.25	ND
	Spiked	1.49	1.56
Zn (mg/kg)	Background	57.1	43.2
	Spiked	130.7	138.7

ND not detected

2.3 Octanol/water partition coefficient

Partition coefficients ($K_{o/w}$) were used to study the change in hydrophobicity of rhamnolipid in metal-spiked solutions at different rhamnolipid concentrations, pH values and metal concentrations. Five millilitres of rhamnolipid at concentrations of 0.3, 3.3 and 7.2 mM were prepared in 15-mL polypropylene centrifuge tubes with or without Cd (0.75 mg/L) or Cd+Zn (0.75 + 75 mg/L). Control samples contained the same combination of rhamnolipid and metal (s), but without the addition of octanol. The solution pH was adjusted to 5.2, 6.6 or 7.9, which spanned the pH range

of soil extracts in the adsorption experiment, using 1 M hydrochloric acid or 1 M sodium hydroxide. One millilitre of *n*-octanol was added to the surface of the solutions. Caps were screwed on firmly and solutions were equilibrated on an end-over-end shaker for 24 h. Samples stood for a further 24 h to allow for phase separation. After the time for phase separation, a needle-attached syringe was inserted in the bottom of the lower phase and 1 mL of the solution removed. The first 0.5 mL solution was used for rhamnolipid analysis. The concentration of rhamnolipid in the octanol phase was calculated using mass balance. The partition coefficient of rhamnolipid was calculated using:

$$K_{o/w} = C_o/C_w \quad (2)$$

where C_o is the rhamnolipid concentration in the octanol phase (mg/L) and C_w is the rhamnolipid concentration in the water phase (mg/L).

2.4 Rhamnolipid phytotoxicity

Sunflower and maize are two high biomass species which have been widely used in chelant-assisted phytoextraction and achieved relatively high phytoextraction efficiency (Chen et al. 2004; Pritsa et al. 2008; Tandy et al. 2006; Turan and Angin 2004); therefore, we selected these two plant species for the study.

Seeds of maize (Hycorn 424, Pacific Seeds Company) were pre-germinated on a Petri dish lined with moistened filter paper for 5 days. Seedlings were then transferred to polypropylene pots containing 500 g uncontaminated Booleroo soil fertilised with N (175 mg/kg), P (57 mg/kg), K (112 mg/kg), Ca (210 mg/kg), Mg (85 mg/kg) and S (135 mg/kg). Plants were grown in a controlled environment growth chamber (17°C (8 h)/20°C (16 h), 45% humidity, high fan speed). Seedlings were thinned to two per pot after 1 week. Rhamnolipid solution dissolved in 50 mL of deionized water was then added to the soil as a single dose 5 days before harvest (5, 20, 50 mmol rhamnolipid/kg soil) or split doses for 4 weeks consecutively, with the total dose matching the single dose (1.25, 5, 12.5 mmol rhamnolipid per kilogram per week × 4 weeks). Decreases in the weight of the pots between watering were assumed to represent evaporation in the pots containing soil only and evapotranspiration (evaporation + plant transpiration) in the pots containing plants (Collins et al. 2002). Plant transpiration was calculated by subtracting the mean evaporation (measured by the loss of weight) of replicate control pots from the mean evapotranspiration values obtained in the pots containing plants. During the experiment, soil moisture content was maintained at 70% maximum water holding capacity (MWHC) by daily additions of DI water. After 6 weeks of growth, plant shoots were harvested approximately 1 cm above the soil. Plant samples were oven-dried at 70°C for 48 h and shoot dry weights recorded.

2.5 Rhamnolipid phytoextraction efficiency

The Cd + Zn-spiked Booleroo and Monarto soils were kept at 50% MWHC and aged for 1 month before being used in the phytoextraction experiment. The moist aged soils, equivalent to a dry weight of 0.8 kg/pot for maize and 0.6 kg/pot for sunflower, were amended with nutrients as outlined above, and the moisture content was brought to 70% MWHC using DI water. Pots were transferred to the controlled growth chamber for equilibration (1 week) and randomised within four blocks. Seeds of maize (Hycorn 424, Pacific Seeds Company) and sunflower (Sunbird 7, Pacific Seeds Company) were germinated on moistened filter paper 3 days before sowing. The germinated seeds were sown at a density of three per pot and thinned to one per pot a week after emergence. Rhamnolipid was then applied at two frequencies, either as multiple small doses or a single dose before harvest. Three rhamnolipid treatments (0.02, 0.2 and 0.4 mmol/kg/week) were applied to the soils on days 1, 7, 13 and 19 (day 1 = the day of the first rhamnolipid application). Single-dose treatments (1.4 mmol/kg) were amended with rhamnolipid 5 days before harvest. Throughout the growth period, the soil moisture content was determined by weight and returned to a constant value with daily addition of DI water. Soil solution was extracted twice, prior to addition of rhamnolipid and before harvest, by soil moisture samplers (Rhizosphere Research Products, Wageningen, The Netherlands) and stored at 4°C until analysis. At harvest (6 weeks after emergence), plant shoots were cut 1 cm above the surface of the soil, washed with DI water and dried at 70°C for 48 h. Ground shoots and soil solution were digested in hot concentrated nitric acid before analysis for Cd and Zn by ICP-OES or ICP-MS. Soil solution was digested in order to avoid rhamnolipid precipitation during acidification for ICP measurement. The transfer factor (TF) from soil solution to plant shoot was calculated using:

$$\mathrm{TF} = \mathrm{Metal}_{shoot}/\mathrm{Metal}_{solution} \quad (3)$$

where Metal_{shoot} is the concentration of Cd or Zn in plant shoot (mg/kg) and $\mathrm{Metal}_{solution}$ is the initial concentration of Cd or Zn in soil solution (mg/L).

2.6 Statistical analysis

Analysis of variance (ANOVA) was performed on all data to determine if significant difference existed between treatments. Least significant difference was used to separate

means where ANOVA showed significance at $P \leq 0.05$. Where variances were proportional to the magnitude of the mean values, data were transformed prior to analysis.

3 Results

3.1 Rhamnolipid adsorption

The pH of a 1 mM rhamnolipid solution was found to be 9.71. The liquid extract used contained considerable amounts of Na (962 mg/L) and S (21 mg/L) and negligible amounts (<5 mg/L) of Ca, Mg, K and P. The sorption of rhamnolipid to vessel walls and membrane filters in this study was found to be negligible (i.e. below detection limits).

Rhamnolipid adsorption showed a similar pattern in both the Booleroo and Monarto soils (Fig. 1). At low initial concentrations (0.1–1.7 mM), increasing rhamnolipid adsorption was found in soils, whilst with increasing rhamnolipid concentration up to 3.6–4.4 mM, rhamnolipid adsorption reduced sharply, by more than 60%. Above 4.4 mM, rhamnolipid adsorption increased slightly, and above 7.9 mM, adsorption increased rapidly. There was a slightly lower mean rhamnolipid adsorption in the Monarto soil (26.8%) than in the Booleroo soil (29%) irrespective of contamination level ($P \leq 0.001$). Generally, the contamination of soil with Cd alone or mixed Cd and Zn did not cause a significant difference in rhamnolipid adsorption compared with the uncontaminated soils. However, an exception was found at a rhamnolipid concentration of 0.1 mM where considerably higher levels of rhamnolipid adsorption were found in the Cd + Zn-spiked Booleroo (93%) and Cd (83%) or Cd + Zn-spiked Monarto soils (86%). The solution pH of the spiked soils was found to be 0.1–0.4 units less than in the uncontaminated soils (data not shown). The pH of the soil extracts was lowered from 7.2 to below 6.6 in the Booleroo soil and from 6.8 to below 6.4 in the Monarto soil with increasing rhamnolipid concentration to 3.6–4.4 mM, after which a gradual pH increase was observed (data not shown). The desorbed Cd in solution (see Fig. 1) did not differ significantly from that in the control samples (DI water extracts) until the rhamnolipid concentration increased to 4.4 mM, at which concentration an increased Cd desorption was found. The highest Cd mobilisation from soil solid phases to soil solution was found in the Monarto soil at a rhamnolipid concentration of 12.2 mM (see Fig. 1), with a 55.7-fold increase compared to the control. There was significantly less Zn desorbed from the Booleroo soil (on average 1.58 mg/L, data not shown) than from the Monarto soil (2.47 mg/L, $P \leq 0.05$, data not shown).

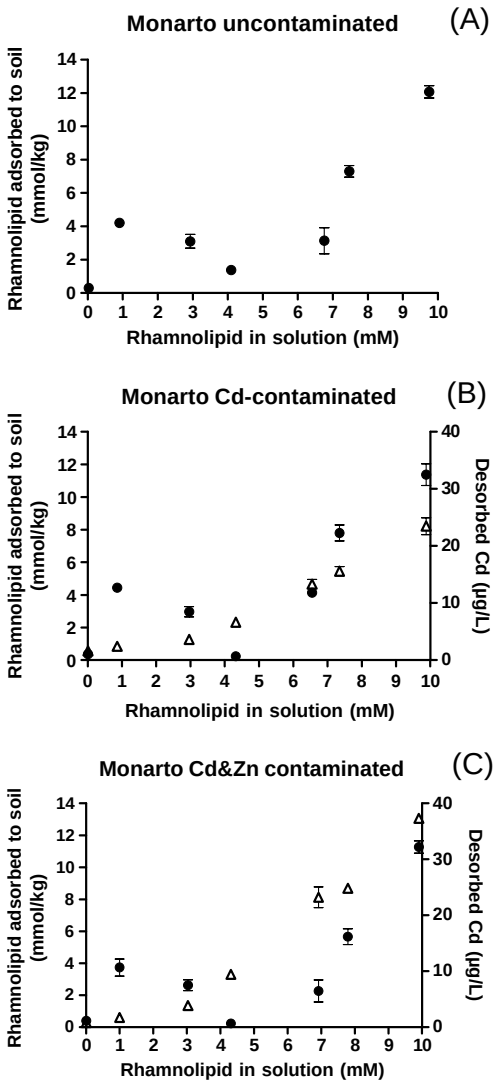

Fig. 1 Rhamnolipid adsorption (*circle*) and Cd desorption (*triangle*) (mean ± 1 SEM) in the Monarto soil (adsorption in Booleroo soil was similar to that in Monarto soil)

3.2 Octanol/water partition coefficient

The concentration of rhamnolipid in the octanol phase increased with increasing initial dose of rhamnolipid. However, the highest mean rhamnolipid $K_{o/w}$ was found at the solution concentration of 0.3 mM (26.3), significantly greater ($P \leq 0.001$) than that at the two higher concentrations (0.83 at 3.3 mM and 0.88 at 7.2 mM, Fig. 2). There was negligible loss of rhamnolipid in the octanol-free samples after shaking, indicating that the high $K_{o/w}$ value at the lowest concentration was not due to the sorption to the vessel walls. Lower pH was associated with higher $K_{o/w}$ values ($P \leq 0.001$, see Fig. 2). In the Cd and Zn co-spiked solution, the $K_{o/w}$ values were on average higher than those in the unspiked or Cd-spiked rhamnolipid solutions ($P \leq 0.001$).

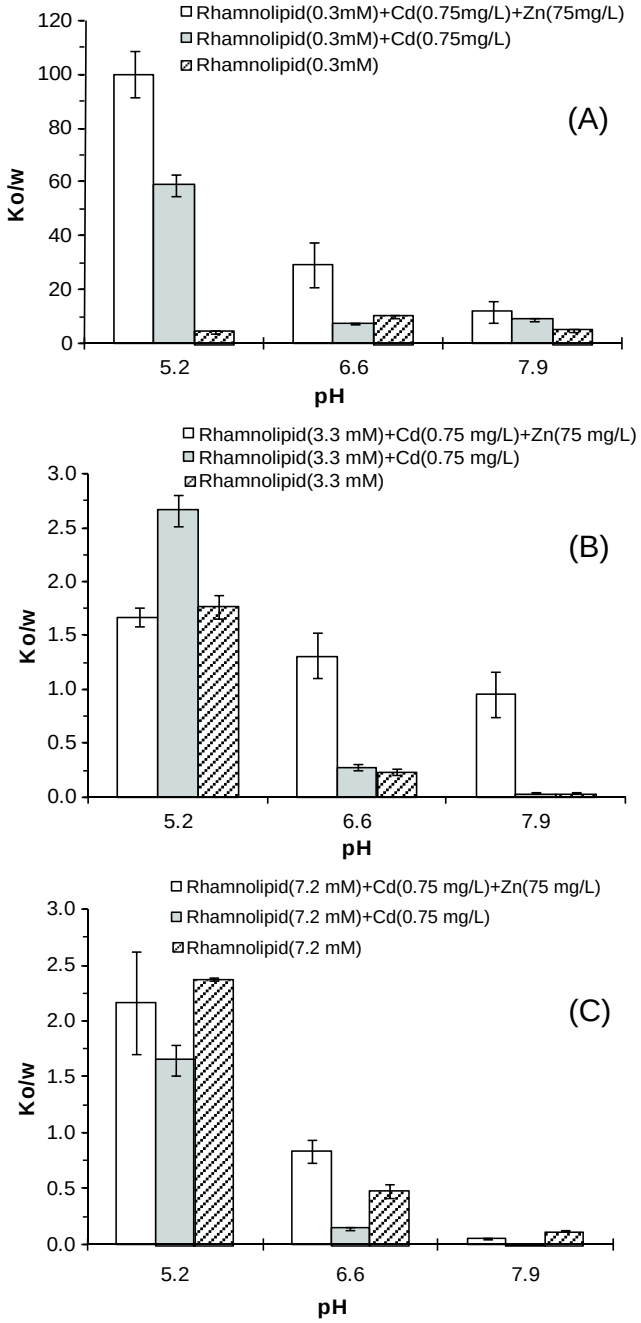

Fig. 2 Partition coefficients (mean ± 1 SEM) of rhamnolipid at concentrations of 0.3 mM (**a**), 3.3 mM (**b**), 7.2 mM (**c**)

3.3 Rhamnolipid phytotoxicity

Rhamnolipid concentrations of 1, 5 and 10 mM (equivalent to 5, 25 and 50 mmol/kg soil) were chosen for the phytotoxicity experiment as these spanned the concentration at which rhamnolipid adsorption was lowest in the batch adsorption experiment (5 mM) and hence would be most likely to facilitate metal complexation (Tan et al. 1994). At all these concentrations, maize plants receiving rhamnolipid application in a weekly dose showed severely stunted growth and decreased concentrations of Cd, Zn and some other nutrient elements in the order of Fe, Ca, K and manganese (Mn) in plant tissue, whilst those receiving the single dose exhibited wilting and necrosis. Irrespective of the application frequency, severely reduced biomass was found in all rhamnolipid-treated plants (Fig. 3). Decreased transpiration compared to the control plants also indicated rhamnolipid-induced phytotoxicity (Fig. 4).

3.4 Phytoextraction efficiency

The lower concentrations of rhamnolipid did not cause a severe reduction in biomass yield when compared to the control, except for the weekly dose of 0.4 mmol/kg (Fig. 5). Nevertheless, lesions on plant leaves were still found in plants receiving the weekly treatment of 0.2 mmol/kg, and plant wilting was seen with the single dose of 1.4 mmol/kg. Plants grown in the Booleroo soil had a significantly higher biomass yield and metal accumulation than in the Monarto soil ($P \leq 0.001$).

The addition of rhamnolipid to the Booleroo soil did not increase the concentrations of Cd and Zn in soil solution at harvest. However, solution Cd concentrations in the Monarto soil were increased threefold after the weekly treatments (0.2 and 0.4 mmol/kg/week) and six to sevenfold after the single-dose treatment (1.4 mmol/kg) compared to the control, possibly due to its sandier texture. The effect of rhamnolipid on solution Zn concentration was less prominent (Table 2). The higher solubility of metals after rhamnolipid application to the Monarto soil did not directly result in higher metal accumulation in plant shoots; only the treatment of 1.4 mmol/kg increased Cd and Zn content in the maize shoot in the Monarto soil 2.4- and 2.7-fold, respectively. Moderate improvement of Cd accumulation was found in the sunflower grown in the Booleroo soil, with an average increase of 1.5-fold in uptake following the

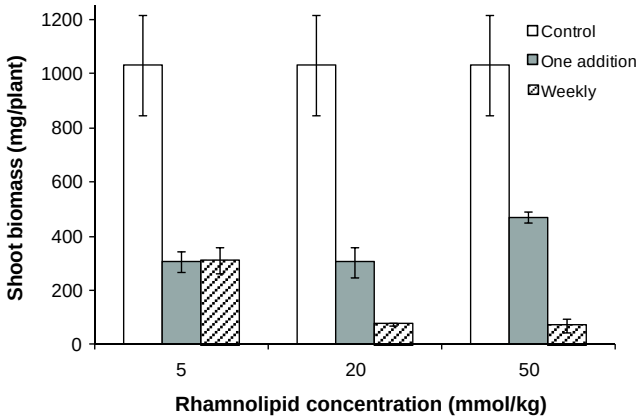

Fig. 3 Shoot dry mass of maize (mean ± 1 SEM) in a phytotoxicity study receiving high rates of rhamnolipid

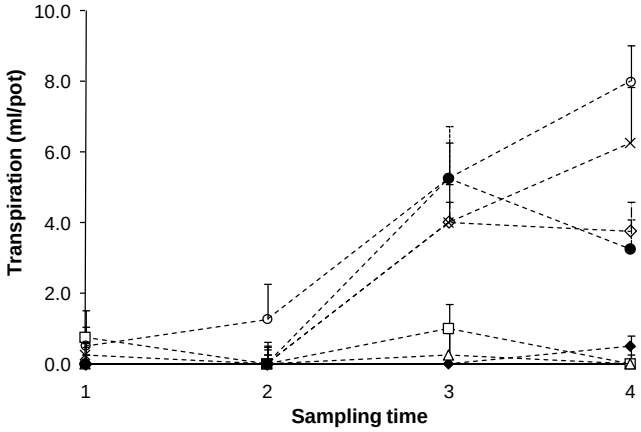

Fig. 4 Transpiration of maize measured 24 h after rhamnolipid addition (*diamond* 1.3 mmol/kg/wk, *square* 5 mmol/kg/wk, *triangle* 12.5 mmol/kg/wk, *ex* 5 mmol/kg, *empty diamond* 20 mmol/kg, *filled circle* 50 mmol/kg, *empty circle* control)

weekly treatments of 0.02 and 0.2 mmol/kg and the single-dose treatment of 1.4 mmol/kg (Fig. 6a). However, these treatments did not affect total shoot Zn uptake in sunflower in the Booleroo soil (Fig. 6b).

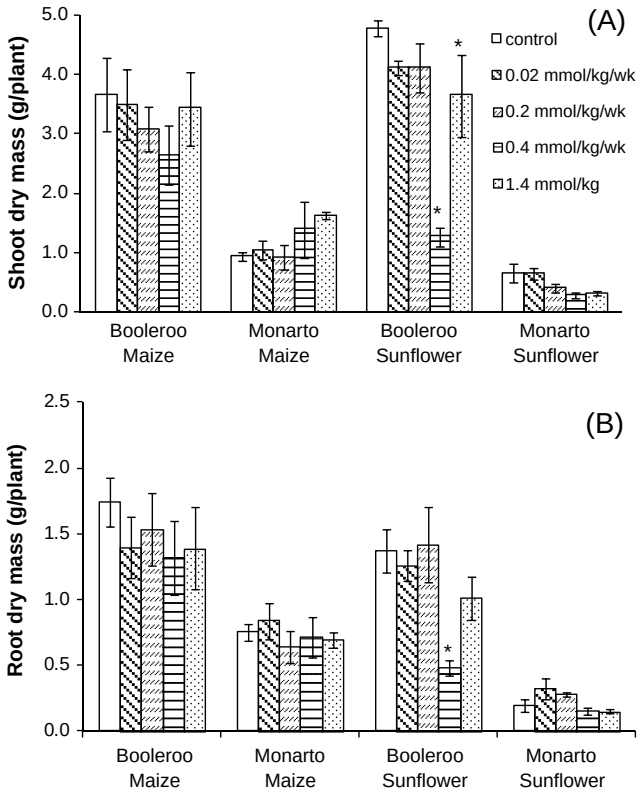

Fig. 5 Shoot (**a**) and root (**b**) dry mass (mean ± 1 SEM) of maize and sunflower in a phytoextraction study (*asterisk* indicates significant difference ($P \leq 0.01$) from the control)

4 Discussion

The successful application of chelant-assisted phytoextraction for significant removal of metals in soils using rhamnolipid requires optimisation of soil solution metal and chelant concentration and plant species (Nowack et al. 2006). In addition, plant metal uptake will also be dependent on the type of ligand in use (McLaughlin et al. 1997). However, generalising from the experimental results given above, the use of rhamnolipid does not seem to be an effective chelant for enhancing Cd phytoextraction in the selected soil. The unsuitability of using rhamnolipid in Cd phytoextraction is associated with its adsorption characteristics in soil, and the lipophilicity changes regulated by pH, metal content and chelant concentration.

Solution or soil pH was found to have an effect on rhamnolipid hydrophobicity (-philicity) and consequently its adsorption behaviour. The spiked soils had higher metal concentrations and lower pH than the unspiked soils. The high $K_{o/w}$ found in the lowest pH solution coincided with the higher rhamnolipid adsorption (at 0.1 mM) in the spiked compared to the unspiked soils. The lower solution pH and higher metal concentration in the co-spiked soil may have led to the increased rhamnolipid hydrophobicity and, hence, high rhamnolipid adsorption. Surprisingly, addition of the alkaline rhamnolipid extracts up to 4.4 mM decreased the soil solution pH, implying that the release of H^+ in the process of metal–rhamnolipid complexation may override the effect of OH^-. However, the reduction of pH did not always result in an increased adsorption, e.g. at rhamnolipid concentrations of 3.6–4.4 mM. Therefore, other factors, including rhamnolipid concentration and lipophilicity changes, may have influenced the adsorption pattern.

The results of the batch adsorption experiment suggested a potentially suitable rhamnolipid concentration range (4–5 mM) where low rhamnolipid retention on soil was observed for the subsequent phytotoxicity and phytoextraction experiments. The adsorption pattern found in our study was also reported in a study by Torrens et al. (1998) under saturated flow conditions, although the lowest rhamnolipid adsorption was found at a different concentration in the alkaline, sandy soil used in their study. They found that more than 95% of rhamnolipid was adsorbed at concentrations between 1.6 and 6.2 mM, whilst only 15% of the rhamnolipid was adsorbed at 12.6 mM. In other studies, rhamnolipid adsorption in soil was demonstrated to follow the Freundlich equation. However, either higher concentrations (12.5–80 mM) or lower concentrations (85–1275 μM) were used in those studies than in the work presented here (Herman et al. 1995; Noordman et al. 2000); thus, the possible changes in rhamnolipid adsorption between these concentration ranges were not reported. We propose that the lipophilicity and morphology change of

Table 2 Concentrations of Cd and Zn in soil solution after rhamnolipid application to Booleroo and Monarto soils spiked with Cd+Zn (1.34 and 134 mg/kg, respectively)

Treatment	Cd (μg/L)		Zn (mg/L)	
	Booleroo	Monarto	Booleroo	Monarto
Maize				
0 mmol/kg	16.3±2.7b	4.9±2.1a	2.3±0.24b	8.1±0.032b
0.02 mmol/kg/wk	5.2±1.7a	6.4±2.0a	1.3±0.12b	4.2±0.32a
0.2 mmol/kg/wk	5.1±1.5a	13.6±4.2b	0.67±0.17b	3.5±0.81a
0.4 mmol/kg/wk	2.9±0.63a	12.6±4.8b	0.27±0.084a	7.6±0.74b
1.4 mmol/kg	3.2±0.60a	27.2±4.4c	0.33±0.051a	7.8±1.8b
Sunflower				
0 mmol/kg	4.3±2.3a	5.5±1.3a	0.44±0.11a	5.1±0.57c
0.02 mmol/kg/wk	3.0±0.94a	5.4±0.0071a	0.41±0.016a	2.8±0.50a
0.2 mmol/kg/wk	2.3±0.72a	13±3.0b	0.34±0.029a	4.0±0.084b
0.4 mmol/kg/wk	5.4±1.4a	19±0.80b	0.67±0.15b	5.5±1.0c
1.4 mmol/kg	4.5±1.1a	28±2.4c	0.47±0.069a	11±1.9d

Letters indicate significant difference ($P \leq 0.05$)

rhamnolipid may account for the adsorption pattern observed and its resultant unsuitability for use in phytoextraction. The normal morphology of rhamnolipid is known to alternate between a small structure (micelle) and a larger structure (vesicle, lamella or lipid aggregate) as a function of chelant concentration, solution pH, temperature or salt content (Zhang and Miller 1992). The micelle-to-vesicle change has been observed with increasing rhamnolipid concentration to 2.5 mM and above in some studies (Sánchez et al. 2007; Guo et al. 2009). In this study, at low rhamnolipid concentrations below or around 1.7 mM where micelle and small aggregates of rhamnolipid could coexist, the adsorption of rhamnolipid to soil was high. This could be due to the adsorption of hydrophobic rhamnolipid micelles or small structures onto soil organic matter, as suggested by the high $K_{o/w}$ values. When the concentration exceeded 2.5 to 4.4 mM, sorption decreased, as did $K_{o/w}$ values of rhamnolipid, suggesting a reduced affinity for soil organic matter. At these concentrations, the formation of vesicles and other larger structures could have occurred. The sharp pH reduction down to below 6.6 at rhamnolipid concentrations of 3.6–4.4 mM would also be favourable for larger vesicle structures to form (Zhang and Miller 1992). Therefore, the low rhamnolipid adsorption to soil observed at these concentrations was likely due to the release of the rhamnolipid sorbed on soil surfaces and the simultaneous formation of vesicles. Higher concentrations (>7.9 mM) may have changed rhamnolipid morphology again to micelle or smaller structures due to a pH increase (Champion et al. 1995), which could lead to rhamnolipid re-adsorption.

Although the low rhamnolipid adsorption found around the concentration of 4.4 mM is desirable for phytoextraction purposes, the high rhamnolipid concentrations introduced in the soil still resulted in phytotoxicity. The freely mobile rhamnolipid ligand or complexed form may have harmed plant roots in such a way that the assimilation of other essential elements (e.g. Fe, Ca, K and Mn) was obstructed. Although higher rhamnolipid application rates increased metal concentration in plant tissue (data not

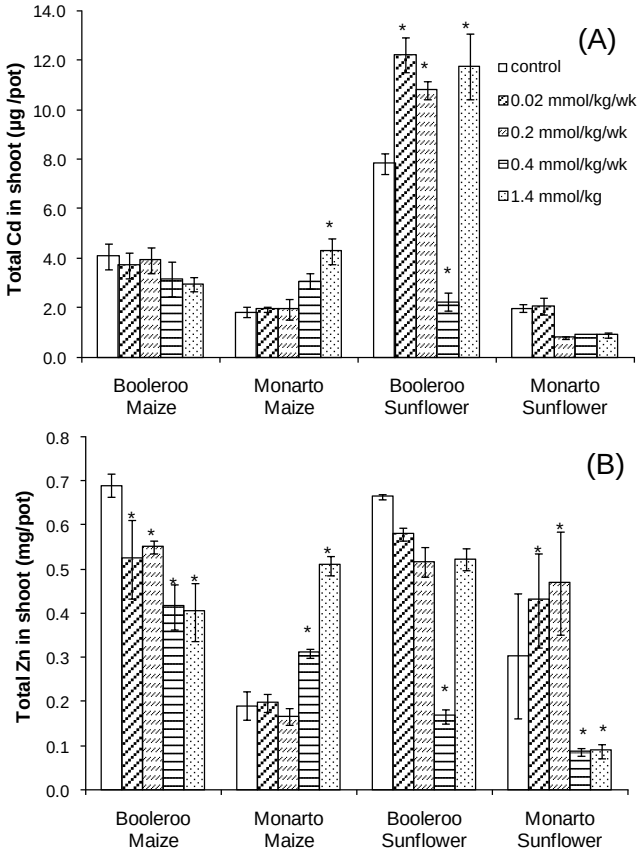

Fig. 6 Total uptake of Cd (**a**) and Zn (**b**) (mean ± 1 SEM) by maize and sunflower shoots (asterisk indicates significant difference ($P \leq 0.01$) from the control)

shown), this was not able to compensate for the severe biomass loss. The failure of metal accumulation at the elevated rhamnolipid application rates could also be due to a change in rhamnolipid morphology. At the higher concentrations, the larger rhamnolipid structures (vesicle) may have been excluded from the plant root cells and been unable to transport the Cd-rhamnolipid complexes into plants.

In contrast, the lower rates of rhamnolipid alleviated the toxicity, but they did not provide a significant enhancement of Cd uptake. In the batch adsorption experiment, there was a high adsorption of rhamnolipid and its metal complexes within the rhamnolipid concentration range of 0.1–3.3 mM. Therefore, in the phytoextraction experiment, rhamnolipid applied at the low rates (equivalent to 0.4 μM–0.28 mM in solution) may have been largely adsorbed on soil particles, especially on clay, organic matter (see Table 1) and the surface of Al mineral oxides (Ochoa-Loza et al. 2001), resulting in a limited metal solubilisation. Therefore, increased Cd accumulation was not observed in all treatments. In the literature, rhamnolipid has been reported not to significantly improve phytoextraction efficiency of other metals, e.g. Cu, Pb and Zn (Johnson et al. 2009). The rhamnolipid concentrations (43 and 347 μM) used in the hydroponics study (pH 6) of Johnson et al. (2009) did not cause any improvement in plant Cu accumulation. Jordan et al. (2002) also found no significant enhancement of Cu, Pb and Zn concentrations in plant tissue using rhamnolipid (5 mmol/kg soil) in an alkaline mixed soil. They both postulated that the failure of using rhamnolipid to effectively enhance metal uptake was due to the relatively large rhamnolipid complex structure which would not be likely to traverse root cell membranes and cortical tissues. It is possible in the study of Johnson et al. (2009) where rhamnolipid morphology may have changed to larger structures at the low pH. However, Jordan et al. (2002) also found that the free rhamnolipid molecule could be assimilated by plant roots. We propose that it is likely the rhamnolipid-induced toxicity at the concentration used in their study which led to the little improvement in metal uptake. It is therefore not appropriate to make the assumption that the metal-bound rhamnolipid complex is excluded from the root entirely as the binding of metals (e.g. Cd) would reduce the rhamnolipid's head diameter by reducing the negative charge repulsion between adjacent polar heads (Champion et al. 1995). In our study, we observed significant increases of Cd uptake at low rhamnolipid application rates using sunflower in the Booleroo soil and maize in the Monarto soil. Nevertheless, these increases were small (on average 1.5-fold) and therefore still insufficient for chelant-assisted phytoextraction purposes.

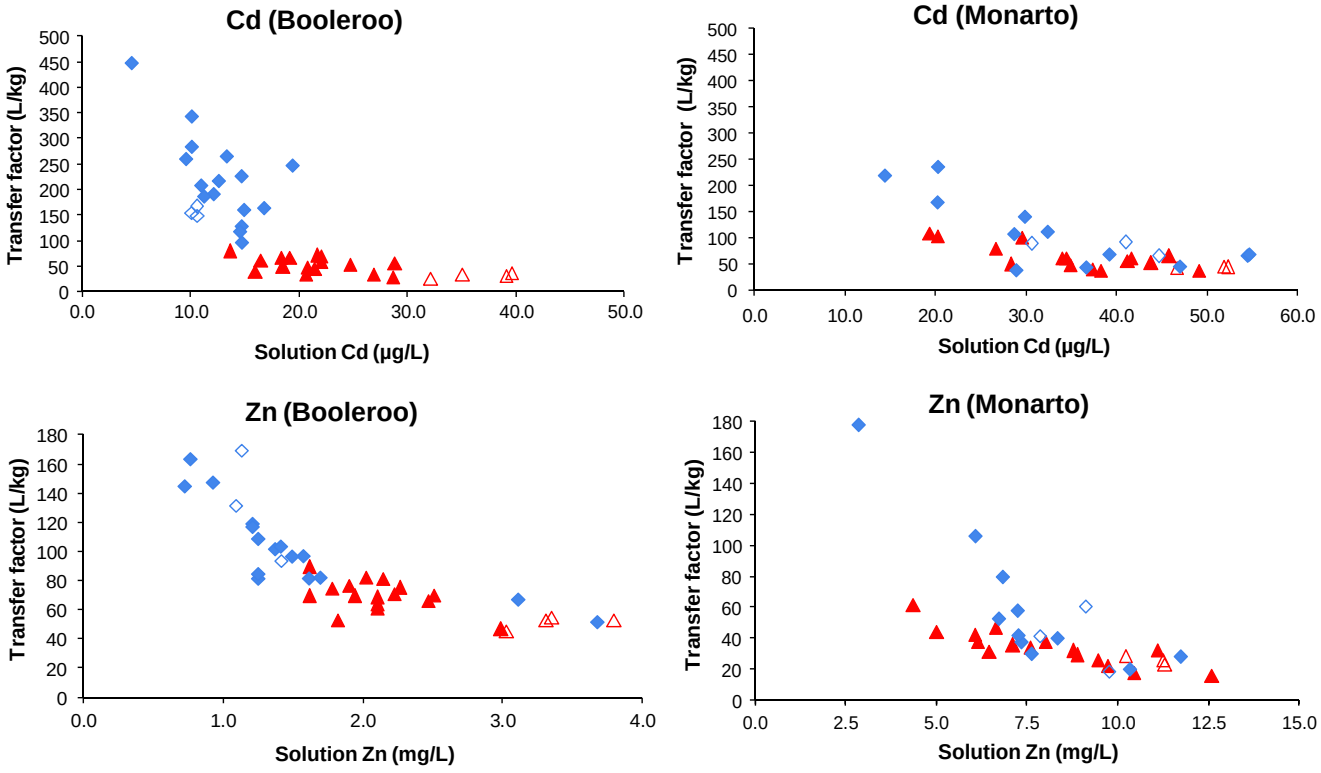

Fig. 7 Relationship between soil solution: plant transfer factors of Cd or Zn and metal concentrations in soil solutions (*empty triangle* maize control, *filled triangle* maize receiving rhamnolipid, *empty diamond* sunflower control, *filled diamond* sunflower receiving rhamnolipid)

By plotting transfer factors against soil solution metal concentrations, we observed different uptake efficiencies for Cd and Zn. Cadmium had higher transfer factors than Zn ($P \leq 0.001$, Fig. 7). Cadmium is less strongly adsorbed to soil than other divalent metals (e.g. Cu, Zn, Ni and Pb; Asami et al. 1995; Bruemmer et al. 1988), which makes it more mobile in soil and therefore more available for plant uptake in the polluted soil as shown in this study. The uptake per unit metal in solution generally declined with solution metal concentration (see Fig. 7). The net uptake of Zn usually responds asymptotically to an increasing Zn concentrations in solution (Rengel and Wheal 1997), whilst the uptake of Cd exhibits a concentration-dependent uptake curve which could be divided into a linear component at low Cd concentrations and a saturated component at higher Cd concentrations (Hart et al. 1998). Therefore, when the uptake rate of Cd or Zn in plants is saturating with increasing solution Cd or Zn concentrations, decreased values of TF would be observed. The TF–concentration relationship also showed that sunflower appeared to be more effective in taking up metals from solution than maize. Generally, the TFs of both metals were higher for sunflower than for maize ($P \leq 0.001$), which is in correspondence to the higher total metal content found in the sunflower shoot than the maize shoot (see Fig. 6a).

Even though no consistent or substantial increase of Cd uptake was observed, some treatments (e.g. 0.02 and 0.2 mmol/kg/wk and 1.4 mmol/kg) rendered Cd more phytoavailable in the Booleroo and Monarto soils (see Fig. 6). Generally, soil solution Cd or Zn in the rhamnolipid-treated soils, with rhamnolipid concentrations ranging from 4 (0.2 mmol/kg/wk) to 280 μM (1.4 mmol/kg), was found to be lower than in the untreated soils (see Table 2), which is in correspondence with the rhamnolipid batch adsorption experiment where increased Cd desorption from soil was only observed at rhamnolipid concentrations >4.4 mM. However, the TFs of Cd or Zn in the rhamnolipid-treated soils were not affected or even increased (see Fig. 7), suggesting that any Cd or Zn–rhamnolipid complexes were effectively taken up by plants. There are data available in the literature suggesting that neutral or lipophilic compounds can be readily absorbed across biological membranes, such as plant roots, via a hydrophobic pathway (Bell et al. 2003; Stacey et al. 2008; Trapp 2002). In this sense, lipophilic rhamnolipid–metal complexes might also be available for plant uptake when smaller structures are favoured to form and able to be taken into plant roots. A recent study showed that at low application rates, Zn–rhamnolipid complexes were found in the roots of canola (*Brassica napus*) as the dominant Zn species, and the uptake of Zn–rhamnolipid was likely through a non-metabolic pathway (Stacey et al. 2008). In the present study, the increased Cd accumulation (see Fig. 6) versus the unchanged or reduced solution Cd (see Table 2) implies the possibility of plant uptake of an intact Cd–rhamnolipid complex that was in soil solution. In addition, in the Booleroo soil where increased Cd uptake was observed, no improvement of Zn uptake was found. By virtue of the higher stability constant of Cd–rhamnolipid (log$K$ 6.9) compared to Zn–rhamnolipid (log$K$ 5.4; Ochoa-Loza et al. 2001), it is expected that Cd–rhamnolipid will be more available for lipophilic uptake.

5 Conclusions

This study supports the conclusion that although rhamnolipid can increase the solubility of Cd in washing of contaminated soils, there is no evidence that rhamnolipid will enhance Cd removal by phytoextraction. Adsorption of rhamnolipid in soil at low application rates resulted in insufficient metal mobilisation into soil solution and subsequently inadequate Cd accumulation by plants for this purpose. High doses of rhamnolipid decreased plant growth significantly. Therefore, the behaviour of rhamnolipid in soils is highly concentration-regulated so that the window where Cd solubilisation is high but phytotoxicity is low is absent or narrow, which limits its use in phytoextraction.

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CHAPTER 4

ASEPTIC HYDROPONICS TO ASSESS RHAMNOLIPID-Cd AND -Zn AVAILABILITY TO SUNFLOWERS (*HELIANTHUS ANNUUS*)

J. WEN[a], M.J. MCLAUGHLIN[ab], S.P. STACEY[a], J.K. KIRBY[b]

[a] Soil and Land Systems, Department of Agriculture, Food and Wine, The University of Adelaide, PMB 1, Glen Osmond, SA 5064, Australia
[b] CSIRO Land and Water, Agricultural Sustainable Flagship, Environmental Biogeochemistry Program, Waite Campus, Urrbrae, SA 5064, Australia

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WEN, J. (Candidate)

Performed analysis on all samples, interpreted data, wrote manuscript and acted as corresponding author
I hereby certify that the statement of contribution is accurate
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……..*Date*……

MCLAUGHLIN, M.J.

Data interpretation and manuscript evaluation
I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis
Signed

……..*Date*……

STACEY, S.P.

Supervised development of work, helped in data interpretation and manuscript evaluation
I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis
Signed

……..*Date*……

KIRBY, J.K.

Helped in data interpretation and manuscript evaluation
I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis
Signed

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Author Affiliation: Jia Wen[1*], Mike J. McLaughlin[1,2], Samuel P. Stacey[1], Jason K. Kirby[2]

1. Soil Sciences, School of Agriculture, Food and Wine, The University of Adelaide, PMB 1, Glen Osmond, SA 5064, Australia.

2. CSIRO Land and Water, Agricultural Sustainable Flagship, Environmental Biogeochemistry Program, Waite Campus, Urrbrae, SA 5064, Australia

[*] **Corresponding Author**: Jia Wen

Address: Soil Sciences, School of Agriculture, Food and Wine, The University of Adelaide, PMB 1, Glen Osmond, SA 5064, Australia.

Telephone number: +61 8 8303 6519

Fax number: +61 8 8303 6511

Email:j.wen@adelaide.edu.au

Abstract

The availability of cadmium (Cd) and zinc (Zn) to sunflower (*Helianthus annuus*) was investigated in rhamnolipid- and EDTA-buffered solutions, in order to evaluate the influence of aqueous speciation of the metals on their uptake by the plant, in relation to predictions of uptake by the free ion activity model (FIAM). Free metal ion activity was estimated using the chemical equilibrium program MINTEQ or measured by Donnan dialysis. The uptake of Cd followed the FIAM for the EDTA-buffered solution at EDTA concentrations below 0.4 μM; for the rhamnolipid-buffered solution, the uptake of both metals in roots was not decreased by increasing rhamnolipid concentrations (reduced free ion activities) in solution. This suggests rhamnolipid might enhance metal accumulation in plant roots per unit free metal in solution, possibly through formation and uptake of lipophilic complexes. It indicates that in soils where free metal ion activities are buffered by the solid phase, complexation by rhamnolipid may lead to higher root uptake due to alleviation of a diffusional limitation to uptake, or due to uptake of the complexed species. However, improved translocation of Cd from roots to shoots was not observed in the presence of rhamnolipid in the short-term. The addition of normal Ca concentrations (low mM range) to the rhamnolipid uptake solutions reduced Cd accumulation in shoots by inhibiting Cd translocation, whereas it significantly increased Zn accumulation in shoots. This study confirms that although rhamnolipid could enhance accumulation of Cd in plants roots at low Ca supply, it is not suitable for Cd phytoextraction in contaminated soil environments where Ca concentrations in soil solution are orders of magnitude greater than those of Cd.

***Keywords*:** Metals; phytoremediation; rhamnolipid; free ion activity model

1. Introduction

Total metal concentrations in soils have been recognised for some time to poorly represent the available and hence the toxic concentration of metal in soils (Campbell, 1995; Checkai et al., 1987; Prokop et al., 2003). The development of methods or models to predict the available pool of metals in soils has stressed that the bioavailability and toxicity of metals are more related to their chemical speciation (Allen, 1993; Allen et al., 1980). The free ion activity model (FIAM) has been used with varying success to predict the phytoavailability and phytotoxicity of metals in hydroponic solutions and soils (Bell et al., 1991; Pavan and Bingham, 1982; Sauvé et al., 1998). This model assumes the rate-limiting step in the uptake of metals is the transport across the plasma membrane into the cytosol (i.e. diffusion of the metal to cell surface binding sites is not rate limiting), and the effect of the metal is proportional to the extent of occupancy of the surface binding sites by free ions. The complexed metal species are assumed not to interact with surface sites, or are not absorbed, and therefore are not bioavailable (Berkelaar and Hale, 2003).

Departures from the FIAM in hydroponics and soils have been reported in many studies with increases in metal uptake correlated with the presence of metal complexes (Berkelaar and Hale, 2003; McLaughlin et al., 1998a; Smolders and McLaughlin, 1996). The increased metal uptake has been suggested to occur due to dissociation of labile metal complexes when the free metal ions become depleted near the membrane surface, and enhanced diffusional flux of metals to sites of uptake (Degryse et al., 2006a; Degryse et al., 2006b; Elgawhary et al., 1970). It is also proposed that uncharged metal complexes may exhibit lipophilicity and be as available to plants as free metal ions (Campbell, 1995; McLaughlin et al., 1998a). This is supported by evidence from marine toxicology that some uncharged metal complexes can move freely across biological membranes (Phinney and Bruland, 1994). The magnitude of the

64 increase of metal uptake in a complexing environment will depend on the ligand type
65 (Degryse et al., 2006a; Degryse et al., 2006b; McLaughlin et al., 1997). Fast-dissociating
66 metal complexes (with low stability constants) could increase metal diffusion from solution
67 to root surfaces to a larger extent by providing a greater flux of free metal ions to the site of
68 uptake.

69 Many organic ligands, such as ethylenediaminetetraacetic acid (EDTA) and ethylenediamine-
70 N,N'-disuccinic acid (EDDS), have been used to increase metal uptake for phytoextraction
71 (Chen et al., 2004; Meers et al., 2005; Tandy et al., 2006). In addition, several studies have
72 shown that surfactants were able to increase metal mobilisation in soils (Juwarkar et al.,
73 2007; Mulligan et al., 2001), which suggests a possible application of using surfactants to
74 increase metal availability to plants and influence their metal phytoremediation potential.
75 However, studies on the subject are scarce. Rhamnolipid is a natural bacterial product that
76 forms stable complexes with metals, such as cadmium (Cd) and zinc (Zn), and it is not highly
77 persistent in soils (Wen et al. 2009). Rhamnolipid biosurfactant has successfully been used in
78 soil-washing procedures to mobilise metal contaminants in soils (Herman et al., 1995;
79 Mulligan, 2005). A few studies have focused on the use of rhamnolipid in phytoremediation,
80 but contradictory results have been obtained. Jordan et al. (2002) and Johnson et al. (2009)
81 showed that rhamnolipid failed to enhance plant uptake of copper (Cu), nickel (Ni) or Zn in
82 hydroponics (Gunawardana et al., 2010; Johnson et al., 2009) and in soil (Jordan et al. 2002)
83 experiments. Jordan et al. (2002) and Johnson et al. (2009) suggested it was unlikely that the
84 relatively large rhamnolipid complex structure would traverse root cell membranes and
85 cortical tissues. However, using ^{14}C-labelled chelants at 20 mM concentrations, Jordan et al.
86 (2002) demonstrated that plants can accumulate significant amounts of rhamnolipid from
87 hydroponic solutions into both roots and shoots, but this was not observed with EDTA. A

recent study using rhamnolipid concentrations < 1.4 mmol/kg showed no consistent or significant increase in the uptake and accumulation of Cd or Zn by sunflower (*Helianthus annuus*) or maize (*Zea mays*) shoots (Wen et al. 2010). These authors suggested the poor enhancement of metal uptake was possibly due to adsorption of the metal-rhamnolipid complex by soils. Nevertheless, the two lowest addition rates of rhamnolipid used in the study (0.02 and 0.2 mmol/kg/wk) still significantly increased Cd and Zn accumulation in the sunflower shoots in the two soils studied. Similarly, Stacey et al. (2008) reported the addition of rhamnolipid at 0.75 to 6 mg/kg increased the uptake of Zn in the roots of Zn-deficient canola (*B. napus* var. Holly), possibly through a non-metabolic, e.g. lipophilic, pathway. Uptake of organic metal complexes through the lipophilic pathway has been shown to be possible (Campbell, 1995; Phinney and Bruland, 1994), and this could be the reason for the increased uptake of Cd or Zn at the μM rhamnolipid concentration range.

In order to investigate the mechanism leading to increased Cd and Zn uptake by plants following the application of rhamnolipid at concentrations < 0.2 mmol/kg/wk (Wen et al. 2010), we used aseptic hydroponics to test the hypotheses that the availability of Cd and Zn for sunflower and their translocation in the plant are increased by rhamnolipid complexation, and that the uptake of Cd or Zn in the presence of rhamnolipid does not fit the FIAM. In this work, EDTA was chosen for comparison due to its strong metal binding ability and hydrophilic properties.

2. **Materials and Methods**

2.1 Overview of study design

Solutions containing different concentrations of rhamnolipid or EDTA (hereafter termed 'uptake solutions') were used as donor solutions for Donnan dialysis to determine "free"

111 metal concentrations. Information on Cd and Zn speciation in the EDTA-buffered uptake
112 solution was obtained using Visual MINTEQ 2.61 (Jon Peter Gustafsson, KTH, Dept. of land
113 and Water Resources Engineering, Stockholm, Sweden). Free Cd and Zn activity in the
114 rhamnolipid-buffered solutions could not be estimated by Visual MINTEQ 2.61 due to lack
115 of information on rhamnolipid in the database. The free Cd and Zn ions were therefore
116 determined using a Donnan dialysis procedure outlined by Nolan et al. (2003). Aseptic
117 hydroponics was used throughout the uptake experiment to prevent biodegradation of the two
118 ligands (Wen et al., 2009).

119 ***2.2 Measurement of Zn(II) and Cd(II) activity in solutions***

120 The rhamnolipid biosurfactant used in this study was a 25% rhamnolipid liquid extract (Jeneil
121 Biosurfactant Company, $C_{26}H_{48}O_9$, $C_{32}H_{58}O_{13}$, CAS no.147858-26-2). The donor solutions
122 (30 mL) for Donnan dialysis contained either 0.4 μM Cd as cadmium nitrate ($Cd(NO_3)_2$) or
123 18 μM Zn as zinc nitrate ($Zn(NO_3)_2$), rhamnolipid concentrations ranging from 0 to 20 μM,
124 0.035 mM Ca as calcium nitrate ($Ca(NO_3)_2$,) and buffer piperazine-N,N'-bis(2-ethane-
125 sulfonic acid) (PIPES, 2 mM) (pH 6.9). The concentrations of the metals in the uptake
126 solutions were equivalent to the total soil solution concentrations in the study of Wen et al.
127 (2010), and the rhamnolipid concentrations used were within the range of the estimated soil
128 solution concentration in that study (based on an average adsorption of 80% to soil and a soil
129 moisture content of 20% w/w). Acceptor solutions (200 μL) contained strontium nitrate
130 ($Sr(NO_3)_2$) to match the ionic strength of donor solutions. All solutions were prepared using
131 ultrapure deionised water (Milli-Q, Millipore). The slight differences in the ionic strength of
132 the donor and acceptor solutions after 2 h were adjusted by the addition of carrier-free ^{22}Na to
133 the donor solution prior to Donnan dialysis. After Donnan equilibrium was reached,
134 subsamples of the acceptor solutions (100 μL) and donor solutions (0.5 mL) were analysed

135 for total Cd and Zn concentrations by inductive coupled plasma-mass spectroscopy (ICP-MS,
136 Agilent 7500ce). Cadmium and Zn concentrations were corrected for differences in ionic
137 strength using the following equation (Nolan et al. 2003):

138 $$\left(\frac{A}{D}\right)_{corrected} = \left(\frac{A}{D}\right)_{measured} \times \left(\frac{D}{A}\right)^{2}_{Na-22} \quad [1]$$

139 where A and D are the activity of the analyte cation or the radioactivity of ^{22}Na in the
140 acceptor and donor solutions, respectively, measured by scintillation counting (LKB Wallac
141 1215 RACKBETA II, Finland). The activity of Cd (II) and Zn (II) in the acceptor solutions
142 was calculated by multiplying their corrected concentrations by their activity coefficients,
143 calculated using the Davies equation.

144 To investigate the effect of Ca concentration on free Cd and Zn concentrations in solutions, a
145 20 μM rhamnolipid and 2 mM Ca solution buffered in 2 mM PIPES (pH 6.9) was dialysed.

146 To assess whether measured free metal concentrations were determined accurately by
147 Donnan dialysis, a solution containing 1.2 μM Cd as $Cd(NO_3)_2$, 1 μM EDTA ($EDTAH_2Na_2$),
148 3 mM Ca as $Ca(NO_3)_2$ and 2 mM PIPES (pH 6.9) was analysed by Donnan dialysis and
149 compared to predicted values determined using Visual MINTEQ.

150 To examine whether non-charged rhamnolipid complexes present in solutions could result in
151 overestimations of free Cd and Zn concentrations by Donnan dialysis, a solution of Cd
152 (1.2 μM) and 2 mM PIPES (pH 6.9) spiked with 20 μM ^{14}C-labelled rhamnolipid was
153 dialysed. The ^{14}C-labelled rhamnolipid was prepared according to Wen et al. (2009). The
154 determination of the "free" metal concentrations was not influenced by the presence of non-
155 charged species in solution if no ^{14}C (e.g. lipophilic ^{14}C-rhamnolipid) was found to be present
156 in the acceptor solution.

157 The influence of the PIPES buffer solution concentration on metal complexation was
158 examined using a donor solution containing 1 μM Cd and with or without 2 mM PIPES (pH
159 6.9).

160 ***2.3 Pre-treatment of sunflower seeds***

161 Sunflower seeds (Sunbird 7, Pacific Seeds Company, Australia) were surface sterilised by
162 rinsing with 95% alcohol, immersing in sodium hypochlorite (NaOCl) containing 4%
163 available chloride (Cl) for 4 min, and rinsing 10 times with deionised water (DI) that had
164 been autoclaved at 121°C for 20 min. The sunflower seeds were pre-germinated on
165 autoclaved Petri dishes that were lined with filter paper moistened with sterilised DI water.
166 On day 6, the thinned seedlings were transferred to a complete nutrient solution in a
167 controlled environment growth chamber (10 h dark at 16°C, 14 h light at 22°C, 41%
168 humidity, and light intensity of 17608 ± 733 lux). The nutrient solution contained Ca
169 (3.43 mM), magnesium (Mg) (1.44 mM), nitrate (8.1 mM), phosphate (0.08 mM), chloride
170 (10 μM), sodium (5.0 mM), potassium (1.3 mM), sulphate (5.8 mM), boron (38 μM),
171 molybdenum (0.6 μM), iron (in the buffered form of iron-EDDHA)(8.2 μM), manganese
172 (Mn) (9.1 μM), Zn (1 μM), and copper (1 μM), buffered at pH 6.9 with 2 mM PIPES.
173 Containers in which seedlings grew were wrapped in aluminium foil so that roots were grown
174 in a dark environment. Sterilised nutrient solutions were aerated using 0.2 μm filtered air and
175 replaced every two days. After 3 weeks, the plants were transferred to pre-treatment solution
176 A (0.5 mM $Ca(NO_3)_2$, 2 mM PIPES, pH 6.9) for 24 h and then pre-treatment solution B
177 (0.035 mM $Ca(NO_3)_2$, 2 mM PIPES, pH 6.9) for 15 min. Pre-treatment solution A contained
178 Ca at 0.5 mM concentration in order to desorb metals apoplastically bound to roots. The pre-
179 treatment solution B was used as a transition to alleviate the effect of the difference in Ca
180 concentrations between pre-treatment solution A and the uptake solution.

2.4 Adsorption and translocation of rhamnolipid-Cd and -Zn complexes

The pre-treated seedlings (thinned to one plant per vial) were transferred to uptake solutions containing Cd or Zn as single metal or complexed metal with increasing concentrations of ligands (EDTA or rhamnolipid). The uptake solution was prepared freshly by addition of 0.4 μM Cd or 18 μM Zn to a ligand solution buffered with 2 mM PIPES and 0.035 mM $Ca(NO_3)_2$ at pH 6.9. Ligand rates were 0, 4, 8, 12, 16, 20 μM rhamnolipid or 0, 0.1, 0.2, 0.3, 0.4, 0.5 μM EDTA. In order to assess the effect of Ca on Cd and Zn uptake in the presence of rhamnolipid, an uptake solution containing 20 μM rhamnolipid and 2 mM Ca was used. The uptake solutions were spiked with ^{109}Cd or ^{65}Zn at 0.037 MBq/L prior to transplanting. The solutions were filtered through 0.2 μm filters to sterilise. Each treatment was replicated three times.

After a 24-h uptake period in a controlled environment growth chamber (10 h dark at 16°C, 14 h light at 22°C, 41% humidity), sunflower plants were removed, roots rinsed with ultra pure deionised water (Millipore) and transferred to isotope desorption solutions at 2°C for 30 min (2 mM PIPES, pH 6.9, 0.5 mM Ca, 200 μM Zn and 5 μM Cd). The remaining uptake solution was analysed for potassium (K) by inductively coupled plasma optical emission spectroscopy (ICP-OES). Roots were rinsed thoroughly in ultrapure deionised water (Millipore) after removal from the desorption solution. Roots and shoots were separated, dried, finely ground and digested using concentrated nitric acid (Zarcinas et al., 1996). The digests were transferred into radioactivity counting vials and the radioactivity measured by gamma spectroscopy (1480 Wizard, Wallac, EG&G Co., Turku, Finland). The free metal uptake efficiency (expressed as a fraction) was calculated by a method modified from that of McLaughlin et al. (1997) and defined as the tissue metal uptake (μmol) divided by the total free metal in the uptake solution (μmol).

$$Cd^{2+}(Zn^{2+})\ \text{uptake efficiency} = \frac{\text{Tissue Cd(Zn) uptake}}{\text{Initial } Cd^{2+}(Zn^{2+}) \text{ in solution}} \quad [2]$$

2.5 Statistical Analysis

Analysis of variance (ANOVA) was performed on all data using Genstat (10th edition, VSN International Ltd.). Least significant differences (LSD) were used to separate means where ANOVA showed significance at $P \leq 0.05$. Where variances were proportional to the magnitude of the mean values, data were transformed prior to analysis.

3. **Results**

3.1 Cd(II) and Zn(II) activity in uptake solutions

Free Cd(II) ion activities in 1 μM EDTA solution measured by Donnan dialysis and predicted by MINTEQ were found to be in good agreement (measured [Cd(II)] activity 17.1 μg/L, calculated [Cd(II)] activity 16.8 μg/L). This finding is similar to that previously published by Nolan et al. (2003) using Donnan dialysis. The possible presence of uncharged rhamnolipid-metal complexes was found to have no effect on free Cd and Zn concentrations by Donnan dialysis with no ^{14}C-rhamolipid found in acceptor solutions following addition to donor solutions.

Free Cd(II) and Zn(II) ion concentrations (expressed as a % of total Cd and Zn concentrations in solutions) decreased with increasing rhamnolipid concentration in the solution (Fig. 1). At the highest rhamnolipid concentration (20 μM), 35% of Cd and 58% of Zn in the solutions was complexed (Fig. 1). In the uptake experiment (Fig. 2), the control samples (no ligand amended) had measured Cd and Zn activities (the *empty circles* with the highest ion activity values) slightly lower than the predicted values (the *solid diamonds* with the highest ion activity values). The deviation was found to be even bigger for Zn; this may be attributed to a

loss of free ions to the vial walls or Donnan system in the absence of the ligands' buffering effect.

The MINTEQ predictions showed that free Cd (%) decreased from 100% in the ligand-free solution to < 1% in the solution with 0.5 μM EDTA. The strong complexation of Cd to EDTA resulted in a wider range of free ion activities in the EDTA-uptake solutions than in the rhamnolipid-uptake solutions (Fig. 2). In contrast, only 3% of Zn was complexed by 0.5 μM EDTA, due to the molarity of Zn (18 μM) being far in excess of that of EDTA (0.1 to 0.5 μM). The conditional stability constants (log K) of rhamnolipid-Cd and rhamnolipid-Zn complexes were calculated according to the method of Ochoa-Loza et al. (2001) and were determined to be -3.1 and -1.2, respectively.

An increase in Ca concentrations from 0.035 mM to 2 mM reduced Cd and Zn complexation by rhamnolipid. The addition of 2 mM Ca increased, relative to the free metal concentrations in 0.035 mM Ca, the free Cd (%) activity in solution from 65% to 73%, and the free Zn (%) concentration from 42% to 68% (Table 1). The stronger complexation of rhamnolipid with Zn than Cd was demonstrated by the lower percentages of free Zn in the uptake solutions at both Ca concentrations (Table 1).

3.2 Adsorption and translocation of rhamnolipid-Cd and -Zn complexes

Plant dry mass of sunflower shoots and roots in the ligand-buffered uptake solutions were not significantly different from the control samples (data not shown), except for a dramatic reduction (60%) in root biomass at the higher EDTA concentrations (0.4 and 0.5 μM) ($P \leq 0.001$). The toxicity of EDTA at the 0.5 μM concentration was also evidenced by a 3-fold increase of K^+ efflux from roots compared to the control (Table 2), as root K^+ efflux can be used as an index of root membrane integrity (Wagatsuma et al., 1987). In contrast, K^+

efflux was unaffected or reduced as rhamnolipid concentrations increased (Table 2). Plant biomass and the rates of K^+ efflux indicate that the concentrations of rhamnolipid used in the uptake solutions were not phytotoxic. Concentrations of nutrient elements in all shoots, such as Ca, K, Mg, Mn, phosphorus and sulphur, were within recommended nutritional requirements (Grundon et al., 1997) (data not shown).

The accumulation of Cd in sunflower roots and shoots significantly decreased with decreasing free Cd(II) activity in EDTA solutions ($P \leq 0.001$, Figs. 2 & 3). In contrast, Zn accumulation in roots and shoots were not significantly different from the control with the addition of increasing concentrations of EDTA ($P > 0.05$, Figs. 2 & 3). The accumulation of Cd and Zn in roots and shoots was not affected by increasing solution concentrations of rhamnolipid (Figs. 2 & 3).

The change in Cd(II) and Zn(II) uptake efficiency showed different patterns for the two ligands (Fig. 4). The Zn(II) uptake efficiency for EDTA-treated plants was not shown because of negligible effects of EDTA on Zn speciation in solution and uptake by plants. The Cd(II) uptake efficiency in both root and shoot remained constant until EDTA concentration reached 0.3 μM, after which an increase of up to 100-fold was found at the two highest EDTA concentrations. There was an increase in the Cd(II) and Zn(II) uptake efficiency in the roots with increasing rhamnolipid concentration, but not in the shoots.

In the rhamnolipid treatment, the increase of Ca concentration to 2 mM reduced root Cd by 43% and halved Cd accumulation in sunflower shoots (Table 1). However, Zn accumulation in the root was not inhibited; shoot Zn increased significantly at the higher solution Ca concentration (Table 1). The shoot Ca concentration significantly increased from 1.5% to 2.0% in the Cd treatment ($P \leq 0.05$), and from 1.7% to 2.6% in the Zn treatment (Table 1).

4. **Discussion**

In this study, increasing EDTA concentrations in solutions reduced the uptake and accumulation of Cd in roots and shoots of sunflower. This decrease in Cd uptake was likely due to the reduction in free Cd(II) ion concentrations in the solution, and possibly increasing the diffusional limitation to Cd uptake in the unstirred layer adjacent to the root or in the root apoplast. The increased uptake efficiency of Cd at 0.4 and 0.5 μM EDTA may have been due to physiological stress or root damage, considering the significantly increased K^+ efflux from roots with increasing EDTA concentration ($P \leq 0.01$, Table 2). Therefore, the negatively charged Cd-EDTA complexes that could not traverse negatively charged root surfaces may have entered roots apoplastically, through breaks in the endodermal barrier induced by EDTA. This damage-induced influx of Cd-EDTA complexes, which was also documented in the study of Collins et al. (2002), may have led to the increased Cd(II) uptake efficiencies at the two highest EDTA concentrations.

The stability constant of rhamnolipid-Cd calculated in the present study was -3.1 (log K unit), similar to the value of -2.5 (log K unit) reported by Tan et al. (1994), but much smaller than the value of 6.9 reported by Ochoa-Loza et al. (2001) using an ion-exchange technique. The log K value for rhamnolipid-Cd in this study is higher than the values reported by Fu et al. (1992) for Cd binding to humic acids (log K -6.0 to -4.9). The addition of rhamnolipid up to 20 μM caused a significant reduction in Cd(II) concentrations ($P \leq 0.001$) in the uptake solution measured immediately after preparation. However, Cd accumulation in plant roots was greater for rhamnolipid treatments than in equivalent EDTA treatments at the same solution Cd^{2+} activities (Fig. 2).

The uptake and accumulation of Cd and Zn in sunflower roots was not affected by the reduced free metal activity in the uptake solutions as rhamnolipid concentrations increased. It

297 has been previously shown that lipophilic metal complexes can increase the absorption of
298 metals in soils by plant roots (Bell et al., 2003; McLaughlin, 2002). The relatively constant
299 Cd and Zn accumulation in roots in the present study with increasing rhamnolipid (and
300 decreasing free Cd(II) and Zn(II) activities) suggests the uptake of lipophilic complexes (Wen
301 et al. 2010) through lipophilic pathways may be important. This hypothesis is supported by
302 previous findings by Stacey et al. (2008) who reported Zn to be predominantly found in roots
303 as a Zn-rhamnolipid complex after rhamnolipid application. However, the very narrow range
304 of Cd activities in the uptake solution precludes making a solid conclusion regarding the
305 uptake of lipophilic Cd complexes.

306 Cadmium and Zn uptake efficiencies for roots increased at higher rhamnolipid concentrations
307 ($P \leq 0.05$) while they were relatively constant for shoots (Fig. 4). This suggests that, at the
308 application rates used and over the short exposure period used (24 h), the rhamnolipid-metal
309 complexes were not transported to any great extent to the xylem and to plant shoots, but they
310 did affect root Cd accumulation. Johnson et al. (2009) and Gunawardana et al. (2010)
311 suggested that rhamnolipid complexes with Cd, Cu and Pb are not readily translocated within
312 plants. These authors attributed the absence of metal-rhamnolipid translocation to the
313 relatively large size of the rhamnolipid complexes. However, Jordan et al. (2002) found
314 significant amounts of rhamnolipid in both roots and shoots in their hydroponic experiments
315 (using ^{14}C-labelled rhamnolipid), and Stacey et al. (2008) directly identified Zn-rhamnolipid
316 complexes in plant roots using synchrotron X-ray techniques. Stacey et al. (2008) also
317 demonstrated a significant enhancement of shoot Zn accumulation when rhamnolipid was
318 applied (once) at low rates (10 μmol/kg) and plants grown for 31 d. Hence, while our short
319 term uptake experiments did not see significant translocation of Cd and Zn to shoots, the
320 increased uptake efficiencies suggest that the lipophilicity of the metal-rhamnolipid

complexes appears to overcome any limitations imposed by complex size, provided the rhamnolipid is not strongly sorbed (Wen et al. 2010) or degraded (Wen et al. 2009).

It is important that effects of chelants on metal uptake determined in hydroponics are considered separately from those observed when applied to soils. Addition of chelants to nutrient solution reduces the free metal ion activity, so that metal uptake is expected to decrease provided the metal-chelant complex is not absorbed, or provided the chelant does not alleviate a diffusional limitation to metal uptake (McLaughlin 2002; Degryse et al. 2006a,b). Hence it is important that careful attention is paid to confirming metal speciation in solutions by direct measurement. Where metal uptake is increased by addition of chelants in nutrient solution, this is often due to damage to root cell membranes (Collins et al., 2002; Epstein et al., 1999; Vassil et al., 1998). When chelants are applied to soils, metals are complexed and desorbed from surface exchange sites, or easily soluble solid phases and, due to buffering of free metal ion activity by soils surfaces, free ion concentrations in solution may not change, but total metal concentrations may increase markedly (Huang et al., 1997). Only if complexed metals are taken up by roots (either as a function of the metal chelate complex or the toxicity of the chelant) will plant accumulations of metals increase (Nowack et al., 2006). In soil experiments, Jordan et al. (2002) found that rhamnolipid was not as effective as EDTA to increase Cu and Zn accumulation by both *Zea mays* and *Atriplex*, and no data on the chelants toxicity were provided. It is likely that the greater accumulation of metals with EDTA was linked to plant phytotoxicity, as the chelants concentration used was at least 5 mM in the soil solution (assuming the soil had a water holding capacity of 20% w/w). In our experiments we used low chelant concentrations ($\leq$ 20 μM rhamnolipid and $\leq$ 0.5 μM EDTA) to minimise the effects of toxicity, but even at 0.4 μM EDTA it was found to impair root integrity which coincided with increased metal uptake.

345 High Ca concentrations in solutions competing with Cd (when Ca/Cd molar ratio > 100) and
346 Zn (when Ca/Zn molar ratio >10) for rhamnolipid complexation has been documented by
347 Ochoa-Loza et al. (2001). Because of a similar radius to that of Cd, Ca has been repetitively
348 reported to suppress Cd uptake by plants, either by competing for exchange sites at root
349 surfaces or by its effect on cell membranes (Jarvis et al., 1976; Lu et al., 2008; Zhao et al.,
350 2002). In the present study, the higher concentration of Ca (2 mM) in uptake solutions,
351 despite displacing Cd from rhamnolipid, did not affect root Cd concentration, which is in
352 agreement with previous results reported by McLaughlin et al. (1998b) and Tyler and
353 McBride (1982), suggesting that any competition from Ca with Cd for absorption at the root
354 surface was balanced by displacement of free Cd(II) from the ligand. However, the reduced
355 shoot Cd concentration and accumulation and increased shoot Ca uptake indicate that there
356 may be a Ca-Cd ion competition for translocation, likely through a competitive inhibition of
357 intracellular Cd uptake (Noraho and Gaur, 1995). In the soil environment, soil solution Ca
358 concentrations (normally in the lower mM range) are far above solution Cd concentrations
359 (usually in the nM in most soils, and possibly low μM range in highly contaminated soils).
360 Therefore, even if rhamnolipid was used at ≤ 20 μM to improve root Cd accumulation at
361 0.035 mM Ca used in this study, the inhibitive effect of Ca at higher concentrations more
362 indicative of soils (e.g. 2 mM) on Cd uptake and translocation reduces its potential for use in
363 Cd phytoextraction. Higher concentrations of rhamnolipid (mM range) are unsuitable for
364 phytoremediation due to phytotoxicity (Wen et al., 2010).

365 Previous short-term uptake studies have reported a suppressive effect of Ca on Zn absorption
366 in roots, due to competition for binding sites on root surfaces (Bell et al., 1989; Chaudhry and
367 Loneragan, 1972). However, increasing Ca concentrations from 0.035 mM to 2 mM (a Ca
368 concentration normally encountered in soil) in this study did not result in any reduction of

root Zn concentration and accumulation; indeed it resulted in a significant increase in shoot Zn accumulation. This increased uptake by sunflower may be due to increased displacement of Zn(II) from the rhamnolipid by Ca, providing the plant roots with more Zn(II) for uptake. The lack of change in Zn concentrations in the roots with increased Ca may be due to the Zn taken up by the root systems being rapidly transported to shoots.

5. **Conclusions**

The study showed there was a good agreement between Cd uptake in the EDTA treatments at the lower concentrations (< 0.4 μM) and Cd(II) ion activities in solution, supporting the FIAM. Significantly enhanced uptake of Cd by high concentrations of EDTA was related to damage to root membranes. In comparison, the pattern of Cd uptake in roots was not in agreement with free Cd(II) ion activities in solution, likely due to root absorption of lipophilic rhamnolipid-Cd complexes. However, improved metal translocation from roots to shoots was not seen in the short exposure period. Rhamnolipid complexes free Cd and Zn ions in solution, but the stability constants of the complexes are low and Ca concentrations in soil solution will therefore play a key role in moderating the complexation capacity of this chelant.

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Figure Legend

Figure 1. Free Cd (A) and Zn (B) (%, based on total metal concentration) concentrations with increasing rhamnolipid concentrations in uptake solution (lines for best-fit linear regression are y=1.6x+99, R^2=0.91 (Cd) and y=1.9x+89, R^2=0.85 (Zn), $P \leq 0.001$. Values are means ± 1 S.E., n =3).

Figure 2. Cd (A) and Zn (B) accumulation in sunflower root treated with rhamnolipid and EDTA (line for best-fit linear regression of Cd in EDTA treatment: y=0.054x+0.26, R^2=0.93; linear regression for other treatments are not shown due to low correlation. Values are means ± 1 S.E., n =3).

Figure 3. Cd (A) and Zn (B) accumulation in sunflower shoot treated with rhamnolipid and EDTA (line for best-fit linear regression of Cd in EDTA treatment is y=0.0067x+0.024, R^2= 0.99; linear regression for other treatments are not shown due to low correlation. Values are means ± 1 S.E., n =3).

Figure 4. Relationship between Cd^{2+} or Zn^{2+} uptake efficiency and concentrations of ligand (A — Cd uptake efficiency affected by EDTA, B — Cd uptake efficiency affected by rhamnolipid, C — Zn uptake efficiency affected by rhamnolipid. Values are means ± 1 S.E., n =3).

534 Table 1. Effect of Ca on free Cd and Zn concentrations in rhamnolipid (20 μM) uptake
535 solution and concentrations in plants (Mean ± STD)*

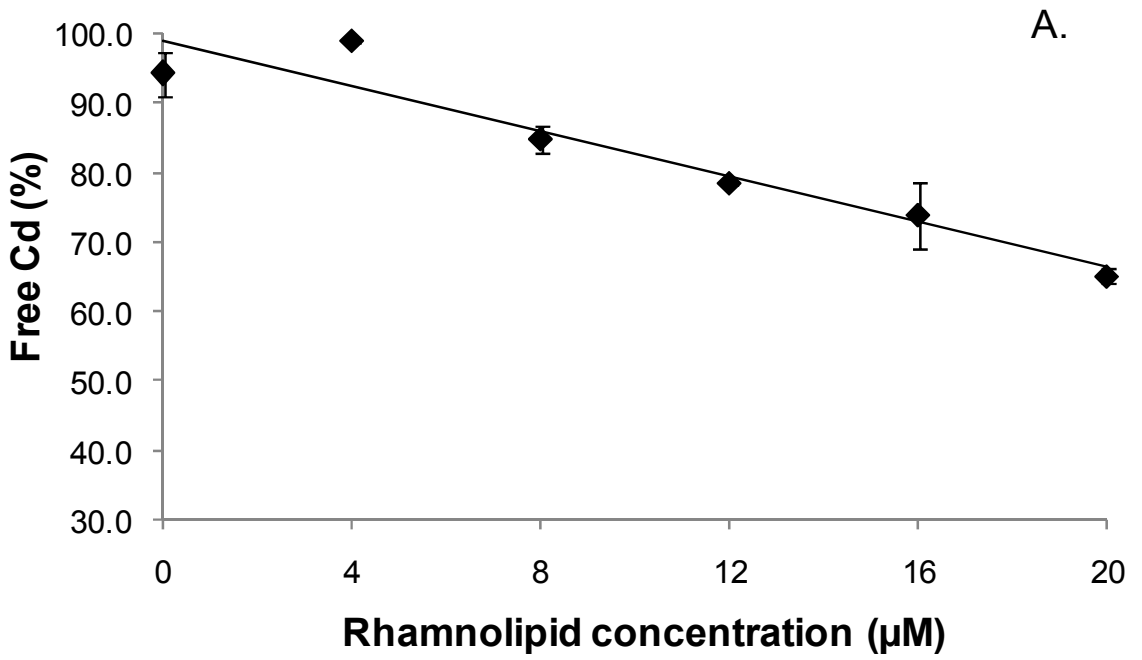
		0.035 mM Ca	2 mM Ca
Free metal (%) measured by Donnan Dialysis	Cd	65.1 ± 1.1^{a}	73.4 ± 1.1^{b}
	Zn	41.9 ± 7.8^{a}	68.3 ± 7.7^{b}
Root concentration (μmol/g DW)	Cd	0.17 ± 0.015^{ns}	0.18 ± 0.052^{ns}
	Zn	2.9 ± 0.55^{ns}	3.5 ± 0.19^{ns}
Shoot concentration (μmol/g DW)	Cd	$(9.5 \pm 1.4) \times 10^{-3b}$	$(6.1 \pm 1.3) \times 10^{-3a}$
	Zn	0.27 ± 0.068^{a}	1.5 ± 0.41^{b}
Root accumulation (μmol/DW plant)	Cd	$(16 \pm 2.7) \times 10^{-3b}$	$(9.2 \pm 1.6) \times 10^{-3a}$
	Zn	0.32 ± 0.072^{ns}	0.31 ± 0.027^{ns}
Shoot accumulation (μmol/DW plant)	Cd	$(1.8 \pm 0.41) \times 10^{-3b}$	$(0.94 \pm 0.14) \times 10^{-3a}$
	Zn	0.068 ± 0.004^{a}	0.20 ± 0.061^{b}
Shoot Ca concentration range (%)	Cd	1.52 ± 0.27^{a}	2.03 ± 0.074^{b}
	Zn	1.71 ± 0.65^{ns}	2.56 ± 0.29^{ns}

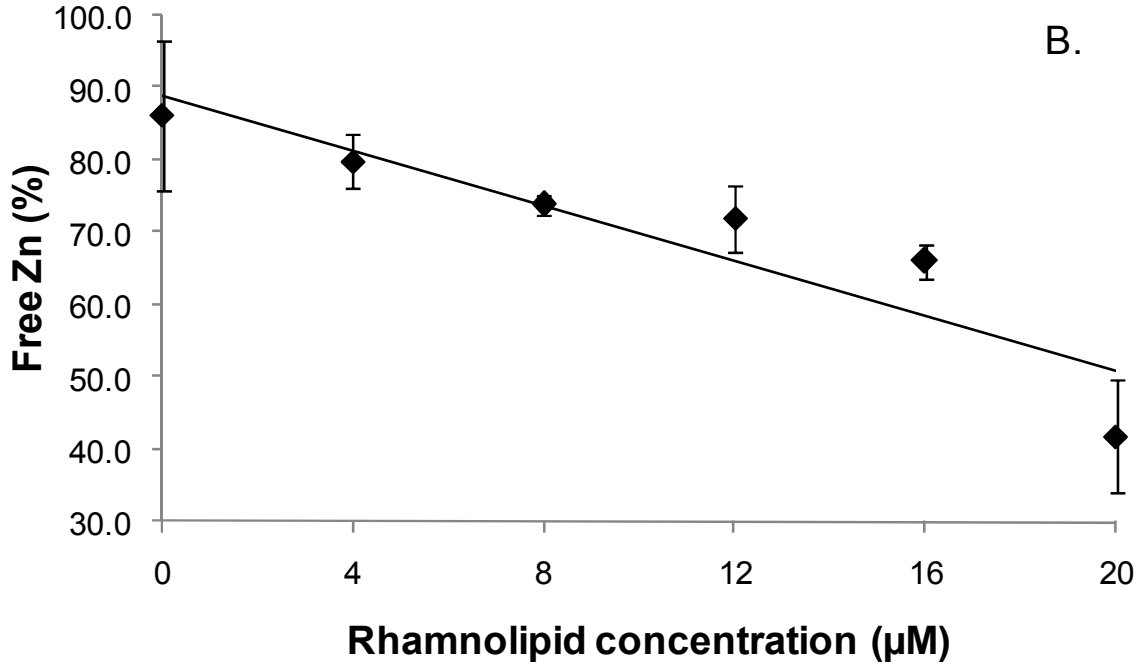
536 *Letters indicate significant differences at $P \leq 0.05$, ^{ns} non-significant difference.

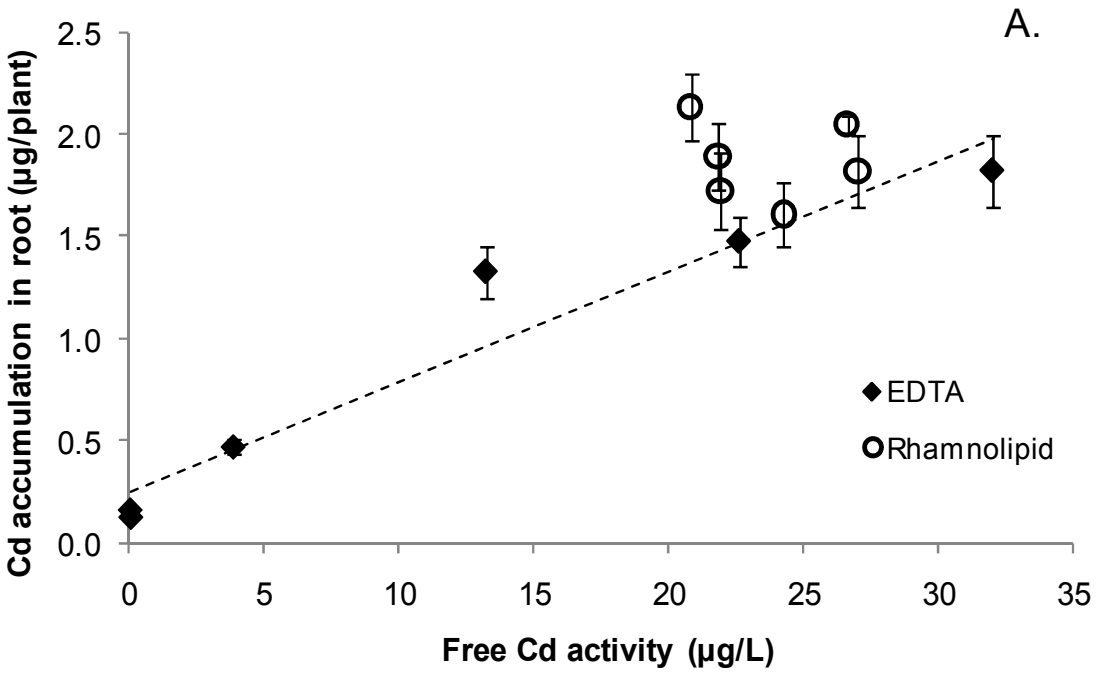
Table 2. Potassium efflux (μmol/g DW)* from sunflower roots over 24 h in uptake solutions containing Cd or Zn in the presence of EDTA or rhamnolipid at different concentrations.

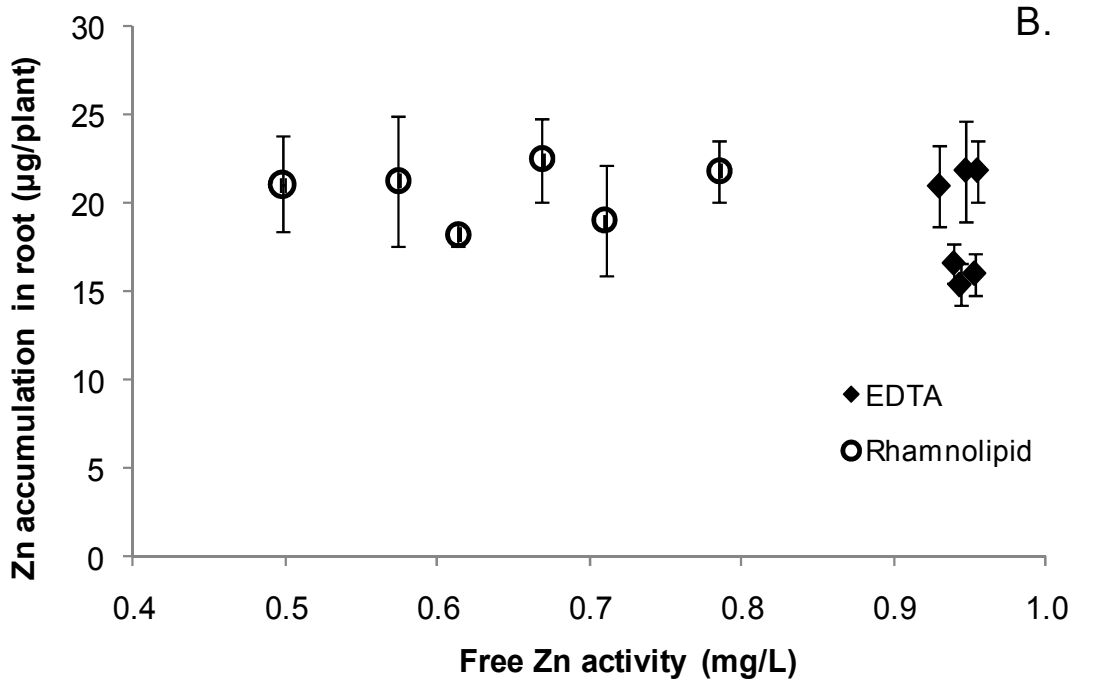
Metal	EDTA concentrations (μM)					
	0	0.1	0.2	0.3	0.4	0.5
Cd	29^{a}	54^{b}	77^{b}	55^{b}	68^{b}	100^{c}
Zn	94^{ns}	81^{ns}	92^{ns}	109^{ns}	75^{ns}	113^{ns}
Metal	Rhamnolipid concentrations (μM)					
	0	4	8	12	16	20
Cd	29^{ab}	23^{ab}	53^{b}	1.6^{ab}	0.1^{a}	0.8^{a}
Zn	94^{b}	3.9^{a}	2.0^{a}	2.6^{a}	1.6^{a}	2.3^{a}

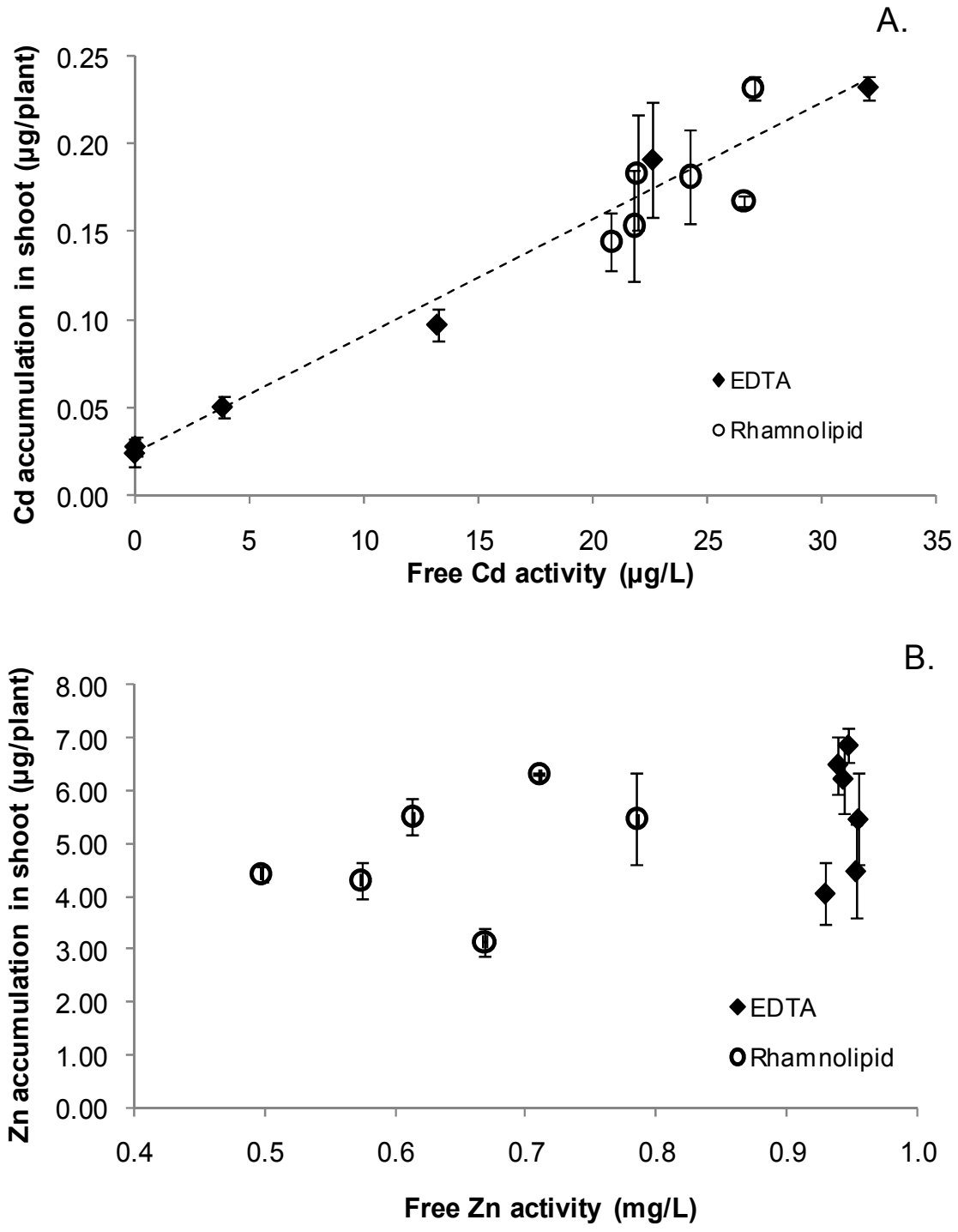
*Letters indicate significant differences at $P \leq 0.01$, ^{ns} non-significant difference

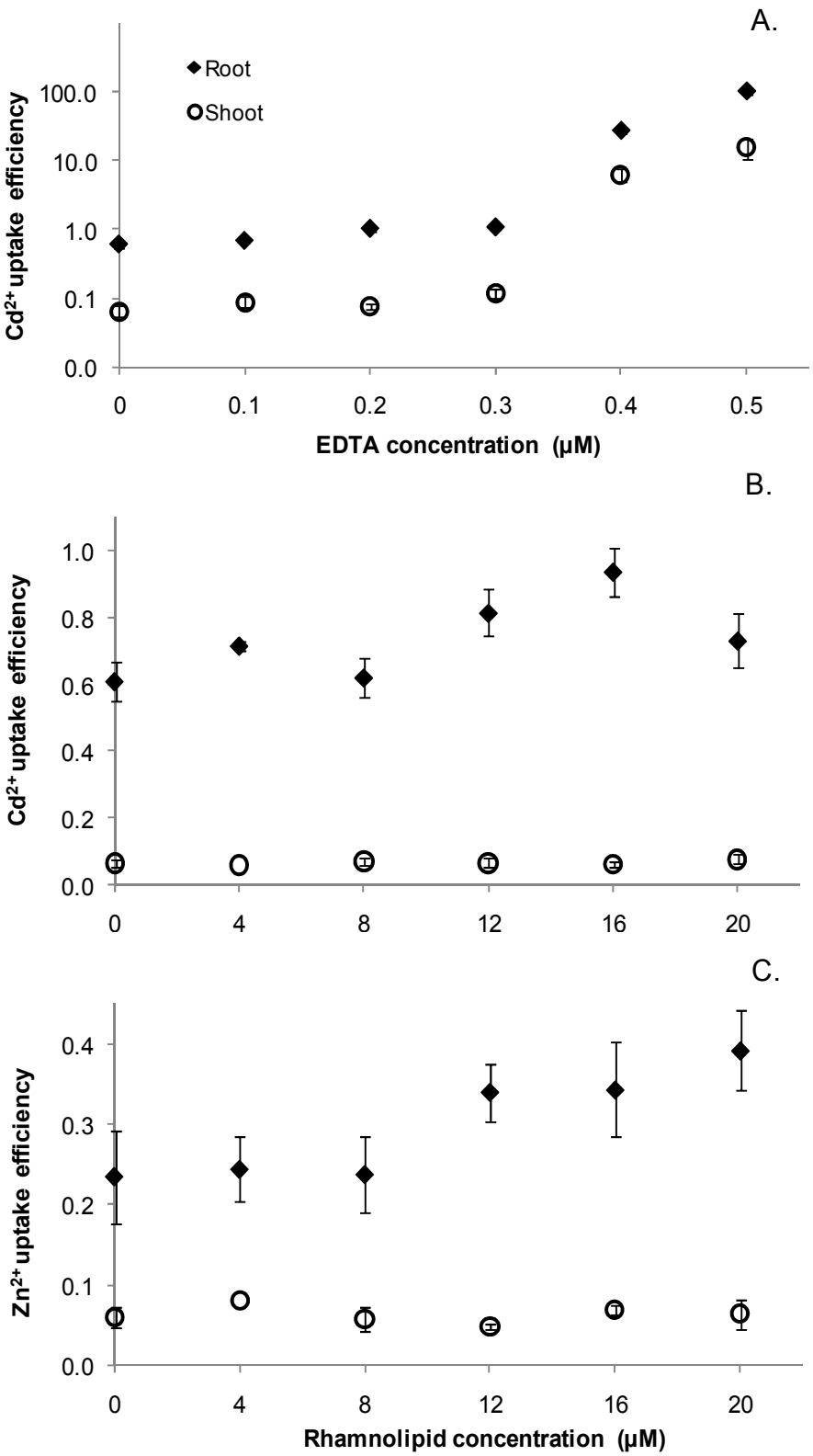

A.
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
Free Cd (%)
0
4
8
12
16
20
Rhamnolipid concentration (µM)


B.
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
Free Zn (%)
0
4
8
12
16
20
Rhamnolipid concentration (µM)


A.
Cd accumulation in root (µg/plant)
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
35
Free Cd activity (µg/L)
EDTA
Rhamnolipid


B.
Zn accumulation in root (µg/plant)
30
25
20
15
10
5
0
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Free Zn activity (mg/L)
EDTA
Rhamnolipid


A.
Cd accumulation in shoot (µg/plant)
0.25
0.20
0.15
0.10
0.05
0.00
0
5
10
15
20
25
30
35
Free Cd activity (µg/L)
EDTA
Rhamnolipid
B.
Zn accumulation in shoot (µg/plant)
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Free Zn activity (mg/L)
EDTA
Rhamnolipid


A.
Root
Shoot
100.0
10.0
1.0
0.1
0.0
Cd2+ uptake efficiency
0
0.1
0.2
0.3
0.4
0.5
EDTA concentration (μM)
B.
1.0
0.8
0.6
0.4
0.2
0.0
Cd2+ uptake efficiency
0
4
8
12
16
20
C.
0.4
0.3
0.2
0.1
0.0
Zn2+ uptake efficiency
0
4
8
12
16
20
Rhamnolipid concentration (μM)

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Cadmium is well known as a metal for its deleterious effect on humans. Long-term inhalation of Cd fumes through air or excessive intake through the food chain will result in diseases or metabolic dysfunction, such as lung cancer, bone lesions and kidney damage (ATSDR 1999). Although the world production of Cd has decreased since 2000, mainly because of a curtailed production in many Western European countries, production from the Asian nations (e.g. China, Korea and India) has expanded, as did production in Eastern Europe (Morrow 2001). The wide use of Cd in Ni-Cd batteries, pigments and intensive smelting or mining activities make land and water contamination by Cd prevalent in many industrialised and mining regions. The issue of soil Cd contamination has gained special attention in Asian countries where rice is a staple crop, as Cd has been found to accumulate in rice grain (Chaney et al. 2004). The cost of remediating Cd-contaminated soil by conventional methods was projected to cost $400 billion in the US alone (Salt et al. 1995). This high cost has fuelled interests in alternative, potentially less expensive, remediation techniques such as phytoextraction. Chelant-assisted phytoextraction has been proposed as an environmentally friendly and promising method for remediation of Cd-contaminated soils, especially in moderately contaminated soils.

Choosing a suitable chelant for metal phytoextraction is critical for effective phytoextraction. Conventional chelants, e.g. EDTA and EDDS, have proven to be useful in enhancing metal availability for plants (Saifullah et al. 2009; Wang et al. 2007). However, many chelants currently being used are either toxic to flora and fauna (e.g. NTA), are mobilising contaminants leading to off-site movement through leaching or run-off (e.g. EDTA), are not easily degraded (e.g. EDTA), or are degraded too fast to sustain phytoextraction (e.g. CA). Therefore, there is a need to identify metal-complexing agents that are both environmentally compatible and have high efficacy.

In recent years, the biosurfactant rhamnolipid has been used in soil washing procedures to remove metals, particularly Cd, Pb and Zn, from contaminated soils (Juwarkar et al. 2007).

Rhamnolipid forms lipophilic complexes with Cd (Ochoa-Loza et al. 2001) and metal bioavailability may be increased when associated with lipophilic compounds (Bell et al. 2003; Phinney and Bruland 1994; Stacey et al. 2008; Trapp 2002). Therefore, rhamnolipid could potentially be used to enhance Cd phytoextraction. The studies reported in this thesis, therefore, examined the behaviour of rhamnolipid in Cd and Zn co-contaminated soils and, evaluated its ability to enhance Cd phytoextraction.

5.1 Biodegradability

The biodegradability of chelants is a key factor to be considered when assessing their suitability for application to soils. The widely used chelant EDTA can greatly improve metal mobility in soils, but its poor biodegradability and strong metal complexing capacity directly result in increased risks of metal leaching and contamination of groundwater (Chen et al. 2004). Although low molecular weight organic acids, such as citric and malic acids, can effectively mobilise soil metals when first applied, their fast degradation may result in re-adsorption of metals on soils and consequently reduced metal availability. For rhamnolipid, its biodegradability has previously not been reported in moderately contaminated soils, but only in non-complex environments, e.g. mixed compost and liquid culture (Zeng et al. 2007), after treatment with specific species of bacteria (Frank et al. 2010), or in single-metal spiked soils with extremely high Cd concentrations (394 to 1777 mg/kg) (Maslin and Maier 2000). The present study confirmed that rhamnolipid is biodegradable in complex soil environments with moderate Cd contamination (10 mg/kg), with a cumulative degradation of 36% and 29% (of total applied) for low (2 mmol/kg) and high (10 mmol/kg) application rates after 20 days, respectively (Chapter 2). By contrast, only 14% of EDTA applied degraded over 20 days, while almost 70% of the applied CA degraded after 20 days. Rhamnolipid was degraded more slowly than CA, but it was not recalcitrant as indicated by the continuing degradation observed at 20 days after application, irrespective of dosage or soil contamination levels. This indicates that the application of rhamnolipid to soils would result in reduced metal leaching

compared to EDTA due to higher rate of degradation, but it is persistent enough compared to CA for potential use in the phytoextraction of Cd-contaminated soils.

5.2 Application strategies for improved metal phytoextraction

A successful phytoextraction strategy requires a suitable concentration of chelant for application to soil where both cost and efficiency constraints can be met. Chelant sorption to soil may reduce its capacity to mobilise metal contaminants. In previous studies, rhamnolipid was readily adsorbed to soils containing high levels of clay and organic matter (Herman et al. 1995; Ochoa-Loza et al. 2007). In this study, low soil pH and high calcium carbonate content were two additional factors found to contribute to high rhamnolipid adsorption in soils (Appendix 1). In this study, rhamnolipid sorption was found to be minimised and Cd desorption enhanced at the initial rhamnolipid solution concentrations between 4 and 5 mM (equivalent to 20 and 25 mmol/kg soil). However, the use of rhamnolipid in this concentration range was found to have deleterious effects on plant growth (Chapter 3). The toxic effect of rhamnolipid found in this study in the low mM range was recently confirmed from a hydroponic study investigating the use of rhamnolipid for Cd, Cu and Pb phytoextraction when applied at 1.5 mM (Gunawardana et al. 2010). This rhamnolipid concentration range was also found to reduce Cd accumulation in plant shoots in this study (Chapter 3), which corresponds with the work of Jordan et al. (2002) where rhamnolipid did not enhance metal uptake when applied at low mM concentrations. The possible reasons for the phenomenon, suggested from this study, are changes in the lipophilicity and morphology of rhamnolipid with increasing concentration, leading to more rhamnolipid partitioning to soil solution and consequent phytotoxicity.

When low concentrations of rhamnolipid ($\leq$ 1.4 mmol/kg, equivalent to $\leq$ 1 mM in soil solution based on an average sorption loss of 80% and soil moisture content of 41% w/w) were used in the phytoextraction study, they were found not to consistently improve plant uptake of Cd and Zn from soils (Chapter 3). This is in contrast to previous work by Stacey et

al. (2008) who found rhamnolipid concentrations of < 0.01 mmol/kg to be effective in enhancing shoot Zn uptake in bread wheat and durum wheat. In this study, the concentrations of Cd and Zn in soil solutions were not increased in most rhamnolipid treatments (Chapter 3). The batch adsorption and octanol/water partition experiments in Chapter 3 found that a large portion of the rhamnolipid applied at these concentrations was sorbed to the soil, probably due to its hydrophobicity. Significant increases in Cd uptake by sunflower were only found at rhamnolipid addition rates of 0.02 and 0.2 mmol/kg/wk, and at the single dose of 1.4 mmol/kg ($P \leq 0.001$). The increases nevertheless were inconsistent and insufficient to markedly increase phytoextraction efficiency. Therefore, the suggestion of using rhamnolipid as an individual treatment for Cd phytoextraction by Gunawardana et al. (2010) based on moderately increased shoot Cd concentration rather than total accumulation is not supported from the findings of this thesis.

Timing of chelant application is crucial for phytoextraction as addition of chelant may directly affect plant growth and yields due to potential toxicity. Many phytoextraction studies have employed a single application of chelant at a time approaching harvest (Jiang et al. 2003; Luo et al. 2005) as early application might reduce biomass production due to metal and/or chelate phytotoxicity (Meers et al. 2004). Occasionally some studies include split doses (Crèman et al. 2001; Meers et al. 2005; Wenzel et al. 2003) or combined the application of different types of chelants (Gunawardana et al. 2010; Wu et al. 2007). Split application was suggested to allow plants to initiate adaption mechanisms to the amended chelants and raise their tolerance (Alkorta et al. 2004). In this study, split doses of rhamnolipid (0.4 mmol/kg/wk) did not increase the uptake of Cd or Zn compared to the single dose (1.4 mmol/kg). In fact, severe phytotoxicity (e.g. stunted plant growth, necrosis and wilting) were observed in the split dose application, while the single dose did not result in significantly decreased plant yields, suggesting the split dose applications are unlikely to increase metal phytoextraction as was hypothesised by Wenzel et al. (2003).

5.3 The lack of enhanced Cd phytoextraction

The hydroponics experiment detailed in Chapter 4 investigated the possible mechanisms of the moderately increased metal uptake observed in Chapter 3, in order to test the hypothesis that the intact rhamnolipid-Cd and rhamnolipid-Zn complexes could be absorbed by plant roots through a lipophilic pathway. This pathway for metal uptake into plants has been documented in several studies (Phinney and Bruland 1994; Trapp 2002). In the present study, Cd and Zn uptake by roots was not inhibited when the free metal ion concentrations were reduced by rhamnolipid complexation and the metal uptake per unit free ion in solution was increased in the rhamnolipid-buffered solutions. This suggests a possible absorption of rhamnolipid-metal complexes into plant roots, possibly through a lipophilic pathway. Indeed, the intact rhamnolipid-Zn complex was found as a dominant Zn species in canola roots, determined by synchrotron x-ray spectroscopy (Stacey et al. 2008). The increased shoot Zn uptake observed by Stacey et al. (2008) also suggests that the rhamnolipid-metal complexes could contribute to enhanced metal translocation. However, it was not observed in our short-term (24 h) uptake experiments.

An increase of Ca concentration from 0.035 mM (low supply) to 2 mM (a normal Ca concentration in soil solution) in the rhamnolipid-buffered solution did not affect Cd uptake in roots, but significantly reduced Cd translocation to shoots ($P \leq 0.001$) (Chapter 4). Although the higher Ca concentration in solutions increased the free Cd ion concentration in solution by displacing it from the rhamnolipid, it still competitively impeded Cd translocation, which could thereby account for the absence of successful Cd phytoextraction in the contaminated soils (Chapter 3), where Ca concentration is normally several orders of magnitude higher than that of Cd.

5.4 Suggestions for future research

Throughout the study, rhamnolipid concentration was measured using the 6-deoxyhexose assay with L-rhamnose as a standard. However, this method is not capable of providing useful information on the complexed rhamnolipid species. There is no proven method to reliably quantify metal-rhamnolipid complexes in simple solutions or complex matrices. In this regard, future research should aim to develop an analytical technique to distinguish and quantify metal-rhamnolipid complexes while producing high separation efficiencies.

In the rhamnolipid degradation experiment (Chapter 2), the half life of rhamnolipid in the soils at different metal-spiking levels could not be determined. The change of Cd partitioning during chelant degradation was not investigated during degradation; this limits our understanding of the chelant's effect on metal bioavailability as affected by time. Therefore, longer duration degradation experiments would be worthwhile where degradation and Cd solubility are determined concurrently.

In the hydroponic experiments (Chapter 4), the binding strength of the rhamnolipid-Cd complex was found to be weak as compared to the value reported by Ochoa-Loza et al. (2001). In order to obtain a more accurate stability constant of rhamnolipid-metal complexes, it would be worthwhile to evaluate the binding strength of the complexes, and one potential method would be to study the formation and dissociation kinetics of the metal-rhamnolipid complexes.

The effect of increased Ca on Zn uptake by sunflower plants in the rhamnolipid-buffered hydroponic experiment is intriguing and requires further investigation. An increased uptake and translocation of Zn to shoots was also observed in the study of Stacey et al. (2008) when plants grew in a rhamnolipid-treated calcareous soil where soil solution was presumably saturated with Ca. Therefore, there could be a Ca-Zn-rhamnolipid interaction which influenced Zn uptake. To investigate more closely the effect of Ca on Zn uptake by plants in the presence of rhamnolipid, solution culture experiments using more plant varieties and

experiments using soils varying in Zn to Ca ratio should be considered. If the positive response of plant Zn uptake to increased Ca in the presence of rhamnolipid is observed in a wider range of plant species and a correlation between Zn uptake and soil Ca can be made, this application would benefit crop Zn uptake in Zn-deficient arable soils.

This study concludes that although rhamnolipid applied at high concentrations has been effective to remove Cd in contaminated soils by soil washing, its application in Cd phytoextraction will be confronted with several obstacles (e.g. adsorption, phytotoxicity, cation competition, etc.) and thereby is unlikely to enhance metal removal efficiency in phytoextraction.

5.5 References

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Appendix 1

Six types of soils were selected for an initial evaluation of rhamnolipid adsorption in soils. Soils varied in pH, organic matter content (OC%), calcium carbonate content ($CaCO_3$%), and cation exchange capacity (CEC) and soil texture (Table A1). The number of soils was too small to develop correlation relationships between rhamnolipid retention and soil properties (due to autocorrelation between properties), but the soils with the highest retention of rhamnolipid had higher concentrations of clay, OC and $CaCO_3$.

Table A1. Physicochemical properties of the soils.

Site	pH	OC	Exch. cations cmol+/kg					$CaCO_3$	CEC (NH_4)	Clay	Silt	Sand
		%	Ca	Mg	Na	K	Total	%	cmol/kg	%	%	%
Lenswood	5.26	3.4	13.10	3.65	0.13	0.58	17.5	< 0.2	23.0	30	44	15
Keith	5.37	0.6	4.56	1.57	0.22	0.52	6.9	< 0.2	8.6	12	2	40
Dooen	8.00	1.1	25.70	4.74	0.55	2.91	33.9	0.6	33.7	57	11	30
Cungena	8.33	0.3	15.39	1.22	<0.1	0.97	17.6	39.0	15.7	6	2	40
Mintaro	6.63	2.4	13.20	3.33	0.13	1.72	18.4	< 0.2	20.3	41	22	37
Booleroo	7.31	1.0	11.10	2.16	0.18	1.65	15.1	< 0.2	13.9	23	22	56

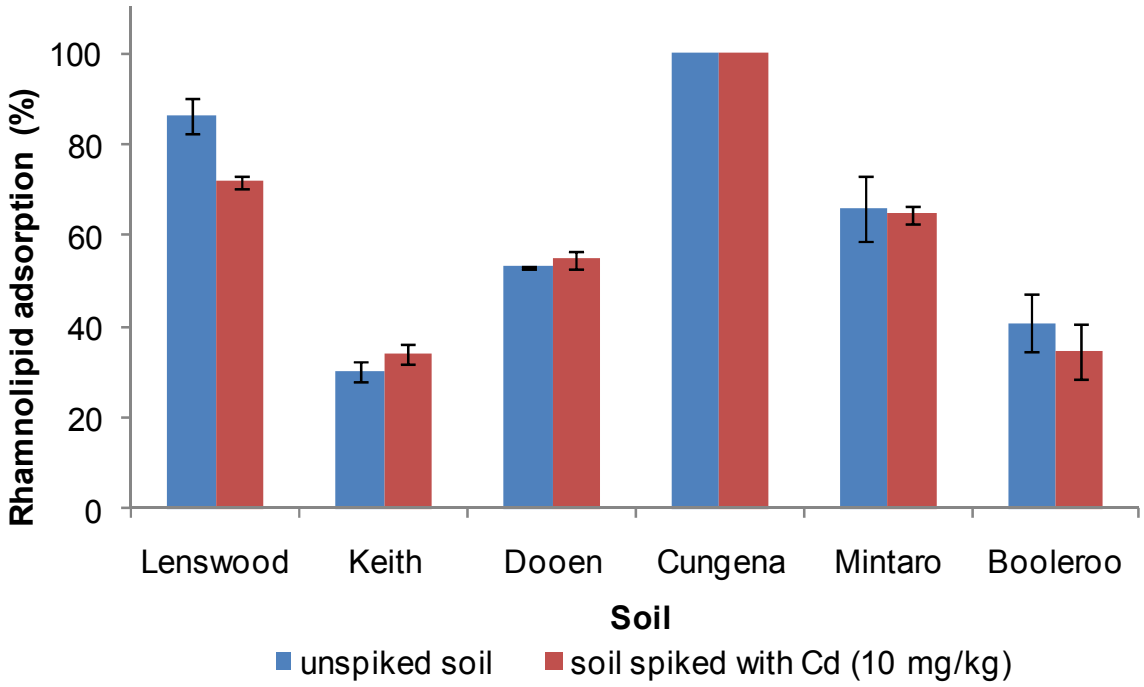

Figure A1. Rhamnolipid adsorption in different soils. Values are the mean of four replicates and error bars indicate standard errors.

Appendix 2

The octanol/water partition coefficient ($K_{o/w}$) is used to assess the lipophilicity of an analyte. In order to assess the likelihood of metal uptake (e.g. Cd and Zn) via a lipophilic pathway, the $K_{o/w}$ values of Cd and Zn were measured in solutions with increasing concentrations of rhamnolipid. The method used was outlined in Chapter 3. Concentrations of Cd and Zn in the aqueous phase were analysed by ICP-OES, those in the non-aqueous (n-octanol) phase were calculated by mass balance. The lipophilicity of both Cd and Zn was increased in line with the increasing concentrations of rhamnolipid, indicating that Cd and Zn complexes with this chelant are likely to be membrane permeable. The lipophilicity of Cd-rhamnolipid complexes was greater than those of Zn-rhamnolipid ($P \leq 0.001$).

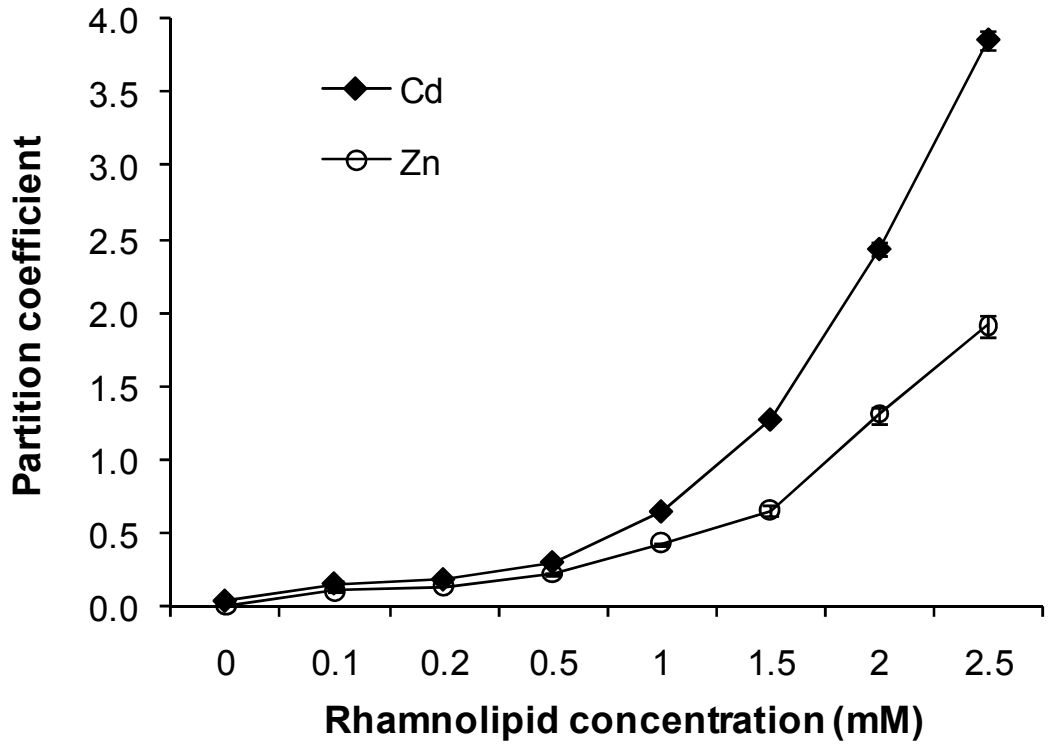

Figure A2. Octanol-water partition coefficients of Cd and Zn with increasing rhamnolipid concentrations. Values are the mean of four replicates and error bars indicate standard errors.

Appendix 3 — Chapter 3 Supplementary Information

The change of soil suspension pH and Cd concentrations with increasing rhamnolipid concentration is shown in Figure A3-1. As discussed in Chapter 3, the pH of soil extracts decreased in both soils at rhamnolipid concentrations of 3.6 – 4.4 mM, which could have contributed to the formation of larger rhamnolipid aggregates at these concentrations. The presence of the additional Zn in soil did not significantly affect Cd desorption in the Booleroo soil. However, a significantly higher Cd desorption in the Cd/Zn-spiked Monarto soil than in the Cd-spiked Monarto soil ($P \leq 0.001$) was observed from rhamnolipid concentrations of 4.4 mM upwards.

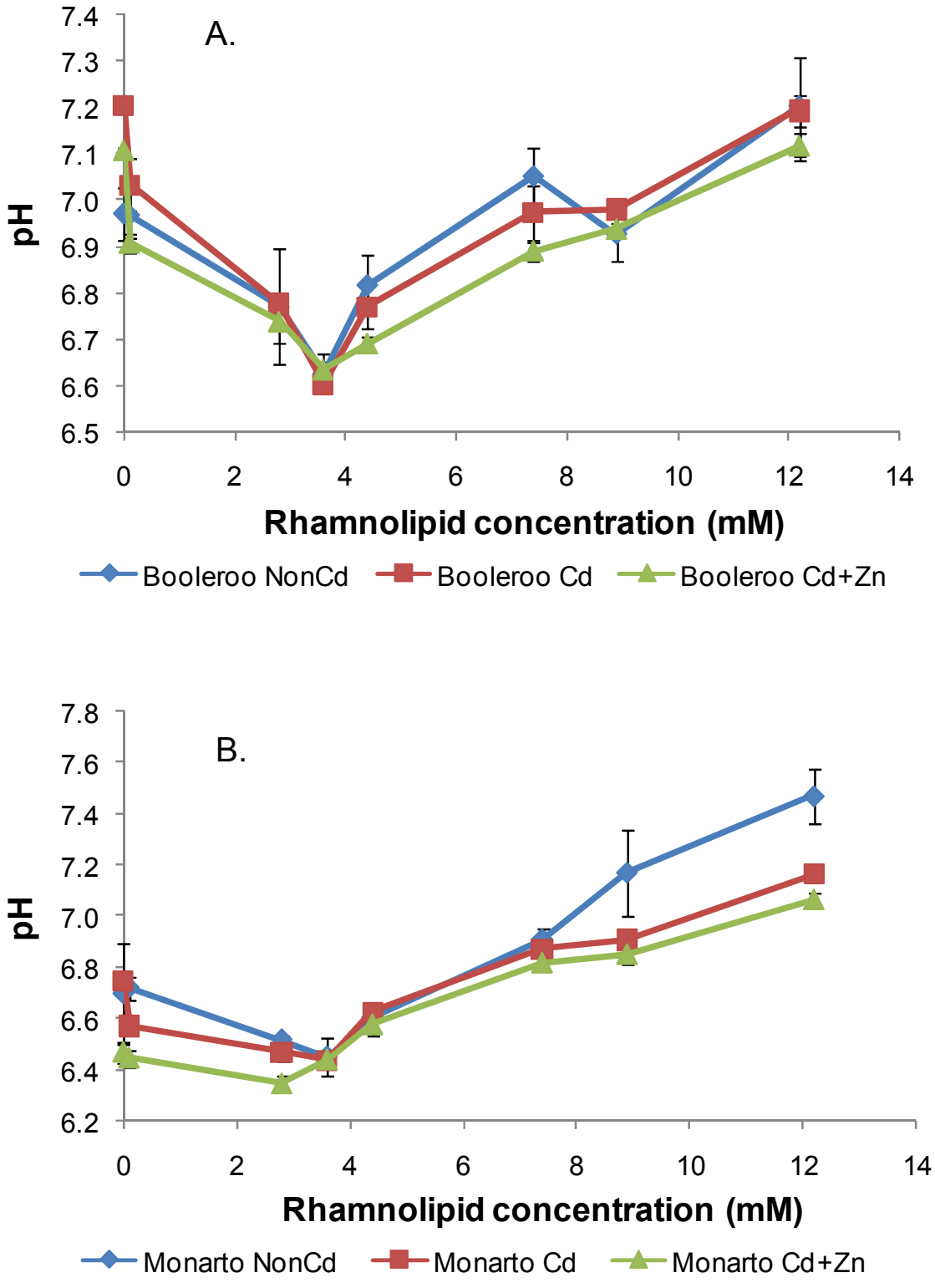

Figure A3-1. The change of pH in soil extracts in Booleroo soil (A) and Monarto soil (B) as a function of added rhamnolipid concentration in the soil suspension. Values are the mean of three replicates, and error bars indicate standard errors.

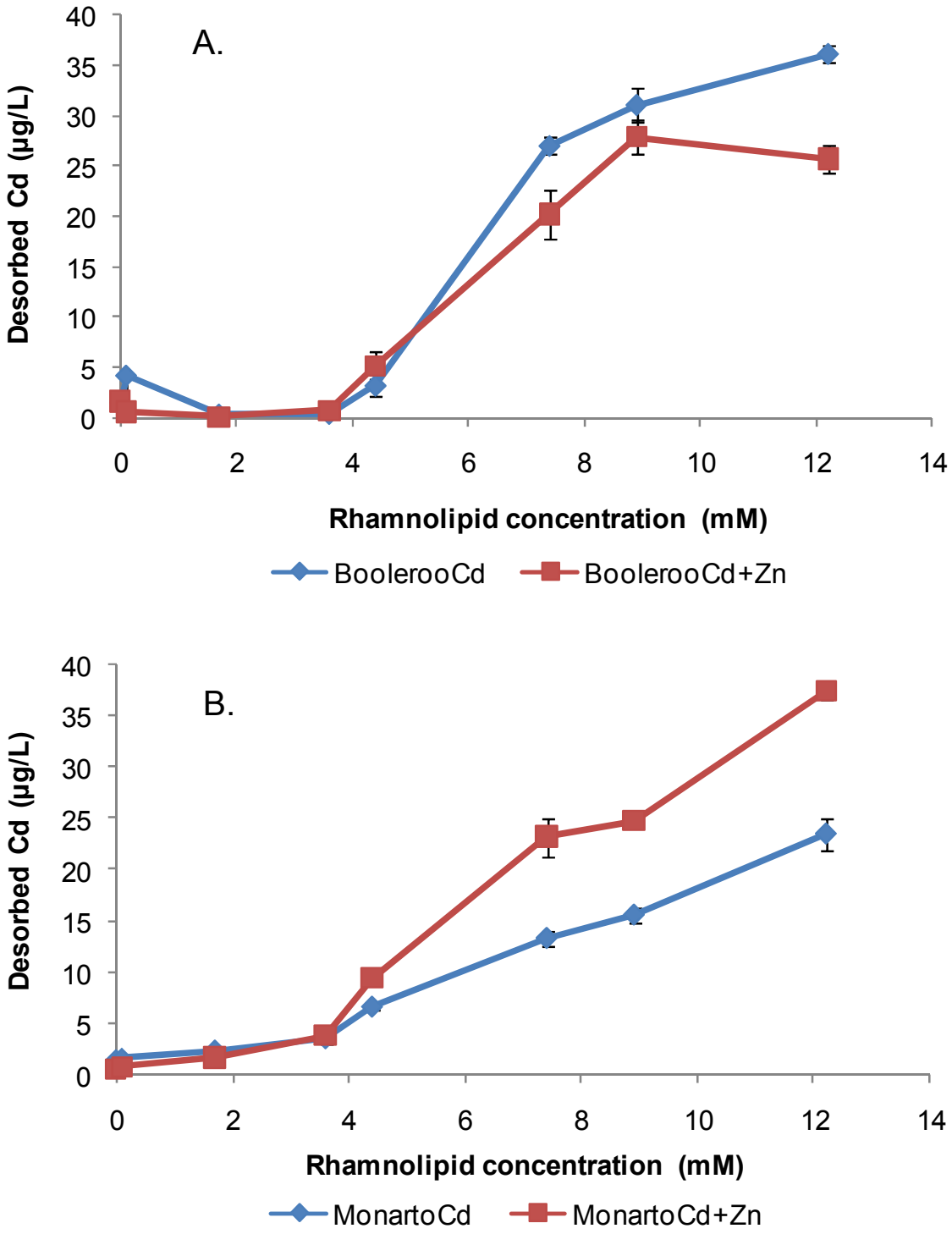

Figure A3-2. Cadmium concentrations in the soil suspensions of the Cd and Cd + Zn spiked Booleroo soil (A) and Monarto soil (B) as a function of added rhamnolipid concentration in the soil suspension. Values are the mean of three replicates, and error bars indicate standard errors.

Appendix 4 — Chapter 3 Supplementary Information

In Chapter 3, a range-finding seedling phytotoxicity experiment was conducted to determine the critical toxicity concentrations for Zn in the soils. Only Zn was studied as Zn is more phytotoxic than Cd, and with Cd:Zn ratios set at 1:100, Zn was the most likely element to limit plant growth. The ambient Zn concentration in the unspiked Booleroo soil was 52 mg/kg. The soil was further spiked to reach Zn concentrations of 359, 585, 838 or 1074 mg/kg. The EC_{25} value for Zn (the effective concentration of Zn in soil which induced a 25% inhibitory effect on seedling emergence) was 134 mg/kg. The spiking rate of Zn which raised soil concentrations to 134 mg/kg was used in subsequent experiments using the Booleroo and Monarto soils.

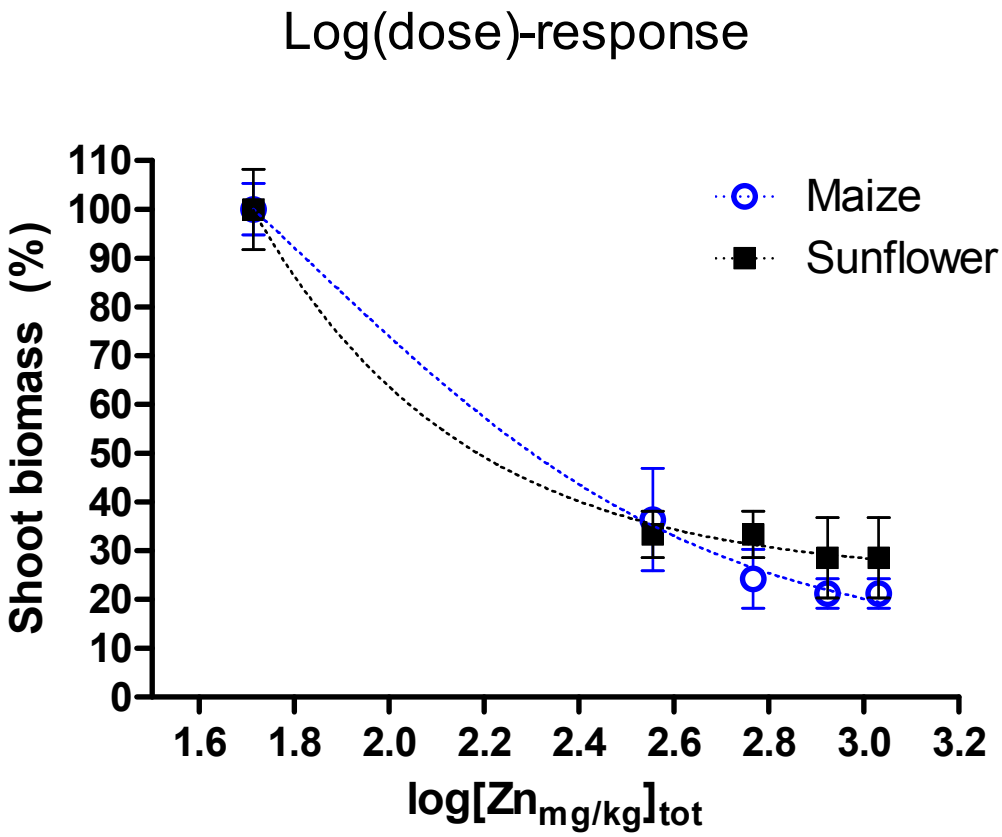

Figure A4. Plant shoot biomass in response to Zn concentrations ($\log[Zn_{mg/kg}]_{tot}$) in the Booleroo soil. Values are the mean of four replicates, and error bars indicate standard errors.

Appendix 5 — Chapter 4 Supplementary Information

Figure A5-A and A5-B are the tissue biomass of sunflower grown in the Cd-spiked and Zn-spiked hydroponics experiment with increasing concentrations of EDTA and rhamnolipid, respectively. The addition of EDTA caused a reduction of root biomass yield ($P \leq 0.001$) in the Cd treatment; the addition of rhamnolipid significantly increased the root biomass yield ($P \leq 0.05$) in the Zn treatment.

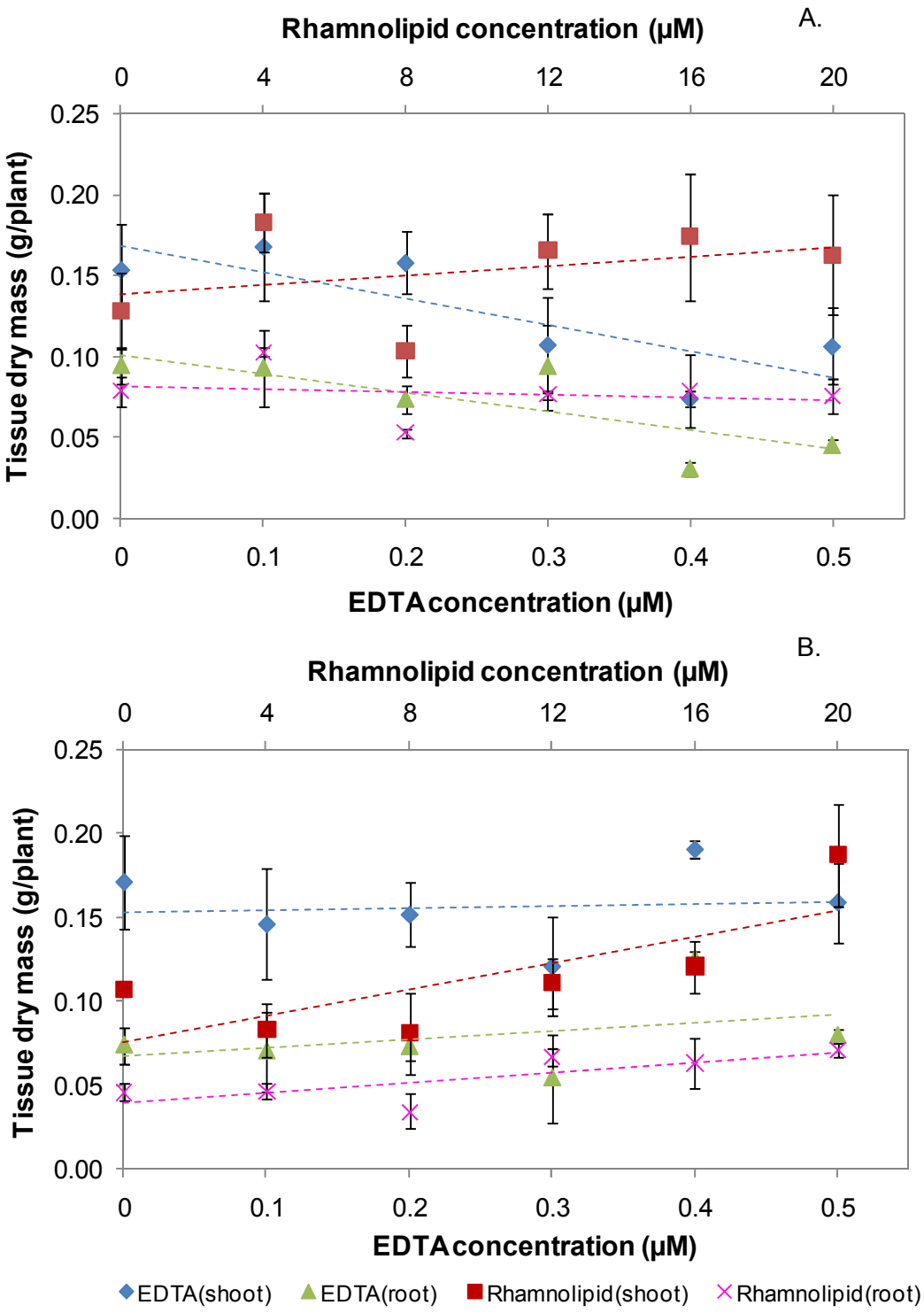

Figure A5. Tissue dry mass of sunflower grew in Cd-spiked (A) and Zn-spiked (B) hydroponics with increasing concentration of EDTA or rhamnolipid. Values are the mean of three replicates, and error bars indicate standard errors.