Chelation in Cd and Zn Phytoextraction

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Well, up until some vague moment in my Ph.D candidature I still didn't quite understand the importance of this page. I guess now, I know.

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Declaration

The content of this thesis is original and does not contain any material which has been accepted by a tertiary institution for the award of any degree or diploma. Material published or written by another person is only included in this thesis after due reference has been made.

After this thesis has been accepted for the degree of Doctor of Philosophy at Adelaide University and deposited in the University library, I fully support it being made available for loan and photocopying.

RICHARD NICHOLAS COLLINS

14th December, 2001

Publications

The work outlined in this thesis has resulted in the following publications:

Journal Articles:

Collins R, B Onisko, M J McLaughlin and G Merrington (2001). Determination of metal-EDTA complexes in soil solution and plant xylem by ion chromatography-electrospray mass spectrometry. *Environ. Sci. Technol. 35 (12):2589-2593.*

Collins R, G Merrington, M J McLaughlin and C Knudsen (2001). Uptake of the intact ZnEDTA complex from soil is dependent on plant species and ZnEDTA concentration. *Environ. Toxicol. Chem. (submitted).*

Collins R, G Merrington, M J McLaughlin, and J-L Morel (2001). Organic ligand and pH effects on isotopically exchangeable Cd in polluted soils. *Soil Sci. Soc. Am J (submitted).*

Conference Articles:

Collins R, M J McLaughlin and G Merrington (2000). The mechanisms of EDTA-induced Zn phytoextraction. Presented at *"Remade Lands 2000, an International Conference on the Remediation and Management of Degraded Lands", Fremantle, WA, Australia, November 2000.*

Collins R, M J McLaughlin, J-L Morel, E Gerard and G Merrington (2001). Effect of organic ligands on isotopically exchangeable Cd (*E* value) in and desorption from two heavy metal contaminated soils. Presented at *"Sixth International Conference on the Biogeochemistry of Trace Elements", Guelph, ON, Canada, July 2001.*

Abstract

The widespread pollution of soils with Cd and Zn in industrialized countries has created the need for well defined remediation strategies that are inexpensive, effective and scientifically proven to reduce risks to health. Presently, the phytoextraction of Cd and Zn from these soils suffers a lack of development in all three areas. Therefore, the primary aims of the research outlined in this thesis were to: 1) develop analytical methodology to assist in the determination of metal-chelate complexes in soil solutions and plant fluids; 2) resolve the processes involved in chelate-assisted Cd and Zn phytoextraction, and also; 3) determine the possible effects of chelation processes on the quantity of phytoavailable Cd and Zn in polluted soils.

An ion chromatography-electrospray mass spectrometry (IC-MS) method was developed to quantify the metal complexes of ethylenediaminetetraacetic acid (EDTA) in soil solution and plant xylem exudate. Metal-EDTA complexes were unequivocally identified by their retention time, mass-to-charge ratio and metal isotopic signature. The number of metal complexes that could be detected was limited by the eluent used for IC (i.e. only those complexes that were stable at high pH), with the metal-EDTA complexes of Al, Cd, Cu, Co, Mn, Ni, Pb and Zn being adequately resolved to detection limits of 0.1 - 1 μM.

In conjunction with the use of this novel analytical technique, pot experiments were conducted with *Hordeum vulgare* L., *Solanum tuberosum* L., *Brassica juncea* L., and *Lupinus albus* L. to determine the nature of Zn mobilization, uptake and root-shoot transport on a Zn contaminated soil in the presence of increasing concentrations of

EDTA (0, 0.034, 0.34 and 3.4 mmole EDTA/kg soil). A significant increase in the concentration of soil solution Zn was only observed after the addition of 3.4 mmole EDTA/kg soil. At this application rate 85 - 104 % of the increase in soil solution Zn could be accounted for by chelation/desorption reactions and 81 - 94 % of total Zn in solution was measured as the ZnEDTA chelate. Furthermore, the accumulation of Zn and the concentration of ZnEDTA in the xylem sap of *B. juncea* were significantly greater than that of *H. vulgare* and *S. tuberosum*. It was, therefore, concluded that there were at least two different mechanisms of ZnEDTA accumulation for these plant species. Based on a review of the existing literature it was hypothesized that uptake of ZnEDTA by *B. juncea* occurred only after physiological damage to it's root system whereas uptake by *H. vulgare* and *S. tuberosum* was via an apoplastic pathway.

Although it was demonstrated that the movement of Zn from the solid to the solution phase was a significant process preceding plant uptake, the quantity of metal that can be solubilized through chelation processes will also be extremely important. In the case of phytoextraction, the use of EDTA, as well as the natural occurrence of other organic ligands in the rhizosphere, may increase the phytoavailable quantity of Cd and Zn in polluted soils. This hypothesis was examined in detail using a batch experimental system with (0.5 and 20 mM) EDTA and 0.25 - 5 mM concentrations of sodium tartrate, the free acid and sodium salt of citrate, histidine and deoxymugineic acid (DMA) in a polluted acidic and calcareous soil. Through the application of isotopic dilution techniques it was determined that the presence of these organic ligands could substantially increase the quantity of isotopically exchangeable Cd and Zn (the *E* value). The ligands increased the

E value through chelation processes and decreases of pH. Hence, it was hypothesized that the application of EDTA or the presence of root exuded organic ligands have the potential to increase the quantity of phytoavailable Cd and Zn (the *L* value) during phytoextraction practices.

Table of Contents

1. Introduction

Significant utilization of products containing the heavy metals - cadmium (Cd) and/or zinc (Zn) - began, about 200 years ago, with the industrialization of western countries (1, 2). Hence, the anthropogenic redistribution of these metals, the largest seen in human history, has resulted in the widespread contamination[1] and pollution[2] of soils (3). For example, over 4 million hectares of land in Europe are contaminated with heavy metals and as far back as 1976 it was estimated that, from mining operations alone, over 2 million hectares of land in the USA were covered by mineral waste (4).

Due to the similar chemical nature of these two metals (both are in Group IIB of the periodic table) their minerals are commonly associated in hydrothermal sulfide ore deposits (2, 4-7). Therefore, it is appropriate to study these two metals together because they are found as co-contaminants in many soils affected by mining, processing and/or smelting activities (8, 9).

Indeed, the activities primarily responsible for Cd and Zn soil pollution in Europe, the USA and Australia have been: 1) industrial atmospheric deposition, particularly from smelting and refining operations but also from coal-fired power plants, and; 2) mining activities and the dispersal of mine wastes (3, 4, 10-12).

However, the disposal of domestic and industrial wastes or by-products have also significantly contributed to soil pollution in these countries (4, 12, 13). For example, although the atmospheric deposition of Cd in England and Wales supplied most Cd to soils from 1995-1997, those soils receiving sewage sludge gained Cd at a 24 fold factor greater than the input from atmospheric deposition (Alloway *et al.* (14) cited in (13)). In addition, the application of phosphate fertilizers and animal manures to areas used for

[1] In this thesis a soil is termed contaminated when the concentration of the contaminant, in this case Cd and/or Zn, is above typical concentrations normally found in soils located in the same geographical region.
[2] A soil becomes polluted when the contaminant exceeds the toxic threshold for some biological system. This biological system could be soil invertebrates, soil microorganisms, soil fauna, soil flora, higher plants, etc.

intensive agriculture have also resulted in the contamination of large areas of soils with low to moderate levels of Cd and Zn in Europe (12), Australia (15) and the USA (11, 16). Indeed, with stricter government regulations being placed on the atmospheric release of these metals, current practices involving the addition of sewage sludges, animal manures and phosphate fertilizers (containing high levels of Cd) to soils are likely to become the most significant sources of Cd and Zn pollution in the future (17).

The pollution of soils with Zn does not normally pose any health risk because it is toxic to pastures or crop plants before it is accumulated to any significant amount that would be detrimental to livestock or humans (18). Cases of acute Zn toxicity have been rare and only observed at intakes of several grams over a short time period (19). Nonetheless, highly polluted soils may pose chronic health risks to local residents, especially children, if the soil is inhaled via dust particles (19). Therefore, the main concern of Zn pollution is its limitation to the economic fertility of the soil, such as disrupting carbon (C) and nitrogen (N) biogeochemical cycles (20, 21). In contrast, Cd can be taken up by pastures or crops in amounts that can be chronically harmful to livestock or humans through the ingestion of excessive amounts through dietary uptake (15). In addition, chronic human exposure to Cd may result from consuming meat products (in particular the kidneys where Cd accumulates) from animals that have grazed on pastures from Cd contaminated soils (3, 22). Therefore, soils polluted or contaminated with Cd and Zn are not only unfit for food production and hazardous to human health, but may also be unsuitable for other forms of sustainable economic development. Furthermore, if these soils are not remediated, off-site migration of these metals can continue to further pollute groundwater or neighboring aquatic systems (11). Therefore, utilization of such land for ecological, agricultural or urban purposes requires a safe and efficient decontamination process.

The remediation of these soils by current physicochemical methods, such as solidification/stabilization, vitrification, soil washing with acids, and pyrometallurgical separation are either expensive or impossible and inevitably render the soil unsuitable for vegetation (11, 13, 23). It has become clear that biological approaches to cleaning up

these soils would be more effective, economic and environmentally desirable. Along this theme, phytoextraction has the potential to become an inexpensive and sustainable in-situ alternative to physicochemical site remediation. Phytoextraction is a strategy which employs hyperaccumulating plants or agricultural crops, with the assistance of chelates, to remove metals from soils (24).

Cd and Zn hyperaccumulating plants have been defined as plants that naturally accumulate these metals in the shoots to concentrations ≥ 100 μg/g and ≥ 10000 μg/g dry weight, respectively (25). Although the study of hyperaccumulators represents a focus of many laboratories, practical phytoextraction with these plants has remained elusive. For example, many of these species either hyperaccumulate Cd or Zn while, as mentioned previously, most sites are contaminated with both metals. However, one species of *Thlaspi caerulescens* - J. & C. Presl is known to hyperaccumulate both metals (26). Yet, like any species that is not normally used in agriculture, little is known about its agronomic management (25, 27). Furthermore, many of these species are slow growing and, although they have high metal concentrations, produce low biomass (25, 28). The factors of slow-growth and low-biomass production by these species is particularly problematic as the measure of phytoextraction success is based on the amount of contaminant removed/hectare.

As such, short-term advances in the phytoextraction of Cd and Zn are likely to come from a better understanding of the mechanisms involved in chelate-assisted Cd and Zn uptake, translocation, and accumulation. In this process plants are grown on contaminated soils to their maximum biomass. At this time a suitable chelate is added to the soil increasing soil solution concentrations of Cd and Zn and hence plant uptake of these metals. The few studies recently published on this area of research have largely focused on Pb (29-34) and only two have been mechanistic studies (30, 34). Despite the lack of literature, good agreement on the plant uptake of Pb during EDTA-assisted Pb phytoextraction exists between these two studies. In both cases, increased Pb uptake was associated with the concentration of Pb and EDTA in the xylem exudate. Unfortunately, these studies were

compromised by the lack of available analytical techniques to measure metal-EDTA complexes as the intact molecule. Therefore, advances in analytical techniques to quantify the metal complexes of EDTA are essential for a better understanding of these mechanisms. This includes identifying the barrier of Cd and Zn translocation from roots to shoots.

Therefore, two major objectives of this thesis were:

1) to determine if a combination of ion chromatography and mass spectrometry (IC-MS) techniques could be used to identify and quantify intact metal-EDTA complexes in soil solution and plant xylem exudate. In this manner the metal-EDTA complexes may be unequivocally distinguished not only by their differences in chromatographic mobilities but also by their mass-to-charge ratio and the isotopic signature of the metal, and;

2) to identify the mechanisms of Cd and Zn mobilization, plant uptake and root-shoot transport of the metal-EDTA complex with a variety of plants that have been proposed for the chelate-assisted phytoextraction of Cd and Zn. More specifically, the hypothesis that differential uptake or exclusion of the intact metal-EDTA complex determines Zn accumulation was examined.

Furthermore, one of the major shortcomings of phytoextraction, if environmental regulations are based on total soil metal concentrations, is the extensive time needed to remediate a soil below action levels (35). Notwithstanding the enormous financial benefits to be gained from reduced rehabilitation costs if regulatory limits are based on metal bioavailability, the fact that soil Cd and Zn can exist in non-phytoavailable pools implies that total concentrations are not a satisfactory criterion to assess soil remediation practices (36-38). Therefore, providing phytoextraction significantly depletes the phytoavailable pool of Cd and Zn, and this pool is not readily replenished by non-

phytoavailable pools, risks to health may be mitigated in a short time even without a significant reduction in the total soil content of metal (39-41).

However, the origin of Cd and Zn that is phytoavailable has yet to be fully elucidated. Some studies using the isotopic labeling of the exchangeable soil metal pool suggest that a wide range of plants access a similar pool of Zn (L value) in agricultural soils (36, 38, 42). The L value or plant labile soil pool of metal is determined by measuring the isotopic ratio of metal in the plant shoot:

$$(1.1)\ L\ (\mu g/g\ soil) = (M_s^*) / (M_p^* / M_p)$$

where M_s^* = carrier free radioactive metal added to soil (kBq/g)

M_p^* = radioactive metal in the plant shoot (kBq)

M_p = soil derived non-radioactive metal in the plant shoot (μg)

However, evidence has also been presented that plants differ in their ability to affect the isotopic dilution of Zn in their root zone (and hence differ in their L values) (43-45). Similarly, there have been conflicting reports on the bioavailability of soil Cd to plants. For example, Hamon *et al.* (36) found that, with the exception of *Brassica napus* L., a wide range of plants accessed a similar pool of Cd. In contrast, Gerard *et al.* (46) observed that the Cd L value of *Lolium perenne* L., *Lactuca sativa* L. and *Thlaspi caerulescens* J. & C. Presl frequently differed from each other and was dependent on soil type, degree of Cd contamination, and time of sampling.

Therefore, the third and final major objective of this thesis was:

3) to determine how plants affect isotopically exchangeable Cd and Zn soil pools. More specifically, to investigate whether a range of plant derived organic ligands have the ability to affect the isotopic exchange properties of soil Cd and Zn in two polluted soils. This knowledge is essential before the

bioavailability of soil Cd and Zn can be considered as a determinant of the health risks associated with soils contaminated by these metals and to further develop suitable laboratory methods that can rapidly and accurately characterize the phytoavailability of soil Cd and Zn.

2. Literature Review

2.1 Toxicology of Cd and Zn

The driving force for the remediation of soils polluted with Cd and Zn is the health risks these metals pose to organisms who either use the soil as a habitat, consume contaminated plant material grown on these soils or absorb Cd and Zn via dust particles. In all living organisms organic ligands have evolved that rely on specific metals to function. The chelation of unsuitable metals, or the binding of metals to reactive sites not normally requiring the metal, is often toxic (47). Therefore, the mechanisms of metal toxicity can generally be classified into the following three categories: 1) blocking of essential biological functional groups in biomolecules/ligands; 2) displacing essential cations from biomolecules/ligands, and; 3) modifying the active conformation of biomolecules/ligands (47). It is possible that Cd and Zn can participate in all three of these general toxicity mechanisms (47).

Cadmium is generally more toxic than Zn at equimolar concentrations in solution (48). However, the level of Cd and Zn toxicity also depends on the solubility and, once in solution, the chemical speciation of the metal in solution (49). For example, if Cd is consumed in an insoluble form it is possible for it to pass through the body without causing any adverse health effects. In the plant and microorganism literature considerable evidence has accumulated suggesting that the free metal ion is the most toxic form in solution (50-52). Although evidence is continually emerging that metal-ligand complexes may be taken up by microorganisms or vegetation, there has been little research to establish what the toxicity of these complexes is to living organisms (53, 54).

2.1.1 Soil biology

The pollution of soils by Cd and Zn disrupts the biological processes upon which the maintenance of soil fertility depends. For example, high concentrations of Cd and Zn can inhibit microbial enzyme activity, C, N and phosphorus (P) mineralization, N_2 fixation and can also reduce the diversity of the soil flora and fauna (3, 55-60). The concentrations of Cd and Zn that affect soil microorganisms have been found to vary with

soil type, source of contamination and the microorganism itself (reviewed in McGrath *et al.* (56) and McGrath (61)). Nevertheless, critical concentrations have been observed to range from 2 - 80 mg Cd/kg soil and 180 - 857 mg Zn/kg soil (56, 61).

2.1.2 Plants

Although Cd is considered to be non-essential to plants, Zn is essential for plant growth (62). The Zn cation is required by higher plants for the activity of various enzymes in several metabolic processes involved in the electron transport system (62, 63).

Many processes in plants are known to be affected by Cd. For example, absorbed Cd (5 - 30 mg/kg DW) can affect the electron and energy transfer reaction in mitochondria (64) and can inhibit photosynthesis (59, 65). However, Cd pollution without co-contamination by Zn is rare (discussed below). Therefore, Cd does not reach phytotoxic levels (~ 0.1 mg/l in soil solution (66)) in many soils before Zn does. Nonetheless, human and animal health can be threatened at Cd concentrations in plant material well below phytotoxicity thresholds (15, 19, 67, 68).

In nutrient solution experiments, Zn concentrations as low as 0.1 - 0.65 mg/l have had adverse effects on the growth of alfalfa (*Medicago sativa* L. cv. Vernal) and maize (*Zea mays* L.) (58, 63). As a comparison, a soil polluted by mill tailings and smelting wastes showed soil solution Zn concentrations of 37 mg/l (69). For plants, critical shoot Zn concentrations vary from 60 - 900 mg/kg DW but most species are affected in the range 200 - 400 mg/kg (21, 66, 70). Generally, growth symptoms of Zn toxicity in plants are similar to those of Zn deficiency. An excess supply of Zn reduces root growth and the shoots often become stunted and chlorotic (66). In addition, the translocation of carbohydrates are markedly restricted by higher Zn levels, leading to the accumulation of sugars and starches in the leaves and reduced transport to growing parts (71). Other well documented symptoms of Zn toxicity for *M. sativa* L. have been the reduction of N metabolism and a decrease in leaf chlorophyll content and rate of photosynthesis (58). Van Assche and Clijsters (72) reported similar observations for bean (*Phaseolus vulgaris*

L.) seedlings, where Zn also negatively affected photophosphorylation. More primary mechanisms of Zn toxicity may result from increased root membrane permeability and, hence, leakage of nutrients (63).

2.1.3 Animals and humans

Zinc is also an essential element for animals and humans since it is required as either a structural component or reaction site in numerous proteins and enzymes (2, 62). Naturally occurring enzymes which possess Zn^{2+} binding sites include carboxypeptidase A and carbonic anhydrase (73). Zinc also plays an integral role in the production of insulin (74) and, as it forms stable complexes with DNA and RNA, high levels of intake may also affect DNA and RNA stability (63). The recommended safe and adequate dietary intake for adults is around 15 mg/day with negative effects being observed at 50 - 150 mg/day (19, 75). Documented cases of acute Zn toxicity have been rare, and where reported have been due to the inhalation of Zn oxide laden dusts from industrial manufacturing (19, 76). As mentioned previously the pollution of soils by Zn are likely to result in phytotoxicity before having any significant effect on grazing animals or humans.

Cadmium, on the other hand, is not an essential element for animals or humans. The condensed free element of Cd is not particularly toxic, however, it is dangerous in the form of its cation or when bonded to short C chains (77). Biochemically, the toxicity of Cd arises from the strong affinity that the cation has for sulfur (S). Thus, sulfhydryl groups (-SH) in enzymes attach themselves to Cd^{2+} and the resultant bonding inactivates the entire enzyme (77). Although acute Cd toxicity caused by food consumption has been rare, it can be fatal. For example, Cd poisoning among rice farmers in the Jintsu Valley of the Toyama Prefecture of Japan resulted in the death of 65 people (78).

The greatest proportion of chronic human exposure to Cd comes from our food supply (15, 68, 77). For example, potatoes and potato products contribute the greatest proportion of the total dietary Cd intake in Australia (15). Exceptions arise for individuals who live

near mines and smelters, particularly those which process nickel (Ni), copper (Cu) or Zn, where the inhalation of dust may double the daily intake of Cd (77). Moreover, tobacco smokers are also exposed to Cd accumulated in the tobacco leaves and this habit can approximately double their daily Cd intake (77). The highest risks associated with the chronic exposure to high Cd levels in food is that of renal failure and bone weakness (79).

2.2 Anthropogenic Cd and Zn pollution of soils

2.2.1 Geological weathering

It is well accepted that many soils are derived from the weathering of the bedrock from which they overlie (1, 21). It follows that areas characterized by metal-bearing formations will have elevated levels of these metals in the soil (80, 81). In this thesis these soils are not regarded as contaminated nor polluted and will not be discussed further. However, some of these soils do show Cd and Zn concentrations equal to or greater than those found in contaminated areas (1, 80).

2.2.2 Atmospheric sources

Despite its original source, aerosols of Cd and Zn usually range in size from 5 nm - 20 μm having an average residence time in the atmosphere of 10 - 30 days (82). As such, the pollution of soils can extend for many kilometers from the atmospheric source (83). The combustion of coal for generating electricity or for industrial and domestic purposes has been a major source of atmospheric Cd and Zn emissions to soils in industrialized countries, such as Western Europe and the USA (1, 84). Cement and phosphate fertilizer production plants have also significantly contributed to the aerosol contamination of soils by Cd and Zn (84). However, the smelting and refining of Cu, Ni, Cd, Zn and iron (Fe) has probably been most responsible for the atmospheric dispersion and soil contamination of Cd and Zn (26, 69, 78, 82, 84-88). As seen from the wealth of literature on this subject the impact of mining and smelting operations on the Cd and Zn contamination of soils has been widespread, well documented and most recently reviewed by Dudka and Adriano (4). Many of these soils are in dire need of remediation.

The three polluted soils used for the experiments reported in this thesis were all polluted by activities associated with the processing of metal ores. The two soils taken from Arras, northern France were polluted by the atmospheric deposition of Cd, Zn and Pb laden sulfide particles. The soil from Richmond, California was polluted by the land application of roasted pyrite wastes (locally termed 'cinders') formed during the manufacture of sulfuric acid.

2.2.3 Domestic and industrial wastes

Soils near mining operations are also affected by the disposal of large volumes of either waste rock that overlies the ore or mine tailings (89). The remediation of these soils, however, is usually limited to stabilization practices using revegetation, commonly with native flora, as many mines in the USA, Canada and Australia are located far from agricultural or residential areas (90, 91). However, if rehabilitation is not conducted at many of these mine sites serious environmental problems may occur. For example, many ores contain sulfide minerals which oxidize when exposed to oxygen and water at the earth's surface. This reaction yields sulfuric acid (acid mine drainage) which increases the solubility of Cd and Zn and promotes their mobility to ground waters or neighboring surface waters (76).

Other site specific sources of soil Cd and Zn pollution include wastes from steelworks, electroplating, metal finishing and chemical industries (84, 92). In addition, the disposal of ash residues from coal combustion, urban refuse and municipal sewage sludge has contributed to the pollution of soils by these elements (84).

The Cd and Zn contamination of soils by the application of sewage sludge (biosolids) has received widespread attention in the literature. As a literature review could solely be based on this source of Cd and Zn contamination and the fact that the soils used in the experiments reported by this thesis were not polluted by sewage sludge this topic will be dealt with briefly. However, it must be noted that the application of sewage sludge to soils remains an important issue in soil contamination. For a current review on the

scientific and regulatory developments on this topic (within Australia and New Zealand but critically reviewed in context to the USA and Western Europe) the reader is referred to McLaughlin *et al.* (68). Sewage sludge has been applied to land as a fertilizer and organic soil conditioner, but primarily as a means of disposal (1). Sewage sludge contains appreciable amounts of Cd and Zn ranging from 1 - 3410 mg Cd/kg and 91 - 49000 mg Zn/kg (1, 21, 56, 68, 84). Due to government regulations, problems associated with sewage sludge disposal are likely to continue in the USA and Western Europe and will probably increase in Australia (68).

2.2.4 Fertilizers

Agricultural and horticultural soils that have been regularly amended with phosphatic fertilizers may contain significant levels of Cd (15, 21). The Cd concentration of phosphatic ores used in fertilizer manufacture is highly variable, but ores from the western United States commonly have high Cd concentrations (up to 156 mg Cd/kg of di-ammonium phosphate) (93). In addition, phosphatic fertilizers that have been used in Australia sourced from the island of Nauru have had similar concentrations to those from the western United States (94). As a result substantial areas of agricultural and horticultural soils have been contaminated with Cd to some degree (68). Although there has been a shift to using phosphatic fertilizers containing low levels of Cd in Australia, they remain the major source of Cd added to Australian agricultural soils (15). Many industrial by-products that are used as Zn fertilizers also contain Cd (95). However, as their application rate is low these fertilizers are not usually a significant source of Cd soil contamination.

Many soils also receive animal wastes as a source of N, P and organic matter (1, 96). Some of these wastes contain Cd, albeit at low concentrations (< 10 mg/kg), but high application rates to soils have resulted in the significant accumulation of Cd in soils and horticultural produce (96).

2.3 Background and anthropogenic Cd and Zn soil concentrations

It is estimated that the earth's crust contains an average of 0.2 mg Cd/kg and 65 - 80 mg Zn/kg (97). Similar values have been reported for soils by Frink (98), who calculated from the results of 11 separate sources (references therein) that the worldwide mean background soil concentration of Cd and Zn to be 0.26 mg/kg and 54.6 mg/kg, respectively (Table 2.1). Holmgren *et al.* (16) have reported that the range of background soil concentrations (including agricultural soils) of Cd and Zn across the USA to be between < 0.01 - 2.0 mg/kg and 1.5 - 264 mg/kg respectively. As such it can be concluded that background concentrations of Cd and Zn in soils are relatively similar throughout the world (Table 2.1).

Table 2.1: Typical examples of background and anthropogenic soil Cd and Zn concentrations.

Source of Cd and Zn	Extraction Technique	Cd (mg/kg)	Zn (mg/kg)	Reference
Background	acid digestion	0.26	54.6	(98)
Background	acid digestion	< 0.01 - 2.0	1.5 - 264	(16)
Background	acid digestion	0.8	45	(99)
P fertilizers	0.1 M EDTA	0.01 - 0.49	nd[1]	(100)
Sewage Sludge	acid digestion	5	447	(99)
Smelter emissions	acid digestion	25.4	1520	(46)
Mine spoil	acid digestion	704	13010	(101)

[1] nd = not determined

The examples given in Table 2.1 indicate that phosphatic fertilizers usually cause the least accumulation of Cd in agricultural soils. Otherwise, the source of pollution causing the greatest accumulation of Cd and Zn in soils generally increases in the following order: sewage sludge < atmospheric deposition < mine spoil. However, this is a general trend

and many exceptions do exist. For example, soils located adjacent to a former Zn smelter in Palmerton, Pennsylvania have been polluted through atmospheric deposition to Cd and Zn concentrations of 1020 mg/kg and 48000 mg/kg, respectively (85).

2.4 Chemistry of Cd and Zn in the soil environment

Cadmium and Zn are metallic elements of the Group IIB series in the Periodic Table with the atomic numbers 48 and 30, respectively. Due to the many stable and radioactive isotopes of Cd and Zn (Cd has 8 and Zn 5 stable isotopes, and Cd has 34 and Zn 23 unstable isotopes and isomers (102)) isotopic techniques have featured frequently in the study of these metals in soils (36-38, 40, 42, 44-46, 101, 103-116). Of these isotopes, the radioactive ^{109}Cd and ^{65}Zn isotopes have been the most commonly used in these studies.

The environmental chemistry of Cd and Zn is restricted to their one oxidation state - that of the divalent cations Cd^{2+} and Zn^{2+} (2, 117, 118). Since the metals of Cd and Zn rank above hydrogen (H_2) in the electrochemical series their metals are not stable in the oxidizing environment of soils (117). The naturally occurring compounds of Cd and Zn in the soil environment have rarely been directly identified. However, in major geological ore bodies Zn is commonly found as ZnS (sphalerite or wurtzite) or $ZnCO_3$ (calamine or smithsonite), whereas Cd is frequently associated with ZnS minerals as CdS (greenockite) (2, 5-7, 119). It has been speculated that Zn can occur in soil minerals where Zn^{2+} has substituted for Fe^{2+} (2). However, this process has, also, yet to be directly observed. In most soils, particularly those that have received anthropogenic inputs by atmospheric deposition, Cd and Zn are generally adsorbed to soil surfaces such as clay minerals, organic matter, manganese (Mn) oxides and Fe hydroxides and oxides (8, 120-122).

In soil solution, Cd^{2+}, like Zn^{2+}, is associated with a sheath of water molecules and both are strong Lewis acids (2, 15, 123). For example, Cd^{2+} can dissociate a proton in the presence of water (15):

$$Cd(H_2O)_{6(aq)}^{2+} \Leftrightarrow Cd(H_2O)_5(OH)_{(aq)}^{+} + H^{+} \quad (pK_1 = 10.1)$$

The high dissociation constant (pK_1) means that in most soils the concentration of $Cd(OH)^+$ is 10^2 - 10^6 lower than Cd^{2+} (15). Although the hydrolysis of Zn^{2+} is more likely than Cd^{2+} to occur in soil solution, Zn^{2+} generally still governs the solution chemistry of Zn (over $Zn(OH)^+$) with a pK_1 estimated to be between 7.7 - 9.05 (124, 125). However, it is believed that the hydroxy metal species of Cd and Zn play an important role in the specific adsorption of these metals to the soil solid phase (discussed in Section 2.5). In the soil environment, Cd and Zn equilibrate between the mineral and organic solid phases (adsorption and precipitation) with the solution phase (free cations, hydroxy cations and organic and inorganic complexes) (126-131). Although Cd and Zn in soil solution are commonly found as the free divalent cations, complexation with soil organic matter (2, 63, 99, 132) or Cl^- and SO_4^{2-} (131, 133-135) can be significant. For example, organically complexed forms of Cd and Zn have accounted for up to 46 and 90 %, respectively, of soil solution concentrations in sludge-amended soils (99) and 99 % of Zn in displaced soil solutions from a calcareous soil (132, 136).

2.5 Adsorption of Cd and Zn to the soil solid phase

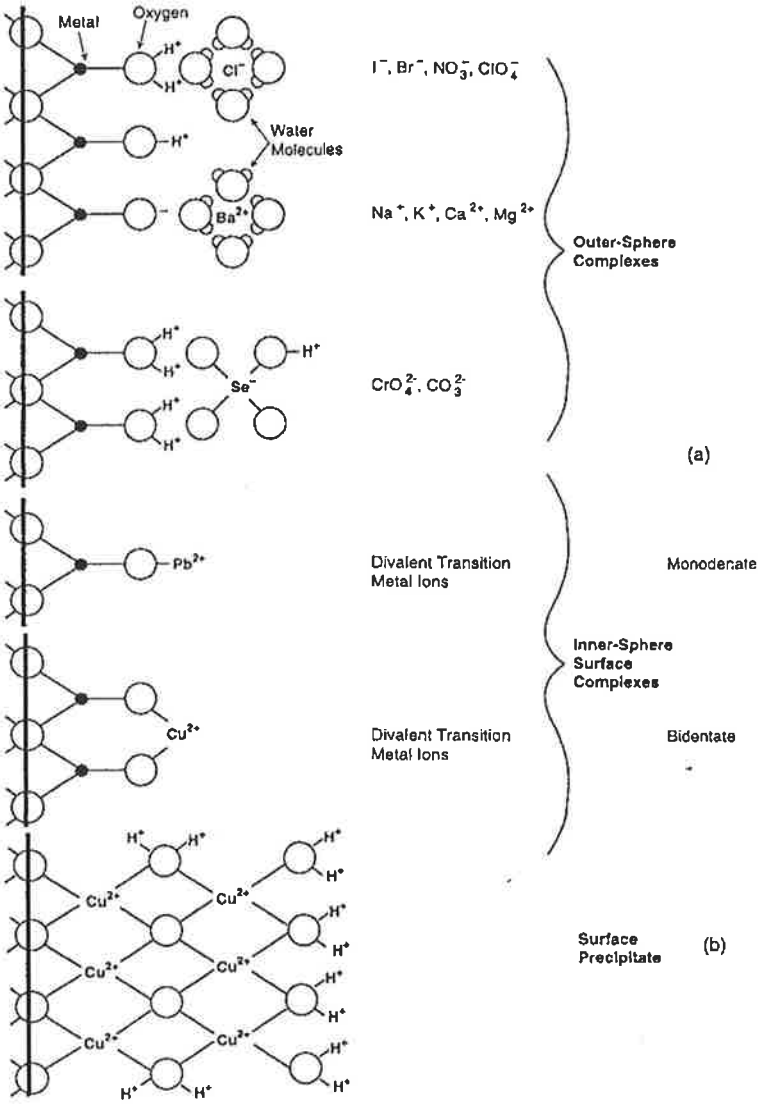
Once in soil solution, Cd and Zn may adsorb to clay minerals (103, 137-139), organic matter (103, 140), calcium carbonate ($CaCO_3$) (103), (hydrous)oxides of Fe (103, 138, 141-144) and aluminum (Al) (103, 142) and Mn oxides (103, 145). Adsorption of Cd and Zn is generally greatest to the metal (hydrous)oxides:

Mn oxides > Al (hydrous)oxides > Fe (hydrous)oxides > organic matter > clay minerals > $CaCO_3$

The forces involved in Cd and Zn (but for all metals as well) adsorption can range from weak electrostatic outer-sphere complexes (e.g. ion exchange) to inner-sphere complexes (e.g. chemisorption, covalent bonding, or complexation due to steric or orientation effects) (146) (Figure 2.1). These processes have also been termed non-specific and

specific adsorption reactions, respectively. At higher pH values and high metal concentrations surface precipitation may also occur (discussed in Section 2.7).

Figure 2.1: Possible sorption complexes at the mineral/water interface indicating outer-sphere adsorption or inner-sphere adsorption (146).



As the name suggests, ion exchange is nonspecific and bonding depends only on the charge and hydration properties of the cation (147):

$$Cd^{2+} + Ca\text{-clay} \Leftrightarrow Cd\text{-clay} + Ca^{2+}$$

As a result, the desorption of Cd and Zn as outer-sphere complexes can be affected by changes in ionic strength (148, 149), alkaline earth cations such as calcium (Ca^{2+}), magnesium (Mg^{2+}), sodium (Na^{+}), potassium (K^{+}) (2, 104, 137, 150, 151), pH (104, 107, 144, 152-154) and complexation by inorganic (148, 151, 155) or organic ligands (128-130).

Inner-sphere complexed Cd and Zn are bound much more tightly to the adsorbent surface (138). The specific sorption of Cd and Zn has been studied on various clays, Mn oxides and the amorphous hydroxides of Fe and Al (138, 141, 156-159). Although inner-sphere complexation of Cd and Zn has not been studied in great detail with soil organic matter, data generated for other metals suggests that it also occurs (reviewed in McBride (147) and Harter and Naidu (128)). However, it must be noted that Campbell *et al.* (160) observed that inner-sphere complexation of Cd to humic acid did not occur and was easily desorbed when the supply of Cd in their column study was removed.

The degree of inner-sphere complexation has been related to the metal's ability to hydrolyze. However, some exceptions have been reported. For example, the metal affinity for amorphous Fe hydroxide has been observed to follow the order (159):

$$Pb^{2+} > Cu^{2+} > Zn^{2+} > Ni^{2+} > Cd^{2+} \geq Co^{2+} > Sr^{2+} > Mg^{2+}$$

Whereas the affinity for Al hydroxide has shown a slightly different order:

$$Cu^{2+} > Pb^{2+} > Zn^{2+} > Ni^{2+} > Co^{2+} \geq Cd^{2+} > Mg^{2+} > Sr^{2+}$$

Therefore, the hydroxide-metal bond is not solely based on electrostatic properties, but also on the electronegative properties of the metal-hydroxy species (147). In the case of inner-sphere complexation desorption may occur via the dissolution of the adsorbing surface (158), displacement by a metal-hydroxy species having a higher affinity to the surface than the adsorbed metal-hydroxy species (e.g. Zn over Cd) (159, 161), or through a lowering in the chemical potential of the free metal in solution (115)

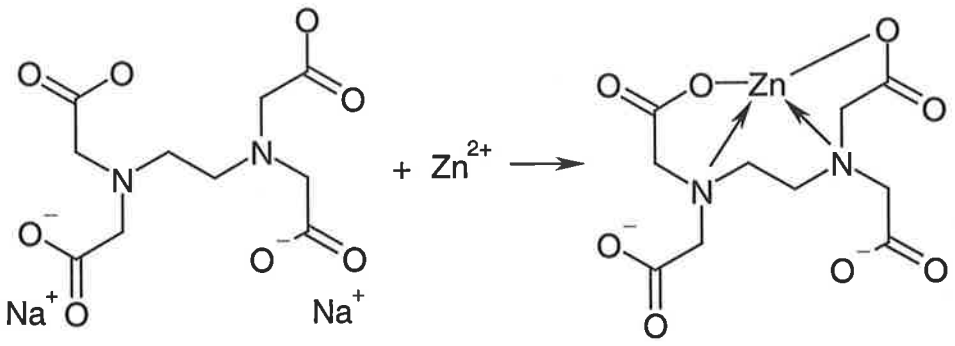
Despite the wide acceptance that metal adsorption in soils is controlled by their metal-hydroxy species, it is important to note that soil solution pH has the most critical influence on the sorption of Cd (104, 107, 144, 152-154). In these studies the sorption of Cd increases very rapidly over a narrow pH range leading to a so-called adsorption edge. Such increases in adsorption are assumed to occur because of the rapid increase in the concentration of the metal-hydroxy species. However, the concentration of $CdOH^+$ is negligible relative to Cd^{2+} at the pH of the adsorption edge. Therefore, for the metal-hydroxy species to be adsorbed, it must have a very high affinity for the soil surface and metal hydrolysis (maintaining the principle of equilibrium) must not be a kinetically limiting step. The surface charge density of soils is also strongly influenced by pH (149). Therefore, increasing solution pH leads to a rapid increase in the net negative surface charge which may also explain the enhanced adsorption in these experiments (162).

2.6 Effect of chelates on the adsorption of Cd and Zn

Many laboratory experiments have shown that the presence of chelates in the soil environment may alter Cd and Zn adsorption/desorption characteristics (128-130, 163-165). In this thesis the term chelate is used interchangeably with organic ligands, naturally or synthetically derived, that are monodentate, bidentate or multidentate. Inorganic anions such as chloride (Cl^-) and sulfate (SO_4^{2-}) are also able to complex Cd and Zn but are not considered in the scope of this review. Therefore, chelates may attach to or co-ordinate with a metal using one, two or more donor atoms. For example, when EDTA, a synthetic multidentate ligand, coordinates with Zn through two carboxyl groups (COOH) a ring structure is formed. This ring includes Zn, the two carboxyl groups

attached to Zn and the N atoms spanning the two carboxyl groups in the chelate - a metal-EDTA complex (Figure 2.2). The formation of such a metal-EDTA complex is referred to as chelation (47).

Figure 2.2: The process of chelation with the disodium salt of EDTA and Zn.

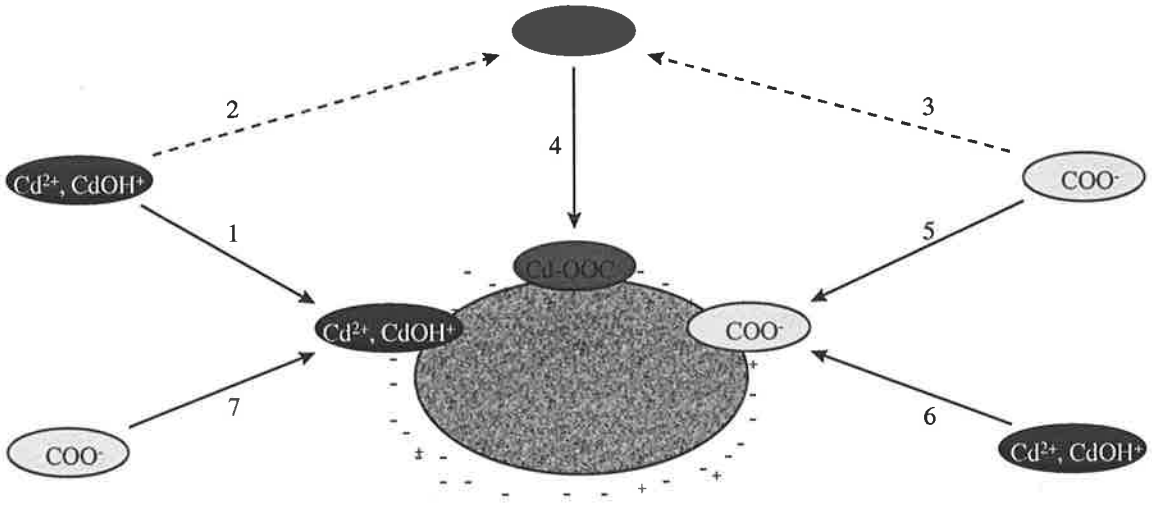


In the presence of chelates, chelation is only one of many processes that may affect the adsorption/desorption characteristics of metals to the soil solid phase. Sposito (166) has proposed the following processes to describe the general effects that chelates may have on metal adsorption/desorption in the soil environment (Figure 2.3):

1) No effect or decreased metal adsorption
    a) The chelate has a low affinity for the metal and for the soil solid phase (no effect on step 1 in Figure 2.3)
    b) The chelate has a high affinity for the soil solid phase and when adsorbed the chelate has a low affinity for the metal (step 5)
    c) The chelate has a high affinity for the metal and forms a soluble complex which has a low affinity for the soil solid phase (steps 2 and 3)
    d) The metal has a high affinity for the soil solid phase and when adsorbed has a high affinity for the chelate (no effect on step 1 but produces step 7)

2) Increased adsorption
    a) The chelate has a high affinity for the metal and forms a soluble complex which has a high affinity for the soil solid phase (steps 2 - 4)
    b) The chelate has a high affinity for the soil solid phase and when adsorbed has a high affinity for the metal (steps 5 and 6)

Figure 2.3: Schematic diagram of the effects that chelates have on metal adsorption according to Sposito (166).



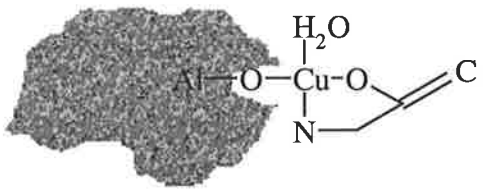
The major failing of Sposito's model, however, is that it does not include chelate-induced changes of soil solution pH. As mentioned previously in section 2.5, soil solution pH has a critical influence on the adsorption of metals, such as Cd (104, 107, 144, 152-154). Therefore, changes in the adsorption of metals to the soil solid phase, in the presence of chelates, may merely reflect changes of pH. However, soil solution pH changes induced by plant-derived chelates are currently difficult to predict because it is largely unknown if they are excreted as the free acid or the alkali metal salt (127). Evidence has been presented for both processes. For example, citrate has been estimated to be excreted by white lupin (*L. albus* L.) solely as the free acid (167), whereas mugineic acid has been determined to be released as the K salt (168). As such, the former will decrease soil

solution pH leading to enhanced desorption, but the latter may induce a rise in pH leading to increased metal adsorption. However, the situation may be more complex as K^+ dissociated from the chelate may induce the dissolution of Mn minerals enhancing the desorption of Cd and Zn (169).

2.6.1 Enhanced adsorption in the presence of chelates

Although it is recognized that all of the proposed mechanisms for increased metal adsorption in the presence of chelates offered by Sposito (166) are possible they have been more difficult to verify experimentally (170-174 and reviewed by Harter and Naidu (128), Morrill *et al.* (175) and Mortland (176)). For example, there has only been one report of the use of electron spin resonance (ESR) to demonstrate the adsorption of metal-chelate complexes (177). In these experiments the adsorption of Cu^{2+} on gibbsite and boehmite was studied in 10 mM aqueous solutions containing 2:1 glycine:Cu ratios. The ESR measurements suggested that Cu^{2+} formed ternary complexes with the surface and one or more glycine molecules (Figure 2.4).

Figure 2.4: Cu^{2+} adsorbed on gibbsite to form a ternary complex in which the metal is coordinated simultaneously with a surface hydroxyl and one glycine molecule (177).



Although the adsorption of Cd- and Zn-chelate complexes have yet to be demonstrated, it has been shown that chelates do adsorb to the soil solid phase and that the extent of this adsorption depends on: 1) the p*K*a of the chelate's functional groups; 2) complexation with metals, and; 3) the variability of the surface charge of the solid phase. As such, chelate adsorption is also greatly influenced by changes of pH. For example, minerals (e.g. Fe, Al and/or Mn oxides) or soils that show a variable surface charge become

increasingly positively charged with decreases in pH and, therefore, attract negatively charged chelates or metal-chelate complexes (129, 172, 178). In either case, an observed enhancement of Cd^{2+} and Zn^{2+} adsorption is seen in the presence of the chelate (adsorption of the negatively charged chelate leads to an increase in the negative surface charge of the soil and, thus, attracts positively charged cations) (130, 164, 170, 174, 179, 180). In fact, it has been reported by Chairidchai and Ritchie (164) that Zn adsorption in the presence of citrate and oxalate was related to the point of zero charge (PZC) of the soil. At pH values below the PZC the soil solid phase carries a net positive charge attracting negatively charged chelates and metal-chelate complexes. At pH values above the PZC the soil has a net negative charge and this substantially reduces its attraction for negatively charged chelates and metal-chelate complexes. Therefore, the presence of negatively charged Zn-citrate complexes enhanced adsorption when the pH was below the PZC and reduced adsorption at pH levels above the PZC (164).

2.6.2 Decreased adsorption

At pH levels above the PZC, soils containing a large variable charge component will react similarly to one dominated by permanent negatively charged surfaces (e.g. clays) and, at constant pH, the presence of chelates favors metal desorption if: 1) they effectively compete for Cd and Zn against sorption sites on the solid phase and; 2) the metal-chelate complex carries a negative charge (129, 130, 164, 165, 181-184). In these cases the strong complexation of Cd and Zn in soil solution reduces Cd^{2+} and Zn^{2+} (or $CdOH^{+}$ and $ZnOH^{+}$) activities and leads to the desorption of solid bound exchangeable metal (163, 182, 185-188). It follows that these effects increase with chelate concentration and are greatest for those chelates which complex Cd and Zn most strongly (189). Furthermore, if the chelate has a positive charge at pH values above or below the PZC of a soil it is also possible that they may compete for metal adsorption sites leading to decreased adsorption - such observations have been made with EDTA (163) and humic acid (190) for Cd.

Chelates may also indirectly promote the desorption of Cd and Zn by dissolving adsorbing surfaces such as clay minerals (120, 191), $CaCO_3$ (167), Mn-oxides (121, 192-

195), and the hydroxides of Fe (121, 192) and Al (192). Dissolution may take place due to a decrease in pH or as a result of chelation. In fact, distinctions between pH and chelation effects have rarely been made in Cd and Zn adsorption or desorption studies as solutions have usually been buffered to maintain constant pH. Therefore, extant research distinguishing these two processes from the overall general effect of chelates is imperative to evaluating Cd and Zn reactions in soil.

2.7 Chelates and the dissolution of Cd and Zn minerals in soils

Chelates have played a major role in the development of the hypothesis that Zn solubility in soils is controlled by the dissolution of simple Zn compounds. Using the thermodynamic solubility products of mineral compounds and stability constants of the metal-chelates of EDTA and diethylenetrinitrilopentaacetic acid (DTPA) work by Lindsay and Norvell (196, 197) provided good evidence that these chelates complexed Zn arising from the dissolution of amorphous $ZnSiO_3$. However, the authors later retracted their conclusions when they realized that they had used the wrong solubility constant for $ZnSiO_3$ and that their results were 'purely coincidental' (198). Nonetheless, further results from other chelation studies have favored the hypothesis that the activity of Zn^{2+} in soil solution, in the presence of EDTA, may be regulated by the dissolution of simple compounds such as ZnS (199) or franklinite ($ZnFe_2O_4$) (200, 201), despite none of these compounds being identified in the soils. Furthermore, the presence of ZnS is unlikely unless the soil is anaerobic (i.e. flooded) (202) or polluted by ZnS mine wastes. Moreover, the solubility of Zn^{2+} in equilibrium with $ZnFe_2O_4$ also depends on the activity of Fe^{3+}. For example, amorphous $Fe(OH)_3$ depresses the solubility of Zn^{2+} supported by $ZnFe_2O_4$, whereas crystalline Fe(III) oxides, such as goethite (α-FeOOH), lower the activity of Fe^{3+} and permit higher equilibrium levels of Zn^{2+} (201). The situation becomes more complex because these differences could also be related to the adsorption properties of amorphous $Fe(OH)_3$ which can adsorb up to 1190 μmole Zn/g (103) - three magnitudes higher than that reported for α-FeOOH (138, 143, 144). These relationships are further confounded by the fact that Zn may be brought into solution by the dissolution of adsorbing Fe-hydroxides by chelates (121, 147, 194-197, 203-205).

Lindsay's work with chelates later extended to Cd in neutral to alkaline soils. However, it was found that the solubility of Cd was at least two orders of magnitude lower than any identified Cd mineral (206). Nonetheless, Santillan-Medrano and Jurinak (207), Street *et al.* (119) and Cavallaro and McBride (151) have provided sufficient evidence to suggest that the solid phases of $CdCO_3$ and/or $Cd_3(PO_4)_2$ most likely limited Cd^{2+} in solution after artificially contaminating alkaline or calcareous soils. On real polluted soils, however, it has been found that soil solutions were undersaturated with respect to $CdCO_3$ but were supersaturated with respect to $Cd_3(PO_4)_2$ (208). Similarly, these Cd compounds could not, or were not, identified. However, studies using X-ray absorption spectroscopy (XAS) in the extended edge X-ray absorption structure (EXAFS) region have shown that the adsorption of Cd on α-FeOOH surfaces can result in the formation of multinuclear surface precipitates (209). These precipitates have been observed at metal surface loading rates far below a theoretical monolayer coverage and at pH values well below where the formation of metal hydroxide precipitates would be expected (146). Although multinuclear metal hydroxides of Zn have yet to be examined by EXAFS, Tiller (210) has already demonstrated that Zn-hydroxides were 2 - 3 orders of magnitude less soluble when precipitated in small amounts on clay minerals.

As there have been no studies examining the effects of chelates on these Cd and Zn compounds it remains to be speculated upon what effect they have on these compounds in the soil environment.

2.8 Phytoavailability of soil Cd and Zn

The degree of Cd and Zn uptake by plants depends not only upon the concentration in soil solution - Intensity (*I*) but also on the buffer power of the soil to replenish the soil solution from the solid phase - Quantity (*Q*) (6, 116, 123, 152, 211). Therefore, a shift from solid-phase forms (*Q*) to that in soil solution (*I*) will increase plant-available Cd and Zn in soil. Most of the factors influencing *I* have been well documented and reviewed earlier in sections 2.6 and 2.7. However, debate still continues on what effects the chelation of Cd and Zn (and metals in general) in soil solution have on the plant uptake of

these metals.

2.8.1 Metal speciation and the FIAM (Intensity)

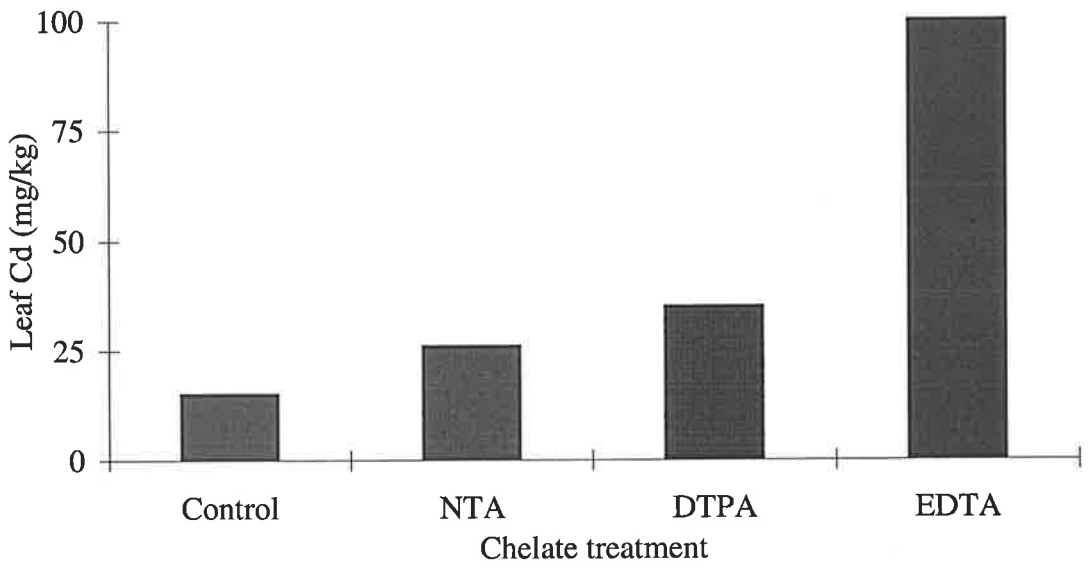
If chelation processes increase Cd and Zn levels in the soil solution then they will play a vital role in transporting these metals to the plant root (212, 213). An increase in concentration of either the free ion (by the dissolution of adsorbing surfaces) or the metal-chelate complex will increase metal flux to the root surface by: 1) mass flow of water to the root to replace transpirational losses and/or; 2) by diffusion of the metal or metal-chelate complex in response to an induced concentration gradient by the selective uptake of the metal by the root (41, 212). Therefore, the flux of metal to the root (J_r) can be estimated by the summation of diffusional (where D_e is the effective diffusion coefficient of the metal in soil, C_s is the concentration of metal on the solid phase that equilibrates with C_l and r is the radial distance) and mass flow (where v_o is the volume of water absorbed and C_l is the concentration of metal, including complexed species, in the soil solution) fluxes (41):

$$(2.1)\ J_r = D_e\,(C_s / r) + v_o C_l$$

For example, Krishnamurti *et al.*(189) observed that the addition of high concentrations (10 mM) of succinic acid and citric acid to three Cd polluted soils could increase the flux of Cd from 0.0009 to 0.2 (μmol/kg)/hr. Similar results have also been obtained for Zn at lower concentrations of DTPA, EDTA and fulvic acid (214-216). For example, Elgawhary *et al.* (215) found that the addition of 1.5×10^{-8} mole EDTA/g soil increased the flux of Zn from 0.36×10^{-9} to 3.0×10^{-9} cm^2/s. At these low rates of application, the synthetic chelate's ability to overcome strong diffusional limitations to Cd and Zn transport to plant roots is the most probable cause for enhanced uptake observed in soil-grown plants (217-222). This hypothesis has also been used to explain enhanced uptake of radionuclides (223-230), Ni (219, 231), Mn (219, 220), Pb (222), Cu (217, 219, 220, 222), cobalt (Co) (232), Fe (219, 220, 233-241) and molybdenum (Mo) (219) after the addition of a range of synthetic chelates to soils. As an example, Wallace *et al.* (221)

demonstrated in a neutral (pH 6.0) soil, contaminated by the addition of 150 mg Cd/kg soil as $CdSO_4$, that the application of nitrilotriacetic acid (NTA), EDTA and DTPA significantly increased Cd uptake by *P. vulgaris* L. (Figure 2.5).

Figure 2.5: Cd concentrations in the shoots of *P. vulgaris* L. after the addition of 100 mg/kg NTA, EDTA or DTPA. Plant dry weights were unaffected by the chelates (221).



This hypothesis inherently assumes that plant uptake of these metals is solely a function of the free ion - The Free Ion Activity Model (FIAM), so that a measure of *I* is purely the free ion in solution. Accordingly, any treatments or changes in soil conditions which affect the concentration (or rather activity) of Cd^{2+} and Zn^{2+} in solution should affect plant accumulation of the metal (15). This hypothesis originally derived from the work of DeKock and Mitchell (242) who found that the toxicity and shoot concentrations of a range of metals, including Zn, decreased after the addition of NTA, EDTA and DTPA to nutrient solutions. The FIAM for the plant uptake of Cd^{2+} and Zn^{2+} has since gained support from other nutrient solution experiments (50, 53, 211, 213, 235-237, 239, 243)

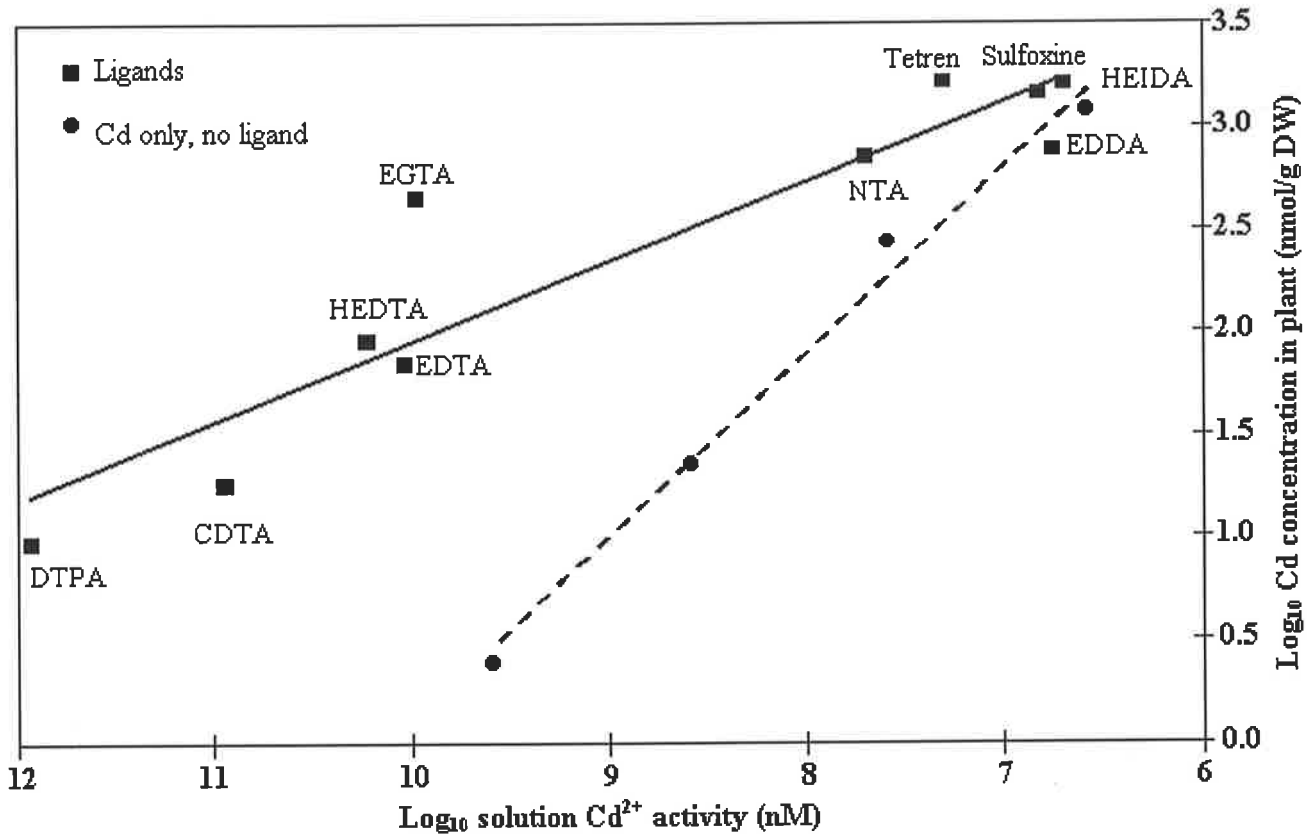
and has been the foundation on which chelator-buffered nutrient solutions for Cd- and Zn-plant research is based (244-251).

These studies have certainly indicated that free metal ions are preferred for plant uptake over metal-chelate complexes. However, decreases in Cd and Zn uptake have hardly ever been in proportion to changes in the free ion activity. For example, when Rengel (245) kept total Zn concentrations constant (4.5 μM) but decreased Zn^{2+} activity 27-fold (1300 - 48 pM), with the addition of N-(2-hydroxyethyl) ethylenedinitrilotriacetic acid (HEDTA), only an approximate 30 % decrease in shoot Zn accumulation by *Triticum aestivum* L. (cv. Aroona) was observed. Rengel (245) suggested that the decreases in shoot Zn concentrations did not parallel Zn^{2+} activities due to a 'sort of homeostatic mechanism' that regulated the accumulation of Zn in the shoots within the range of Zn^{2+} activities studied. This mechanism may also explain the results of Checkai *et al.* (252) who examined the uptake of Cd and Zn by tomato plants (*Lycopersicon esculentum* L.) at constant metal activity with the use of a chelating resin. Although the addition of EDTA to these solutions increased the total concentration of the metals in solution (the increase being metal-EDTA complexes), concentrations of Cd and Zn in the plant shoots were unaffected. However, this mechanism seems highly unlikely for the non-essential Cd where evidence to date suggests that plant uptake is not highly regulated (253).

In studies where Cd and Zn uptake have been compared at a given Cd^{2+} or Zn^{2+} activity, plant uptake has been shown to be dependent on the nature of the chelate (254-257), and in the study of McLaughlin *et al.* (257) uptake of Cd and Zn by lettuce (*Lactuca sativa* L.) was actually greater in the presence of synthetic chelates (Figure 2.6).

McLaughlin *et al.* (257) also examined the role of the charge of the metal-chelate complex on Cd and Zn uptake. Despite the earlier results of DeKock and Mitchell (242) for a variety of metals and those of Iwasaki and Takahashi (258) for Cu, no relationship was found between the charge of the metal-chelate complex and Cd and Zn uptake (257). Rather it was observed that Cd and Zn uptake efficiency (a measure of shoot metal

Figure 2.6: Relationship between *L. sativa* L. Cd concentrations and the negative logarithm of solution Cd^{2+} activity in molar units in the absence and presence of ligands (257).



(CDTA) = trans-1,2-cyclohexyl-diamine-N,N,N',N'-tetraacetate

(EGTA) = ethylene-bis-(oxyethylenenitrilo)-tetraacetate

(HEIDA) = hydroxyethyl-imino-diacetate

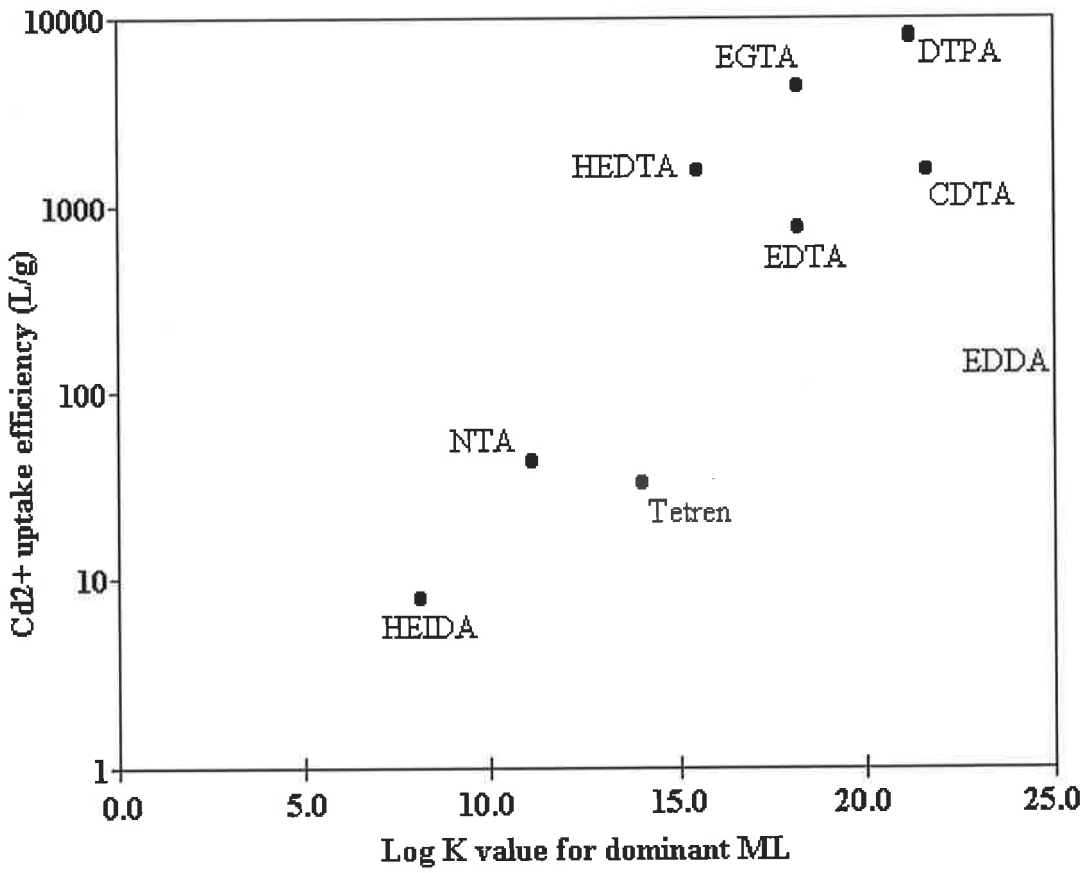
(EDDA) = N,N-ethylene-diamine-diacetate

(Tetren) = 1,4,7,10,13-pentaazatridecane

(Sulfoxine) = 8-hydroxyquinoline-5-sulfonate

concentration / free ion activity in solution) was positively correlated with the thermodynamic stability constant of the Cd- and Zn-chelate complexes (Figure 2.7). The relationship between uptake efficiency and metal-chelate complex stability suggests that Cd and Zn uptake was diffusionally limited within the root apoplast and that the chelates were able to buffer free ion activities at the site of uptake. Such a relationship has also been observed for Fe and Al in the presence of EDTA (259). This hypothesis is plausible considering that the diffusion length in the apoplast may be greater than 100 μm due to the tortuous nature of the pores in the cell walls (260, 261).

Figure 2.7: Relationship between Cd^{2+} uptake efficiency, calculated as plant Cd concentration per unit solution Cd^{2+} activity, and binding constant of dominant metal-chelate complex (257).



On the contrary, a relationship between uptake efficiency and the thermodynamic stability constant of metal-chelate complexes may suggest uptake of the intact metal-chelate complex. For example, there have been a number of studies suggesting the plant uptake of a variety of metals complexed to synthetic chelates at concentrations ≤ 500 μM (54, 215, 262-277). Of those that have attempted to verify the presence of the intact metal-chelate complex within the plant (264-267, 273, 275-277) only one has focused on either Cd or Zn (273). From these experiments, it has been postulated that breaks in the endodermal barrier at root apices and at the sites of lateral root initiation might permit passive, convective uptake of the intact metal-chelate complex (254).

Generally, plant uptake of the intact metal-chelate complex was studied using isotopically labeled chelates (e.g. ^{15}N or ^{14}C) and metals. As such, uptake of the intact metal-chelate was indirectly estimated by comparing the ratios of the two radioisotopes. However, uncertainties arise with what compound the ^{15}N or ^{14}C radioisotope actually represents and does not necessarily indicate that the intact metal-chelate complex was present in the plants. For example, Hill-Cottingham and Lloyd-Jones (264) determined, using paper chromatography, that less than 50 % of the recovered ^{14}C from plants supplied with ^{14}C-labeled EDTA was present as the unchanged chelate. The only attempts to directly verify the presence of intact metal-chelate complexes have been that of Jeffreys and Wallace (277) and Hill-Cottingham (278). In both cases, UV-absorbance was used to detect the presence of iron ethylenediamine di(O-hydroxyphenylacetate) (FeEDDHA) or Fe(III)EDTA in plant tissue. Studies at higher concentrations of synthetic chelates (> 500 μM) have also resulted in suggestions of the plant uptake of intact metal-chelate complexes. However, these concentrations may physiologically stress plants and it is possible that different mechanisms were operating (268, 272, 279) and will be discussed further in Section 2.9.2.

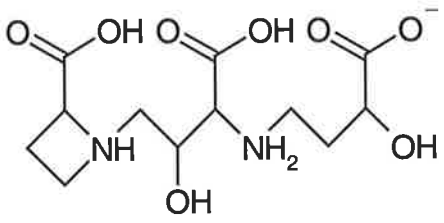
In addition to the synthetic chelates, there is evidence that plant excreted phytosiderophores from the graminaceous plant species are absorbed as intact metal-chelate complexes (280-287). These substances have been characterized chemically as

nonproteinogenic amino acids of the mugineic acid family (Figure 2.8) and are excreted by plant roots in response to Fe- and Zn-deficiency (284, 286, 288, 289).

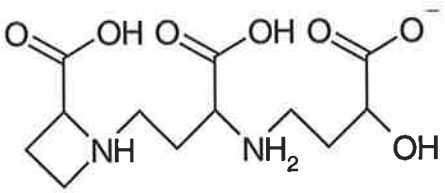
Short-term uptake studies with double-labeled Fe-phytosiderophores have implied the existence of an uptake system for nondissociated Fe- and Zn-phytosiderophores at the root plasma membrane (280, 290, 291). This phytosiderophore transporter is sensitive to chilling and proton uncouplers (292) and has a high affinity for Fe-phytosiderophores (292) but the data for Zn-phytosiderophores remains controversial (281, 289, 293).

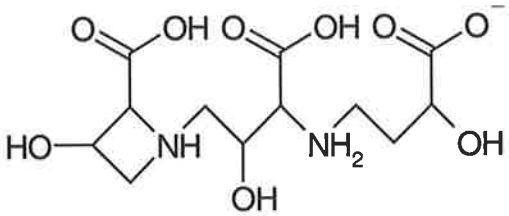
Figure 2.8: Chemical structures of: (A) mugineic acid; (B) deoxymugineic acid, and; (C) 3-epi-hydroxymugineic acid.

A



B





C

Work with other naturally occurring chelates have generally indicated that an active uptake mechanism for intact metal-chelate complexes may be restricted to the mugineic acid family of phytosiderophores. For example, Jones and Darrah have demonstrated that *Z. mays* L. cannot absorb Fe-citrate (294). However, they did observe the uptake of free citrate at solution concentrations ≥ 1 mM or from solutions with low ionic strength. In an

earlier study the same authors also found an active uptake mechanism for free amino acids in the same plant (295). Although the uptake of metal-amino acid complexes have not been studied, it may be worthy of future research since it has been observed that: 1) histidine is involved in Ni hyperaccumulation by *Alyssum lesbiacum*, and; 2) supplying histidine to a non-accumulating species (*Alyssum montanum*) greatly increased its capacity for Ni transport to the shoot (296).

Regardless of whether it is the free ion that is taken up by the plant, or uptake is via some complexed form through direct uptake or alleviation of diffusional limitations, it is evident that the relationship between free metal activity in solution and plant metal uptake is not as close a relationship as has been assumed. One barrier to such studies is the lack of simple and convenient analytical methods for unambiguously quantifying metal-chelate complexes. Progress in this area would greatly facilitate studies examining metal speciation in soil solutions providing useful information on the phytoavailability of metals in both the free and complexed form.

2.8.2 Isotopically exchangeable metal pools (Quantity)

The *I* of Cd and Zn in soil solution (free ion and complexed forms) is largely controlled by its equilibrium with the quantity of metal associated with the soil solid phase (*Q*). Estimates of *Q* (the quantity of metal that is phytoavailable) have ranged from very conservative predictions based on the total quantity of soil Cd and Zn (complete acid digestion) to discrete chemical fractions based on the use of chemical extractants, such as 5 mM DTPA (297, 298). The most serious limitation of complete acid digestion to measure *Q* is that it invariably measures Cd and Zn associated with insoluble minerals or within stable crystal structures that, in a reasonable time frame, will have no impact on the plant uptake of these metals. It is, therefore, surprising that total metal concentrations are still frequently used as a measure of *Q* when the concept of phytoavailability has been recognized for over 130 years - 'we are informed how much phosphoric acid, potash, magnesia, etc., exist in the soil, but get from the analysis no clue to the amount of any of these substances which is at the disposition of the present crop.' - (Johnson, 1870) cited

in Frink (98).

Chemical extraction methods to determine Q have ranged from single extractions, using pH buffered solutions usually containing $CaCl_2$ and a chelating agent such as EDTA or DTPA (297-299), to chemical fractionation methods that allocate metals into operationally defined pools of distinct biogeochemical form (e.g. water soluble, weakly adsorbed, specifically sorbed, carbonate, organic, hydrous oxide, or crystal lattice forms, etc.) (105, 111, 116, 300-302). The latter extraction procedures use increasingly severe reagents in a sequential fashion to extract increasingly refractory forms of the metals. Commonly, the first one, two or occasionally three extracted fractions are taken to be the pool of metal that is phytoavailable (116, 301). Much less frequently, however, this assumption has actually been tested by comparing the extracted pools with plant uptake of the metal (111, 116).

A complementary approach to estimating the size of Q has been to stress the overall chemical interactions between the solid and solution phases in soils (303). Radioisotopic procedures have proved invaluable in this area of research and the technique of isotope dilution has now become the reference standard against which chemical extraction procedures for determining Q are compared. Three isotopic dilution procedures have been developed to estimate the amount of phytoavailable soil metal and, depending upon the system used for equilibration, have been designated as E, L and A values. The E value is measured by sampling the solution phase of a soil suspension that has reached isotopic equilibrium. In essence, the E value is a measure of the size of the isotopically exchangeable 'pool' of element in the soil:

$$(2.2)\ E\ (\mu g/g\ soil) = (M_s^*) / (M_l^* / M_l)$$

where M_s^* = radioactive metal associated with the solid phase (kBq/g)

M_l^* = radioactive metal added to soil suspension remaining in solution (kBq)

M_l = soil derived non-radioactive metal in solution (μg)

The L value has already been introduced in chapter 1, and is determined by growing plants in an isotopically-labeled soil that has reached isotopic equilibrium at moisture and temperature conditions suitable for plant growth (equation 1.1). The A value is measured under similar experimental conditions to those of the L value. However, the A value is a measure of Q in terms of a reference source, usually an isotopically-labeled fertilizer material, that is taken as the standard of phytoavailability (304, 305). The mathematical equation used to calculate the A value is almost identical to that used for the L value, with the exception that the amount of added element (M_a in equation 2.3) is taken into consideration. Thus, equation 1.1 becomes:

$$(2.3)\ A\ (\mu g/g\ soil) = \{(M_s^*)M_a / (M_p^* / M_p)\} - M_a$$

where M_s^* = radioactive metal added to soil (kBq/g)

M_a = non-radioactive metal (e.g. fertilizer) added to soil (μg/g)

M_p^* = radioactive metal in the plant shoot (kBq)

M_p = soil derived non-radioactive metal in the plant shoot (μg)

As the medium of measurement for A and L values is the plant material any effect that the plants may have on the isotopic exchange properties of the soil is reflected in the measurements. In A value measurements, ideally, the reference source does not interact with the soil and maintains the same standard of phytoavailability over the growth period of the plant in different types of soil. The A value has been most widely applied to N and P fertilization research with only a few instances of its use for Zn fertilization studies (105, 305). A similar approach, but more applicable to environmental pollution studies, has also been applied to quantify the phytoavailability of Cd and Zn when added to soils in sewage sludges (303). Unlike the A value fertilization studies it is the soil that is labeled (L value) and not the reference source (i.e. sludge). In this way the untreated soil becomes the reference against which the effects of the sludge are compared. It is surprising that this technique has not been utilized more often since it would provide valuable information on: 1) the phytoavailability of contaminants added to soils through

sewage sludge or other wastes and; 2) the effectiveness of immobilizing techniques to decrease contaminant phytoavailability.

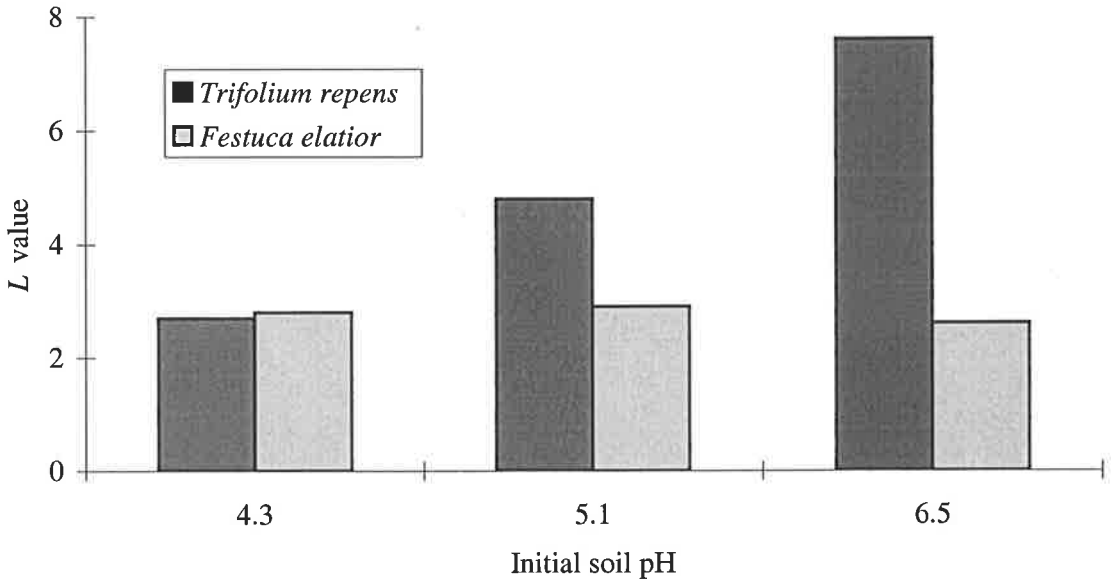
Studies examining the *L* values of Cd and Zn have proved extremely useful for determining the *Q* of these metals because many have indicated that a wide range of plants access the same pool of metal (i.e. they show the same *L* value) (36, 38, 42, 44, 46, 101, 305). For example, Hamon *et al.* (36) found that a wide range of plants accessed the statistically same pool of Zn and, with the exception of one plant - *Brassica napus* L., the same pool of Cd. Further practical importance has been demonstrated by many of these studies when it was found that the *L* value was statistically equal to the isotopically exchangeable metal in the soil - the *E* value (38, 45, 46, 101). This equality is significant because the determination of *L* values is time consuming and cannot be practically used for large sets of soil samples.

However, close agreement between the Cd and Zn *L* values of different plant species have not always been observed suggesting that some plants may have the ability to alter the isotopic exchange properties of the soil in the root zone (and hence differ in their *L* values) (43-46). For example, Rule and Graham (43) found that *Trifolium repens* L. and *Festuca elatior* L. (cv. Ky 31) only accessed the same Zn pool at pH 4.3 whereas at pH 5.1 and 6.5 the former accessed a larger pool of Zn not available to the latter (Figure 2.9). Similar results for Cd have also been reported by Gerard *et al.* (46) who observed that the Cd *L* value of *Lolium perenne* L., *L. sativa* L. and *T. caerulescens* J. & C. Presl frequently differed from each other and was dependent on soil type, degree of Cd contamination, and time of sampling.

Often when *L* values have been compared to *E* values it also appears that plant-related factors may increase the size of the isotopically exchangeable pool of Cd and Zn in the rhizosphere (37, 38, 43, 45, 46, 101). For example, Smolders *et al.* (37) not only observed the Zn *L* value of *T. aestivum* L. to always exceed the corresponding Zn *E* value

(in 10 mM $CaCl_2$) but also found this result applied to Cd on 10 soils varying in pH (4.5 - 7.2) and Cd contamination.

Figure 2.9: Zn *L* values (mg/kg) of *T. repens* L. and *F. elatior* L. (cv. Ky 31) grown on a soil at different pH values. Measurements taken on one cutting of *T. repens* L. after 60 days of growth and a second cutting of *F. elatior* L. after 70 days (43).



Therefore, it is quite plausible that plant uptake of Cd and Zn involves non-isotopically exchangeable Cd and Zn brought into solution by processes associated with the rhizosphere, such as acidification and chelation by root excreted ligands (38, 162). In the experiments of Rule and Graham (43) no differences in soil pH were observed between *T. repens* and *F. elatior* suggesting that factors other than pH contributed to the former having a greater *L* value. Furthermore, where the effect of pH on Cd and Zn *E* values have been conducted most have shown that the size of the pool of isotopically exchangeable metal was independent of pH (110, 306, 307). However, this was not found to be true by Sinha *et al.* (44) who observed that Zn *E* values on some calcareous soils increased when the pH of the DTPA extracting solution was decreased by more than

two pH units. Similarly, extractions made with 5 mM EDTA have been shown to increase the isotopically exchangeable pool of Cd in polluted soils (110) and also of Zn in a sewage sludge (308). Nonetheless, it is still unknown as to what effect natural chelates, at rhizosphere concentrations, may have on the isotopically exchangeable pools of Cd and Zn.

2.9 Chelate-assisted Cd and Zn phytoextraction

Phytoextraction has generated immense interest in the scientific and wider community with the number of reviews almost overwhelming the number of scientific articles (11, 309-319). Two general strategies of Cd and Zn phytoextraction have emerged since Chaney (320) first proposed the use of metal hyperaccumulator plants to extract large quantities of metals from polluted soils. The hyperaccumulating plant that has shown most promise for the remediation of Cd and Zn polluted soils is *T. caerulescens* J. & C. Presl. However, practical phytoextraction with this species is still in its developmental stages with knowledge on optimizing soil conditions for their agronomic management (e.g. P fertilization (87)) and a fundamental understanding of the mechanisms responsible for Cd and Zn hyperaccumulation (321) still lacking in the literature. Furthermore, phytoextraction of Cd and Zn employing *T. caerulescens* has been hindered by the low biomass produced by the plant (35, 322, 323) and its slow growth habits (35, 86, 324, 325).

The second strategy that has been proposed for the phytoextraction of Cd and Zn, that would eliminate the factors of slow-growth and low-biomass production by *T. caerulescens*, has been to employ techniques that increase metal accumulation by high biomass crops (29, 32, 33). The technique of adding synthetic chelates to enhance metal accumulation by these plants has been most popular (29, 32, 33, 88, 326). For example, Huang and Cunningham (31) and Huang *et al.* (32) have shown that the chelation of Pb with EDTA renders this element much more available to *Z. mays* so that a metal content of > 10000 mg Pb/kg DW can be obtained in the shoots. Promising results have also been found with Cd and Zn (29, 88), however, a systematic study of the processes of

EDTA-assisted Cd and Zn phytoextraction has still not been published.

2.9.1 Mobilization of metals into soil solution

As discussed earlier in section 2.6.2 the presence of chelates in the soil environment can increase the concentration of Cd and Zn in soil solution. These effect is generally greatest at high chelate concentrations and for those chelates which complex Cd and Zn most strongly (29). Increases in Cd and Zn soil solution concentrations leads to greater metal transport to plant roots and, desirably (for phytoextraction), greater potential for enhanced metal uptake. It is these two principles of greater transport in the soil matrix and subsequent enhanced metal uptake that have underpinned the studies of not only Cd and Zn chelate-assisted phytoextraction (29, 88, 327, 328) but also of other metals such as Pb (29-33, 329-332), and U (326, 333).

Most of the studies reported on chelate-assisted phytoextraction have focused on the removal of Pb from polluted soils. Typically, chelate concentrations added to the soil have exceeded 3 mmole/kg soil resulting in large increases of Pb concentrations in soil solution. For example, remarkable results have been reported by Huang and Cunningham (31) when 24 hours after the addition of 5.6 mmole Na_3HEDTA/kg to a soil contaminated with 2500 mg Pb/kg, the concentration of Pb in soil solution increased from 3.6 mg/L to 4050 mg/L. In a later study, Huang *et al.* (32) demonstrated that under similar conditions Pb accumulation in the shoots of pea plants (*Pisum sativum* L. cv. Sparkle) reached over 6000 mg Pb/kg, whereas that of *Z. mays* L. (cv. Fiesta) was 2000 mg Pb/kg. Interestingly, more than 98% of the increase in shoot Pb accumulation by the *Z. mays* plants could be accounted for by increases in soil solution concentrations of Pb. In another study, Blaylock *et al.* (29) found the effectiveness of various chelates to solubilize Pb to decrease in the order EDTA ~ DTPA < CDTA < EGTA ~ citric acid. Similarly, a close correlation between Pb solubilization and enhanced Pb uptake by *B. juncea* (cv. 426308) was observed regardless of the chelate used.

Of the studies examining Zn chelate-assisted phytoextraction only 2 different chelates

have been examined for their effectiveness in solubilizing this metal - NTA and EDTA (88, 328). Similar to the studies examining Pb polluted soils the application of high concentrations of these chelates resulted in increased soil solution concentrations of Zn. For example, Ebbs and Kochian (88) found the addition of 6.1 mmole EDTA/kg to a polluted soil containing 3100 mg Zn/kg increased Zn solubility in pots containing *B. juncea* (cv. 184290) dramatically from 0.1 - 90 mg Zn/kg (88). Interestingly, there were significant differences in the amount of Zn solubilized between pots containing plants of *B. juncea* and those containing oat (*Avena sativa* L.) or barley (*H. vulgare* L.) plants, suggesting that plants may also determine the effectiveness of chelates to solubilize soil Zn.

Although all of these studies have reported similar observations, none have attempted to determine the mechanisms of metal mobilization from the soil solid phase into the soil solution. This question is not just of academic importance as the mechanism of solubilization may determine which chelate is most suitable for chelate-assisted phytoextraction. For example, if the solubilization of Cd and Zn is a result of strong complexation with Cd^{2+} and Zn^{2+} in soil solution, reducing Cd^{2+} and Zn^{2+} solution activities and increasing the desorption of solid bound exchangeable metal (163, 182, 185-188), then chelates having a high affinity for these metals should be selected. Alternatively, if the chelate promotes the desorption of Cd and Zn by dissolving adsorbing surfaces such as clay minerals (120, 191), $CaCO_3$ (167), Mn-oxides (121, 192-195), and the hydroxides of Fe (121, 192) and Al (192), then chelates most effective at dissolving these surfaces should be selected.

Furthermore, the use of chelates for Cd and Zn phytoextraction raises the question of what effects they may have on the phytoavailable pools of these metals (*L* value). For example, if environmental regulations are based on total soil metal concentrations, it maybe desirable to use chelates (or concentrations) that solubilize non-phytoavailable pools of Cd and Zn.

2.9.2 Mechanisms of plant metal uptake

Although the addition of chelates is known to increase metal flux to the root surface by mass flow and diffusion, the physiological basis for enhanced metal uptake by plants at concentrations used in chelate-assisted Cd and Zn phytoextraction still remains, largely, speculative. At lower chelate concentrations, as discussed in section 2.8.1, conflicting evidence for metal uptake via the free ion or as the metal-chelate complex has been presented.

Studies at higher metal-chelate concentrations have generally indicated that at least some metal uptake is via the intact metal-chelate complex. For example, Wallace (272) demonstrated that the conducting tissue of *S. tuberosum* contained the purple color of the intact metal-chelate complex after being exposed to solutions containing 5 mM FeEDDHA. Corroborating results for uptake of the intact metal-chelate complex were observed more recently for the 426308 cultivar of *B. juncea* in nutrient solutions containing PbEDTA and in studies of EDTA-assisted Pb phytoextraction (30, 34). Vassil *et al.*'s (34) evidence consisted of Pb accumulation in the shoots of *B. juncea* being positively correlated with EDTA accumulation, having a ratio of 1:0.67 (EDTA:Pb), and a High Pressure Liquid Chromatography (HPLC) profile of the xylem exudate indicating that at least some of the Pb taken up by the plant may be the intact PbEDTA complex. Similarly, Epstein *et al.* (30) provided indirect evidence for this mechanism with soil grown plants when ^{14}C-labeled compounds accumulated in the shoots of *B. juncea* after adding ^{14}C-labeled EDTA (10 mmole EDTA/kg soil). However, it must be pointed out that these studies have similar limitations to those discussed earlier in section 2.8.1 because uncertainties arise with what compound the ^{14}C radioisotope represents.

What has been important for determining metal uptake in these recent studies on chelate-assisted phytoextraction is that threshold concentrations of the chelate seem to induce dramatic increases in shoot metal concentrations. For example, Blaylock *et al.* (29) found a dramatic increase in shoot Pb concentrations between 1 and 5 mmole EDTA/kg soil treatment. In nutrient solution experiments, Vassil *et al.* (34) observed that a threshold concentration of 0.5 mM PbEDTA was required to induce the accumulation of high

concentrations of Pb in the shoots. It is likely that at these high chelate concentrations, and those used in earlier studies (268, 272, 334, 335), the normal mechanisms for regulating metal transport into the root was damaged leading to indiscriminate plant uptake. Indeed, Vassil *et al.* (34) speculated that at these threshold concentrations, EDTA destroys the physiological barrier(s) in roots that normally function to control uptake and translocation of solutes. This is possible as it is known that both Zn^{2+} and Ca^{2+} ions are involved in stabilizing plasma membranes providing a barrier to root cells (336, 337). Therefore, EDTA may induce the uptake of metal-EDTA complexes by chelating the stabilizing Zn^{2+} and Ca^{2+} cations from the plasma membrane.

Nonetheless, these studies have only been limited to the one cultivar of *B. juncea* - (cv. 426308) and PbEDTA. It is, therefore, still unknown if these mechanisms apply to other plant species or other metals. Such a problem has been highlighted by the results of Ebbs and Kochian (88) who observed that the addition of EDTA to a Zn contaminated soil increased Zn uptake by *B. juncea* but not by *H. vulgare* or *A. sativa*. As such, a mechanistic study of EDTA induced Cd and Zn uptake during EDTA-assisted phytoextraction is needed to further develop this technology for soils polluted with these metals. More specifically, the hypothesis that differential uptake or exclusion of the intact metal-EDTA chelate needs to be examined.

2.9.3 Limitations to determining metal-EDTA complexes in environmental samples

One of the major hindrances to understanding the mechanisms of how EDTA mobilizes metals from soil and affects plant uptake has been the lack of suitable techniques to measure the intact metal complexes of EDTA. Maybe due to its importance in Fe nutrition, early attempts to determine intact metal-EDTA complexes in soil and plant extracts were limited to the measurement of Fe(III)EDTA by UV-absorbance (278). Other earlier attempts to measure the complexation of EDTA with metals were limited to indirect techniques such as metal specific ion electrodes, titration, and/or flame absorption spectrometry (196, 197, 338, 339). Indeed, the equilibrium constants used for metal-EDTA complexes in programs such as GEOCHEM-PC (124) are commonly based

on these indirect methods.

More recently, capillary zone electrophoresis (CZE) and ion chromatography (IC) have proven to be reliable direct detection methods for the quantification of metal-EDTA standards in simple solutions (340-342). However, CZE has yet to demonstrate its usefulness in environmental matrices and the use of IC has been restricted due to high limits of detection (343, 344). Nevertheless, Miller *et al.* (344) were able to demonstrate the presence of EDTA, as Fe(III)EDTA and/or Al(III)EDTA, below 14 μM by using negative and/or positive ion mode electrospray liquid chromatography-tandem mass spectrometry (LC-MS-MS). More recently, LC-MS-MS has also been used to detect the intact complexes of CuEDTA, PbEDTA and CdEDTA (345) to 1-2 μM in Millipore water. However, these methods are of limited value for quantifying metal-EDTA complexes in environmental matrices that vary in ionic strength and pH because these two parameters dramatically affect the electrospray ionization process (346). Therefore, large errors in quantitation will be observed when environmental samples are compared to external standards that have been acidified to pH 1 (345, 347).

However, the coupling of IC (with post-column suppression) to MS has been used to determine EDTA-H^+ (m/z: 291) in river water samples down to a concentration of 7 nM (348). This is three orders of magnitude lower than that reported for CZE (341) or IC (342, 344). Furthermore, when a groundwater sample was spiked with EDTA it was found that m/z: 291 had multiple retention times (Bauer, *et al.*, 1997 cited in (348)). However, of the three metal-EDTA complexes separated, only two could be empirically identified as CaEDTA and Fe(II)EDTA. Further progress in this analytical methodology could lead to major improvements in the measurement of intact metal-EDTA complexes in the environment because they may be distinguished not only by differences in their chromatographic mobilities but also by their mass-to-charge ratio (m/z) and the isotopic signature of the metal.

2.10 Summary

The mining, smelting and processing of nonferrous metals has been largely responsible for the ubiquitous contamination and pollution of soils with Cd and Zn. Although cases of acute toxicity have been rare, chronic exposure to these metals, as well as economic losses, will be sustained in the future if: 1) these soils are not remediated and; 2) current practices of applying sewage sludge, fertilizers or other amendments, contaminated with Cd and Zn, to soils is not modified.

In soil solution the concentration of Cd and Zn is highly pH dependent and largely controlled by adsorption/desorption processes, whereas dissolution processes may only be of importance at higher pH values. Although it has been demonstrated that chelates may affect the desorption of Cd and Zn from soils, less work has been undertaken on polluted soils to differ between true chelation effects and pH. Knowledge on the factors affecting desorption and dissolution of Cd and Zn is needed to better understand the phytoavailability of these metals (*L* values). Phytoavailability predictions are not only needed for risk assessment studies but to also to evaluate the success of phytoextraction practices.

The success of chelate-assisted Cd and Zn phytoextraction also depends on furthering our present understanding of the mechanisms involved in metal mobilization as well as the plant uptake and root-shoot transport of these metals. In particular, the hypothesis that differential uptake or exclusion of the intact metal-EDTA complex determines metal accumulation by different plant species needs critical examination. This can only be accomplished with advances in novel methods to determine intact metal-chelate complexes in environmental matrices. Of these IC-MS appears to have the most potential to be used to identify and quantitate not only intact metal-EDTA complexes but other metal-chelate complexes as well.

3. General materials and methods

3.1 Introduction

The aims of this chapter are to: 1) describe the general properties of the soils used in the experiments outlined by this thesis; 2) provide a succinct account of the methodology employed to conduct these experiments and; 3) outline statistical and analytical techniques and, when needed, verification procedures used to ensure the reliability and accuracy of results. To ease reference in subsequent chapters the section describing the applicable method or material will be cited along with the page number where it is located.

3.2 Soils

In total, four different soils were utilized in the experiments described by this thesis. Before the soils were used in any experiments their general physical and chemical properties were determined as described below and are provided in Table 3.1. After the soils had been collected from the field they were air-dried, sieved to < 2 mm and stored in polyethylene bags until analyses or experiments commenced.

The first soil 'Pinole' was used in preliminary experiments aimed at obtaining soil solution and plant xylem samples containing metal-EDTA complexes (Chapter 4). These samples were used to develop a suitable analytical method to determine metal-EDTA complexes in these matrices. The Pinole soil was obtained from an undisclosed agricultural area in northern California which was routinely used by Zeneca Ag. Product's Western Research Center, Richmond, California for agrochemical research. This soil was selected due to the ease with which it could be accessed for preliminary pot experiments.

The second soil, termed Bazar soil, was sampled from a former industrial site in Richmond, California. This soil was used for the experiments described in Chapter 5. The soil was polluted with arsenic (As), Cd, Cu and Zn between approximately 1896 - 1946 by the land application of roasted pyrite wastes (locally termed 'cinders') formed during the manufacture of sulfuric acid. This site is located on the former shoreline of the

San Francisco Bay that was reclaimed during the late 1800's. As a result, the soil lacks any well developed pedological characteristics.

The final two soils (Hapludalfs) were obtained from Région Nord - Pas de Calais, France. One of these soils was called the 'acidic soil' (pH 6.2) while the other soil, containing significantly more $CaCO_3$, was termed the 'calcareous soil'. The acidic soil was located adjacent to a Zn smelter, near the village of Auby, that has been in operation since 1869. The calcareous soil was also taken from the proximity of a Pb and Zn smelter operating near Noyelles-Godault. Both soils have been subject to Cd, Pb and Zn pollution by the deposition of atmospheric particles. These soils were used in the experiments outlined in Chapter 6.

3.2.1 Soil analyses

3.2.1.1 Macronutrients

The basic chemical properties of the soils were determined using standard procedures. Powdered soil samples were combusted in a LECO C/S 244 analyzer (LECO Corp., St. Joseph, Michigan) to determine inorganic and total organic carbon (TOC) contents. Prior to the analysis of TOC, inorganic C was removed by reacting soils with 1 M hydrochloric acid (HCl) in polyethylene triangle beakers for 12 hours. The contents of each beaker were filtered through a glass microfiber filter (Whatmann GF/C, 1.2 μm retention rating) and rinsed with hot 1 M HCl to ensure the complete removal of carbonates. Total carbon was also determined on non-acid treated soils and the difference between TOC values was assumed to represent inorganic C, as $CaCO_3$, present in the soil.

Total N was determined by ammonia distillation after reduction to ammonium sulfate using 'Kjeldahl' digestion with sulfuric acid (H_2SO_4). Ammonium N in the sodium hydroxide (NaOH) distillate was determined by a titrimetric procedure using 0.01 M HCl (349). Plant available P was determined by extraction with 0.5 M sodium bicarbonate ($NaHCO_3$) before colorimetric determination of P in solution (350).

Table 3.1: Selected chemical and physical properties of the soils used in the experiments described by this thesis.

	Pinole	Bazar	Acidic	Calcareous
pH (H_2O)	5.5	5.0	6.2	8.0
Organic C (g/kg)	26	13	25	16
Total N (g/kg)	3.2	1.8	1.5	1.2
PO_4 (g/kg)	0.2	0.4	0.1	0.5
Total $CaCO_3$ (%)	<0.1	<0.1	<0.1	1.4
CEC ($cmol_c/kg$)	24.8	42.6	13.1	9.7
Ca^{2+} ($cmol_c/kg$)	18.3	37.6	7.8	29.8
Mg^{2+} ($cmol_c/kg$)	6.9	5.8	1.2	0.6
K^+ ($cmol_c/kg$)	0.2	0.2	0.6	0.6
Cd (mg/kg)	nq	6.2	20.3	18.7
Zn (mg/kg)	23	880	3300	1400
Pb (mg/kg)	nq	nd	990	960
Cu (mg/kg)	2	410	84	38
Mn (mg/kg)	92	380	nq	nq
Ni (mg/kg)	nq	nq	23	15
WHC (0,01 Mpa)	28	22	25	31

nq = not quantified, nd = not detected

The exchangeable cations - Ca, Mg and K - were estimated by extraction with 1 M ammonium chloride (NH_4Cl). Subsequently, the cation exchange capacity (CEC) of the soils were determined by leaching with 1 M potassium chloride (KCl) (351). All metals were analyzed by flame atomic absorption spectroscopy (FAAS) or inductively coupled plasma-atomic emission spectroscopy (ICP-AES). The pH of the soils were measured in high purity (18 MΩ) water using a soil-to-liquid ratio of 1:5 after one hour of equilibration.

3.2.1.2 Trace metals

Total soil concentrations of Cd, Cu, Mn, Pb and Zn were also determined by FAAS or ICP-AES after digesting 1.0 g of finely ground oven-dried soil samples in 25 ml of concentrated HNO_3:HCl (aqua-regia). After digestion, solutions were evaporated to near dryness before being taken up to 5 ml with 18 MΩ water. The DTPA extractable contents of Cu, Mn and Zn for the Pinole soil had already been determined by the methodology described by Lindsay and Norvell (297). Therefore, total metal concentrations were not determined for this soil as it was only used during preliminary experiments.

3.3 Protocol for determining plant uptake of metal-EDTA complexes

Two different pot experiments are described in this thesis. The first was conducted with *H. vulgare* L. (cv. Harrington) plants on the Pinole soil to obtain metal-EDTA complexes in soil solution and plant xylem exudate for method development (Chapter 4). In the second experiment a larger selection of plants were grown on the Bazar soil to determine the nature of Zn mobilization, uptake and root-shoot transport in the presence of increasing concentrations of EDTA (Chapter 5).

3.3.1 Fertilization

The commercial fertilizer - Tagline™ (12:12:12) - was used as a supplemental source of N, P and K for plant growth in pot experiments. The fertilizer was thoroughly mixed with the Pinole soil before sowing seeds of *H. vulgare*. In subsequent experiments, using

the Bazar soil, the fertilizer was applied in three portions throughout the experiment to minimize the phytotoxicity of heavy metals that may be displaced by the fertilizer from the soil solid phase (352). In both cases, by the end of the experiment, a total of 150 mg fertilizer/kg had been added to the soil. This amount supplied an additional 75 mg of N, P and K to pots containing 500 g of the Pinole soil. In experiments using the Bazar soil the fertilizer supplement provided an additional 105 and 90 mg of N, P and K to pots containing *S. tuberosum* L. plants (700 g of soil) and the remaining pots (600 g of soil), respectively.

3.3.2 Selection of plant species

Preliminary experiments were conducted with two monocotyledons (*H. vulgare* L. and *Z. mays* L.) and three dicotyledons (*B. juncea* L., *L. albus* L. and *S. tuberosum* L.) to select species and cultivars most tolerant to growing on the polluted Bazar soil. The only cultivar of *Z. mays* (cv. Zeamx, PI3394, Zeneca Ag. Products, Richmond, California) tested did not survive when planted in this soil. *Hordeum vulgare* and *B. juncea* plants were initially selected as they have previously been demonstrated to differ in their ability to accumulate Zn after the soil application of EDTA (88). As such, it was believed that further study with these two species would lead to a better understanding of the mechanisms responsible for enhanced Zn uptake in the presence of EDTA. The cultivar of *B. juncea* (cv. 426308) used by Ebbs and Kochian (88) did not survive when grown on the Bazar soil. Therefore, another cultivar (cv. 182921, Northern Central Regional Plant Introduction Station, Ames, Iowa) better adapted to growing on this soil was used for further experiments. *Solanum tuberosum* was included in these experiments mainly because of the large above-ground biomass that this plant produces. However, it was also selected because of the ease with which xylem exudate could be sampled from this plant. The first cultivar of *S. tuberosum* (cv. Yukon Gold) tested showed no signs of toxicity when planted in the Bazar soil. Of the *L. albus* cultivars obtained from the Western Regional Plant Introduction Station, Pullman, Washington, the cultivar 606483 was chosen based on the relatively high amount of above-ground biomass it produced.

3.3.3 Plant germination

The seeds of *H. vulgare*, *B. juncea* and *L. albus* were sown directly into the pots and watered according to the protocols described below. Upon germination these plants were thinned so that each pot contained 5 seedlings. In contrast, the sprouts of *S. tuberosum* were cut from tubers, placed in pots and the attached tuber covered with soil. Due to the large size of the sprouts, and attached tuber, only one was planted in each pot.

3.3.4 Environmental conditions for plant growth

The experiments used to select the plant cultivars suitable for growing on the Bazar soil were conducted in a greenhouse. The same greenhouse was used to grow *H. vulgare* plants on the Pinole soil to obtain soil solution and plant xylem samples containing metal-EDTA complexes. The temperature of the greenhouse was controlled to give a night/day temperature range of 18 - 27 °C. Pots were watered regularly to the field capacity of the soil.

In the experiment reported in Chapter 5 the environmental conditions for plant growth were more precisely controlled by using a growth chamber. The experiment was conducted using a completely randomized block design and artificial lighting was supplied at 300 - 400 μmol/m^{2}s with a mixture of fluorescent and incandescent lights for 16 hours a day. The day/night temperature regime of the growth chamber was maintained at 22/18 °C. Throughout this experiment the pots were watered daily, twice daily when needed, to 76 % of the water holding capacity of the soil. Plant transpiration was estimated by subtracting the evaporation of triplicate control pots, containing soil only, from the total water loss observed from pots containing plants. This calculation could be accurately made because pots were not allowed to freely drain.

3.3.5 Addition of EDTA and the sampling of soil solutions and plant materials

The *H. vulgare* plants used to obtain metal-EDTA complexes in soil solution and plant xylem exudate were grown in the greenhouse for 5 weeks. At this time, the potassium salt of EDTA (Fisher Scientific, Fair Lawn, New Jersey) was added to duplicate pots at

the rate of 2.5 mmole EDTA/kg soil, with watering. Twenty-four hours later plant xylem was extracted for one hour by vacuum extraction (353). After xylem extraction had been completed the soil solution from the same pot was collected by displacement with water (354). Xylem exudate and soil solution samples were immediately frozen until metal-EDTA complexes were determined by IC-MS (Chapter 4).

In the subsequent experiment, described in Chapter 5, EDTA was applied to pots (in triplicate) after 4 weeks of plant growth. The amount of EDTA added in this experiment varied and resulted in four different application rates: 0.0; 0.034; 0.34 and 3.4 mmole EDTA/kg soil. Concentrations of EDTA, corresponding to these application rates, were made by diluting a 250 mM stock solution of the free acid of EDTA (Fisher Scientific, Fair Lawn, New Jersey) that had been neutralized to pH 7 in 18 MΩ water with 29 % ammonium hydroxide (NH_4OH). Ammonium hydroxide was used to neutralize EDTA as Na^+ interfered with the electrospray process during IC-MS analyses. As the aim of this experiment was to examine relationships between the plant uptake of metals and metal-EDTA complexes the harvesting of plant shoots and sampling of soil solutions and plant xylem were delayed until five days had elapsed after the addition of EDTA.

Plant shoots were harvested by cutting stems approximately 1 cm above the soil surface. The shoots were weighed and washed with 18 MΩ water before being oven-dried to constant weight at 80 °C. Subsequently, plant material was ground in an agate mortar and pestle and microwave digested in concentrated HNO_3 according to US EPA method 3052. After digestion, solutions were evaporated to near dryness before being taken up to 5 ml with 18 MΩ water and refrigerated prior to metal analysis by FAAS.

After harvesting shoots, xylem exudate was collected for only one hour to ensure that the plants remained metabolically active during sampling (353). Preliminary experiments with *H. vulgare* and *Z. mays* indicated that the previously used vacuum extraction method was only successful at obtaining xylem exudate from *H. vulgare*. Therefore, in this experiment plants were left to exude at atmospheric pressure. Due to the larger size of

this experiment, and the difficulties encountered with using water displacement to obtain soil solution, the methodology for sampling the soil solution was also modified. In this case, soil solution samples were obtained by applying a small vacuum, for up to 16 hours, to non-porous rhizosphere samplers (Rhizosphere Research Products, Netherlands) that had been inserted into the soil at the beginning of the experiment. One ml was taken from the soil solution samples for metal-EDTA complex determination and was frozen with the xylem exudate samples until IC-MS analysis. The remaining portion of the soil solutions were acidified with a small quantity of concentrated HNO_3 and refrigerated prior to metal analysis with FAAS.

3.4 Protocol for examining organic ligand and H^+ effects on soil Cd and Zn

The role played by organic ligands in the desorption of Cd and Zn from the two polluted French soils was examined by batch experimentation (Chapter 6). At the same time the effects of these ligands on the isotopically exchangeable pools of soil Cd and Zn (*E* values) were examined.

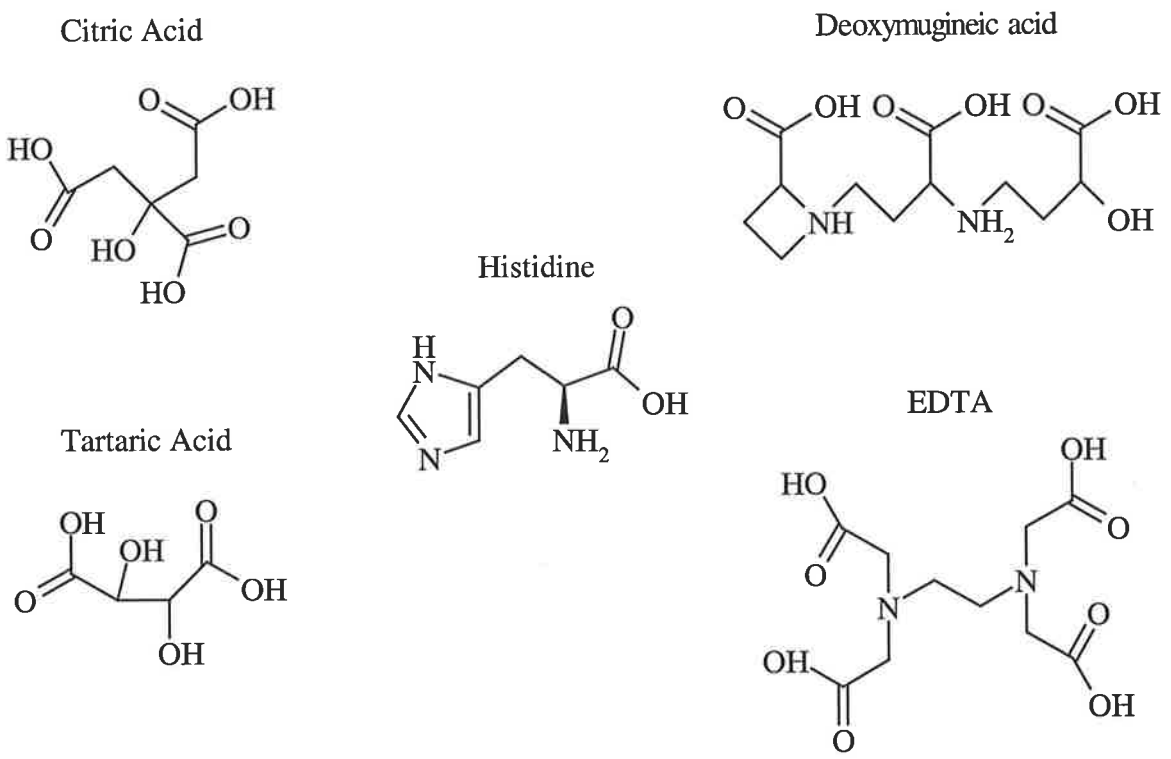
3.4.1 Selection of organic ligands

A range of organic ligands (organic and amino acids - Figure 3.1) were selected based on: 1) their ability to complex Cd and Zn (citrate, histidine, DMA and EDTA - Table 3.2); 2) their ubiquitous occurrence in the rhizosphere (citrate and tartrate)(127); 3) their role in metal uptake by plants (citrate and DMA) (122, 284, 291, 355); 4) their role in natural (histidine and DMA) or induced (EDTA and citrate) metal hyperaccumulation (32, 284, 296, 326), or; 5) simply the lack of available data on the effects that these ligands may have on the chemistry of Cd and Zn in the soil environment.

The Na-salts of citrate and *L*-tartrate (Aldrich-Chemie, Steinheim, Germany) were used as available evidence tends to suggest that organic acids are not excreted by plant roots as the free acid, but as the alkali metal salt (127). For comparison purposes, however, the free acid of citrate (Merck, Damstadt, Germany) was included in these experiments. No literature exists on the root exudation of histidine, therefore, the free base of *L*-histidine

was used as received from the vendor (Sigma, Steinheim, Germany). It has recently been demonstrated that mugineic acid is excreted by *H. vulgare* plants as a K-salt (168). Therefore, based on inference, it could be assumed that the same would apply for DMA. However, due to the limitations imposed by the methodology to collect DMA (as root exudates, described below) only the free acid was used in these experiments. A stock solution of EDTA was made by dissolving the free acid (Prolabo, Paris, France) in 18 MΩ water buffered to pH 7 with 29 % NH_4OH. This solution was identical to the EDTA solution used in the pot experiment described in Chapter 5.

Figure 3.1: Chemical structure of the organic ligands used in this thesis.



Although the range of organic ligand concentrations in soil solution has generally been found to be between 10 - 100 μM (reviewed by Jones (127)), it is not unusual to find much higher concentrations in the rhizosphere. For example, citric acid has been measured in the rhizosphere of *L. albus* L. at concentrations of up to 5 mM (167, 356,

Table 3.2: Chemical properties of the organic ligands used in this thesis. The values for pK_a (- log of H^+ dissociation constant) and log K (log of the thermodynamic stability constant of the metal-ligand complex), with the exception of DMA, were derived from the GEOCHEM-PC, version 2 database (359) that had been updated, where possible, with values from the NIST 2001 Critically Selected Stability Constants of Metal Complexes Database (US Dept. of Commerce, Gaithersburg, Maryland). All values have been corrected to zero ionic strength and 25 °C.

Organic Ligand	Molecular Weight	Isoelectric Point (pH)	pK_a (pH)[1]	Metal-Ligand Complex	log K Cd	log K Zn
Citrate	192		3.1 (COOH)	$(M\text{-Citrate})^-$	5.0	6.3
			4.8 (COOH)	$(M\text{-Citrate}_2)^{4-}$	6.0	7.5
			6.4 (COOH)	$(M\text{-HCitrate})^0$	9.5	10.3
				$(M\text{-H}_2\text{Citrate})^+$	12.9	12.8
				$(MOH_2\text{-Citrate}_2)^{4-}$		-2.0
Tartrate	150		3.0 (COOH)	$(M\text{-Tartrate})^0$	3.9	3.6
			4.4 (COOH)	$(M\text{-HTartrate})^+$		5.6
Histidine	155	7.56	1.6 (COOH)	$(M\text{-Histidine})^+$	6.1	7.0
			6.0 ($>N^+H$)	$(M\text{-Histidine}_2)^0$	10.6	12.7
			9.3 ($-N^+H_2-$)	$(M\text{-HHistidine})^{2+}$	11.4	11.6
DMA	304	unknown	2.39 (COOH)	$(M\text{-DMA})^-$	unknown	12.8[2]
			2.76 (COOH)			
			3.4 (COOH)			
			7.78 ($>N^+H$)			
			9.55 ($-N^+H_2-$)			
EDTA	292		2.2 (COOH)	$(M\text{-EDTA})^{2-}$	18.1	18.0
			3.2 (COOH)	$(M\text{-HEDTA})^-$	21.5	21.5
			6.3 (COOH)	$(M\text{-H}_2\text{EDTA})^0$	23.6	23.3
			11.0 (COOH)	$(MOH\text{-EDTA})^{3-}$	4.1	6.0

[1] pK_a values for DMA were sourced from Sugiura *et al.* (283)

[2] The log K of $(Zn\text{-DMA})^-$ was taken from Murakami *et al.* (360).

357). Furthermore, in all but one of the studies (358) reviewed by Jones (127) the total amount of ligand sorbed to the soil solid phase was not taken into account. Therefore, to the extent that sorption and possibly degradation may occur in the experiments described below, a range of organic ligand concentrations from 250 μM - 5 mM were used to represent those concentrations likely to be found in the rhizosphere of a variety of plant species. All solutions were made in 18 MΩ water.

3.4.2 Acquisition of DMA

Deoxymugineic acid was collected as root exudates from *Z. mays* L. (cv. Zeamx, PI3394, Zeneca Ag. Products, Richmond, California) plants grown hydroponically under Fe-deficient conditions (280).

3.4.2.1 Nutrient solutions

The aerated nutrient solution used to induce Fe-deficiency in *Z. mays* consisted of 2.0 mM $Ca(NO_3)_2$, 0.7 mM K_2SO_4, 0.5 mM $MgSO_4$, 0.1 mM KH_2PO_4, 0.1 mM KCl, 10 μM H_3BO_3, 0.5 μM $ZnSO_4$, 0.2 μM $CuSO_4$, and 0.01 μM $(NH_4)_6Mo_7O_{24}$. Stock solutions of these nutrients were made in 18 MΩ water and were appropriately diluted with the same high purity water to create 3 liters of nutrient solution. The nutrient solution containers were covered with aluminum foil to limit root exposure to light.

3.4.2.2 Plant germination and growth

Seeds of *Z. mays* were sown in trays containing a mixture of acid washed quartz sand and vermiculite. The trays were placed in the greenhouse and watered regularly to stimulate germination. After the seedlings had reached about 5 cm in height the sand and vermiculite were washed from the roots using deionized water. Seedlings were bunched into groups of about 70 and transferred to the nutrient solutions. The plants were cultured in a growth chamber under the same conditions as described previously. Nutrient solutions were changed every 48 - 72 hours depending on the amount of plant transpiration. After about 3 weeks of growth plants started to exhibit Fe-deficiency symptoms.

3.4.2.3 Collection of root exudates

Root exudates were collected for one week following the onset of Fe-deficiency chlorosis. Each day, two hours after the commencement of the light period, seedlings were removed from the nutrient solutions, the roots washed with water and then immersed in 500 ml of 18 MΩ water (361). Root exudates were collected for a 4 hour period at which time the seedlings were returned to the nutrient solutions until the following day. The collection volume was treated with 0.1 % Micropur (Black Mountain Stores Inc., Odessa, Texas) to prevent microbial degradation and then refrigerated at 4 °C until purification as described below.

3.4.2.4 Purification of DMA

After acidifying the crude root exudates DMA was concentrated on a cation exchange column (AG 50W-X8 resin in the H^+ form, 200-400 mesh, Bio-Rad, Richmond, California), washed with 0.2 M HCl and eluted with 2.0 M HCl (modified methodology of Takagi *et al.* (362)). The protonated DMA was desalted in autoclaved 18 MΩ water by three short dialysis periods using a 100 MWCO membrane (Spectrum, Laguna Hills, California). The identity of DMA was confirmed by electrospray mass spectrometry (ES-MS) and its purity by IC.

3.4.3 Measurement of organic ligand sorption

Sorption of the organic ligands to the soil solid phase was determined by adapting the methodology of Jones and Brassington (172) to the systems used for determining the sorption coefficients and *E* values of Cd and Zn (i.e. 1:10 soil-to-solution ratio, described below). Briefly, 25 ml of the organic ligand solutions and 50 μl of chloroform were added to 60 ml polyethylene bottles containing air-dried soil (equivalent to 2.5 g of oven-dried soil) and shaken at 320 rpm for 10 minutes.

The pH of the soil solutions were immediately measured prior to 5 minutes of centrifugation at 1000 RCF to aid phase separation. Aliquots of 100 μl were taken from the supernatant and the concentration of organic ligand remaining in solution was

quantified by IC or IC-MS. The amount of sorption was calculated by subtracting the amount of organic ligand measured in solution from the initial concentration added. The sorption of DMA was not measured in these experiments due to the lack of available instrumentation for accurate quantification (ES-MS is commonly only used for qualification purposes). Initial attempts to use IC-MS to quantify DMA, using anion exchange (363) and eluent suppression, were unsuccessful. Although further work to develop this method was not continued due to time constraints, it may be a research area worthy of continued efforts because of the lack of suitable analytical techniques to measure these types of phytosiderophores.

3.4.4 Determination of Cd and Zn sorption coefficients and *E* values

Sorption coefficients (K_d - the distribution of metal between the solid and solution phases) and *E* values of Cd and Zn in the soils were measured by batch experiments using the same bottles as those used for measuring organic ligand sorption. Similarly, air-dry soil, equivalent to 2.5 g of oven-dry soil, was added to these bottles with 25 ml of solution (e.g. 0.5 mM organic ligand). Calcium chloride ($CaCl_2$) and calcium nitrate ($Ca(NO_3)_2$) solutions at 10 mM were also used to determine K_d and *E* values because this concentration has commonly be used for experiments with Cd (37, 108) and Zn (38, 42, 108, 116). When examined, variations of pH were facilitated by the addition of small amounts of either 100 mM NaOH or HNO_3 to 18 MΩ water. The highest volume of acid used in these experiments resulted in an ionic strength of 0.004. To minimize bacterial activity during equilibration 50 μl of chloroform was also added.

The soils were allowed to equilibrate at room temperature with the added solution by shaking the bottles (in triplicate) on an end-over-end shaker (40 rpm) for 24 hours. At this time 1 ml of carrier-free ^{109}Cd (0.35 - 4.9 kBq/g soil) and/or ^{65}Zn (0.14 - 11.0 kBq/g soil) was added to the bottles and equilibrated for a further 24 hours. The maximum amount of Cd (2.76×10^{-4} μg) added to the bottles with a ^{109}Cd spike (Specific activity: 44.4 MBq/μg Cd, Amersham Pharmacia Biotech UK limited, Little Chalfont, Buckinghamshire, England) represented 1.1 % of the lowest concentration of stable Cd

measured in solution after equilibration. Therefore, it was assumed that the ^{109}Cd spike did not affect the equilibrium that had been attained prior to adding the radioisotope. In contrast, the maximum quantity of Zn (0.26 μg) added to the bottles with ^{65}Zn (Specific activity: 105.3 MBq/mg Zn, NEN Life Science Products Inc., Boston, Massachusetts) amounted to 20.6 % of the minimum solution concentration of Zn measured after radioisotope equilibration. However, after equilibration < 1 % of the added ^{65}Zn remained in solution. As > 99 % of the radioisotope was rapidly sorbed to the solid phase under these conditions it was, therefore, also assumed that carrier Zn added with the ^{65}Zn neither contributed significantly to the measured solution concentration of Zn nor significantly altered the distribution of stable Zn between the solid and solution phases.

After equilibration the bottles were centrifuged at 1000 RCF for 5 minutes and the supernatant filtered through non-sorbing cellulose nitrate (0.025 μm) into 20 ml polyethylene bottles (46). The solutions were acidified with concentrated HNO_3 and 1 ml was taken for measuring the concentration of ^{109}Cd and/or ^{65}Zn (γ-spectrometry) while the remainder was refrigerated until analysis for stable Cd, Fe and Zn by ICP-AES. Solution concentrations of organic ligands and H^+ were also quantified after 48 hours as described above.

3.4.4.1 Equations

Sorption coefficients (K_d in L/kg soil) of Cd and Zn were calculated using the following equation:

$$(3.1)\ K_d = \frac{R\text{-}r}{r} \times \frac{L}{S}$$

where R = the *total* quantity of carrier free ^{109}Cd or ^{65}Zn added (kBq)

r = the *total* quantity of ^{109}Cd or ^{65}Zn remaining in solution after equilibration (kBq)

L/S = the liquid to solid ratio (L/kg soil)

The value for (*R-r*) was used to represent the amount of added radioisotope that is associated with the soil solid phase. When this value is divided by the measured amount of total radioisotope remaining in solution (*r*) this ratio represents the distribution of the radioisotope between the solid and solution phases (at equilibrium) *only* under the experimental conditions used to determine these values. Therefore, this ratio is multiplied by the liquid-to-solid ratio to give K_d values that are normalized to conditions of a 1:1 liquid-solid ratio.

Thus, the isotopically exchangeable metal on the solid phase, the *E* value (mg/kg soil), is simply the product of K_d and the stable metal (*M* in mg/L) in solution:

$$(3.2)\ E = M \times K_d$$

However, the *E* value only represents the metal associated with the soil solid phase. Therefore, the total solution concentration of metal must be added to the *E* value to attain the *total* isotopically dilutable metal in the soil-solution system (recently termed the E_a value (364) in mg/kg soil):

$$(3.3)\ E_a = E + M\left(L/S\right)$$

Throughout this thesis the *E* values reported actually represent values of E_a but will simply be referred to as the *E* value.

3.4.4.2 Selection of equilibration period

To determine if dynamic equilibrium had, in fact, been attained after 48 hours (24 hours before and after the addition of the isotope), preliminary experiments were conducted to examine the effect of increasing equilibration times to 96 hours (24 hours before and 76 hours after the addition of the isotope). An analysis of variance indicated that there was no statistical difference ($P \leq 0.05$) between the Cd *E* and K_d values measured with the

acidic soil at these two equilibration periods suggesting that equilibration had been reached after 48 hours (Table 3.3).

Table 3.3: The effect of equilibration time on the K_d and *E* value of Cd in the acidic and calcareous soil. Values represent the mean and (standard deviation) of triplicate measurements. Values in *italics* indicate decreases of K_d as a result of a lower pH. Values underlined indicate decreases of K_d due to a reduction in the solution concentration of the organic ligand.

Cadmium				
	48 hours	96 hours	48 hours	96 hours
Acidic Soil	K_d (L/kg)		*E* (mg/kg)	% of *E* at 48 hrs[1]
10mM $CaCl_2$	20.4 (1.0)	17.4 (0.8)	13.5 (0.3)	105.5
10 mM $CaNO_3$	31.2 (0.6)	32.8 (0.5)	13.3 (0.4)	101.5
0.5 mM NaCitrate	174 (22)	212 (15)	14.1 (0.8)	100.7
0.5 mM Tartrate	1020 (50)	1100 (100)	14.0 (0.6)	96.4
0.5 mM EDTA	75.6 (1.2)	76.6 (4.2)	14.8 (0.6)	101.6
0.5 mM Citric Acid	350 (7.1)	337 (4)	14.7 (0.2)	86.6
5 mM Citric Acid	29.4 (1.1)	27.7 (2.2)	15.4 (0.2)	100.9
			Mean (n=21)	**99.0**
Calcareous Soil				
10mM $CaCl_2$	*258 (10)*	*199 (1)*	6.0 (0.1)	117.6
10 mM $CaNO_3$	*456 (18)*	*385 (11)*	4.5 (0.2)	105.3
0.5 mM NaCitrate	1220 (75)	1350 (12)	4.9 (0.3)	97.2
0.5 mM Tartrate	3170 (43)	3520 (260)	3.2 (0.0)	110.9
0.5 mM EDTA	0.5 (0.1)	0.0 (0.3)	6.1 (0.1)	-[2]
0.5 mM Citric Acid	444 (10)	855 (25)	5.7 (0.2)	90.3
5 mM Citric Acid	28 (1.1)	27.9 (0.9)	9.5 (0.2)	-
			Mean (n=15)	**104.3**

[1] % of *E* at 48 hrs was determined using the mean of triplicate measurements.

[2] The Cd *E* values after 96 hours of equilibration were not determined in the presence of these ligands.

Similar results were generally obtained in experiments examining the calcareous soil. Although slight decreases in the K_d of Cd in the presence of the calcium salts were observed, this change was synchronous with a reduction in solution pH (data not shown). In addition, a lowering of K_d in the presence of 0.5 mM citric acid could be accounted for by decreases in the solution concentration of this ligand. Therefore, as changes of K_d were also compatible with variations in pH and organic ligand concentrations (indicating equilibration under these conditions), the shorter equilibration time was also used for experiments with the calcareous soil.

Due to the results obtained with Cd a smaller number of treatments were used to examine the effect of equilibration time on the K_d and *E* values of Zn in these soils (Table 3.4). It was observed, with the exception of the 0.5 mM citric acid and histidine treatments in the calcareous soil, that the solution concentrations of stable Zn did not vary between equilibration times. However, the solution concentrations of citric acid and histidine decreased, respectively, from 0.48 - 0.39 mM and 0.13 - < 0.01 mM and, therefore, would explain the decrease of solution Zn seen in these treatments.

In contrast to Cd, it was found that increasing the radioisotopic equilibration time to 72 hours resulted in higher K_d values of Zn for both soils. These increases of K_d inflated average *E* values of the acidic and calcareous soils by 117 and 134 %, respectively. This result may somewhat be expected as *E* values increase with the time of contact that the radioisotope tracer has with the soil (Figure 3.2). It has previously been observed that ^{65}Zn is slower than ^{109}Cd to attain equilibrium under similar experimental conditions (141).

Nonetheless, at the conclusion of these experiments a 24 hour pre-isotope and 24 hour post-isotope equilibration time was chosen, despite the results obtained for ^{65}Zn, for subsequent experiments. This decision was based on a number of factors that included: 1) the solution concentration of stable Cd and Zn had reached equilibrium; 2) the concentration of ^{109}Cd had also reached equilibrium; 3) extended shaking times lead to

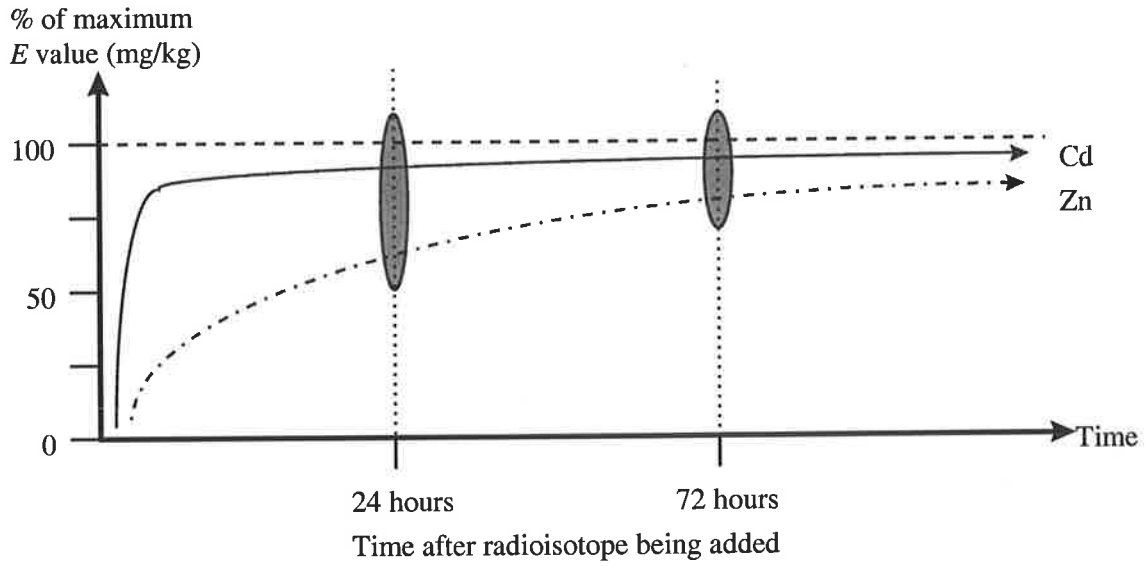
significant losses of organic ligands from solution and; 4) using one equilibration time halves the size of the experiment. As such, it should be noted that the reported Zn *E* values do not reflect maximum values. However, as the aim of these experiments was to compare relative differences between treatments, this fact does not affect the overall significance of these results.

Table 3.4: The effect of equilibration time on the K_d and *E* value of Zn in the soils. Values represent the mean and (standard deviation) of triplicate measurements. Values underlined indicate decreases in the solution concentration of stable Zn and K_d with a reduction in ligand concentration.

Zinc				
	48 hours	96 hours	48 hours	96 hours
Acidic Soil	K_d (L/kg)		*E* (mg/kg)	% of *E* at 48 hrs[1]
0.5 mM NaCitrate	115 (6)	151 (3)	1200 (19)	123.3
0.5 mM Tartrate	370 (18)	420 (13)	1297 (62)	108.8
0.5 mM Citric Acid	122 (3)	156 (6)	1350 (33)	115.4
0.5 mM Histidine	515 (42)	637 (65)	1274 (102)	119.1
			Mean (n=12)	**116.7**
Calcareous Soil				
0.5 mM NaCitrate	1402 (30)	1634 (220)	282 (17)	125.1
0.5 mM Tartrate	3185 (200)	3260 (340)	288 (19)	122.2
0.5 mM Citric Acid	496 (25)	872 (41)	238 (18)	132.7
0.5 mM Histidine	89.5 (4.3)	896 (19)	217 (5)	156.9
			Mean (n=12)	**134.2**

[1] % of *E* at 48 hrs was determined using the mean of triplicate measurements.

Figure 3.2: Schematic diagram depicting the rates at which ^{109}Cd and ^{65}Zn reach equilibrium with pre-equilibrated soil suspensions. (The figure is not drawn to scale)



3.5 Analytical methodology

3.5.1 Reagents

3.5.1.1 IC and ES-MS eluents

The eluents used for IC or IC-MS were made by adding the appropriate amount of 0.5 M Na_2CO_3, 0.5 M ammonium carbonate (both made in 18 MΩ water), 50 % (v/v) NaOH, 1 M tetrabutylammonium hydroxide, 29% (v/v) NH_4OH, 0.1 % formic acid, methanol and/or acetonitrile (all of American Chemical Society or High Pressure Liquid Chromatography grade) to helium degassed 18 MΩ water. It was not necessary to degas eluents when only (ES-MS) was used.

3.5.1.2 Standards

Quantitative metal standards were created by diluting acidified 1000 mg/l atomic absorption metal standards (EM Science, Gibbstown, New Jersey) in 18 MΩ water.

Standards were made in polyethylene bottles and refrigerated at ~ 4 °C between analyses. Before commencing analyses, standards were brought to room temperature.

To ensure that the solution concentration of organic ligand standards were not compromised by microbial degradation new standards were created just prior to each analysis session. Stock solutions of tartrate, citrate and histidine were made by adding an appropriate amount of solid (Aldrich-Chemie, Steinheim, Germany) to 18 MΩ water. These stock solutions were further diluted with 18 MΩ water to make quantitative standards.

Stock solutions of metal-EDTA complexes were prepared by equimolar additions of the 1000 mg/l atomic absorption metal standards and 0.25 M of the free acid of EDTA (Fisher Scientific, Fair Lawn, New Jersey), neutralized to pH 7 with 29% (v/v) NH_4OH, to 18 MΩ water (365). Chelation of the metals by EDTA was ensured by: 1) increasing the stock solution pH to 7 - 9 with 100 mM NH_4OH and; 2) equilibration for 24 hours, before refrigeration at 4 °C. Quantitative standards were produced by diluting with 18 MΩ water.

3.5.2 Quantification using standard curves

Linear regressions of metal, organic ligand or metal-EDTA complex concentration against the units of measurement (e.g. absorbance, conductivity, etc.) were used to develop standard curves. These curves were calculated using the results from at least 4 external standards and were employed to relate the units of measurement determined in sample solutions to solution concentrations of the metal, organic ligand or metal-EDTA complex. Standard curves always had R^2 ($P \leq 0.01$) values > 0.95 and were generally > 0.99.

To ensure analytical precision the analyses of samples were always bracketed with analyses of external standards. If the measurements between the external standards varied by less than 5 % the results for the bracketed samples were considered accurate. If

concentrations of metals or metal-EDTA complexes in the samples exceeded the linear range they were appropriately diluted and reanalyzed.

3.5.3 Metal analyses of soil solutions and plant materials

The Zn content of digested plant material was determined by FAAS (Thermo Jarrel Ash, Waltham, Massachusetts) and background corrected using a Smith-Hieftje background corrector. A standard reference material (citrus leaves: 1572, National Bureau of Standards, Washington, District of Columbia) was included with the digests and values for Zn concentrations matched the stated ranges, ensuring reliability of the analyses (certified Zn value = 29 ± 2, measured Zn value = 30 ± 3). Acidified soil solutions were analyzed by either FAAS, as stated above, or ICP-AES (JY 238, Jobin Yvon, Paris, France) according to standard procedures. During the analyses of soil solutions, samples were periodically reanalyzed after the addition of a metal standard to validate the accuracy of the measurements.

3.5.4 Metal speciation calculations with GEOCHEM-PC and MINTEQA2

Metal speciation calculations were made with either of the two speciation programs: GEOCHEM-PC, version 2 (359) or MINTEQA2 (366). The database of GEOCHEM-PC was updated using values obtained from the NIST 2001 Critically Selected Stability Constants of Metal Complexes Database (US Dept. of Commerce, Gaithersburg, Maryland).

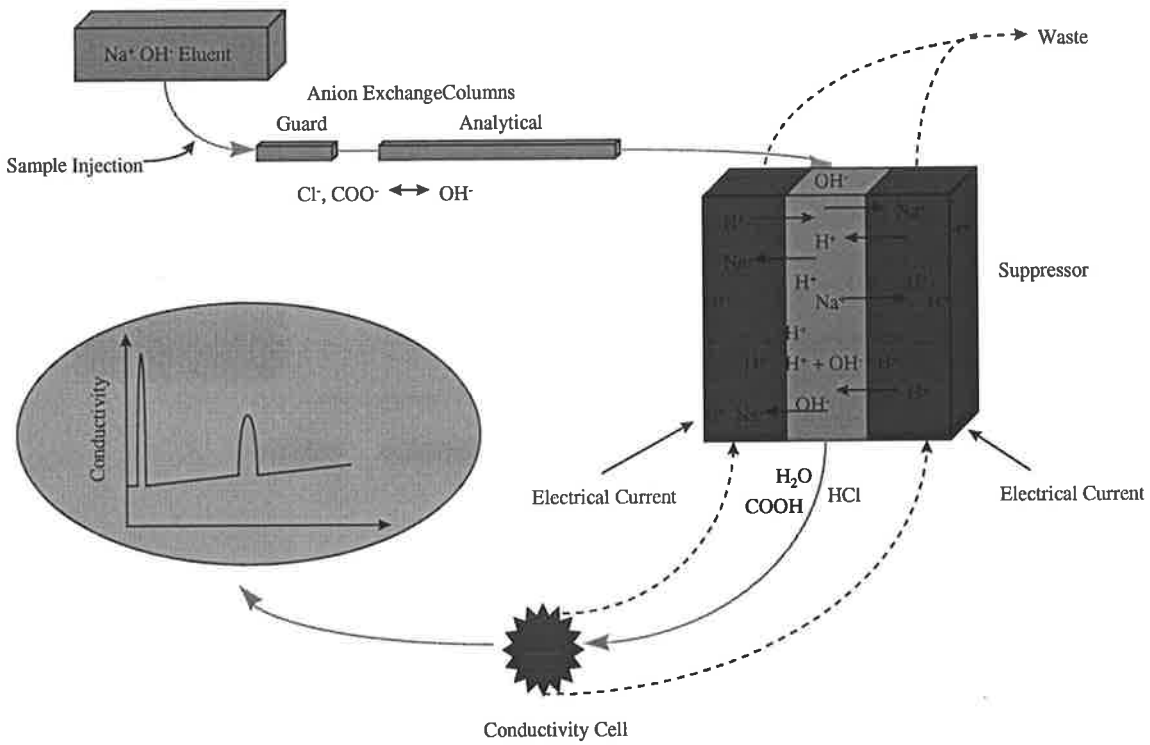
3.5.5 Anion ion chromatography

A large number of inorganic anions and organic acids can be resolved using IC with suppressed conductivity detection. Figure 3.3 represents an example of IC using a NaOH eluent. Organic acids (e.g. -COOH) and inorganic anions (e.g. Cl^-) become fully deprotonated and ionized, respectively, when injected into the alkaline NaOH eluent. Separation on the analytical columns is achieved through exchange with the anion-exchange layer (OH^-) that is (usually) functionalized with quaternary ammonium groups. When the analytes elute from the columns they are still in the NaOH eluent which is fully

ionized and highly conducting. Therefore, to reduce background conductivity the eluent and analytes are passed through a suppressor. The suppressor behaves like a cation exchanger and replaces the Na^+ counterions with H^+ ions. Thus, when the analytes leave the suppressor they are in a weakly ionized solution (H_2O) which has a low background conductivity. Analyte response is further enhanced as the hydronium counterion is about 7 times more conductive than the sodium counterion (367).

After passing through the conductivity cell the eluent can be recycled through the suppressor to be used as a source of water for the further creation of H^+ ions. The H^+ ions are generated by passing an electrical current through the suppressor. Alternatively, chemical suppression, utilizing dilute H_2SO_4 as a source of H^+ ions, may be used.

Figure 3.3: Schematic diagram of anion ion chromatography (IC) using eluent suppressed conductivity detection.



3.5.5.1 Organic ligand quantification

Concentrations of citrate and tartrate in the experiments described in Chapter 6 were quantified by suppressed conductivity detection after IC separation. The IC system (Dionex 500X, Dionex Corp., Sunnyvale, California) was equipped with a Dionex ASRS-Ultra suppressor and CD20 conductivity detector. In all cases, samples (50 μl) were injected onto the analytical columns at 30 °C. The organic acids were chromatographed using NaOH gradient elution (Table 3.5) at 2 ml/min on a Dionex AS11 analytical column fitted with a Dionex AG11 guard column. Histidine was resolved in 2.6 minutes using the same analytical columns with an 8 mM NaOH eluent run isocratically at 1ml/min. Amino acids have a low conductance and cannot be quantified using conductivity detection. Therefore, histidine was detected by direct-UV absorbance at 210 nm (Varian 9065 Polychrom, Varian Inc., Walnut Creek, California).

Table 3.5: NaOH gradient elution conditions used in the quantification of citric and tartaric acid by IC.

Citrate (retention time = 5.6 min.)			**Tartrate** (retention time = 2.2 min.)		
Time (minutes)	7.5 mM NaOH (%)	150 mM NaOH (%)	Time (minutes)	7.5 mM NaOH (%)	150 mM NaOH (%)
0	100	0	0	100	0
1	100	0	1	100	0
7	79	21	3	93	7
7.2	100	0	3.2	100	0
10.2	100	0	5.2	100	0

Samples taken from the soil suspensions (10 - 200 μl) were appropriately diluted in 18 MΩ water to ensure that concentrations were within the linear range established by the standards and analyzed immediately. Dilution resulted in the analyzed samples having maximum concentrations of 50 μM for citrate or tartrate and 250 μM for histidine. Linear ranges from 13 μM - 104 μM, 17 μM - 133 μM and 62.5 μM - 1000 μM were established for citrate, tartrate and histidine, respectively. Although, histidine was only

quantified down to 62.5 μM the limit of detection for this compound was approximately six times lower at 10 μM.

During the analysis of citrate neither an interference from co-eluting peaks nor with changing peak retention times was observed when analyzing the soil suspensions (Figures 3.4 - 3.6). Identical results were obtained for tartaric acid and histidine (data not shown).

3.5.5.2 Validating IC for organic ligand quantification using GEOCHEM-PC

As the organic ligands were separated by anion exchange the pH of the NaOH eluent was always higher than the pH of either the standards or the environmental samples. Although this increase in pH would be expected to dissociate many metal-ligand complexes it is possible that some metal-ligand complexes may remain intact. Thus, during anion exchange, metal-ligand complexes would have different mobilities to the uncomplexed organic ligand due to charge, mass or structural changes of the ligand and, hence, would not be quantified as part of the total organic ligand concentration. In effect, an underestimation of the total organic ligand remaining in solution would be the result.

Calculations with GEOCHEM-PC, version 2 (359) indicated that all metal-ligand complexes of tartrate and citrate (at 1:1 metal:ligand molar ratios up to 50 μM) completely dissociate (100 %) under IC conditions for commencing gradient elution (7.5 mM NaOH, pH = 11.9). A concentration of 50 μM was the maximum concentration of organic ligand in the diluted samples, taken from the soil suspensions, and used for analysis. Therefore, it was concluded that IC analyses quantitatively reflected total solution concentrations of tartrate and citrate.

Figure 3.4: Anion chromatogram of a 52 μM citric acid standard made in 18 MΩ water.

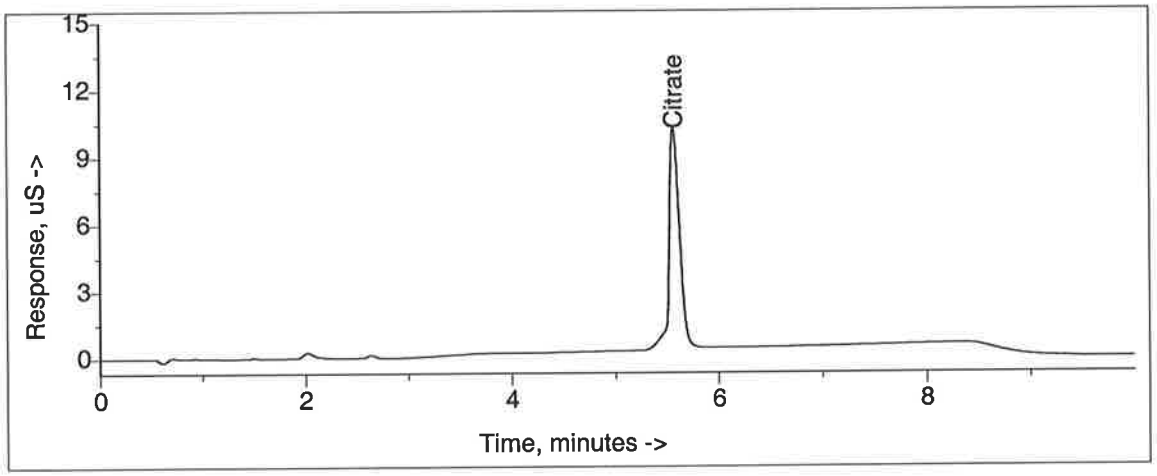


Figure 3.5: Anion chromatogram of a diluted (factor of 5) calcareous soil suspension sample that initially contained 250 μM of citric acid. The sample was taken during ligand sorption experiments.

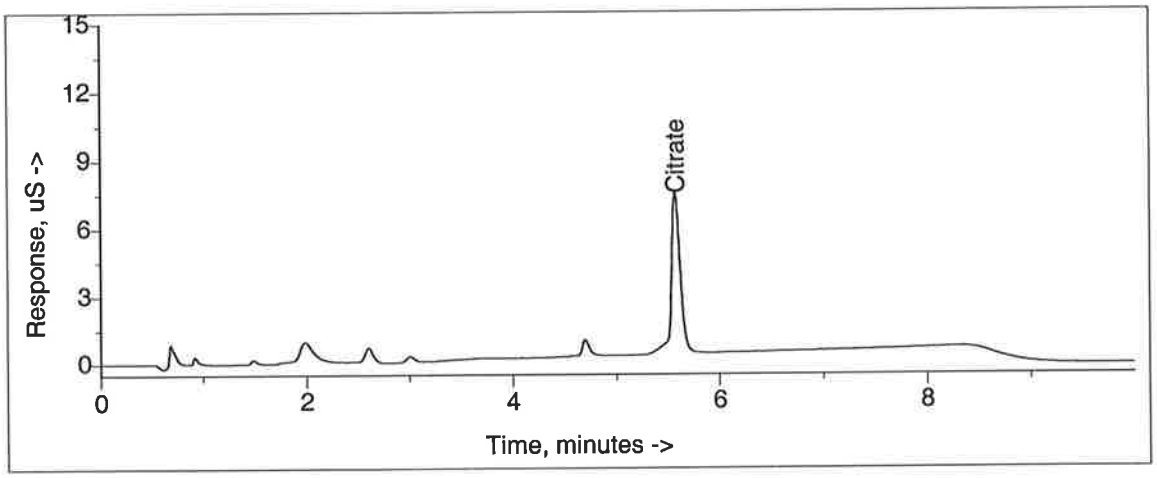
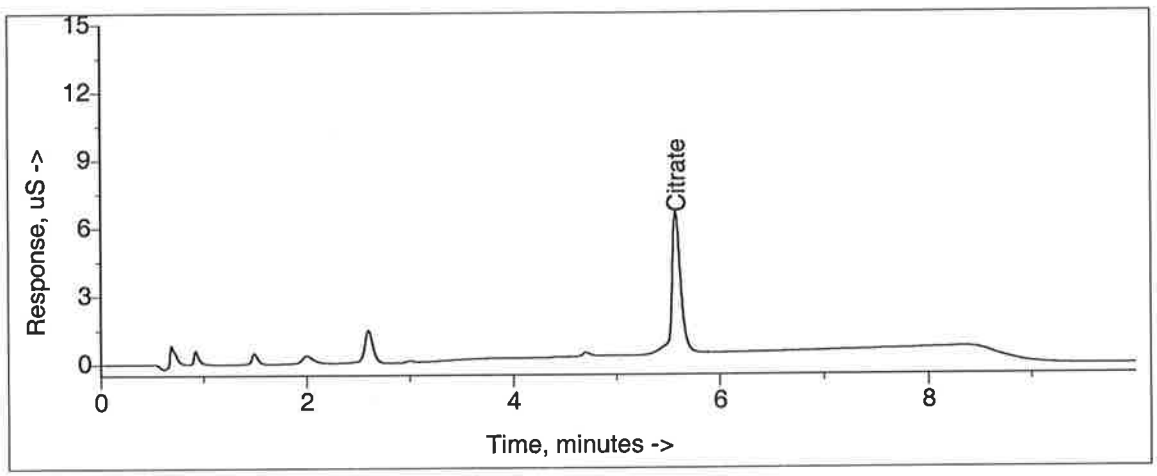


Figure 3.6: Anion chromatogram of a diluted (factor of 5) acidic soil suspension sample that initially contained 250 μM of citric acid. The sample was taken during ligand sorption experiments.



Identical calculations with histidine indicated similar dissociation phenomenon as that predicted for the other two organic ligands with the exception that Cu- and Ni-histidine complexes would not completely dissociate (92 - 100 % and 91 - 100 %, for Cu- and Ni-histidine, respectively, for concentrations ranging from 10 μM - 132 μM and 10 μM - 44 μM. In effect, dissociation increases as the concentration of the metal-histidine complex decreases). The values of 132 μM and 44 μM represent the maximum concentrations of Cu- and Ni-histidine that could be in solution *if* all of the metal in the acidic soil was available to be chelated. These values were examined because the acidic soil contained higher concentrations of Cu and Ni than the calcareous soil (Table 3.1).

Hypothetically, therefore, the lowest recovery of histidine (as measured by IC) would be obtained in the bottles containing the acidic soil and 250 μM solutions of histidine. For example, only 92 % of (132 μM) Cu-histidine and 91 % of (44 μM) Ni-histidine would dissociate in the NaOH eluent. Therefore, the recovery of histidine in this situation would be {(132 μM x 92 %) + (44 μM x 91 %) + (74 μM uncomplexed histidine or other

metal-histidine complexes x 100 %)} / 250 μM = 94 % of the solution concentration of histidine.

However, it is rather unlikely that the total concentration of the Cu and Ni in the acidic soil would be available to be chelated and simply by halving this amount of 'chelatable' Cu and Ni to more realistic levels increases the percentage of histidine analyzed by IC to 100 %. Therefore, although the solution concentrations of Cu and Ni were not quantified, it was concluded that IC analyses also quantitatively reflected solution concentrations of histidine.

3.5.5.3 Analysis of purified DMA samples

Conductivity detection after anion chromatography and eluent suppression was also used to determine that purified DMA samples were free from the presence of other organic acids and was sufficiently desalted by dialysis. The IC system used for these experiments has already been described. Inorganic anions and organic acids were separated on a Dionex AS11 analytical column equipped with an AG11 guard column over 13.5 minutes using a 7.5 - 35 mM NaOH gradient at 1 ml/min (modified from Neumann *et al.* (363)). No other organic acids were detected in the DMA samples that had been purified from the root exudates of *Z. mays* (Figure 3.7). Although mugineic acids elute under these conditions (363) they were not detected by conductivity because of their low electrical conductance. Therefore, further analyses utilizing ES-MS were conducted to confirm the identity of DMA.

In addition, the quantity of inorganic anions (early eluting peaks) in the purified DMA samples were well below the amounts determined in a quantitative DMA standard that had been kindly provided by Professor Satoshi Mori, Laboratory of Plant Molecular Physiology, The University of Tokyo, Tokyo, Japan (Figure 3.8). Therefore, lyophilized extracts of the purified DMA samples were considered to quantitatively represent DMA.

Figure 3.7: Conductivity chromatogram of a purified DMA sample collected as root exudates from *Z. mays* plants grown hydroponically under Fe-deficiency conditions. The only significant peak represents Cl^- (0.6 mg/l).

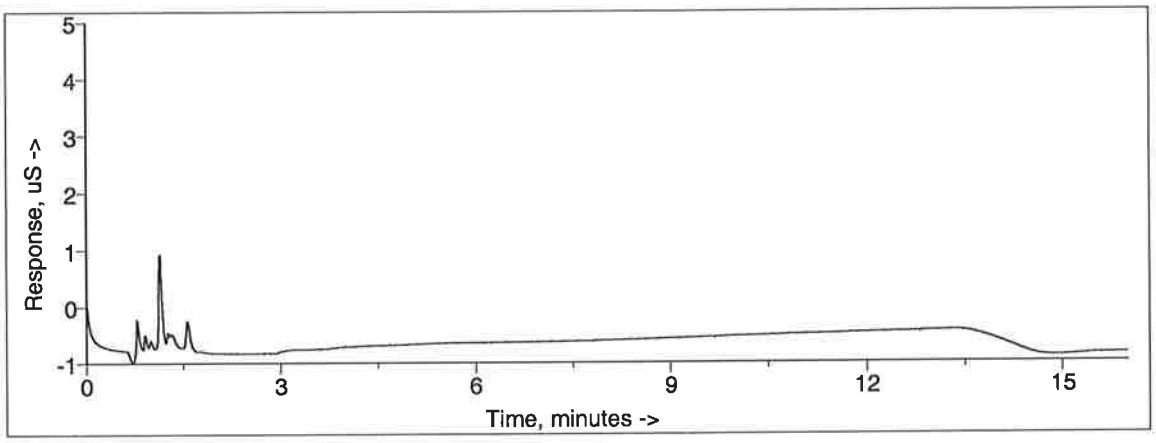
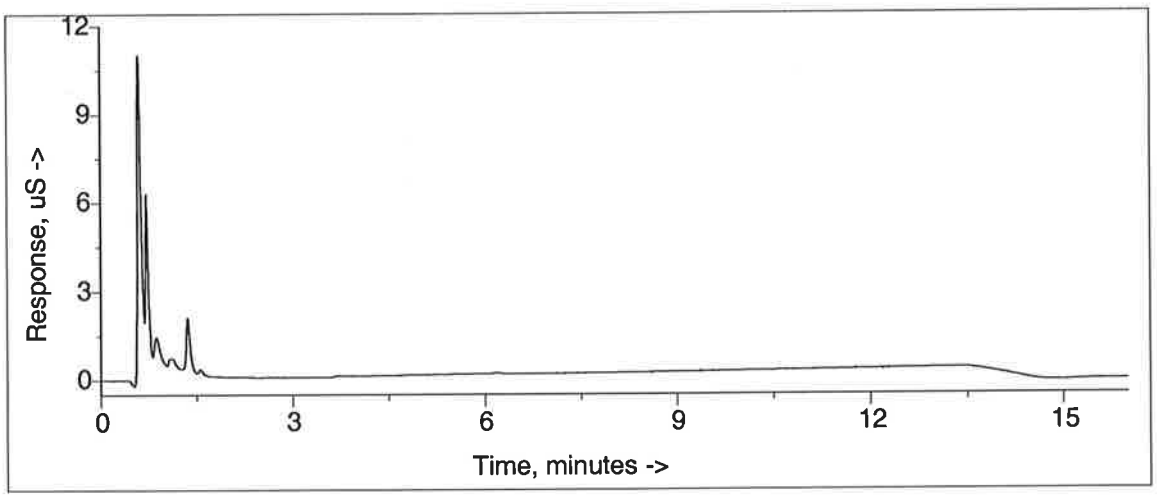


Figure 3.8: Conductivity chromatogram of the DMA standard provided by Professor Mori. The anions producing the two major peaks were neither identified nor quantified.



3.5.5.4 Separation of metal-EDTA complexes

EDTA forms negatively charged complexes with mono-, di- and trivalent metal cations. Therefore, separation of these complexes on IC columns is via anion exchange. In the experiments described in Chapter 4, IC was examined for separating the metal chelates of EDTA before detection by electrospray mass spectrometry. The same Dionex 500X ion chromatograph system, as described previously, was also used in these experiments when post-column eluent suppression or conductivity detection were utilized. Various Dionex anion exchange columns were tested in the process of method development and included the 4-mm AS5/AG5, 4-mm AS11/AG11 and the 4-mm AS9/AG9 columns. Reversed-phase ion-pair chromatography using a Hewlett Packard C_{18} column was also studied using the Dionex 500X system. When neither post column suppression nor conductivity detection were needed the analytical columns were simply placed in-line of the ES-MS system described below.

In all cases the sample (50 μl) was injected onto the analytical columns at room temperature. Flow rates varied from 2 ml/min with the Dionex columns down to 0.3 ml/min for the C_{18} column. For IC-MS analyses the flow rate to the electrospray interface was adjusted to 200 - 280 μl/min, after the analytical columns, by means of a zero dead volume T-piece.

3.5.6 Electrospray mass spectrometry

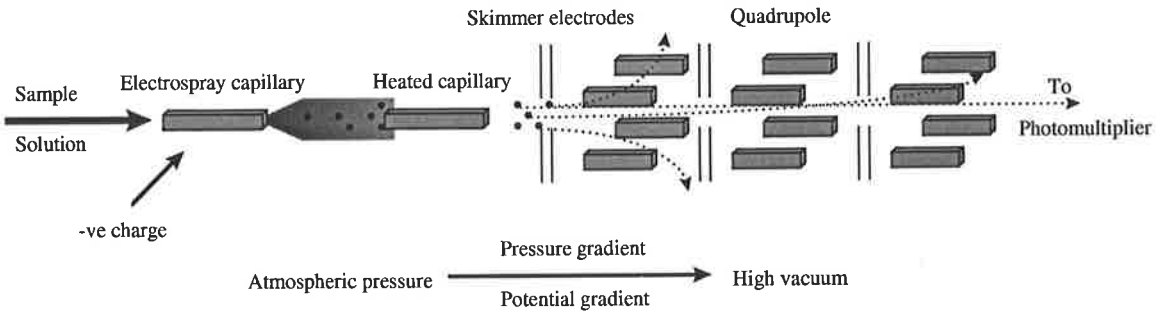
The following is a brief outline of the basic principles underlying ES-MS. This section is provided as background information for following the methodology reported in Chapter 4.

A mass spectrometer consists of three components: 1) an ionization source; 2) a mass analyzer and; 3) a detector. However, technically speaking, ES-MS does not have an ionization source. Rather, the electrospray process transfers analyte species, already ionized in the liquid phase, into the gas phase as isolated entities (368). Therefore, the important underlying mechanism of this process is that the charge states of the gaseous

ions reflects the charge states of the aqueous ions. Nonetheless, this mechanism is somewhat modified following ion-molecule collisions in the electrospray interface and, therefore, a multiple of charged gaseous ions are commonly observed. The number of charges retained by an analyte can then depend on factors such as the composition and pH of the electrosprayed solvent as well as the chemical nature of the sample (346).

Introduction of aqueous samples to the electrospray interface is usually through a capillary held at high potential - typically 3.5 kV (Figure 3.9). The sign (+ or -) of the potential applied to the capillary tip is determined by which charged species are being examined. For example, a negative potential is applied when examining negatively charged analytes. Regardless of the sign of the potential, the effect of the high electric field on the solution as it emerges from the capillary is to generate a fine mist of highly charged droplets. These droplets reduce in size by either solvent evaporation or droplet subdivision (resulting from high charge densities). Controlled heating of the interface is usually applied to promote these processes and, ultimately, the fully desolvated charged gaseous ions are produced. Sampling of the fully or partially desolvated ions is made using a capillary or a skimmer device and the ions pass down a potential and pressure gradient towards the analyzer portion of the mass spectrometer.

Figure 3.9: Schematic diagram of the underlying mechanisms of electrospray mass spectrometry.



The mass analyzer portion of the mass spectrometer used in the experiments outlined by this thesis consisted of three quadrupole mass filters (termed triple quadrupole). A quadrupole mass filter consists of four parallel metal rods arranged to allow ions to pass lengthwise between the rods. Two opposing rods have both DC and AC voltages applied, while the other two rods have the same potential applied, but of opposite polarity. The oscillation of these voltages affects the trajectory of charged ions traveling down the flight path centered between the four rods. For given DC and AC voltages, only ions of a certain mass-to-charge ratio (m/z) pass through the quadrupole filter. All other ions are ejected from their original path and eliminated. Variations in the potential applied to the quadrupole rods over time, and the consequential selection of ions of specific m/z ratios is referred to as scanning. Therefore, a mass spectrum is obtained by monitoring the ions passing through the quadrupole filters as the voltages on the rods are varied. In a triple quadrupole mass spectrometer the function of the first and third quadrupoles is to filter ions of a specific m/z ratio by scanning the potentials applied to the quadrupole rods. The second serves as a collision cell and is constructed to transmit ions without selection. When an inert collision gas is introduced into the collision cell the third quadrupole is then used to record the m/z ratios of the fragment ions that originate from the fragmentation of the precursor ion selected by the first quadrupole.

Once the ion passes through the quadrupoles it is then detected by the ion detector, the final element of the mass spectrometer. The detector allows a MS to create a signal (current) from ions by generating secondary electrons, which are further amplified in a photomultiplier. A photomultiplier is a sealed evacuated glass tube which contains a series of electrodes. Initially, ions strike a dynode surface (electrode) that induces the ejection and temporal displacement of several secondary electrons. The electrons are then directed towards a phosphorus screen which releases photons, as in the photoelectric effect. These photons strike the inner surface of the front of the photomultiplier which absorbs the photons and re-emits the energy in the form of electrons. At this stage the electrons are then accelerated by a DC voltage to the first electrode within the photomultiplier. Each electron striking the electrode emits two or three secondary

electrons which, in turn, are accelerated to the next electrode where the process is repeated. The last electrode produces the final output pulse which is now a cascade of electrons. A typical photomultiplier has an amplification of between 10^4 and 10^6.

3.5.6.1 Detecting metal complexes of EDTA

The ES-MS system used to detect the metal complexes of EDTA (Chapter 4) consisted of a Hewlett-Packard 1050 quaternary LC with autosampler connected to a Finnigan MAT TSQ 700 triple-stage quadrupole MS using a Finnigan electrospray interface. Preliminary investigations revealed that the negative ion mode resulted in significantly better detection limits and was, therefore, used throughout the rest of the experimental period. During analyses the voltage at the capillary entrance was fixed at 4 kV and the temperature of the heated capillary at 250 °C. Other parameters of the electrospray interface were optimized, as needed, for the detection of the metal-EDTA complexes. Nitrogen was used as the drying and nebulizing gas and for MS-MS investigations argon was used as the collision gas. In all cases the photomultiplier voltage was set to 1.3 kV and data acquisition was performed after the third quadrupole in the full scan mode generally over the m/z range of 250 - 550.

Standard curves were developed for the metal-EDTA complexes from the limit of detection to 250 μM. In addition, NiEDTA, due to its extremely slow exchange kinetics for other metals (369), was used as an internal standard when environmental matrices were analyzed. Measurements of the soil solution and plant xylem spiked with 1 - 250 μM NiEDTA, just prior to analysis, resulted in average NiEDTA recoveries of 95.6 ± 4.4 % and 94.5 ± 2.7 % respectively. The small volume of NiEDTA added had insignificant effects on the pH and ionic strength of the environmental samples.

3.5.6.2 Identification of DMA

Electrospray-mass spectrometry was also used to identify the mugineic acid collected from the root exudates of *Z. mays*. Samples were flow injected (10 μl) into a 50 % (v/v) methanol eluent containing 0.1 % formic acid. Analyses were conducted over the m/z

range of 180 - 450 in the positive ion mode. Deoxymugneic acid was the only mugineic acid phytosiderophore to be detected (m/z of DMA^+ = 305, Figure 3.10).

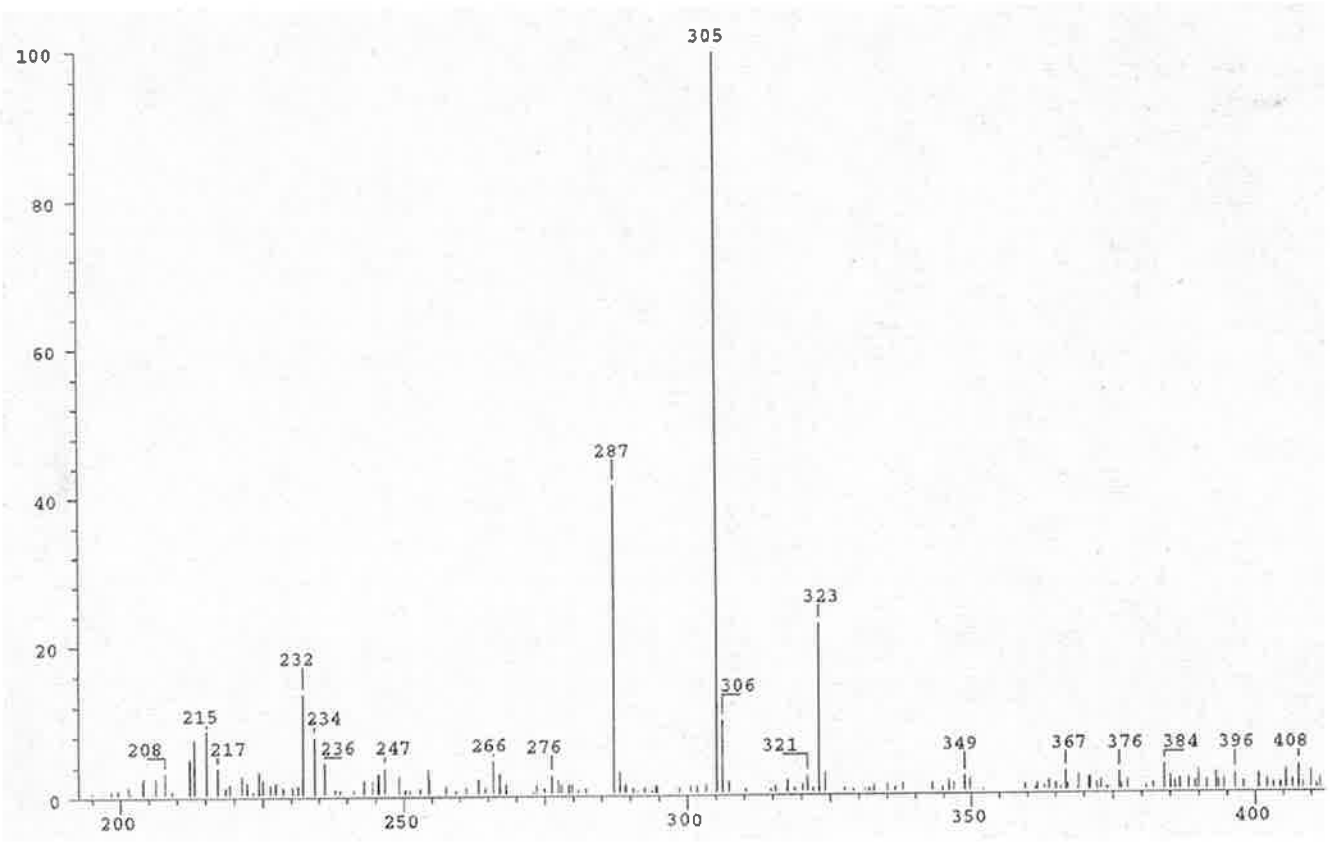
3.5.7 Analyses of radioisotopes

The amount of radioactive metal added to soil solutions and that remaining in solution after equilibration was quantified by γ-spectrometry (Cobra 5003, Packard, Meriden, Connecticut). The ^{109}Cd isotope was counted in the window range of 15 - 110 keV while ^{65}Zn was counted in the 900 - 1200 keV window range. Counting efficiency was estimated to be approximately 85 % for ^{109}Cd and 29 % for ^{65}Zn using a three inch NaI crystal. When the isotopes were examined simultaneously, corrections were made for overlapping energies with a spilldown % of 18.28 and a spillup % of 1.4. No background γ-radioactivity, within these window ranges, were detected in non-labeled soil suspensions.

3.5.8 Statistical analyses

Statistical differences were calculated using ANOVA with the STATITCF, version 5 computer program (ITCF, Biogneville, France). Linear and nonlinear regressions were determined using SIGMAPLOT, version 5.0 (SPSS Science, Chicago, Illinois).

Figure 3.10: Positive-ion mass spectrum of a purified DMA sample collected from the root exudates of *Z. mays* plants grown under Fe-deficient conditions. The y-axis = % relative to base peak (m/z 305). The m/z ion of 305 = DMA + H^+, m/z 323 = DMA + H_2O and m/z 287 = DMA - H_2O. The single positively charged molecules of mugineic acid (m/z = 321) and epi-hydroxymugineic acid (m/z = 337) were not detected.



4. Determination of metal-EDTA complexes in soil solution and plant xylem by ion chromatography-electrospray mass spectrometry

4.1 Introduction

From a review of the literature it was identified that further progress in chelate-assisted Cd and Zn phytoextraction could be made with a better understanding of the mechanisms involved. It was also considered that the development of a technique to unambiguously determine intact metal-chelate complexes in environmental matrices was essential to accomplish this task.

Most progress in this area of analytical chemistry has been made with the metal complexes of EDTA. Presently, there have been no reports on direct detection methods for the metal complexes of naturally occurring organic ligands. As a better foundation of analytical techniques existed for metal-EDTA complexes (340, 341, 344, 345, 348, 370) and as EDTA has been the major ligand used for chelate-assisted phytoextraction (30-32, 88, 332) the development of an analytical technique for these complexes was considered within the scope of this thesis.

Of the methods reviewed IC-MS appeared to have the most potential to eliminate analytical interferences caused by environmental matrices while still producing high separation efficiencies and reduced limits of detection. Thus, experiments were conducted to develop an IC-MS method that could be used to identify and quantitate intact metal-EDTA complexes in soil solution and plant xylem samples.

4.2 Materials and methods

The protocols used to obtain soil solution and *H. vulgare* xylem samples have been outlined in section 3.3, pp. 47 - 51 and the analytical instrumentation in section 3.5, pp. 62 -75.

4.3 Results and discussion

4.3.1 Optimizing IC for the separation and detection of metal-EDTA complexes

Anion chromatographic separation of metal-EDTA complexes has been reported on 4 anion exchange columns made by Dionex: the AS4A (342), AS5 (343), AS9 (370) and AS11 (343). To limit unnecessary expense, the AS4A column was not considered in this study as two columns, in tandem, are needed to separate the metal complexes of EDTA (342). Attempts to separate the complexes with either the AS9 or AS11 column using the techniques described elsewhere (343, 370) were not successful. However, a number of metal-EDTA chelates were easily detected by suppressed conductivity detection, with the AS5 column using 2 mM Na_2CO_3 as an eluent (data not shown). The only metal-EDTA complexes that could not be detected by conductivity were Ca, Mg and Fe(III)EDTA (discussed below).

In an attempt to detect Fe(III)EDTA simultaneously with the other metal-EDTA complexes, reversed-phase ion-pair chromatography with post-column suppression on a C_{18} column was used. However, using 1 - 5 mM tetrabutylammonium hydroxide and an acetonitrile concentration ranging from 5 - 40 % (v/v) it was not possible to separate any of the metal-EDTA complexes within 20 minutes (data not shown). Therefore, the AS5 analytical column with 2 mM Na_2CO_3 as eluent was chosen for further experiments with ES-MS detection.

When IC-MS (with post-column eluent suppression) was attempted with the AS5 column the metal-EDTA complexes could neither be detected in the positive nor negative ion detection mode (data not shown). As has been found with capillary electrophoresis-MS it was believed that the non-volatile Na_2CO_3 eluent was suppressing analyte volatilization and hence detection by ES-MS (365). Therefore, to increase the volatility of the eluent the combination of the more volatile ammonium compounds $(NH_4)_2CO_3$ and NH_4OH were used. The final eluent of 2.25 mM $(NH_4)_2CO_3$, 9.7 mM NH_4OH (pH 9.9) achieved high separation efficiency but minimized ionic strength. The latter may suppress analyte ionization in the electrospray interface (Kebarle and Tang, 1993, cited in (344)). The

addition of organic solvents to an eluent enhances MS detection by: 1) assisting the liquid to gas phase process (346) and; 2) providing a stable spray in the electrospray interface. Therefore, 4 % (v/v) methanol was added to the eluent (a maximum of 5 % (v/v) organic solvents can be used with the AS5 column). Acetonitrile was first examined as the organic solvent but resulted in peak broadening of the CdEDTA peak (Figure 4.1), whereas methanol had no effect (Figure 4.2).

Unlike the Na_2CO_3 eluent, the $(NH_4)_2CO_3/NH_4OH$/methanol eluent permitted metal-EDTA complexes to be detected by either negative or positive ion ES-MS. Analyses using negative ion ES-MS detection resulted in greater detection limits and was, therefore, used throughout the entirety of the experimental period. It was also observed that post-column eluent suppression increased ES-MS detection limits by a factor of 4 over non-suppressed conditions. However, the latter system was chosen for further development as the metal-EDTA complexes could be detected as the intact molecular species.

4.3.2 Validating IC-MS for analyzing metal-EDTA complexes using MINTEQA2

As separation of the metal-EDTA complexes was achieved by anion exchange chromatography using a carbonate/hydroxide eluent the pH during analyses was always higher than the pH of the standards and the environmental samples. Due to this change of pH it was possible that: 1) there was no change in the distribution or quantity of metal-EDTA complexes; 2) free metal ions in environmental samples displaced metal ions complexed with EDTA; 3) chelated metals precipitated due to the low solubility products of their respective carbonates/(hydro-)oxides or; 4) free EDTA (resulting from the precipitation of complexed metals) could chelate free metal ions in the environmental samples.

Figure 4.1: Conductivity chromatogram of a 25 μM CdEDTA standard using 4 % (v/v) acetonitrile as organic modifier.

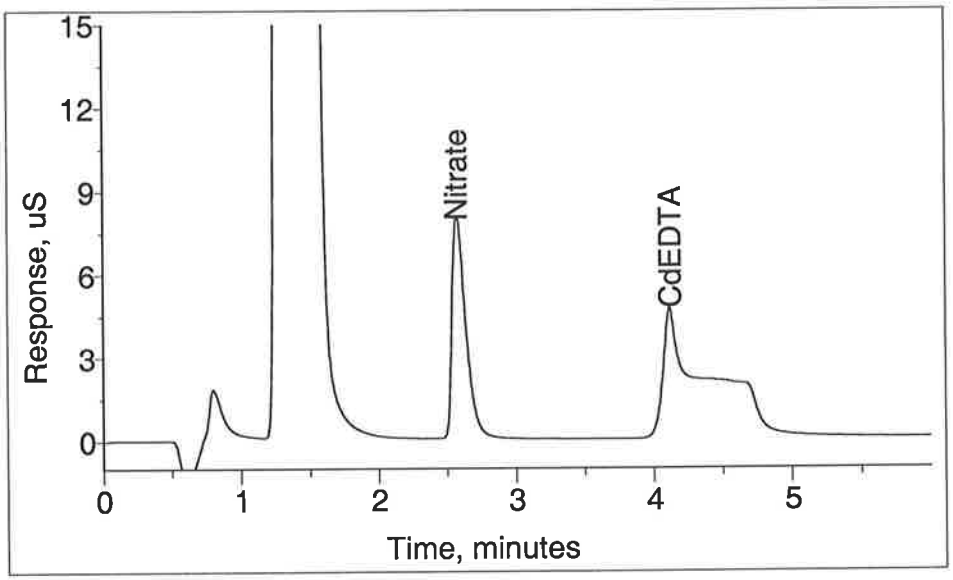
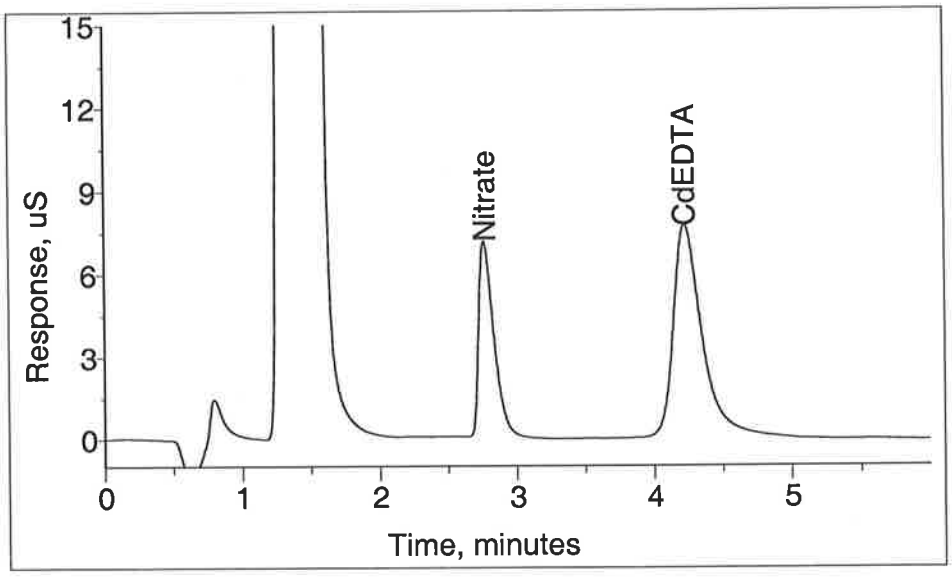


Figure 4.2: Conductivity chromatogram of a 25 μM CdEDTA standard using 4 % (v/v) methanol as organic modifier.



Speciation calculations performed using MINTEQA2 (366) under conditions of IC-MS analysis (2.5 mM $(NH_4)_2CO_3$, 9.7 mM NH_4OH, 4 % (v/v) methanol, pH 9.9 in a closed system with respect to O_2 and CO_2) indicated that the metal-EDTA complexes of Ca, Cd, Co, Cu, Mg, Mn, Ni, Pb and Zn remain thermodynamically stable. On the contrary, it was predicted that 94 % of the Fe(III) complexed to EDTA would precipitate as hematite (α-Fe_2O_3). In agreement with these calculations Fe(III)EDTA was not detected ≤ 250 μM. Similarly, MINTEQA2 calculations estimated that 90 % of the Al complexed to EDTA would precipitate as diaspore (α-AlOOH). However, unlike Fe(III)EDTA, the EDTA complex with Al was detected by IC-MS and, in fact, had the lowest detection limit of all the metal-EDTA complexes identified in the environmental samples (Table 4.1).

Table 4.1: Thermodynamic stability constants (*K*), limits of detection (LOD) using IC-MS and concentrations of the metal-EDTA complexes detected in soil solution and *H. vulgare* xylem exudate.

Metal-EDTA complex	log K^1	LOD^2 (μM)	Soil Solution (μM)	*H. vulgare* xylem (μM)
MnEDTA	15.6	0.5	3800 ± 260	730 ± 57
ZnEDTA	18.2	1.0	830 ± 71	95 ± 4.5
AlEDTA	19.1	0.1	230 ± 57	10 ± 9.0
CdEDTA	18.2	0.5	30 ± 2.7	9.0 ± 1.4
CuEDTA	20.5	1.0	< 1.0	11 ± 16

[1] Thermodynamic stability constants derived from GEOCHEM-PC, version 2 (359).

[2] LOD established as the concentration whose peak area was > 3 times the average background area count.

Similar discrepancies between GEOCHEM-PC, version 2 (359) speciation calculations and CZE analyses of the metal-NTA complexes of Pb and Zn have also been observed by Owens *et al.* (341). These authors suggested that the short analysis times of CZE were insufficient to allow precipitation of Pb and Zn or that any precipitates formed were

unstable under the conditions employed in their analyses. Considering the results of Owens *et al.* (341) it is, therefore, possible that α-AlOOH was thermodynamically unstable or its formation kinetically limited during IC-MS analyses. Other possibilities include: 1) the process of anion exchange with the analytical column precludes the precipitation of Al or; 2) the thermodynamic stability constant of AlEDTA and/or the solubility product of α-AlOOH in the MINTEQA2 database is incorrect for conditions experienced during IC-MS analyses.

Furthermore, CaEDTA and MgEDTA, despite speciation calculations suggesting they remained stable in the eluent, were neither detected ≤ 250 μM by suppressed-conductivity or electrospray-mass spectrometry. High detection limits of CaEDTA (> 500 μM) have been previously observed with CZE and IC (341, 342). However, it is possible that these two complexes become unstable when introduced to the electrospray interface and, therefore, are not detected as the intact metal-EDTA complex by MS. Nonetheless, during the analyses of standards containing these two complexes a peak of m/z 291 ($EDTA^-$) could not be consistently attributed to either CaEDTA or MgEDTA.

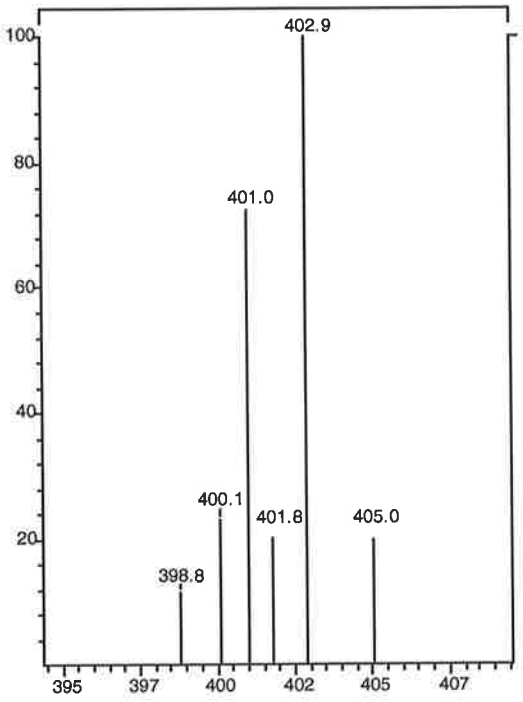
Further speciation calculations with MINTEQA2 also predicted that any free metal ions in environmental samples or standards, that also contain metal-EDTA complexes, immediately precipitate upon contact with the eluent. Therefore, free metal ions (in particular Ca^{2+}) should not affect the overall distribution of metal-EDTA complexes present in environmental samples. This mechanism was actually confirmed using equimolar concentrations of Mn^{2+} and EDTA in solutions of varying pH. For example, at pH 5.5 only 88 % of Mn was predicted to be complexed with EDTA, whereas under conditions employed for IC-MS complexation was predicted to increase to 100 %. When the MnEDTA solution of pH 5.5 (88 % complexation) was compared to one that had been adjusted to pH 7 (100 % complexation) a reduction in the amount of MnEDTA measured, approximately equal to that estimated by MINTEQA2, was observed. Therefore, neither free metal ions nor excess EDTA affected the quantification of metal-EDTA complexes when analyzed by IC-MS.

4.3.3 Determination of metal-EDTA complexes by IC-MS

All of the metal-EDTA complexes could be detected as the singly charged molecule (loss of H^+) and the doubly charged molecule (loss of 2 H^+). For example the Zn^{64} isotope EDTA complex was detected at m/z 353 (Zn^{64}EDTA - H^+), and the doubly charged ion at m/z 176 (Zn^{64}EDTA - $2H^+$). Furthermore, fragment ions could also be detected with the loss of CO_2, for example m/z 309 (Zn^{64}EDTA - CO_2), or two COOH groups m/z 263 (Zn^{64}EDTA - 2COOH). Therefore, for preliminary analyses the molecular ion, the diagnostic fragmentation pattern and the isotopic signature of the metals were all used to positively identify the metal-EDTA complexes (Figure 4.3). However, for quantitation purposes it was desirable to reduce fragmentation. Therefore, conditions in the electrospray interface were optimized to enhance the detection of the singly charged metal-EDTA molecular ion. Optimization resulted in electrospray conditions that gave the desired molecular ion as the base peak of the spectrum.

Once the IC-MS method had been optimized for quantitation the metal-EDTA complexes (- H^+), as seen in Figures 4.4, 4.5 and 4.6, of the following metals were detected as: Al^{3+}(m/z 315), Cd^{2+}(m/z 399>403), Cu^{2+}(m/z 352+354), Zn^{2+}(m/z 353+355), Ni^{2+}(m/z 347+349), Pb^{2+}(m/z 495>497), Co^{2+}(m/z 348) and Mn^{3+}(m/z 343). MnEDTA was detected as the Mn(III) and not the Mn(II) complex. However, this was assumed to be an artifact of the method because off-line flow injection analysis (FIA) with 50 % (v/v) methanol detected MnEDTA as Mn(II)EDTA with a m/z of 344. Complete separation of the metal-EDTA complexes was achieved with the exception of ZnEDTA and Co(II)EDTA. Although these two species co-eluted, they could be still be identified by their specific molecular ions (Figure 4.4) and injection of a series of solutions containing different ratios of the two chelates indicated that neither had an effect on the quantitation of the other up to 250 μM (data not shown).

Figure 4.3: Negative ion MS spectrum of CdEDTA demonstrating the isotopic signature of Cd. X-axis = m/z (mass-to-charge ratio) and Y-axis = % relative to base peak (m/z 403).



For quantitation purposes the limit of detection was established as a response > 3 times the average background area count. The metals that have one major isotope usually resulted in better detection limits of their corresponding EDTA complex. However, for the other metals, such as Cd, detection limits were improved by adding their major isotopes together for quantification (for example, CdEDTA at 399>403) and are noted in the Figures. The limit of detection for the tested metal-EDTA complexes ranged from 0.1 - 1 μM (Table 4.1). A practical working linear range from the limit of detection up to 250 μM was established ($R^2 > 0.99$, $P \leq 0.01$) for simple solutions that contained all the metal-EDTA standards. The detection limits established for this method are similar to those for MS (345) but significantly higher than eluent suppressed IC-MS (348).

Figure 4.4: Negative ion MS chromatogram of seven 50 μM metal-EDTA standards.

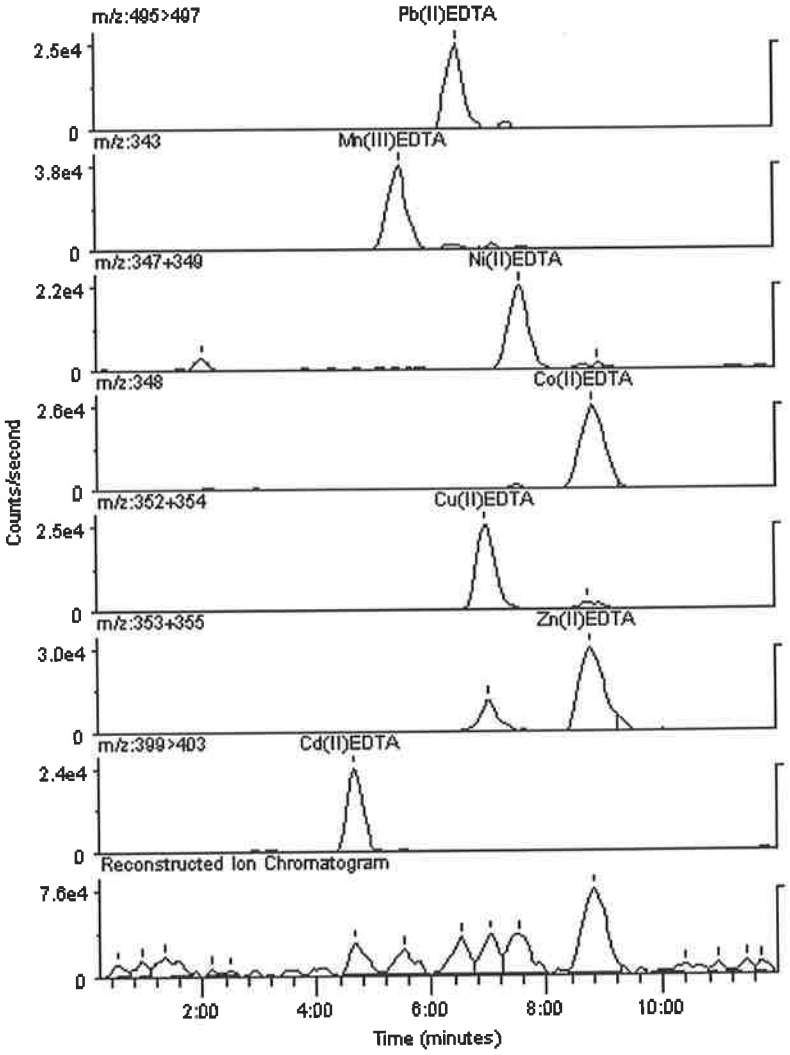


Figure 4.5: Negative ion MS chromatogram of an undiluted *H. vulgare* xylem sample.

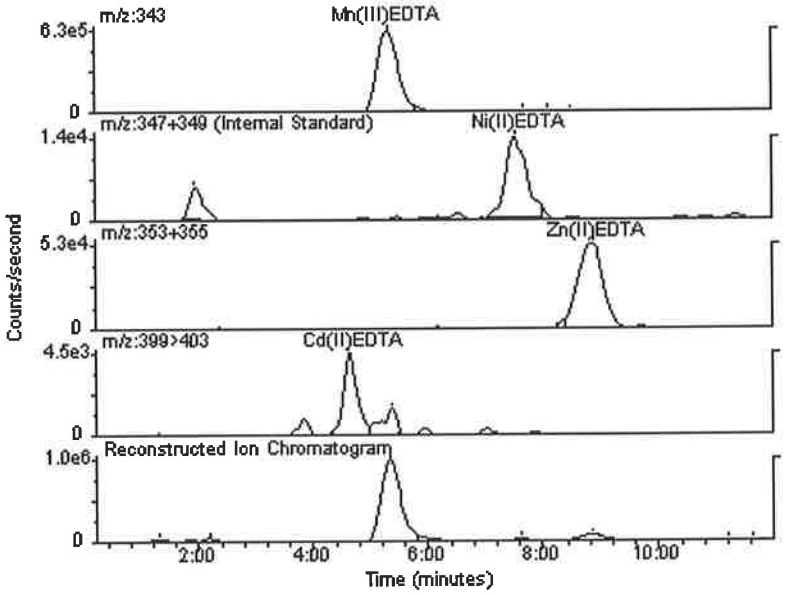
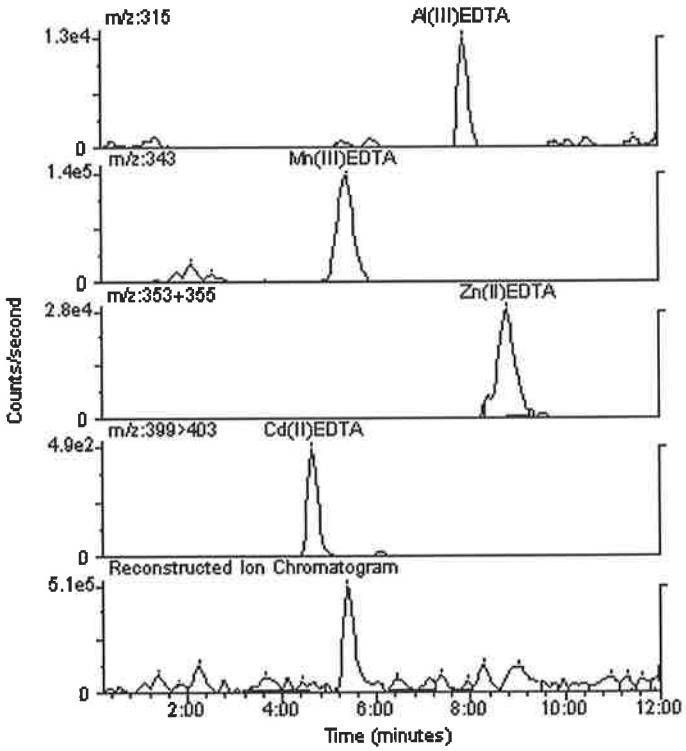


Figure 4.6: Negative ion MS chromatogram of a diluted (20x) soil solution sample. (NiEDTA was added at 5 μM before dilution and is not observed in this chromatogram)



4.3.4 Metal-EDTA complexes in environmental samples

Soil solutions and plant xylem were analyzed to test the suitability of IC-MS for quantifying the metal-EDTA molecules in complex environmental matrices. Representative chromatograms taken from the analysis of soil solution and the xylem of *H. vulgare* are presented in Figures 4.5 and 4.6 and demonstrate that there was neither an interference from co-eluting peaks nor with changing peak retention times on the analysis of the metal-EDTA complexes present.

MnEDTA accounted for the majority of metal-EDTA complexes detected in soil solution followed by Zn, Al and CdEDTA (Table 4.1). This order of metal-EDTA complex formation bears little resemblance to the thermodynamic stability constants of the metals for EDTA and suggests that the mineralogical composition of the soil, in conjunction with pH, may have an overriding effect of metal-EDTA complexation in soil solution. All of the metal-EDTA complexes detected in soil solution were also observed in the xylem of *H. vulgare*. However, the Cu complex of EDTA was only detected in the xylem of *H. vulgare*, suggesting that this complex either formed in the xylem or the methodology to collect the soil solutions may have resulted in a small redistribution of the metal complexes of EDTA. Although the latter could be overcome in the future by sampling with non-porous rhizosphere samplers (Rhizosphere Research Products, Netherlands) the former is, technically, more challenging to resolve.

4.4 Conclusions

An analytical method, using a combination of anion ion chromatography to separate and electrospray-mass spectrometry to detect (IC-MS), was successfully developed to simultaneously quantify the metal-EDTA complexes of Al, Cd, Cu, Co, Mn, Ni, Pb and Zn. Detection limits for the various complexes ranged from 0.1-1 μM, however, this may be improved (after suitable separation) by using eluent suppressed IC-MS.

The robustness of the method was tested by quantifying a large range of metal-EDTA complexes in soil solution and *H. vulgare* exudate. Analyses were neither affected by co-

eluting peaks nor changing peak retention times. High recoveries of an internal standard (NiEDTA) further validated the method for analyzing metal-EDTA complexes in these environmental matrices.

4.5 Further research

It has been postulated that intact metal-EDTA complexes may be absorbed by plant roots via an apoplastic pathway (54, 275, 279), unless, of course, at high concentrations these chelates physiologically damage the root membranes that normally would impede the uptake of intact metal-EDTA complexes (34). Due to the short sampling time (24 hours) after the addition of 2.5 mmole EDTA to the soil it was not possible to detect any adverse effects (e.g. leaf necrosis, reduced transpiration, etc.) in the present experiments. However, as the distribution of the metal-EDTA complexes in the xylem generally reflected that seen in soil solution, although diluted, it suggests that uptake in this case was via an apoplastic pathway. As water is absorbed both symplastically and apoplastically the uptake of metal-EDTA complexes *only* via the latter would dilute their concentration but not affect their distribution.

These results confirm the observations of Vassil *et al.* (34) and Epstein *et al.* (30) who have shown, using largely indirect techniques, that a cultivar (cv. 426308) of *B. juncea* is likely to accumulate Pb as the intact PbEDTA complex (when grown in nutrient solution containing high levels of PbEDTA or on artificially Pb contaminated soils to which large quantities of EDTA have been applied).

However, these results do *not* support the hypothesis of Vassil *et al.* (34) that the uptake of PbEDTA by *B. juncea* only occurred after unidentified physiological damage to the root membranes. Therefore, further experiments were planned, using this technique, to establish the conditions under which intact metal-EDTA complexes are taken up by plants (e.g. EDTA concentrations, plant species) and if so by which mechanism (e.g. apoplastically or indiscriminate uptake after root membrane damage).

5. Uptake of the intact ZnEDTA complex from soil is dependent on plant species and ZnEDTA concentration

5.1 Introduction

The mechanisms of metal uptake by plants in the presence of ethylenediaminetetraacetic acid (EDTA) have been debated since its introduction to plant nutrition in the 1950's (371). The major debate has centered on whether the metal-ligand complex is actually absorbed by the plant. The majority of research dealing with this issue has focused on the complex of Fe - Fe(III)EDTA. In this case, the evidence has suggested that Fe is taken up by the plant as Fe(III)EDTA (268) or as Fe(II) after a two step process of Fe(III)EDTA reduction to Fe(II)EDTA and displacement of Fe(II) at the root surface by a mechanism analogous to competitive chelation (372). However, these results are difficult to resolve because of the use of different plant species as well as different Fe(III)EDTA concentrations between experiments. Furthermore, reliable conclusions based on the observations of these experiments have oftentimes been confounded by the Fe-nutritional status of the plants.

Similarly, research on other metals chelated to EDTA has produced conflicting results. As discussed in Chapter 2, data from both nutrient solution and pot experiments suggest Cd, Cu, Pb, Sr and Zn chelated to EDTA is unavailable for plant uptake (50, 211, 373-375). Under these conditions, it has been hypothesized that the addition of EDTA enhances metal uptake *only* by overcoming strong diffusional transport limitations within the soil or root apoplast. On the contrary, in other nutrient solution experiments the enhancement of metal uptake by EDTA has been related to the direct absorption of intact metal-EDTA complexes (255, 257). Indeed, PbEDTA has even been successfully used to determine the pathways of water transport in *T. aestivuum* L. (cv. Kloka) (334). Once again, differences in metal-EDTA complex concentrations and plant species may have been factors responsible for these discrepencies.

Despite these uncertainties, it has now been proposed that high additions of EDTA to soils contaminated with heavy metals such as Pb, Cd, Cu and Zn may assist in the

phytoextraction process (29, 31, 32, 331). To date, good agreement on the form of Pb absorbed by plants in the presence of high concentrations of PbEDTA exists between nutrient solution studies (34) and those on artificially contaminated soil (30). In either case, increased Pb uptake was associated with xylem concentrations of Pb and EDTA and it was hypothesized that physiological stress induced the uptake of the intact PbEDTA complex. However, these studies were limited to one cultivar of *B. juncea* - (cv. 426308) and PbEDTA. It is, therefore, still unknown if this mechanism applies to other plant species or to other metal complexes of EDTA. The significance of these questions to the EDTA-assisted phytoextraction of Zn have recently been highlighted by Ebbs and Kochian (88) and Sarret *et al.* (376). The former demonstrated that the addition of EDTA to a Zn contaminated soil only increased Zn uptake by *B. juncea* (and not by *H. vulgare* or *A. sativa*) while the latter reported that *P. vulgaris* only accumulated PbEDTA (and not ZnEDTA) from nutrient solution. Thus, it appears that there may be real differences in: 1) Zn uptake between plant species in the presence of ZnEDTA and; 2) the uptake of Zn and Pb when present as the intact metal-EDTA complex.

With the development of an IC-MS method to unequivocally detect these complexes in soil solution and plant xylem exudate in the preceding chapter it is now possible to establish for EDTA-assisted phytoextraction of Zn contaminated soils: 1) the mechanisms by which Zn is mobilized from the soil solid phase into solution (e.g. chelation); 2) conditions under which intact ZnEDTA complexes are taken up plants (e.g. concentration of the metal-ligand complex) and; 3) when ZnEDTA is taken up by plant species then it is by which process (e.g. apoplastically or indiscriminate uptake after root membrane damage). This last process needs clarification as the results from Chapter 4 do not support the views of Vassil *et al.* (34) who postulated that the uptake of PbEDTA by *B. juncea* (cv. 426308) only occurs after unidentified physiological damage to the root membrane.

5.2 Materials and methods

The protocols used to conduct this experiment are described in section 3.3, pp. 47 - 51.

Metal analyses were conducted by FAAS as outlined in section 3.5, pp. 62 - 64 and intact metal-EDTA complexes were detected using the IC-MS methodology specified in Chapter 4.

5.3 Results and discussion

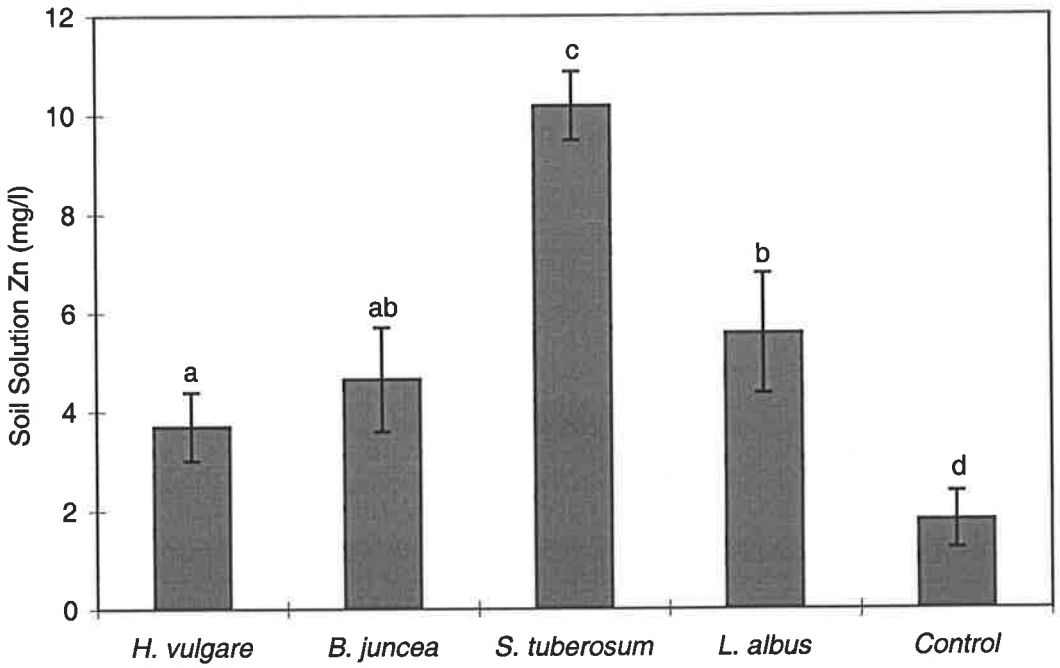
5.3.1 Effect of plants and EDTA on soil solution Zn

The soil solution from pots containing plants (0 mmole EDTA/kg) had significantly higher Zn concentrations than the control soil solutions (Figure 5.1). In addition, the amount of Zn in soil solution from the pots containing *S. tuberosum* was significantly higher than that found for the other plants. This result is unexpected considering that most research on Zn contaminated soils has generally shown that there is either no change or a decrease in soil solution Zn in the presence of agricultural crops (352, 377) or Zn hyperaccumulating plants (321, 323). However, soil solution Zn has been observed to increase in the presence of plants on a Zn contaminated calcareous soil and, in this case, was attributed to a decrease in pH (378). In addition, it was observed by Ebbs and Kochian (88) that concentrations of soil solution Zn from a contaminated soil (pH 7.7) differed between *A. sativa, H. vulgare* and *B. juncea.* Nevertheless, as data for control soil solutions was not reported it is unknown if the concentration of Zn increased due to the presence of these plants. Although the present soil had a pH of 5.0 and the measurement of pH or root exudates was not conducted on the soil solutions, it is likely that one of these two parameters were responsible for these results.

After the addition of 0.034 and 0.34 mmole EDTA/kg a small increase in soil solution Zn was observed for *H. vulgare* (Figure 5.2). A similar increase was observed for *B. juncea* at 0.34 mmole EDTA/kg. However, significant increases in soil solution Zn for all plants were observed only after the addition of 3.4 mmole EDTA/kg. Due to the negligible effects of the lower two EDTA concentrations on soil solution Zn it was possible that EDTA was: 1) chelating/desorbing other metals from the soil solid phase; 2) being sorbed to the soil solid phase or; 3) overcoming diffusional limitations to active Zn uptake.

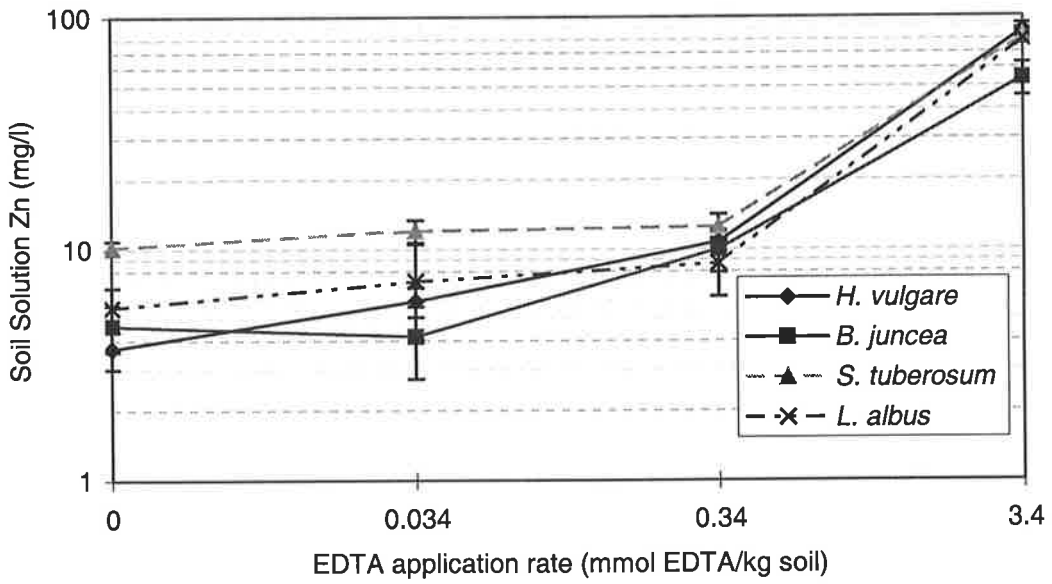
To determine whether low concentrations of EDTA resulted in the chelation/desorption of other metals from the soil solid phase the metals Cu, Cd, Fe, Mn and Al were also measured in soil solution. Of these metals only Fe was observed to significantly increase in concentration after the addition of 0.034 mmole EDTA/kg. Without the addition of EDTA the concentration of Fe was below the limit of detection. However, at the rate of 0.034 mmole EDTA/kg the concentration of Fe increased to 3.2 - 8.1 mg/l. In addition, there were significant increases in the soil solution concentrations of Cu, Fe and Mn at 0.34 mmole EDTA/kg (data not shown). Therefore, EDTA, at these lower application rates, was not effective at increasing Zn in soil solution because it was chelating/desorbing other metals from the soil solid phase.

Figure 5.1: Soil solution Zn concentrations in pots containing plants (0 mmole EDTA/kg) and control pots. Values represent the mean of three pots and error bars represent ± 1 standard deviation (SD). Columns assigned with the same letter are not significantly different ($P \leq 0.05$).



When the soil solution was analyzed for metal-EDTA complexes, regardless of plant species, the only complexes to be detected were ZnEDTA, CuEDTA and MnEDTA. Fe(III)EDTA could not be detected due to the analytical limitations of the IC-MS methodology. Although it has been reported that neither ZnEDTA nor MnEDTA are sorbed in acidic soils (379, 380) there have been a number of studies demonstrating the adsorption of EDTA (381) and Fe(III)EDTA (241, 379, 380, 382). In this experiment CuEDTA, ZnEDTA and MnEDTA accounted for 29 - 83 % and 47 - 80 % of the total EDTA added to the soil at the 0.034 and 0.34 mmole EDTA/kg application rates, respectively. As total EDTA remaining in soil solution was not measured it is, therefore, possible that the sorption of EDTA or its metal complexes may have been contributing to the results seen at these lower application rates.

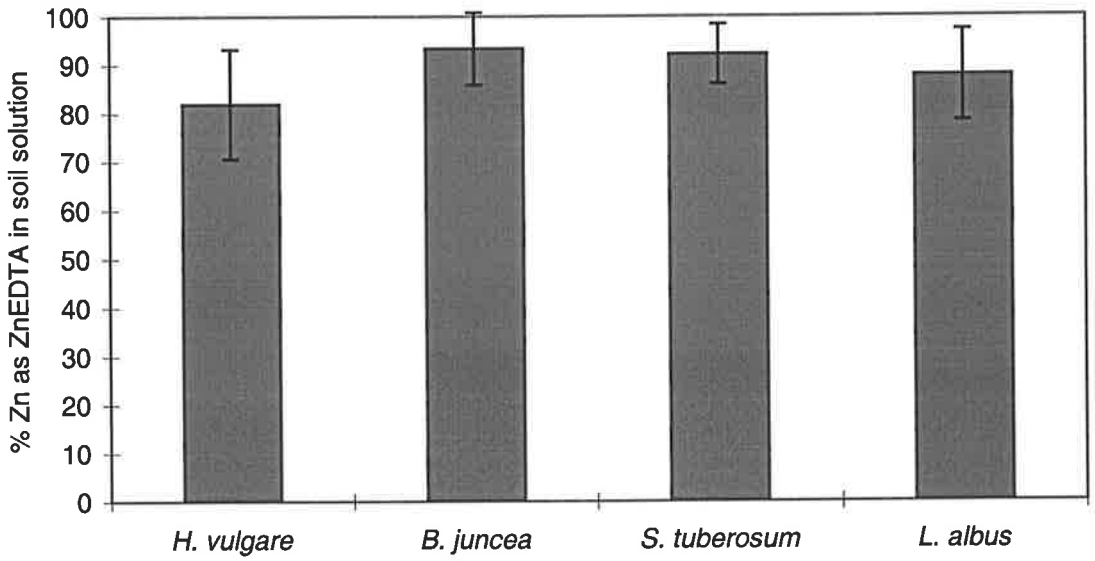
Figure 5.2: Soil solution Zn concentrations as affected by plant species and EDTA application rate. Values represent the mean of three pots and error bars ± 1 SD.



Finally, it is possible that any increases in soil solution Zn at the lower EDTA application rates may have been negated by an increase in plant uptake. This mechanism requires active uptake by the plant as purely passive uptake would have little or no effect on the concentration of Zn in solution. Such a mechanism could occur if there was a diffusional limitation to the uptake of Zn in this soil (162). However, due to the high concentrations of soil solution Zn observed in the presence of plants (Figure 5.1) and an increase in plant Zn content not being observed at these concentrations (discussed below) this mechanism is unlikely.

Although there were only insignificant or small increases of Zn in solution at 0.034 and 0.34 mmole EDTA/kg, the proportion of Zn as ZnEDTA increased. For example, ZnEDTA accounted for only 4 - 17 % of soil solution Zn at 0.034 mmole EDTA/kg but increased to 47 - 94 % after the addition of 0.34 mmole EDTA/kg. In contrast, after the addition of 3.4 mmole EDTA/kg, a concomitant increase in ZnEDTA with soil solution Zn was observed. In fact, 85 - 104 % of the increase in soil solution Zn could be accounted for by chelation/desorption with EDTA. At this EDTA concentration there was no significant difference in the percentage of soil solution Zn as ZnEDTA between plant species and the metal-EDTA complex accounted for 82 - 93 % of Zn present in soil solution (Figure 5.3).

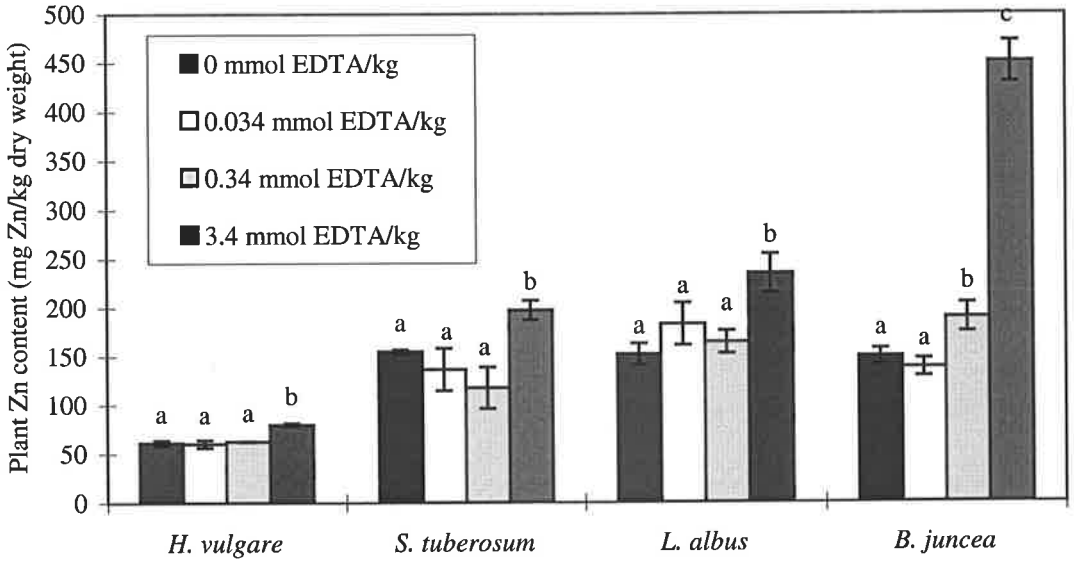
Figure 5.3: Percentage of soil solution Zn detected as the ZnEDTA complex in pots containing plants after the addition of 3.4 mmole EDTA/kg. Values represent the mean values from three pots and error bars ± 1 SD.



5.3.2 Effect of EDTA on plant transpiration and Zn uptake

At all EDTA concentrations *H. vulgare* contained significantly less Zn than the other plants (Figure 5.4). The Zn contents of the other three plants were similar at all EDTA concentrations until the 3.4 mmole EDTA/kg application rate. At this concentration a significant increase in Zn uptake by all plants was observed. Although minimal for the other three plants, *B. juncea*'s Zn concentration approximately doubled at this application rate (Figure 5.4). Similar patterns of Zn uptake by different cultivars of *H. vulgare* and *B. juncea* in the presence of high concentrations of EDTA have been previously observed (88). However, no attempt was made by the authors to determine the processes responsible for their observations.

Figure 5.4: Zn content of plant shoots harvested 5 days after the application of various concentrations of EDTA. Values represent mean values of three pots, and error bars ± 1 SD. Columns assigned with the same letter for treatments with the same plant species are not significantly different ($P \leq 0.05$).



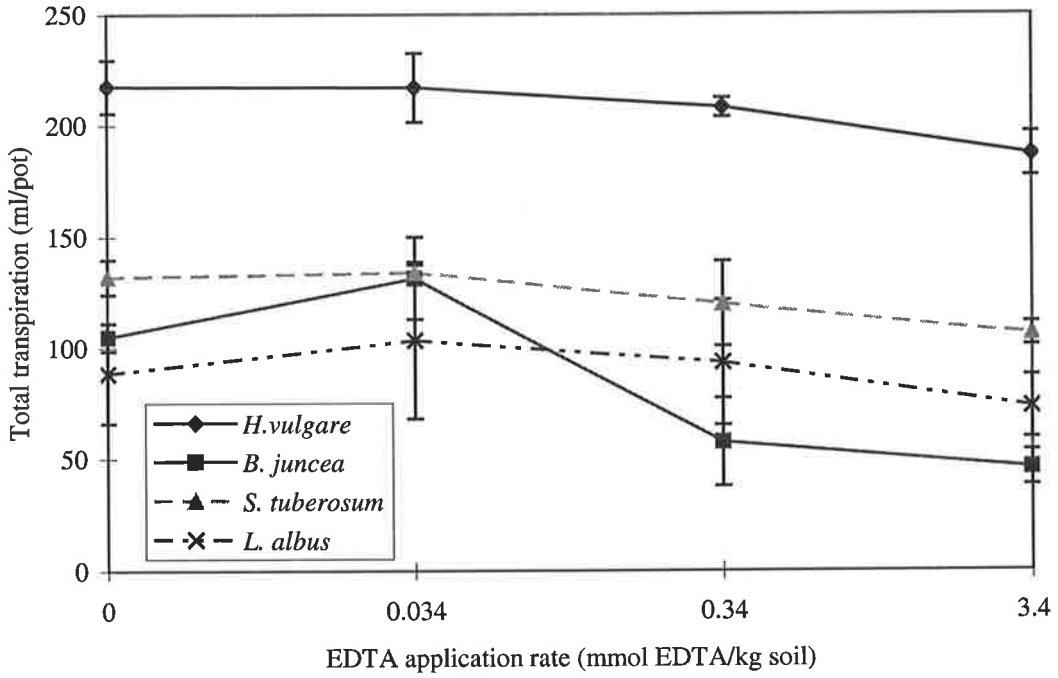
Upon the addition of EDTA, plant Zn uptake was associated with increases in soil solution Zn. For example, a significant increase in Zn uptake by all the plants was only observed after the addition of 3.4 mmole EDTA/kg. As mentioned previously, it is unlikely that the addition of EDTA overcame diffusional limitations to plant Zn uptake. Therefore, increases in Zn uptake were either due to the passive uptake of higher soil solution concentrations of ZnEDTA or high concentrations of EDTA physiologically damaged the plant roots leading to indiscriminate ZnEDTA uptake (34). If passive uptake was responsible for the observed results then a relationship would exist between the concentration of ZnEDTA in soil solution, enhanced Zn accumulation and transpiration (383).

Increasing EDTA concentrations only had a slightly negative effect on the transpiration of

H. vulgare, *S. tuberosum* and *L. albus* (Figure 5.5). This result, in conjunction with similar soil solution ZnEDTA concentrations and modest increases in plant Zn accumulation observed from the 0.34 mmole EDTA/kg to 3.4 mmole EDTA/kg application rate, suggests that a mechanism such as passive uptake was likely to be in progress for these species. In contrast, there was a significant decline in the transpiration of *B. juncea* at 0.34 and 3.4 mmole EDTA/kg. As high increases of Zn accumulation by *B. juncea* were associated with significantly lower transpiration rates it implies that another mechanism was occurring.

Decreases in transpiration indicate that the plants were suffering from physiological stress and *B. juncea* was more susceptible to this stress than the other species. Therefore, it is reasonable to postulate that the degree of this stress could be responsible for the observed differences in plant Zn uptake between *B. juncea* and the other plants. Although the exact nature of this damage could not be identified from the results of this experiment, similar observations for another *B. juncea* cultivar - (cv. 426308) - have been obtained after EDTA has been added in large amounts to nutrient solutions or artificially Pb contaminated soils (30, 34). Vassil *et al.* (34) postulated that high concentrations of EDTA were damaging root membranes and increases in plant Pb content were due to the uptake and xylem transport of the intact PbEDTA complex. Therefore, we analyzed plant xylem for the intact ZnEDTA complex to test this hypothesis and to examine whether differences in Zn uptake could be explained by this mechanism.

Figure 5.5: Total plant transpiration (5 days) per pot after the addition of various concentrations of EDTA.

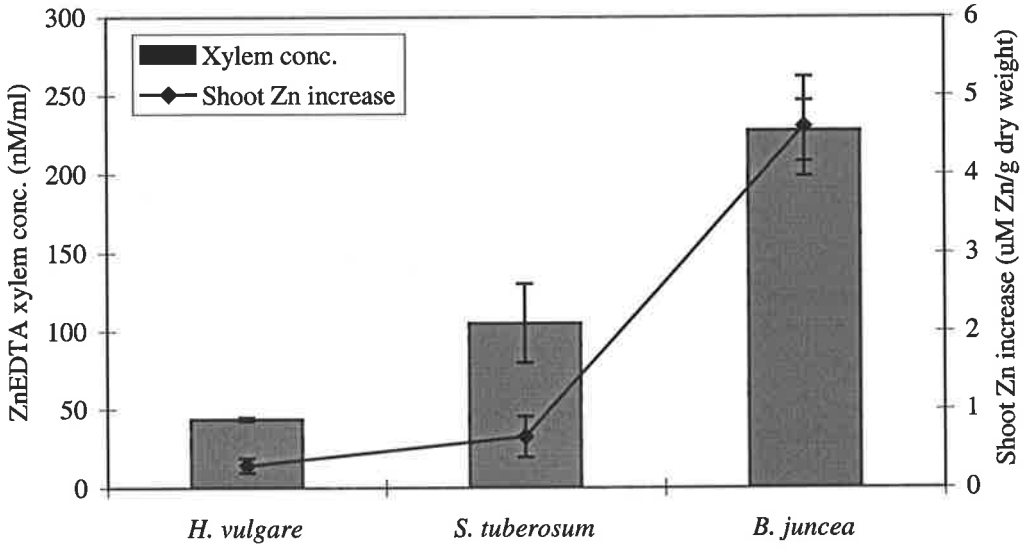


5.3.3 Measurement of intact metal-EDTA complexes in plant xylem exudate

Attempts to extract exudate from the xylem from *L. albus* were unsuccessful and hence no data are reported for this species. After the addition of 0.034 mmole EDTA/kg, no metal-EDTA complexes nor EDTA itself were detected in the xylem of any of the plants. At 0.34 mmole EDTA/kg ZnEDTA was only detected in the xylem of *B. juncea.* However, ZnEDTA was detected in the xylem of all plant species after the addition of 3.4 mmole EDTA/kg (Figure 5.6). The complexes of MnEDTA and CuEDTA were also present in the xylem exudate when ZnEDTA was detected (data not shown). At the 3.4 mmole EDTA/kg application rate the xylem exudate of *B. juncea* showed significantly higher concentrations of ZnEDTA than that of *S. tuberosum* and both had significantly higher concentrations than *H. vulgare* (Figure 5.6). This observation was consistent with increases in plant Zn and a positive linear relationship existed between these two

measurements (R^2 = 0.93, $P \leq 0.01$). Therefore, regardless of the exact mechanism, the uptake of ZnEDTA appeared to be the limiting factor to enhanced plant Zn accumulation.

Figure 5.6: ZnEDTA xylem concentrations in relation to enhanced Zn uptake. Values represent the mean of three pots and error bars ± 1 SD. Shoot Zn increase = Zn content in plants after the addition of 3.4 mmole EDTA/kg - Zn content in plants without EDTA addition).



Nonetheless, the concentration of ZnEDTA in the xylem of *H. vulgare* and *S. tuberosum* represented only 4.0 and 9.1 %, respectively, of the concentration of ZnEDTA measured in soil solution. These values are similar to that of Crowdy and Tanton (334) who estimated that 3.5 % of a 12.5 mM PbEDTA solution 'entered' *T. aestivuum* L. (cv. Kloka) plants. On precipitation of the lead as the lead sulfide, Crowdy and Tanton (334) illustrated that PbEDTA uptake by these plants was restricted to the region between 3-140 mm from the root tip where suberisation of the cell walls had not yet occurred. This pathway would allow metal-EDTA complexes to circumnavigate the impermeable Casparian strip, allowing for the apoplastic uptake of the intact complex to proceed.

Similarly, apoplastic uptake of the intact ZnEDTA complex could occur at breaks in the Casparian strip, along the primary root, that are initiated by the budding of lateral roots (384-387). Due to the fact that high concentrations of EDTA had little effect on the transpiration of *S. tuberosum* and *H. vulgare* and both showed low xylem ZnEDTA concentrations, it confirms that a similar mechanism of uptake was occurring for these plants. In contrast, *B. juncea*'s xylem concentration of ZnEDTA represented 30 % of that detected in soil solution after the addition of 3.4 mmole EDTA/kg. As mentioned previously, this species exhibited significant decreases in transpiration and increased Zn uptake at this EDTA concentration. These results are in agreement with the findings of Epstein *et al.* (30) and Vassil *et al.* (34) and support the hypothesis that, for *B. juncea only*, high concentrations of EDTA damage root membranes leading to uptake and xylem transport of high concentrations of intact metal-EDTA complexes.

However, the observed increases in plant Zn only accounted for 9 - 16 % of that estimated if ZnEDTA concentration in the xylem is multiplied by transpiration. Due to the short sampling time of the xylem (1 hr) it is unlikely that this led to a concentration effect as has been seen with longer sampling times (388-391). However, if root damage precedes ZnEDTA uptake, then it is possible that there was a delay in ZnEDTA transport to the xylem while this damage was occurring or, for either mechanism of uptake, while sorption sites on the (damaged) roots were saturated with respect to the metal-EDTA complex. Evidence for such a delay in the uptake of metal-EDTA complexes has been reported by Wu *et al.* (331). These authors observed that Pb concentrations in the shoots of *Z. mays* grown hydroponically, and supplemented with 1.5 mM PbEDTA, did not significantly increase until 72 hours after the addition of the chelate. Therefore, the amount of ZnEDTA measured in the xylem in this experiment may be representative of the maximum concentration experienced over the 5 day period and could explain the discrepancy between actual Zn uptake and that predicted by ZnEDTA in the xylem and transpiration.

5.4 Conclusions

The application of EDTA to a Zn contaminated soil was only effective at increasing concentrations of Zn in soil solution after the addition of 3.4 mmole EDTA/kg soil. Although a greater percentage of Zn in soil solution was found as the ZnEDTA complex at lower application rates, the concentration of Zn did not increase as EDTA chelated/desorbed other metals or was sorbed to the soil solid phase. Despite the ZnEDTA complex being present in soil solution at all EDTA application rates the intact metal complex was only detected in the xylem exudate of all plant species at the highest EDTA concentration. Although the detection of ZnEDTA in xylem exudate was coincident with elevated levels of Zn in the plant shoots, the results indicated that *H. vulgare* and *S. tuberosum* may have only taken up ZnEDTA apoplastically. In contrast, *B. juncea*, as well as having significantly higher concentrations of ZnEDTA in the xylem exudate and Zn levels in the shoots than the other species, exhibited signs of phytotoxicity. Therefore, it was hypothesized that physiological damage to this plant's root system preceded the indiscriminate uptake of soil solution. The different ZnEDTA uptake patterns observed between these plant species suggest that EDTA-assisted phytoextraction of Zn will only be successful if high application rates of EDTA (> 3.4 mmole EDTA/kg soil) are used in combination with plants that are susceptible to the toxic effects of EDTA. However, these types of application rates may lead to further environmental consequences, such as the off-site migration of metals, because it was demonstrated that plants only absorb a small quantity of the metal-EDTA complexes present in the soil solution.

5.5 Further research

Although the results of this experiment indicate that there are likely to be different mechanisms of ZnEDTA uptake between plant species, the exact pathways of uptake were not *directly* identified. Therefore, further research could be aimed at determining whether root membrane damage is the cause of *B. juncea*'s capacity to accumulate Zn and ZnEDTA over the other plant species tested. This could be achieved using nutrient solutions containing various concentrations of the ZnEDTA complex. Using this

experimental system root membrane damage could be inferred from measuring the leakage of K^+ from the plant's root system into nutrient solution. However, now that the evidence from these experiments strongly infer the mechanisms of ZnEDTA uptake, further research may only be of academic importance. Therefore, it was concluded that further experiments in this area of research area would not significantly benefit the utilization of this technology for remediating metal polluted soils.

As discussed in Chapter 2, one of the major shortcomings of phytoextraction, if current environmental regulations are based on total soil metal concentrations, is the extensive time needed to remediate a soil below action levels (35). Based on the evidence that soil Cd and Zn can exist in non-phytoavailable pools (36-38) it has been argued that total concentrations are not a satisfactory criterion to assess soil remediation efforts (36). Although a valid argument, the forms of Cd and Zn that are phytoavailable have yet to be fully elucidated. Therefore, it was considered that the use of phytoextraction as a remedial tool would substantially profit from research aimed at identifying the phytoavailable pools of Cd and Zn as well as the plant/soil factors controlling its chemical, and hence biological, behavior.

6. Influence of chelation and pH on isotopically exchangeable soil Cd and Zn

6.1 Introduction

It is well known that plants may modify the physicochemical reactions of Cd and Zn in the soil environment (162). Plants are able to induce changes of pH in the rhizosphere which, in turn, has a critical influence on the sorption of these metals to the soil surface (103, 144, 149, 152-154). In addition, the development of novel analytical techniques over the last decade has revealed the ubiquitous nature of organic ligands in the rhizosphere (127, 128). Although the free acid of some of these organic ligands may alter Cd and Zn sorption characteristics (106, 129, 130, 163-165, 182), few studies have attempted to discern between the importance of pH or chelation processes (130, 164).

As discussed in Chapter 2, the movement of metal from the solid to the solution phase is a significant process preceding plant uptake. However, the quantity of metal that can be brought into solution through rhizosphere processes is also extremely important. The application of radioisotopic techniques to plant and soil research has proved invaluable for determining the amount of Cd and Zn associated with the soil solid phase that is potentially available for plant uptake (*L* values) (36-38, 43, 44, 46, 101, 305). Most often these studies have demonstrated that the quantity of solid phase metal available for plant uptake (*L* value) is invariable between plant species. However, enough evidence has accumulated to substantiate claims that processes occurring in the rhizosphere may be able to influence the quantity of soil Cd and Zn that is available for plant uptake.

Moreover, to alleviate the potential health risks of polluted soils, in which the risk is primarily associated with soil-plant-animal/human transfers, it may only be necessary for phytoextraction strategies to deplete the phytoavailable pool of metal. Along this theme, therefore, it is desirable to ascertain the effects of EDTA, during chelate-assisted phytoextraction, on these metal pools. Although, it has recently been demonstrated that EDTA application rates up to 10 mmole/kg do not increase the isotopically exchangeable

pool of Cd to *B. juncea* (114), further studies are needed on soils polluted through other means than the application of sewage sludge.

Considering the literature on the sorption characteristics of these metals, the presence of organic ligands and plant-induced changes of pH may arguably be two of the most important variables likely to affect the quantity of phytoavailable Cd and Zn in the rhizosphere (Chapter 2). Therefore, the following batch experiments were conducted to examine the effects that these two variables have on the distribution and quantity of isotopically exchangeable Cd and Zn (*E* value) in two - one acidic and one calcareous - polluted soils.

6.2 Materials and methods

The protocols used to carry out these experiments are outlined in section 3.4, pp. 51 - 62 and analyses were conducted according to the methodologies reported in section 3.5, pp. 62 - 77 and Chapter 4.

6.3 Results and discussion

6.3.1 Mobilization of Cd and Zn into solution by pH

The K_d values of Cd and Zn in the soils were strongly pH-dependent (Figures 6.1 and 6.2), confirming previous observations on the sorption of Cd (104, 107, 126, 144, 149) and Zn (103, 129, 144, 154, 164, 165, 392-394) to soils. Over the pH ranges examined in these soils, the partitioning of both metals between the solid and solution phases were characterized by sigmoidal shaped curves. At lower pH values these curves were distinguished by steep changes in the quantity of metal associated with the solid phase. The natural pH of the acidic soil (pH 5.7) was located within the pH range with the steepest change in K_d, whereas the natural pH of the calcareous soil (pH 7.5) represented the end point of this range. The K_d measurements above these ranges were largely unaffected by changes in the solution concentration of H^+ since > 98 % and > 99 % of the added radioisotope (either ^{109}Cd or ^{65}Zn) was found to be associated with the solid phase of the acidic and the calcareous soil, respectively.

Figure 6.1: Sorption of Cd to the soils as affected by changes of pH. The 5 parameter sigmoidal regression fitted for the acidic soil was $K_d = 48.4 + 2499/(1 + e^{-(pH-5.77)/0.18})^{0.86}$: $R^2 = 0.99$, $P \leq 0.0001$ and for the calcareous soil was $K_d = 112.4 + 3421/(1 + e^{-(pH-7.49)/0.0034})^{0.0112}$: $R^2 = 1.00$, $P \leq 0.0001$.

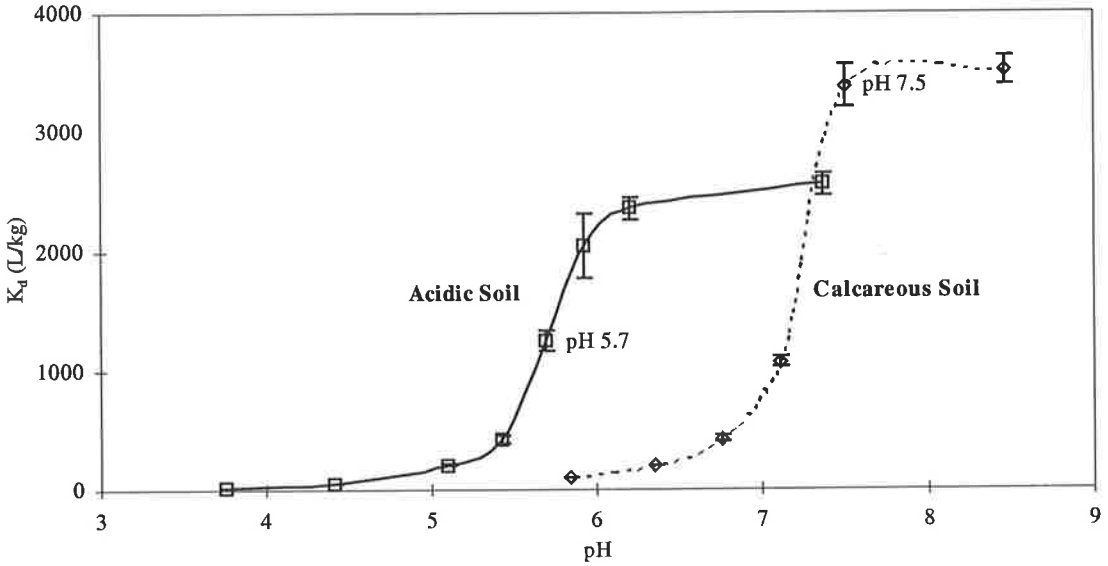
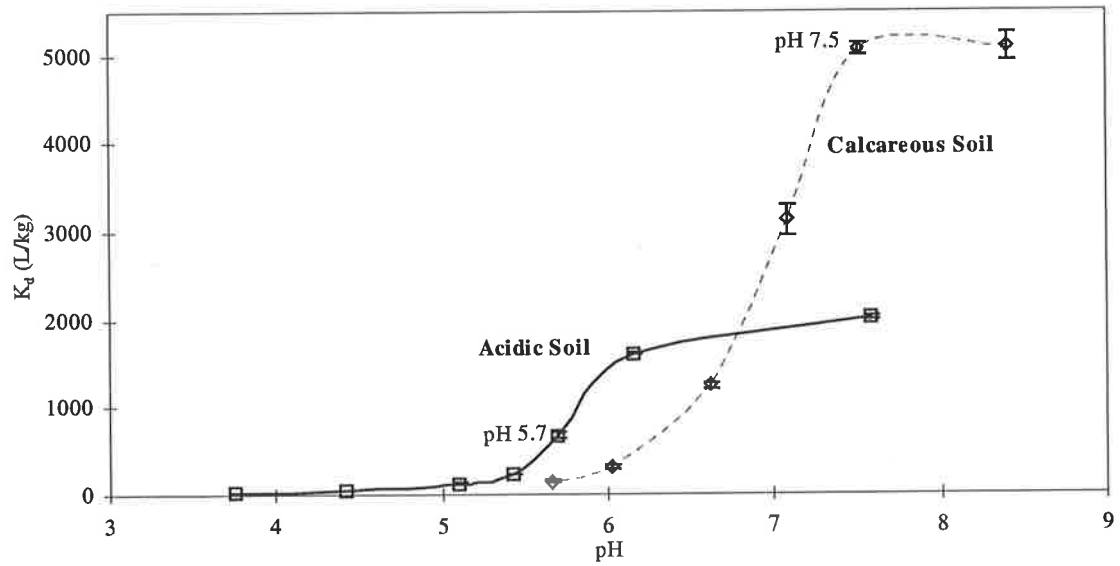


Figure 6.2: Sorption of Zn to the soils as affected by changes of pH. The 4 parameter sigmoidal regression fitted for the acidic soil was $K_d = 31.7 + 1985/(1 + e^{-(pH-5.87)/0.22})$: $R^2 = 1.00$, $P \leq 0.0001$ and for the calcareous soil was $K_d = 215.3 + 5041/(1 + e^{-(pH-6.97)/0.23})$: $R^2 = 0.99$, $P \leq 0.0001$.



Sharp variations in the distribution of Cd between the solid and solution phases, with small increases of pH, have commonly been attributed to the preferential adsorption of Cd hydroxy species ($CdOH^+$) (39, 130, 141). Elsewhere, experiments have indicated that the soil solution concentration of Cd is controlled by the adsorption of the divalent cation (Cd^{2+}) (144, 392, 393). Nonetheless, all these studies have stressed adsorption reactions whereas the important reaction for most environmental applications depends on desorption (39). Therefore, most hypotheses for the desorption mechanisms of Cd have been developed based on inference from sorption experiments. Of these hypotheses, proton competition for sorption sites (155), acid catalyzed dissolution of reactive oxide sites (395) and variations in the negative surface-charge density of the soil (149) have had most support in the literature. In the present experiments when the logarithm of the K_d data were plotted against pH, a linear relationship existed at pH values below that of the natural pH of the soil (i.e. at pH values where desorption was occurring) (Figure 6.3). The slopes of these regressions were extremely similar and indicated that a decrease of one pH unit (10-fold increase in H^+ concentration), over the range of pH values examined, resulted in an approximate 9.1-fold increase in the solution concentration of Cd. Therefore, these results are consistent with a mechanism whereby Cd desorption occurred via proton exchange for monovalent cation species of Cd (e.g. $CdOH^+$).

However, a relationship between K_d and pH is simply a description of macroscopic data and, therefore, does not definitely prove that a desorption mechanism was occurring (146). In fact, based exclusively on these K_d values, the desorption of Cd is experimentally indistinguishable from the dissolution of Cd in a mixed solid phase that has a dissolution slope of unity with pH. Furthermore, as the first hydrolysis constant of Cd is at pH 10.1, the solution concentration of $CdOH^+$ in the acidic soil will, theoretically, be at least 4 orders of magnitude lower than Cd^{2+} at pH values ≤ 5.8 (pH_{50}). These elements tend to detract from a mechanism of $CdOH^+$ desorption.

Figure 6.3: Relationship between the logarithm of the K_d of Cd in the soils with pH. Fitted lines are log K_d = 0.921pH - 2.3: R^2 = 0.98, $P \leq 0.001$ (acidic soil) and log K_d = 0.916pH - 3.45: R^2 = 0.97, $P \leq 0.001$ (calcareous soil).

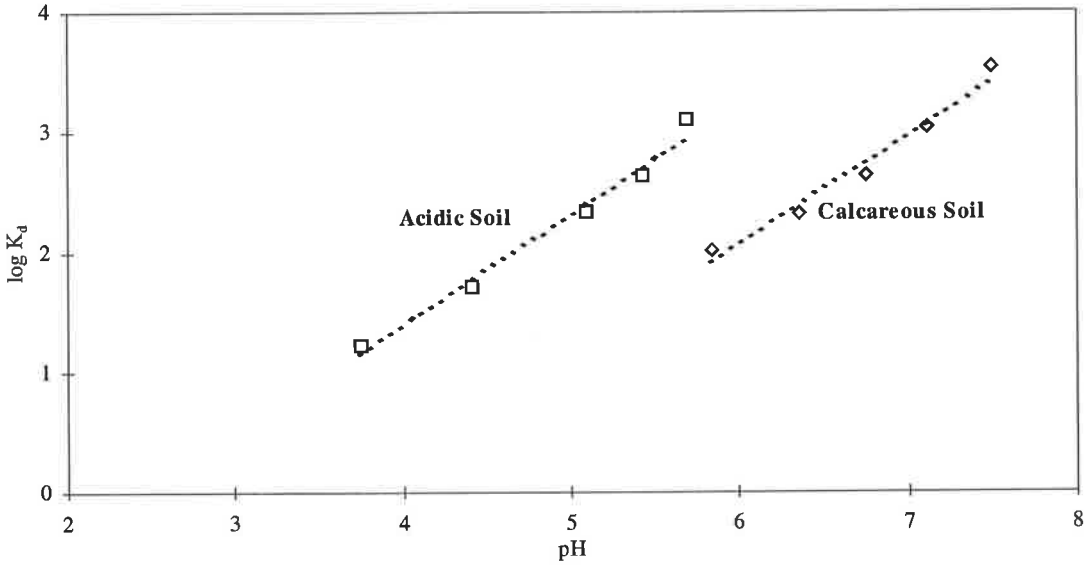
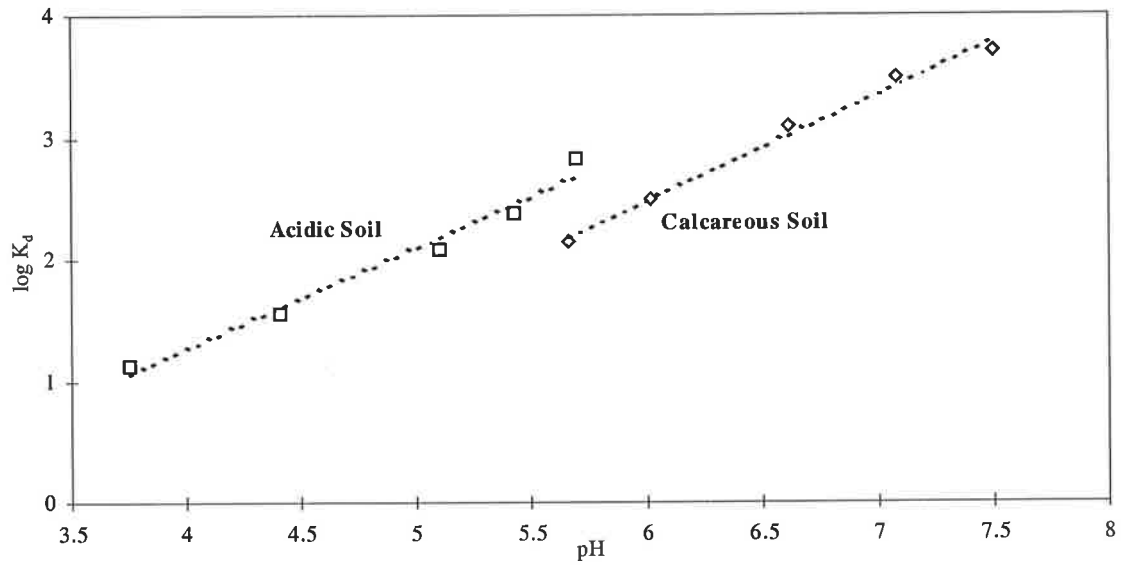


Figure 6.4: Relationship between the logarithm of the K_d of Zn with pH. Fitted lines are log K_d = 0.831pH - 2.06: R^2 = 0.98, $P \leq 0.001$ (acidic soil) and log K_d = 0.871pH - 2.74: R^2 = 0.99, $P \leq 0.001$ (calcareous soil).



As was the case for Cd, when the K_d data for Zn was log transformed linear relationships existed at pH values where desorption/dissolution was occurring (Figure 6.4). The slope of the regression for Zn desorption/dissolution with pH in the calcareous soil was closer to a 1-to-1 relationship than that for the acidic soil. However, neither of the regressions were as close to the 1-to-1 relationship observed between the mobilization of Cd into solution and pH. This observation may be due to the concentration of Zn being two orders of magnitude higher than Cd in these soils (Table 3.1). It has previously been demonstrated that mobilization reactions involving the divalent cation of Zn are more pronounced at elevated soil Zn concentrations (103, 154). Evidence that dissolution reactions (involving divalent Zn cations) were contributing to the mobilization of Zn into the solution phase of the acidic soil was the greater affinity of Cd, over Zn, for the solid phase. If the distribution of Zn between the solid and solution phases was being controlled solely by hydrolysis reactions then the opposite selectivity would be observed (Chapter 2). Nevertheless, conclusions derived from these results carry the same uncertainty as those made for Cd because the relationship between K_d and pH does not, by itself, confirm a reaction mechanism.

6.3.2 Mobilization of soil solid phase Cd and Zn by organic ligands

6.3.2.1 Sorption of organic ligands

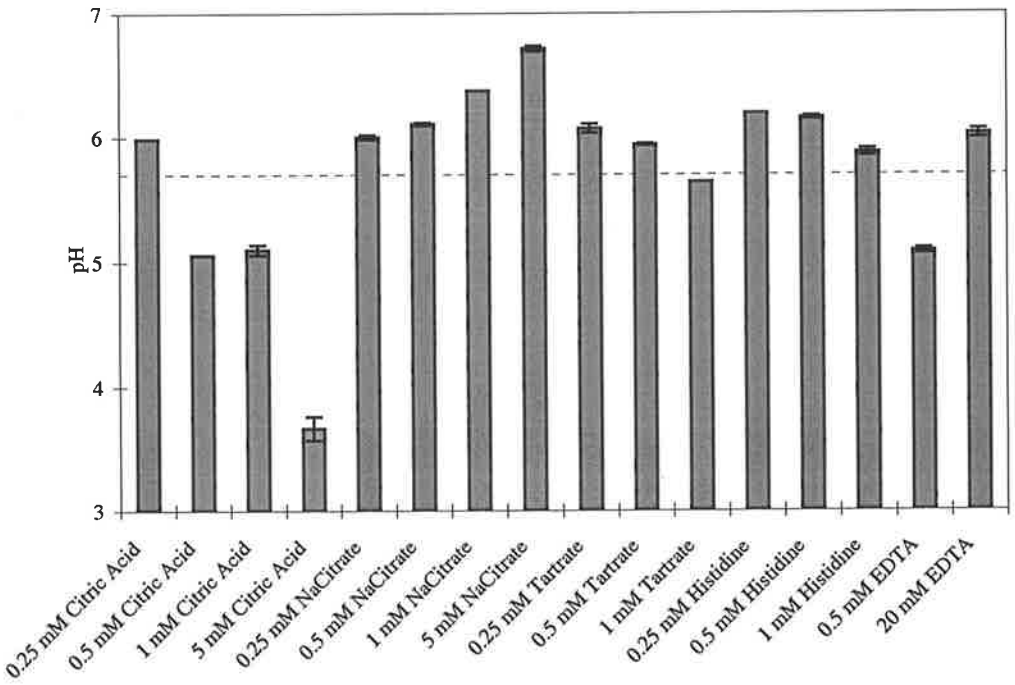
In general, there was little sorption of the organic ligands, except histidine, in both soils (Table 6.1). For most ligands, sorption was positively related to increases in the solution concentration of the ligand. However, as pH was not buffered in these experiments varying the concentration of most ligands resulted in changes of pH (Figures 6.5 and 6.6). Nonetheless, despite previous results indicating that pH may effect citric acid and sodium citrate sorption to acidic soils at concentrations > 2 mM (173), there were no significant differences between the sorption of these ligands to the soils used in these experiments. The sorption of tartrate and EDTA was also unaffected by soil type. As sorption of these ligands was unaffected by soil type and changes of pH it suggests that neither the pK_a of the ligand functional groups, complexation with metals nor the surface charge of the soils played a significant role in these experiments.

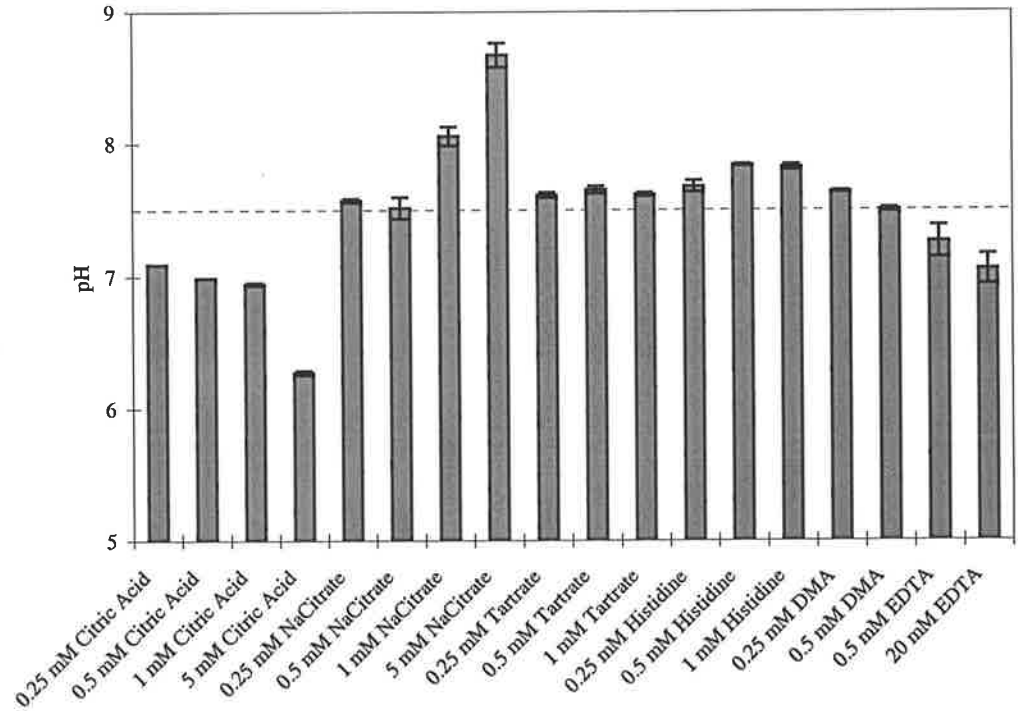
Table 6.1: Concentration of organic ligand remaining in solution after sorption (10 min) and isotopic equilibration (48 hr). The standard deviation for all measurements was less than 5 %.

	Initial Conc.	After sorption		After Equilibration	
		Acidic Soil	Calcareous Soil	Acidic Soil	Calcareous Soil
	---mM---				
Citric Acid	0.25	0.21	0.22	0.15[1]	0.19[1]
	0.5	0.43	0.45	0.34[1]	0.48
	1.0	0.92	0.92	0.73[1]	0.92
	5.0	4.7	4.8	4.7	4.7
Sodium Citrate	0.25	0.21	0.22	0.16[1]	0.19[1]
	0.5	0.46	0.47	0.42	0.46
	1.0	0.96	0.96	0.93	0.96
	5.0	4.9	4.6	5.0	4.8
Tartrate	0.25	0.22	0.24	0.19[1]	0.24
	0.5	0.44	0.46	0.41	0.46
	1.0	0.90	0.93	0.85	0.93
Histidine	0.25	< 0.01	0.13	< 0.01	< 0.01[1]
	0.5	< 0.01	0.38	< 0.01	0.13[1]
	1.0	0.13	0.90	< 0.01[1]	0.22[1]
EDTA	0.5	0.44	0.47	0.45	0.49
	20	18.96	18.78	17.87	18.28

[1] Significant at the 0.05 probability level between sorption and after equilibration in the same soil for the same organic ligand.

Figures 6.5 and 6.6: Equilibrium pH of organic ligand solutions with the acidic soil (top) and calcareous soil (bottom). The pH of soil suspensions containing water only are indicated by the hashed lines at pH 5.7 (acidic soil) and pH 7.5 (calcareous soil).





In contrast to the organic acids, histidine had a much stronger affinity to the solid phase of the soils. High levels of histidine sorption to the acidic soil, in comparison to the calcareous soil, were probably related to the isoelectric point of histidine and pH differences between the soils. The isoelectric point of an amino acid is the pH at which the ligand carries a net zero charge. As such, histidine is positively charged at pH values < 7.47 and negatively charged above this pH despite the values of pK_2 (pH 6.0) and pK_3 (pH 9.3). Therefore, in suspensions of the acidic soil (pH range 6.5 - 6.7) histidine was being sorbed as a positively charged ligand. In contrast, the pH of the suspensions of the calcareous soil (pH range 8.2 - 8.4) were all higher than the isoelectric point of histidine which coincided with less sorption to this soil.

Although the sorption of DMA was not measured, its sorption behavior would be similar to that observed for the negatively charged organic acids because its isoelectric point would be at a pH between pK_2 (pH 2.76) and pK_3 (pH 3.4) (283).

6.3.2.2 Organic ligand concentrations after isotopic equilibration

Preliminary results indicated that the addition of 50 μl of chloroform to the soil suspensions only minimized microbial activity. Therefore, to accurately assess the influence of organic ligands on the chemistry of Cd and Zn in these soils, the solution concentration of the ligand after isotopic equilibration was also measured (Table 6.1).

Significant decreases in the solution concentration of sodium citrate and tartrate at 0.25 mM and citric acid from 0.25 - 1.0 mM were observed in the experiments using the acidic soil. In addition, histidine was not detected in any treatments after the equilibration period. In the calcareous soil, significant decreases in the concentration of citric acid and sodium citrate at 0.25 mM and histidine at all concentrations were observed.

When compared to measurements taken during ligand sorption experiments it was noted that the pH of these solutions after equilibration had decreased by as much as one pH unit. Therefore, decreases in pH may have: 1) increased the quantity of positively

charged histidine molecules and, thus, enhanced histidine sorption, or; 2) increased the positive surface charge of the soils enhancing sorption of negatively charged metal-ligand complexes or the ligands themselves. It was not ultimately verified whether these observations were a result of biodegradation or of continued sorption to the soil solid phase.

6.3.2.3 Effect of organic ligands on the K_d of Cd and Zn in the acidic soil

The type and concentration of organic ligand affected the distribution of Cd and Zn between the solid and solution phases of the acidic soil (Figures 6.7 and 6.8). To distinguish between pH and other processes (e.g. chelation) affecting K_d the data were compared to those values predicted at the same pH in the absence of the ligand. The values of K_d (y) for Cd over the pH range examined in this soil were obtained by fitting the data presented in Figure 6.1 by a 5 parameter sigmoidal regression:

$$(6.1)\quad y = y0 + \frac{a}{(1 + e^{-(x-x0)/b})^c}$$

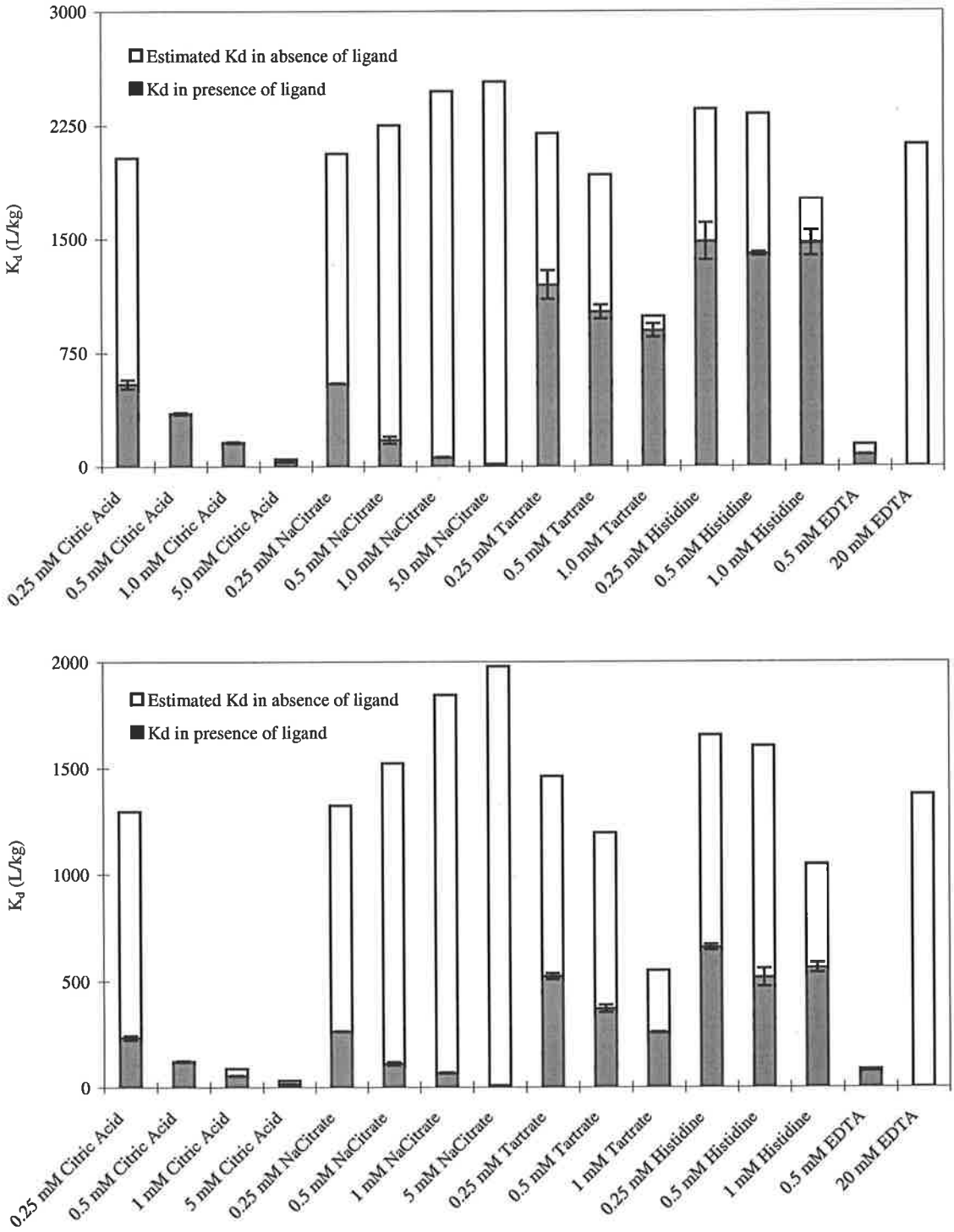
where $y0$ is the minimum K_d value, a is (maximum K_d - minimum K_d), x is pH, $x0$ is pH_{50}, b is a constant describing the linearity of the regression and c is a measure indicating the sigmoidal quality of the regression.

A 4 parameter sigmoidal regression was used to fit the data presented in Figure 6.2 to model the relationship between the K_d of Zn (y) and pH:

$$(6.2)\quad y = y0 + \frac{a}{(1 + e^{-(x-x0)/b})}$$

The fifth parameter (c) was not used to model the Zn data because of the more symmetrical nature of the data.

Figures 6.7 and 6.8: The K_d of Cd (top) and Zn (bottom) in the acidic soil as affected by organic ligands compared to estimated values at the same equilibrium pH in the absence of the ligand.



When these values were compared to the results obtained for the organic ligands it was found that histidine *increased* the solution concentration of Cd and Zn over that estimated at the same pH in the absence of the ligand. This is an interesting result considering that histidine was significantly sorbed to the soil solid phase at all concentrations and was not detected in solution after equilibration. Therefore, as histidine was sorbed as a positively charged ligand, it was desorbing Cd and Zn by directly competing for the same adsorption sites.

Although a general increase in the solution concentration of Cd and Zn occurred as concentrations of citric acid and tartrate increased the *proportion* accounted for by pH also increased. In conjunction, increasing concentrations of these ligands resulted in a lowering of the solution pH (Figure 6.5). Similarly, EDTA, at a concentration of 0.5 mM, decreased the solution pH and produced a K_d of Cd and Zn that was indistinguishable from that estimated at the same pH in the absence of the ligand. Therefore, if the presence of a ligand was associated with a decrease in pH then the K_d of Cd and Zn apparently became controlled by the solution concentration of H^+.

At higher pH values, where the K_d was lower than that predicted by pH alone, there are a number of processes that could have been responsible for increased Cd and Zn desorption in the presence of sodium citrate, tartrate and 20 mM EDTA. For example, higher solution concentrations of the metals may have been the result of: 1) direct competition for adsorption sites - as demonstrated for histidine; 2) increases in ionic strength (as Na^+); 3) the ligand forming non-sorbing soluble metal complexes reducing Cd^{2+} and Zn^{2+} activities (and possibly the activity of hydrolysis species) in soil solution leading to the desorption of solid bound exchangeable metal, and/or; 4) the ligand dissolving Cd and Zn mixed solids or adsorbing surfaces (e.g. Fe oxides) via a process similar to {3}.

Calculations made with the computer program GEOCHEM-PC, version 2.0 (359) indicated that the metal-ligand complexes of citrate, tartrate and EDTA either carried a net zero or negative charge in the pH range examined in these experiments. Similar

calculations predicted that the ligands themselves had a net negative charge. Therefore, the desorption of Cd and Zn in the presence of these ligands was not via a process similar to that observed for histidine.

It is highly unlikely that Na^+ from the organic ligand solutions was contributing to enhanced Cd desorption based on the results of Naidu and Harter (130) and Naidu *et al.* (149). These authors noted, using a total of 10 soils varying widely in chemical and physical characteristics, that for soils having pH values > 5 the amount of Cd desorbed by $NaNO_3$ was insignificant or non-detectable at concentrations up to 30 mM $NaNO_3$. In the experiments reported here the maximum concentration of Na^+ (5 mM sodium citrate) was less than and the pH of the soil greater than that reported by these authors. Further evidence that Na^+ had little effect on the solution concentration of Cd and Zn was the fact that the K_d between the 0.25 mM citric acid and sodium citrate treatments were not significantly different (both samples had the same pH).

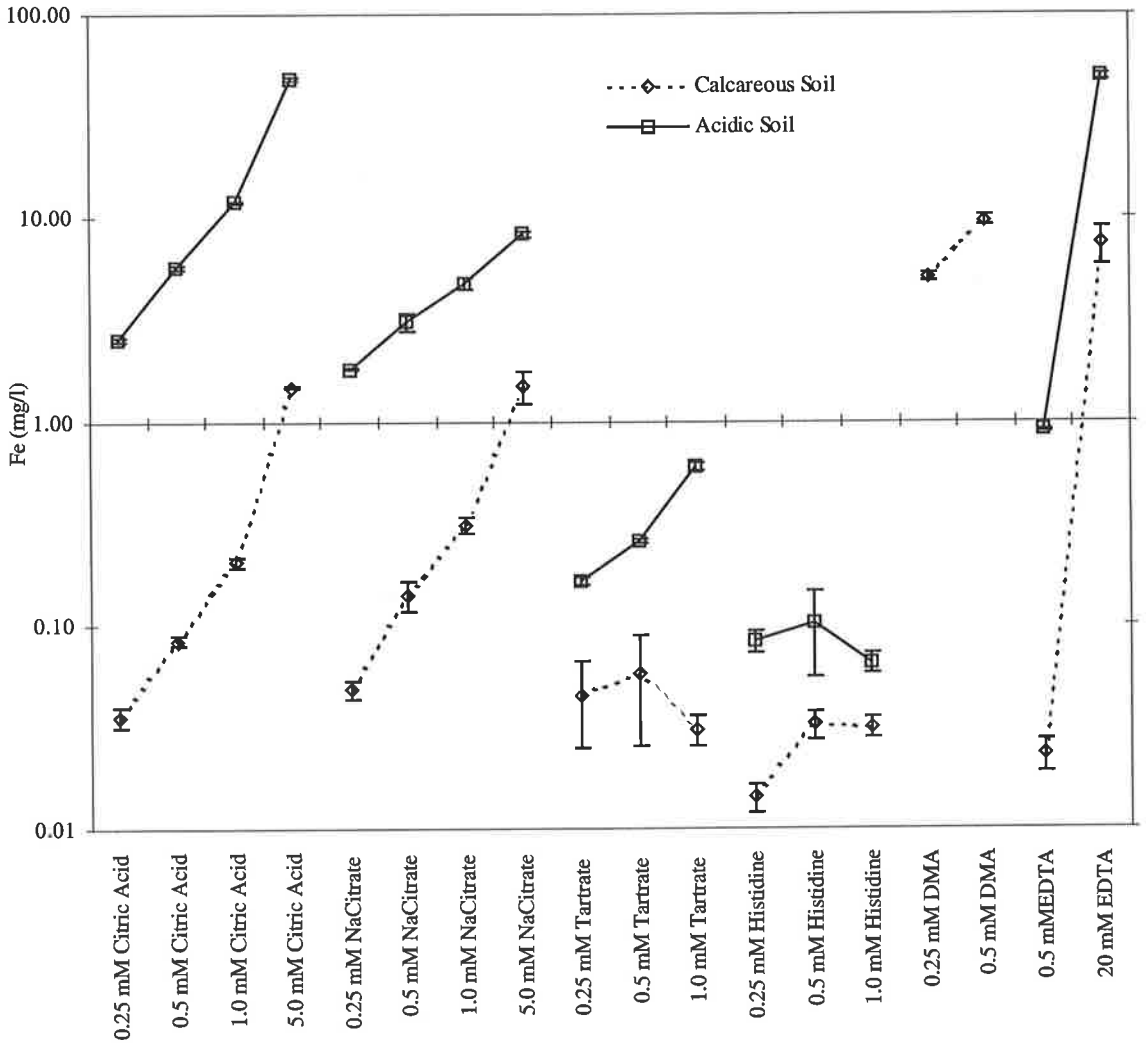
Therefore, sodium citrate, tartrate and EDTA were increasing the solution concentrations of Cd and Zn by the processes of {3} or {4} described above. In the 20 mM EDTA solution > 96 % of the Cd and Zn measured in solution was detected as the metal-EDTA complex. Therefore, EDTA, at this concentration, may have been desorbing Cd and Zn through a process analogous to {3}. However, concomitant with increases in the solution concentration of these metals, an increase in the concentration of Fe was also observed in the presence of all these ligands (Figure 6.9). Therefore, the ligands were desorbing/dissolving Cd and Zn by forming soluble complexes with Cd, Zn and/or other metals constituting the solid phase of the soil.

6.3.2.4 Effect of organic ligands on the K_d of Cd and Zn in the calcareous soil

The presence of organic ligands also decreased the K_d of Cd and Zn in the calcareous soil (Figures 6.10 and 6.11). Similar regressions to that used for the experiments with the acidic soil were applied to the data in Figures 6.1 and 6.2 to distinguish between pH and

other possible mechanisms that may have increased the solution concentrations of Cd and Zn.

Figure 6.9: The solution concentration of Fe (mg/l) in the presence of organic ligands.



As for the acidic soil, increasing concentrations of citric acid decreased pH. Similarly, the K_d values of Cd were not significantly different to those values calculated at the same pH in the absence of the ligand. However, it was not until 5 mM citric acid was used that pH became the dominate parameter controlling Zn desorption. These observations are

consistent with the results from the experiments with the acidic soil when Cd and Zn desorption became controlled by the solution concentration of H^+ when the presence of ligands generated a decrease of pH. The presence of EDTA also decreased the solution pH. In contrast to citric acid, however, EDTA reduced the K_d of Cd and Zn to a greater extent than that predicted at the same pH in the absence of the ligand. This may be a result of: 1) competitive chelation processes or; 2) the higher stability constants that Cd and Zn have with EDTA.

It was more appropriate to compare the ability of the other ligands to bring Cd and Zn into solution, in comparison to the acidic soil, because the pH of these solutions were in the range where K_d values were not affected by pH (i.e. > 99 % of the ^{109}Cd or ^{65}Zn was adsorbed). At the same initial concentration the capacity of these ligands to bring Cd and Zn into solution followed the order:

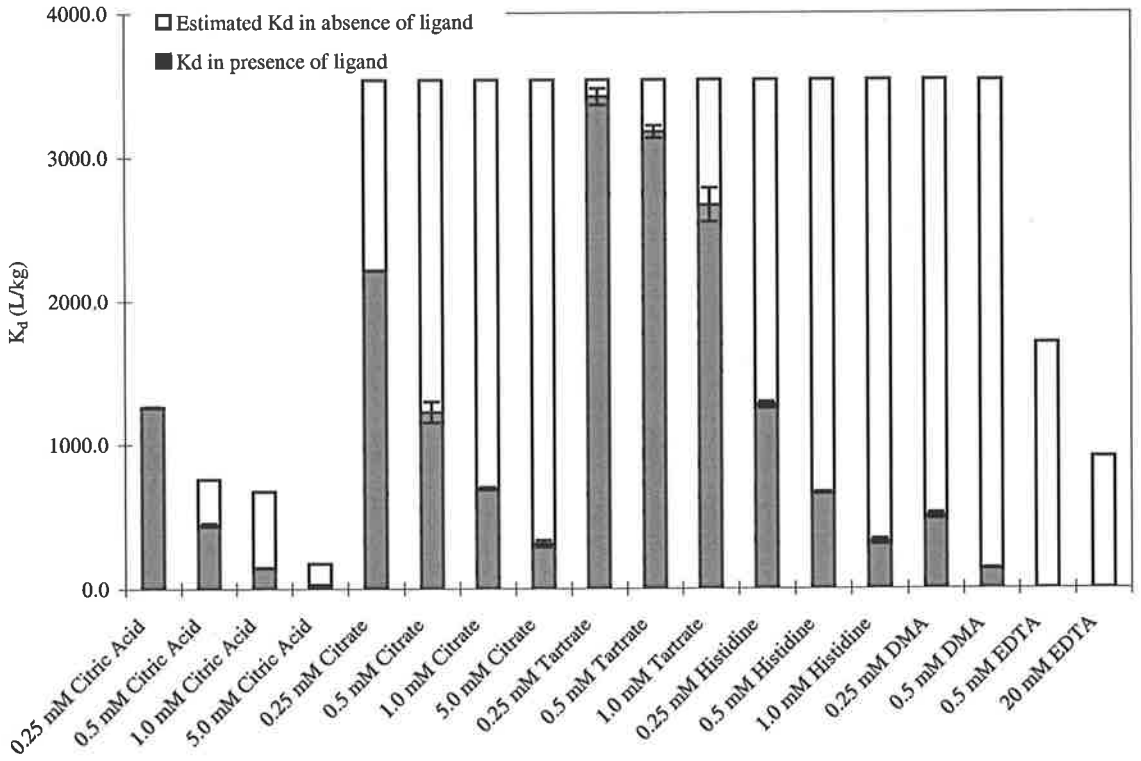
DMA > histidine > citrate > tartrate

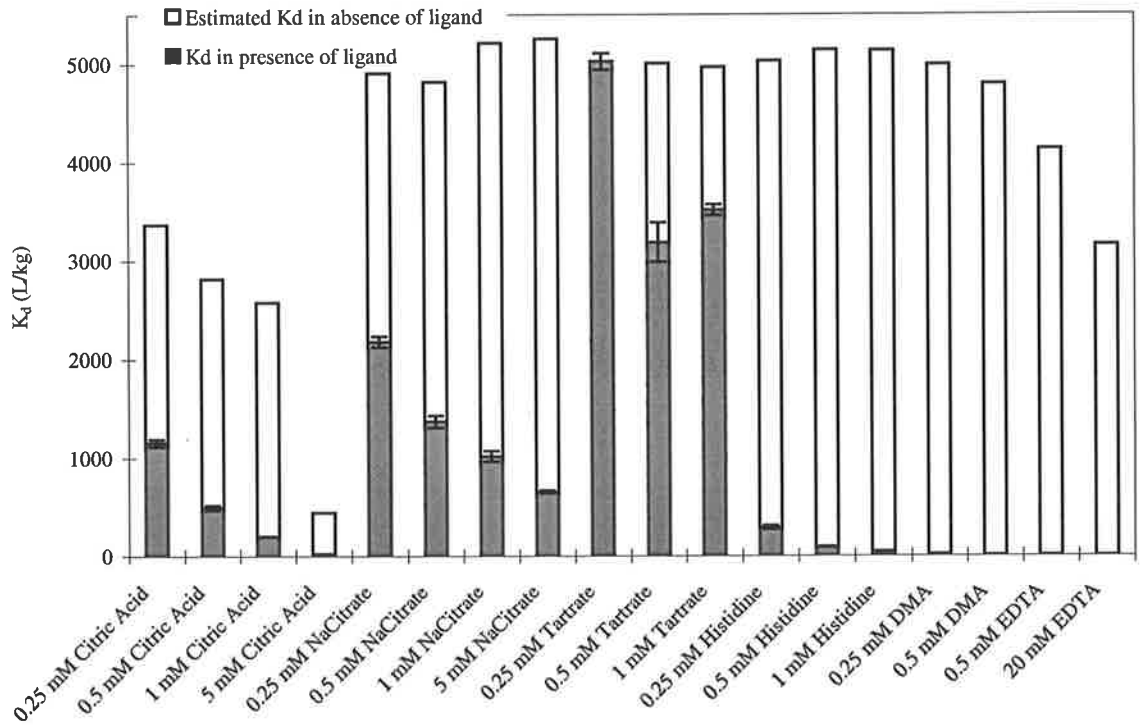
This order provides good evidence that chelation processes were controlling metal desorption as it reflects the known conditional stability constants of the dominant Cd- and Zn-ligand complexes within this pH range (359). Although the thermodynamic stability constants (log *K*) of Cd-DMA complexes are unknown, the conditional log *K* (conditional as they are adjusted for pH) of the dominant Cd- and Zn-ligand complexes with the other chelates are (359, 360):

6.08 (> 97 % $CdHistidine^+$) > 4.93 (100 % $CdCitrate^-$) > 3.85 (100 % $CdTartrate^\circ$)

12.8 (100% $ZnDMA^-$) > 7.0 (69 - 73 % $ZnHistidine^+$) - 13.0 (27 - 31 % $ZnHistidine_2^0$) > 6.25 (> 97 % $ZnCitrate^-$) > 3.55 (100 % $ZnTartrate^\circ$)

Figures 6.10 and 6.11: The K_d of Cd (top) and Zn (bottom) in the calcareous soil as affected by organic ligands compared to estimated values at the same equilibrium pH in the absence of the ligand.





Furthermore, these relationships would not be confounded by the fact that a significant quantity of histidine, at initial concentrations of 0.5 and 1 mM, was sorbed to the soil (Table 6.1). For example, despite the solution concentrations of histidine being significantly lower than those of sodium citrate, the conditional stability constants of the Cd- and Zn-histidine complexes are more than 14- and 300-fold higher, respectively, than that for the dominant Cd- and Zn-citrate complexes. In addition, it seems unlikely that cation exchange processes in the presence of 0.25 mM histidine were solely responsible for the large desorption of Cd and Zn observed in this treatment (in comparison to the acidic soil). The solution concentration of Zn in this treatment was approximately 10 μM. As this Zn concentration was approximately equal to the detection limit of histidine, it is quite plausible that this ligand, at levels below the limit of detection, was also causing desorption through chelation processes.

Further evidence that chelation was affecting the desorption of Cd and Zn, and not the dissolution of adsorbing surfaces, was found with increases in the concentration of Fe following a slightly different order (Figure 6.9):

$$\text{DMA} > \text{citrate} > \text{histidine} \approx \text{tartrate}$$

6.3.3 Isotopically exchangeable metal pools of Cd and Zn

6.3.3.1 Cd and Zn *E* values in the acidic soil as affected by pH and organic ligands

Despite the large effect of pH on the K_d of Cd and Zn in the acidic soil, variations from the natural pH of the soil only significantly changed the *E* value of Zn (Table 6.2). For example, the Zn *E* value at pH values ≤ 4.4 and ≥ 6.2 were statistically higher and lower, respectively, than that measured at the natural pH of the soil. In contrast, only at pH 3.8 was the *E* value of Cd significantly higher than that measured at pH values > 5.9.

The presence of organic ligands did not produce a Cd *E* value that was statistically different to that measured in water at the same pH (Figure 6.12). Similarly, the *E* value of Zn was generally unaffected by the presence of the ligands. However, solutions of 5 mM

citric acid and 0.5 - 5 mM sodium citrate were able to increase and decrease, respectively, the Zn *E* value in this soil. However, when the pH of these solutions were taken into account, these values were also indistinguishable from those calculated at the same pH in the absence of the ligands (Figure 6.13). Experiments using 20 mM EDTA confirmed that, even at high concentrations, the capacity of these organic ligands to alter the *E* value of Zn in this soil was due solely to their ability to induce changes of pH (i.e. and not through chelation processes).

Table 6.2: The *E* value of Cd and Zn in the acidic soil as affected by changes in the soil suspension pH.

Cadmium			Zinc		
pH	*E* (mg/kg)[1]	% of *E*[2]	pH	*E* (mg/kg)[1]	% of *E*[2]
3.8	15.30 (0.48) a	117	3.8	1920 (23) a	139
4.4	14.11 (0.17) ab	108	4.4	1810 (54) b	131
5.1	13.87 (0.45) ab	106	5.1	1440 (28) c	104
5.4	13.18 (0.60) ab	101	5.4	1410 (11) c	102
5.7	13.10 (0.46) ab	100	5.7	1380 (25) c	100
5.9	13.52 (1.29) ab	103	6.2	1050 (36) d	76.0
6.2	12.60 (0.93) b	96.2	7.6	944 (26) e	68.4
7.4	11.98 (1.57) b	91.5			

[1] % of *E* was calculated by normalizing all values to that of the soil at its natural pH (underlined).

[2] *E* values followed by the same letter are not significantly different at the 0.05 probability level for the same soil type.

The experiments conducted with 20 mM EDTA also indicated that the *E* value of Cd was indifferent to high concentrations of strong metal-complexing organic ligands (*E* = 15.1 mg/kg). This result, in conjunction with the fact that a reduction of pH to 3.8 only

increased the Cd *E* value by 17 %, indicates that the quantity of Cd that was isotopically exchangeable in this soil was a relatively stable pool of metal despite it only representing 65 % of the total content.

Figure 6.12: The *E* value of Cd in the acidic soil as affected by pH and the presence of organic ligands. The hashed lines represent 95 % confidence limits of the linear regression $E = -0.8683pH + 18.223$: $R^2 = 0.91$, $P \leq 0.001$ established for variations of the Cd *E* value with pH.

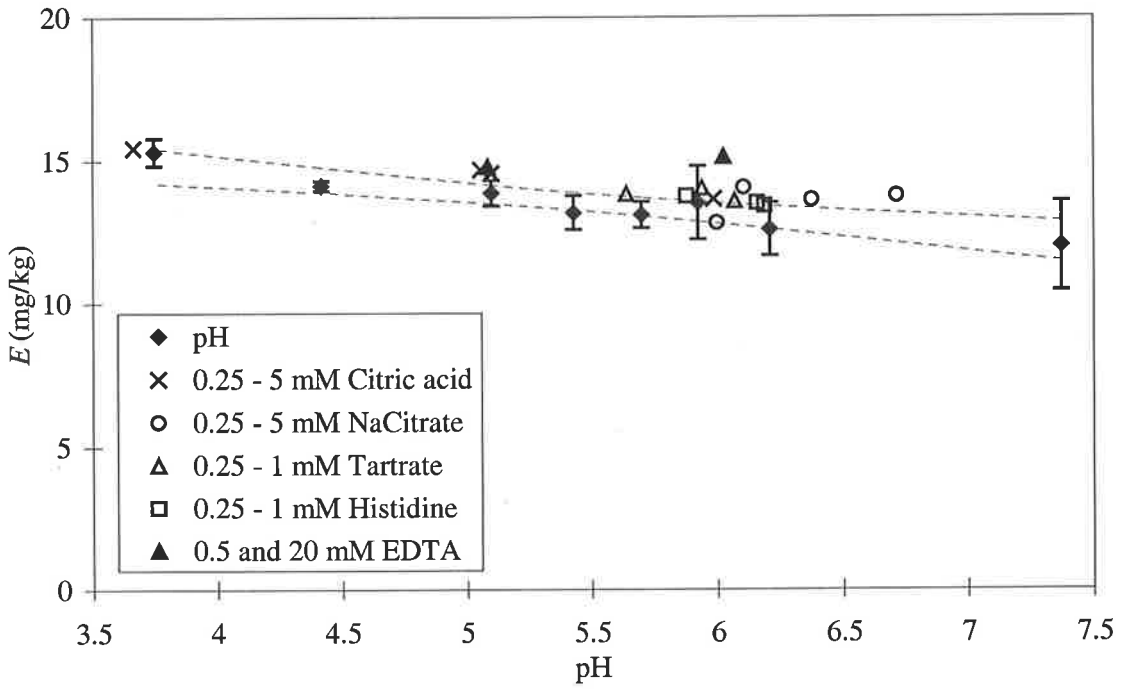
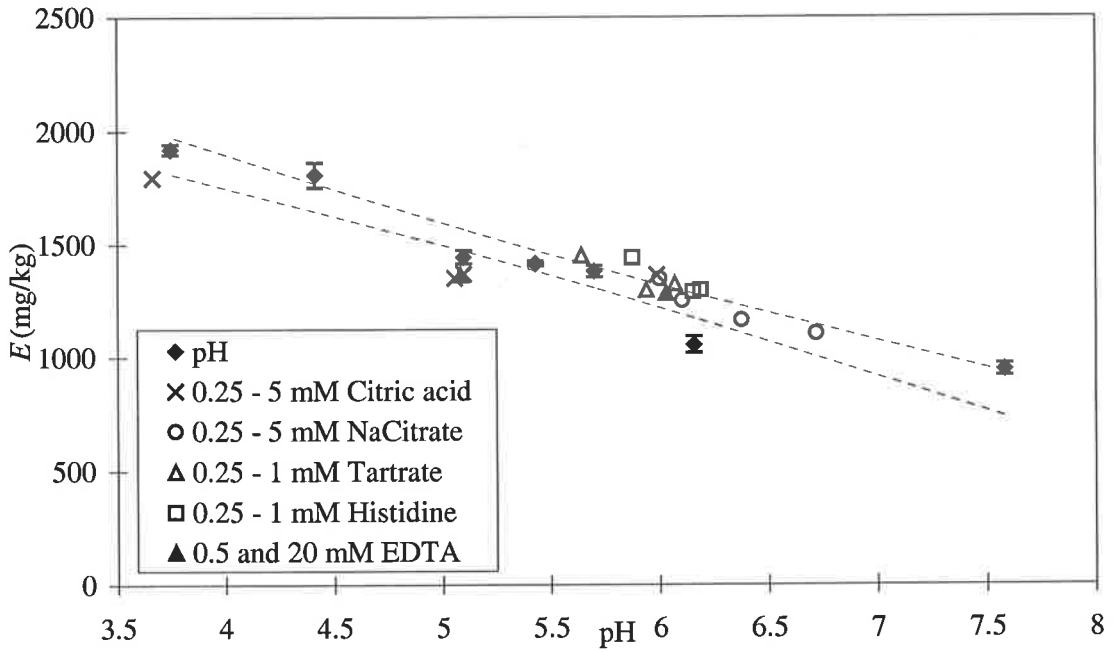


Figure 6.13: The *E* value of Zn in the acidic soil as affected by pH and the presence of organic ligands. The hashed lines represent 95 % confidence limits of the linear regression $E = -276pH + 2929$: $R^2 = 0.92$, $P \leq 0.001$ established for variations of the Zn *E* value with pH.



In contrast, the quantity of isotopically exchangeable Zn was sensitive to decreases in pH. This indicates that H^+ was liberating non-isotopically exchangeable Zn from interior or occluded sites within the soil. An increase of pH also had the ability to reduce the size of the isotopically exchangeable pool of Zn. This indicates that isotopically exchangeable Zn can also undergo reactions that transform the chemical state of the metal to one that is not in equilibrium with the solution (i.e. precipitation). This phenomenon is likely to be one of the major factors contributing to decreased Zn phytoavailability observed in soils that have either been limed or, naturally, have an alkaline pH (118, 119, 152, 396-402). Therefore, in the context of managing Zn polluted soils, the practice of increasing field pH will not only alleviate Zn phytotoxicity through a reduction of *I* but also through a *direct* reduction of the potentially phytoavailable Zn pool.

6.3.3.2 Cd and Zn *E* values in the calcareous soil as affected by pH and organic ligands

Despite having a similar total Cd content to the acidic soil, only 19 % of the Cd in the calcareous soil was isotopically exchangeable in water (Table 6.3). Similarly, after 24 hours of isotopic equilibration, the *E* value of Zn consisted a small fraction of the total soil Zn (~ 17 %). Nevertheless, a reduction of pH from 7.5 to 5.8 *tripled* the size of the Cd *E* value and almost *doubled* the *E* value of Zn. Unlike the acidic soil, an increase in pH did not significantly reduce the size of the isotopically exchangeable pools of these metals.

Variations of pH in the calcareous soil resulted in Cd and Zn *E* values that could be characterized, respectively, by a 5 parameter sigmoidal regression (as used for the K_d data) and a second order inverse polynomial regression. Although all the concentrations of citric acid increased the *E* value of Cd and at 5 mM that of Zn (compared to the *E* value measured at the natural pH of the soil), when these measurements were compared to those generated by the regressions (i.e. at the same pH without the ligand) no statistical difference between these data were observed (Figures 6.14 and 6.15). Therefore, citric acid was only increasing the quantity of isotopically exchangeable Cd and Zn through its ability to supply H^+ ions to the system.

As observed for Cd and Zn in the acidic soil, 0.5 mM EDTA did not increase the *E* value of Zn in this soil. If this concentration of EDTA is scaled up to field conditions it is approximately equal to an application rate of 5 mmole EDTA/kg. Therefore, these results suggest that the EDTA application rates typically applied during chelate-assisted phytoextraction may not increase the isotopically exchangeable pools of Cd and Zn in the acidic soil and of Zn in the calcareous soil. This tentatively extends the conclusions of Stanhope *et al.* (114) to Zn and other soil types. Furthermore, Cd *E* values measured with 0.5 mM EDTA were not statistically different to those predicted at the same pH (Figure 6.14). Measurements conducted with 10 mM $Ca(NO_3)_2$ also returned statistically identical results to the EDTA solution. However, when compared to values measured in water at pH 7.5 it appeared that 0.5 mM EDTA was able to increase the *E* value of Cd.

Therefore, assuming that a 0.5 mM EDTA solution will produce the same result as 5 mmole EDTA/kg, the solution used for determining Cd *E* values will determine whether the application of 5 mmole EDTA/kg apparently increases the *L* value of Cd in this soil.

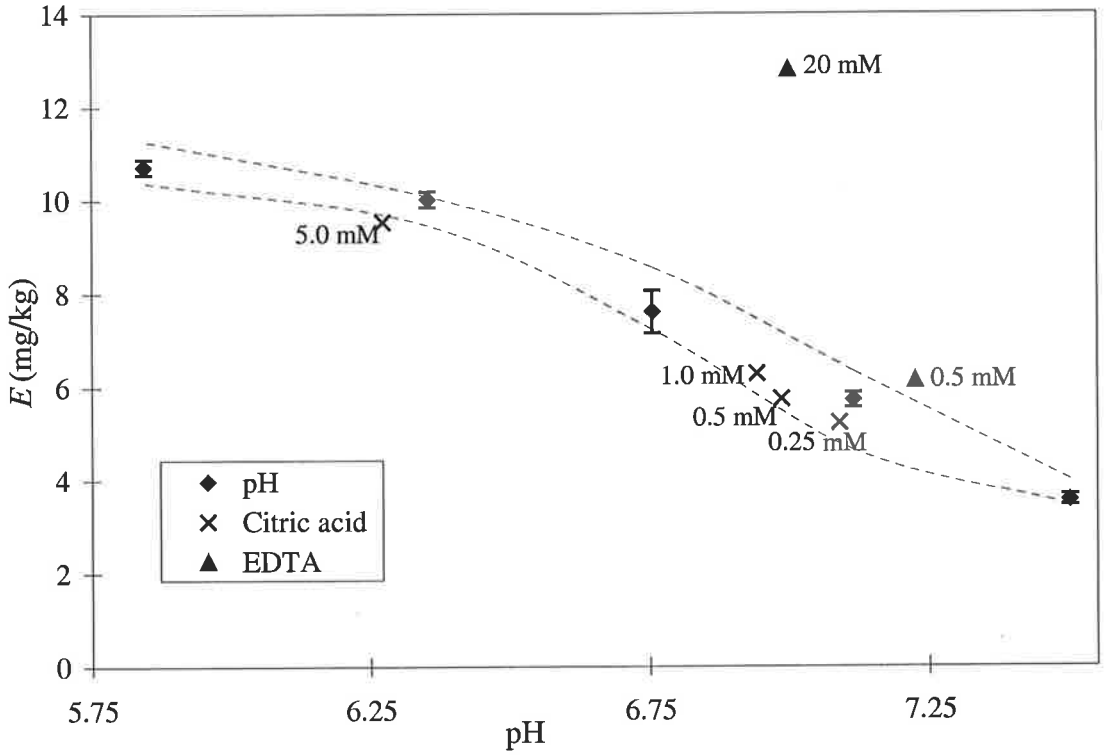
Table 6.3: The *E* value of Cd and Zn in the calcareous soil as affected by changes in the soil suspension pH.

Cadmium			Zinc		
pH	*E* (mg/kg)[1]	% of *E*[2]	pH	*E* (mg/kg)[1]	% of *E*[2]
5.8	10.71 (0.09) a	301	5.7	421 (3.2) a	180
6.4	10.01 (0.17) b	281	6.0	344 (1.6) b	147
6.8	7.61 (0.17) c	214	6.6	285 (8.9) c	122
7.1	5.73 (0.45) d	161	7.1	262 (20) cd	111
7.5	3.56 (0.16) e	100	7.5	234 (19) de	100
8.5	3.53 (0.12) e	99.1	8.4	221 (4.7) e	94.4

[1] % of *E* was calculated by normalizing all values to that of the soil at its natural pH (underlined).

[2] *E* values followed by the same letter are not significantly different at the 0.05 probability level for the same soil type.

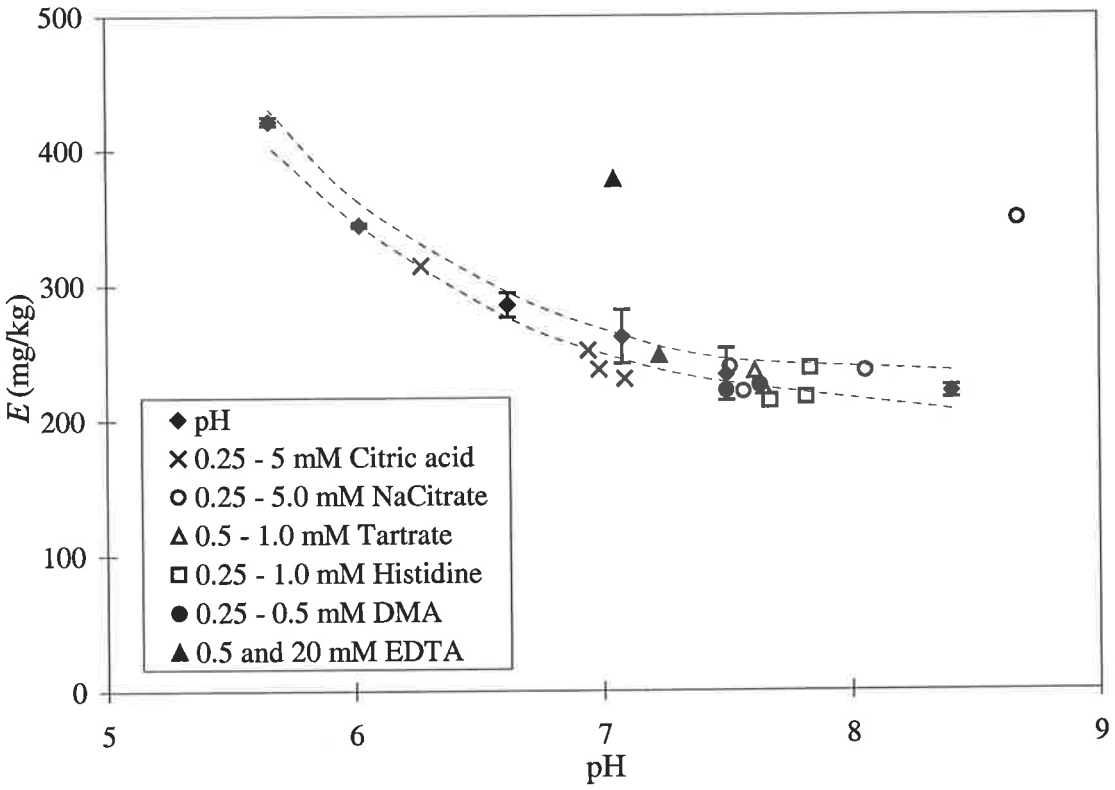
Figure 6.14: The *E* value of Cd in the calcareous soil as affected by pH and the presence of citric acid. The hashed lines represent 95 % confidence limits of the fitted 5 parameter sigmoidal regression established for variations of the Cd *E* value with pH - $E = 3.44 + 7.83/(1 + e^{-(pH-8.23)/-0.41})^{21}$: $R^2 = 0.99$, $P \leq 0.0001$.



The other organic ligands, with the exception of 0.5 mM tartrate, also had the capability to increase the quantity of isotopically exchangeable Cd in the calcareous soil (Figure 6.16). Variability in the results between the replicates of 0.25 mM tartrate were high and an inspection of the solution concentrations of Cd, Zn and Fe indicated that these solutions had been contaminated with stable metals. As a result, the Cd (and Zn) *E* values for this treatment are not reported. Despite the Cd *E* value generally increasing with the concentration of the other organic ligands, no comprehensive relationship could be ascertained. In contrast, with the exception of 5 mM sodium citrate and 20 mM EDTA, these organic ligands did *not* increase the *E* value of Zn above that predicted at

the same pH in the absence of the ligand (Figure 6.15). This result would be expected considering that the pool of isotopically exchangeable Zn within this pH range was at least 63-fold larger than that of Cd. Therefore, only at high concentrations would these organic ligands exhibit the same ability to increase the *E* value of Zn through chelation processes.

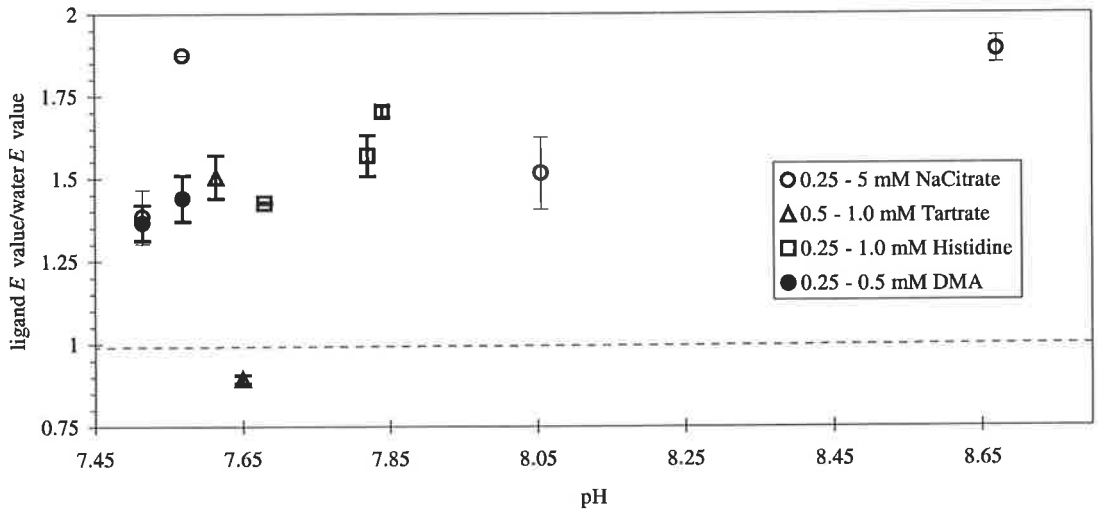
Figure 6.15: The *E* value of Zn in the calcareous soil as affected by pH and the presence of organic ligands. The hashed lines represent 95 % confidence limits of the second order inverse polynomial regression - $E = 955 + (-12586/pH) + (53965/pH^2)$: $R^2 = 0.97$, $P \leq 0.0001$ - established for variations of the Zn *E* value with pH.



Based on - 1) the premise that the *E* value represents the *total* quantity of surface-adsorbed element that is in equilibrium with the solution phase and; 2) the result that the

K_d of Cd and Zn both had an approximate (negative) 1:1 relationship with the solution concentration of H^+ - it maybe inferred that the increase of the *E* value in this soil was a result of the dissolution of monovalent cations of Cd and Zn from precipitated minerals or mixed solids.

Figure 6.16: The effect of organic ligands on the *E* value of Cd in the calcareous soil. Values represent the ratio of the *E* value measured in the presence of the ligand against that measured in the absence of the ligand at pH 7.5. Symbols and error bars represent the mean and standard deviation of triplicate measurements.



Nevertheless, this data may also be interpreted as the desorption of non-isotopically exchangeable surface-adsorbed Cd and Zn, since strongly adsorbed cations that are not isotopically exchangeable are also not in equilibrium with the solution phase (147). This latter possibility was examined in further detail with experiments using a 10 mM solution of $Ca(NO_3)_2$. This solution only produced a higher Cd *E* value (4.5 mg/kg). However, the ionic strength of this solution decreased the equilibrium pH to 7.1 and when this was taken into account no significant difference between *E* values were observed. Therefore, due to the confounding decreases of pH for Cd, and possibly the need for higher

concentrations of $Ca(NO_3)_2$ to influence the *E* value of Zn, the hypothesis that surface-adsorbed Cd and Zn can exist in non-isotopically exchangeable forms awaits further confirmation.

6.4 Summary

In these experiments it was demonstrated that the *E* value of the acidic soil measured in water represented a major portion, if not all, of the surface-adsorbed Cd. This conclusion was supported by the fact that decreases of pH and variations of solution composition, although greatly affecting K_d, had no significant effect on the size of the isotopically exchangeable pool of Cd. Based on these observations it is unlikely that conditions in the rhizosphere would alter the isotopic exchange properties of Cd in this soil. Therefore, from a practical point of view, the *E* value, regardless of the solution composition used for isotopic equilibration, would be a good indication of the total quantity of Cd that would be phytoavailable (*L* value) in this soil.

In contrast, variations of the Zn *E* value in the acidic soil and of both metals in the calcareous soil indicated that isotopically exchangeable Cd and Zn measured in water may not represent all of the metal potentially available upon acidification or in the presence of organic ligands (assuming that the complexed metal is completely labile). Therefore, an observed increase in the *E* value could indicate either:

1) a process of dissolution from interior or occluded sites or;
2) desorption of strongly adsorbed (chemisorbed) cations that are not isotopically exchangeable and not in equilibrium with the solution phase (147).

To determine which process is dominant is problematic, but may be investigated by determining *E* values in the presence of metals that readily displace chemisorbed Cd and Zn, such as Pb^{2+} and Cu^{2+} (assuming the sites are competitive for all metals) (403).

In conclusion, the results of this chapter highlight the fact that the *E* value will only be a reliable indication of the quantity of phytoavailable Cd or Zn (*L* value) under one condition - when the isotopically exchangeable pool of metal is insensitive to changes of K_d induced by environmental perturbations (e.g. pH, chelation, etc.). This condition was only met for Cd in the acidic soil. However, in soils where isotopically exchangeable Cd and Zn varies with K_d it will also be possible, but much more difficult, to predict the quantity of phytoavailable metal, but only if the K_d measured in the laboratory determined *E* values is exactly the same as the K_d in the rhizosphere. As such, plant-induced decreases of pH and/or the exudation of organic ligands may increase the quantity of isotopically exchangeable metal in these types of soils and, therefore, may possibly account for previously observed differences between *L* and *E* values of Cd and Zn (37, 43-46, 101).

7. Conclusions and further studies

7.1 Introduction

The following is a reiteration of the major conclusions derived from the data presented in this thesis. In addition, a discussion is provided on further studies necessary to compliment the existing scientific understanding of the behavior of Cd and Zn in polluted soils and, therefore, that need to be considered before the implementation of a remediation strategy.

7.2 Analytical techniques for the measurement of intact metal-ligand complexes

IC-MS can be used to unequivocally identify and quantify a large number of metal-EDTA complexes in soil solution and plant xylem exudate.

This methodology has the potential to measure an even larger range of metal-ligand complexes, provided that the complexes have a high thermodynamic stability constant and also remain stable at pH values experienced during anion exchange.

For example, Cd and Zn complexation by the phytosiderophores of the mugineic acid family are areas of research that could greatly benefit from the use of this methodology. The log *K*'s of Zn with the mugineic acids are high, but are unknown for Cd, and their complexes appear to be stable at alkaline pH values (283). Indeed, the results from Chapter 6 indicated that DMA was one of the most effective organic ligand to mobilize Cd and Zn from the solid phase of the calcareous soil into solution. Therefore, IC-MS may be able to quantify these metal-ligand complexes and eliminate the difficulties involved with the current cumbersome HPLC techniques (404, 405). In fact, Neumann *et al.* (363) have already reported the separation of 4 mugineic acids on the identical IC column used to quantify histidine, citrate and tartrate in the experiments detailed in Chapter 3 - the Dionex AS11. This type of methodology will be essential to resolve the current controversy surrounding the plant uptake of intact Zn-phytosiderophores (281, 289, 293).

In addition, the range of metal-ligand complexes that can be quantified by IC-MS may be extended to those complexes having lower log *K*'s, and/or which are unstable at high pH values, by using a less alkaline eluent. The longer retention times resulting from the use of this eluent could be overcome through the use of shorter IC columns (e.g. the sole use of a guard IC column).

The development of this method also has broad implications for research on the metal speciation of EDTA in the environment. IC-MS may potentially be used to determine the role of EDTA in the migration of ^{60}Co from the USA Department of Energy's Oak Ridge, Tennessee and Hanford, Washington sites (343, 406, 407) and the remobilization of heavy metals from river (408) and estuarine (369) sediments. Furthermore, this analytical procedure has the capacity to determine the exchange kinetics of strongly-chelated metals that up until now have been difficult to predict in the environment beyond the generalization that they react much more slowly than aqua or mono-dentate ligand complexes (52). This information would supply critical data for the FIAM of biotic metal uptake in aquatic systems because it is generally not known whether metal transport is under equilibrium or kinetic control.

7.3 The processes involved in EDTA-assisted Zn phytoextraction

During the course of EDTA-assisted Zn phytoextraction:

1) EDTA mobilizes soil Zn into solution through chelation/desorption processes and;
2) plant uptake of ZnEDTA from soil is dependent on the concentration of the complex and the plant species.

As mentioned in Chapter 5, further studies may be aimed at determining the exact mechanisms responsible for the differences observed in the plant uptake of Zn between species (at a given ZnEDTA concentration). However, this knowledge may or may not

significantly contribute to utilizing EDTA-assisted Zn phytoextraction as a remedial tool. For example, high concentrations of EDTA are needed to induce Zn accumulation, therefore, regardless of the nature of ZnEDTA accumulation, a large proportion of the metal-EDTA complexes remain in the soil and represent an environmental risk through off-site migration. Although this point has often been cited as a flaw to chelate-assisted phytoextraction, research on this topic remains scant.

Furthermore, the plant accumulation of Zn using EDTA-assisted phytoextraction observed in this research and elsewhere (29, 88) has never reached the levels reported for Pb - > 10000 mg Pb/kg dry weight (29, 31, 32, 330, 331). This lack of induced 'hyperaccumulation' needs to be addressed in the future as this will be one of the determining factors in the success, or otherwise, of this technology.

These results also have ramifications for using EDTA, or other similar aminocarboxylic acids, to buffer metal activities in nutrient solutions that are used for plant research (Chapter 2). These studies have routinely used these chelates based on the assumption that only the free ion is available for plant uptake (FIAM). The evidence presented in this thesis suggests that this maybe a valid assumption when using low concentrations of EDTA. However, interpretational errors will occur at higher concentrations and, furthermore, the magnitude of this error will be dependent on the plant species being tested.

7.4 Isotopically exchangeable soil Cd and Zn

Variations of pH and the use of organic ligands modify the *E* value of Cd and Zn in polluted soils.

In addition:

1) a portion of Cd and Zn remains non-isotopically exchangeable;

2) if an organic ligand decreases pH then it is the relationship between pH and the chemical form of the metal in the soil which controls the quantity of isotopically exchangeable Cd and Zn;
3) higher concentrations of organic ligands are needed to increase the *E* value when the quantity of isotopically exchangeable metal is also high and;
4) if the concentration of an organic ligand is not high enough to affect the *E* value then the size of the isotopically exchangeable pool of metal is also controlled by the relationship between pH and the chemical form of the metal in the soil.

Therefore, only in soils that have similar characteristics to the acidic soil - where isotopically exchangeable Cd was indifferent to chemical conditions likely to be experienced in the rhizosphere and, furthermore, was not readily replenished by non-isotopically exchangeable pools - will phytoextraction based on metal phytoavailability have the potential to mitigate risks associated with these soils over a short time period.

Another significant finding from the experiments outlined in Chapter 6 was the sorption behavior of histidine. These results indicate that histidine, released into the rhizosphere, at pH values below its isoelectric point will be strongly sorbed to the soil surface. This confirms the data of Jones *et al.* (171) who noted that the sorption of four other amino acids were related to their isoelectric point. This relationship raises a serious doubt to the hypothesis that histidine is involved in 'high rates of nickel transport into the xylem required for hyperaccumulation in the shoot' of the Ni-hyperaccumulator *A. lesbiacum* or non-hyperaccumulator *A. montanum* (296). This conclusion was based on the results that adding 0.3 mM histidine to the solution of a hydroponically grown *A. montanum* plant increased Ni flux through its xylem. However, considered in light of the sorption behavior of histidine in the soil environment, it is highly unlikely that these types of concentrations would be observed at pH values below 7.47. Therefore, the involvement of histidine in Ni hyperaccumulation will either be limited to within the plant or to soils

with pH values > 7.5. This highlights the limitations of hypotheses developed from nutrient solution studies when they have not been validated in a soil medium.

Finally, the data reported in this thesis offer a solution to the observed differences between *E* and *L* values and *L* values between plant species (36, 37, 43-46, 101). It was demonstrated that the quantity of isotopically exchangeable Cd and Zn in soils may vary with chemical conditions likely to be experienced in the rhizosphere. Therefore, the *E* value will only be a reliable indication of the *L* value when the isotopically exchangeable pool of metal is insensitive to these changes. Furthermore, if conditions in the rhizosphere affect the isotopic exchange properties of a soil, such as the calcareous soil, then metal phytoavailability (*L* value) will simply be a relative term dependent on the plant species. Therefore, with this knowledge, it may now be necessary to reevaluate conclusions that have been made from previous studies. For example, rather than concluding that *B. napus* was unable to access a pool of non-isotopically exchangeable Cd that was available to other plant species (36), *B. napus* may simply have decreased the size of the isotopically exchangeable pool of Cd through inducing a rise of pH in its rhizosphere. Nevertheless, field experiments are now ultimately needed to verify that the processes identified in these experiments also occur in the rhizosphere.

8. References

1. Alloway B J (1990). The origin of heavy metals in soils. In *Heavy metals in soils*, Alloway B J, Editor. *Blackie: London. pp. 29-39.*
2. Barak P and P A Helmke (1993). The chemistry of zinc. In *Zinc in soils and plants*, Robson A D, Editor. *Kluwer Academic Publishers: Dordrecht, Germany. pp. 1-13.*
3. Allen H E (1995). Metal speciation and contamination of soil. *Lewis publishers: Boca Raton, FL, USA. p. 358*
4. Dudka S and D C Adriano (1997). Environmental impacts of metal ore mining and processing: a review. *J. Environ. Qual.* ***26****: 590-602.*
5. Shuman L M (1980). Zinc in soils. In *Zinc in the Environment, Part 1. Ecological Cycling*, Nriagu J O, Editor. *John Wiley and Sons: New York, NY, USA. pp. 39-69.*
6. Page A L, F T Bingham and A C Chang (1981). Cadmium. In *Effect of heavy metal pollution on plants, Vol 1: Effects of trace metals on plant function*, Lepp N W, Editor. *Applied Science Publishers: Barking, Essex, England. pp. 77-109.*
7. Moore J W (1991). Inorganic contaminants of surface waters. Research and monitoring priorities. *Springer-Verlag: New York, NY, USA. p. 334.*
8. Thornton I (1981). Geochemical aspects of the distribution and forms of heavy metals in soils. In *Effect of heavy metal pollution on plants, Vol 2: Metals in the environment*, Lepp N W, Editor. *Applied Science Publishers: Barking, Essex, England. pp. 1-34.*
9. Watanabe M (1997). Phytoremediation on the brink of commercialization. *Environ. Sci. Technol.* ***3****(4): 182A-186A.*
10. Meulen-Smidt G R B ter (1995). Regional differences in potentials for delayed mobilisation of chemicals in Europe. In *Biogeodynamics of pollutants in soils and sediments: Risk assessment of delayed and non-linear responses*, Salomons W and W M Stigliani, Editors. *Springer-Verlag: Berlin, Germany. pp. 135-169.*

11. Cunningham S D, J R Shann, D E Crowley and T A Anderson (1998). Phytoremediation of contaminated water and soil. In *Phytoremediation of soil and water contaminants*, Kruger E L, T A Anderson, and J R Coats, Editors. *American Chemical Society: Danvers, MA, USA. pp. 3-16.*

12. Salomons W (1995). Long-term strategies for handling contaminated sites and large-scale areas. In *Biogeodynamics of pollutants in soils and sediments: Risk assessment of delayed and non-linear responses*, Salomons W and W M Stigliani, Editors. *Springer-Verlag: Berlin, Germany. pp. 1-30.*

13. McGrath S P (2000). Soil Contamination. In *Remade Lands 2000. International conference on the remediation and management of degraded lands. Fremantle, WA, Australia: Promaco Conventions Pty Ltd.*

14. Alloway B J, *et al.* (1999). MAFF OC09325 Contract Report. *Ministry of Agriculture, Fisheries and Food: London, England.*

15. McLaughlin M J, K G Tiller, R Naidu and D P Stevens (1996). Review: The behaviour and environmental impact of contaminants in fertilisers. *Aust. J. Soil Res.* ***34****: 1-56.*

16. Holmgren G G S, M W Meyer, R L Chaney and R B Daniels (1993). Cadmium, lead, zinc, copper, and nickel in agricultural soils of the United States of America. *J. Environ. Qual.* ***22****: 335-348.*

17. Johnston A E and K C Jones (1995). The origin and fate of cadmium in soil. *The Fertiliser Society: Thorpe Wood, Peterborough, England. p. 34.*

18. Chaney R L and D P Oliver (1996). Sources, potential adverse effects and remediation of agricultural soil contaminants. In *Contaminants and the soil environment in the Australiasia-Pacific region*, Naidu R, *et al.*, Editors. *Kluwer Academic Publishers: Dordrecht. Germany. pp. 323-359.*

19. Gupta U C and S C Gupta (1998). Trace element toxicity relationships to crop production and livestock and human health: implications for management. *Commun. Soil Sci. Plant Anal.* ***29****(11-14): 1491-1522.*

20. Maxwell C D (1995). Microbial ecology of Sudbury soils. In *Environmental restoration of the industrial city*, Lal F and B A Stewart, Editors. *Springer-Verlag: Berlin, Germany. pp. 219-231.*

21. Kabata-Pendias A and H Pendias (1991). Trace elements in soils and plants, 2nd Edition. *CRC Press: Boca Raton, FL, USA. p. 365.*

22. Beeftink W G and J Nieuwenhuize (1982). Heavy-metal accumulation in salt marshesfrom the western and eastern Scheldt. *Sci. Total Environ.* ***25****: 199-223.*

23. Evanko C R and D A Dzombak (1997). Remediation of metals-contaminated soils and groundwater. Technology Evaluation Report. *Ground-Water Remediation Technologies Analysis Center: Pittsburgh, PA, USA. p. 53.*

24. Khan A G, T M Chaudhury, C Khoo and W J Hayes (1998). Role of plants, mycorrhizae and phytochelators in heavy metal contaminated land assessment and remediation. In *International conference on environmental contamination, toxicology and health. Hong Kong: Perkin Elmer.*

25. Brooks R R (1998). Plants that hyperaccumulate heavy metals. *CAB International: Wallingford, Oxon, UK. p. 380.*

26. Brown S L, R L Chaney, J S Angle and A J M Baker (1995). Zinc and cadmium uptake by the hyperaccumulator *Thlaspi caerulescens* grown in nutrient solution. *Soil Sci. Soc. Am. J.* ***59****: 125-133.*

27. Kumar N P B A, V Dushenkov, H Motto and I Raskin (1995). Phytoextraction: The use of plants to remove heavy metals from soils. *Environ. Sci. Technol.* ***29****: 1232-1238.*

28. Ebbs S D and L V Kochian (1997). Toxicity of zinc and copper to *Brassica* species: Implications for phytoremediation. *J. Environ. Qual.* ***26****: 776-781.*

29. Blaylock M, D E Salt, S Dushnekov, O Zakharova, C Gussman, Y Kapulnik, B D Ensley and I Raskin (1997). Enhanced accumulation of Pb in Indian Mustard by soil-applied chelating agents. *Environ. Sci. Technol.* ***31****: 860-865.*

30. Epstein A L, C D Gussman, M J Blaylock, U Yermiyahu, J W Huang, Y Kapulnik and C S Orser (1999). EDTA and Pb-EDTA accumulation in *Brassica juncea* grown in Pb-amended soil. *Plant Soil* ***208****: 87-94.*

31. Huang J W and S D Cunningham (1996). Lead phytoextraction: species variation in lead uptake and translocation. *New Phytol.* ***134****: 75-84.*

32. Huang J W, J Chen, W R Berti and S D Cunningham (1997). Phytoremediation of lead-contaminated soils: role of synthetic chelates in lead phytoextraction. *Environ. Sci. Technol.* ***31****: 800-805.*

33. Huang J W, J Chen and S D Cunningham (1997). Phytoextraction of lead from contaminated soils. In *Phytoremediation of soil and water contaminants*, Kruger E L, T A Anderson, and J R Coats, Editors. *American Chemical Society: Washington DC, USA. pp. 283-306.*

34. Vassil A D, Y Kapulnik, I Raskin and D E Salt (1998). The role of EDTA in lead transport and accumulation by Indian Mustard. *Plant Physiol.* ***117****: 447-453.*

35. Felix H (1997). Field trials for *in situ* decontamination of heavy metal polluted soils using crops of metal-accumulating plants. *Z. Pflanzenernahr. Bodenk.* ***160****: 525-529.*

36. Hamon R, J Wundke, M McLaughlin and R Naidu (1997). Availability of zinc and cadmium to different plant species. *Aust. J. Soil Res.* ***35****: 1267-1277.*

37. Smolders E, K Brans, A Foldi and R Merckx (1999). Cadmium fixation in soils measured by isotopic dilution. *Soil Sci. Soc. Am. J.* ***63****: 78-85.*

38. Tiller K G, J L Honeysett and M P C deVries (1972). Soil zinc and its uptake by plants. I. Isotopic exchange equilibria and the application of tracer techniques. *Aust. J. Soil Res.* ***10****: 151-164.*

39. Tiller K G (1996). Soil contamination issues: past, present and future, a personal perspective. In *Contaminants and the soil environment in the Australasia-Pacific region*, Naidu R, *et al.* Editors. *Kluwer Academic Publishers: Dordrecht. Germany. pp. 1-27.*

40. Hamon R E, M J McLaughlin, R Naidu and R Correll (1998). Long-term changes in cadmium bioavailability in soil. *Environ. Sci. Technol.* ***32****: 3699-3703.*

41. McLaughlin M J (2001). Bioavailability of metals to terrestrial plants. In *Bioavailability of metals in terrestrial ecosystems. Importance of partitioning for bioavailability to invertebrates, microbes and plants*, Allen H E, Editor. *Society of Environmental Toxicology and Chemistry: Pensacola, FL, USA. (In press).*

42. Tiller K G and P Wassermann (1971). Radioisotopic techniques and zinc availability in soil. In *Symposium on the use of isotopes and radiation in research on soil-plant relationships, including applications in forestry. Vienna, Austria: International Atomic Energy Agency.*

43. Rule J H and E R Graham (1976). Soil labile pools of manganese, iron, and zinc as measured by plant uptake and DTPA equilibrium. *Soil Sci. Soc. Am. J.* ***40****: 853-857.*

44. Sinha M K, K S Dhillon and S K Dhillon (1977). Labile pool and selective distribution of zinc in soils. I. Comparison of laboratory and greenhouse measurements of labile zinc in alkaline soils. *Plant Soil* ***48****: 369-385.*

45. Dhillon K S, S K Dhillon and M K Sinha (1984). Selective distribution and labile pool of zinc in some alkaline soils of the Punjab state. *J. Nuclear Ag. Biol.* ***13****(2): 40-43.*

46. Gerard E, G Echevarria, T Sterckeman and J L Morel (2000). Cadmium availability to three plant species varying in cadmium accumulation pattern. *J. Environ. Qual.* ***29****: 1117-1123.*

47. Nieboer E and D H S Richardson (1980). The replacement of the nondescript term 'heavy metals' by a biologically and chemically significant classification of metal ions. *Environ. Pollut. (series B)* ***1****: 3-26.*

48. Venugopal B and T P Luckey (1975). Toxicology of non-radioactive heavy metals and their salts. In *Heavy metal toxicity, safety and hormology*, Luckey T D, B Venugopal, and D Hutcheson, Editors. *George Thieme: Stuttgart, West Germany. pp. 4-73.*

49. Piscator M (1986). The dependence of toxic reactions on the chemical species of elements. In *The importance of chemical 'speciation' in environmental processes*, Bernhard M, R E Brinkman, and P J Sadler, Editors. *Springer-Verlag: Berlin, West Germany. pp. 59-70.*

50. Halvorson A D and W L Lindsay (1977). The critical Zn^{2+} concentration for corn and the nonabsorption of chelated zinc. *Soil Sci. Soc. Am. J.* ***41****: 531-534.*

51. Marschner H (1995). Mineral nutrition of higher plants, second edition. *Academic Press: San Diego, CA, USA. p. 889.*

52. Hudson R J M (1998). Which aqueous species control the rates of trace metal uptake by aquatic biota? Observations and predictions of non-equilibrium effects. *Sci. Total Environ.* ***219****: 95-115.*

53. Van Der Werff M M and T Out (1981). The effect of humic acid as Zn complexing agent on water cultures of *Holcus lanatus. Biochem. Physiol. Pflanzen.* ***176****: 274-282.*

54. Taylor G J and C D Foy (1985). Differential uptake and toxicity of ionic and chelated copper in *Triticum aestivum. Can. J. Bot.* ***63****: 1271-1275.*

55. McGrath S P and A M Chaudri (1999). Long term effects of metal contamination on *Rhizobium. Soil Biol. Biochem.* ***31****: 1205-1207.*

56. McGrath S P, A M Chaudri and K E Giller (1995). Long-term effects of metals in sewage sludge on soils, microorganisms and plants. *J. Indust. Microbiol.* ***14****: 94-104.*

57. Hartenstein R, E F Neuhauser and A Narahara (1981). Effects of heavy metal and other elemental additives to activated sludge on growth of *Eisenia fetida. J. Environ. Qual.* ***10****: 372-376.*

58. Porter J R and R P Sheridan (1981). Inhibition of nitrogen fixation in alfalfa by arsenate, heavy metals, fluoride, and simulated acid rain. *Plant Physiol.* ***68****: 143-148.*

59. Huang C-Y, F A Bazzaz and L N Vanderhoef (1974). The inhibition of soybean metabolism by cadmium and lead. *Plant Physiol.* ***54****: 122-124.*

60. Davies B E (1990). Lead. In *Heavy metals in soils*, Alloway B J, Editor. *Blackie: London, England. pp. 177-196.*

61. McGrath S P (1999). Adverse effects of cadmium on soil microflora and fauna. In *Cadmium in soils and plants*, McLaughlin M J and B R Singh, Editors. *Kluwer Academic Publishers: Dordrecht, Germany. pp. 199-218.*

62. Kiekens L (1990). Zinc. In *Heavy metals in soils*, Alloway B J, Editor. *Blackie: London, England. pp. 284-305.*

63. Collins J C (1981). Zinc. In *Effect of heavy metal pollution on plants, Vol I: Effects of trace metals on plant function*, Lepp N W, Editor. *Applied Science Publishers: Barking, Essex, England. pp. 145-169.*

64. Miller R J, J E Bittell and D E Koeppe (1973). The effect of cadmium on electron and energy transfer reactions in corn mitochondria. *Physiol. Plant.* ***28****: 166-171.*

65. Marchiol L, L Leita, M Martin, A Peressotti and G Zerbi (1996). Physiological responses of two soybean cultivars to cadmium. *J. Environ. Qual.* ***25****: 562-566.*

66. Pahlsson A-M B (1989). Toxicity of heavy metals (Zn, Cu, Cd, Pb) to vascular plants. *Water Air Soil Pollut.* ***47****: 287-319.*

67. Mortvedt J J (1996). Heavy metal contaminants in inorganic and organic fertilizers. *Fert. Res.* ***43****: 55-61.*

68. McLaughlin M J, R E Hamon, R G McLaren, T W Speir and S L Rogers (2000). Review: A bioavailability-based rationale for controlling metal and metalloid contamination of agricultural land in Australia and New Zealand. *Aust. J. Soil Res.* ***38****: 1037-1086.*

69. Ebbs S D, M M Lasat, K J Brady, J Cornish, R Gordon and L V Kochian (1997). Phytoextraction of cadmium and zinc from a contaminated soil. *J. Environ. Qual.* ***26****: 1424-1430.*

70. Takkar P N and M S Mann (1978). Toxic levels of soil and plant zinc for maize and wheat. *Plant Soil* ***49****: 667-669.*

71. Rauser W E and A B Samarakoon (1980). Vein loading in seedlings of *Phaseolus vulgaris* exposed to excess cobalt, nickel, and zinc. *Plant Physiol.* ***65****: 578-583.*

72. VanAssche F and H Clijsters (1986). Inhibition of photosynthesis in *Phaseolus vulgaris* by treatment with toxic concentrations of zinc: effects on electron transport and photophosphorylation. *Physiol. Plant.* ***66****: 717-721.*

73. Ochiai E (1977). Bioinorganic chemistry: An introduction. *Allyn and Bacon: Boston, MA, USA. p. 515.*

74. Blundell T L, J F Cutfield, S M Cutfield, E J Dodson, G G Dodson, D C Hodgkin, D A Mercola and M Vijayan (1971). Atomic positions in rhombohedral 2-zinc insulin crystals. *Nature* ***231****: 506-511.*

75. Mertz W (1981). The essential trace elements. *Sci.* ***213****: 1332.*

76. Yong R N, A M O Mohamed and B P Warkentin (1992). Principles of contaminant transport in soils. *Elsevier Science Publishers: Amsterdam, The Netherlands. p. 327.*

77. Baird C (1995). Environmental Chemistry. *W.H. Freeman and Company: New York, NY, USA. p. 484.*

78. Alloway B J (1990). Cadmium. In *Heavy Metals in Soils*, Alloway B J, Editor. *London, England. pp. 100-124.*

79. Stewart-Pinkham S M (1989). The relative role of cadmium and lead in disease. *Int. J. Biosocial. Med. Res.* ***11****: 121-133.*

80. Freedman B and T C Hutchinson (1981). Sources of metal and elemental contamination of terrestrial environments. In *Effect of heavy metal pollution on plants, Vol 2: Metals in the environment*, Lepp N W, Editor. *Applied Science Publishers: Barking, Essex, England. pp. 35-93.*

81. Forstner U and G T W Wittmann (1981). Metal pollution in the aquatic environment, Second Revised Edition. *Springer-Verlag: Berlin, West Germany. p. 486.*

82. Bowen H J M (1979). Environmental chemistry of the elements. *Academic Press: London, England. p. 333.*

83. Cartwright B, R H Merry and K G Tiller (1976). Heavy metal contamination of soils around a lead smelter at Port Pirie, South Australia. *Aust. J. Soil Sci.* ***15****: 69-81.*

84. Nriagu J O and J M Pacyna (1988). Quantitative assessment of worldwide contamination of air, water and soil by trace metals. *Nature **333**: 134-139.*

85. Brown S L, R L Chaney, J S Angle and A J M Baker (1994). Phytoremediation potential of *Thlaspi caerulescens* and Bladder Campion for zinc- and cadmium-contaminated soil. *J. Environ. Qual. **23**: 1151-1157.*

86. Robinson B H, M Leblanc, D Petit, R R Brooks, J H Kirkman and P E H Gregg (1998). The potential of *Thlaspi caerulescens* for the phytoremediation of contaminated soils. *Plant Soil **203**: 46-56.*

87. Schwartz C and J L Morel (1998). How can agricultural practices improve phytoremediation of soils contaminated by heavy metals? In *Contaminated Soil '98. London, England: Thomas Telford.*

88. Ebbs S D and L V Kochian (1998). Phytoextraction of zinc by oat (*Avena sativa*), barley (*Hordeum vulgare*), and indian mustard (*Brassica juncea*). *Environ. Sci. Technol. **32**: 802-806.*

89. Salomons W and U Forstner (1988). Chemistry and biology of solid waste: dredged materials and mine tailings. *Springer Verlag: Berlin, West Germany. p. 305.*

90. Pichtel J and C A Salt (1998). Vegetative growth and trace metal accumulation on metalliferous wastes. *J. Environ. Qual. **27**: 618-624.*

91. Moore T R and R C Zimmerman (1977). Establishment of vegetation on serpentine asbestos mine wastes, southeastern Quebec, Canada. *J. Appl. Ecol. **14**: 589-599.*

92. Khan A G, T M Chaudhry, W J Gayes, C S Khoo, L Hill, R Fernandez and P Gallardo (1998). Physical, chemical and biological characterisation of a steelworks waste site at Port Kembla, NSW, Australia. *Water Air Soil Pollut. **104**: 389-402.*

93. Reuss J O, H L Dooley and W Griffis (1978). Uptake of cadmium from phosphate fertilizers by peas, radishes, and lettuce. *J. Environ. Qual. 7(1): 128.*

94. Zarcinas B and R O Nable (1992). Boron and other impurities in South Australian fertilisers and soil amendments. *Divisional Report 118, CSIRO Division of Soils: Adelaide, SA, Australia. p. 10.*

95. Mortvedt J J and R J Gilkes (1993). Zinc fertilizers. In *Zinc in soils and plants*, Robson A D, Editor. *Kluwer Academic Publishers: Dordrecht, West Germany. pp. 33-44.*

96. Jinadasa K B P N, P J Milham, C A Hawkins, P S Cornish, P A Williams, C J Kaldor and J P Conroy (1997). Survey of cadmium concentrations in vegetables and soils of Greater Sydney, Australia. *J. Environ. Qual.* ***26****: 924-933.*

97. Craig P J (1980). Metal cycles and biological methylation. In *The Handbook of Environmental Chemistry, Vol. 1. Part A, The Natural Environment and the Biogeochemical Cycles*, Hutzinger O, Editor. *Springer-Verlag: Berlin, West Germany. p. 169-227.*

98. Frink C R (1996). A perspective on metals in soils. *J. Soil Cont.* ***5****(4): 329-359.*

99. De Villarroel J R, A C Chang and C Amrhein (1993). Cd and Zn phytoavailability of a field-stabilized sludge-treated soil. *Soil Sci.* ***155****(3): 197-205.*

100. Merry R H and K G Tiller (1991). Distribution and budget of cadmium and lead in an agricultural region near Adelaide, South Australia. *Water Air Soil Pollut.* ***57/58****: 171-180.*

101. Hutchinson J J, S D Young, S P McGrath, H M West, C R Black and A J M Baker (2000). Determining uptake of 'non-labile' soil cadmium by *Thlaspi caerulescens* using isotopic dilution techniques. *New Phytol.* ***146****: 453-460.*

102. Lide D R (1998-1999). CRC Handbook of Chemistry and Physics, 79th Edition. Lide D R, Editor. *CRC Press LLC: Boca Raton, FL, USA.*

103. Brummer G, K G Tiller, U Herms and P M Clayton (1983). Adsorption-desorption and/or precipitation-dissolution processes of zinc in soils. *Geoderma* ***31****: 337-354.*

104. Christensen T H (1984). Cadmium soil sorption at low concentrations: I. effect of time, cadmium load, pH, and calcium. *Water Air Soil Pollut.* ***21****: 105-114.*

105. Deb D L, G N Gupta, M B Meisheri, R K Rattan and A K Sarkar (1986). Radioisotope aided micronutrient research for increasing fertiliser use efficiency. *Fert. News.* ***February****: 21-29.*

106. Elgawhary S M, W L Lindsay and W D Kemper (1970). Effect of complexing agents and acids on the diffusion of zinc to a simulated root. *Soil Sci. Soc. Am. Proc.* ***34****: 211-214.*

107. Filius A, T Streck and J Richter (1998). Cadmium sorption and desorption in limed topsoils as influenced by pH: isotherms and simulated leaching. *J. Environ. Qual.* ***27****: 12-18.*

108. Fuji R and R B Corey (1986). Estimation of isotopically exchangeable cadmium and zinc in soils. *Soil Sci. Soc. Am. J.* ***50****: 306-308.*

109. Graham E R (1973). Selective distribution and labile pools of micronutrient elements as factors affecting plant uptake. *Soil Sci. Soc. Am. Proc.* ***37****: 70-74.*

110. Nakhone L N and S D Young (1993). The significance of (radio-) labile cadmium pools in soil. *Environ. Pollut.* ***82****: 73-77.*

111. Pandeya S B, A K Singh and P Jha (1998). Labile pool of cadmium in sludge-treated soils. *Plant Soil* ***203****: 1-13.*

112. Qitang W, J L Morel and A Guckert (1993). Study on transfer of cadmium in soil-plant systems with the isotopic dilution method. *Acta Ag. Nucleatae Sinica. 7(2): 110-116.*

113. Sinaj S, F Machler and E Frossard (1999). Assessment of isotopically exchangeable zinc in polluted and nonpolluted soils. *Soil Sci. Soc. Am. J.* ***63****: 1618-1625.*

114. Stanhope K G, S D Young, J J Hutchinson and R Kamath (2000). Use of isotopic dilution techniques to assess the mobilization of nonlabile Cd by chelating agents in phytoremediation. *Environ. Sci. Technol.* ***34****: 4123-4127.*

115. Young S D, A Tye, A Carstensen, L Resende and N Crout (2000). Methods for determining labile cadmium and zinc in soil. *Euro. J. Soil Sci.* ***51****: 129-136.*

116. Tiller K G, J L Honeysett and M P C deVries (1972). Soil zinc and its uptake by plants. II. Soil chemistry in relation to prediction of availability. *Aust. J. Soil Res.* ***10****: 165-182.*

117. Dean J A (1999). Lange's handbook of chemistry. *McGraw-Hill Inc: New York, NY, USA. Various pagings.*

118. Banuelos G S and H A Ajwa (1999). Trace elements in soils and plants: an overview. *J. Environ. Sci. Health.* ***A34****(4): 951-974.*

119. Street J J, W L Lindsay and B R Sabey (1977). Solubility and plant uptake of cadmium in soils amended with cadmium and sewage sludge. *J. Environ. Qual.* ***6****(1): 72-77.*

120. Huang W H and W D Keller (1972). Dissolution of clay minerals in dilute organic acids at room temperature. *Am. Mineral.* ***56****: 1082-1095.*

121. Jauregui M A and H M Reisenauer (1982). Dissolution of oxides of manganese and iron by root exudate components. *Soil Sci. Soc. Am. J.* ***46****: 314-317.*

122. Jones D L, P R Darrah and L V Kochian (1996). Critical evaluation of organic acid mediated iron dissolution in the rhizosphere and its potential role in root iron uptake. *Plant Soil* ***180****: 57-66.*

123. Barrow N J (1993). Mechanisms of reaction of zinc with soil and soil components. In *Zinc in soils and plants*, Robson A D, Editor. *Kluwer Academic Publishers: Dordrecht, Germany. pp. 15-31.*

124. Parker D R, R L Chaney and W A Norvell (1995). Chemical equilibrium models: Applications to plant nutrition research. In *Chemical equilibrium and reaction models*, Loeppert R H, Editor. *Soil Sci. Soc. Am.: Madison, WI, USA. pp. 163-200.*

125. Paulson A J, M M Benjamin and J F Ferguson (1989). Zn solubility in low carbonate solutions. *Water Res.* ***23****(12): 1563-1569.*

126. Elliott H A, M R Liberati and C P Huang (1986). Competitive adsorption of heavy metals by soils. *J. Environ. Qual.* ***1****(3): 214-219.*

127. Jones D L (1998). Organic acids in the rhizosphere - a critical review. *Plant Soil.* ***205****: 25-44.*

128. Harter R D and R Naidu (1995). Role of metal-organic complexation in metal sorption by soils. *Adv. Agron.* ***55****: 219-263.*

129. Chairidchai P and G S P Ritchie (1990). Zinc adsorption by a lateritic soil in the presence of organic ligands. *Soil Sci. Soc. Am. J.* ***54****: 1242-1248.*

130. Naidu R and R D Harter (1998). Effect of different organic ligands on cadmium sorption by and extractability from soils. *Soil Sci. Soc. Am. J.* ***62****: 644-650.*

131. McLaughlin M J, L T Palmer, K G Tiller, T A Beech and M K Smart (1994). Increased soil salinity causes elevated cadmium concentrations in field-grown potato tubers. *J. Environ. Qual.* ***23****: 1013-1018.*

132. Hodgson J F, W L Lindsay and J F Trierweiler (1966). Micronutrient cation complexing in soil solution. II. Complexing of Zn and Cu in displacing solution from calcareous soils. *Soil Sci. Soc. Am. Proc.* ***30****: 723-726.*

133. Li Y-M, R L Chaney and A A Schneiter (1994). Effect of soil chloride level on cadmium concentration in sunflower kernels. *Plant Soil* ***167****: 275-280.*

134. Doner H E (1978). Chloride as a factor in mobilities of Ni(II), Cu(II), and Cd(II) in soil. *Soil Sci. Soc. Am. J.* ***42****: 882-885.*

135. Tills A R and B J Alloway (1983). The use of liquid chromatography in the study of cadmium speciation in soil solutions from polluted soils. *J. Soil Sci.* ***34****: 769-781.*

136. Hodgson J F, H R Geering and W A Norvell (1965). Micronutrient cation complexes in soil solution: Partition between complexed and uncomplexed forms by solvent extraction. *Soil Sci. Soc. Am. Proc.* ***29****: 665-669.*

137. Garcia-Miragaya J and A L Page (1977). Influence of exchangeable cation on the sorption of trace amounts of cadmium by montmorillonite. *Soil Sci. Soc. Am. J.* ***41****(4): 718-721.*

138. Tiller K G, J Gerth and G Brummer (1984). The relative affinities of Cd, Ni and Zn for different soil clay fractions and goethite. *Geoderma.* ***34****: 17-35.*

139. Inskeep W P and J Baham (1983). Adsorption of Cd(II) and Cu(II) by Na-montmorillonite at low surface coverage. *Soil Sci. Soc. Am. J.* ***47****(660-665).*

140. Polo M J, R Ordonez and J V Giraldez (1999). Copper and zinc adsorption by sewage sludge-treated soil in southern Spain. *Commun. Soil Sci. Plant Anal.* ***30****(7&8): 1063-1079.*

141. Bruemmer G W, J Gerth and K G Tiller (1988). Reaction kinetics of the adsorption and desorption of nickel, zinc and cadmium by goethite. I. Adsorption and diffusion of metals. *J. Soil Sci.* ***39****: 37-52.*

142. Shuman L M (1977). Adsorption of Zn by Fe and Al hydrous oxides as influenced by aging and pH. *Soil Sci. Soc. Am. J.* ***41****: 703-706.*

143. Padmanabham M (1983). Comparative study of the adsorption-desorption behaviour of copper(II), zinc(II), cobalt(II) and lead(II) at the goethite-solution interface. *Aust. J. Soil Res.* ***21****: 515-525.*

144. Barrow N J and B R Whelan (1998). Comparing the effects of pH on the sorption of metals by soil and by goethite, and on uptake by plants. *Euro. J. Soil Sci.* ***49****: 683-692.*

145. McKenzie R M (1980). The adsorption of lead and other heavy metals on oxides of manganese and iron. *Aust. J. Soil Res.* ***18****: 61-73.*

146. Scheidegger A M and D L Sparks (1996). A critical assessment of sorption-desorption mechanisms at the soil mineral/water interface. *Soil Sci.* ***161****(12): 813-831.*

147. McBride M B (1989). Reactions controlling heavy metal solubility in soils. *Adv. Agron.* ***10****: 1-56.*

148. Garcia-Miragaya J and A L Page (1976). Influence of ionic strength and inorganic complex formation on the sorption of trace amounts of Cd by montmorillonite. *Soil Sci. Soc. Am. J.* ***40****: 658-663.*

149. Naidu R, N S Bolan, R S Kookana and K G Tiller (1994). Ionic-strength and pH effects on the sorption of cadmium and the surface charge of soils. *Euro. J. Soil Sci.* ***45****: 419-429.*

150. McBride M B, L D Tyler and D A Hovde (1981). Cadmium adsorption by soils and uptake by plants as affected by soil chemical properties. *Soil Sci. Soc. Am. J.* ***45****: 739-744.*

151. Cavallaro N and M B McBride (1978). Copper and cadmium adsorption characteristics of selected acid and calcareous soils. *Soil Sci. Soc. Am. J.* ***42****: 550-556.*

152. Eriksson J E (1989). The influence of pH, soil type and time of adsorption and uptake by plants of Cd added to the soil. *Water Air Soil Pollut.* ***48****: 317-335.*

153. Harter R D (1983). Effect of soil pH on adsorption of lead, copper, zinc and nickel. *Soil Sci. Soc. Am. J.* ***47****: 47-51.*

154. Basta N T and M A Tabatabai (1992). Effect of cropping systems on adsorption of metals by soils: II. Effect of pH. *Soil Sci.* ***153****(3): 195-204.*

155. Boekhold A E, E J M Temminghoff and S E A T M Van Der Zee (1993). Influence of electrolyte composition and pH on cadmium sorption by an acid sandy soil. *J. Soil Sci.* ***44****: 85-96.*

156. Kalbasi M, G J Racz and L A Loewen-Rudgers (1978). Mechanism of zinc adsorption by iron and aluminum oxides. *Soil Sci.* ***125****: 146-150.*

157. Zasoski R J and R G Burau (1988). Sorption and sorptive interaction of cadmium and zinc on hydrous manganese oxide. *Soil Sci. Soc. Am. J.* ***52****: 81-87.*

158. Tiller K G and J F Hodgson (1962). The specific sorption of cobalt and zinc by layer silicates. *Clay Mineral.* ***9****: 393-403.*

159. Kinniburgh D G, M L Jackson and J K Syers (1976). Adsorption of alkaline earth, transition and heavy metal cations by hydrous oxide gels of iron and aluminum. *Soil Sci. Soc. Am. J.* ***40****: 796-799.*

160. Campbell G D, H F Galicia and P W Schindler (1987). Binding of cadmium by montmorillonite-humic acid mixtures: miscible-displacement experiments. *Aust. J. Soil Res.* ***25****: 391-403.*

161. Christensen T H (1987). Cadmium soil sorption at low concentrations: V. Evidence of competition by other heavy metals. *Water Air Soil Pollut.* ***34****: 293-303.*

162. McLaughlin M J, E Smolders and R Merckx (1998). Soil-Root interface: physicochemical processes. In *Soil Chemistry and Ecosystem Health*, Huang P M, Editor. *Soil Sci. Soc. Am.: Madison, WI, USA. pp. 233-277.*

163. Chubin R G and J J Street (1981). Adsorption of cadmium on soil constituents in the presence of complexing ligands. *J. Environ. Qual.* ***10****: 225-228.*

164. Chairidchai P and G S P Ritchie (1992). The effect of pH on zinc adsorption by a lateritic soil in the presence of citrate and oxalate. *J. Soil Sci.* ***43****: 723-728.*

165. Chairidchai P and G S P Ritchie (1993). Zinc adsorption by sterilized and non-sterilized soil in the presence of citrate and catechol. *Commun. Soil Sci. Plant Anal.* ***24****(3&4): 261-275.*

166. Sposito G (1989). The chemistry of soils. *Oxford University Press: New York, NY, USA. p. 277.*

167. Dinkelaker B, V Romheld and H Marschner (1989). Citric acid excretion and precipitation of calcium citrate in the rhizosphere of white lupin (*Lupinus albus* L.). *Plant Cell Environ.* ***12****: 285-292.*

168. Sakaguchi T, N K Nishizawa, H Nakanishi, E Yoshimura and S Mori (1999). The role of potassium in the secretion of mugineic acids family phytosiderophores from iron-deficient barley roots. *Plant Soil.* ***215****: 221-227.*

169. Krishnamurti G S R and P M Huang (1992). Dynamics of potassium chloride induced manganese release in different soil orders. *Soil Sci. Soc. Am. J.* ***56****: 1115-1123.*

170. Wallace G A and A Wallace (1983). Clay fixation of metal chelates as a factor in their usability by soil application to correct micronutrient deficiencies. *J. Plant Nutr.* ***6****(6): 439-446.*

171. Jones D L, A C Edwards, K Donachie and P R Darrah (1994). Role of proteinaceous amino acids released in root exudates in nutrient acquisition from the rhizosphere. *Plant Soil.* ***158****: 183-192.*

172. Jones D L and D S Brassington (1998). Sorption of organic acids in acid soils and its implication in the rhizosphere. *Euro. J. Soil Sci.* ***49****: 447-455.*

173. Jones D L and P R Darrah (1994). Role of root derived organic acids in the mobilization of nutrients from the rhizosphere. *Plant Soil.* ***166****: 247-257.*

174. Haas C N and N D Horowitz (1986). Adsorption of cadmium to kaolinite in the presence of organic material. *Water Air Soil Pollut.* ***27****: 131-140.*

175. Morrill L G, B C Mahilum and S H Mohiuddin (1982). Organic compounds in soils: sorption, degradation and persistence*: Ann Arbor Science: Ann Arbor, MI, USA. p. 326..*

176. Mortland M M (1970). Clay-organic complexes and interactions. *Adv. Agron.* ***22:*** *75-117.*

177. McBride M B (1985). Influence of glycine on Cu^{2+} adsorption by microcrystalline gibbsite and boehmite. *Clays Clay Miner.* ***30:*** *397-402.*

178. Girvin D C, P L Gassman and H B Jr (1993). Adsorption of aqueous cobalt ethylenediaminetetraacetate by δ-Al_2O_3. *Soil Sci. Soc. Am. J.* ***57:*** *47-57.*

179. Rueda E H, R L Grassi and M A Blesa (1985). Adsorption and dissolution in the system goethite/aqueous EDTA. *J. Colloid Interface Sci.* ***106****(1): 243-246.*

180. Xue J and P M Huang (1995). Zinc-adsorption-desorption on short-range ordered iron oxide as influenced by citric acid during its formation. *Geoderma.* ***64:*** *343-356.*

181. Mench M and E Martin (1991). Mobilization of cadmium and other metals from two soils by root exudates of *Zea mays* L., *Nicotiana tabacum* L. and *Nicotiana rustica* L. *Plant Soil.* ***132:*** *187-196.*

182. Elliott H A and C M Denneny (1982). Soil adsorption of cadmium from solutions containing organic ligands. *J. Environ. Qual.* ***11****(4): 658-663.*

183. Farrah H and W F Pickering (1979). pH effects in the adsorption of heavy metal ions by clays. *Chem. Geol.* ***25****(4): 317-326.*

184. Hong J and P N Pintauro (1996). Selective removal of heavy metals from contaminated kaolin by chelators. *Water Air Soil Pollut.* ***87:*** *73-91.*

185. Neal R H and G Sposito (1986). Effects of soluble organic matter and sewage sludge amendments on cadmium sorption by soils at low cadmium concentrations. *Soil Sci.* ***142:*** *164-172.*

186. Inskeep W P and J Baham (1983). Competitive complexation of Cd(II) and Cu(II) by water-soluble organic ligands and Na-montmorillonite. *Soil Sci. Soc. Am. J.* ***47:*** *1109-1115.*

187. Asher L E and B Bar-Yosef (1982). Effects of pyrophosphate, EDTA and DTPA on zinc sorption by montmorillonite. *Soil Sci. Soc. Am. J.* ***46****: 271-276.*

188. Sinha M K, S K Dhillon and K S Dhillon (1977). Zinc chelate reactions in alkaline soils. *Aust. J. Soil Res.* ***15****: 103-113.*

189. Krishnamurti G S R, G Cieslinski, P M Huang and K C J Van Rees (1997). Kinetics of cadmium release from soils as influenced by organic acids: implication in cadmium availability. *J. Environ. Qual.* ***26****: 271-277.*

190. Levy R and C W Francis (1976). Adsorption and desorption of cadmium by synthetic and natural organo-clay complexes. *Geoderma* ***15****: 361-370.*

191. Boyle J R and G K Voigt (1973). Biological weathering of silicate minerals. Implications for tree nutrition and soil genesis. *Plant Soil* ***38****: 191-201.*

192. Pohlman A A and J G McColl (1986). Kinetics of metal dissolution from forest soils by soluble organic acids. *J. Environ. Qual.* ***15****(1): 86-92.*

193. Stone A T and J J Morgan (1984). Reduction and dissolution of manganese(III) and manganese(IV) oxides by organics. 1. Reaction with hydroquinone. *Environ. Sci. Technol.* ***18****(6): 450-456.*

194. Stone A T and J J Morgan (1984). Reduction and dissolution of manganese(III) and manganese(IV) oxides by organics. 2. Survey of the reactivity of organics. *Environ. Sci. Technol.* ***18****: 617-624.*

195. Youssef R A and M Chino (1990). Effects of rhizosphere processes on the solubilization of manganese as revealed with radioisotope techniques. In *Plant nutrition - Physiology and Applications.* van Beusichem M L, Editor. *Kluwer Academic Publishers: Wageningen, The Netherlands. pp.229-233.*

196. Norvell W A and W L Lindsay (1969). Reactions of EDTA complexes of Fe, Zn, Mn, and Cu with soils. *Soil Sci. Soc. Am. Proc.* ***33****: 86-91.*

197. Lindsay W L and W A Norvell (1969). Equilibrium relationships of Zn^{2+}, Fe^{3+}, Ca^{2+}, and H^{+} with EDTA and DTPA in soils. *Soil Sci. Soc. Am. Proc.* ***33****: 62-81.*

198. Norvell W A and W L Lindsay (1970). Lack of evidence for $ZnSiO_3$ in soils. *Soil Sci. Soc. Am. Proc.* ***34****: 360-361.*

199. Singh S P (1982). Control of Zn, Cu and Mn activities in alkaline and calcareous soils equilibrated with EDTA. *J. Agric. Sci.* ***98****: 203-207.*

200. Ma Q and W L Lindsay (1990). Divalent zinc activity in arid-zone soils obtained by chelation. *Soil Sci. Soc. Am. J.* ***54****: 719-722.*

201. Sachdev P, W L Lindsay and D L Deb (1992). Activity measurements of zinc in soils of different pH using EDTA. *Geoderma* ***55****: 247-257.*

202. Gilmore J T and J A Kittrick (1979). Solubility and equilibria of zinc in a flooded soil. *Soil Sci. Soc. Am. J.* ***43****: 890-892.*

203. Lindsay W L (1979). Chemical Equilibria in Soils. Lindsay W L, Editor. *John Wiley & Sons: New York, NY, USA. p. 449.*

204. Hering J G (1996). Implications of complexation, sorption and dissolution kinetics for metal transport in soils. In *Metal speciation and contamination of soil*, Allen H E, *et al.*, Editors. *Lewis Publishers: Boca Raton, FL, USA. pp. 59-86.*

205. Bromfield S M (1958). The solution of γ-MnO_2 by substances released from soil and from the roots of oats and vetch in relation to manganese availability. *Plant Soil* ***10****: 147-160.*

206. Workman S M and W L Lindsay (1990). Estimating divalent cadmium activities measured in arid-zone soils using competitive chelation. *Soil Sci. Soc. Am. J.* ***54****: 987-993.*

207. Santillan-Medrano J and J J Jurinak (1975). The chemistry of lead and cadmium in soil: solid phase formation. *Soil Sci. Soc. Am. Proc.* ***39****: 851-855.*

208. Jopony M and S D Young (1994). The solid/solution equilibria of lead and cadmium in polluted soils. *Euro. J. Soil Sci.* ***45****: 59-70.*

209. Spadini L, A Manceau, P W Schindler and L Charlet (1994). Structure and stability of Cd^{2+} surface complexes on ferric oxides. I. Results from EXAFS spectroscopy. *J. Colloid. Interface Sci.* ***168****: 73-86.*

210. Tiller K G (1968). Stability of hectorite in weakly acidic solutions. III. Adsorption of heavy metal cations and hectorite stability. *Clay Miner. 7: 409-419.*

211. Hardiman R T and B Jacoby (1984). Absorption and translocation of Cd in bush beans (*Phaseolus vulgaris*). *Physiol. Plant.* ***61****: 670-674.*

212. Hodgson J F (1981). Contribution of metal-organic complexing agents to the transport of metals to roots. *Soil Sci. Soc. Am. Proc* ***33****:68-75*

213. Chaney R L (1988). Metal speciation and interactions among elements affect trace element transfer in agricultural and environmental food-chains. In *Metal speciation: theory, analysis and application*, Kramer J R and H E Allen, Editors. *Lewis Publishers, Inc: Chelsea, MI, USA. pp. 219-260.*

214. Gupta G N and D L Deb (1983). Effect of EDTA on diffusive supply of zinc to maize and rice roots in soils. *J. Nuclear Agric. Biol.* ***12****: 53-57.*

215. Elgawhary S M and S A Barber (1973). Measurement of uptake of chelated and unchelated Ca and Sr from solution culture. *Plant Soil* ***39****: 581-590.*

216. Prasad B, M K Sinha and N S Randhawa (1976). Effect of mobile chelating agents on diffusion of zinc in soils. *Soil Sci.* ***122****(5): 260-266.*

217. Butler P C and R H Bray (1956). Effect of the zinc chelate of ethylenediaminetetraacetic acid on plant uptake of zinc and other heavy metals. *Soil Sci. Soc. Am. Proc.* ***20****: 348-351.*

218. Wallihan E F, T W Embleton and W Printy (1958). Zinc deficiency in the avocado. *Calif. Ag.* ***(Sept.)****: 4-5.*

219. Wallace A, R T Mueller, J W Cha and G V Alexander (1974). Soil pH, excess lime, and chelating agent on micronutrients in soybeans and bush beans. *Agron. J.* ***66****: 698-700.*

220. Wallace A and G A Wallace (1983). DTPA as a source of Zn, Mn, Cu and Fe in calcareous soil. *J. Plant Nutr.* ***6****(6): 451-455.*

221. Wallace A, E M Romney, G V Alexander, S M Soufi and P M Patel (1977). Some interactions in plants among cadmium, other heavy metals, and chelating agents. *Agron. J.* ***69****(1): 18-20.*

222. Patel P M, A Wallace and E M Romney (1977). Effect of chelating agents on phytotoxicity of lead and lead transport. *Commun. Soil Sci. Plant Anal. 8(9): 733-740.*

223. Essington E, H Nishita and A Wallace (1962). Influence of chelates on availability of fission products to plants grown in a contaminated soil. *Soil Sci.* ***94****: 96-105.*

224. Essington E, H Nishita and A Wallace (1963). Effect of chelating agents on the uptake of Y91, Ru106, Ce144 and Pm147 by beans grown in a calcareous soil. *Soil Sci. **95**: 331-337.*

225. Adriano D C, M Delaney and D Paine (1977). Availability of cobalt-60 to corn and bean seedlings as influenced by soil type, lime, and DTPA. *Commun. Soil Sci. Plant Anal. 8(8): 615-628.*

226. Adriano D C, M S Delaney, G D Hoyt and D Paine (1977). Availability to plants and soil extraction of americium-241 as influenced by chelating agent, lime, and soil type. *Environ. Exp. Bot. **17**: 69-77.*

227. Adriano D C (1979). Factors affecting the availability of Americium-241 to the rice plant. *J. Agric. Food Chem. 27(6): 1369-1375.*

228. Vyas B N and K B Mistry (1983). Influence of chelating agents on the uptake of 239Pu and 241Am by plants. *Plant Soil **73**: 345-353.*

229. Wallace A (1972). Effect of soil pH and chelating agent (DTPA) on uptake by and distribution of ^{241}Am in plant parts of Bush Beans. *Rad. Bot. **12**: 433-435.*

230. Hale V Q and A Wallace (1970). Effect of chelates on uptake of some heavy metal radionuclides from soil by bush beans. *Soil Sci. **109**(4): 262-263.*

231. Wallace A, E M Romney, J W Cha and S M Soufi (1977). Nickel phytotoxicity in relationship to soil pH manipulation and chelating agents. *Commun. Soil Sci. Plant Anal. 8(9): 757-764.*

232. Wallace A and T R Mueller (1973). Effects of chelated and nonchelated cobalt and copper on yields and microelement composition of bush beans grown on calcareous soil in a glasshouse. *Soil Sci. Soc. Am. Proc. **37**: 907-908.*

233. Tiffin L O, J C Brown and R W Krauss (1960). Differential absorption of metal chelate components by plant roots. *Plant Physiol. **35**: 362-367.*

234. Tiffin L O, J C Brown and R S Holmes (1960). Chelating agent and plant nutrient interactions affecting the iron nutrition of soybeans. *Soil Sci. Soc. Am. Proc. **24**: 120-123.*

235. Tiffin L O and J C Brown (1959). Absorption of iron from iron chelate by sunflower roots. *Sci. **130**: 274-275.*

236. Brown J C, L O Tiffin, A W Specht and J W Resnicky (1961). Iron absorption by roots as affected by plant species and concentration of chelating agent. *Agron. J.* ***53****: 81-85.*

237. Brown J C, L O Tiffin, A W Specht and J W Resnicky (1961). Stability and concentration of metal chelates, factors in iron chlorosis of plants. *Agron. J.* ***53****: 85-90.*

238. Brown J C (1969). Agricultural use of synthetic metal chelates. *Soil Sci. Soc. Am. Proc.* ***33****: 59-61.*

239. Chaney R L, J C Brown and L O Tiffin (1972). Obligatory reduction of ferric chelates in iron uptake by soybeans. *Plant Physiol.* ***50****: 208-213.*

240. Holmes R S and J C Brown (1955). Chelates as correctives for chlorosis. *Soil Sci.* ***80****(3): 167-179.*

241. Lunt O R and A Wallace (1955). Use of iron chelates. Supplying plants with iron through soil treatment limited to high-value plantings. *Calif. Ag.* ***9****(June): 4.*

242. DeKock P C and R L Mitchell (1957). Uptake of chelated metals by plants. *Soil Sci.* ***84****: 55-62.*

243. Parker D R (1997). Responses of six crop species to solution $zinc^{2+}$ activities buffered with HEDTA. *Soil Sci. Soc. Am. J.* ***61****: 167-176.*

244. Parker D R and W A Norvell (1999). Advances in solution culture methods for plant mineral nutrition research. *Adv. Agron.* ***65****: 151-213.*

245. Rengel Z (1999). Physiological responses of wheat genotypes grown in chelator-buffered nutrient solutions with increasing concentrations of excess HEDTA. *Plant Soil* ***215****: 193-202.*

246. Rengel Z and R K Graham (1995). Wheat genotypes differ in Zn efficiency when grown in chelate-buffered nutrient solution. II. Nutrient uptake. *Plant Soil* ***176****: 317-324.*

247. Rengel Z and R D Graham (1995). Wheat genotypes differ in Zn efficiency when grown in chelate-buffered nutrient solution. I. Growth. *Plant Soil* ***176****: 307-316.*

248. Rengel Z and R D Graham (1996). Uptake of zinc from chelate-buffered nutrient solutions by wheat genotypes differing in zinc efficiency. *J. Exp. Bot.* ***47****(295): 217-226.*

249. Welch R M, J J Hart, W A Norvell, L A Sullivan and L V Kochian (1999). Effects of nutrient solution zinc activity on net uptake, translocation, and root export of cadmium and zinc by separated sections of intact durum wheat (*Triticum turgidum* L. var *durum*) seedling roots. *Plant Soil* ***208****: 243-250.*

250. Welch R M and W A Norvell (1993). Growth and nutrient uptake by Barley (*Hordeum vulgare* L. cv Herta): studies using an N-(2-Hydroxyethyl)ethylenedinitrilotriacetic acid-buffered nutrient solution technique. II. Role of zinc in the uptake and root leakage of mineral nutrients. *Plant Physiol.* ***101****: 627-631.*

251. Norvell W A and R M Welch (1993). Growth and nutrient uptake by Barley (*Hordeum vulgare* L. cv Herta): studies using an N-(2-Hydroxyethyl)ethylenedinitrilotriacetic acid-buffered nutrient solution technique. I. Zinc ion requirements. *Plant Physiol.* ***101****: 619-625.*

252. Checkai R T, R B Corey and P A Helmke (1987). Effects of ionic and complexed metal concentrations on plant uptake of cadmium and micronutrient metals from solution. *Plant Soil* ***99****: 335-345.*

253. Salt D, R Prince, I Pickering and I Raskin (1995). Mechanisms of cadmium mobility and accumulation in Indian mustard. *Plant Physiol.* ***109****: 1427-1433.*

254. Bell P F, R L Chaney and J S Angle (1991). Free metal activity and total metal concentrations as indices of micronutrient availability to barley [*Hordeum vulgare* (L.) 'Klages']. *Plant Soil* ***130****: 51-62.*

255. Laurie S H, N P Tancock, S P McGrath and J R Sanders (1991). Influence of complexation on the uptake by plants of iron, manganese, copper and zinc. I. Effect of EDTA in a multi-metal and computer simulation study. *J. Exp. Bot.* ***42****(237): 509-513.*

256. Laurie S H, N P Tancock, S P McGrath and J R Sanders (1991). Influence of complexation on the uptake by plants of iron, manganese, copper and zinc. II. Effect of DPTA in a multi-metal and computer simulation study. *J. Exp. Bot.* ***42****(237): 515-519.*

257. McLaughlin M J, E Smolders, R Merckx and A Maes (1997). Plant uptake of Cd and Zn in chelator-buffered nutrient solution depends on ligand type. In *Plant nutrition - for sustainable food production and environment,* Ando T, *et al.*, Editors. *Kluwer Academic Publishers, Dordrecht, Germany. p. 113-117.*

258. Iwasaki K and E Takahashi (1989). Effects of charge characteristics of Cu-chelates on the Cu uptake from the solution by Italian ryegrass and red clover. *Soil Sci. Plant Nutr.* ***35****(1): 145-150.*

259. Hartikainen H (1981). Uptake of soil P, Al, Fe, Mn, Mg and Ca by Italian rye grass (*Lolium multiflorum* L.) induced by synthetic chelating agent. *J. Scientific Ag. Soc. Finland.* ***53****: 152-160.*

260. Clarkson D T and J Sanderson (1978). Sites of absorption and translocation of iron in Barley roots. Tracer and microautoradiographic studies. *Plant Physiol.* ***61****: 731-736.*

261. Clarkson D T and U Luttge (1989). Mineral nutrition: divalent cations, transport and compartmentalization. *Prog. Bot.* ***51****: 93-112.*

262. Weinstein L H, E R Purvis, A N Meiss and R L Uhler (1954). Absorption and translocation of ethylenediamine-tetraacetic acid by sunflower plants. *J. Ag. Food Chem.* ***2****(8): 421-425.*

263. Hill-Cottingham D G (1957). A spectrophotometric method of analysis of chelate solutions and its application to the study of iron chelates in soils and plants. *Soil Sci.* ***84****: 43-49.*

264. Hill-Cottingham D G and C P Lloyd-Jones (1965). The behaviour of iron chelating agents with plants. *J. Exp. Bot.* ***16****(47): 233-242.*

265. Hill-Cottingham D G and C P Lloyd-Jones (1961). Absorption and breakdown of iron-ethylenediamine tetraacetic acid by tomato plants. *Nature* ***189****(January 28): 312.*

266. Jeffreys R A, V Q Hale and A Wallace (1961). Uptake and translocation in plants of labeled iron and labeled chelating agents. *Soil Sci.* ***92****: 268-273.*

267. Wallace A and V Q Hale (1961). Effect of varying concentrations of iron and ethylenediamine di(o-hydroxyphenyl acetate) on concentrations of each in soybeans. *Soil Sci.* ***92****: 404-407.*

268. Beckett J T and W P Anderson (1973). Ferric-EDTA Absorption by Maize Roots. In *Ion Transport in Plants. Proceedings of an International Meeting, Liverpool, July 1972*, Anderson W P, Editor. *Academic Press: London, England. p. 595-607.*

269. Parker D R, J J Aguilera and D N Thomason (1992). Zinc-phosphorus interactions in two cultivars of tomato (*Lycopersicon esculentum* L.) grown in chelator-buffered nutrient solutions. *Plant Soil* ***143****: 163-177.*

270. Smeulders F and S C Van De Geijn (1983). *In situ* immobilzation of heavy metals with tetraethylenepentamine (tetren) in natural soils and its effect on toxicity and plant growth. III. Uptake and mobility of copper and its tetren-complex in corn plants. *Plant Soil* ***70****: 59-61.*

271. Smeulders F, J Sinnaeve and A Cremers (1983). *In situ* immobilization of heavy metals with tetraethylenepentamine (tetren) in natural soils and its effect on toxicity and plant growth. II. Effect of complex formation with tetren on copper and zinc uptake in corn from nutrient solutions. *Plant Soil* ***70****: 49-57.*

272. Wallace A (1963). Role of chelating agents on the availability of nutrients to plants. *Soil Sci. Soc. Am. Proc.* ***27****: 176-179.*

273. Wallace A and R T Mueller (1959). Responses of plants to zinc and manganese chelates. *Soil Sci. Soc. Am. Proc.* ***23****: 79.*

274. Simons J N, R Swidler and H M Benedict (1962). Absorption of chelated iron by soybean roots in nutrient solution. *Plant Physiol.* ***37****: 460-466.*

275. Romheld V and H Marschner (1981). Effect of Fe stress on utilization of Fe chelates by efficient and inefficient plant species. *J. Plant Nutr.* ***3****(1-4): 551-560.*

276. Wallace A and C P North (1953). Lime-induced chlorosis - chelating agents a possible means of control in citrus, avocado, and other subtropicals. *Calif. Ag.* ***7****(August): 10.*

277. Jeffreys R A and A Wallace (1968). Detection of iron ethylenediamine di(o-hydroxyphenylacetate) in plant tissue. *Agron. J.* ***60****: 613-616.*

278. Hill-Cottingham D G (1955). Spectrophotometric determination of iron chelates. *Anal.* ***80****: 906-908.*

279. Tanton T W and S H Crowdy (1971). The distribution of lead chelate in the transpiration stream of higher plants. *Pestic. Sci.* ***2****: 211-213.*

280. von Wiren N, H Marschner and V Romheld (1996). Roots of iron-efficient maize also absorb phytosiderophore chelated zinc. *Plant Physiol.* ***111****: 1119-1125.*

281. Walter A, V Romheld, H Marschner and S Mori (1994). Is the release of phytosiderophores in zinc-deficient wheat plants a response to imparied iron utilization? *Physiol. Plant.* ***92****: 493-500.*

282. Takagi S-I, S Kamei and M-H Yu (1988). Efficiency of iron extraction from soil by mugineic acid family phytosiderophores. *J. Plant Nutr.* ***11****(6-11): 643-651.*

283. Sugiura Y, H Tanaka, Y Mino, T Ishida, N Ota, M Inoue, K Nomoto, H Yoshioka and T Takemoto (1981). Structure, properties, and transport mechanism of iron(III) complex of mugineic acid, a possible phytosiderophore. *J. Am. Chem. Soc.* ***103****: 6979-6982.*

284. Romheld V (1991). The role of phytosiderophores in acquisition of iron and other micronutrients in graminaceous species: An ecological approach. *Plant Soil* ***130****: 127-134.*

285. Marschner H, V Romheld and M Kissel (1987). Localization of phytosiderophore release and of iron uptake along intact barley roots. *Physiol. Plant.* ***71****: 157-162.*

286. Cakmak I, K Y Gulut, H Marschner and R D Graham (1994). Effect of zinc and iron deficiency on phytosiderophore release in wheat genotypes differing in zinc efficiency. *J. Plant Nutr.* ***17****(1): 1-17.*

287. Crowley D E, Y C Wang, C P P Reid and P J Szaniszlo (1991). Mechanisms of iron acquisition from siderophores by microorganisms and plants. *Plant Soil* ***130****: 179-198.*

288. Cakmak I, B Erenoglu, K Y Gulut, R Derici and V Romheld (1998). Light-mediated release of phytosiderophores in wheat and barley under iron or zinc deficiency. *Plant Soil **202**: 309-315.*

289. Gries D, S Brunn, D E Crowley and D R Parker (1995). Phytosiderophore release in relation to micronutrient metal deficiencies in barley. *Plant Soil **172**: 299-308.*

290. Marschner H, M Treeby and V Romheld (1989). Role of root-induced changes in the rhizosphere for iron acquisition in higher plants. *Z. Pflanzenernahr. Bodenk. **152**: 197-204.*

291. Romheld V and H Marschner (1986). Evidence for a specific uptake system for iron phytosidersphores in roots of grasses. *Plant Physiol. **80**: 175-180.*

292. von Wiren N, H Marschner and V Romheld (1995). Uptake kinetics of iron-phytosiderophores in two maize genotypes differing in iron efficiency. *Physiol. Plant. **93**: 611-616.*

293. Erenoglu B, I Cakmak, H Marschner, V Romheld, S Eker, H Daghan, M Kalayci and H Ekiz (1996). Phytosiderophore release does not relate well with zinc efficiency in different bread wheat genotypes. *J. Plant Nutr. **19**(12): 1569-1580.*

294. Jones D L and P R Darrah (1995). Influx and efflux of organic acids across the soil-root interface of *Zea mays* L. and its implication in rhizosphere C flow. *Plant Soil **173**: 103-109.*

295. Jones D L and P R Darrah (1994). Amino-acid influx at the soil-root interface of *Zea mays* L. and its implications in the rhizosphere. *Plant Soil **163**: 1-12.*

296. Kramer U, J D Cotter-Howells, J M Charnock, A J M Baker and J A C Smith (1996). Free histidine as a metal chelator in plants that accumulate nickel. *Nature **379**(15 February): 635-638.*

297. Lindsay W L and W A Norvell (1978). Development of a DTPA soil test for zinc, iron, manganese, and copper. *Soil Sci. Soc. Am. J. **42**: 421-428.*

298. Norvell W A (1984). Comparison of chelating agents as extractants for metals in diverse soil materials. *Soil Sci. Soc. Am. J. **48**: 1285-1292.*

299. Clayton P M and K G Tiller (1979). A chemical method for the determination of the heavy metal content of soils in environmental studies. *Division of Soils Technical Paper 41, CSIRO: Glen Osmond, SA, Australia.*

300. Almas A, B R Singh and B Salbu (1999). Mobility of cadmium-109 and zinc-65 in soil influenced by equilibration time, temperature, and organic matter. *J. Environ. Qual. **28**: 1742-1750.*

301. Ribeiro A, A Villumsen, A Refega, J S Vieira and G Bech-Nielsen (1996). Looking at each step of a sequential extraction procedure applied to a contaminated soil before and after an electrodialytic remediation experiment. In *16th World Congress of Soil Science. Montpellier, France: Int. Soil Sci. Soc.*

302. Ahnstrom Z A S and D R Parker (2001). Cadmium reactivity in metal-contaminated soils using a coupled stable isotope dilution-sequential extraction procedure. *Environ. Sci. Technol. **35**: 121-126.*

303. Tiller K (1978). Applications of isotopes to micronutrient studies. In *Isotopes and radiation in research on soil-plant relationships. Colombo, Sri Lanka: International Atomic Energy Agency.*

304. Fried M and L A Dean (1952). Concept concerning the measurement of available soil nutrients. *Soil Sci. **73**: 256-271.*

305. Shaw E, R G Menzel and L A Dean (1954). Plant uptake of zinc65 from soils and fertilizers in the greenhouse. *Soil Sci. **77**: 205-214.*

306. Lopez P L and E R Graham (1970). Isotopic exchange studies of micronutrients in soils. *Soil Sci. **110**: 24-30.*

307. Lopez P L and E R Graham (1972). Labile pool and plant uptake of micronutrients: 1. Determination of labile pool of Mn, Fe, Zn, Co, and Cu in deficient soils by isotopic exchange. *Soil Sci. **114**(4): 295-299.*

308. Stacey S (1999). Measuring plant available and exchangeable Cd and Zn in sewage sludge and the progress of ageing reactions using isotopic tracers. *The University of Adelaide, Adelaide. B.Sc Honours Thesis. p. 51*

309. McIntyre T and G M Lewis (1997). The advancement of phytoremediation as an innovative environmental technology for stabilization, remediation, or restoration of contaminated sites in Canada: A discussion paper. *J. Soil Cont.* ***6****(3): 227-241.*

310. Raskin I, R D Smith and D E Salt (1997). Phytoremediation of metals: using plants to remove pollutants from the environment. *Curr. Opin. Biotech.* ***8****: 221-226.*

311. Chaney R L, M Malik, Y M Li, S L Brown, E P Brewer, J S Angle and A J M Baker (1997). Phytoremediation of soil metals. *Cur. Opin. Biotech.* ***8****: 279-284.*

312. McGrath S P (1998). Phytoextraction for Soil Remediation. In *Plants that hyperaccumulate heavy metals*, Brooks R R, Editor. *CAB International: Wallingford, Oxon, UK. pp. 261-287*

313. McLaughlin M J, R E Hamon, N A Maier, M K Smart, R Genderson, G D Cozens, R L Correll and C D Grant (1998). An overview of phytoremediation and *in-situ* immobilisation techniques to remediate cadmium-contaminated agricultural soils. *ASSI National Soils Conference 1998. Environmental Benefits of Soil. Brisbane, Queensland, Australia: ASSI.*

314. Schnoor J L (1997). Phytoremediation. *Technology Evaluation Report. Ground-Water Redmediation Technologies Analysis Center: Pittsburgh, PA, USA. p. 29.*

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

316. Salt D E, R D Smith and I Raskin (1998). Phytoremediation. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* ***49****: 643-668.*

317. Cunningham S D and D W Ow (1996). Promises and prospects of phytoremediation. *Plant Physiol.* ***110****: 715-719.*

318. White P J (2001). Phytoremediation assisted by microorganisms. *Trends Plant Sci. 6(11):502.*

319. Kumar P B A N, V Dushenkov, H Motto and I Raskin (1995). Phytoextraction: The use of plants to remove heavy metals from soils. *Environ. Sci. Technol.* ***29****: 1232-1238.*

320. Chaney R L (1983). Plant uptake of inorganic waste constituents. In *Land treatment of hazardous wastes*, Parr J F, P D Marsh, and J M Kla, Editors. *Noyes Data Corporation: Park Ridge, NJ, USA. pp. 50-76.*

321. McGrath S P, Z G Shen and F J Zhao (1997). Heavy metal uptake and chemical changes in the rhizosphere of *Thalspi Caerulescens* and *Thlaspi Ochroleucum* grown in contaminated soils. *Plant Soil **188**(1): 153-159.*

322. Brown S L, R L Chaney, J S Angle and A J M Baker (1995). Zinc and cadmium uptake by hyperaccumulator *Thlaspi caerulescens* and metal tolerant *Silene vulgaris* grown on sludge-amended soils. *Environ. Sci. Technol. **29**(6): 1581-1585.*

323. Luo Y M, P Christie and A J M Baker (2000). Soil solution Zn and pH dynamics in non-rhizosphere soil and in the rhizosphere of *Thlaspi caerulescens* grown in a Zn/Cd-contaminated soil. *Chemo. **41**(1-2): 161-164.*

324. Baker A J M and R R Brooks (1989). Terrestrial higher plants which hyperaccumulate metallic elements-A review of their distribution, ecology and phytochemistry. *Biorecovery **1**: 81-126.*

325. Knight B, F J Zhao, S P McGrath and Z G Shen (1997). Zinc and cadmium uptake by the hyperaccumulator *Thlaspi caerulescens* in contaminated soils and its effects on the concentration and chemical speciation of metals in soil solution. *Plant Soil **197**: 71-78.*

326. Huang J W, M J Blaylock, Y Kapulnik and B D Ensley (1998). Phytoremediation of uranium-contaminated soils: role of organic acids in triggering uranium hyperaccumulation in plants. *Environ. Sci. Technol. **32**: 2004-2008.*

327. Kayser A, K Wenger, A Keller, W Attinger, H R Felix, S K Gupta and R Schulin (2000). Enhancement of phytoextraction of Zn, Cd, and Cu from calcareous soil: the use of NTA and sulfur amendments. *Environ. Sci. Technol. **34**: 1778-1783.*

328. Kulli B, M Balmer, R Krebs, B Lotherbach, G Geiger and R Schulin (1999). The influence of nitrilotriacetate on heavy metal uptake of lettuce and ryegrass. *J. Environ. Qual. **28**: 1699-1705.*

329. Deram A, D Petit, B Robinson, R R Brooks, P Gregg and C van Halluwyn (2000). Natural and induced heavy-metal accumulation by *Arrhenatherum elatius*: implications for phytoremediation. *Commun. Soil Sci. Plant Anal.* ***31****(3&4): 413-421.*

330. Cooper E M, J T Sims, S D Cunningham, J W Huang and W R Berti (1999). Chelate-assisted phytoextraction of lead from contaminated soils. *J. Environ. Qual.* ***28****: 1709-1719.*

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

332. Jorgensen S E (1993). Removal of heavy metals from compost and soil by ecotechnological methods. *Ecol. Engineer.* ***2****: 89-100.*

333. Ebbs S D, D J Brady and L V Kochian (1998). Role of uranium speciation in the uptake and translocation of uranium by plants. *J. Exp. Bot.* ***49****(324): 1183-1190.*

334. Crowdy S H and T W Tanton (1970). Water pathways in wheat leaves. I free space in wheat leaves. *J. Exp. Bot.* ***21****(66): 102-111.*

335. Tanton T W and S H Crowdy (1972). Water pathways in higher plants. II. Water pathways in roots. *J. Exp. Bot.* ***23****(76): 600-618.*

336. Kaszuba M and G R A Hunt (1990). Protection against membrane damage: a H-NMR investigation of the effect of Zn^{2+} and Ca^{2+} on the permeability of phospholipid vesicles. *J. Inorganic Biochem.* ***40****: 217-225.*

337. Pasternak C A (1988). A novel role of Ca^{2+} and Zn^{2+}: protection of cells against membrane damage. *Bioscience Reports* ***8****: 578-583.*

338. Lindsay W L (1979). Chelate Equilibria. In *Chemical Equilibria in Soils*, Lindsay W L, Editor. *John Wiley & Sons: New York, NY, USA. pp. 238-265.*

339. Martell A E and M Calvin (1952). Chemistry of the metal chelate compounds. *Prentice-Hall Chemistry Series, Prentice-Hall, Inc: New York NY, USA. p. 613.*

340. Burgisser C S and A T Stone (1997). Determination of EDTA, NTA and other amino carboxylic acids and their Co(II) and Co(III) complexes by capillary electrophoresis. *Environ. Sci. Technol.* ***31****: 2656-2664.*

341. Owens G, V K Ferguson, M J McLaughlin, I Singleton, R J Reid and F A Smith (2000). Determination of NTA and EDTA and speciation of their metal complexes in aqueous solution by capillary electrophoresis. *Environ. Sci. Technol.* ***34****: 885-891.*

342. Krokhin O V, A V Adamov, H Hoshino, O A Shpigun and T Yotsuyanagi (1999). Separation selectivity of some ethylenediaminetetraacetic acid and cyclohexane-1,2-diaminetetraacetic acid complexes in column and ion electrokinetic chromatography. *J. Chromatogr. A.* ***850****: 269-276.*

343. Jardine P M and D L Taylor (1995). Fate and transport of ethylenediaminetetraacetate chelated contaminants in subsurface environments. *Geoderma* ***67****: 125-140.*

344. Miller M L, B R McCord, R Martz and B Budowle (1997). The analysis of EDTA in dried bloodstains by electrospray LC-MS-MS and ion chromatography. *J. Anal. Toxicol.* ***21****: 521-528.*

345. Baron D and J G Hering (1998). Analysis of metal-EDTA complexes by electrospray mass spectrometry. *J. Environ. Qual.* ***27****: 844-850.*

346. Niessen W M A (1999). State-of-the-art in liquid chromatography-mass spectrometry. *J. Chromatogr. A.* ***856****: 179-197.*

347. Wang H J and G R Agnes (1999). Evaluation of electrospray mass spectrometry as a technique for quantitative analysis of kinetically labile solution species. *Anal. Chem.* ***71****(17): 3785-3792.*

348. Bauer K H, T P Knepper, A Maes, V Schatz and M Voihsel (1999). Analysis of polar organic micropollutants in water with ion chromatography-electrospray mass spectrometry. *J. Chromatogr. A.* ***837****: 117-128.*

349. Bremner J M and C S Mulvaney (1982). Nitrogen-Total. In *Methods of Soil Analysis. Part 2: Chemical and microbiological properties. 2nd Edition*, Page A L, R H Miller, and D R Keeney, Editors. *Am. Soc. Agron., Inc. and Soil Sci. Soc. Am., Inc: Madison, WI, USA. pp. 595-624.*

350. Olsen S R and L E Sommers (1982). Phosphorus. In *Methods of Soil Analysis. Part 2: Chemical and microbiological properties. 2nd Edition*, Page A L, R H Miller, and D R Keeney, Editors. *Am. Soc. Agron., Inc. and Soil Sci. Soc. Am., Inc: Madison, WI, USA. pp. 403-430.*

351. Thomas T (1982). Exchangeable cations. In *Methods of Soil Analysis. Part 2 - Chemical and microbiological properties. 2nd Edition*, Page A L, R H Miller, and D R Keeney, Editors. *Soil Sci. Soc. Am.: Madison, WI, USA. pp. 159-165.*

352. Hamon R E, S E Lorenz, P E Holm, T H Christensen and S P McGrath (1995). Changes in trace metal species and other components of the rhizosphere during growth of radish. *Plant Cell Environ.* ***18****(7): 749-756.*

353. GildeCarrasco C, M Guzman, F A Lorente and M Urrestarazu (1994). Xylem sap extraction: A method. *Commun. Soil Sci. Plant Anal.* ***25****(9&10): 1829-1839.*

354. Lorenz S E, R E Hamon and S P McGrath (1994). Differences between soil solutions obtained from rhizosphere and non-rhizosphere soils by water displacement and soil centrifugation. *Euro. J. Soil Sci.* ***45****: 431-438.*

355. Marschner H, V Romheld, W J Horst and P Martin (1986). Root-induced changes in the rhizosphere: Importance for the mineral nutrition of plants. *Z. Pflanzenernaehr. Bodenk.* ***149****: 441-456.*

356. Hoffland E, G R Findenegg and J A Nelemans (1989). Solubilization of rock phosphate by rape. *Plant Soil* ***113****: 161-165.*

357. Hoffland E (1992). Quantitative evaluation of the role of organic acid exudation in the mobilization of rock phosphate by rape. *Plant Soil* ***140****: 279-289.*

358. Szmigielska A M, K C J V Rees, G Cieslinski and P M Huang (1996). Low molecular weight dicarboxylic acids in rhizosphere soil of durum wheat. *J. Agric. Food Chem.* ***44****: 1036-1040.*

359. Parker D R, W A Norvell and R L Chaney (1995). GEOCHEM-PC- A chemical speciation program for IBM and compatible personal computers. In *Chemical equilibrium and reaction models*, Loeppert R H, A P Schwab, and S Goldberg, Editors. *Soil Sci. Soc. Am., Inc., Am. Soc. Agron., Inc.: Madison, WI, USA. pp. 253-270.*

360. Murakami T, K Ise, M Hayakawa, S Kamei and S-I Takagi (1989). Stabilities of metal complexes of mugineic acids and their specific affinities for iron(III). *Chem. Letters: 2137-2140.*

361. von Wiren N, S Mori, H Marschner and V Romheld (1994). Iron inefficiency in maize mutant ys1 (*Zea mays* L. cv Yellow-stripe) is caused by a defect in uptake of iron phytosiderphores. *Plant Physiol.* ***106****: 71-77.*

362. Takagi S-I, K Nomoto and T Takemoto (1984). Physiological aspect of mugineic acid, a possible phytosiderophore of graminaceous plants. *J. Plant Nutr.* ***7****(1-5): 469-477.*

363. Neumann G, C Haake and V Romheld (1999). Improved HPLC method for determination of phytosiderophores in root washings and tissue extracts. *J. Plant Nutr.* ***22****(9): 1389-1402.*

364. Hamon R E, I Bertrand and M J McLaughlin (2001). Clarification of the derivation and application of E-values in soil chemistry and plant nutrition. *Soil Sci. Soc. Am. J. (submitted).*

365. Schramel O, B Michalke and A Kettrup (1998). Analysis of metal species by using electrospray ionization mass spectrometry and capillary electrophoresis-electrospray ionization mass spectrometry. *J. Chromatogr. A.* ***819****: 231-242.*

366. Allison J D and D S Brown (1995). MINTEQA2/PRODEFA2-A geochemical speciation model and interactive preprocessor. In *Chemical equilibrium and reaction models*, Loeppert R, A P Schwab, and S Goldberg, Editors. *Soil Sci. Soc. Am, Inc., Am. Soc. Agron., Inc.: Madison, WI, USA. pp. 241-252.*

367. Anon. (1999). Installation instructions and trouble shooting guide for the IONPAC AS11. *Dionex Corp.: Sunnyvale, California.*

368. Gaskell S J (1997). Electrospray: Principles and Practice. *J. Mass Spec.* ***32****: 677-688.*

369. Bedsworth W W and D L Sedlak (1999). Sources and environmental fate of strongly complexed nickel in estuarine waters: the role of ethylenediaminetetraacetate. *Environ. Sci. Technol.* ***33****: 926-931.*

370. Hajos P, G Revesz, O Horvath, J Peear and C Sarzanini (1996). The simultaneous analysis of metal-EDTA complexes and inorganic anions by suppressed ion chromatography. *J. Chromatogr. Sci.* ***34****: 291-299.*

371. Stewart I and C D Leonard (1952). Chelates as sources of iron for plants growing in the field. *Sci.* ***116****: 564-566.*

372. Norvell W A, R M Welch, M L Adams and L V Kochian (1993). Reduction of Fe(III), Mn(III), and Cu(II) chelates by roots of pea (*Pisum sativum* L.) or soybean (*Glycine max*). *Plant Soil* ***155/156****: 123-126.*

373. Denduluri S (1994). Ameliorative effects of ethylenediaminetetraacetic acid and nitrilotriacetic acid on lead toxicity in okra (*Abelmoschus esculentus* L.) grown in sewage-irrigated soil. *Bull. Environ. Contam. Toxicol.* ***52****: 516-522.*

374. Harrison S J, N W Lepp and D A Phipps (1984). Uptake of copper by excised roots. IV. Copper uptake from complexed sources. *Z. Pflanzenphysiol. Bodenk.* ***113****: 445-450.*

375. Malzer G L and S A Barber (1976). Calcium and strontium absorption by corn roots in the presence of chelates. *Soil Sci. Soc. Am. J.* ***40****: 727-731.*

376. Sarret G, J Vangronsveld, A Manceau, M Musso, J K'Haen, J-L Menthonnex, J-L Hazemann (2001). Accumulation forms of Zn and Pb in *Phaseolus vulgaris* in the presence and absence of EDTA. *Environ. Sci. Technol.* ***35****: 2854-2859.*

377. Lorenz S E, R E Hamon, P E Holm, H C Domingues, E M Sequeira, T H Christensen, S P McGrath (1997). Cadmium and zinc in plants and soil solutions from contaminated soils. *Plant Soil* ***189****: 21-31.*

378. Gerard E (2000). Caracterisation du cadmium phytodisponible des sols par des methodes isotopiques. *Institut National Polytechnique de Lorraine, Vandoeuvre-les-Nancy, France. Ph.D Thesis. p. 146.*

379. Lahav N and M Hochberg (1975). Fixation of iron and zinc applied as chelates into a soil column during leaching. *Soil Sci. Soc. Am. Proc.* ***39****: 1213-1215.*

380. Wallace A and O R Lunt (1956). Some reactions of iron, zinc, and manganese chelates in various soils. *Soil Sci. Soc. Am. Proc.* ***20****: 479-482.*

381. Stewart I and C D Leonard (1954). Chelated metals for growing plants. In *Fruit nutrition*, Childs N F, Editor. *Horticultural Publications: New Brunswick, NJ, USA. pp. 775-809.*

382. Lunt O R, N Hemaidan and A Wallace (1956). Reactions of some polyamine polyacetate iron chelates in various soils. *Soil Sci. Soc. Am. Proc.* ***20****: 172-175.*

383. Grifferty A and S Barrington (2000). Zinc uptake by young wheat plants under two transpiration regimes. *J. Environ. Qual.* ***29****: 443-446.*

384. Peterson C A and L V Edgington (1975). Uptake of the systemic fungicide methyl 2-benzimidazolecarbamate and the fluorescent dye PTS by onion roots. *Phytopath.* ***65****: 1254-1259.*

385. Romheld V and H Marschner (1981). Rhythmic iron stress reactions in sunflower at suboptimal iron supply. *Physiol. Plant.* ***53****: 347-353.*

386. Sanderson J (1983). Water uptake by different regions of the barley root. Pathways of radial flow in relation to development of the endodermis. *J. Exp. Bot.* ***34****(140): 240-253.*

387. Sattelmacher B, K-H Muhling and K PennewiB (1998). The apoplast - its significance for the nutrition of higher plants. *Z. Pflanzenernahr. Bodenk.* ***161****(5): 485-498.*

388. Else M A, K C Hall, G M Arnold, W J Davies and M B Jackson (1995). Export of abscisic acid, 1-aminocyclopropane-1-carboxylic acid, phosphate, and nitrate from roots to shoots of flooded tomato plants. *Plant Physiol.* ***107****: 377-384.*

389. Liang J and J Zhang (1997). Collection of xylem sap at flow rate similar to in vivo transpiration flux. *Plant Cell Physiol.* ***38****(12): 1375-1381.*

390. Schurr U (1998). Xylem sap sampling - new approaches to an old topic. *Trends Plant Sci.* ***3****(8): 293-298.*

391. White M C, R L Chaney and A M Decker (1981). Metal complexation in xylem fluid. III. Electrophoretic evidence. *Plant Physiol.* ***67****: 311-315.*

392. Anderson P R and T H Christensen (1988). Distribution coefficients of Cd, Co, Ni, and Zn in soils. *J. Soil Sci.* ***39****: 15-22.*

393. Sauve S, W Hendershot and H E Allen (2000). Soil-solution partitioning of metals in contaminated soils: dependence on pH, total metal burden, and organic matter. *Environ. Sci. Technol.* ***34****(7): 1125-1135.*

394. Shuman L M (1975). The effect of soil properties on zinc adsorption by soils. *Soil Sci. Am. Proc.* ***39****: 454-458.*

395. Elliott H A and C P Huang (1979). The adsorption characteristics of Cu(II) in the presence of chelating agents. *J. Colloid Interface Sci.* ***70****(1): 29-.*

396. Lehoczky E, P Marth, I Szabados and A Szomolanyi (1996). Effect of liming on the heavy metal uptake of lettuce. In *16th World Congress of Soil Science. Montpellier, France: Int. Soil Sci. Soc.*

397. Pepper I L, D F Bezdicek, A S Baker and J M Sims (1983). Silage corn uptake of sludge-applied zinc and cadmium as affected by soil pH. *J. Environ. Qual.* ***12****: 270-275.*

398. Scotti I A, S Silva and C Baffi (1999). Effects of fly ash pH on the uptake of heavy metals by chicory. *Water Air Soil Pollut.* ***109****: 397-406.*

399. Chairidchai P and G S P Ritchie (1993). The effect of citrate and pH on zinc uptake by wheat. *Agron. J.* ***85****: 322-328.*

400. Pierzynski G M and A P Schwab (1993). Bioavailability of zinc, cadmium, and lead in a metal-contaminated alluvial soil. *J. Environ. Qual.* ***22****: 247-254.*

401. Jackson A P and B J Alloway (1991). The transfer of cadmium from sewage sludge amended soils into the edible components of food crops. *Water Air Soil Pollut.* ***57-58****: 873-881.*

402. Mordvedt J J, D A Mays and G Osborn (1981). Uptake by wheat of cadmium and other heavy metal contaminants in phosphate fertilizers. *J. Environ. Qual.* ***10****: 193-197.*

403. Tiller K G, V K Nayyar and P M Clayton (1979). Specific and non-specific sorption of cadmium by soil clays as influenced by zinc and calcium. *Aust. J. Soil Res.* ***17****: 17-28.*

404. Howe J A, Y H Choi, R H Loeppert, L C Wei, S A Senseman and A S R Juo (1999). Column chromatography and verification of phytosiderophores by phenylisothiocyanate derivatization and UV detection. *J. Chromatogr. A.* ***841:*** *155-164.*

405. Mori S, N Nishizawa, S Kawai, Y Sato and S Takagi (1987). Dynamic state of mugineic acid and analogous phytosiderophores in Fe-deficient Barley. *J. Plant Nutr.* ***10****(9-16): 1003-1011.*

406. Taylor D L and P M Jardine (1995). Analysis of cobalt(II)EDTA and cobalt(III)EDTA in pore water by ion chromatography. *J. Environ. Qual.* ***24:*** *789-792.*

407. Means J L, D A Crerar and J O Duguid (1978). Migration of radioactive wastes: radionuclide mobilization by complexing agents. *Sci.* ***200:*** *1477-1481.*

408. Nowack B, F G Kari, S U Hilger and L Sigg (1996). Determination of dissolved and adsorbed EDTA species in water and sediments by HPLC. *Anal. Chem.* ***68:*** *561-566.*