PHYTOEXTRACTION OF CADMIUM FROM SOILS TREATED WITH SEWAGE SLUDGE

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ABSTRACT

The efficacy of phytoextraction strategies were tested by pot and field trials on soil contaminated with heavy metals, including Cd, derived from long-term disposal of sewage sludge. The strategies investigated were: i) the use of hyperaccumulators; ii) chemically-enhanced uptake using arable species and iii) the use of short rotation coppice (SRC). Chemical interventions including EDTA, chloride salts, HCl and herbicide were used to enhance uptake by arable and SRC species.

Tissue Cd concentrations in the Ganges population of *Thlaspi caerulescens* were lower than reported in other studies; the mean Cd concentration was 265 mg kg^{-1}. It was deduced that Cd uptake was limited by a low Cd^{2+} concentration in soil and the rate at which solution Cd was replenished. High rates of plant mortality were observed, raising questions over the successful husbandry of *T. caerulescens* for phytoextraction. Chemical interventions produced significant increases in metal uptake by arable and SRC species. For example, Cd uptake by *Z. mays* following application of 10 mmol EDTA kg^{-1} and by *Salix caprea x cineria x viminalis* following combined application of EDTA and HCl. However, concentrations were still well below those required for successful remediation. Furthermore downward migration of metal was observed through the soil profile following EDTA application. For example, the soil Cd concentration in the 0 - 10 cm profile was reduced from 32.0 to 25.5 mg kg^{-1} seven months after application of 10 mmol EDTA kg^{-1}, yet only 1 % of this reduction could be accounted for by *Z. mays* Cd off-take.

Realistic estimates for phytoextraction timescales and costs were made in line with legislative thresholds. Overall the time required to reduce total soil Cd concentrations below 3 mg kg^{-1} was large and the costs were prohibitive. For example, although Cd off-take by Ganges was greater than for any of the other species tested, it was estimated that well over one century would be required to reach target metal concentrations.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Contamination of soil by cadmium

1.1.1 Principal sources of cadmium to soil

Cadmium (Cd) in soil originates from either geological parent material, following weathering, or from anthropogenic sources (Kabata-Pendias and Pendias, 1992). Estimates for worldwide anthropogenic Cd production is shown in Table 1.1. The greatest anthropogenic sources of Cd, after fly ash production, is atmospheric deposition resulting from metalliferous mining and smelting, metal-using industries, phosphatic fertiliser manufacture, general urban emissions, incineration of municipal waste, coal combustion and road dust (Alloway and Steinnes, 1999). The next most important Cd inputs to soil come from applications of phosphatic fertiliser and sewage sludge. The Cd concentration of phosphatic fertilisers, present as a natural constituent in rock phosphates, may be up to 300 mg Cd kg^{-1}. By contrast, N and K fertilisers generally contain less than 9 mg Cd kg^{-1} (Fergusson, 1990). However, the input of Cd to soils amended with sewage sludge is of major importance (Alloway and Jackson, 1991a). Cd is reported to pose the greatest toxicity threat to humans resulting from Cd uptake by plants where sewage sludge is applied to agricultural land (Smith, 1996). The production and sustainable disposal of sewage sludge are discussed in Sections 1.1.4 and 1.1.5.

Table 1.1. Worldwide production of Cd from anthropogenic sources (From Nriagu and Pacyna, 1988).

Sources	Production (10^6 kg y^{-1})	Percentage of total
Agricultural (including fertiliser)	0.2 - 4.5	11.7
Logging and wood wastes	0.2 - 2.2	5.8
Urban refuse and sludge	0.7 - 7.8	20.6
Fly-ash	1.5 - 13.0	34.2
Atmospheric deposition	2.2 - 8.4	22.2
Others	0.7 - 2.2	5.5

1.1.2 Cd toxicity to humans

Cd is toxic to humans at low concentrations and food crops may accumulate potentially harmful concentrations without exhibiting phytotoxic symptoms (Singh and McLaughlin, 1999). The critical organ for long-term exposure in humans is the kidney, for which the biological half-life of Cd is believed to be 18 years (Forstner, 1991). The first observation of Cd-induced disease was reported for subsistence farmers growing rice in the Jintsu River Valley, Japan in 1964, in an area where Cd-rich wastewater was used for irrigation (Wagner, 1993). The source of Cd was acidic drainage water from a Pb/Zn mine. The disease ("Itai itai") was reported where Cd intake was estimated to be 300 - 600 μg day^{-1} (Ryan *et al.*, 1982). Diseases caused by Cd toxicity have also been reported among subsistence rice farmers elsewhere in Japan and China, even when soil concentrations were as low as 2 mg kg^{-1} (Nogawa, 1984). The use of flooded soils for rice production is believed to increase Cd transfer to the grain to an extent not seen in conventional crop production systems on more aerobic soils (Chaney *et al.*, 1999). Tobacco is the other major crop known to induce disease resulting from the accumulation of Cd, to potentially harmful concentrations, as smoking has been shown to double Cd concentration in the kidneys of smokers with low Cd diets (Chaney *et al.*, 1999). The Cd content of one cigarette is reported to be 1 - 2 μg (DEFRA, 2002a), while the estimated daily uptake associated with smoking 20 cigarettes per day is 1 - 4 μg Cd (WHO, 1992).

WHO/FAO (1972) considered that damage may occur when the Cd concentration in the renal cortex exceeds 200 μg g^{-1}. However, a provisional tolerable weekly intake (PTWI) of 400 - 500 μg Cd per person was recommended. US EPA (2001) also reported that the highest concentration of Cd in the renal cortex which was not associated with significant proteinuria was 200 μg Cd g^{-1} and WHO (2001) subsequently revised their recommendations. The critical concentration of Cd in the renal cortex was therefore amended to 50 μg g^{-1} (WHO, 2001). WHO (2001) also suggested a PTWI of 7 μg kg^{-1} body weight $week^{-1}$. Current UK guidelines for tolerable daily oral intake (TDI_{oral}), the mean daily oral intake (MDI) and the tolerable daily oral soil intake (TDSI) are shown in Table 1.2. TDSI is defined as the difference

between TDI_{oral} and MDI. A TDI_{oral} of 0.77 μg kg^{-1} body weight d^{-1} for an adult is equivalent to 7 μg kg^{-1} bw $week^{-1}$, i.e. the PTWI set out by WHO (2001).

Table 1.2. Tolerable Daily Intake (TDI_{oral}), Mean Daily Intake (MDI) and Tolerable Daily Soil Intake (TDSI) for an adult and six year old child (DEFRA, 2002a). Values are expressed per unit bodyweight.

TDI_{oral} (μg kg^{-1} d^{-1})	Oral MDI (μg d^{-1})	TDSI for an adult (μg kg^{-1} d^{-1})	TDSI for a six year old child (μg kg^{-1} d^{-1})
1	16	0.77	0.5

Nogawa (1984) reported that the typical daily intake of Cd in the USA and Canada is between 50 and 80 μg Cd d^{-1} from food, although Smith (1996) suggested that the average daily intake for non-smokers is 16 μg d^{-1}. DEFRA (2002a) reported that the approximate current mean daily intake of Cd from food and water combined is 16 μg Cd $d^{-1}$for an adult weighing approximately 70 kg in the UK (based on data from MAFF, 1999).

1.1.3 Cd toxicity to plants

The most common symptoms of Cd toxicity are stunting and chlorosis which may resemble Fe deficiency (Das *et al.*, 1997). Interactions between Cd and Fe have been widely studied and Haghiri (1973) reported that Fe uptake was suppressed by high Cd concentrations in the growing medium. Cd adversely affects enzyme systems (Forstner, 1991) and strongly interferes with the functions of metalloproteins, metallo-enzymes, metallothioneins and phospholipids (Forstner, 1991; Das *et al.*, 1997).

1.1.4 Production of sewage sludge

Sewage disposal traditionally involved applications of raw sewage to dedicated land, but this method became inadequate as conurbations expanded during the last century (DETR, 1998). Around 35 Mt of raw sewage sludge are produced in the UK each year

(DETR, 2000a). A range of different sludge treatment processes exist including pasteurisation, dewatering and storage, composting and mesophilic anaerobic digestion (MAD; DETR, 1998). Rowlands and Sweet (1997) reported that MAD was the most widely used sludge treatment process in the UK. This process essentially digests the sludge under anaerobic conditions at 30 - 37 ^{0}C, with a retention period of at least 15 days. Secondary digestion is required to reduce pathogen content, which encompasses a 14 day retention period (DETR, 1998). End products include clean effluent, which can be returned to the river system, and sludge containing most of the organic load of the original raw sewage (DETR, 1998). Treatment processes significantly reduce the pathogen content of sludge, but heavy metals remain, so the disposal of treated sludge must be controlled (Section 1.1.5). Quantities of treated sludge are usually expressed as dry solids, of which approximately 1.1 Mt are currently produced in the UK, although this is expected to rise to 1.5 Mt by 2005 (DETR, 2000a).

The origins of heavy metals in wastewater and sewage are varied, and can include both industrial and domestic sources. Cu, for example, is derived mainly from Cu piping within domestic plumbing (Coppoolse, 1992). Pb plumbing can contribute 14 mg y^{-1}, compared to an average 9 mg y^{-1} (Koch and Rotard, 2001). However, Cd is more likely to be derived from industrial activities such as the manufacture of batteries or electroplating (Alloway and Steinnes, 1999). The Zn content of sewage sludge results mainly from domestic activities such as the use of washing machines (20 %) and faeces (50 %); the corresponding values for Ni are 19 and 40 % (Smith, 1996). Smith (1996) extensively reviewed the sources and composition of heavy metals in sludge. Metal concentrations in sludge have declined markedly in the last forty years, mainly as a result of a decline in traditional manufacturing, the adoption of cleaner technologies, and improved control of wastewater discharges. Rowlands (1992) reported a reduction of 98 % in the Cd concentration of sewage sludge produced in Nottingham between 1962 and 1992, coupled with a reduction of 80 % in Zn over the same period. The median Cd concentration of sewage sludge used in agriculture in the UK in 1996/97 was 1.6 mg kg^{-1}, with a 90 percentile of 3.4 mg kg^{-1} (Rowlands and Sweet, 1997).

1.1.5 Legislation and the 'sustainable' disposal of sewage sludge to agricultural land

Disposal options for sewage sludge are varied and controversial; the main disposal routes used in the UK are illustrated in Table 1.3. Marine disposal of sludge ceased in 1998 following changes in EU legislation, and it is estimated that land disposal will account for 60 % of sewage sludge by 2005 (DETR, 2000a). Indeed, the current UK government 'Waste Strategy' describes the spreading of sewage sludge on agricultural land as the 'best practicable environmental option' (DETR, 2000a).

Table 1.3. Disposal routes for sewage sludge in UK in 1994, 1996/97 and projected for 2005.

Disposal Route	**1994** [1] **%**	**1996/97** [2] **%**	**2005** [3] **%**
Agricultural land	44	52	60
Landfill	8	10	4
Sea	30	19	0
Incineration	7	9	36
Other	11	10	Not provided

[1] Hall and Dalimer (1994)
[2] Rowlands (1998).
[3] DETR (2000a)

As heavy metals persist in the environment and do not migrate through the soil profile (McGrath and Lane, 1989), their maximum concentrations in both sludge and soil must be controlled by legislation; without appropriate legislation, uptake of Cd may exceed safe limits as described in Section 1.1.2. The current EU legislation (Directive 86/278/EEC; CEC, 1986) has been enforced in England, Scotland and Wales by *The Sludge (Use in Agriculture) Regulations, 1989* (SI, 1989). The maximum concentrations of heavy metals permitted in agricultural soil following the application of sewage sludge and the maximum annual inputs allowed are shown in Table 1.4. Regulations include provision for the application of sludge to dedicated sites, where crop production must be exclusively used for animal production, and where periodic site sampling and monitoring are required as part of a management plan approved by the Ministry of Agriculture, Fisheries and Foods (MAFF) (now Department for

Environment, Fisheries and Rural Affairs (DEFRA); DoE, 1989). DEFRA (2002b) outlined proposed changes to the 1989 Sludge Regulations, suggesting that the maximum Pb concentration should be reduced to 200 mg kg^{-1} for soil with a pH ≤5.0 and Zn to 200 mg kg^{-1} for soil with a pH 5.5 - 7.0.

Table 1.4. Maximum permissible concentrations of heavy metals in soil after applying sewage sludge and maximum annual rates of addition; as set out by the 1989 Sludge Regulations (SI, 1989).

Heavy metal	Maximum permissible concentration in soil (mg kg^{-1} dry solids)				Maximum permissible mean annual application rate over a 10 year period (kg ha^{-1})
	pH 5.0 < 5.5	pH 5.5 < 6.0	pH 6.0 < 7.0	pH >7.0	
Zn	200	250	300	450	15
Cu	80	100	135	200	7.5
Ni	50	60	75	110	3
	For pH 5.0 and above				
Cd	3				0.15
Pb	300				15

However, even with legislation in place, the sustainability of the long-term application of sewage sludge to land is unclear. Smith (1996) calculated the minimum number of years required for soil metal concentrations to reach the current maximum soil limits (3 mg kg^{-1} for Cd in the UK) assuming an average background soil Cd concentration of 0.8 mg kg^{-1}, a soil contamination depth of 20 cm, and a bulk density of 1.0 g cm^{-3}. This analysis showed that, if the maximum permitted annual sludge inputs (0.15 kg Cd ha^{-1} y^{-1}) were applied each year, all elements would reach the permitted concentrations within 50 years (Cd in 29 years). A similar calculation by Keller and Desaules (1997) estimated that the Swiss guide values would be reached within 100 years for Pb and Cu, and that almost 65000 ha of land in Switzerland exceeded their guide values for Cd as a result of sludge application.

Table 1.5 outlines equivalent US and European metal thresholds for land treated with sewage sludge. In general, European legislation has taken a more precautionary

approach and some concern has been expressed in the literature over the US regulatory values. Furthermore, the draft EU proposal (ENV.E3/LM 2000) suggests that future European legislation will be tightened (Table 1.6), thus decreasing the time taken for soil metal concentrations to reach the statutory maximum levels. Taking the proposed upper limit for Cd of 1.0 mg kg^{-1} for soil with a pH range 6.0 - 7.0 (Table 1.6) and using the assumptions described by Smith (1996), it would take just 13 years for soil Cd concentration to reach the proposed maximum limit if the proposed annual rate of Cd application was adopted (0.03 kg ha^{-1} y^{-1}).

Table 1.5. Maximum permissible concentrations of heavy metals in soil (mg kg^{-1}) following application of sewage sludge permitted by US and European legislation (from McBride, 1995).

Metal	USEPA - 503	UK (pH 5.5 < 6.0)	Germany	Netherlands
Zn	1400	250	600	1000
Cu	750	100	200	200
Ni	210	60	100	200
Cd	20	3	6	10
Pb	150	300	200	300

Table 1.6. Maximum permissible concentrations of heavy metals in soil after applying sewage sludge and maximum annual rates of addition, as proposed by the Working Document on Sludge Third Draft (ENV.E3/LM 2000).

Heavy metal	Maximum permissible concentration in soil (mg kg dry solids)			Maximum permissible average annual rate over a 10 year period (kg ha^{-1})
	pH 5.0 < 6.0	pH 6.0 < 7.0	pH >7.0	
Zn	60	150	200	7.5
Cu	20	50	100	3.0
Ni	15	50	70	0.9
Cd	0.5	1	1.5	0.03
Pb	70	70	100	2.25

1.1.6 Contaminated land: Industrial and arable sites

In addition to agricultural sites contaminated with heavy metals, there are numerous contaminated former industrial sites worldwide. In Europe, there are reported to be 1.4 million contaminated sites (ETCS, 1998), and estimates for the UK range from 100000 - 300000 ha of contaminated land (Holgate, 2000). The British Government has projected a requirement for 4.4 million new houses by 2016, with a minimum target of 60 % of these to be built on brownfield land (UK Parliament, 1998). The consequent demand for remediation of contaminated land is large, with around £500 million being spent each year in the UK alone (Bardos *et al.*, 1999).

The definition of contaminated land, as outlined by Part IIA of the Environment Protection Act 1990 which came into force in 2000 is "land which appears to the Local Authority to be in such a condition, by research of substances in, on or under the land, that significant harm is being caused, or there is a significant possibility of such harm being caused, or that pollution of controlled water is being, or is likely to be caused" (DETR, 2000b).

Local Authorities with responsibility for assessing contaminated land in the UK, must consider the concept of 'pollutant linkages'. A linkage between a contaminant and a receptor must be established by means of a pathway for a site to be classified as contaminated (Holgate, 2000). To complete this process, Local Authorities may use a risk assessment model in future, most likely the 'Contaminated Land Exposure Assessment' (CLEA) model (DEFRA, 2002c). However, as risk assessment is highly objective, it is likely that the Local Authorities will adopt a highly precautionary approach, probably relying on the new CLEA (generic) trigger values.

The CLEA 'trigger values' (Table 1.7) replace the ICRCL 'trigger concentrations' (Table 1.8) and utilise total metal thresholds, in much the same was as the 1989 Sludge Regulations (Section 1.1.5). In the case of the 1989 Sludge Regulations, threshold values are designed to set upper limits for total soil metal concentrations in agricultural soil following application of sewage sludge. Where ICRCL 'trigger concentrations' are used to assess whether former industrial soils require remediation, this is recommended if 'trigger concentrations' are exceeded. The new CLEA 'trigger values' will indicate whether a full risk assessment is warranted. For Cd, the 1989 Sludge Regulations and ICRCL rely on the value of 3 mg kg^{-1} as either an upper limit for total soil Cd concentration or a 'trigger concentration'. The CLEA value for Cd on allotments or residential land where plants are grown is 1 mg kg^{-1} (pH 6), which is in line with proposed changes to the 1989 Sludge Regulations (ENV.E3/LM 2000; Table 1.6). A full risk assessment is required if this value is exceeded.

Table 1.7. Soil guideline values for Cd as a function of land use (DEFRA, 2002d).

Standard land use	**Soil Guideline Value (mg kg^{-1} air-dried soil)**		
	pH 6	**pH 7**	**pH 8**
Residential with plant uptake / allotments	1	2	8
Residential without plant uptake		30	
Commercial / industrial		1400	

Table 1.8. Selected ICRCL trigger concentrations for contaminated land assessment (ICRCL, 1987).

Phytotoxic contaminants not normally hazardous to health	Planned use for site	Trigger concentrations (mg kg^{-1} air-dried soil)
Zn	Any uses where plants are to be grown	300
Cu	Any uses where plants are to be grown	130
Ni	Any uses where plants are to be grown	70
Contaminants posing health hazards		
Cd	Domestic gardens, allotments	3
	Parks, playing fields, open space	15
Pb	Domestic gardens, allotments	500
	Parks, playing fields, open space	2000

According to a survey of the heavy metal content of UK soils presented in the Soil Geochemical Atlas of England and Wales (McGrath and Loveland, 1992; Table 1.9), 10 % of UK soils exceed a Cd concentration of 1.4 mg kg^{-1}. This concentration exceeds the proposed upper limit for Cd concentrations in soil (pH 6.0 – 7.0) following sludge application as set out by (ENV.E3/LM 2000; Table 1.6). Furthermore, Rowlands and Sweet (1997) reported that the mean Cd concentration of soil used for sewage sludge disposal is 0.5 mg kg^{-1}, with a ninety percentile of 1.7 mg kg^{-1}. Therefore, over 10 % of soil currently used for sludge application would also exceed the proposed upper limit for soil Cd concentrations set out by (ENV.E3/LM 2000; Table 1.6).

Table 1.9. Metal concentrations (mg kg^{-1} air-dried soil) in soils of England and Wales (McGrath and Loveland, 1992).

Metal	Ten percentile	Median	Ninety percentile	Arithmetic mean
Zn	38	82	147	97
Cu	9	18	37	23
Ni	7	2.3	42	25
Cd	0.2	0.7	1.4	0.8
Pb	20	40	131	74

1.1.7 Options for remediating contaminated land

Various methods have been developed for the remediation of metal contaminated soils; these broadly fit into four categories: removal, containment, *ex-situ* treatment, and *in situ* treatment. Examples of each method are outlined in Table 1.10, from which it is clear that these remediation methods are costly. Taking, for example, the cheapest treatment process listed, i.e. soil washing, and assuming a remediation depth of only 20 cm, with a soil bulk density of 1.25 g cm^{-3}, the cost would be US \$62500 ha^{-1}, excluding excavation and transport costs.

Table 1.10. Summary of principal soil remedial techniques and their estimated costs (Mulligan *et al.*, 2001).

Technology	Description	Cost US \$ t^{-1} (£ t^{-1})[1]	
Removal[2]	Excavation and disposal in landfill	65 – 120	(45 – 85)
Containment			
Physical	Prevent movement of fluid flow	10 – 90	(7 – 63)
Encapsulation	Creation of an inert waste	60 – 290	(42 –204)
Vitrification	Application of electrical energy to vitrify contaminant	400 – 870	(281 – 613)
Ex-situ treatment			
Physical separation	Various, e.g. gravity separation, screening, froth flotation	60 – 245	(42 – 173)
Soil washing	Addition of surfactants and other additives to solubilise contaminants	25 – 300	(18 – 211)
Pyrometallurgical	Elevated temperature extraction and processing for metal removal	200 – 1000	(140 – 704)
In-situ treatment			
Reactive barriers	Creation of permeable barrier	60 – 245	(42 – 173)
Soil flushing	Water flushing to leach contaminants	100 – 200	(70 – 140)
Electrokinetic[3]	Application of electrical current	120 – 170	(86 – 120)

[1]Currency conversion US \$ to £ sterling @ 0.7043 on 20/5/2002.
[2](Churngold Remediation Ltd, Bristol. *pers. comm.*)
[3](www.geokinetics.com) example cost for small-scale project.

In the UK, the most widely used remediation technique involves removal and disposal of contaminated soil in landfill sites; this approach accounts for around 50 % of all remediation (Wallace, 1998). However, this is not a long-term solution, as the contaminant is simply relocated. Similarly, the cheapest option, containment, does not provide a long-term solution, and restricts the land use options for the site in question (Mulligan *et al.*, 2001).

In addition, the processes listed in Table 1.10 are unlikely to be suitable for the remediation of contaminated agricultural land, as expensive remedial processes are difficult to justify on sites where profit margins are often low. In an attempt to resolve the problems, associated with traditional remediation techniques, the novel technique of 'phytoremediation' has been investigated.

1.1.8 Phytoremediation

Phytoremediation is a term used to describe a set of technologies in which plants are used to clean contaminated sites (US EPA, 2000). In this context, plants are often described as 'solar driven pumping and filtering systems' (Cunningham *et al.*, 1995), whereby solar energy is used to power a useful remediation process. This approach is reported to be more acceptable to the public than conventional remediation methods (Matso, 1995), and is also estimated to be up to 80 % cheaper (US EPA, 2000). The main techniques associated with phytoremediation involve:

1) Phytodegradation of organic compounds, including pesticides (Nyer and Gatliff, 1996)
2) Phytostabilisation of polluted soil, using plants to establish vegetative cover (Salt *et al.*, 1995)
3) Phytofiltration, where metals are precipitated and absorbed from solution (Matso, 1995)
4) Phytoextraction, the uptake of metals into harvestable plant tissue (Raskin *et al.*, 1997)

The principles involved in phytoextraction are discussed in detail in Section 1.3.

1.2 Factors affecting uptake of Cd by plants from soils treated with sewage sludge

1.2.1 Introduction

Uptake of Cd may be controlled primarily by its concentration in the soil solution accessed by crop roots (Mullins *et al.*, 1986). However, the Cd cycle in soil is complex and may be influenced by a number of biotic and abiotic factors (Fig. 1.1). Several studies of metal uptake by plants following application of sewage sludge indicate important characteristics common to soil under this land use. For example, the total quantity of Cd present in soil and that present in forms available to plants are closely correlated with metal additions derived from sewage sludge applications (Kelling *et al.*, 1977; Chumbley and Unwin, 1982; Lund *et al.*, 1985; Miller *et al.*, 1995; Moreno *et al.*, 1996; Sloan *et al.*, 1997; Canet *et al.*, 1998). Gardiner *et al.* (1995), for example, reported that the Cd content of Swiss chard increased from 0.7 mg kg^{-1} to 4.4 mg kg^{-1} in control plants following application of sludge containing 21.2 kg Cd ha^{-1}.

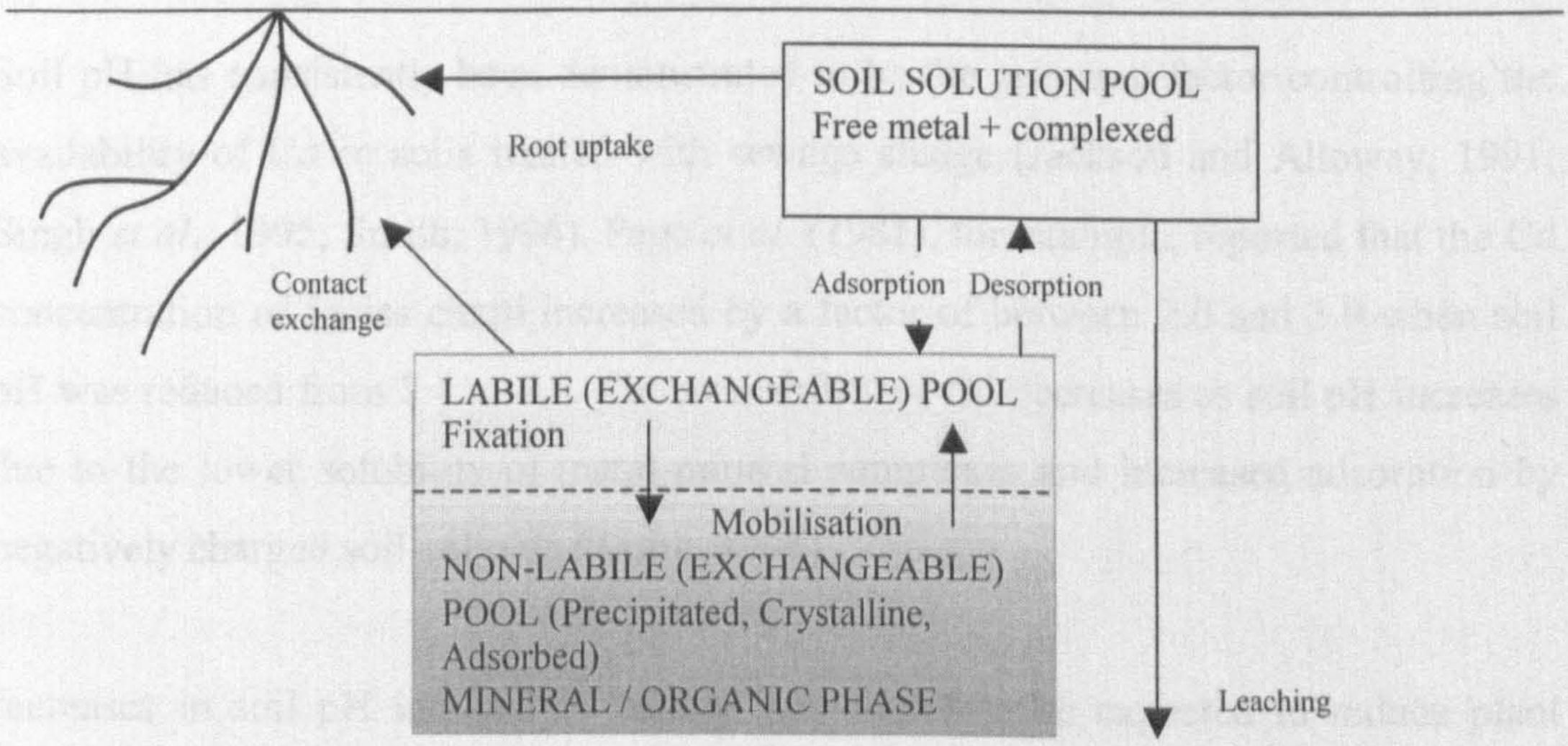


Figure 1.1. The main fluxes of Cd in soils (from McLaughlin and Singh, 1999).

Cd is reported to be the most bio-available metal following sludge application to soil even several years after cessation of sludge application. For example, Alloway and

Jackson (1991a), suggested that 'almost all' authors assessing the bio-availability of metals in sludged soils concluded that high concentrations of metals in plant organs were observed for many years after application relative to plants grown on non-contaminated sites. Villarroel *et al.* (1993) reported significantly higher concentrations of Cd in Swiss chard for nine consecutive years following the termination of sludge application in comparison to non-contaminated soils.

In addition, metals are reported to remain within the rooting zone for long periods (McGrath and Lane, 1989; McBride, 1995). For example, Chang *et al.* (1984) reported that over 90 % of metal from sewage sludge application remained in the 0 - 15 cm soil profile, six years after sludge application. Metal contamination in soils treated with sewage sludge is therefore characteristically located in the surface soil horizons and is mainly restricted to the plough layer.

1.2.2 Principal soil factors affecting metal bioavailability

Soil pH

Soil pH has consistently been demonstrated to be the principal factor controlling the availability of Cd in soils treated with sewage sludge (Jackson and Alloway, 1991; Singh *et al.*, 1995; Smith, 1996). Page *et al.* (1981), for example, reported that the Cd concentration of Swiss chard increased by a factor of between 2.0 and 3.9 when soil pH was reduced from 7.4 to 4.5. The availability of Cd decreases as soil pH increases due to the lower solubility of metal mineral complexes and increased adsorption by negatively charged soil colloids (Smith, 1994).

Increases in soil pH induced by liming may therefore be expected to reduce plant metal uptake (Maclean, 1976). Krebs *et al.* (1998) demonstrated that the aerial parts of pea grown on limed soils contained lower concentrations of Cu, Zn and Cd than plants grown on unlimed soils. The greatest contrast between the limed and unlimed treatments was for plant Cd concentrations, as a value of 213 mg kg^{-1} plant dry weight was obtained for plants grown in unlimed plots compared to 67 mg kg^{-1} on limed plots. Alloway and Jackson (1990) found that liming of 18 soils previously treated

with sewage sludge raised their pH to a value of 7 and reduced the Cd content of lettuce by an average of 41 % and cabbage by 43 %. Alloway and Jackson (1991b) also demonstrated a significant negative correlation between soil pH and plant Cd concentration, whereby the Cd concentration of lettuce was reduced from 13.8 to 4.4 μg kg^{-1} following the application of lime.

Organic and inorganic fractions

Organic constituents play an important role in affecting the availability of Cd in soil by adding adsorptive properties (Antoniadis and Alloway, 2000; Li *et al.*, 2001; Moreno *et al.*, 2001). Soluble organic components (fulvic acids) raise the carrying capacity of soil solutions by forming soluble metal organic complexes. Such complexes may not be directly bioavailable. However, they may increase uptake of metals by effectively lowering the diffusion distance to the root surface provided the complex can dissociate in response to depletion of Cd^{2+} in the rhizosphere solution. By contrast, insoluble organic components (e.g. flocculated humic acids) effectively inhibit uptake by raising the overall distribution coefficient (kd) of the soil for trace metals (Petrussell *et al.*, 1994; McBride, 1995; Christensen and Huang, 1999). The soil kd is defined as the ratio of total metal bound to solids relative to that found in soluble phases (Sauve *et al.*, 2000a).

Although, the annual contribution of organic matter (OM) to soil by sewage sludge application is small, long-term application of sewage sludge gradually increases OM levels to more significant values. A soil with a 3 % OM content contains around 100 t ha^{-1} of OM in the plough layer (Smith, 1996). A typical application of 5 t ha^{-1} (dry solids) of sewage sludge would therefore add about 2.5 t of OM to the soil and would raise the soil OM content to 3.07 %. However, decomposition will occur and the half-life of OM decomposition is ≈10 years (Bell *et al.*, 1991).

The overall effect of sewage sludge addition and decomposition on the uptake of heavy metals by plants is controversial and little consensus can be found (Stacey *et al.*, 2001). One theory suggests that the slow mineralisation of OM in sludge following termination of sludge application could release metals in more soluble

forms, thus increasing Cd plant uptake with time; the sludge 'time bomb' hypothesis (Fig. 1.2, curve C; Beckett and Davis, 1979; McBride, 1995; Smith, 1996; Chang *et al.*, 1997). Alternatively the 'sludge protection' or 'plateau hypothesis' suggests that metal uptake by plants reaches a maximum as sludge applications increase and does not continue to rise even if metal loadings continue to increase (Fig. 1.2, curve b; Chaney and Ryan, 1993; McBride 1995; Chang *et al.*, 1997). The 'sludge protection' hypothesis essentially relies on metal solubility being maintained at very low levels due to the increased number of adsorption sites provided by the addition of sludge residue. Curve a in Figure 1.2 assumes that metal uptake is a linear function of total heavy metal content derived from the cumulative application of sludge (McBride, 1995). Data for metal uptake by plants from an experiment in the USA where sewage sludge was applied for 10 years were assessed by Chang *et al.* (1997); this analysis indicated that neither a 'time bomb' nor a 'plateau' effect was present. Hyun *et al.* (1998) also found no evidence for either hypothesis after 10 years of applying sewage sludge, despite a 40 % decrease of organic carbon during the same period. This observation is also consistent with results reported by Brown *et al.* (1998). Hyun *et al.* (1998) reported that, although plant tissue concentrations of Cd continued to rise with increasing mass loadings of Cd following applications of sewage sludge, plant uptake did not exhibit a sharp increase for up to 10 years after the cessation of sludge application (the length of the experiment), as might be expected according to the 'time bomb' hypothesis. It is possible that the decomposition time for OM added in the form of sewage sludge is relatively long, a theory supported by Johnston *et al.* (1989). Alternatively, components of the OM fraction may resist decomposition altogether and continue to provide protection against increased metal solubility.

However, it is likely that inorganic residues, such as phosphates, silicates and Fe, Al and Mn oxides provide long term retention capacity for some heavy metals, thus exerting greater control over their solubility as organic matter decays (McBride, 1995). Li *et al.* (2001) suggested that sewage sludge contains from 30 to 60 % of inorganic mineral factions and that large P concentrations for example, would reduce Cd solubility by precipitation in the form of various phosphates. Li *et al.* (2001) also reported that, in the five experimental soils used, soil Cd adsorption capacity was increased by an average of 125 % following sewage sludge application. The inorganic

fractions continued to maintain Cd at low levels of solubility, equivalent to those of controls, even when the organic fraction was degraded in the sewage sludge treated soils.

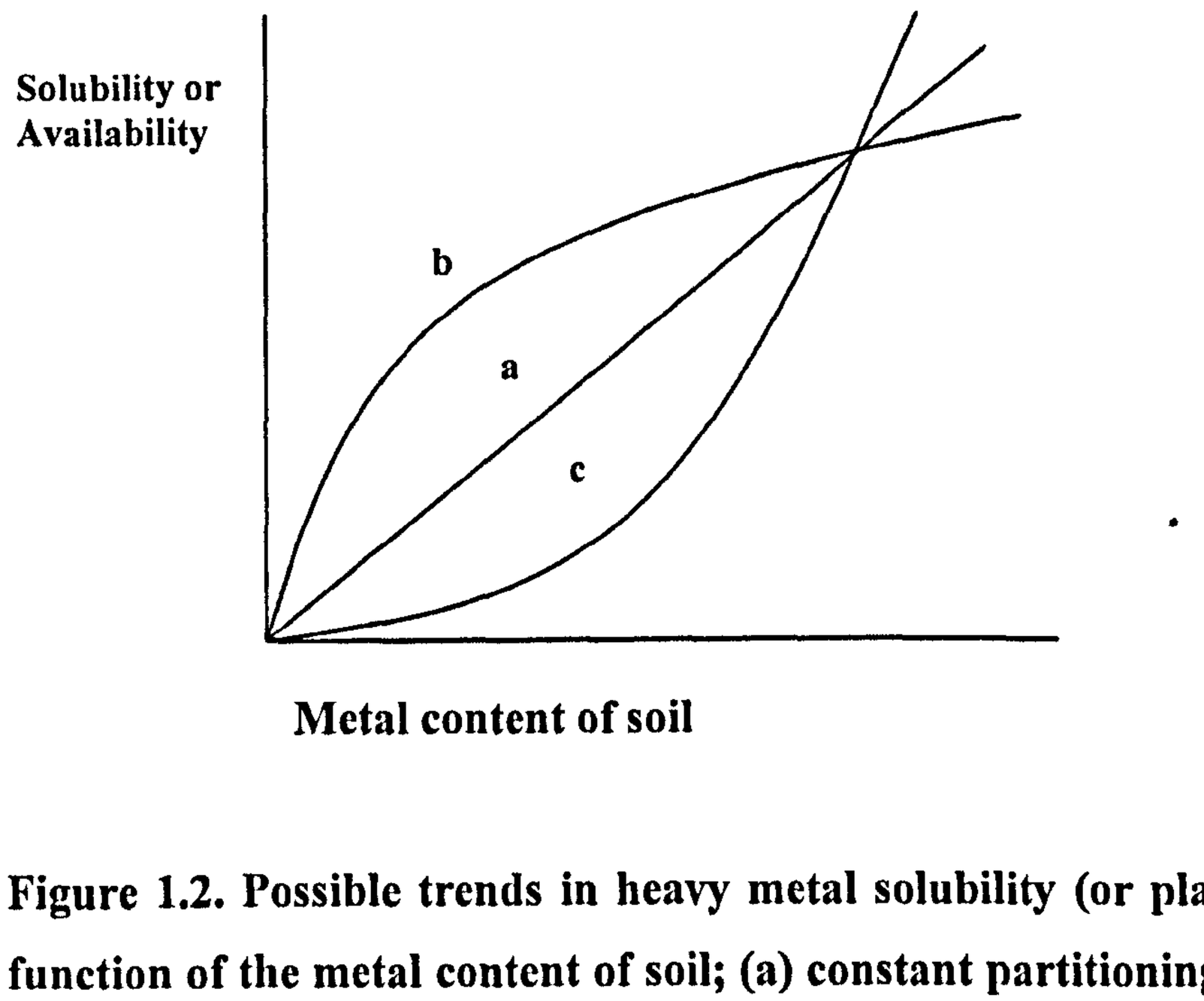


Figure 1.2. Possible trends in heavy metal solubility (or plant availability) as a function of the metal content of soil; (a) constant partitioning model, (b) the 'soil protection' model and (c) the sludge 'time bomb' hypothesis (McBride, 1995).

Speciation

As described earlier, the uptake of Cd by plants is primarily influenced by its concentration in the soil solution accessed by crop roots. Cd in soil solution can be present in a number of different chemical forms, principally as free cations or as species complexed with organic or inorganic ligands (Helmke, 1999). Alloway and Jackson (1991a) cited several studies in which Cd speciation in sludged soils was predicted using computer speciation models. Mahler *et al.* (1980) for example, suggested that the free metal ion was the predominant species, while Mullins and Sommers (1988) predicted that 85 % of Cd and 91 % of Zn were present as free metal ions in soils to which sewage sludge had been applied five years previously. However, complexed Cd species may also be important in determining uptake of Cd by plants. Many attempts to model uptake have relied on the free ion activity model (FIAM),

which correlates well with metal bioavailability, but does not take into account the uptake of complexed metal species (Brown and Markich, 2000; Sauve *et al.*, 2000b).

Perhaps one of the most widely researched complexes of Cd is with chloride. Much research on Cd uptake in relation to soil chloride has focused on the consequences of growing crops on saline soils in Southern Australia, where elevated Cd concentrations in crops have been reported (McLaughlin *et al.*, 1994). These workers sampled 89 commercial potato crops and soils between February and July 1992. The greatest concentrations of Cd in tubers were found predominantly in saline soils, even at neutral or slightly alkaline pH levels (6 – 7.5). Cd concentrations in tubers were positively correlated with the concentrations of water-extractable chloride in the soil. The high chloride concentrations resulted from the use of saline irrigation waters (McLaughlin *et al.*, 1994). Increased plant Cd concentrations associated with elevated soil chloride concentrations, were also reported by Li *et al.* (1994) for sunflower kernels and Norvell *et al.* (2000) for durum wheat grain. Weggler-Beaton *et al.* (2000) demonstrated that soil Cd originating from inputs of phosphatic fertiliser, could increase plant Cd concentrations in the presence of elevated chloride concentrations, derived from bio-solids (sewage sludge). Sewage sludge in Australia is often rich in sodium chloride (NaCl). In a pot experiment, Weggler-Beaton et al. (2000) leached sewage sludge with de-ionised water to remove NaCl, thereby permitting germination of sugar beet and wheat. Five concentrations of NaCl were then applied at concentrations of 0, 400, 800, 1200 and 1400 mg L^{-1}. Although the use of de-ionised water to leach NaCl from the soil prior to germination did not reduce soil Cd concentrations, increasing chloride concentrations by applying NaCl significantly increased Cd concentrations in the shoots of both species. The salt treatments applied in this experiment had no effect on soil pH. Some authors have suggested that increasing soil chloride concentrations may override the beneficial effect of lime in reducing plant Cd uptake. Li *et al.* (1994), for example, showed that plant Cd concentrations were increased in plants grown on soil containing elevated chloride concentrations under alkaline conditions (7.3 – 8.1).

$CdCl^+$ and $CdCl_2^0$ complexes predominate at chloride concentrations below 100 mM in solution; both complexes are less strongly sorbed to soil than the free Cd^{2+} ion

(Weggler-Beaton *et al.*, 2000). Therefore the concentration of Cd in solution at the soil-root interface is increased within this chloride concentration range (Weggler-Beaton *et al.*, 2000). Several hypotheses have been proposed to explain the increase in plant Cd associated with high soil chloride concentrations (Grant *et al.*, 1999).

1) An increase in NaCl increases the severity of osmotic stress experienced by plant roots and affects membrane function, allowing more Cd to be absorbed.
2) Increasing complementary cation (Na^+) concentrations displaces Cd^{2+} into soil solution, increasing its availability for uptake by roots.
3) Chloride ions complex Cd^{2+} bound to soil colloids and increase the total Cd concentration in solution, resulting in the subsequent uptake of complexed Cd ($CdCl^+$, $CdCl_2^0$) by roots.
4) Increasing chloride concentrations in solution complex Cd^{2+} and increase the effective diffusion of Cd to sites of uptake in the roots.

The first hypothesis was discounted by Smolders *et al.* (1998), as addition of $NaNO_3$ to soil had no effect on Cd uptake by plants, whereas addition of NaCl had a marked effect. Smolders and McLaughlin (1996a) demonstrated that $CdCl_2$ species might be able to cross the plasma membrane in root cells or be taken up through breaks in the endodermis. Smolders and McLaughlin (1996b) demonstrated that Cd uptake by plants increased as chloride concentrations increased and Cd^{2+} activity was kept constant. Plants were grown in nutrient solution, which was continuously aerated and recirculated over a resin column adjacent to the pot. Four chloride treatments were used (0.01, 40, 80 and 120 mM) using NaCl. It was therefore concluded that the third and fourth hypotheses (described above) are the most likely, with $CdCl_2$ complexes in solution being available for plant uptake (in addition to Cd^{2+}). Other possible explanations include; an increase in diffusion through the apoplast to sites of Cd uptake within roots or dissociation of $CdCl^+$ in response to a reduced concentration of Cd^{2+} at the root surface.

1.2.3 Plant Cd uptake mechanisms

The processes involved in the transfer of metals from the soil solution into roots, transport across the root cells of plasma membranes and the transport to the shoots are

complex. At a basic level, the movement of metal ions to the root surface will be driven by three processes: i) mass flow, or the movement of ions in the water flow resulting from transpiration; ii) diffusion, the passive movement of ions down a concentration or electrochemical gradient; and iii) root interception, where roots move into previously un-occupied pores within the soil (Marschner, 1995; Lack and Evans, 2001).

The second stage in the uptake of metal ions by plants, movement from the root surface to the xylem, involves transfer across the plasma membrane. This movement is driven by two main processes. Firstly, heavy metal or CPx type ATPases (primary transporters) use ATP to pump metal ions across the plasma membrane by creating an electrochemical gradient (Guerinot, 2000; Williams *et al.*, 2000). Specific metal uptake ATPases have not so far been identified in plants, although the RAN1 CPx-ATPase is believed to have a role in transporting Cu in yeast (Williams *et al.*, 2000). However, the negative membrane potential created by ATPase pumps is sufficient to drive Cd^{2+} uptake, even at low soil solution activities (Welch and Norvell, 1999). The specific mechanisms for Cd uptake are poorly understood, but are likely to involve: i) movement via a saturable cation transporter in the plasma membrane, such as via a carrier for another divalent cation like Zn^{2+}, Cu^{2+} or Fe^{2+}; and ii) diffusive movement through a divalent cation membrane channel with linear concentration kinetics, such as for Ca^{2+} or Mg^{2+} (Welch and Norvell, 1999). In addition, it is likely that Cd^{2+} could follow a similar influx pathway as Zn^{2+} due to their chemical similarities.

Further transport systems (secondary transporters) including symporters, antiporters and ion channels or uniporters also play an essential role in the transfer of metal ions across the plasma membrane (Guerinot, 2000; Lack and Evans, 2001). The Nramp (Natural resistance associated macrophage proteins) and the cation diffusion facilitator (CDF) family of proteins are believed to be important in facilitating metal uptake by plants. For example, AtNramp3 has been shown to be involved with Cd^{2+} sensitivity in *Arabidopsis* (Clemens, 2000; Thomine *et al.*, 2000). In addition, a number of genes involved in ion transport in plants have been identified, particularly for Zn and Cu. These include ZAT1 (zinc transporter of *Arabidopsis thaliana*; VanDer Zaal *et al.*, 1999) and COPT1 (copper transporter 1) also from *Arabidopsis* (Williams *et al.*,

2000). However, COPT1 is not expressed in roots so, although available data suggests that it has an important role in the uptake of Cu by plants, it is not specifically responsible for transfer of Cu^{2+} across the plasma membrane (Guerinot, 2000).

It has been suggested that transporters for essential cationic nutrients such as Fe may also facilitate the transport of Cd into plant cells (Thomine *et al.*, 2000). Fe deficiency in pea is known to stimulate high affinity Fe uptake and also stimulates Cd^{2+} uptake (Cohen *et al.*, 1998). Progress has recently been made in elucidating specific Cd uptake and transport mechanisms in the hyperaccumulator species *Thlaspi caerulescens*, including work addressing Cd^{2+} uptake in association with Fe deficiency (Lombi *et al.*, 2002; Section 1.3).

Metal ions are transported passively to the shoots via the xylem in the transpiration stream. Cd may move in the xylem as Cd^{2+} or in complexes with organic molecules (Welch and Norvell, 1999). Cd competes with other metals which bind preferentially with sulphur, such as Zn and Ni (Welch and Novell, 1999), and citric acid is hypothesised to be a major ligand of Cd^{2+} in the xylem even at low Cd^{2+} concentrations (Wagner, 1993).

1.3 Phytoextraction

Phytoextraction is defined by the USEPA (2000) as the uptake of contaminants by roots, transport within the plant and the subsequent harvest of the contaminated plant material. The process concentrates the contaminant and leaves a much smaller mass to be disposed of than more traditional soil remediation methods, such as soil excavation. Phytoextraction is mainly regarded as a technique for removing metals from contaminated soil, but can also be used to extract metalloids and radionuclides. The main advantages cited for phytoextraction in comparison to other remedial processes include low capital costs, low ongoing operational costs, easy implementation and high public acceptance; it is also considered to be non-invasive (Boyajian and Carreira, 1997). Salt *et al.* (1995) estimated that using phytoextraction to clean up one acre of soil to a depth of 50 cm, would cost approximately US $60,000 - 100,000, compared to at least US $400,000 for soil excavation. Glass (2000) estimated that the

potential US market opportunity for phytoextraction of metal contaminated soils was just below US $ 1 billion y^{-1}. Although these figures are largely speculative, the potential of phytoextraction as a means of soil remediation is probably substantial and a great deal of research effort has been directed at this subject in recent years. Furthermore, phytoextraction may be more suited for the remediation of agricultural land treated with sewage sludge than to other remedial techniques and may help to facilitate more sustainable disposal of sewage sludge to land.

Phytoextraction can be sub-divided into three main strategies (McGrath *et al.*, 2001) involving the use of:

1. hyperaccumulator plants.
2. 'large biomass crops', which are induced to take up large amounts of metal when the mobility of metals in soil is enhanced with chemical treatments.
3. fast growing trees (e.g. *Salix* species).

1.3.1 Strategy 1: Phytoextraction using hyperaccumulators

Discovery and description of hyperaccumulators

Plants containing large concentrations of heavy metals were first discovered in 1885, although Brooks *et al.* (1977) first used the term hyperaccumulator to describe plants containing >1000 μg g^{-1} Ni in their dried leaves. Definitions for hyperaccumulation of other metals include >10000 μg g^{-1} for Zn and >100 μg g^{-1} for Cd (Baker and Brooks, 1989). The potential for plants capable of hyperaccumulating metals for use in soil remediation was postulated by Chaney (1983), and in the last decade there has been a rapid expansion of research in this area. Recent reviews of hyperaccumulation include Baker *et al.* (2000), McGrath *et al.* (2000), Reeves and Baker (2000) and Salt and Kramer (2000).

Three basic strategies were proposed by Baker (1981) to explain plant responses to large concentrations of heavy metals in their environment. These were: i) **accumulators**, in which metals are concentrated in the shoots; ii) **indicators**, in which metal uptake to the shoot reflects the soil metal concentration; and iii) **excluders**, in

which metal concentrations in the shoot are restricted. However, this model was revised by McGrath *et al.* (2000), who suggested that the term hyperaccumulators should be used to replace the original "accumulator" model, as this plant ideotype is capable of accumulating substantially greater concentrations of metal than either indicator or excluder species. In addition, a cut-off point was added to the hyperaccumulator strategy, as hyperaccumulators have a physiological requirement for some metals at very large concentrations, for example Zn by *T. caerulescens* (Fig. 1.3).

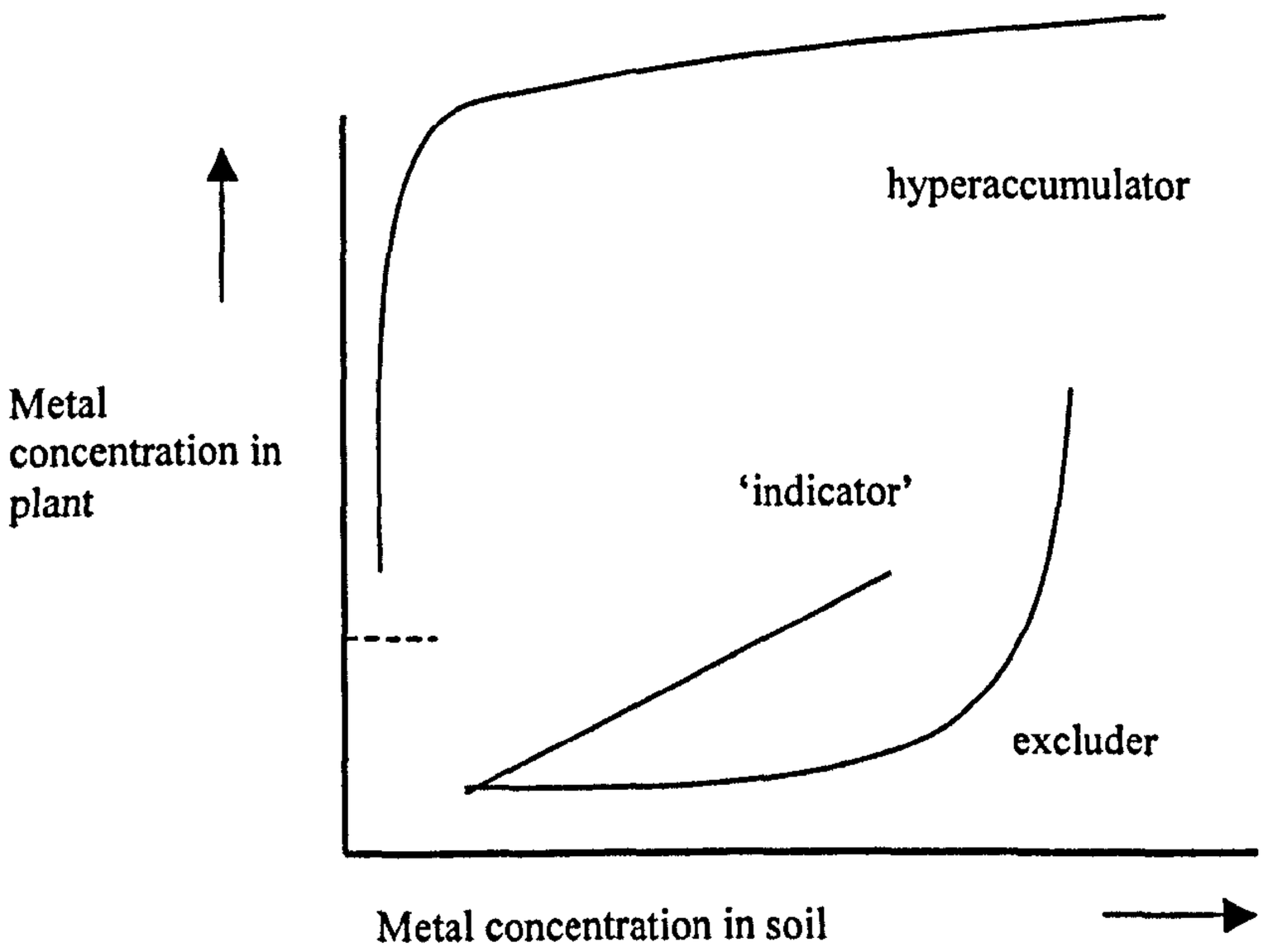


Figure 1.3. Conceptual response strategies of metal concentrations in the above-ground plant parts in relation to increasing total metal concentrations in the soil (McGrath *et al.*, 2000).

Hyperaccumulation of metals is not a common characteristic and is believed to be an extreme evolutionary response to the presence of large metal concentrations in the soil (Baker *et al.*, 2000). The occurrence of distinct vegetation types on metal-contaminated areas has long been recognised (Antonovics *et al.*, 1971); metal-rich soils include Calamine (Zn) soils in Western and Central Europe and Serpentine (Ni and Cr) soils worldwide. These soils are often associated with the occurrence of

specific hyperaccumulator species (Baker, 1981). In total, around 400 species have been identified as metal hyperaccumulators, although only eleven are recognised as Zn hyperaccumulators and only one as a Cd hyperaccumulator (Baker *et al.*, 2000). The evolutionary development of the hyperaccumulation trait is thought to contribute to protection against fungal and insect attack; for example, recent studies have demonstrated the anti-herbivory effect of Zn in *T. caerulescens* (Pollard and Baker, 1997).

Mechanisms of Cd and Zn hyperaccumulation by *Thlaspi caerulescens*

Relatively few hyperaccumulators accumulate either Zn or Cd. *Thlaspi caerulescens* has been widely reported as the best known example of a Zn hyperaccumulator (Shen *et al.*, 1997; Baker *et al.*, 2000; Zhao *et al.*, 2001). In addition, Lombi *et al.* (2000) demonstrated the potential for a population of *T. caerulescens* found near Ganges, France to accumulate large concentrations of Cd.

In recent years, the understanding of hyperaccumulation mechanisms has improved. One theory suggests that hyperaccumulators solubilize metals within their rhizosphere by releasing metal chelating compounds (Salt and Kramer, 2000). However, Zhao *et al.* (2001) demonstrated that *T. caerulescens* is not capable of releasing significantly greater quantities of exudates (Dissolved Organic Carbon (DOC) basis) than other species. In addition, Zhao *et al.* (2001) found that the root exudates of *T. caerulescens* do not contain significantly greater amounts of chelating compounds with a high affinity for metal than the non-hyperaccumulators, wheat and oil seed rape.

However, there is some evidence that *T. caerulescens* can access insoluble Zn (McGrath *et al.*, 1997; Knight *et al.*, 1997; Whiting *et al.*, 2001a). However, Whiting *et al.* (2001a) suggested that high root density resulting from the limited volume of the pots used, might have caused greater dissolution of insoluble Zn. Other work to assess which metal pools are accessed by *T. caerulescens* for metal uptake provides some support for the findings of Zhao *et al.* (2001). Work by Gerrard *et al.* (2000), for example, compared the IC_p (isotopic composition of Cd in plants) for several species including *T. caerulescens* and the non-hyperaccumulator plants, ryegrass and lettuce.

Although Cd uptake by the different species was large, the IC_p values were similar for the same soil, indicating that *T. caerulescens* was accessing the same soil pools of Cd as both non-hyperaccumulators. However, depletion of the isotopically exchangeable pool (phytoavailable pool of soil Cd) was less than 1 % for ryegrass and lettuce and up to 22.5 % for *T. caerulescens*. Similar results were reported by Hutchinson *et al.* (2000) who compared the L value (biological estimate of labile pool; comparable with ICp) for *T. caerulescens* and the non-hyperaccumulator *Lepidium heterophyllum*. The L value was comparable for both species. Hutchinson *et al.* (2000) also compared the L and E value (a chemical estimate of the radio-labile metal pool) for several populations of *T. caerulescens*. If *T. caerulescens* was able to mobilise non-labile metal pools the L and E values, would have been expected to differ. However, the L and E values were similar for each population. Although this work does not exclude the possibility that *T. caerulescens* can release metal chelating compounds, it does indicate that it is not capable of accessing non-labile metal pools. Further evidence that *T. caerulescens* does not mobilise Zn in the rhizosphere was reported by Whiting *et al.* (2001b), as Zn uptake by the non-hyperaccumulators, *Thlaspi arvense* and *Festuca rubra* was not increased when their roots shared the same soil rhizosphere as *T. caerulescens*. This result is also consistent with Gove *et al.* (2002).

The ability of hyperaccumulators to alter soil pH has been ruled out as a mechanism for increasing the uptake of Cd or Zn by *T. caerulescens* (Bernal and McGrath, 1994; Knight *et al.*, 1997; McGrath *et al.*, 1997; Luo *et al.*, 2000). Knight *et al.* (1997), for example, measured the pH of soil solution following the growth of *T. caerulescens* and reported no significant changes despite very large shoot Zn concentrations. Other work by Schwartz *et al.* (1999) and Whiting *et al.* (2000) showed that the roots of *T. caerulescens* proliferate in locally enriched areas of Zn and Cd, and may actually be capable of foraging for such metal hotspots.

It is recognised that the capacity for Zn influx across the cell plasma membrane of *T. caerulescens* roots is much greater than in other species. Lasat *et al.* (1996) reported a value for V_{max} (maximum transport rate; μmol g fresh wt^{-1} h^{-1}) for Zn^{2+} in *T. caerulescens* which was 4.5 times greater than in *T. arvense*, suggesting a higher density of Zn transporters per unit membrane area in *T. caerulescens*. This finding

was supported by Pence *et al.* (2000) and Assuncao *et al.* (2001), who demonstrated the over-expression of the ZNT1 gene in roots and shoot tissue of *T. caerulescens and* suggested that this could explain the increased transport of Zn to the leaf cells. In the case of Cd, Lombi *et al.* (2001a) showed that the value of V_{max} for Cd^{2+} in the Ganges population of *T. caerulescens* was five times greater than in the Prayon population. This observation again suggests a greater density or activation of Cd transporters in the root cell membranes.

Recent work by Lombi *et al.* (2002) demonstrated that the activity of the Tc IRT-G gene was greatly increased in response to Fe deficiency in the Ganges population of *T. caerulescens*, but not found in the Prayon population. Fe deficiency has also been shown to induce a marked increase in the V_{max} for Cd influx in Ganges, but not in Prayon. However, it is not known whether the Tc IRT-G gene is responsible for the large difference in Cd uptake between the Ganges and Prayon populations when Fe is sufficient.

Work by White *et al.* (2002) suggests that the transfer of Zn to the xylem in *T. caerulescens* may occur through both the apoplastic and symplastic pathways. The mechanisms for Cd and Zn uptake through the apoplastic pathway have not yet been fully identified. However, it is likely that hyperaccumulators produce organic ligands which complex Zn^{2+} in the cytoplasm, rendering it non-toxic and facilitating its transport in the xylem (Lasat *et al.*, 1998). Work considering this process is ongoing, but Salt *et al.* (2000) demonstrated that histidine and citrate complexes of Zn appear to be the major species associated with transport to the shoots. Further work is required to resolve the mechanisms of hyperaccumulation.

The use of hyperaccumulator plants for phytoextraction

Although the potential for soil remediation using hyperaccumulators has long been recognised, relatively few field experiments have been conducted to test the efficacy of this remediation strategy (Baker and Whiting, 2002). However, a number of hyperaccumulator species, including two populations of *T. caerulescens*, have been

grown in the Woburn Market Garden Experiment in North London, England. Using data from this experiment, it is possible to calculate the time required for phytoextraction using hyperaccumulator plants. For example the Whitesake population of *T. caerulescens* accumulated up to 40 mg Cd kg^{-1} when soil Cd was 8 mg kg^{-1} (McGrath *et al.*, 2000). As the highest removal of Cd by this population was 0.250 kg ha^{-1}, 50 years would be required to reduce Cd content in the surface 20 cm soil horizon from 8 to 3 mg kg^{-1}, assuming a bulk density of 1.25 g cm^{-3}. However, this does assume that all metal is present in a plant-available form and that uptake remains constant throughout the remediation period. A number of similar calculations can be found in the literature, but unfortunately they have included the same unrealistic assumptions (Baker *et al.*, 1994; Brown *et al.*, 1994: McGrath *et al.*, 2000).

Robinson *et al.* (1998) collected examples of wild *T. caerulescens* plants growing in two regions of Southern France with average soil Cd concentrations of 163 mg kg^{-1}. However, calculations by these authors of the time taken to remove soil Cd using *T. caerulescens* were based on estimated biomass values assuming positive effects of fertilisation, and the assumption that Cd uptake by plants was a linear function of soil Cd concentration. As the actual biomass obtained in this study was 2.6 t ha^{-1}, a 99 year period would be required to reduce Cd concentration to 3 mg kg^{-1} in the surface 20 cm of soil, assuming a bulk density of 1.25 g cm^{-3}. However, the value used for soil Cd content of 163 mg kg^{-1} represented total soil Cd and is unlikely to have been entirely plant-available.

Disadvantages of using hyperaccumulator plants for phytoextraction

There are a number of disadvantages associated with using hyperaccumulators for phytoextraction. Firstly, hyperaccumulator plants are slow growing and produce a limited biomass (Brown *et al.*, 1995; Shen *et al.*, 1997). Therefore a very long time period may be required for successful soil remediation (Brown *et al.*, 1994; Knight *et al.*, 1997). Secondly, hyperaccumulator plants are often rare, occur in remote geographical areas and have very small populations (Baker *et al.*, 2000). Consequently, the costly establishment of seed banks will be necessary before commercial phytoextraction can occur. Thirdly, hyperaccumulation can be metal-

specific, especially for target metals such as Cd (Lombi *et al.*, 2000). Fourthly, McLaughlin and Henderson, (1999) reported that *T. caerulescens* is sensitive to Cu, therefore potentially restricting its use for phytoextraction to contaminated soils with low concentrations of Cu.

Finally, much research into the possible future use of hyperaccumulator plants relies on genetic modification of plants whereby, plants are manipulated to increase their capacity to accumulate high concentrations (Baker *et al.*, 1994; Guerinot, 2000; Karenlampi *et al.*, 2000; Williams *et al.*, 2000; Lasat, 2002). However, public acceptance of this technology is far from certain (Baker and Whiting, 2002) and many concerns over the safety and long-term success of this approach exist (Soil Association, 2002).

1.3.2 Strategy 2: Chemically-enhanced phytoextraction

Description and mechanisms of chemically-enhanced phytoextraction

Due to the disadvantages associated with using hyperaccumulators for phytoextraction, alternative strategies have been investigated. Chemically-enhanced phytoextraction is perhaps the most widely researched alternative. This strategy relies on the growth of large biomass crops on contaminated soil, to which metal solubilizing agents, such as synthetic chelates, are added to increase metal solubility. Arable crops are suitable for this approach as they are easy to grow and the technology required for their production is usually widely available. Most research has focused on the uptake of Pb by *Brassica juncea* (Indian Mustard; Huang *et al.*, 1997a; Huang *et al.*, 1997b; Wu *et al.*, 1999; Vassil *et al.*, 1998; Epstein *et al.*, 1999. However, arable crops do not generally possess metal uptake traits (Blaylock and Huang, 2000), and Blaylock *et al.* (1997) proposed that transpiration is the major force driving Pb accumulation in the shoots of *B. juncea.* Increased metal solubility in the rhizosphere might therefore be expected to increase metal uptake. Vassil *et al.* (1998) demonstrated that the Pb-EDTA complex was taken up by the plant following analysis of xylem sap, while Huang *et al.* (1997a), after examining the efficiency of a range of chelates, showed that EDTA (diaminoethanetetra acetic acid) was most efficient at

increasing plant Pb uptake (Fig. 1.4). In addition to an increase in passive metal uptake via the transpiration stream, Wu *et al.* (1999) reported that changes occur in the transport pathway within roots following chelate application to soil. The integrity of the plasma membrane surrounding living root cells relies on the presence of Zn^{2+} and Ca^{2+} ions. Synthetic chelates may remove these stabilising ions, leading to a rapid equilibrium of the soil solution and xylem sap (Vassil *et al.*, 1998). An increase in metal solubility following chelate application will therefore induce the transfer of metals across the root membranes, facilitating greater uptake of metals by plants.

Work by Collins *et al.* (2002) demonstrated that the Zn-EDTA complex was detected in xylem exudates from species examined, including *B. juncea*, only when applied at 3.4 mmol $kg^{-1,}$ but not when applied at lower EDTA concentrations. Furthermore, the uptake of Zn-EDTA was significantly increased in *B. juncea* despite a significant decrease in transpiration. Thus, Collins *et al.* (2002) suggested that physiological damage to *B. juncea* roots, led to the indiscriminate uptake of soil solution following applications of EDTA above a critical threshold concentration.

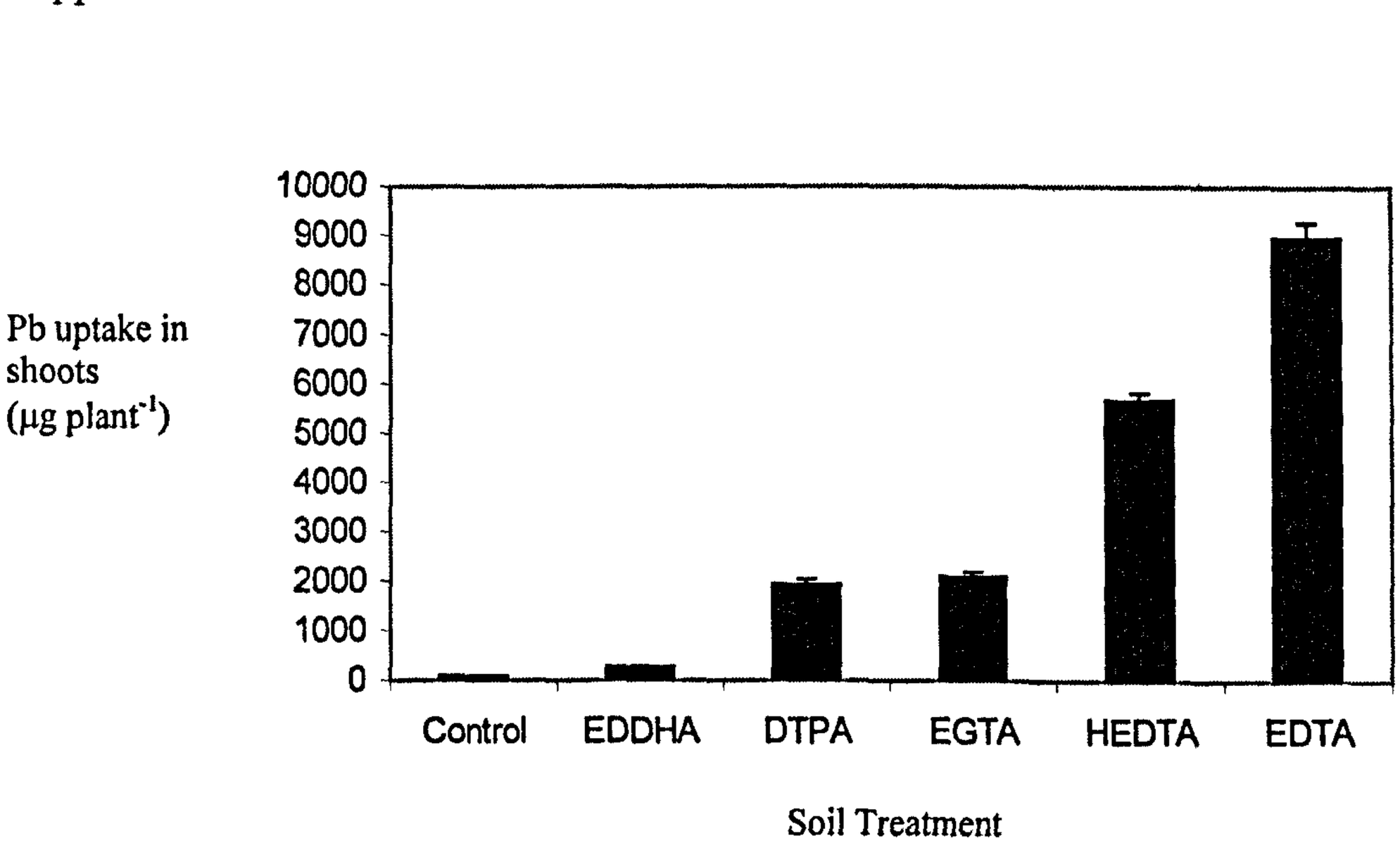


Figure 1.4. The effect of adding five different chelates at (0.5 g kg^{-1} soil) to a contaminated soil on Pb uptake (µg plant $^{-1}$) by pea plants (From Huang *et al.*, 1997a).

The use of other chemical agents capable of increasing metal uptake by plants has also been investigated; for example, some studies have attempted to incorporate modifications of soil pH within a chemically enhanced phytoextraction system. For example, Blaylock *et al.* (1997) reported an increase in tissue Pb concentration from 28 mg kg^{-1} in control to 785 mg kg^{-1} following EDTA application, and 1471 mg kg^{-1} following EDTA and acetic acid application. Soil pH was reduced from 8.3 to 7.8.

Other chemical agents capable of disrupting the plant root transport pathways within roots, as reported following chelate application, have also been investigated. For example, Ensley *et al.* (1999) reported that the use of herbicide increases metal uptake by plants if used in conjunction with chelates. However, published information concerning the effect of herbicide on metal uptake is very limited.

Examples of chemically-enhanced phytoextraction

Most studies of the potential for chemically enhanced phytoextraction have relied on pot or laboratory-based experiments, often involving soils spiked with metals. However, pot experiments do not accurately represent field conditions, as the extraction potential of roots per unit soil volume is much greater when confined to pots (Hamon *et al.*, 1998). Moreover, soils spiked with heavy metals often provide a greater metal availability to plants than non-treated soils (Romkens *et al.*, 2002).

A field study by Kayser *et al.* (2000) tested several crops including *B. juncea* and *Zea mays* (forage maize) grown on a contaminated calcareous soil in Switzerland. However, the Cd concentration in the soil at this site was <3 mg kg^{-1}, which is within the legislative thresholds for agricultural soil in the UK. The easily biodegradable chelate Nitrilotriacetate (NTA) was used in an attempt to enhance metal uptake. Although solubility of Cd and Zn within the soil increased significantly following treatment, metal uptake was increased only by a factor of 2 or 3. Metal uptake by plants was reported to be 'far below' that required to produce a useful remediation effect. It is likely that NTA was degraded too rapidly to produce a significant increase in metal uptake, as was also reported following applications of citric acid to soil (see below).

Blaylock *et al.* (2000) described a field experiment to examine Pb uptake by *B. juncea* grown on two Pb-contaminated sites in the USA. EDTA was applied as 2 mmol EDTA kg^{-1} using soil irrigation equipment, although the timing of application in relation to planting and harvest was not described. Average surface soil (0 - 15 cm) Pb concentrations were reported to have decreased from 2055 to 960 mg kg^{-1}, and from 984 to 644 mg kg^{-1} for the two sites examined following three crops of *B. juncea,* although, details of plant biomass and metal uptake were not presented. Van Der Lelie *et al.* (2001) reported the successful use of phytoextraction by Endenspace Corporation, a US company specialising in phytoremediation, to remove Pb from three sites in the USA. However, it is believed that the technique used to remediate these sites involved a joint soil washing and phytoextraction system (Blaylock and Elless, 2001). A soil liner was installed, requiring the removal of the soil from the site, and the soil water was then recycled following application of EDTA. Although this system featured the successive cropping of *B. juncea*, it is difficult to ascertain the relative importance of the phytoextraction element as compared to the *in situ* soil washing process.

Disadvantages associated with chemically-enhanced phytoextraction

The application of chelates, such as EDTA to the soil, presents some environmental concerns. EDTA forms highly stable metal complexes and is used extensively by industry, for example, in the bleaching of textiles and the processing of photographic materials (Henneken *et al.*, 1998). However, degradation of EDTA in the environment is generally reported to be slow (Means *et al.*, 1980; Bolton *et al.*, 1993; Hong *et al.*, 1999), although some more recent reports indicate that oxidation by microorganisms can occur (Nortemann, 1999). The presence of EDTA has been reported in surface and drinking water (Nortemann, 1999), and for this reason much attention has been directed at developing biological treatments to remove EDTA from industrial discharges (Henneken *et al.*, 1998). For these reasons, the deliberate application of EDTA to contaminated soils may appear unacceptable.

The risk of metal leaching and ground water contamination has been raised widely in the literature (Abruzzese *et al.*, 2001; Barona *et al.*, 2001; Greman *et al.*, 2001;

McGrath *et al.*, 2001). Greman *et al.* (2001) conducted column-based experiments and reported that leaching following application of EDTA to soil was significant. Similar results were reported for movement of Pb-EDTA within the soil profile by Abruzzese *et al.* (2001) and Barona *et al.* (2001), although both studies used soil columns rather than field based systems. The use of biodegradable chelates such as NTA or citric acid to reduce metal leaching has produced mixed results. The rapid degradation of these chelates generally results in limited enhancement of plant metal uptake; citric acid, for example, has been reported to have very limited effect on the uptake of Pb by plants due to rapid microbial mineralisation (Romkens *et al.*, 2002).

Apart from the concerns surrounding the application of chelate to soils, metal uptake resulting from chemically-enhanced phytoextraction is generally insufficient to produce a viable remediation system, although, the number of field experiments testing chemically-enhanced phytoextraction is limited, as discussed above. Chaney *et al.* (1999) suggested that the difference in tissue metal concentrations between hyperaccumulators and high biomass arable crops is approximately 50-fold, whereas the difference in biomass is around 10-fold. EDTA is also an expensive product at around £16 kg^{-1} and may not be economically competitive with other technologies, even when applied at low concentrations (Chen and Cutright, 2001).

1.3.3 Strategy 3: Phytoextraction using short rotation willow coppice

Justification for using willow as a phytoextraction crop

The use of willow for phytoextraction offers several key advantages compared to either hyperaccumulators or high biomass arable crops. Firstly, as an energy crop, willow contributes to the environmental objective of reducing global carbon dioxide (CO_2) emissions. As an energy source, willow can be regarded as 'CO_2-neutral', as carbon assimilated during growth is released during combustion, and so the net contribution to the atmospheric CO_2 budget is zero (Perttu, 1998; Perttu, 1999). Recent EU legislation, formed partly in response to the Kyoto Protocol, has set a target for electricity produced from renewable energy sources in the UK as 10 % of the total by 2010 (CEC, 2001). In the UK, the 'Energy Crops Scheme' provides grants

of up to 50 % of the establishment costs (MAFF, 2000). This is important as it provides a significant and expanding market for willow production, supported by the EU and national governments.

Secondly, willow is easy to propagate, fast growing, inexpensive to establish and produces a large biomass (Ledin, 1996). Estimates for annual biomass yields vary from $\leq$10 t ha^{-1} y^{-1} on reclaimed land (Bardos *et al.*, 2001) to $\leq$ 30 t ha^{-1} y^{-1} under optimal agricultural conditions (Labrecque *et al.*, 1998).

Thirdly, nutrient and water use by high yielding willow plantations can be large, especially in comparison to other tree crops (Ericsson, 1994a, b), and elements such as Ca, K, Mg and P accumulate in the ash following biomass combustion. It has been suggested that a sustainable energy biomass system would need to recycle this ash as a fertiliser (Narodoslawsky and Obernberger, 1996). Conventional biomass combustion is achieved during several stages, producing a mixture of bottom ash (75 - 85 % of ash), cyclone fly ash (15 - 25 % of ash) and filter fly ash (1 to 4 % of ash). Apart from N, which is volatilised during combustion, the macronutrients are concentrated in the bottom ash. However, trace metals such as Cd and Zn are concentrated in the cyclone and filter fly ash (Narodoslawsky and Obernberger, 1996; Obernberger *et al.*, 1997). It is therefore possible to return up to 85 % of the fuel ash to the soil, whilst retaining metal contaminants in a concentrated form.

The main areas of research concerning willow and phytoextraction

The use of willow within wastewater filter systems has long been recognised as an economic treatment for a variety of waste products and, in Sweden, there are currently over forty installations for the treatment of landfill leachate or wastewater. However, these installations are mainly used to remove nutrients such as N and K (Perttu and Kowalik, 1997; Aronsson and Perttu, 2001). Research concerning the disposal of sewage sludge to land under short rotation coppice (SRC) is also ongoing (Labrecque *et al.*, 1997; Riddell-Black, 1998; Sims and Riddell-Black, 1998; Hasselgren, 1998, 1999; Moffat *et al.*, 2001). Labrecque *et al.* (1995), for example, suggested that

applications of sludge equivalent to 200 kg of 'available' N ha^{-1} can increase biomass by up to seven-fold.

The use of willow to extract heavy metals from soil is an emerging technology as it is recognised that trees are reasonably tolerant of heavy metal contamination (Dickinson *et al.*, 1991a, b; Eltrop *et al.*, 1991; Turner *et al.*, 1991). The main tolerance mechanisms observed in trees include immobilisation, avoidance, excretion and exclusion, and ecotypic variation in tolerance has also been observed in willow (Kahle,1993). In addition, some workers have demonstrated the possibility of inducing acclimation of willow to contaminated conditions (Dickinson *et al.*, 1992; Punshon *et al.*, 1996; Punshon and Dickinson, 1997).

However, most research seems to have focused on the variability of metal uptake between willow species (Riddell-Black, 1994; Landberg and Greger, 1994; Nissen and Lepp, 1997; Greger and Landberg, 1999). Riddell-Black (1994), for example, showed significant differences in Cd uptake between four willow species grown under the same conditions, while Landberg and Greger (1994) found substantial variation in heavy metal accumulation and tolerance between 108 willow clones. Landberg and Greger (1996) reported a greater variation in metal uptake *within* than *between* species of willow. The concentration of Cd, for example, varied by a factor of 25 within *Salix viminalis* species, whereas the variation in median values within *Salix daphnoides* was less than two-fold. One Swedish grower germinates up to 15000 new willow species annually before conducting field tests with selected types (Larsson, 1994). However, as effective field testing requires several years of growth, recent work has attempted to develop a rapid screening test to assess the suitability of willow species for heavy metal uptake (Pulford, 2001; Bertholdsson, 2001). Hydroponic tests, in which young plants are exposed to a cocktail of heavy metals, may provide a suitable approach (Watson *et al.*, 1999).

Examples of phytoextraction using willow

Several field experiments have been conducted in recent years to assess the potential of willow for removing metals from contaminated soil, although many have reported limited metal uptake. Eriksson and Ledin (1999), for example, observed only a slight decrease in soil Cd content using willow. Pulford *et al.* (2001) also found limited metal uptake by willow grown on heavily contaminated soil, and concluded that phytoextraction by this species may only be suitable for 'polishing' marginally contaminated sites. Greger and Landberg (1997) calculated that it would take over 12 years to remove typical Cd loadings in Swedish soils resulting from Cd deposition during the last century. This assumed removal of 33 g Cd ha^{-1} y^{-1} from a soil pool of 400 g Cd ha^{-1} and an annual deposition rate of 1.2 g ha^{-1} y^{-1}. Annual biomass production by willow was estimated to be 15 t ha^{-1} y^{-1} from stem material. As a typical SRC rotation exceeds 20 years, a remediation period of only 12 years is potentially of value. A possible limitation is that it is unlikely that all soil Cd will be plant-available, or that plant metal uptake as a proportion of soil metal would remain constant throughout the remediation period. However, the concentration of Cd in willow leaves is often two to five times greater than in the stem (Greger and Landberg, 1997; Robinson *et al.*, 2000), harvesting the leafy material in addition to stem material may therefore reduce the time required to reach remediation targets.

Possible role for mycorrhizal fungi within willow phytoextraction systems

Mycorrhizal fungi are symbiants, which form mutualistic associations with plant roots. This association can increase acquisition of nutrients and water by the host and provide enhanced protection against soil pollutants (Punshon, 1996). About 95 % of the world's plant species form mutualistic mycorrhizal associations (Pawlowska *et al.*, 2000), of which there are two main types; endo- and ecto-mycorrhizas (van der Heijden, 2001). Usually plants only form mycorrhizas with one type or the other, although some species, including *Salix*, can form associations with both forms (Lodge, 1989; Dhillion, 1994; van der Heijden, 2001). Under natural conditions, trees are almost always mycorrhizal (Ahonen-Jonnarth and Finlay, 2001) and several studies have reported that mycorrhizal associations may ameliorate the toxicity of heavy

metals in soil. For example, Colpaert and Van Assche (1993) found that some mycorrhizal fungi can ameliorate Cd-induced inhibition of water use in *Pinus sylvestris* seedlings. Jentschke *et al.* (1999) demonstrated that species *Paxillus involutus* ameliorated the toxic effects of Cd on *Picea abies* (Norway Spruce), whereas the mycorrhizal species, *Laccaria bicolor,* did not. Furthermore, some studies have demonstrated increased uptake of heavy metals where mycorrhizal associations are present, thus providing potential for enhanced phytoextraction. For example, Lambert and Weidensaul (1991) showed that inoculation of soybean with arbuscular mycorrhiza fungi (AMF) increased accumulation of Zn and Cu. Similarly, Oudeh *et al.* (2002) showed that inoculation with AMF generally increased metal concentrations in leek plants.

Most investigations of mycorrhizal effects on heavy metal uptake by plants have been pot-based using soils spiked with metal salts (e.g. Jentschke *et al.*, 1999; Colpaert and Van Assche, 1993). Adding metals in the form of salts in pot experiments will clearly enhance metal solubility compared to field conditions, which may in turn influence the response of any mycorrhizal associations. Some studies have demonstrated reduced mycorrhizal growth in soils contaminated with heavy metal. For example, Gildon and Tinker (1981) reported that high concentrations of heavy metal could eliminate arbuscular mycorrhizal spores. Val *et al.* (1999) demonstrated a significant decrease in arbuscular mycorrhizal colonisation following application of sewage sludges containing heavy metals. In addition, no beneficial effects on plant growth were recorded following mycorrhiza inoculation, perhaps as a result of increased soil fertility following sewage sludge application (Val *et al.*, 1999).

Although mycorrhizal associations have been shown to provide protection against heavy metals, there is also evidence that no beneficial effects may be induced. Protection may involve sequestration of metal in mycorrhizal roots and therefore reduce transfer of metal to above-ground tissue (Pawlowska *et al.*, 2000). Furthermore, several reports indicate that mycorrhizal survival in soils contaminated with heavy metals is restricted, further field-based work will be required, before a beneficial role for mycorrhizal within phytoextraction systems can be established.

1.4 Summary

Soils contaminated with heavy metals are a widespread concern and the demand for effective, low cost remedial techniques is large. One significant source of heavy metals is sewage sludge, of which 1.5 Mt (dry solids) are expected to be produced in the UK by 2005 per year (DETR, 2000a). Up to 60 % of this will be deposited on agricultural land in the UK (DETR, 2000a). Cd contamination poses a risk to human health and is perhaps the most problematic contaminant present in sewage sludge. Furthermore, legislative guidelines for Cd loadings in sewage sludge, maximum permissible soil thresholds and human intake levels have been or are in the process of being updated and tightened. The sustainability of long-term sewage sludge disposal to agricultural soil is therefore uncertain. In addition, traditional soil remediation techniques are not suited for use on agricultural land due to their prohibitive costs.

Phytoextraction has been suggested as a potential low cost, *in situ* alternative to other remediation methods. There are three main strategies for phytoextraction; i) the use of hyperaccumulator plants, ii) chemically-enhanced metal uptake using arable crops; and iii) the use of short rotation coppice. Research into phytoextraction has expanded rapidly in recent years. However, very few field experiments have so far been conducted and only one study has assessed all three strategies on the same site, although this was only conducted on a slightly contaminated soil. Furthermore, calculations assessing the timescale required for successful remediation using phytoextraction have generally been over simplistic and very few assessments of realistic phytoextraction costs have been made. Much research has focused on the genetic modification of crops, a technique that still has not received widespread public support. Therefore, greater evaluation of the potential for phytoextraction is required utilising realistic crop scenarios.

1.5 Experimental aims and objectives

The primary aim of this work was to test the efficacy of phytoextraction for the remediation of soils contaminated by the application of sewage sludge. Specific objectives were to undertake pot and field experiments involving all three phytoextraction strategies, attempting to optimise and enhance these strategies to maximise off-take of Cd by plants, thereby enabling realistic estimates for the timescale of metal removal and a more accurate appreciation of the system cost to be obtained. Specific experimental hypotheses are outlined in individual results chapters.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 Description of field site

Field trials were established within a 5 ha arable field (Plate 2.1; Fig 2.1) which forms part of a dedicated sewage sludge disposal facility located at Stoke Bardolph near Nottingham, England (GR: SK643 406). The site is operated by Severn Trent Water and consists of over 630 ha of agricultural land. The site is managed within the guidelines governing 'dedicated sites' set out by the 1989 Sludge Regulations (SI, 1989), and is utilised for dairying and production of livestock feed. Sewage sludge has been applied to parts of this land for approximately 100 years. The field chosen for experimental work was originally used as a lagoon for sludge de-watering (Plate 2.1). Sewage sludge is currently applied to fields via sub-surface injection from tractor-mounted equipment, and towed pumps (Heaven and Delve, 1997). An underground ring main has been installed throughout the site to facilitate the transport of sludge around the farm. An intensive geochemical survey of the farm is conducted at five year intervals; this involves collecting soil from the, 0 - 25 and 25 - 50 cm horizons and conducting analysis for a wide range of metals and metalloids (Heaven and Delve, 1997). These data show that the test field contains amongst the highest concentrations of metals, particularly Cd, from the entire site (Table 2.1; Fig. 2.1).

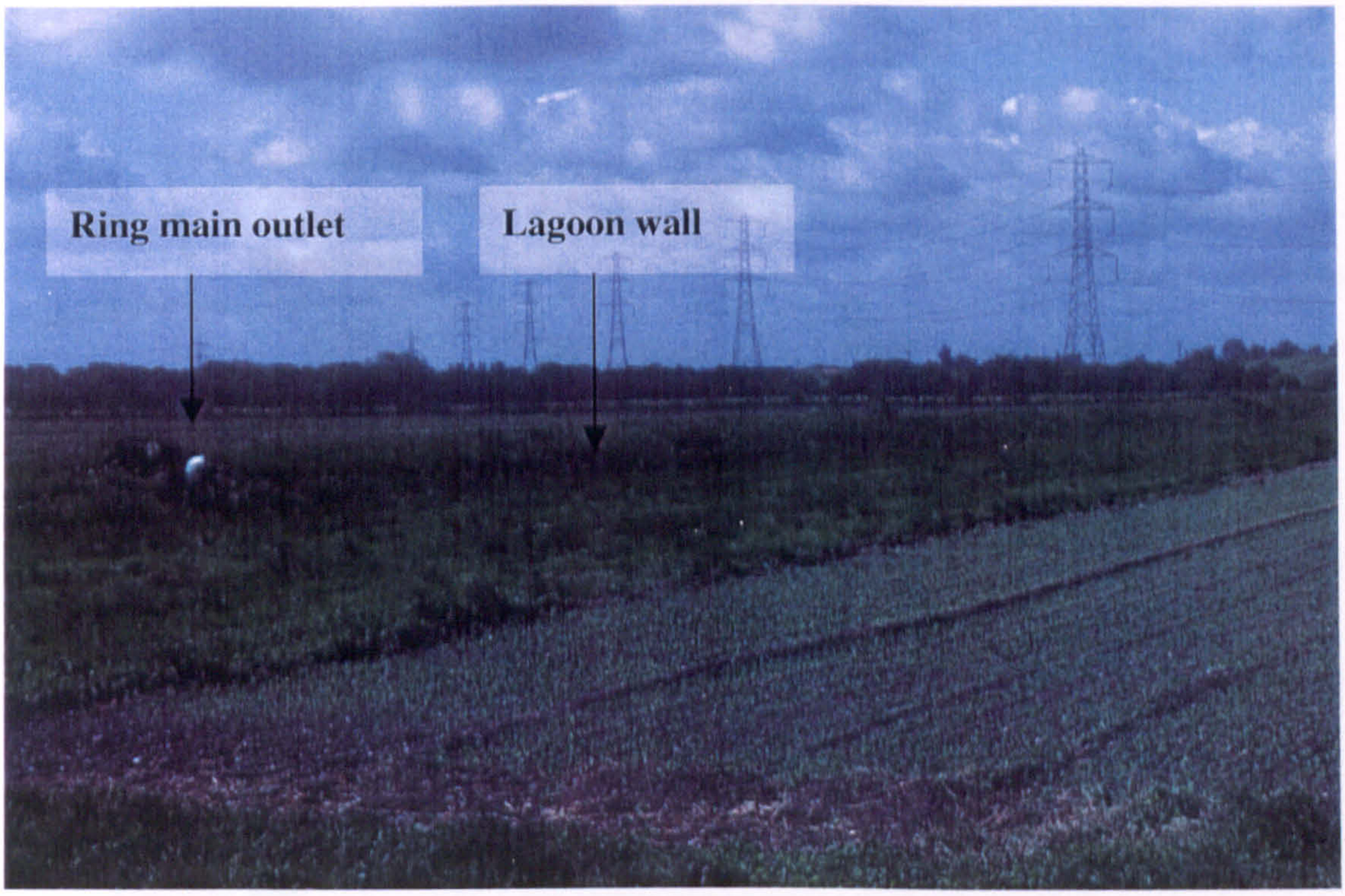


Plate 2.1. View of test field; the old lagoon boundary and a ring main outlet for sludge disposal are visible.

Table 2.1. Range of metal concentrations found within the test field and across the entire Stoke Bardolph field site.

Heavy metal	Range occurring across site[1] (n = 475) (mg kg^{-1})	Range occurring in test field[1] (n = 8) (mg kg^{-1})	Maximum permissible concentrations in 'normal' agricultural soil (pH 6.0 - 7.0) [2] (mg kg^{-1})
Cd	0.2 - 75	46 - 71	3
Cu	15.6 - 1120	855 - 1011	135
Pb	26 - 870	696 - 774	300
Ni	10 - 630	499 - 591	75
Zn	82 - 2700	2129 - 2516	300

[1]Heaven and Delve (1997).
[2]The 1989 Sludge Regulations (SI, 1989).

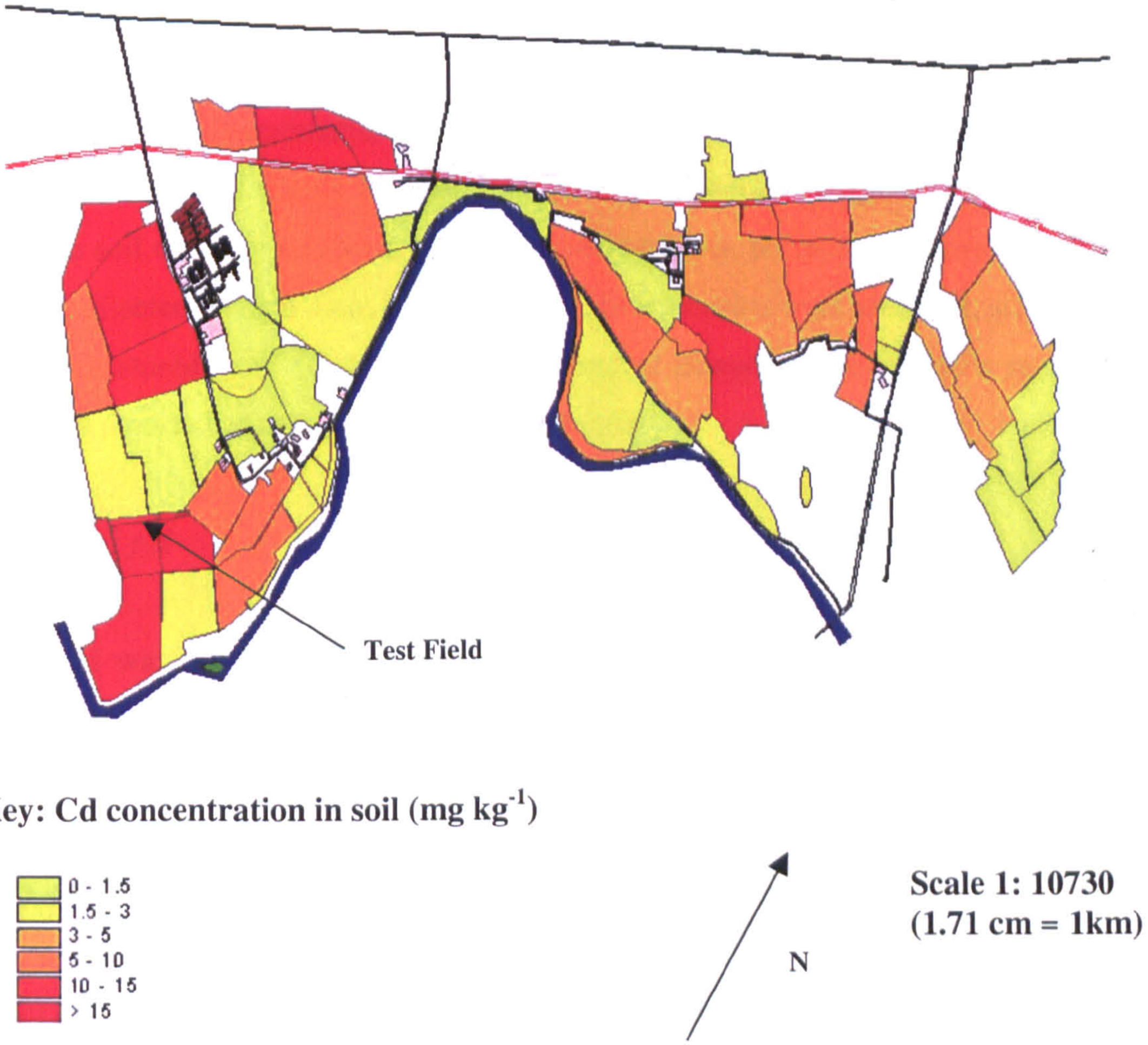


Figure 2.1. Geochemical map of the Stoke Bardolph estate illustrating the mean Cd concentration of soil (0 - 25 cm), based on field averages (Heaven and Delve, 1987). The test field is indicated.

2.2 Soil analysis and preparation

2.2.1 Soil sampling methods

For use in pot experiments, soil was sampled from the 0 - 20 cm soil horizon from multiple locations across the test field in a standard 'W transect' (Rowell, 1997). Where soil was sampled from field experimental plots, multiple soil cores (0 - 20 cm) were collected using a 4 cm diameter auger. Soil samples were air-dried, sieved to < 2 mm and stored at 15 ^{0}C. Normally, all chemical assays were undertaken in triplicate and are reported as mean values ± standard error.

2.2.2 Soil pH

Soil pH was determined using air-dried soil mixed with de-ionised water to form a slurry with a soil: liquid ratio of 1:2.5 (W: V) after 15 min shaking. The pH of the suspension was recorded, using a combined pH electrode, after 30 s (Rowell, 1997).

2.2.3 Soil moisture determination

Soil samples were oven dried at 105 ^{0}C for 24 h. Water content was calculated from the loss of weight, expressed on a soil 'dry weight basis'.

2.2.4 Loss on ignition

Approximately 10 g of oven dry soil was combusted in a muffle furnace at 550 ^{0}C for 6 h and samples were allowed to cool. Loss on ignition was calculated from the loss of weight and expressed in percentage terms.

2.2.5 Available phosphate content

Available phosphate was determined by extraction with sodium bicarbonate followed by colorimetric assay using a reduced phospho-molybdate method (Rowell, 1997). Samples of air-dried soil (c. 5.0 g) were extracted for 30 min with 0.5 M $NaHCO_3$; a

teaspoonful of activated charcoal was shaken with the suspension to remove dissolved organic carbon from solution. Samples were filtered using Whatman No. 25 filter papers. A molybdate stock solution was prepared by dissolving 1.5 g ammonium molybdate ($(NH_4)_6Mo_7)_{24}.4H_2O$), 0.086 g potassium antimony tartrate ($KSbOC_4H_4O_6$) and 17.6 mL of concentrated sulphuric acid (H_2SO_4) in 200 mL deionised water. The reducing reagent stock solution was 2.7 g ascorbic acid ($C_6H_8O_6$), dissolved and made up to a volume of 50 mL with deionised water. A colour-forming reagent (CFR), was made freshly each day, by combining the molybdate and ascorbic acid solutions in a ratio of 4:1. Sample and standard solutions for colorimetric assay were prepared by adding 1 mL of 1.5 M H_2SO_4 and 4 mL of CFR to 5 mL of sample then diluting to 50 mL with deionised water. Absorbance of the coloured solutions at 880 nm was determined in a 1 cm quartz cell in a *Cecil* CE1011 spectrophotometer. Calibration standards contained 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg P L^{-1}.

2.2.6 Soil carbonate content

Soil $CaCO_3$ content was determined barometrically from the CO_2 evolved from 2.5 g of finely ground air-dried soil acidified with 10 mL of 4 M HCL in a 'Collin's calcimeter' (Black, 1965).

2.2.7 Labile Cd by extraction with 1 M $CaCl_2$

Labile soil Cd content was determined by extraction with 1 M $CaCl_2$ (Young *et al.*, 2000). Air dried soil (c. 5.0 g) was shaken with 25 mL of 1 M $CaCl_2$ for 48 h. Samples were centrifuged at 2200 g for 20 min and 5 mL of the supernatant acidified to give 0.1 M HNO_3. Metal concentrations were determined by Flame Atomic Absorption Spectroscopy (FAAS) using a *Varian* Spectra AA220 instrument calibrated with matrix-matched standards.

2.2.8 Labile metal by extraction with 0.05 M EDTA

Labile soil metal content was determined by extraction with 0.05 M EDTA (MAFF, 1986). Air dried soil (c. 5.0 g) was shaken with 25 mL of 0.05 M EDTA for 48 h

before samples were centrifuged at 2200 g for 20 min. Determination of metal content was as described in section 2.2.7.

2.2.9 Total soil metal content by Aqua-regia digestion

Finely ground soil samples (c. 1.0 g) were digested with 20 mL of 'Aqua Regia' (conc. HCl and HNO_3 in a ratio of 1:3) on a hot plate. The digest volume was reduced to < 5 mL, allowed to cool, diluted with ≈5 mL deionised water and filtered through Whatman No. 25 filter paper. Samples were made up to 50 mL with deionised water. Digest blanks were conducted for each acid batch in which the acid digest process was repeated with no soil present. Determination of metal content was as described in section 2.2.7.

2.2.10 Total plant metal content

Dried plant samples were digested in 20 mL conc. HNO_3 on a hot plate. In some experiments the whole plant (up to 4 g) was digested. In others, where plant biomass was > 4 g, a uniform mass (c. 1.0 g) of finely ground material was used. Appropriate acid digest blanks were used for all experiments. Digest volumes were reduced on a hotplate to < 5 mL, allowed to cool, diluted with ≈5 mL deionised water and filtered using Whatman No. 25 filter paper. Filtered samples were made up to 50 mL using deionised water. Determination of metal content was as described in section 2.2.7.

2.2.11 Description of plants used

A number of standard commercially available arable and short rotation coppice energy crops have been used throughout experimental work as described in individual chapters. In addition, two populations of *T. caerulescens* were used: the 'Ganges' population, collected from the Cevennes region of Southern France, and the 'Prayon' population collected from Prayon, province of Liege, E. Belgium (Meerts and Van Isacker, 1997). The Ganges population has been shown to accumulate up to 3700 mg Cd kg^{-1} (Lombi *et al.*, 2000) and is believed to possess specific Cd transporters in its

roots (Section 1.3). By contrast Prayon has no apparent ability to accumulate Cd, but hyperaccumulates Zn to a level similar to that of the Ganges population.

2.2.12 Statistical analysis

Analysis of variance (ANOVA) and correlations were performed using GenStat package (5th Edition) as described in individual chapters. An example of an ANOVA table is shown in Appendix 1 for two experiments discussed in Chapter 3. In addition, T-tests were also performed using Microsoft Excel as described in individual chapters.

CHAPTER 3: THE USE OF CHLORIDE AND EDTA TO ENHANCE METAL UPTAKE BY HYPERACCUMULATOR AND NON-HYPERACCUMULATOR PLANTS.

3.1 Introduction

Phytoextraction has been described as a novel remediation tool that may offer advantages over other soil remedial procedures (Boyajian and Carreira, 1997; Section 1.3). Furthermore, the most widely researched phytoextraction strategies involve the use of hyperaccumulator plants or the use of large biomass crops that are induced to take up metal following soil chemical treatments (McGrath *et al.*, 2001). These phytoextraction strategies were discussed in detail in Sections 1.3.1 and 1.3.2 respectively.

The aim of this project was to test the efficacy of phytoextraction for the remediation of Cd-contaminated soils treated with sewage sludge. The current section aims to evaluate different types of crop for use on the Stoke Bardolph field site by testing their response to the addition of metal-solubilizing agents such as EDTA and chloride. A number of common arable crop types were selected, based partly on their inclusion in previous phytoextraction research or their suitability for growing at the Stoke Bardolph field site and for producing a large biomass. *Brassica juncea* (Indian mustard) has been researched extensively for use in phytoextraction (Salt *et al.*, 1995; Huang *et al.*, 1997b; Vassil *et al.*, 1998; Blaylock *et al.*, 2000; Kayser *et al.*, 2000). Similarly, *Zea mays* (forage maize) has also been considered widely in phytoextraction research (Blaylock *et al.*, 1997; Wu *et al.*, 1999; Kayser *et al.*, 2000; Lombi *et al.*, 2001b; Wenger *et al.*, 2002). *Brassica napus* (oil seed rape) and *Linum usitatissimum* (linseed) may offer advantages over other arable crops, for it may be possible to produce an oil crop from such species within a phytoextraction system, as metals are unlikely to pass to the lipid phase. *Beta vulgaris* (sugar beet) is a crop that has been used in a number of studies addressing the enhancement of plant uptake of Cd from soil containing high chloride concentrations (Section 1.2.2) and is known to be salt tolerant (Marschner, 1995). Finally two populations of the Zn-hyperaccumulator *Thlaspi caerulescens* (Ganges and Prayon) were also grown for

comparison with the non-hyperaccumulator crops. Ganges is also a hyperaccumulator for Cd, whereas Prayon is not (Lombi *et al.*, 2000; Section 1.3.3).

3.1.1 EDTA treatments

The enhancement of metal uptake by non-hyperaccumulator crops using soil chemical inputs has been researched extensively (Section 1.3.2) and the chelate EDTA has been reported as the most efficient agent for this purpose (Huang *et al.*, 1997b). The concentration of EDTA used was selected following an extraction experiment testing the amount of Cd and other metals solubilized by a range of EDTA concentrations. However, as EDTA is expensive and some concerns over its use in phytoextraction have been reported (Section 1.3.2), alternative methods of inducing uptake of Cd by crops were also assessed.

3.1.2 Chloride treatments

Work addressing the uptake of Cd by crop plants on chloride-rich soils has been previously undertaken, particularly involving the crop *B. vulgaris* (Section 1.2.2). Recent work by Xiong and Feng (2001) suggests that chloride could be used to increase plant Pb uptake through complexation. However, the use of chloride to actively induce plant uptake of Cd has not been previously studied. Both $CaCl_2$ and KCl were assessed for their possible use within phytoextraction systems. Depending on the relative importance of Cd^{2+} uptake via calcium channels (Section 1.2.2), application of KCl to soil, may increase plant Cd concentrations to a greater extent than application of $CaCl_2$, due to less competition for uptake between Cd^{2+} and Ca^{2+}. Cd uptake is expected to increase through chloro-complexation for at least two reasons: i) additional assimilation of intact complexes such as $CdCl_2$ and ii) a greater rate of Cd^{2+} absorption into roots due to the reduced diffusion path afforded by the local solution reservoir of Cd chloro-complexes (Grant *et al.*, 1999; Section 1.2.2).

3.1.3 Experimental hypothesis

The experimental hypothesis to be tested by this section was that plant uptake by the selected crops could be significantly increased through application of the chelate EDTA or the complexing agents $CaCl_2$ or KCl.

3.2 Materials and Methods

3.2.1 Soil measurements

Soil was collected from the 0 - 20 cm horizon from the Stoke Bardolph field site in October 1999 (Section 2.2.1). Analyses were conducted for total and EDTA-extractable Cd, Zn, Pb, Cu and Ni, $CaCl_2$-extractable Cd, bicarbonate-extractable phosphate, loss on ignition and $CaCO_3$ content (Section 2.2).

3.2.2 Metal solubility following extraction with EDTA and $CaCl_2$.

Ten concentrations of $CaCl_2$ were prepared, on a log scale between 10^{-3} and 1 M. Similarly, ten stock solutions of EDTA were prepared between 10^{-4} and 10^{-1} M (Range 1) and between 2×10^{-3} and 4×10^{-2} M (Range 2). Aliquots of 25 mL of EDTA or $CaCl_2$ were added to 5 g of air-dried soil and shaken for 48 h. Samples were then centrifuged at 2200 g for 20 min and the supernatant was filtered using 'Whatman 42' filter paper (Ure *et al.*, 1993; Quevauviller *et al.*, 1994). For samples 1 to 8 (Table 3.1) 10 mL of the filtrate from range one was added to 30 mL polythene bottles containing 10 mL of 0.1 M EDTA or $CaCl_2$. For samples 9 and 10 (Table 3.1) 5 mL of 0.2 M extractant was added to 5 mL of deionised water to provide a consistent matrix. Samples in $CaCl_2$ were acidified with 1 mL of 1 M HNO_3 to prevent adsorption to container walls. Samples were analysed for Cu, Zn, Pb, Ni and Cd by F-AAS as described in Section 2.2.

Table 3.1. Concentrations of EDTA and $CaCl_2$ used in soil extraction experiments.

Solution Number	EDTA Range 1 (M)	EDTA Range 2 (M)	$CaCl_2$ (M)
1	0.0001	0.002	0.001
2	0.0002	0.003	0.002
3	0.0004	0.004	0.004
4	0.0009	0.005	0.009
5	0.002	0.007	0.02
6	0.004	0.01	0.04
7	0.009	0.015	0.09
8	0.02	0.02	0.2
9	0.04	0.03	0.4
10	0.1	0.04	1.0

3.2.3 Pot experiments: General details

A number of pot experiments were conducted under glasshouse conditions, set to provide 15 - 18 ^{0}C, 16 h light / 8 h dark. Pots of 12.7 cm diameter were loosely packed with moist soil collected from the Stoke Bardolph field site as described above, equivalent to a dry weight of 400 g, and arranged in a fully randomised block design to give five-fold replication of treatments. A layer of perlite was added to the soil surface to reduce evaporation. Two representative pots were weighed every other day for each treatment and in each block and watering was carried out to return pots to water content of 50 % (dry weight basis). The EDTA and chloride treatments were made up to 20 mL solution using deionised water. All treatments were slowly watered into pots using a measuring cylinder. Plant shoots were harvested by cutting them ≈ 1 cm above the perlite surface; yield and total metal uptake were determined, as described in Section 2.2.10.

3.2.4 Plant metal uptake for seven selected plant species, in response to a range of EDTA and KCl application rates

One hundred and forty pots were sown with one of seven different plant species (Table 3.2). Plants were sown at greater than commercial densities and thinned prior to chemical application (Table 3.2).

Five pots of each species were harvested and analysed for plant metal content after eight weeks of growth. At this point five pots of each species were treated with 20 mmol EDTA kg^{-1} and a further five pots with 25 mmol KCl kg^{-1}. The EDTA-treated plants were harvested one week after treatment following substantial desiccation. The KCl-treated pots were harvested three weeks after treatment, following slight desiccation. For comparison, five additional control pots of each species were also harvested at the same time of harvesting the KCl treated plants.

Table 3.2. Species and planting densities used for pot experiments.

Species	Variety or (population)	Commercial planting densities (m^{-2}) [1]	Number of plants sown (12.7 cm pots)	Final density at time of treatment (plants pot^{-1})
Alpine Pennygrass (*Thlaspi caerulescens*)	Prayon (population)*	N/A	12	8 (mean density)
Alpine Pennygrass (*Thlaspi caerulescens*)	Ganges (population)*	N/A	12	9 (mean density)
Maize (*Zea mays*)	Lincoln	12	3	1
Indian Mustard *(Brassica juncea)*	Cornell*	120	6	2
Sugar Beet *(Beta vulgaris)*	Madison	7.5	3	1
Oil Seed Rape *(Brassica napus)*	Star	120	6	2
Linseed *(Linum usitatissimum)*	Norlin	400	12	6

All seeds provided by a commercial supplier except*, supplied by IACR- Rothamsted, Harpenden, England.

[1] Soffe (1995).

3.2.5 Metal uptake by B. napus *in response to a range of EDTA application rates*

Plants of *B. napus* were grown using the experimental conditions and design described in Section 3.2.4. After 12 weeks of growth, five pots per treatment were treated with 20, 30, 40 or 50 mmol EDTA kg^{-1} respectively. In addition, five pots were left untreated as replicated controls. All plants were harvested one week after treatment.

3.2.6 Influence of two chloride salts on plant metal uptake

Three plant species, *T. caerulescens* (Ganges population), *B. vulgaris* and *B. napus,* were grown as described in Section 3.2.4. After seven weeks growth, five pots per treatment were treated with either 20 or 50 mmol kg^{-1} $CaCl_2$ or KCl. Five replicate pots were left untreated as controls. All plants were harvested two weeks after treatment.

3.2.7 Influence of $CaCl_2$ application rate on plant metal uptake

Three plant species (Section 3.2.6) were grown as described in Section 3.2.4. After seven weeks of growth, five pots per treatment were treated with 20, 40, 60 or 80 mmol $CaCl_2$ kg^{-1}. Five replicate pots were left untreated as controls. All plants were harvested at death or two weeks after treatment.

3.2.8 Statistical analysis

An analysis of variance (ANOVA) was undertaken using GenStat package (5th Edition) to establish treatment effects. Where hyperaccumulator and non-hyperaccumulator plants were grown in the same experiment, ANOVA was designed to test treatment differences, but not plant differences. The very large differences observed in plant metal concentrations between hyperaccumulator and non-hyperaccumulator plants might have biased the ANOVA results. Therefore in some cases, hyperaccumulator plants were assessed by separate ANOVA to the non-hyperaccumulator plants.

3.3 Results and Discussion

3.3.1 Soil characteristics and EDTA extraction experiment

The main soil characteristics that affect plant uptake of metal are discussed in Section 1.2; and Tables 3.3 and 3.4 show the characteristics of soil used in this section. The most important variables are generally accepted to be soil pH, organic matter (OM)

and inorganic constituents. Much discussion exists in the literature concerning the long-term availability of metals from soil treated with sewage sludge and the relative importance of the organic or inorganic soil fractions (Section 1.2.2). For example, OM decomposition over time may result in increased metal availability, a theory described as the sludge 'time bomb hypothesis' (McBride, 1995; Smith, 1996; Chang *et al.*, 1997). By contrast, the 'sludge protection' hypothesis, suggests that plant metal may reach a threshold, where plant uptake does not increase despite further additions of sewage sludge, due to the adsorptive properties of sludge residues (Chaney and Ryan, 1993; McBride, 1995; Chang *et al.*, 1997). Consequentially, metal availability following sludge application may remain restricted in the long term (Hooda and Alloway, 1994; McBride, 1995; Section 1.2).

At the Stoke Bardolph field site, sewage sludge application has taken place regularly for approximately 100 years and the OM fraction in the test field was measured as 27 %, approximately 7 times greater than usually measured in typical UK agricultural soil (MAFF, 1995). Extractable P was > 200 mg kg^{-1} (MAFF index 9), which is also substantially greater than usually recorded in typical UK agricultural soil; extractable P concentrations in most arable soils are more likely to fall within index 0 - 3 (MAFF, 1995). In addition, large volumes of lime have been applied to the site to maintain pH within legislative limits, a process likely to add further adsorptive sites to the soil (Krebs *et al.*, 1998). Soil pH was measured as 6.00, well within guideline values according to the 1989 Sludge Regulations (SI, 1989). It is likely that the magnitude of the OM content and the large extractable P concentration, are among the most important factors restricting metal availability at the Stoke Bardolph field site. Both are a consequence of sludge application.

Table 3.3. Total, EDTA-extractable and $CaCl_2$-extractable metal content from soil used for solubility experiments and pot experiments discussed in Chapter 3. Values are means (n=3). Values in parenthesis are standard error of the mean.

	Total soil metal content (mg kg^{-1})		EDTA-extractable metal content (mg kg^{-1})		$CaCl_2$-extractable metal content (mg kg^{-1})	
Cd	34.6	(0.90)	23.1	(0.30)	18.4	(1.09)
Zn	1470	(284)	807	(54.9)	-	
Cu	603	(109)	424	(28.9)	-	
Pb	524	(41.9)	246	(6.39)	-	
Ni	344	(18.8)	261	(7.90)	-	

Table 3.4. Selected soil characteristics for soil used in experiments discussed in Chapter 3. Values are means (n=3). Values in parenthesis are standard error of the mean.

	pH	[a]LOI %	$CaCO_3$ (mg kg^{-1})	[b]Available P (mg kg^{-1})
Mean	6.00 (0.03)	27.3 (0.11)	131 (1.00)	214 (0.42)

[a]LOI, Loss on ignition. [b] Bicarbonate-extractable phosphate.

The concentration of Cd extractable using 1 M $CaCl_2$, which is known to provide a good representation of the labile Cd pool (Young *et al.*, 2000), was 18.5 mg kg^{-1} (Table 3.3). Results from the EDTA extraction experiment (Section 3.2.2) using concentrations of between 0.5 and 500 mmol EDTA kg^{-1}, show a distinct shift in the resultant trend, which occurs at approximately 18 mg Cd kg^{-1} (Fig. 3.1). This response trend confirms that the labile Cd phase is the most readily complexed phase and also confirms a fundamental difference in chemical form between the labile and non-labile Cd. Once the labile Cd phase has been extracted, in this case above approximately 18 mg Cd kg^{-1}, it is clear that greater concentrations of EDTA would be required to extract the remaining more stable metal fraction. This result is consistent with Kamath (1999) where a linear response was shown for Cd extraction by EDTA up to 20 mmol

EDTA kg^{-1} using soil also collected from the Stoke Bardolph field site. A similar trend is shown for Cu, Zn, Pb and Ni (Fig. 3.1), but an apparent asymptote was reached in the case of Pb above applications of approximately 220 mmol EDTA kg^{-1}.

Some workers have suggested a remediation strategy, which only aims to decrease the plant-available metal fraction, termed 'bioavailable metal stripping' (Hamon and McLaughlin, 1999). However, total metal concentrations are usually the main measure used by regulatory authorities (Sauve *et al.*, 2000b). For example the metal thresholds set out by the 1989 Sludge Regulations (SI, 1989) are set in terms of total soil concentrations. Furthermore, direct inhalation and ingestion of soil may still occur after the removal of plant-available metal (Stanhope *et al., 2000*). Therefore, simply removing the plant-available metal fraction may not be sufficient to achieve successful soil remediation within the parameters of a planning authority's licence. For this reason, the results discussed above may have important implications for chemically-enhanced phytoextraction using EDTA. If the labile phase were successfully depleted following phytoextraction, i.e. successive plant cropping combined with EDTA application; the efficiency of such a system may reduce as continuing to apply EDTA at a consistent concentration would induce a smaller increase in metal solubility and the rate of metal removal would be reduced. However, replenishment of the labile phase may also take place over time, although predicting the rate and extent of this process on land subject to long term chemically-enhanced phytoextraction is so far unquantified.

Selecting a suitable dose of chelate for use in phytoextraction must address a number of issues, not least the balance between root metal influx and the risk of metal leaching through the soil profile. In this study, the EDTA concentration selected was that shown to extract virtually the complete labile Cd pool: 20 mmol EDTA kg^{-1} (Fig. 3.1). Unlike EDTA, there is no precedent for applying chloride to produce increases in Cd uptake. The concentration of $CaCl_2$ required to mobilise the complete labile Cd pool from the extraction experiments, in comparison to EDTA, was large (500 mmol kg^{-1}; Fig. 3.1). For this reason a chloride concentration comparable to the EDTA solution was selected for use in the first pot experiment as a basis for direct comparison of the efficiencies of the complexing agents.

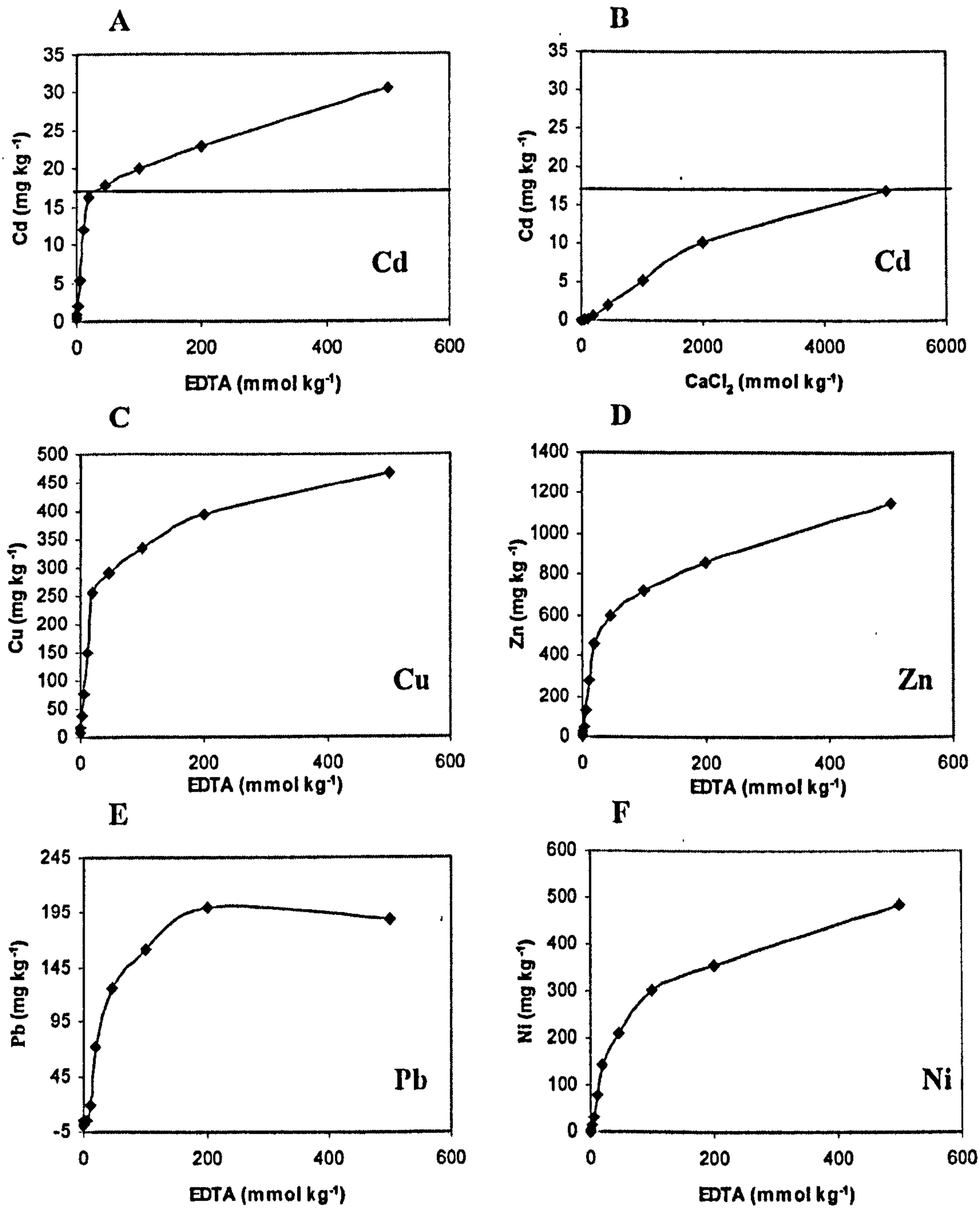


Figure 3.1. A, Cd concentration extracted by EDTA (0.5 - 500 mmol kg^{-1}); B, Cd concentration extracted by $CaCl_2$ (5 - 5000 mmol kg^{-1}); C - F, Cu, Zn, Pb and Ni concentrations extracted by EDTA (0.5 - 500 mmol kg^{-1}). The solid line in A and B corresponds to Cd extractable with 1 M $CaCl_2$.

3.3.2 Uptake of Cd and Zn following application of EDTA

Non- hyperaccumulator plants

A significant increase ($P<0.01$) in the Cd and Zn concentration of all arable crops was observed as a result of EDTA application except in the case of Cd uptake by *Z. mays* (Fig. 3.2). The processes influencing metal uptake by plants following EDTA application are discussed in Section 1.3.2. Essentially passive metal uptake via the transpiration stream is increased following solubilization of soil metal by EDTA complexation (Blaylock *et al.*, 1997). Furthermore, the EDTA metal complex has been shown to pass across root membranes of selected crops (Vassil *et al.*, 1998). In addition, EDTA may damage root membranes, thus increasing the influx of metal ions through root membranes and enhancing transport from roots to shoots (Vassil *et al.*, 1998). The Cd concentration in the arable crops was in the order *L. usitatissimum* > *B. juncea* > *B. vulgaris* > *B. napus* > *Z. mays*.

However, typical biomass values for crops such as *L. usitatissimum* and *B. juncea* are substantially lower than for crops such as *Z. mays*. For example, an average yield for *L. usitatissimum* grown on typical agricultural soil is 1.8 t ha^{-1} y^{-1} (dry weight basis) compared to 12.5 t ha^{-1} y^{-1} for *Z. mays* (dry weight basis; Soffe, 1995). These differences in biomass values would mean that a *Z. mays* crop could remove over twice the amount of Cd removed by *L. usitatissimum*, assuming the largest Cd concentrations observed from the pot experiment following application of 20 mmol EDTA kg^{-1} could be achieved under field conditions.

A significant increase ($P<0.01$) in both Cd and Zn uptake was also observed by *B. napus* following EDTA application of up to 50 mmol kg^{-1} (Fig 3.3) compared to control plants. The greatest rate of uptake per mmol EDTA applied was observed following application of 20 mmol kg^{-1} compared to control plants. This concentration was shown by the EDTA extraction experiment to extract virtually the complete labile Cd pool. Applications of greater than 20 mmol kg^{-1} up to 50 mmol kg^{-1} were shown to increase Cd solubility to a lesser extent, although solubility continued to increase even

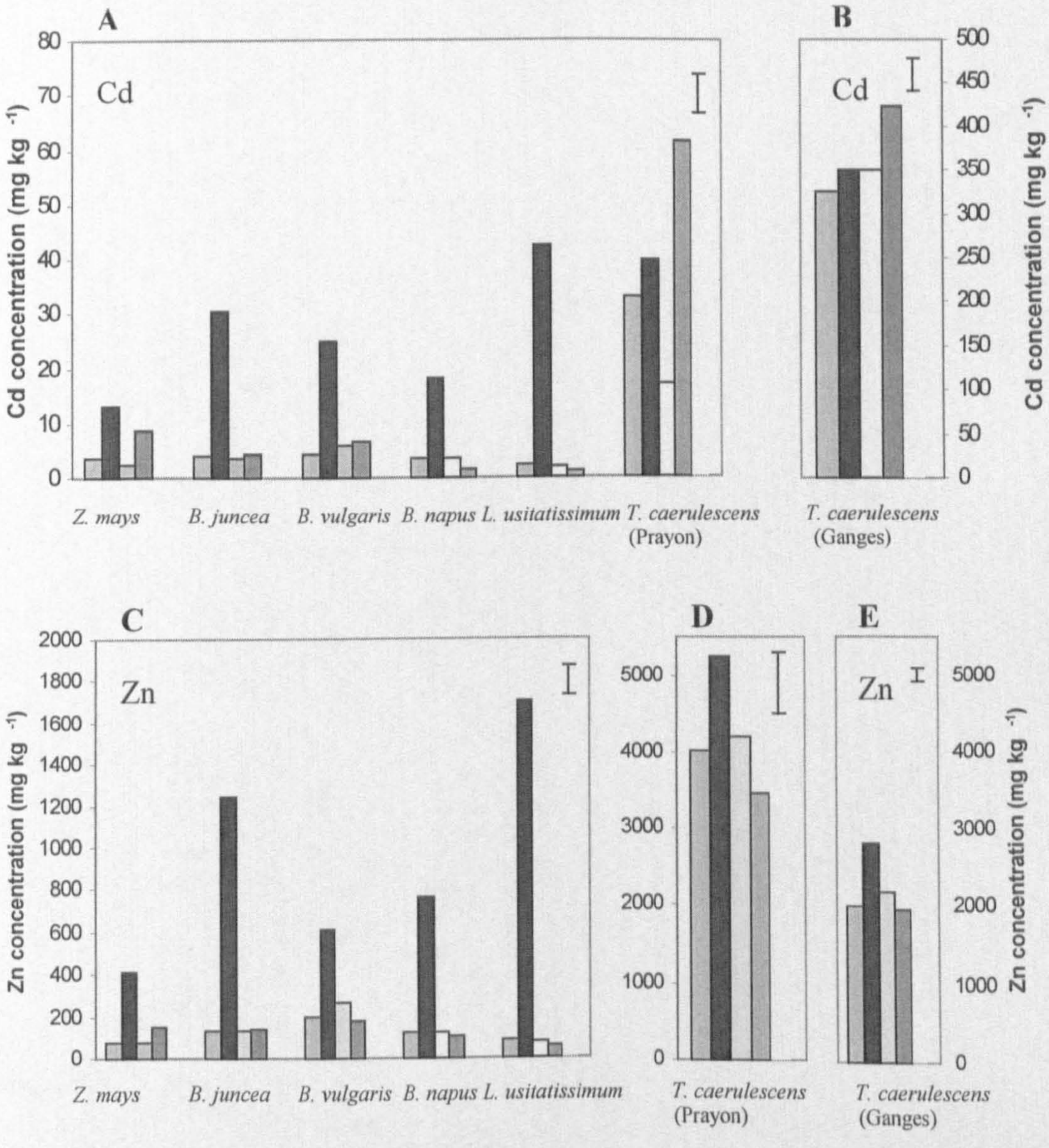


Figure 3.2. Cd and Zn concentration in shoot material of seven crop types following application of EDTA and KCl after 8 weeks of growth. Treatments include: (Control 1 harvested after 8 weeks growth. (□); 20 mmol EDTA kg^{-1} harvested after 9 weeks growth (■); Control 2 harvested after 11 weeks growth (▨) and 25 mmol KCl kg^{-1} harvested after 11 weeks growth(▨)). Vertical bars show the SED value.

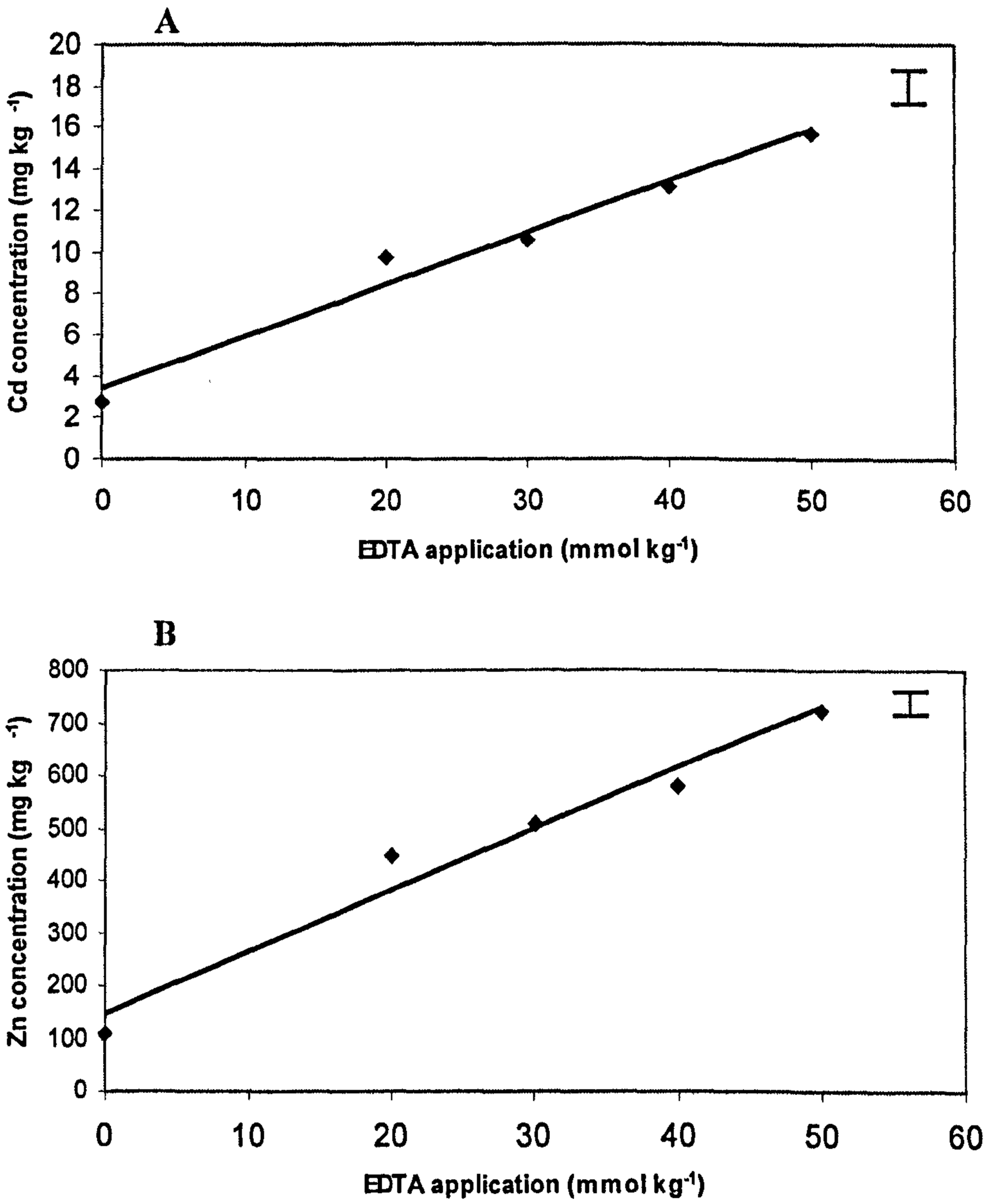


Figure 3.3. Cd and Zn concentration in shoot material of *B. napus* following application of EDTA at 0, 20, 30, 40 and 50 mmol kg^{-1} after 12 weeks growth. All plants were harvested after 13 weeks growth. Vertical bars show the SED value.

up to 500 mmol EDTA kg^{-1} (Fig. 3.1). The observed increases of Cd and Zn concentrations of *B. napus* were almost linear following application of up to 50 mmol EDTA kg^{-1}. However, this trend does not reflect the response of Cd and Zn extracted from soil (Fig. 3.1) and it is therefore likely that increases in solubility do not provide the only explanation for the observed increases in plant metal concentrations. It is likely that above 20 mmol EDTA kg^{-1} plant physiological effects rather than simply increases in solubility in the soil are responsible for the increased concentration of tissue Cd and Zn observed in *B. napus*. Collins *et al.* (2002) suggested that physiological damage to *B. juncea* roots occurred above critical EDTA concentrations (Section 1.3.2), thus supporting the above hypothesis.

No significant change in biomass, dry weight basis, was observed following EDTA applications up to 50 mmol kg^{-1} to *B. napus* (Fig. 3.4), one week after treatment. However, a significant reduction in biomass, wet weight basis, was observed ($P<0.01$; Fig. 3.4) clearly demonstrating a desiccation effect.

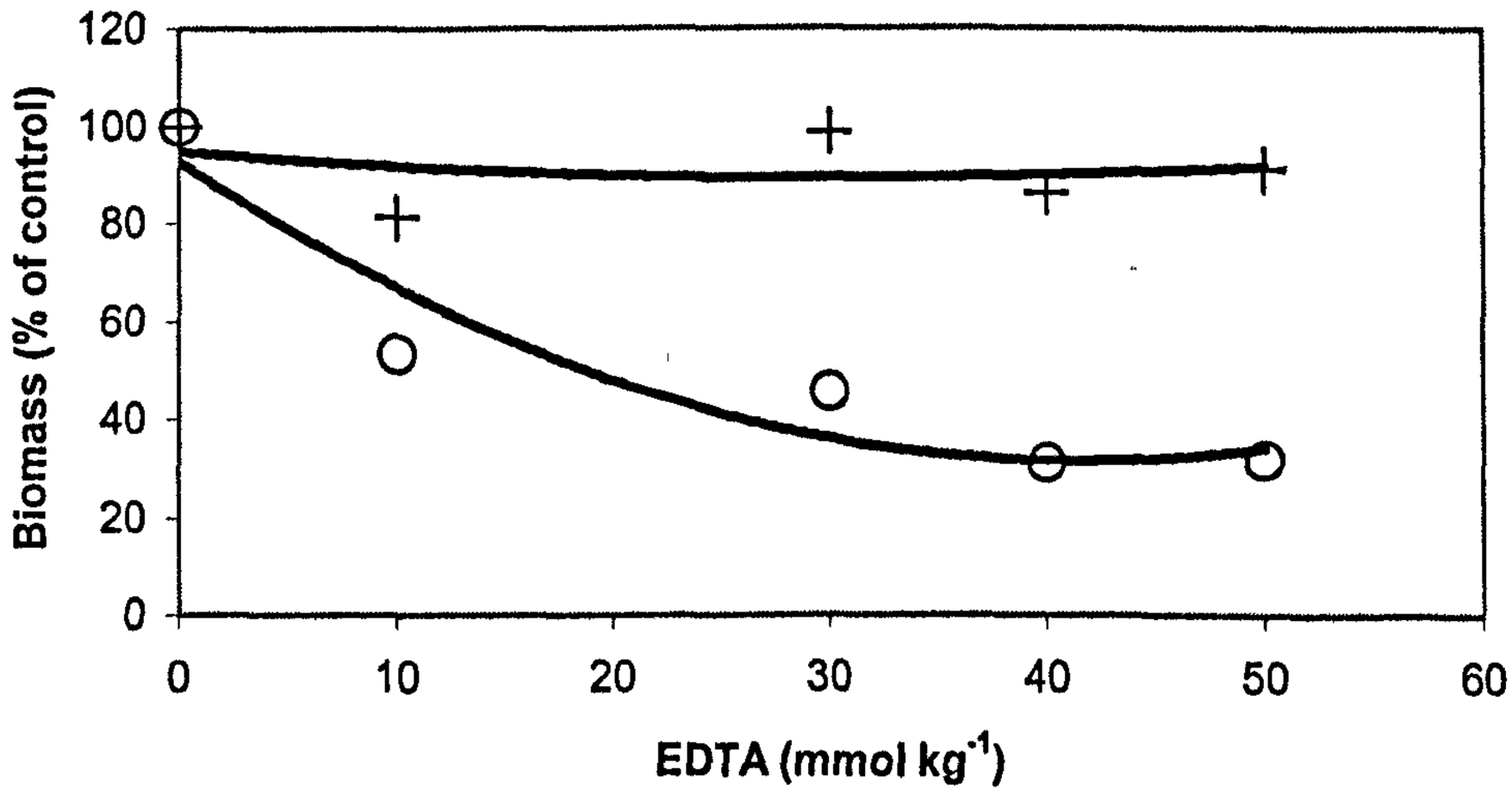


Figure 3.4. Dry weight (+); wet weight (○) shoot biomass of *B. napus* (g $plant^{-1}$) as % of control following application of EDTA at 0, 20, 30, 40 and 50 mmol kg^{-1} after 12 weeks growth. All plants were harvested after 13 weeks growth.

Hyperaccumulator plants

Differences in Cd uptake between the Ganges and Prayon populations of *T. caerulescens* have been reported in the literature (Section 1.3.2; Lombi *et al.*, 2000), and are also clearly shown in Figure 3.2. However, no significant increases in Cd uptake were observed following application of EDTA to the hyperaccumulator plants (Fig. 3.3). As EDTA will strongly complex with Cd^{2+}, it is assumed that the Cd^{2+} concentration in solution will decline, even though the overall concentration of Cd in the soil pore water will increase. Cd uptake by Ganges is reported to be through specific Cd^{2+} transporters (Lombi *et al.*, 2001a; Lombi *et al.*, 2002) and it can be assumed that the passive uptake of Cd-EDTA complex is relatively minor. Furthermore, metal uptake via transpiration is not dominant in hyperaccumulator plants. Consequently, EDTA application to soil does not significantly increase Cd uptake by Ganges.

Cd uptake by Ganges was much greater than observed for any other plant species grown in the pot experiments. For example, the Cd concentration of control plants harvested after eight weeks was 326 mg kg^{-1}. However, biomass production is also important for estimating the overall mass of metal removed from a given site. McGrath *et al.* (2000) reports an annual yield for the Prayon population of *T. caerulescens* of 4.47 t ha^{-1}. If this figure is comparable to biomass production by Ganges, and if the Cd uptake from the pot experiments is transferable to field conditions, then Cd off-take by Ganges would be almost nine times greater than for *Z. mays*. Of course establishing values for metal uptake and biomass production from field grown crops would provide a much more realistic estimate.

Uptake of Cd by Prayon is reported to be much lower than for Ganges (Lombi *et al.*, 2000), which is confirmed by the present study. No significant increases in Cd uptake were observed by Prayon following EDTA application and although Cd uptake by Prayon is so far poorly defined, the presence of specific Cd transporters identified in Ganges have not been identified in Prayon (Lombi *et al.* 2001a). Further investigation of the mechanisms responsible for Cd uptake by Prayon would be useful. However,

given that EDTA application has not significantly increased Cd uptake by Prayon, Cd^{2+} uptake is more likely to be prominent.

The uptake of Zn by both hyperaccumulators increased significantly ($P<0.01$) following EDTA application. Zn uptake by Prayon is facilitated by a specific Zn^{2+} transporter, labelled ZNT1 (Pence *et al.*, 2000; Assuncao *et al.*, 2001). Furthermore, some evidence exists that Zn uptake by other *T. caerulescens* populations also is governed by this transporter (Assuncao *et al.*, 2001). Consequently Zn uptake by both Ganges and Prayon is unlikely to involve significant uptake of Zn-EDTA. Zn uptake following EDTA application may be the result of a shortened diffusion pathway between Zn pools and root surfaces following dissociation of Zn-EDTA.

3.3.3 Uptake of Cd and Zn following application of chloride

Non-hyperaccumulator plants

Figure 3.2 shows the results of applying 20 mmol KCl kg^{-1} to a number of different arable crops (described above). As expected, no significant increase in plant Zn concentrations were observed following application of KCl, as Zn complexation with chloride is minimal. However, there was also no significant increase in plant Cd uptake observed following KCl application. The tissue Cd concentration was greater after KCl application to *Z. mays*, compared to control plants, but this was not statistically significant.

Only *B. vulgaris* and *B. napus* were grown in the pot experiments testing the influence of $CaCl_2$ and KCl on plant metal uptake (Sections 3.2.6 and 3.2.7). In both cases the Zn concentration of plant tissue did not increase following chloride application (Figs 3.5 and 3.6). However, the Cd concentration of *B. vulgaris* increased significantly following application of 20 mmol kg^{-1} of either $CaCl_2$ or KCl compared to control plants ($P<0.01$; Fig. 3.5). The Cd concentration of *B. vulgaris* also increased significantly following application of 50 mmol kg^{-1} of either $CaCl_2$ or KCl compared to both the control plants and those treated with 20 mmol kg^{-1} of either $CaCl_2$ or KCl respectively ($P<0.01$; Fig. 3.5). The tissue Cd concentrations of *B. vulgaris* following

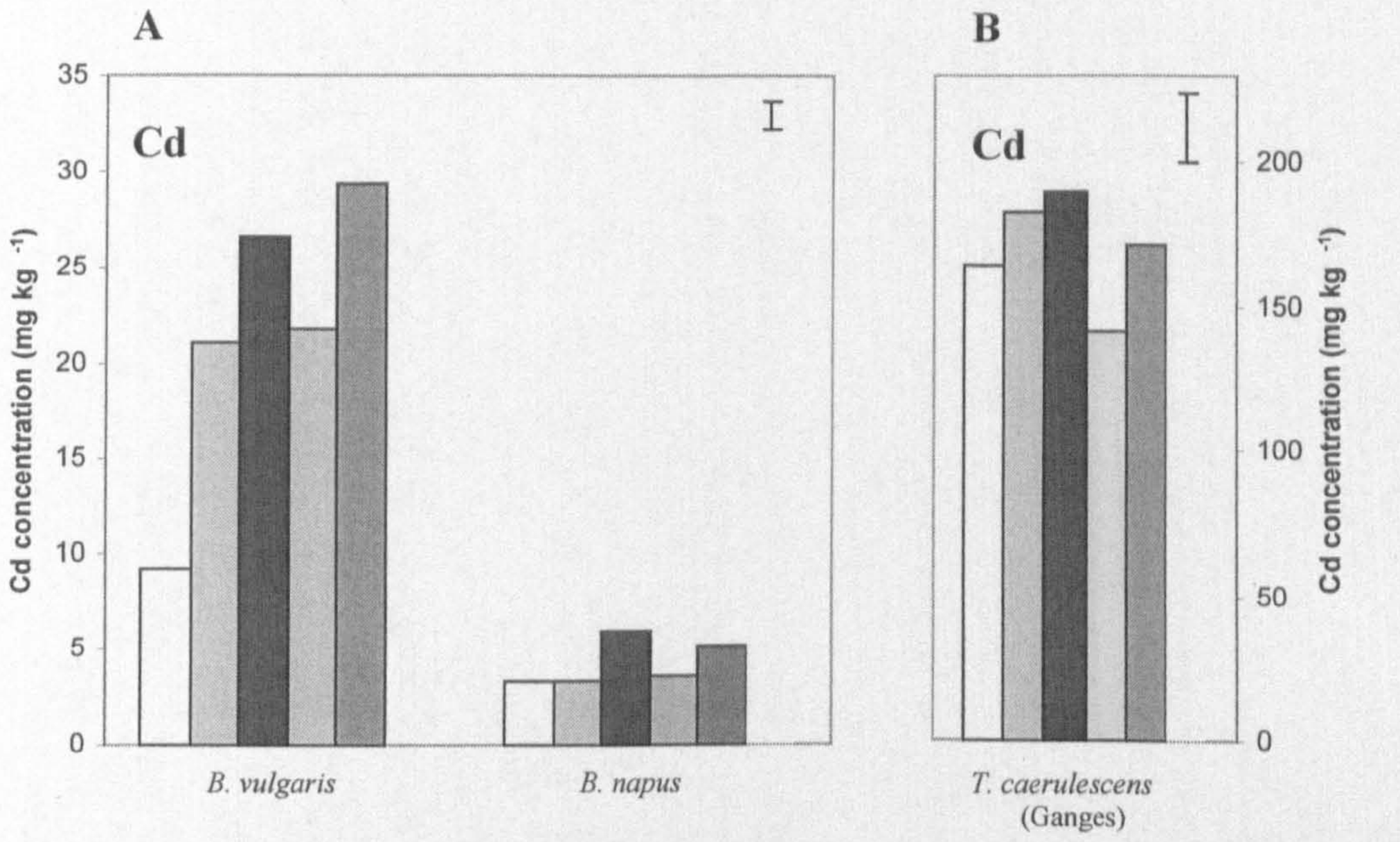


Figure 3.5. Cd and Zn concentration in shoot material of *B. vulgaris*, *B. napus* and *T. caerulescens* (Ganges population) following application with either $CaCl_2$ or KCl after 7 weeks growth. Treatments include: (Control (□); 20 mmol $CaCl_2$ kg^{-1} (▫); 50 mmol $CaCl_2$ kg^{-1} (■); 20 mmol KCl kg^{-1} (▨) and 50 mmol KCl kg^{-1} (▨)). All plants were harvested after 9 weeks growth. Vertical bars show the SED value.

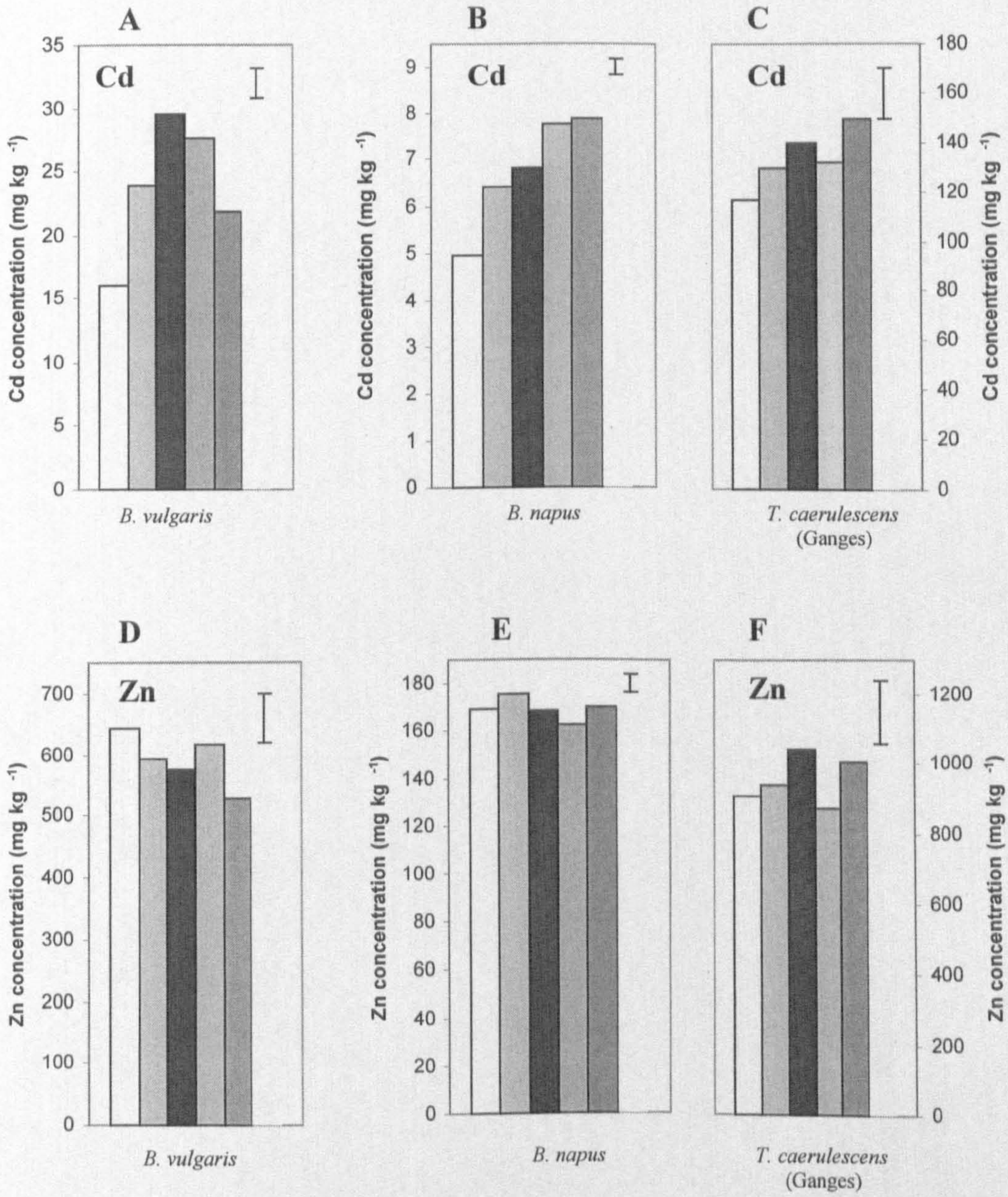


Figure 3.6. Cd and Zn concentration in shoot material of *B. vulgaris*, B. napus and *T caerulescens* (Ganges population) following application with $CaCl_2$ after 7 weeks growth. Treatments include: (Control (□);20 mmol $CaCl_2$ kg^{-1} (▢); 40 mmol $CaCl_2$ kg^{-1}(■); 60 mmol $CaCl_2$ kg^{-1} (▨) and 80 mmol $CaCl_2$ kg^{-1} (▨)). All plants were harvested at death or after 9 weeks growth. Vertical bars show the SED value.

larger doses of $CaCl_2$ are shown in Figure 3.6. Applications of 60 and 80 mmol $CaCl_2$ kg^{-1} did significantly increase the Cd concentration of *B. vulgaris* compared to control plants ($P<0.01$; Fig. 3.6) but not compared to plants treated with 40 mmol $CaCl_2$ kg^{-1}. In fact, the Cd concentration of plants treated with 60 and 80 mmol $CaCl_2$ kg^{-1} was lower than for those plants treated with 40 mmol $CaCl_2$ kg^{-1}. Thus increasing Cd uptake by application of chloride in *B. vulgaris* appears to be limited to relatively small chloride treatments.

No significant increases in plant Cd concentrations in *B. napus* were observed following application of either 20 or 50 mmol kg^{-1} of either $CaCl_2$ or KCl (Fig. 3.5). However, a significant increase ($P<0.01$) in the tissue Cd concentration of *B. napus* compared to control plants was observed following applications of $CaCl_2$ in the experiment testing plant response to four application rates of $CaCl_2$ (Fig. 3.6; Section 3.2.7). The soil and growing conditions for all experiments were the same. Therefore it is possible that slight changes in conditions such as light levels and temperature accounted for the different responses observed in *B. napus* to Cd uptake following $CaCl_2$ application between different experiments. Further work is required to clarify the response of Cd uptake by *B. napus* following application of chloride.

There was no significant difference observed in plant Cd uptake following application of $CaCl_2$ compared to application of KCl (Fig. 3.5) even though the additions of both salts are probably sufficient to significantly affect the cation balance in the soil. Although competition between Cd^{2+} and Ca^{2+} is possible, the relative importance of Ca channels were not investigated. Furthermore, uptake of $CdCl^{+}$ may be via K^{+} channels (Welch and Norvell, 1999) and so may provide an explanation for the similarities between Cd uptake following application of either KCl or $CaCl_2$. More detailed investigations are required to elucidate how different chloride species are influenced by specific metal uptake mechanisms.

Hyperaccumulator plants

No significant increases in uptake of Cd or Zn were observed in the Ganges population of *T. caerulescens* grown in any of the pot experiments where chloride was

applied. This result is not unexpected as Cd uptake by Ganges is predominantly of Cd^{2+} by specific transporters (Lombi *et al.*, 2001a; Lombi *et al.*, 2002; Section 3.3.2). However, Cd uptake by Prayon increased significantly following application of 20 mmol KCl kg^{-1} ($P<0.01$; Fig. 3.2). This result may be an anomaly as the Cd concentration observed in the two different experimental controls, harvested after eight and eleven weeks of growth, were not consistent. Zhao *et al.* (2002) increased chloride concentrations in solution culture to decrease the activity of free Cd^{2+} by 50% through chloro-complexation and observed a decrease in Cd uptake in Prayon, but not in Ganges. As Cd supply was not limiting, Zhao *et al.* (2002) concluded that Ca channels might therefore contribute significantly to Cd uptake in Prayon, but not in Ganges. This is consistent with Cd^{2+} uptake in Ganges being facilitated by specific transporters. However, no soil buffering will occur in solution culture experiments. Therefore, small additions of chloride to soils will result in increased Cd concentrations in solution, without significantly reducing the Cd^{2+} concentration. It is likely that any increase in the Cd concentration of Prayon, observed by the current study, is the result of a shortened diffusion pathway, rather than uptake of Cd-chloride complexes.

3.4 Conclusions

A distinct shift in the trend in metal solubility produced following extraction with EDTA using concentrations of between 0.5 and 500 mmol kg^{-1} was observed. This shift occurred at approximately 18 mg Cd kg^{-1} following application of 20 mmol EDTA kg^{-1}. The Cd concentration extracted using a 1M $CaCl_2$ solution, which is known to provide a good representation of the labile Cd pool, was consistent with the above observation at 18.5 mg kg^{-1}. Consequently, metal removal by plants, as part of a phytoextraction system relying on the repeated application of EDTA, may be substantially less efficient following complete removal of the labile Cd pool.

Tissue Cd concentrations following application of chloride salts produced inconsistent results between individual experiments, although some significant increases in the Cd uptake by plants were observed. For example, Cd uptake by *B. vulgaris* following application of both KCl and $CaCl_2$ at concentrations of 20 and 50 mmol kg^{-1} ($P<0.01$)

was observed. The maximum Cd concentration observed in *B. vulgaris* followed chloride application of 50 mmol kg^{-1}. Applying concentrations of either 60 or 80 mmol kg^{-1} resulted in lower tissue Cd concentrations being recorded. It is therefore possible that use of chloride to enhance plant Cd will be limited to relatively low concentrations.

A significant increase in plant Cd was observed following EDTA application. Increases in Cd uptake per mmol EDTA applied in *B. napus* were greatest following application of 20 mmol kg^{-1}, the concentration shown to remove the complete labile fraction, compared to applications of 30, 40 or 50 mmol kg^{-1}. The greatest Cd concentration was observed for the Ganges population of *T. caerulescens*, however, metal concentrations observed from field grown crops are likely to be more realistic than from pot based studies.

CHAPTER 4: COMPARISON OF CADMIUM UPTAKE BY TWO POPULATIONS OF *THLASPI CAERULESCENS*

4.1 Introduction

Research addressing the use of hyperaccumulator plants for phytoextraction was discussed in detail in Section 1.3.1. As many hyperaccumulator plants are slow-growing and produce a low biomass (Brown *et al.*, 1995; Shen *et al.*, 1997), much research has focused on understanding the specific uptake mechanisms responsible for hyperaccumulation. It has been postulated that the genetic modification of hyperaccumulator plants, may in the future be used to create genotypes suitable for particular phytoextraction purposes (Baker *et al.*, 1994; Guerinot, 2000; Karenlampi *et al.*, 2000; Williams *et al.*, 2000; Lasat, 2002). However, in addition to the requirement for further mechanistic investigation, more field-based work is required to evaluate the overall suitability of hyperaccumulator plants for phytoextraction. Baker and Whiting (2002) recently suggested that the race to identify genes responsible for metal uptake traits in hyperaccumulator plants, has resulted in important issues being overlooked, including plant performance under natural field conditions. In addition, questions remain over the future public acceptance of genetically modified plants (Baker and Whiting, 2002).

The work described in this Chapter was designed to test two populations of *T. caerulescens*, Ganges and Prayon, for their ability to phytoextract Cd and Zn from a contaminated field site. Both populations are known to accumulate Zn, but Ganges is also a hyperaccumulator of Cd (Lombi *et al.*, 2000). Both populations were grown at the Stoke Bardolph field site, which is heavily contaminated with Cd and Zn derived from long-term disposal of sewage sludge. Field experiments were conducted to assess the viability of Ganges and Prayon as phytoextraction tools.

4.1.1 Experimental hypothesis

The experimental hypothesis being tested was that growth of the Ganges and Prayon populations of *T. caerulescens* would result in sufficient metal uptake for the successful phytoextraction of the Stoke Bardolph field site.

4.2 Materials and methods

4.2.1 Crop establishment

Two populations of *T. caerulescens* were germinated under glasshouse conditions before being transplanted to the field site on several planting dates between April and July 2000. Plants were grown in three batches: in Batch 1 plants of both populations were grown (Plates 4.1 and 4.2), whereas Batches 2 and 3 comprised only Ganges. Seed for Batch 1 were initially germinated in P60 micro-pots (25 cm^3 volume) before being transplanted after two to three weeks to P15 micro-pots (100 cm^3 volume). Batches 2 and 3 were germinated in 12.7 cm diameter pots before being transplanted to P15 micro-pots after the same time period. All plants were grown in soil collected from the field site (Section 2.2) under the glasshouse conditions used for the pot experiments described in Chapter 3. After approximately five weeks of growth, the seedlings were moved to an unlit and unheated polythene tunnel before being transplanted into the field after a further two to three weeks of growth. Each batch was planted in a single fenced plot (2 x 2 m) and consisted of 200 equally spaced plants. Batch 1 was sown on 11 March 2000 and transplanted to the field on 28 April 2000; Batches 2 and 3 were sown on 10 April and transplanted to the field on 1 and 7 June 2000 respectively. All plants were sprayed regularly with "Fastac" (100 g L^{-1}) containing alphacypermethrin (insecticide) to protect against the suspected presence of flea beetle.

Batch 1 flowered from mid-June and substantial losses of plants were observed during this period. As the problem was thought to be caused by cutworm (Sarah Dunham, *pers. comm.*), the plants were sprayed with "Dursban" (480 g L^{-1}) containing Chloropyrifos (Organophosphorus insecticide). However, this treatment is best applied as a soil drench prior to planting, and so was not wholly effective in eliminating the problem. Plant losses also started to appear in Batches 2 and 3 from the beginning of July.

Plate 4.1. A: *T. caerulescens*, Ganges population. B: *T. caerulescens* Prayon population.

Plate 4.2. Batch 1 plants May 2000 after approximately two months of growth.

4.2.2. Crop harvest

Batch 1 was harvested in the first week of July due to the plant losses described above and Batches 2 and 3 were harvested at the end of July for the same reason. At harvest, the plants were severed • 1 cm above ground level. The harvested material was rinsed in deionised water to remove any surface contamination and dried at 80 ^{0}C for 48 h. Plant material was ground using a pestle and mortar and mixed, before digesting 1 g of shoot material in concentrated HNO_3. Analysis for Cd, Zn, and Cu was conducted

as described in Section 2.2 using F-AAS. Ten randomly selected plants of both populations were digested in triplicate from Batch 1. Single digests were conducted for each plant from Batch 2; 159 individual plants. Eight randomly selected plants from Batch 2 were also digested in triplicate to test the reliability of the single digest analytical approach. Batch 3 plants were not analysed.

4.2.3 Soil measurements

Following harvest, six soil cores (0 - 20 cm; 4 cm diameter) were collected and combined for each of the three plots. Analysis was conducted for pH, total and EDTA extractable Cd, Zn, Pb, Cu and Ni, $CaCl_2$ extractable Cd, phosphate extractable in bicarbonate and loss on ignition using the approaches described in Section 2.2.

4.2.4 Statistical analysis

T-tests were conducted to compare metal concentrations between the two populations of *T. caerulescens* using Microsoft Excel. Correlations assessing the relationship between plant biomass and the ratios of Cd:Zn uptake with plant Cd, Zn, Pb and Cu concentration were performed using GenStat package (5th Edition).

4.3 Results and Discussion

4.3.1 Soil characteristics

Soil characteristics at the Stoke Bardolph field site are discussed in detail in Section 3.3 and the corresponding details for the soil used in the current chapter are presented in Tables 4.1 and 4.2. The total soil metal concentrations were substantially greater than those reported in other chapters of this thesis. For example, total soil Cd was 54 mg kg^{-1} in this chapter compared to 35 - 44 mg kg^{-1} for the other field studies (Chapters 5 and 7). As metals in sludged soils are expected to remain where they are incorporated over extended periods (McGrath and Lane, 1989); it is possible that the application of metal-rich sludge to the study area has been uneven over the prolonged period during which this field has been used for sludge disposal. The location chosen

for growing *T. caerulescens* may therefore have represented a contamination hotspot. The concentration of Cd extractable using 1 M $CaCl_2$ was 29 mg kg^{-1}. This result is consistent with Hutchinson *et al.* (2000) for soil from the same field who reported a total Cd concentration of 59.3 mg kg^{-1}, a value of 27.1 mg Cd kg^{-1} extractable with 1 M $CaCl_2$, and a radio labile Cd content of 29 mg kg^{-1}.

Table 4.1. Total, EDTA-extractable and $CaCl_2$-extractable metal content in topsoil from the field site used for the *T. caerulescens* trials. Values are means (n = 3). Values in parenthesis show standard error of the mean.

	Total soil metal content (mg kg^{-1})	EDTA extractable metal content (mg kg^{-1})	$CaCl_2$ extractable metal content mg kg^{-1})
Cd	54.2 (3.23)	33.7 (2.26)	30.0 (1.84)
Zn	2160 (136.0)	1099 (68.0)	-
Cu	860 (48.0)	611 (33.0)	-
Pb	601 (21.0)	277 (4.33)	-
Ni	497 (27.0)	420 (27.0)	-

Table 4.2. Selected soil characteristics for the field site used for the *T. caerulescens* trials. Values are means (n = 3). Values in parenthesis show standard error of the mean.

	pH	[a]LOI (%)	[b]Available P (mg kg^{-1})
Mean	6.00 (0.03)	34.5 (0.93)	302 (54.0)

[a]LOI, loss on ignition. [b]Bicarbonate-extractable phosphate

4.3.2 Plant biomass

Although seed from wild populations of *T. caerulescens* can produce highly variable shoot biomass (Lombi *et al.*, 2000), the quantities obtained in the present study were fairly consistent. Plants from Batch 1 produced a mean shoot biomass of 5.02 g $plant^{-1}$

(SE 0.589) for Ganges, and 6.06 g plant^{-1} (SE 0.701) for Prayon. The difference in biomass between populations was not statistically significant. Estimates for plant yield were 2.52 t ha^{-1} for Ganges and 3.03 t ha^{-1} for Prayon. These values are consistent with Robinson *et al.* (1998), who reported an annual biomass production by wild *T. caerulescens* in Southern France (reported to be Ganges by Lombi *et al.*, 2000) of 2.6 t ha^{-1}. McGrath *et al.* (2000) reported a yield of 4.47 t ha^{-1} for Prayon after 4 months of growth at the Woburn Market Garden experiment in 1991. However, the planting density at Woburn is 90 plants m^{-2} compared to 50 plants m^{-2} in the present study. Annual biomass for all populations of *T. caerulescens* grown at Woburn ranges from 4.4 to 9.9 t ha^{-1} (McGrath, 1998).

4.3.3 Plant metal uptake

Metal concentrations in the shoots did not differ significantly between Ganges and Prayon with the exception of Cd ($P<0.01$; Table 4.3 and Fig. 4.1). This is consistent with published work reporting the ability of Ganges to accumulate large concentrations of Cd relative to other populations of *T. caerulescens* (Lombi *et al.*, 2000; Lombi *et al.*, 2001a, b; Lombi *et al.*, 2002).

Table 4.3. Concentrations of Cd, Zn and Cu in shoot material from two populations of *T. caerulescens* grown at the Stoke Bardolph field site (Batch 1 plants). Postscript letters represent significant between population differences ($P<0.01$) for individual elements.

	Ganges (TcG; n = 9)			Prayon (TcP; n = 10)		
	Mean (mg kg^{-1})		SE	Mean (mg kg^{-1})		SE
Cd	265	**a**	17.0	20.5	**b**	1.71
Zn	1009	**c**	53.0	844	**c**	62.0
Pb	4.70	**c**	1.05	2.70	**c**	1.03
Cu	16.0	**c**	1.09	16.4	**c**	1.96

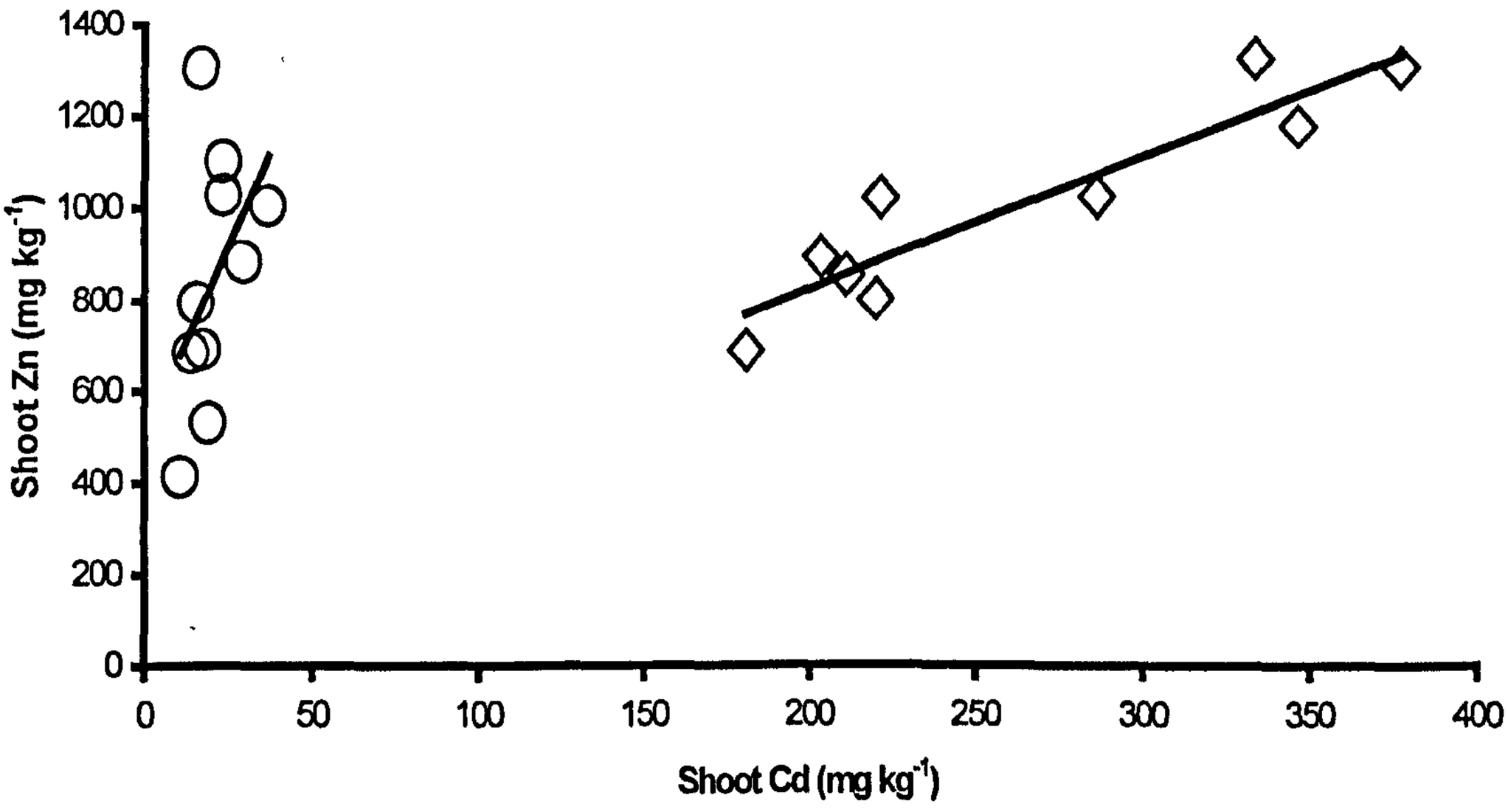


Figure 4.1. Concentration of Cd and Zn in the shoots of Prayon (O) and Ganges (◇) grown at the Stoke Bardolph field site.

The biomass of plants from Batch 2 was lower than that for Batch 1 due to the shorter growing period used. However, Cd and Zn concentrations in Ganges plants from Batch 2 were also lower than in plants from Batch 1 (Table 4.4). This observation is inconsistent with a dilution effect, whereby lower metal concentrations might be expected in larger plants. Work by Schwartz *et al.* (2001) supports this observation, as very slight dilution effect accompanied increases in plant biomass induced by fertilisation applications. In this study, a population of *T. caerulescens* from southern France, probably Ganges, was grown in a field experiment where sewage applications of up to 15 t ha^{-1} significantly increased plant biomass. Unfortunately, biomass values for the fertilised plants were not published. Furthermore, Angle *et al.* (2001) suggested from extensive experience of growing a range of *T. caerulescens* populations for use in Ni mining that the uptake of metal reaches a maximum during the mid-flowering stage. This may help to explain the greater uptake of metals by the Batch 1 plants, as they were harvested during this growth stage. The metal concentrations for Batch 2 plants analysed following single acid digests correspond closely with those for a random set of eight plants digested in triplicate (Table 4.4).

Table 4.4. Concentrations of Cd, Zn and Cu in shoot material from the Ganges population of *T. caerulescens* grown at the Stoke Bardolph field site (Batch 2 plants). A: single acid digest per plant; n = 159; B: triplicate acid digest per plant; n = 8.

	Ganges (A)		Ganges (B)	
	Mean (mg kg^{-1})	SE	Mean (mg kg^{-1})	SE
Cd	124	3.39	122	8.88
Zn	980	21.8	995	65.6
Cu	13.8	0.437	11.1	0.879

The mean Cd concentration for Batch 1 plants of 265 mg kg^{-1} is substantially lower than that reported for wild plants by Robinson *et al.* (1998) of 1000 mg kg^{-1}. Even the maximum individual plant Cd concentration of 378 mg kg^{-1} was still far lower than that reported for wild plants. Ganges has been shown to be capable of accumulating up to 3700 mg Cd kg^{-1} in pot trials and up to 1.4 % Cd in dry tissue in hydroponic trials (Lombi *et al.*, 2000). In fact, 265 mg Cd kg^{-1} is below the accepted threshold of 0.1 % Cd in dry tissue often used to define Cd hyperaccumulation (Baker and Brooks, 1989). Field data reported by Lombi *et al.* (2000) showed an uptake of 516 mg kg^{-1} when Ganges plants were grown on soil containing a total Cd concentration of 12.5 mg kg^{-1} at the Woburn field site. Although this represents a much greater level of Cd uptake from a soil with a lower total Cd concentration than in the present study, the tissue concentration reported by Lombi *et al.* (2000) was still below 0.1 % Cd in dry tissue. The Cd concentration reported by Lombi *et al.* (2000) for the shoots of field grown Prayon plants is consistent with the present study, although soil Cd concentrations were much greater at Stoke Bardolph.

The differences in the quantity of Zn taken up by Prayon and Ganges were much smaller than those for Cd (Lombi *et al.*, 2000). However, Lombi *et al.* (2000) reported concentrations of 1500 and 2700 mg kg^{-1} respectively in the shoots of Prayon and Ganges plants grown on soil with a total Zn concentration of 500 mg kg^{-1}.

The very large extractable phosphate and relatively high organic matter values for the Stoke Bardolph soil clearly have an important role in reducing metal solubility (Section 3.3). It is reasonable to assume that the low concentration of Cd^{2+} at this site is therefore a major limitation on uptake by the Ganges population. The V_{max} values for this population of *T. caerulescens* are reported to be large (Lombi *et al.*, 2001a; Section 1.3.1) and Cd uptake by Ganges is substantial when grown in hydroponic solution (Lombi *et al.*, 2000). Thus, if the rate of Cd uptake by Ganges exceeds the rate of replenishment from the labile pool, which offers a likely explanation for the limited Cd uptake at the Stoke Bardolph field site, then certain genetic modification approaches would be unlikely to increase Cd uptake by this population. For example, over-expressing the genes responsible for producing metal transporter proteins (Section 1.3.1) might increase the *rate* of metal transfer, but would not increase overall metal *uptake*. An alternative approach, may be to enhance the soil pool of metal accessible to the roots, thereby increasing overall metal uptake. For example, Whiting *et al.* (2000) demonstrated proliferation of lateral roots in *T. caerulescens* in areas of localised Zn enrichment. If proliferation of fine lateral roots could be induced in homogeneously contaminated soil, metal supply to the roots might be enhanced. Investigations of hairy roots in *T. caerulescens* have been conducted (Nedelkoska and Doran, 2000a, b) and their survival at high Cd solution concentrations demonstrated. Further studies are required to establish whether increasing the growth and number of hairy roots would increase Cd influx by increasing the pool of soluble Cd available to the plant.

4.3.4 Influence of soil Cu on the uptake of Cd and Zn by plants

T. caerulescens has been reported to be sensitive to soil Cu (McLaughlin and Henderson, 1999). This work showed that increasing Cu^{2+} activities in solution culture experiments significantly reduced root and shoot growth in the Ganges population of *T. caerulescens*. Furthermore, Lombi *et al.* (2001b) suggested that Ganges is no more tolerant of Cu toxicity than normal crop species. They also found that Cd uptake by Ganges from Cu rich soil was much lower than from soil with a low Cu concentration. For example, Cd uptake by Ganges was up to 140 mg kg^{-1} when grown on soil with a Cu concentration of 1245 mg kg^{-1}. By contrast uptake of Cd was up to 576 mg kg^{-1} in

soil containing only 78 mg Cu kg^{-1}. Tissue Zn concentration was also much greater in plants grown on the low Cu soil. Similarly, the uptake of Cd and Zn by *T. caerulescens* in a study by Ebbs *et al.* (1997) was relatively low when plants were grown on a soil with a total Cu concentration of 3420 mg kg^{-1}. However, Lombi *et al.* (2001b) also reported significant differences in the Cd concentrations of soil capillary water from the two soils studied, which is likely to have had an important influence on the uptake of Cd by plants.

In the present study, total and EDTA-extractable soil Cu concentrations were over 100 mg kg^{-1} greater in the Batch 2 plot than in the Batch 1 plot, providing a possible explanation for why Cd accumulation was significantly greater in Batch 1 than in Batch 2 plants. Furthermore, the total soil Cu concentration for the Stoke Bardolph site was much greater than the ICRCL guideline value for Cu contamination (130 mg Cu kg^{-1}) and the normal threshold concentration for Cu toxicity for most crop species (30 mg kg^{-1}; Marshner, 1995). The tissue Cu concentrations for Ganges reported by Lombi *et al.* (2001b) were 36 - 92 mg kg^{-1} for the high Cu soil and 9.1 - 23.9 mg kg^{-1} for the low Cu soil. At Stoke Bardolph, tissue Cu concentrations in Ganges were 11.3 - 27.5 mg kg^{-1}. Thus, tissue Cu concentrations were similar irrespective of whether Ganges was grown on either the low Cu soil used by Lombi *et al.* (2001b), or the high Cu soil from the Stoke Bardolph site. It is therefore possible that plant sensitivity to Cu is less important in influencing Cd uptake than Cd solubility.

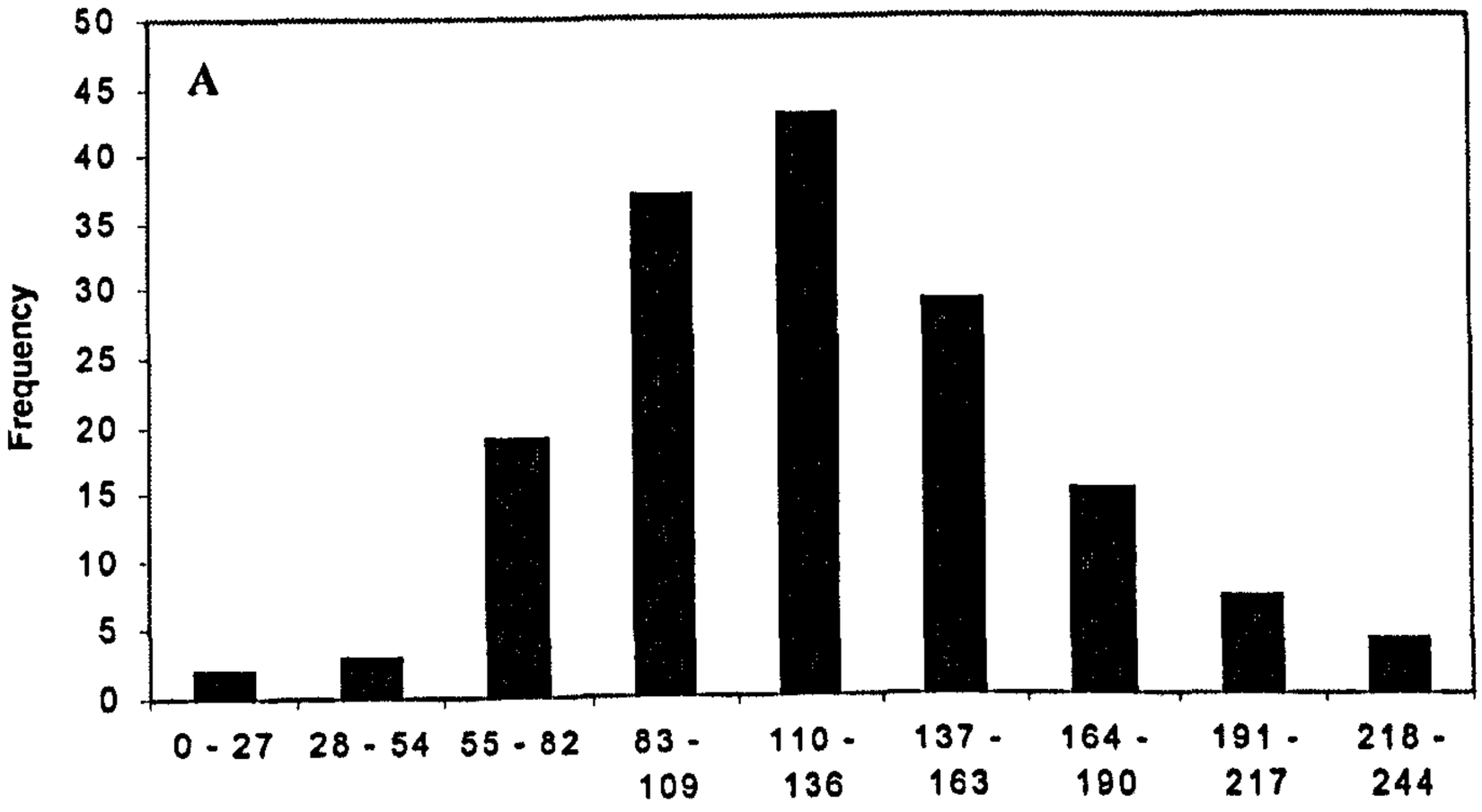
4.3.5 Growth and production of T. caerulescens

Previous studies have reported that the metal uptake trait exhibited by *T. caerulescens* may be part of an anti-herbivory mechanism developed in response to evolutionary pressures (Pollard and Baker, 1997). Furthermore, Angle *et al.* (2001) observed no evidence of herbivory on hyperaccumulators, despite extensive grazing of adjacent weeds. Angle *et al.* (2001) suggested that the large metal content of the shoots of hyperaccumulators reduced their palatability to insects or animals. However, plants grown in the current study were attacked by flea beetle and required regular spraying with insecticide to avoid extensive damage. The source of flea beetle was an oil seed rape crop grown in close proximity. For *T. caerulescens* to be suitable for the

phytoextraction of agricultural soil, it will necessarily have to be grown in close proximity to other crops. Given the limited number of field experiments conducted with *T. caerulescens* to date, it is probable that other pests of common arable crops may also affect its performance. Regular use of insecticide may therefore be required for the commercial production of *T. caerulescens*.

Plants from Batches 1 and 2 were harvested after 10 and 7 weeks of growth in the field respectively, following substantial losses of plants. Cutworm larvae have been reported to be a problem associated with growing *T. caerulescens* (McGrath *et al.*, 2000) and were suggested to be a likely cause of our plant losses (Sara Dunham. *pers comm.*). However, no evidence of cutworm was found during soil sampling. Visual examination of individual plants revealed no evidence of pests or disease apart from the flea beetle damage, described above. No explanation was found for the sudden loss of plants after approximately two months of growth. Extensive plant mortality of field grown *T. caerulescens* plants was also reported by Kayser *et al.* (2000), although no explanation was given.

The uptake of Cd and Zn by Batch 2 plants showed a normal distribution (Fig. 4.2) based on a population of 159 plants. This may be important, as commercial phytoextraction would require the efficacy of metal removal across a given site to be uniform, or at least, within target parameters. However, as phytoextraction is likely to extend over a period of many years, regular soil ploughing and the resultant mixing of soil would compensate for any local variation in metal uptake by plants. It is likely that the observed variation in metal uptake within the Ganges plants grown in the present study reflects intrinsic variation in the characteristic of seed collected from a wild population. This hypothesis is supported by the work by Angle *et al.* (2001), who detected significant variation in growth and metal uptake parameters for *T. caerulescens,* even when seed were collected from wild plants grown in a very small geographical area.



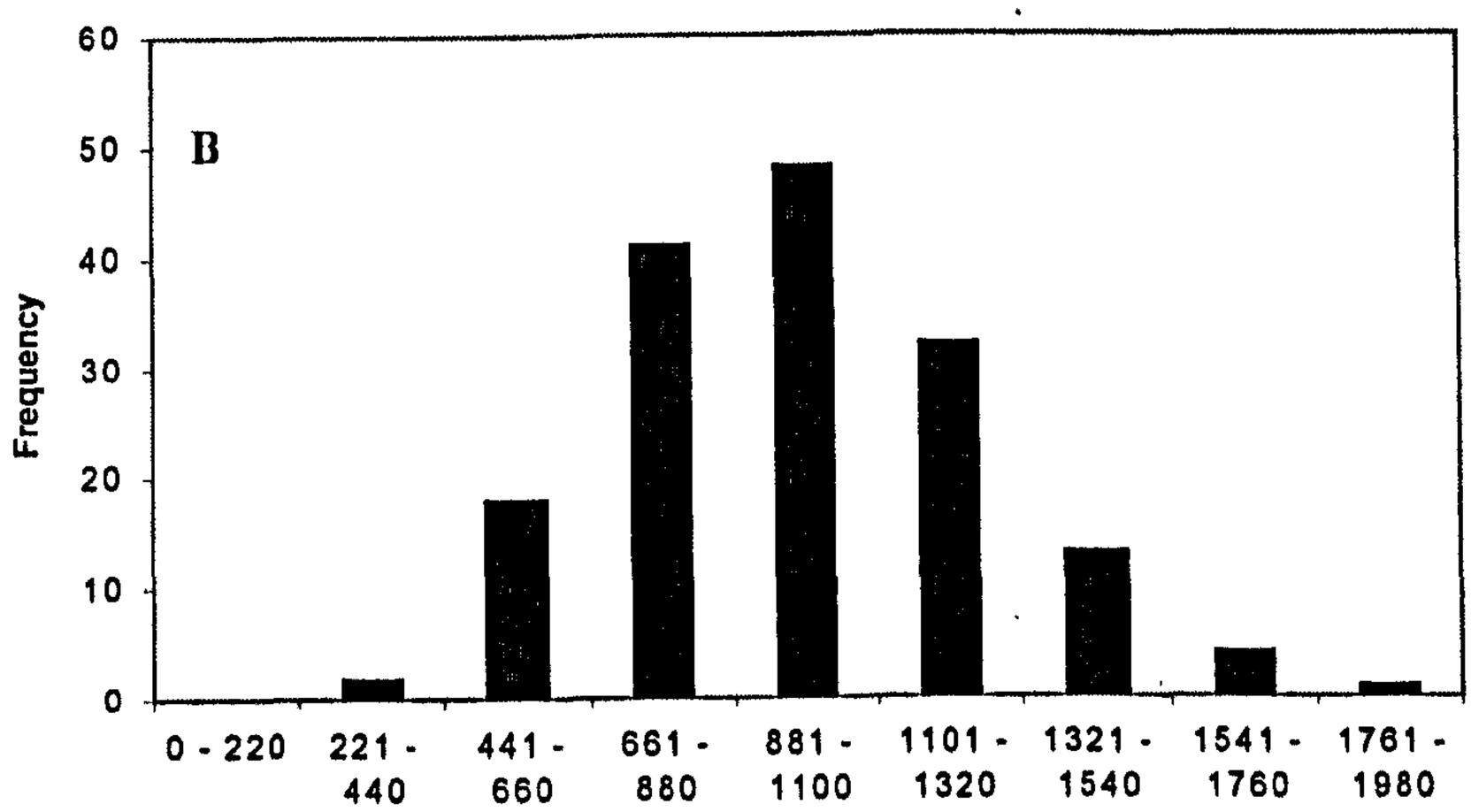


Figure 4.2. Frequency distribution of (A) Cd concentration and (B) Zn concentration in the shoots of the Ganges population of *T. caerulescens* grown at the Stoke Bardolph field site (n = 159).

4.3.6 Estimation of the potential of Ganges and Prayon for phytoextraction of Cd and Zn from the Stoke Bardolph field site

The number of years required to reduce total soil Cd and Zn concentrations below 3 and 300 mg kg^{-1} respectively were calculated for the test site (Tables 4.5 and 4.6). These concentrations are the maximum permitted according to the 1989 Sludge Regulations (SI, 1989). The calculation was performed using Microsoft Excel, where the total soil metal concentration was amended following each cropping assuming a

constant soil → plant transfer factor throughout the remediation period. Therefore plant off-take of metal also reduced for each cropping in line with the same assumption. It was also assumed that soil contamination extended to a depth of 20 cm and the bulk density was 1.25 g cm^{-3}. The solution phase of Cd and Zn were assumed to continuously replenish from other phases during the remediation period.

Although Ganges removed substantially greater quantities of Cd than Prayon, the time period required to reduce soil Cd concentration to the required target was still large. However, as plants from Batch 1 were only grown for 10 weeks in the field, two crops could be grown each year, thus reducing the time taken to reach the target concentration for Cd. If a planting density of 90 plants m^{-2} was used and two crops were harvested per year, the target could be reached in 163 years using Ganges. This calculation assumes that biomass production increases linearly with population density, based on data obtained for planting density of 50 plants m^{-2}. As the plants in Batch 2 were grown in the field for only 7 weeks, the production of three crops per year might be possible. However, the lower biomass and metal uptake values for plants from Batch 2 relative to Batch 1 suggest that growing two crops for a longer period may offer a more effective strategy. Indeed plant metal uptake reaches a maximum during the mid flowering stage (Angle *et al.*, 2001; Section 4.3.3) which requires longer than 7 weeks growth to achieve.

The cost of growing *T. caerulescens* on a commercial basis is estimated to be approximately £923 ha^{-1} for a single crop (Table 4.7), based on the assumption that direct drilling would be successful. This estimate is consistent with McGrath *et al.* (2000) who calculated the cost of producing a hypothetical phytoextraction crop based on the production of a single agricultural crop per year. However, in the present project, seven-week-old seedlings were hand-planted in the field, which is an extremely expensive practice if attempted commercially (Table 4.7; Angle *et al.*, 2001). The cost of using Ganges to reduce total soil Cd to 3 mg Cd kg^{-1} at the Stoke Bardolph field site was also estimated (Table 4.8). As the production of two crops each year would require planting seedlings, due to the relatively short growing season in the UK, this strategy would be very expensive. The cost of growing two crops of *T. caerulescens* per year for 163 years at a planting density of 90 plants m^{-2} is estimated

to be almost £6 m. Even if the target soil Cd concentration could be reached in 10 years, growing two crops of Ganges per year at a planting density of 90 plants m^{-2} would still cost over £360000 (Table 4.9). For this reason, alternative remediation strategies are likely to offer more favourable options. The most widely used soil remediation method in the UK is excavation and disposal (Wallace, 1998). To remediate the Stoke Bardolph field site using this method would cost between £120000 and £220000 ha^{-1} if the 0 - 20 cm soil profile was removed and a bulk density of 1.25 g cm^{-3} is assumed (Section 1.1.7). Consequently, based on Cd uptake by the Ganges plants grown in the field studies reported here, phytoextraction is unlikely to be cost-effective even if conducted over a prolonged period. However, remediation of agricultural soil using the excavation and burial technique would involve importing clean topsoil, thereby incurring additional expense.

The calculation of the remediation costs involved in using *T. caerulescens* clearly demonstrates the need to increase metal uptake by field grown plants. To reduce total soil Cd to 3 mg kg^{-1} within 10 years using a planting density of 90 plants m^{-2} and growing two crops each year would require a tissue Cd concentration of 3775 mg kg^{-1} (Table 4.9). To avoid the costly use of planting seedlings, a plant Cd concentration of over 7500 mg kg^{-1} would be required if a single crop was grown each year.

Table 4.5. Removal of soil Cd by two populations of *T. caerulescens* grown under different planting regimes and the number of years required to reduce total soil Cd below 3 mg kg^{-1} (maximum permitted Cd concentration prescribed by the 1989 Sludge Regulations; SI, 1989).

Crop	Regime	Density (plants m^{-2})	Concentration in shoot (mg kg^{-1})	Above-ground biomass (t ha^{-1})	Removal (kg ha^{-1})	Years to target [1]
Ganges	1 Crop	50	265	2.51	0.66	589
		90		4.51	1.20	327
	2 Crops	50		5.02	1.33	294
		90		9.03	2.40	163
Prayon	1 Crop	50	20.5	3.03	0.06	6312
		90		5.45	0.11	3509
	2 Crops	50		6.06	0.12	3156
		90		10.9	0.22	1754

[1] The calculation is based on soil contamination to a 20 cm depth, a bulk density of 1.25 g cm^{-3} and a constant soil → plant transfer factor throughout the remediation period.

Table 4.6. Removal of soil Zn y^{-1} by two populations of *T. caerulescens* and the number of years required to reduce total soil Zn below 300 mg kg^{-1} (maximum permitted Zn concentration prescribed by the 1989 Sludge Regulations for soil with a pH of 6 – 7; SI, 1989).

Crop	Regime	Density (plants m^{-2})	Concentration in shoot (mg kg^{-1})	Above-ground biomass (t ha^{-1})	Removal (kg ha^{-1})	Years to target [1]
Ganges	2 Crops	90	1009	9.03	9.12	1169
Prayon	2 Crops	90	844	10.9	9.20	1158

[1] The calculation is based on soil contamination to a 20 cm depth, a bulk density of 1.25 g cm^{-3} and a constant soil → plant transfer factor throughout the remediation period.

Table 4.7. Estimated cost of cultivating and processing a single crop of *T. caerulescens* for phytoextraction at a planting density of 50 plants m^{-2}, based on a typical agricultural site.

Cost Description	£ ha^{-1} (US $ ha^{-1})[1]
Seed Cost[2]	120 (170)
Based on cost of *T. arvense* from Herbi Seed Ltd; £120 kg^{-1} @ 800 – 1000 seed g^{-1}	
Farming Cost	451 (635)
Ploughing £40 ha^{-1} (average cost to farmer)[3]	
Rotovating £57 ha^{-1} (")[3]	
Drilling £40 ha^{-1} (based on maize crop)[3]	
Spraying £90 ha^{-1} (")[3]	
Weeding £102ha^{-1} (based on lettuce crop)[4]	
Harvest £122 ha^{-1} (based on cabbage crop)[3]	
Ashing[5]	320 (450)
Disposal Cost	
Landfill Cost [6] £54.05 t^{-1} for vegetable waste contaminated with heavy metals	32 (45)
Transport Cost[7] £200 $load^{-1}$ (19 t) transporting 50 miles	
Total Cost (direct drilling)	**923 (1300)**
Purchase cost for seedlings £15 for 1000 plants (based on lettuce crop)[8]	7500 (10565)
Planting cost £3.50 for 1000 plants (based on lettuce crop)[8]	1750 (2465)
Total Cost (seedlings)	**10173 (14330)**

[1] Currency conversion, £ sterling to US $ @ 1.4087 on 20/5/2002.

[2] (Herbi Seed Ltd. *pers. comm*).

[3] (Nix, 2001).

[4] (Lambkin and Measures, 2001).

[5] (McGrath *et al.*, 2000).

[6] Assumes that ashing will reduce biomass of 5 t ha^{-1} to 10 % original (Tom Diggle, Waste Recycling Group PLC. *pers. comm*).

[7] (Carl Wright Haulage. *pers. comm*).

[8] (Ian Gillott, *pers. comm*).

Table 4.8. Estimated cost of reducing total soil Cd below 3 mg kg^{-1} (maximum permitted concentration prescribed by the 1989 Sludge Regulations; SI, 1989) at the Stoke Bardolph field site using various planting regimes for *T. caerulescens* (Ganges population).

Regime	Density (plants m^{-2})	Years to target	Cost of production (£ y^{-1})	Overall cost of remediation (£ ha^{-1})[1]
1 crop	50	589	923	543647
	90	327	923	301821
2 crops	50	294	20346[1]	5,981724
	90	163	36622[1]	5,969386

[1] The costs of producing *T. caerulescens* crops with a planting density of either 50 or 90 plants m^{-2} by direct drilling are identical when only one crop is grown each year; the estimate assumes that 1 kg of seed containing approximately 1 m seed is sufficient to provide either planting density of an area of 1 ha. Other costs are based on standard agricultural costs per ha.

[2] Figure derived from Table 4.7.

Table 4.9. Required shoot Cd concentrations for reducing total soil Cd below 3 mg kg^{-1} (maximum permitted concentration prescribed by the 1989 Sludge Regulations; SI, 1989) at the Stoke Bardolph field site by *T. caerulescens* (Ganges population) and estimated cost.

Regime	Density (plants m^{-2})	Biomass (t ha^{-1})	Years to target	Required shoot concentration (mg kg^{-1})	Overall cost of remediation (£ ha^{-1}) [1]
1 crop	50	2.51	10	13575	9910
	90	4.51	10	7550	9910
2 crops	50	5.02	10	6785	204020
	90	9.03	10	3775	367230

[1] The costs of producing *T. caerulescens* crops with a planting density of either 50 or 90 plants m^{-2} by direct drilling are identical when only one crop is grown each year; the estimate assumes that 1 kg of seed containing approximately 1 m seed is sufficient to provide either planting density of an area of 1 ha. Other costs are based on standard agricultural costs per ha.

4.4 Conclusions

Despite substantial research interest in the use of hyperaccumulator plants for phytoextraction in recent years, relatively few field experiments have been conducted. This study evaluated the phytoextraction potential of two populations of *T. caerulescens,* Prayon and Ganges, at a field site heavily contaminated with heavy metals following the long-term disposal of sewage sludge. Plant biomass was consistent with other studies, as Ganges and Prayon produced 2.52 and 3.03 t ha^{-1} respectively. No significant differences in metal uptake were observed between the two populations except for Cd, which is also consistent with previous work. Ganges is known to possess specific Cd uptake traits. However, metal uptake by both populations was much lower than in previous studies, including the Woburn field experiment. The low soluble Cd levels associated with the substantial extractable phosphate and OM fractions, provides a plausible explanation for this effect. Consequently, the rate of Cd uptake by Ganges was either limited by the low concentration of Cd^{2+} in soil or by the rate solution Cd was replenished from the labile pool. Therefore, certain genetic manipulation techniques maybe unsuitable for enhancing Cd uptake. For example, over-expressing the genes responsible for producing transporter proteins might only increase the rate of metal uptake, but not the overall off-take of metals by plants. Increased root proliferation, thereby extending the soluble pool of metals accessible to roots, may offer a more suitable approach.

Although some reports have suggested that hyperaccumulation may protect plants from herbivory, plant growth in the present study was severely affected by flea beetle. Frequent application of insecticide was therefore necessary. Substantial plant mortality was also observed, possibly as a result of infestation by cutworm, although no visual evidence was obtained to support this theory.

The estimated number of years required to reduce soil Cd and Zn concentrations below legislative thresholds was large and closely dependent on planting density and cropping regime. If maximum metal uptake occurs during the mid-flowering stage, as observed in this study, the production of more than one crop per season would require the use of pre-germinated seedlings. As this practice is costly, the use of

hyperaccumulator plants to remediate the Stoke Bardolph field site may be no more cost-effective than other remediation techniques. The estimated time required to reduce soil Cd below 3 mg kg^{-1} was 163 years, using a planting density of 90 plants m^{-2} and two crops per year. The remediation costs were estimated to be approximately £ 6 m ha^{-1}. Although hyperaccumulator plants may provide a useful remediation strategy for marginally contaminated sites, further enhancement of metal uptake is required if the remediation of heavily contaminated sites or sites with low metal solubility, is to be practical.

CHAPTER 5: CHEMICALLY ENHANCED PHYTOEXTRACTION OF CADMIUM USING ARABLE CROPS

5.1 Introduction

The principles of chemically-enhanced phytoextraction were discussed in detail in Section 1.3.2 along with the main problems and concerns associated with this form of remediation. To date, very few field experiments have been conducted to test this technique and the validity of pot-based studies is restricted by several limitations. The aim of the work in this section was to test the viability of chemically-enhanced phytoextraction with field experiments. Specifically metal off-take for important, arable crops, following the application of a range of chemical treatment regimes, was compared and the associated risk of metal leaching was assessed.

Three arable crops widely grown in the UK were chosen as a follow on from pot experiments discussed in Chapter 3. *Beta vulgaris* was selected due to its response to applications of chloride when grown in pots, as significant increases in Cd uptake were observed following application of both $CaCl_2$ and KCl (Section 3.3). *Zea mays* and *Brassica napus* also performed well in pot experiments when biomass data was considered and are regularly grown at Stoke Bardolph; hence advice on husbandry under local conditions was available. *B. napus* is also an oil crop, therefore testing if phytoextraction could be carried out while producing a saleable oil product. The other arable crops grown in pot experiments (Chapter 3), *L. usitatissimum* and *B. juncea* were precluded from being grown at Stoke Bardolph due to a lack of suitable agricultural equipment.

Two chemicals were applied in an attempt to increase metal availability to the plant, EDTA and KCl. In addition, the broad-spectrum herbicide glyphosate was applied to the crop canopy as a potential 'inducing agent', in an attempt to increase transport of metal from roots to shoots (Section 1.3.2).

5.1.1 EDTA treatment

The chelate EDTA was included in the present field experiment mainly for comparison with previous work (Section 1.3.2) and pot trials conducted within this study (Chapter 3). The lower application rate chosen, 2 mmol EDTA kg^{-1}, reflects rates commonly used in previous studies (Section 1.3.2). One of the largest application rates referred to in the literature is 10 mmol EDTA kg^{-1} (Blaylock *et al.*, 1997). This was also included in the current study, firstly to investigate whether any increase in metal uptake following a large chelate dose was sufficient for successful phytoextraction, and secondly to establish if this treatment increased leaching of metal through the soil profile. The use of a larger EDTA dose, in line with rates used in pot experiments discussed in Chapter 3, i.e. 20 mmol kg^{-1}, was precluded due to excessive cost.

5.1.2 KCl treatment

Enhancement of Cd uptake by plants when grown in soils containing large chloride concentrations has been researched extensively and is discussed in Section 1.2.2. Chloride is also substantially cheaper than the more widely researched EDTA, and may be especially useful for selective plant uptake of Cd following chloro-complexation (Maxted *et al.*, 2001). The application rates of KCl used in the current study were selected for consistency with the pot experiments discussed in Chapter 3, i.e. 20 and 40 mmol kg^{-1}.

5.1.3 Glyphosate treatment

The degree of metal uptake observed by the arable crops grown in pot experiments discussed in Chapter 3 was insufficient for viable phytoextraction. Therefore, additional chemical treatments were considered. The possible use of herbicide to 'induce' the transport of metal from roots to shoots is reported by Ensley *et al.* (1999; Section 1.3.2). Although published information on this process is limited (Section 1.3.2), glyphosate is the main herbicide reported to increase metal uptake by plants (Ensley *et al.*, 1999; Mathis and Kayser, 2001), and was therefore selected for use in

this experiment. Glyphosate is also non-toxic to humans and wildlife (Baylis, 2000) and consequently, its use may be accepted in large-scale phytoextraction systems.

5.1.4 Measurement of metal leaching through the soil profile

The risk of metal leaching and groundwater contamination following application of solubilizing agents was reviewed in Section 1.3.2. Although previous experiments using soil columns have demonstrated significant metal leaching (Section 1.3.2), no extensive field experiments have been carried out to test this concept (Greman *et al.*, 2002). The use of biodegradable chelates in an effort to reduce metal leaching has also been tested previously, but has so far only produced a limited increase in metal uptake by plants (Section 1.3.2). Detailed soil profile sampling was therefore considered important, as significant leaching could form a major limitation to the commercial application of chemically-enhanced phytoextraction.

5.1.5 Experimental hypothesis

The experimental hypothesis being tested was that Cd uptake by *Z. mays*, *B. napus* and *B. vulgaris* would be significantly enhanced following application with either EDTA or KCl as single treatments or in combination with glyphosate; thus providing a realistic soil remediation strategy for soils treated with sewage sludge.

5.2 Materials and Methods

5.2.1 Crop establishment

A field trial was conducted at the Stoke Bardolph field site (Section 2.1) between March and September 2000. Two strips (12 x 140 m) were ploughed to a depth of 15 cm and power-harrowed to a depth of 10 cm. The first area was drilled with *B. napus* (cv. "Rebel") on 17 March 2000 at a sowing density of 120 seeds m^{-2}. Spraying of "Benozalox" (0.75 L ha^{-1}) containing Benazolin and Clopyralid (post emergence herbicide) and "Butisan-5" (0.75 L ha^{-1}) containing Metazachlor (residual herbicide) was carried out on 16 of May 2000, while "Fastac" (250 mL ha^{-1}) containing

alphacypermethrin (insecticide) was applied on 24 May 2000. Flowering of *B. napus* occurred during mid to late June 2000. The second area of prepared soil was drilled with *Z. mays* (cv. "Renard") at a sowing density of 4.2 seeds m^{-2} on 18 May 2000 and sprayed with "Jesgrim 500" (3. L ha^{-1}) containing Atrazine (triazine herbicide) on 24 May 2000. Flowering of *Z. mays* occurred during mid August 2000.

A total of forty plots (4 x 4 m) were established within each of the *B. napus* and *Z. mays* crops, arranged into four blocks each containing ten plots. A third area of soil (50 x 8 m) was drilled with *B. vulgaris* (cv. "Madison") on 19 April 2000 at a density of 10 seeds per m^{-2}. Spraying with Mn solution "Jett" (1.5 L ha^{-1}) and Fe solution "Ferrosol" (3 L ha^{-1}) took place at regular intervals during plant development. Plant growth of *B. vulgaris* was poor and only three blocks of three plots (2 x 2 m) became fully established.

5.2.2 Soil measurements

Three soil cores (0 - 20 cm; 4 cm diameter) collected from each plot were combined for measurement of soil pH. Soil samples from plots within each block were combined and mixed for soil analysis. Analyses were conducted for total and EDTA extractable Cd, Zn, Pb, Cu and Ni, $CaCl_2$ extractable Cd, bicarbonate extractable phosphate and loss on ignition (as described in Section 2.2). Soil cores were also collected for the 0 - 10, 10 - 20, 20 - 30 and 30 - 40 cm horizons from five locations within the *B. napus* and *Z. mays* crops for soil moisture content determinations (Section 2.2.3) immediately prior to application of the soil chemical treatments. Soil moisture was determined to establish the change induced by application of the soil chemical treatments.

5.2.3 Soil chemical treatments: EDTA and KCl

Sub-plots (1.5 x 1.5 m) for soil chemical treatment were established in the centre of all plots, thus providing a guard area of 1.25 m in the *B. napus* and *Z. mays* plots and 0.5 m in the *B. vulgaris* plots. 50 L of irrigation water were applied to each plot of *B. napus* and *Z. mays* to increase soil water content one or two days before chemical

treatment application. Chemical treatments were applied to the soil surface in the *B. napus* and *Z. mays* plots during flowering using irrigation piping (Plate 5.1). Treatments to each sub-plot consisted of 50 L solution (equivalent to 2.22 cm rainfall): 25 L chemical treatment followed by 25 L water. The water was added to improve treatment penetration within the plough layer. Treatments applied to *B. napus* included EDTA, applied at rates of 2 and 10 mmol EDTA kg^{-1}, KCl, applied at rates of 20 and 40 mmol KCl kg^{-1}, and control plots treated with 50 L water. Treatments were applied during the week commencing 20 June 2000 and harvest occurred three weeks after treatment. Chemical applications to *Z. mays* and *B. vulgaris* were applied during the week commencing 14 August 2000. Chemical treatments to *Z. mays* were as for *B. napus*. Soil chemical treatments for *B. vulgaris* only included KCl at the rates of 20 mmol KCl kg^{-1} and 40 mmol KCl kg^{-1}; EDTA applications were omitted due to poor growth and the limited plots available.

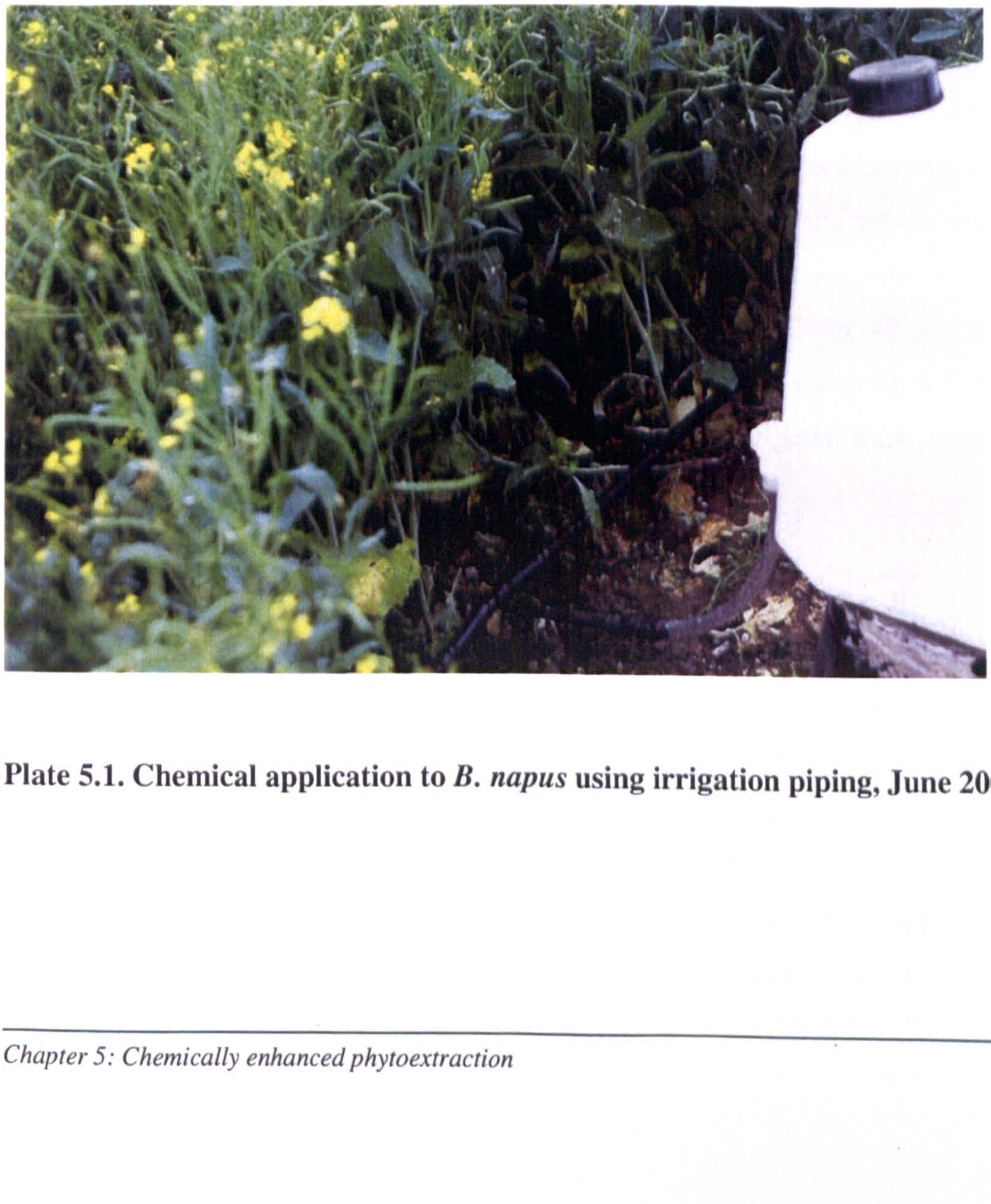


Plate 5.1. Chemical application to *B. napus* using irrigation piping, June 2000.

5.2.4 Glyphosate treatments

All soil chemical amendments to *Z. mays* were duplicated to accommodate the application of glyphosate (broad spectrum herbicide), i.e. control, KCl at both rates and EDTA at both rates were applied in the presence and absence of 480 g ha^{-1} of glyphosate containing isoproylamine salt of glyphosate and polyoxyethylene amine surfactant. The application rate of 480 g ha^{-1} represents a low dose of glyphosate when utilised in normal agricultural production and was used to minimise crop damage. Glyphosate was applied to the crop canopy 24 h after EDTA or KCl treatment using a Cooper Pegler Series 2000 CP15 sprayer.

5.2.5 Crop harvest

B. napus and *Z. mays* were harvested four weeks after treatment, while *B. vulgaris* was harvested two weeks after treatment. Shoot and foliar tissue was collected for all plants species, harvested ≈ 10 cm above ground level to minimise surface soil contamination with soil particles. In addition, *Z. mays* cobs were collected and analysed separately. Harvested material from individual plots was shredded using an AL-CO Kober Silent Power 3500 shredder, thoroughly mixed and sub-sampled. Fresh weights were determined for the complete sample and sub-samples to determine biomass. Sub-samples of plant material were dried at 80 ^{0}C for 48 h. Dried plant material was milled, 1 g was digested in concentrated HNO_3 and analysed for Cd, Zn and Cu as described in Section 2.2 using F-AAS. Digests were conducted in duplicate.

5.2.6 Soil profile measurements from Z. mays *crop*

In order to assess the extent of metal leaching over winter, soil cores (0 - 10, 10 - 20, 20 - 30, 30 - 40, 40 - 60 and 60 - 80 cm depth; 4 cm diameter) were collected in triplicate from selected treatments. Soil cores were collected from the *Z. mays* plots during March 2001, approximately seven months after soil chemical treatment application. Treatments selected included the control plots, low and high EDTA treatments (2 and 10 mmol kg^{-1}) and the high KCl treatment (40 mmol kg^{-1}).

Herbicide treated plots were not included as glyphosate was applied to the crop canopy and therefore would not influence soil metal leaching. Soil cores were collected from within the treated sub-plots. Total soil metal determination was conducted following methodology described in Section 2.2.9.

5.2.7 Statistical analysis

An analysis of variance (ANOVA) was undertaken using GenStat package (5th Edition) to establish treatment effects.

5.3 Results and Discussion

5.3.1 Soil characteristics

Soil characteristics are presented in Tables 5.1 and 5.2 for *B. napus* and *Z. mays* and 5.3 for *B. vulgaris*. Some soil measurements are missing for *B. vulgaris* due to an oversight. Characteristics are consistent with soil used in the pot experiments described in Section 3.3 with pH around 6, and a large organic matter (OM) fraction and extractable phosphate content. Mean total soil Cd concentrations under all three crop species ranged between 35 - 41 mg Cd kg^{-1}. Mean gravimetric soil moisture content, determined immediately prior to application of solubilizing agents, for the 0 - 10, 10 - 20, 20 - 30 and 30 - 40 cm horizons were 42.0 % (SE 0.86) for *B. napus* and 41.8 % (SE 1.89) for *Z. mays*. This value equates to approximately 55 % of field capacity prior to application of the soil chemical treatments. Application of solubilising agents consisting of 25 L solution followed by 25 L water, increased the soil moisture content to 66 % of field capacity in the 0 - 20 cm soil horizon.

Table 5.1. Total, EDTA extractable and $CaCl_2$ extractable metal content at the Stoke Bardolph field site used to grow *B. napus and Z. mays*. Values are means (n=4). Values in parenthesis are standard error of the mean.

		Total soil metal content (mg kg^{-1})	EDTA extractable metal content (mg kg^{-1})	$CaCl_2$ extractable metal content (mg kg^{-1})
B. napus	Cd	39.1 (1.45)	25.2 (1.23)	22.4 (1.10)
	Zn	1880 (164)	190 (80.1)	-
	Cu	819 (70.0)	557 (19.0)	-
	Pb	606 (47.8)	241 (6.19)	-
	Ni	409 (27.7)	360 (7.20)	-
Z. mays	Cd	35.3 (0.56)	22.9 (2.41)	18.6 (1.61)
	Zn	2280 (83.3)	957 (65.8)	-
	Cu	915 (37.6)	577 (26.9)	-
	Pb	662 (26.7)	253 (1.73)	-
	Ni	311 (10.5)	296 (18.60)	-

Table 5.2. Soil characteristics at the Stoke Bardolph field site used to grow *B. napus* and *Z. mays*. Values are means (n = 4). Values in parenthesis are standard error of the mean.

	pH	[a]LOI (%)	[b]Available P (mg kg^{-1})
B. napus	6.00 (0.05)	30.5 (1.76)	230 (27.2)
Z. mays	6.00 (0.02)	32.1 (1.37)	228 (17.1)

[a] LOI, Loss on ignition. [b] Bicarbonate-extractable phosphate.

Table 5.3. Total and $CaCl_2$ extractable metal content at the Stoke Bardolph field site used to grow *B. vulgaris*. Values are means (n=4).

	Total soil metal content (mg kg^{-1})	$CaCl_2$ extractable metal content (mg kg^{-1})
Cd	41.5	19.2
Zn	2056	-
Cu	590	-
Pb	605	-

5.3.2 Plant growth and uptake of heavy metals in the absence of treatment with solubilizing agents

Growth of both *B. napus* and *Z. mays* was generally good although *Z. mays* displayed some symptoms of Zn toxicity during early growth stages, with chlorosis clearly being visible on the young leaves. However, as the toxicity symptoms did not persist, it is assumed that they were related to the relatively shallow root system at the time and the spatial location of Zn contamination in the surface soil horizon. By contrast, the growth of *B. vulgaris* was very poor, with an overall ground cover of < 20 %. *B. vulgaris* displayed symptoms that were consistent with Mn deficiency as the older leaves developed interveinal yellow mottling and the leaf margins curled inwards (MAFF, 1980). High Zn availability is known to decrease Mn content of plants markedly. Furthermore, the critical toxicity levels for Zn in the leaves of crop plants are between 100 and 300 mg Zn kg^{-1} (dry weight basis; Marschner, 1995) and the mean leaf Zn content of *B. vulgaris* plants in the present study was 422 mg Zn kg^{-1}, providing a likely explanation for its poor growth. Fe deficiency can also be induced in the presence of higher tissue Zn concentrations, due to the similar ion radius of Zn^{2+} and Fe^{2+} (Woolhouse, 1983). Cd, Zn and Cu concentrations in the shoots of *B. napus*, *B vulgaris* and *Z. mays* are shown by Figures 5.1 - 5.5. Shoot Cu concentration in all species was c. 16 mg Cu kg^{-1}, well below the critical concentration required to induce

visible symptoms of Cu toxicity, which occur between 20 and 30 mg Cu kg^{-1} (Marschner, 1995).

5.3.3 Uptake of heavy metals following application of KCl

In general, Cd uptake for *B. vulgaris* and *Z. mays* increased following KCl application (Figs 5.2 and 5.3). However, this increase was only statistically significant ($P<0.05$) following the 40 mmol KCl kg^{-1} application to *B. vulgaris* compared to control plots. The tissue Cd concentration of control plants was 8.16 mg kg^{-1}, whereas the tissue Cd concentration following 40 mmol KCl kg^{-1} was 11.6 mg kg^{-1}. Shoot Cd concentration of *B. napus* declined following KCl application although this decline was not statistically significant. Observed tissue Cd concentrations were greater for pot experiments (Section 3.3.3) compared to field experiments. For example, the tissue Cd concentration in *B. vulgaris* following application of 50 mmol KCl kg^{-1} grown in pots was 29.2 compared to 11.6 mg kg^{-1} following application of 40 mmol KCl kg^{-1} grown in the field. The contrast in metal uptake by plants between pot and field grown crops is however reported in the literature (Section 1.3.2) and reflects the importance of undertaking field scale investigations.

5.3.4 Uptake of heavy metals following application of EDTA

Shoot Cd, Zn and Cu concentrations of *B. napus* following application of solubilizing agents are shown by Figure 5.1. There was a significant increase in shoot Cu concentrations following both 2 and 10 mmol EDTA kg^{-1} applications ($P<0.05$). A similar increase in shoot Cd and Zn concentrations was also observed following the same EDTA treatments, although in this case the increase was not statistically significant. The Cd, Zn and Cu concentrations of *Z. mays* shoots, following application of solubilizing agents, are shown in Figure 5.3. Although shoot Zn and Cu concentrations were greater following chemical treatment, this was not statistically significant. Shoot Cd concentration following both EDTA treatments did increase significantly compared to control plots ($P<0.01$). No significant increases in *Z. mays* cob or root metal concentrations were observed following the application of EDTA.

Despite increases in shoot metal concentrations following EDTA application, compared to control plots metal concentrations are still much lower than required for successful phytoextraction. The maximum shoot Cd concentration of *Z. mays* was 7.62 mg kg^{-1} following application with 10 mmol EDTA kg^{-1}. If a typical biomass of 12.5 t ha^{-1} is assumed this would produce Cd off-take of only 0.09 kg ha^{-1} removed $crop^{-1}$. A comparable figure for the Ganges population of *T. caerulescens* is 0.66 kg Cd ha^{-1} removed $crop^{-1}$, yet even at this level of Cd removal, several hundred years would be required to remediate the field site to within current maximum soil contamination values (Section 4.3).

Lombi *et al.* (2001b) demonstrated that metal concentrations in *Z. mays* roots were much greater than in the shoots following application of 2.7 mmol EDTA kg^{-1}. However, this result was derived from a pot trial, where root exposure to soil solution amended by the chelate application, is likely to be greater than for field grown crops. The current study shows that root metal concentrations following EDTA were lower than the shoot concentrations suggesting that the transfer of metal across root membranes was not adequately overcome. Metal uptake following application of EDTA from pot grown plants (Section 3.3) were greater than for the field grown crops as observed following KCl application (Section 5.3.3).

The results from the current study are consistent with the limited number of other field trials conducted (Section 1.3.2) and the application of chemically-enhanced phytoextraction to the field has so far been unsuccessful. Work reported by Blaylock and Elless (2001; Section 1.3.2) showed an apparently large removal of Pb from a contaminated site in the US using *B. juncea.* However this system is believed to involve the burial of a impermeable soil liner and the recycling of soil water, thus soil remediation is not achieved exclusively by phytoextraction. Alternative strategies have been tested, for example the specific targeting of a chelate treatment to the rhizosphere was tested by Kayser *et al.* (2000) who used a Shell fertiliser injector to inject the chelate treatment into the surface 20 cm of soil. However, this strategy only produced a slight increase in shoot metal concentrations (Section 1.3.2). It is likely that the extensive nature of the soil profile in field conditions, compared to the restrictive nature of a pot system, provides the greatest challenge for the scale up of

phytoextraction using arable crops. Targeting increases in soil solubility to root surfaces, whilst avoiding soil metal leaching will be an important hurdle to overcome. The associated risk of metal leaching following chelate treatment is discussed in Section 5.3.5.

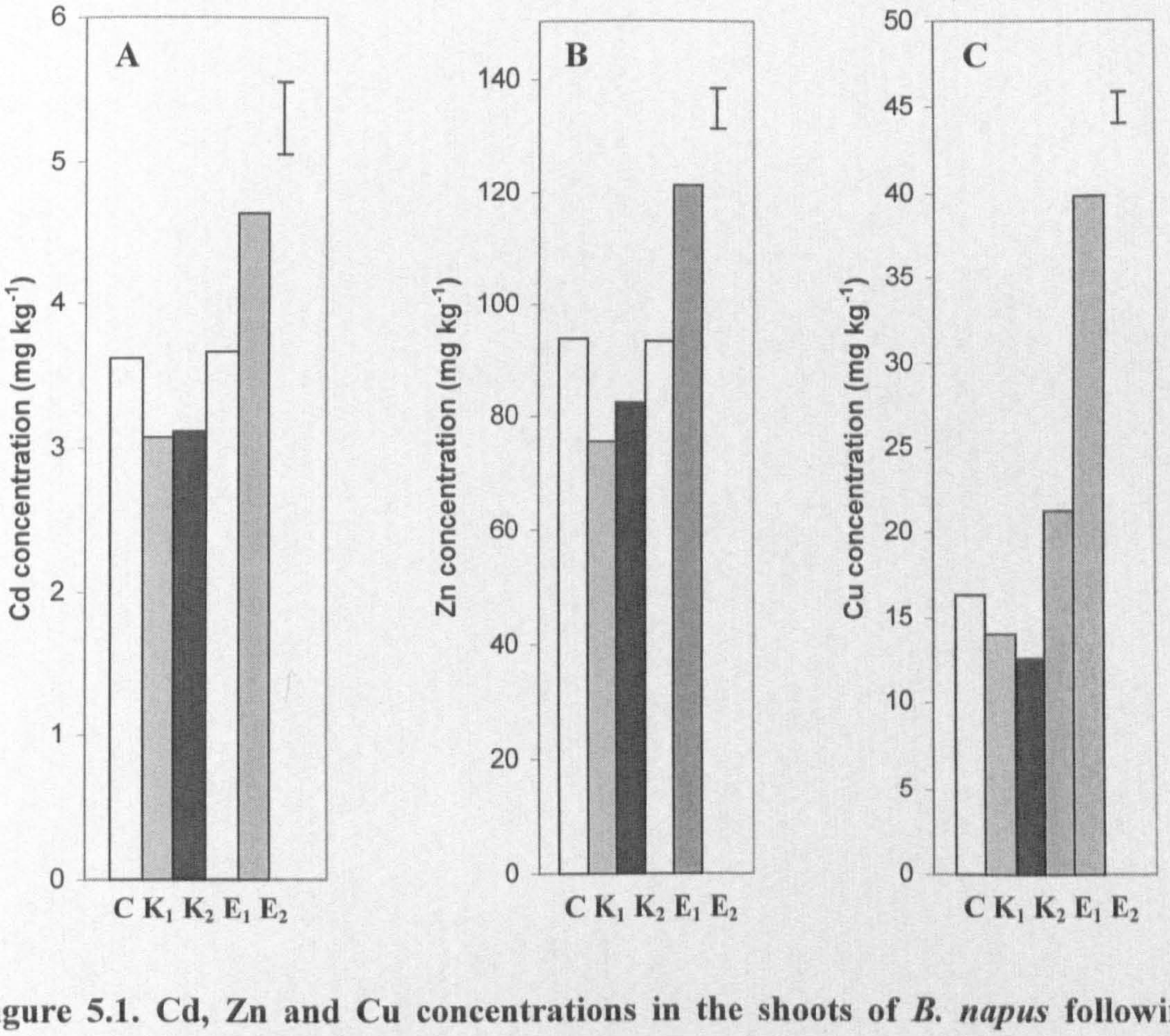


Figure 5.1. Cd, Zn and Cu concentrations in the shoots of *B. napus* following treatment with KCl and EDTA after 14 weeks growth. Treatments include: (Control (C); KCl 20 mmol kg^{-1} (K_1); KCl 40 mmol kg^{-1} (K^2); EDTA 2 mmol kg^{-1} (E^1) and EDTA 10 mmol kg^{-1} (E_2)). Plants were harvested after 18 weeks. Vertical bars show the SED values.

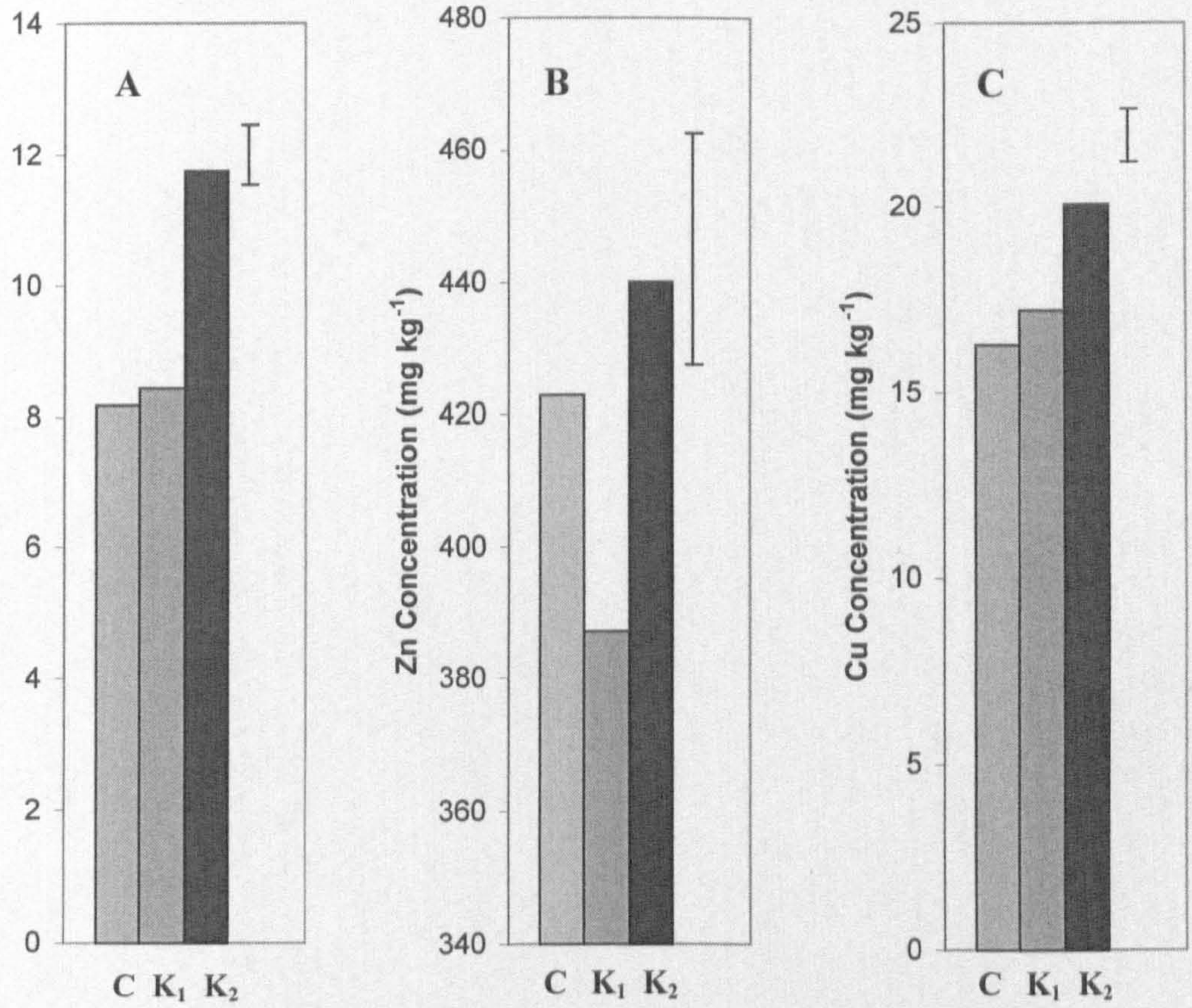


Figure 5.2. Cd, Zn and Cu concentrations in the shoots of *B. vulgaris* following treatment with KCl after 17 weeks growth. Treatments include: (Control (C); KCl 20 mmol kg^{-1} (K_1) and KCl 40 mmol kg^{-1} (K_2)). Plants were harvested after 19 weeks. Vertical bars show the SED values.

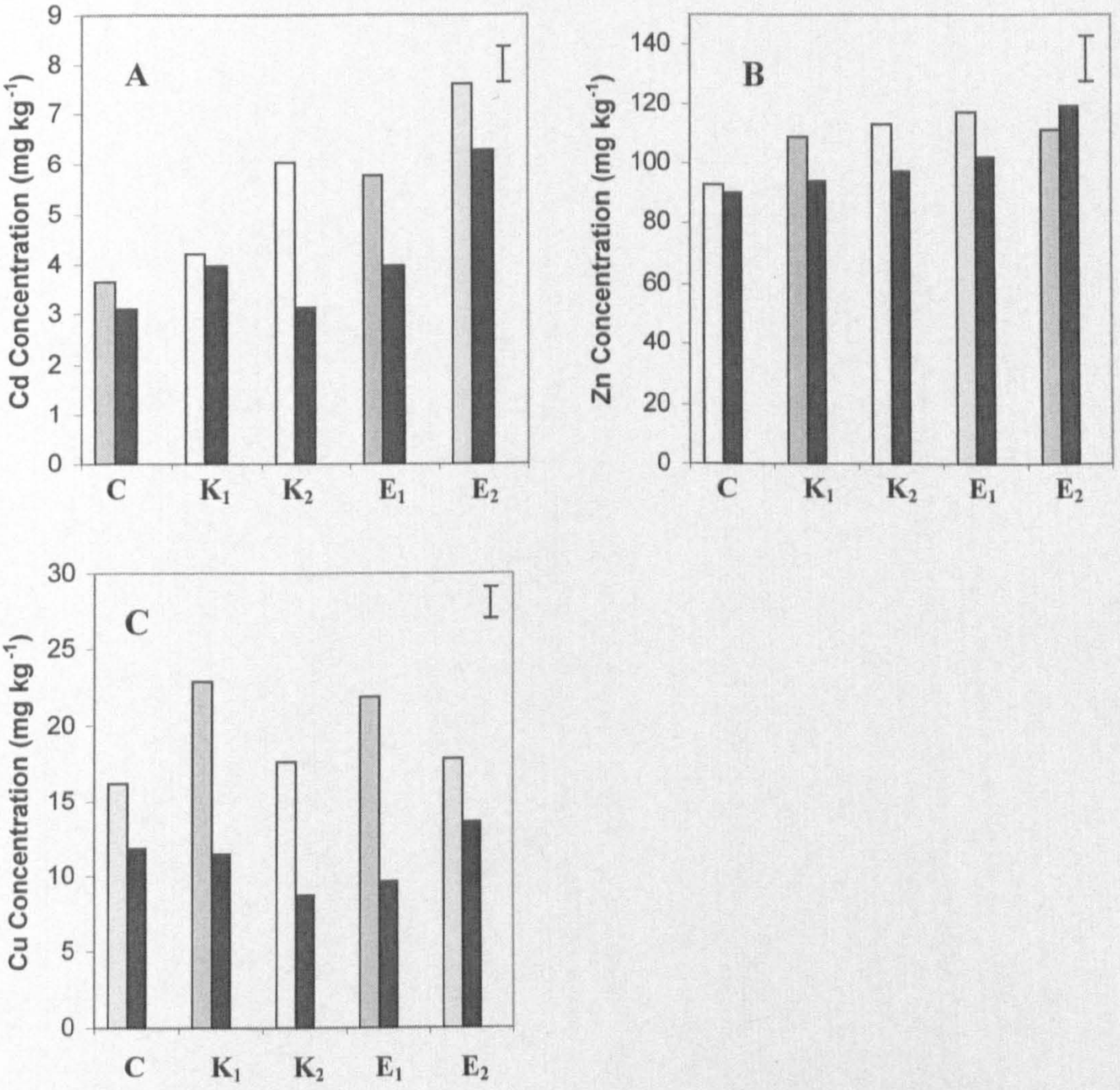


Figure 5.3. Cd, Zn and Cu concentrations in the shoots of *Z. mays* following treatment with KCl or EDTA after 13 weeks growth. Treatments include: (Control (C); KCl 20 mmol kg^{-1} (K_1); KCl 40 mmol kg^{-1} (K_2); EDTA 2 mmol kg^{-1} (E_1) and EDTA 10 mmol kg^{-1} (E_2); with glyphosate applied at a rate of 1L ha^{-1} (■), and without glyphosate (▨). Plants were harvested after 17 weeks. Vertical bars show the SED values.

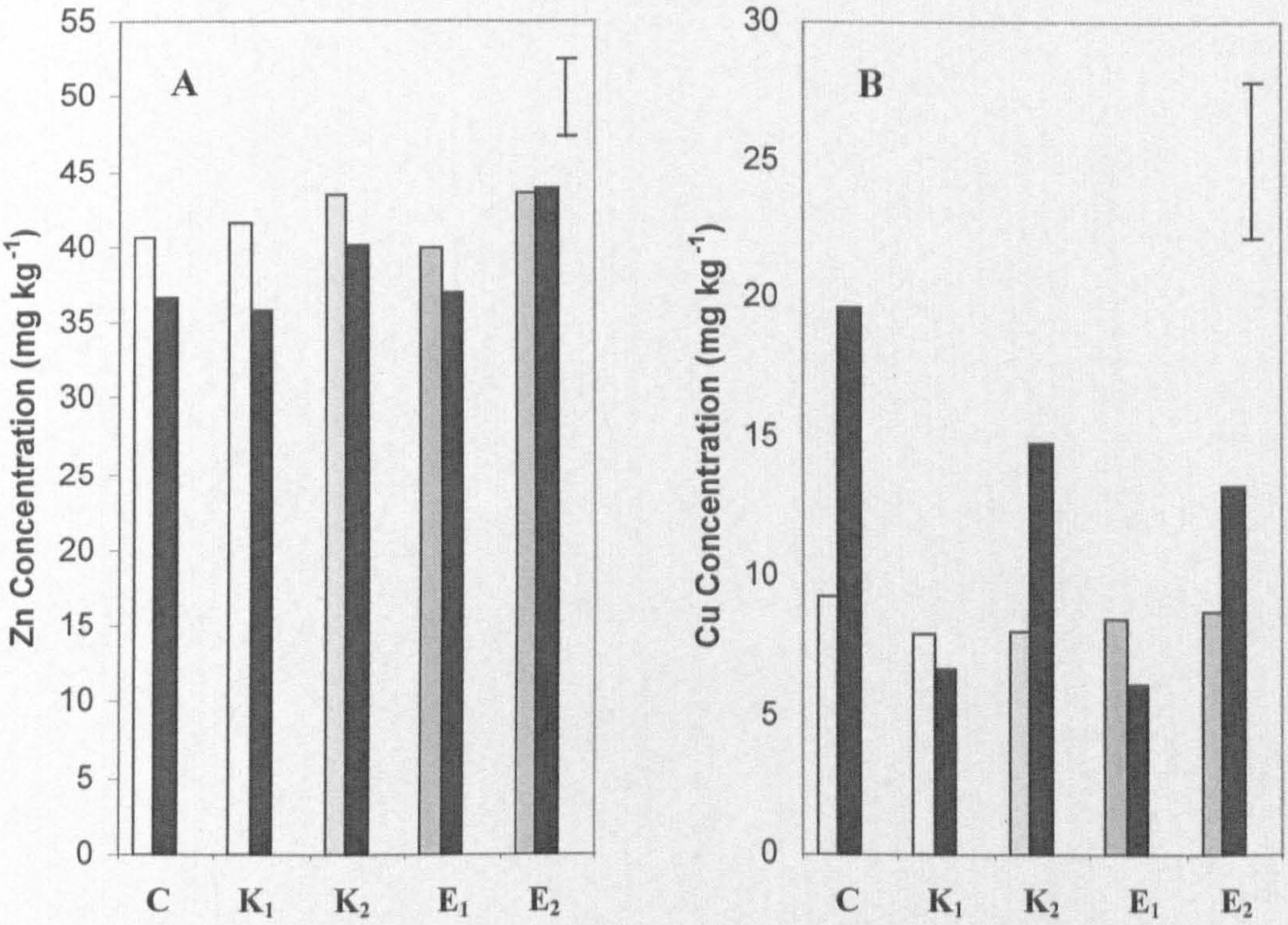


Figure 5.4. Zn and Cu concentrations in the cobs of *Z. mays* following treatment with KCl or EDTA after 13 weeks growth. Treatments include: (Control (C); KCl 20 mmol kg^{-1} (K_1); KCl 40 mmol kg^{-1} (K_2); EDTA 2 mmol kg^{-1} (E_1) and EDTA 10 mmol kg^{-1} (E_2); with glyphosate at the rate of 1L ha^{-1} (■) and without glyphosate (▨). Plants were harvested after 17 weeks. Vertical bars show the SED values.

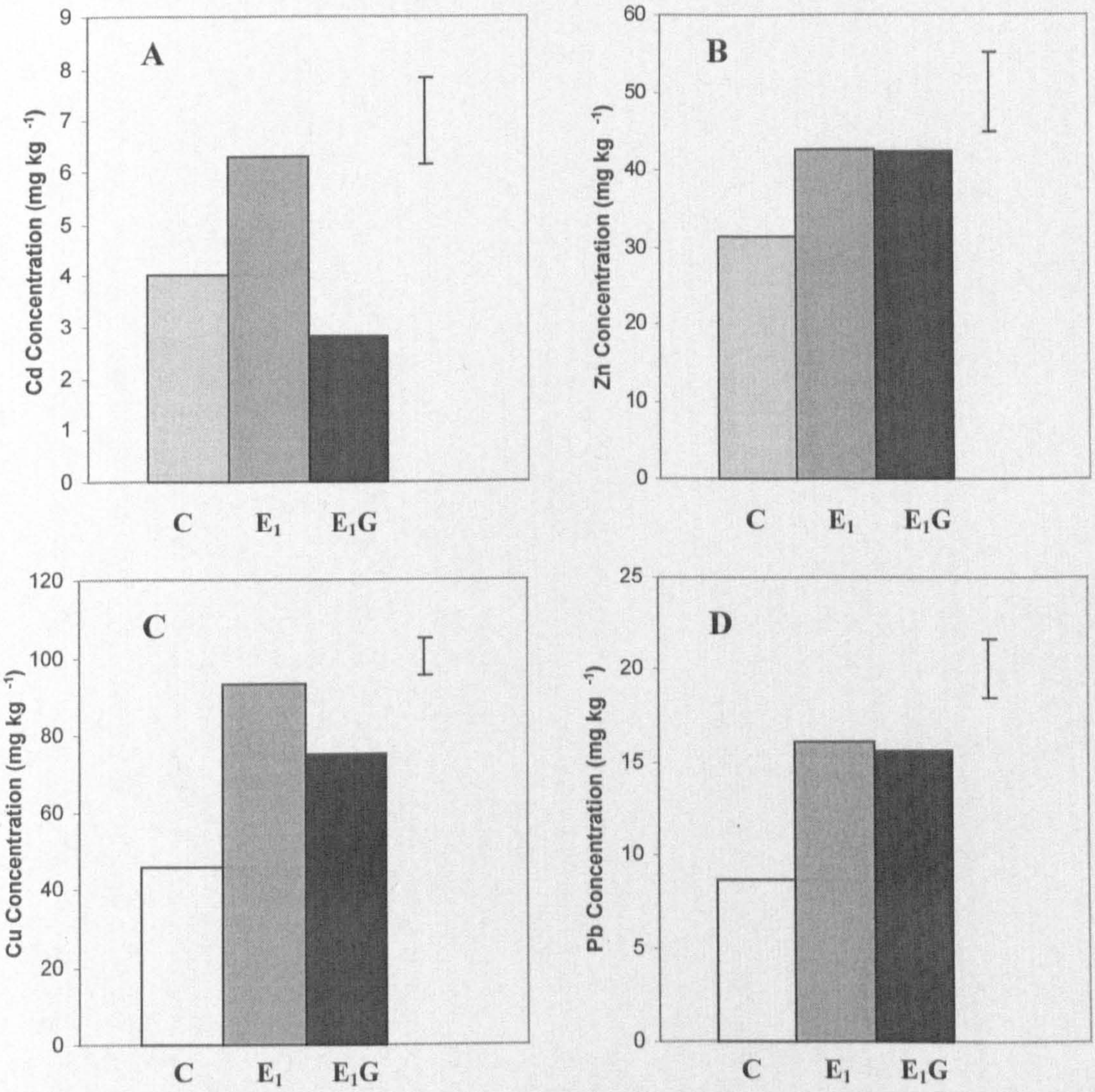


Figure 5.5. Cd, Zn, Cu and Pb concentrations in the roots of *Z. mays* following treatment with EDTA and /or glyphosate after 13 weeks growth. Treatments include: (Control (C); EDTA 10 mmol kg^{-1} (E_1) and EDTA 10 mmol kg^{-1} and glyphosate (1L ha^{-1}; E_1G). Plants were harvested after 17 weeks. Vertical bars show the SED values.

5.3.5 Uptake of heavy metals following application of glyphosate

Figure 5.3 shows the Cd, Zn and Cu concentrations in shoots of *Z. mays*, following application of solubilizing agents, in the presence and absence of glyphosate. Overall, there was a significant decrease in shoot Cd and Cu concentration following glyphosate application ($P<0.01$). Tissue Zn concentration was also lower following glyphosate application, although the reduction was not statistically significant. Treatment with glyphosate had no statistically significant effect on the Zn and Cu concentrations of *Z. mays* cobs (Fig. 5.4) although the Cu concentrations were higher in the cobs of glyphosate treated plants. Application of glyphosate also had no statistically significant effect on Cd, Zn, Cu and Pb concentrations in the roots of *Z. mays* (Fig. 5.5), although the value for Cd tended to be lower following glyphosate treatment.

Previous research to examine the potential role of herbicide to induce heavy metal uptake by plants is discussed in Section 1.3.2. Both Ensley *et al.* (1999) and Mathis and Kayser (2001) reported a significant increase in the concentrations of selected heavy metals in the shoots of *B. juncea*, following the combined application of chelates and glyphosate. However, both studies were pot-based, with chelate being applied after only three and four weeks of growth respectively. Glyphosate application in the current study was administered 24 h after the chelate treatment to *Z. mays* during flowering after approximately three months growth. In addition, the rate of glyphosate application was greater in both the earlier studies compared to the present work. Ensley *et al.* (1999) used application rates of 1.5, 5 and 10 % glyphosate solution, while Mathis and Kayser (2001) used rates of 0, 1 and 100 % glyphosate solution. In the current study an application rate of 480 g ha^{-1} was applied equating to 0.4 % glyphosate solution. It is likely that the action of glyphosate on young plants grown in pots is more rapid than on mature field-grown plants as the application of glyphosate to larger plants will effectively reduce the dose of herbicide solution, per unit tissue mass, thereby reducing its efficiency. Therefore the combination of greater application rates and the use of young pot grown plants may therefore explain the contrast in findings with the current work, in which the low application rate of 480 g ha^{-1} equating to a 0.4 % glyphosate solution, may have been insufficient to cause

significant root damage. The application of glyphosate is discussed in greater detail in Chapter 6.

5.3.6 Soil metal contamination profiles following application of solubilizing agents

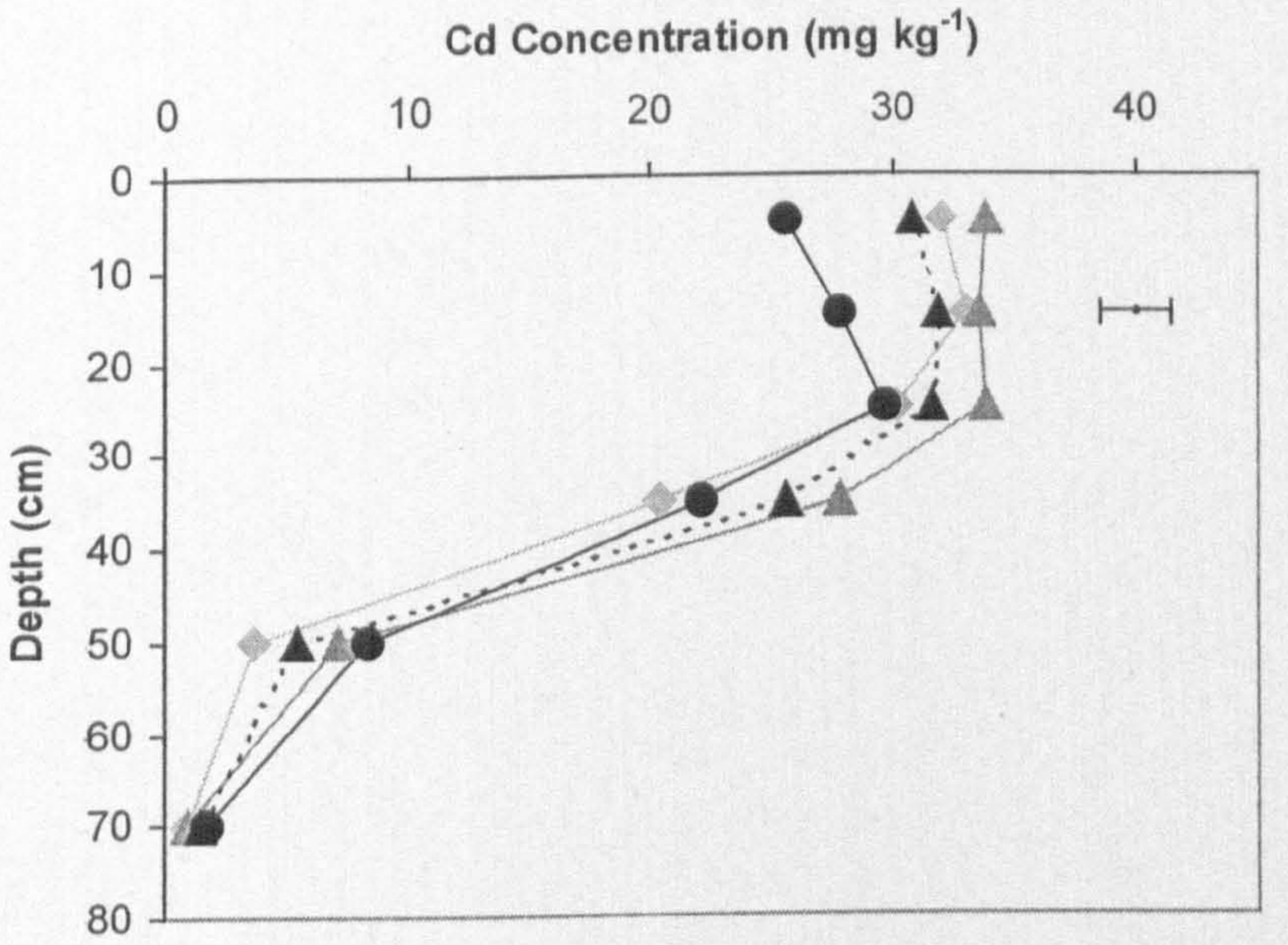
Total Cd, Zn, Cu, Pb and Ni concentrations within the 0 - 80 cm soil profile are shown in Figures 5.6 - 5.8. All elements exhibited a significant decline in total metal concentrations with depth ($P < 0.01$). This result reflects the nature of metal contamination typical of long-term sewage sludge disposal, as the soil metal content does not migrate below the level of incorporation (Section 1.2.2). Treatment with 10 mmol EDTA kg^{-1} significantly reduced total soil Ni concentration ($P<0.01$) relative to untreated plots in the 0 - 10, 10 - 20 and 20 - 30 cm horizons (Fig. 5.8). Similar reductions in total soil Cd, Zn and Cu concentrations were also apparent in the 0 - 10 and 10 - 20 cm horizons following treatment with 10 mmol kg^{-1} EDTA, although were not statistically significant.

However, these reductions in metal content in the surface horizons were accompanied by a tendency for values to be greater in the deeper horizons of plots treated with the higher EDTA concentrations, suggesting a downwards migration of metals had been induced. Although the apparent reductions in total metal content in the surface horizons were not statistically significant, they nevertheless reflect an important decline in heavy metal content within the upper part of the soil profile. For example, total Cd concentration in the 0 - 10 cm soil horizon following application of 10 mmol EDTA kg^{-1} was reduced from 32.0 to 25.5 mg kg^{-1}. Yet, the off-take of Cd by the *Z. mays* crop only accounted for c. 1 % of this reduction in soil total Cd. The lower EDTA concentration applied, 2 mmol kg^{-1}, which is perhaps a more realistic treatment concentration, both economically and environmentally (Section 1.3.2), reduced soil total Cd from 32.0 to 30.8 mg kg^{-1} in the 0 - 10 cm horizon. Yet, Cd off-take by *Z. mays* only accounted for 4.6 % of this Cd loss from the surface soil horizon.

These results are consistent with leaching experiments using soil columns, in which reduction in total soil metal concentrations occurred from the surface soil following application of EDTA (Abruzzese *et al.*, 2001; Barona *et al.*, 2001; Greman *et al.*,

2001; Section 1.3.2). However, work presented in the current study is the first demonstration of leaching from direct measurements taken from an extensive phytoextraction experiment.

Recent work by Greman *et al.* (2002) compared the use of EDTA with the chelate ethylenediaminedissucinate (EDDS). EDDS is described as environmentally safe, as it is much more readily biodegradable than EDTA (Bolton *et al.*, 1993; Greman *et al* 2002). However, the work by Greman *et al.* (2002) was based on pot trials and therefore does not provide adequate evidence that EDDS is suited to field scale phytoextraction. Previous attempts to use biodegradable chelates such as NTA have produced only limited plant metal uptake from field conditions (Kayser *et al.*, 2000). It is likely that without some additional mechanism, the use of biodegradable chelates alone will not produce a viable phytoextraction system using arable crops.



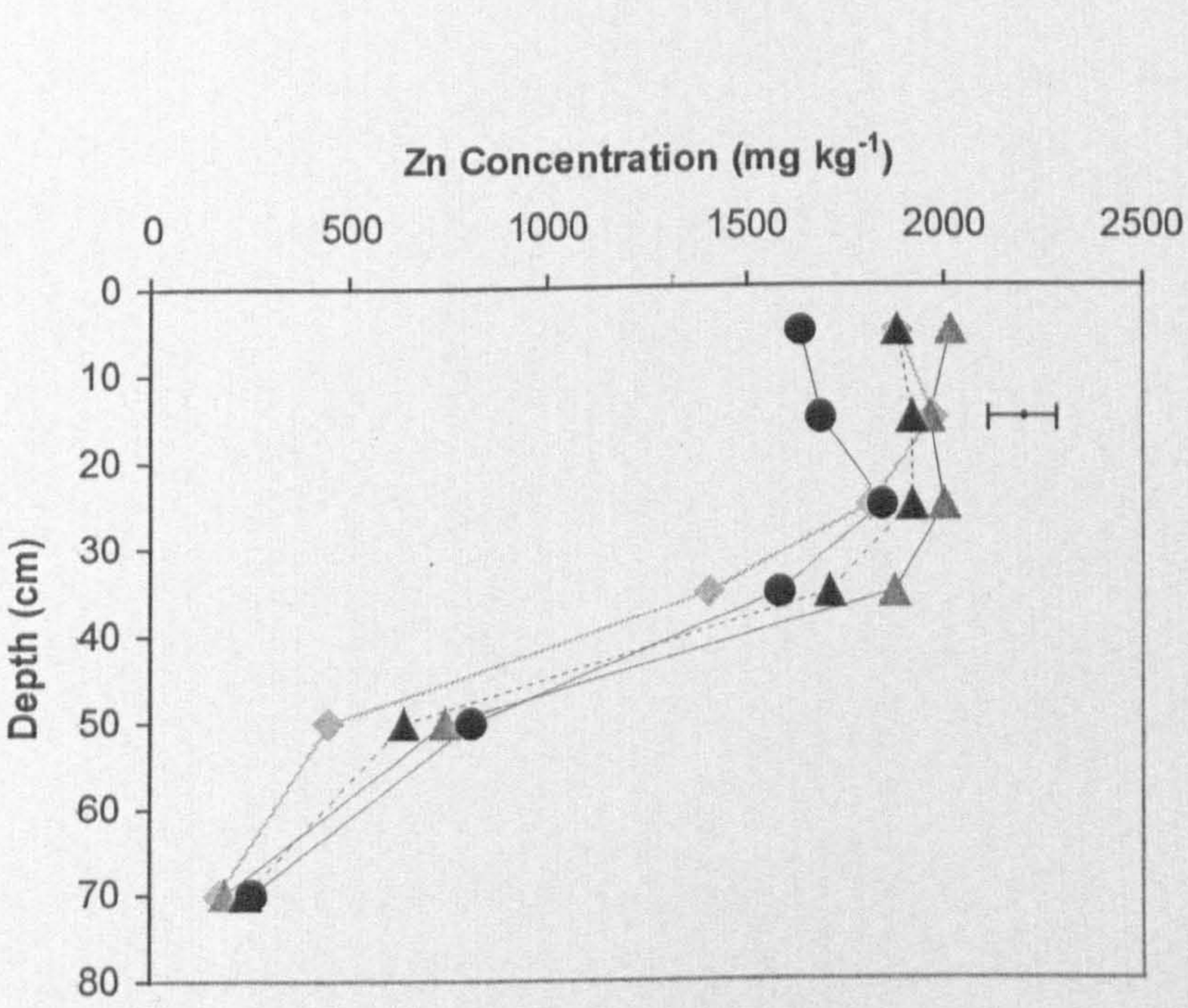
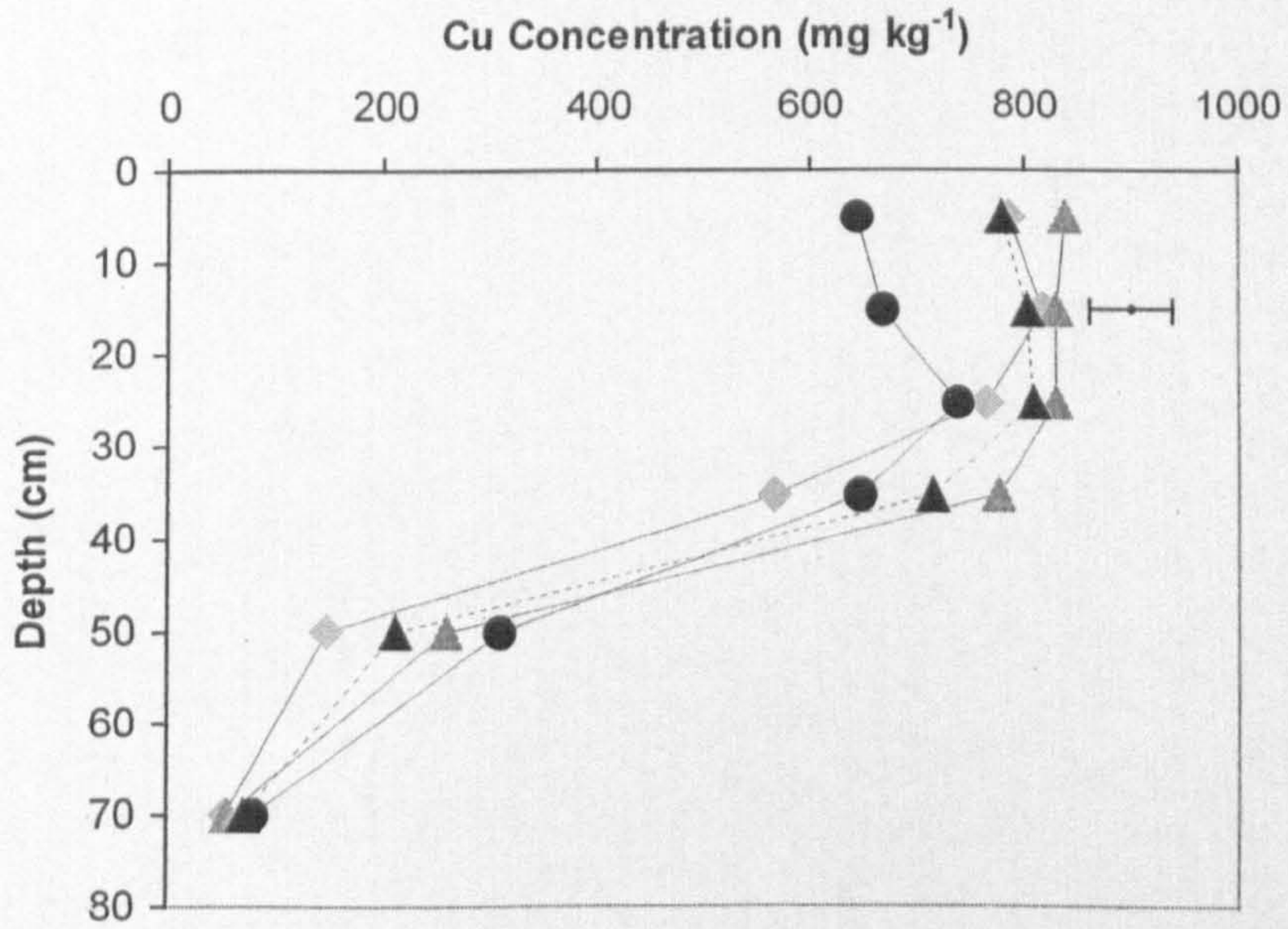


Figure 5.6. Total Cd and Zn concentrations in the 0 - 10, 10 - 20, 20 - 30, 30 - 40, 40 - 60 and 60 - 80 cm horizons of the soil profile approximately six months after harvesting *Z. mays*. EDTA or KCl were applied after 13 weeks of growth, and the plants were harvested after 17 weeks of growth. Treatments include: (Control (◆); EDTA 2 mmol kg^{-1} (▲); EDTA 10 mmol kg^{-1} (●) and KCl 40 mmol kg^{-1} (▲). Horizontal bars show the SED values.



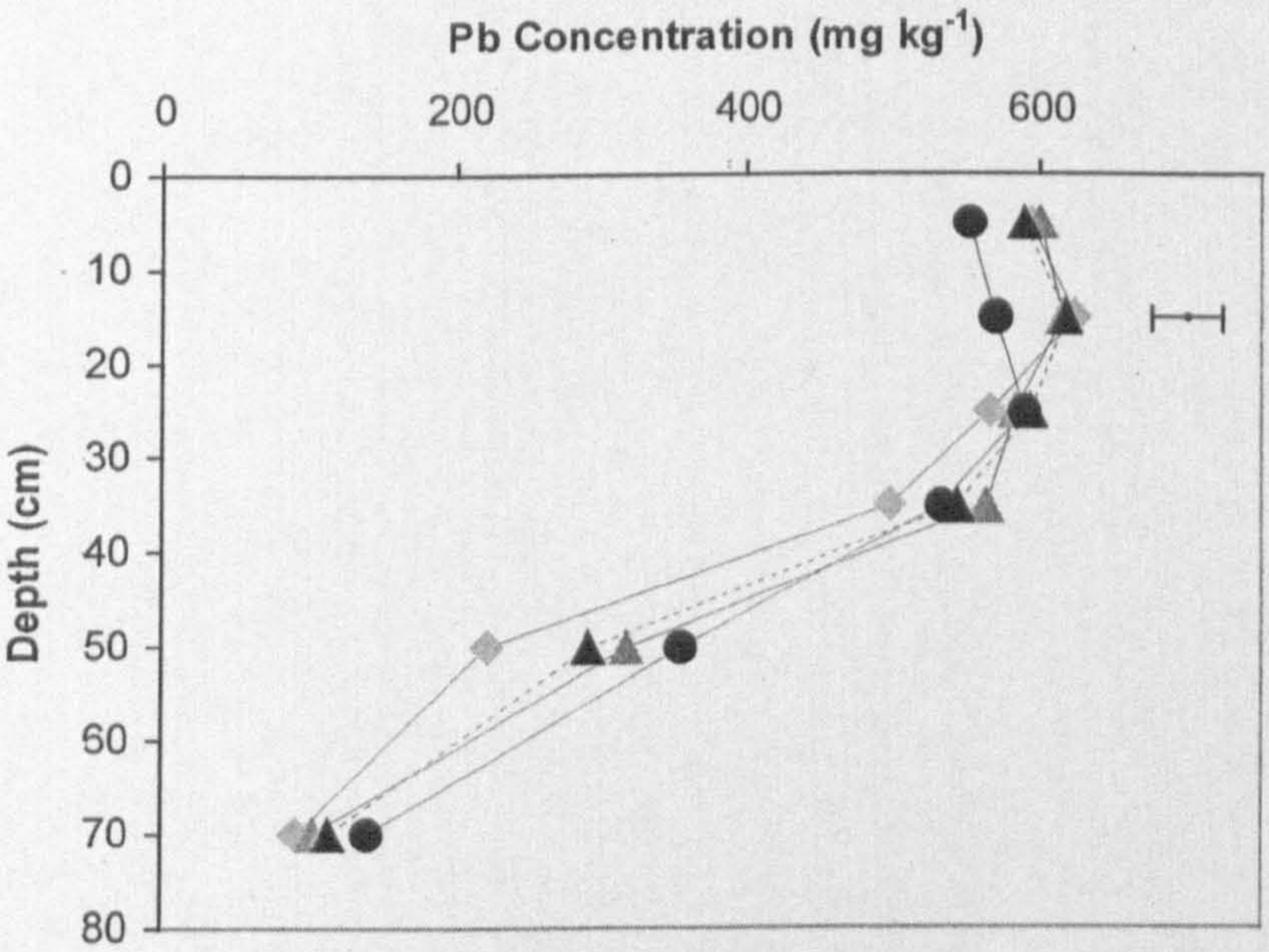


Figure 5.7. Total Cu and Pb concentrations in the 0 - 10, 10 - 20, 20 - 30, 30 - 40, 40 - 60 and 60 - 80 cm horizons of the soil profile approximately six months after harvesting *Z. mays*. EDTA or KCl were applied after 13 weeks of growth, and the plants were harvested after 17 weeks of growth. Treatments include: (Control (◆); EDTA 2 mmol kg^{-1} (▲); EDTA 10 mmol kg^{-1} (●) and KCl 40 mmol kg^{-1} (▲). Horizontal bars show the SED values.

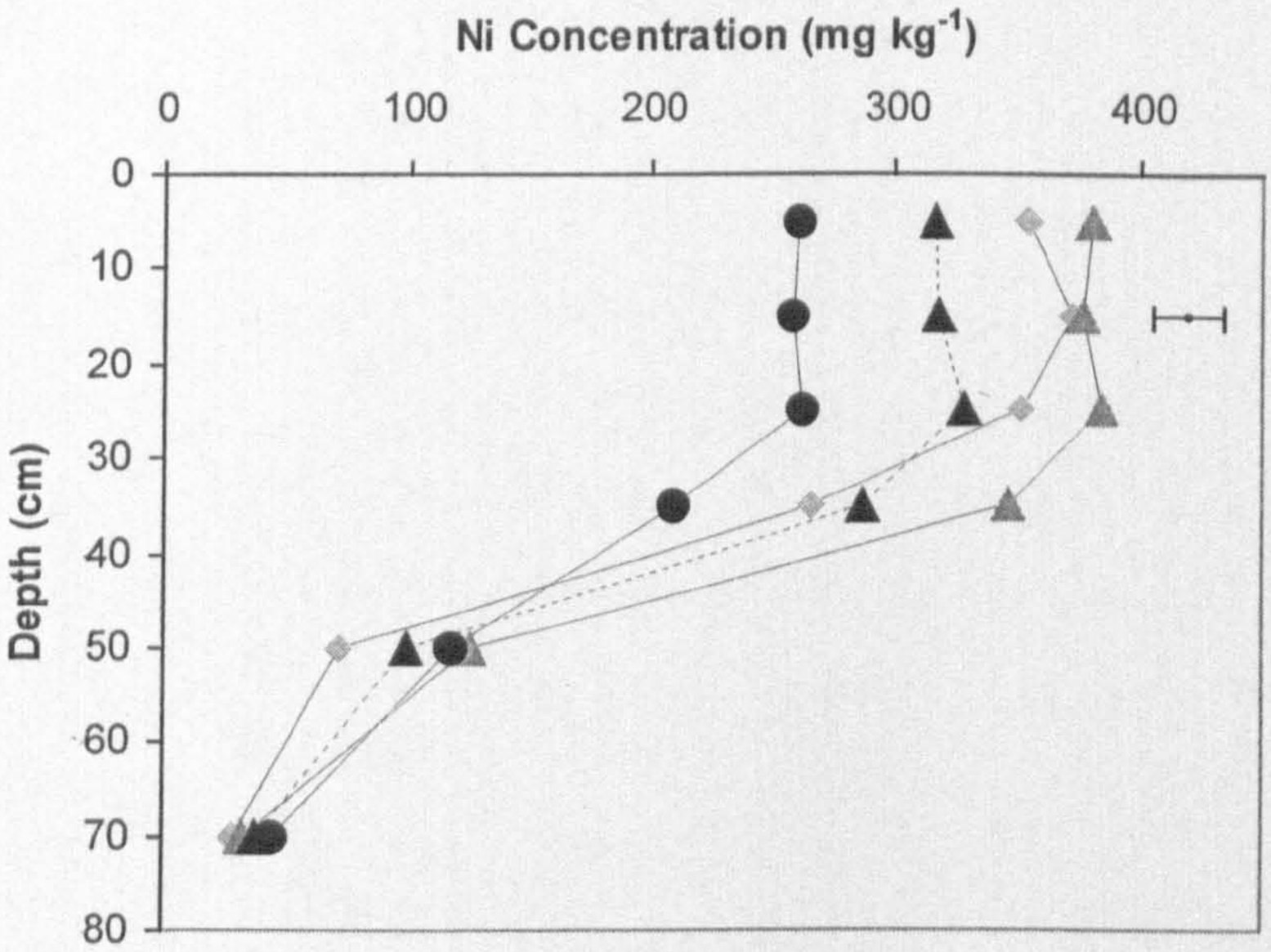


Figure 5.8. Total Ni concentrations in the 0 - 10, 10 - 20, 20 - 30, 30 - 40, 40 - 60 and 60 - 80 cm horizons of the soil profile approximately six months after harvesting *Z. mays*. EDTA or KCl were applied after 13 weeks of growth, and the plants were harvested after 17 weeks of growth. Treatments include: (Control (◆); EDTA 2 mmol kg^{-1} (▲); EDTA 10 mmol kg^{-1} (●) and KCl 40 mmol kg^{-1} (▲). Horizontal bars show the SED values.

5.3.7 Estimate for phytoextraction of Cd and Zn from the Stoke Bardolph field site using Z. mays

The estimated times required to reduce soil total Cd and Zn concentrations to the maximum permitted by the 1989 Sludge Regulations (SI, 1989) are shown by Tables 5.7 and 5.8. During these calculations it was assumed that a constant soil → plant transfer factor would exist throughout the remediation period; it was also assumed that the labile phase would be replenished throughout the same period. This calculation is explained in detail in Section 4.3.6. The various KCl and EDTA treatments applied had very little effect on tissue Zn concentration in *Z. mays*. For this reason, only the treatment producing the greatest tissue Zn concentration is presented (Table 5.8). The values obtained make it clear that very long time periods would be required to remediate the soil, and that the current system is not realistic. Similar findings were reported by Kayser *et al.* (2000), Lombi *et al.* (2001b) and Wenger *et al.* (2002). Wenger *et al.* (2002) calculated the remediation time required to remove soil Zn for a field site located in Switzerland. This work amended the soil Zn concentration by adding Zn in sulphate form and may therefore have not adequately represented realistic field conditions. However, several decades were still reported to be necessary to reduce the soil Zn concentration to within legislative thresholds.

Although very few field experiments testing phytoextraction have been conducted, previous attempts to estimate the cost of different phytoextraction strategies are even more limited. The estimated cost of producing *Z. mays* for phytoextraction is shown in Table 5.9 and indicates that the cost of EDTA contributes the largest single factor. Even a single application of 2 mmol EDTA kg^{-1} would cost around £30000 ha^{-1}. KCl is much cheaper than EDTA and mureate of potash only costs around £0.11 kg^{-1}. However, scientific grade KCl from Fisher Scientific costing £2.70 kg^{-1} was used in the present study, and in any case, metal uptake following KCl treatment was far below that required for a viable soil remediation system. The overall estimated costs for reducing soil total Cd below 3 mg kg^{-1} are shown in Table 5.10. Remediation of the Stoke Bardolph field site using an application of 10 mmol EDTA kg^{-1} is estimated to cost c. £340 m. To reduce the soil Cd concentration to 3 mg kg^{-1} within ten years would require a shoot Cd concentration of 1545 mg kg^{-1}, approximately 200 times the

existing maximum concentration obtained by this study, following application of 10 mmol EDTA kg^{-1}.

Table 5.4. Cd removal by a *Z. mays* crops grown under various treatment regimes, and the number of years required to reduce total soil Cd below 3 mg kg^{-1} (maximum permitted concentration prescribed by the 1989 Sludge Regulations; SI, 1989).

Treatment Regime[1]	Concentration in shoot (mg kg^{-1})	Biomass (t ha^{-1} y^{-1})[2]	Removal (kg ha^{-1} y^{-1})	Years to target[3]
20 mmol KCl kg^{-1}	4.20	12.5	0.05	4143
40 mmol KCl kg^{-1}	6.40	12.5	0.08	2719
2 mmol EDTA kg^{-1}	5.77	12.5	0.07	3016
10 mmol EDTA kg^{-1}	7.62	12.5	0.09	2283

[1] No herbicides were applied in any treatment.

[2] Biomass figures are means for *Z. mays* grown at the field site (John Jackson. *pers. Comm.*).

[3] This calculation is based on soil contamination to a 20 cm depth, a bulk density of 1.25 g cm^{-3} and a constant soil → plant transfer factor.

Table 5.5. Zn removal by a *Z. mays* crop treated with 2 mmol EDTA kg^{-1} 13 weeks after planting and the number of years required to reduce total soil Zn below 300 mg kg^{-1} (maximum permitted concentration prescribed by the 1989 Sludge Regulations; SI, 1989) for soil with pII 6 – 7).

Treatment Regime[1]	Concentration in shoot (mg kg^{-1})	Biomass (t ha^{-1} y^{-1})[2]	Removal (kg ha^{-1} y^{-1})	Years to target[3]
2 mmol EDTA kg^{-1}	117	12.5	1.46	7904

[1] No herbicides were applied.

[2] Biomass figures are means for *Z. mays* grown at the field site (John Jackson. *pers. Comm.*).[3] This calculation is based on soil contamination to a 20 cm depth, a bulk density of 1.25 g cm^{-3} and a constant soil → plant transfer factor.

Table 5.6. Estimated cost of producing a single *Z. mays* crop for phytoextraction.

Cost Description	£ ha^{-1} (US $ ha^{-1})[1]
Seed Cost[2]	120 (170)
Based on planting density of 4.2 plants m^2	
Farming Cost[2]	307 (432)
Ploughing £40	
Rotavating £57	
Drilling £40	
Spraying £90	
Harvest £80	
Ashing[3]	320 (450)
Disposal Cost[4]	80 (112)
Landfill cost £54.05 t^{-1} for vegetable waste contaminated with heavy metals [5]	
Transport Cost [6] £200 load^{-1} (19 t) transporting 50 miles	
Total Cost (not including solubilising agents)	**847 (1193)**
Total Cost (including solubilising agents[7]**)**	
KCl @ 20 mmol kg^{-1} (£410.0 ha^{-1})	1256 (1770)
KCl @ 40 mmol kg^{-1} (£820.0 ha^{-1})	1666 (2346)
EDTA @ 2 mmol kg^{-1} (£29 712 ha^{-1})	30 559 (43048)
EDTA @ 10 mmol kg^{-1} (£148 608 ha^{-1})	149 455 (210537)
Additional Cost[8]	
Irrigation pump and piping ¼ ha^{-1}	7000 (9860)

[1] Currency conversion, £ sterling to US $ @ 1.4087 on 20/5/2002.

[2] Figures for maize crop (Nix, 2001).

[3] (McGrath *et al.*, 2000).

[4] Assumes that ashing will reduce biomass to 10 % original.

[5] (Tom Diggle, Waste Recycling Group PLC. *pers. comm*).

[6] (Carl Wright Haulage. *pers. comm*).

[7] (EDTA cost £16 kg^{-1} for minimum of 50 kg, from Fisher Scientific, 2001. KCl cost £0.11 kg^{-1} (Mureate of potash).

[8] Based on piping at 1.5 m spacing at £4 m^{-2} (Nix, 2001).

Table 5.7. Estimated cost of reducing total soil Cd to 3 mg kg^{-1} (maximum permitted concentration prescribed by the 1989 Sludge Regulations; SI, 1989) at the Stoke Bardolph field site using *Z. mays* grown under various treatment regimes.

Treatment Regime[1]	Years to target[3]	Cost of Production (£ ha^{-1} y^{-1})	Overall cost of remediation (£ m ha^{-1})
20 mmol KCl kg^{-1}	4143	1256	5.2
40 mmol KCl kg^{-1}	2719	1666	4.5
2 mmol EDTA kg^{-1}	3016	30559	92.1
10 mmol EDTA kg^{-1}	2283	149455	341.2

5.4 Conclusions

The use of chemically-enhanced phytoextraction using large biomass arable crops is often presented as an alternative to using hyperaccumulator plants. However, as is the case with hyperaccumulator plants, relatively few field experiments have been conducted. This study aimed to test the viability of three arable crops, for successful phytoextraction of the Stoke Bardolph field site, using chemical enhancement of metal uptake.

KCl and EDTA were used at two rates of application, in an effort to enhance metal solubility. Some significant increases in plant metal uptake were observed, for example Cd uptake by *Z. mays* following the application of 10 mmol EDTA kg^{-1}. However, overall plant metal concentrations were well below those required for successful remediation, as reported by the limited number of previous field experiments. The use of glyphosate as a potential inducing agent was also tested, but did not significantly increase plant metal uptake. The tissue Cd concentration of *Z. mays* roots was lower than the shoot concentration, indicating only limited transfer of Cd across root membranes.

Intensive sampling of the soil profile several months after chemical treatment showed the downward migration of metal in the soil profile. For example, the Cd concentration in the 0 - 10 cm profile was reduced from 32.0 to 25.5 mg kg^{-1} following application of 10 mmol EDTA kg^{-1}. Only around 1 % of this reduction could be accounted for by *Z. mays* Cd off-take.

The time required to remediate the field site to within legislative limits was calculated to be very large. For example, over 2000 years would be required to reduce Cd to below 3 mg kg^{-1} and over 7000 years to reduce Zn to below 300 mg kg^{-1}. Phytoextraction costs were also calculated to be inhibitive, especially for EDTA at approximately £30000 for a single treatment of 2 mmol kg^{-1}. To reduce soil Cd to below 3 mg kg^{-1} in 10 years would require a shoot Cd concentration approximately 200 times greater than the maximum observed concentration in this study. Clearly this method is not suited to the remediation of the Stoke Bardolph field site without further enhancement of metal uptake by plants, and by controlling the degree of metal leaching through the soil profile.

CHAPTER 6: ENHANCING UPTAKE OF CADMIUM BY *Z. MAYS* USING COMBINED APPLICATIONS OF HERBICIDE, HYDROCHLORIC ACID AND CHELATES

6.1 Introduction

In chapter 5 it was demonstrated that the application of EDTA alone is insufficient to induce the substantial uptake of Cd and Zn by *Z. mays* plants, required for successful phytoextraction, based on field data at the Stoke Bardolph field site. In fact, less than 5 % of the observed reduction in topsoil Cd, following application of EDTA, could be accounted for by uptake of Cd by the plants. Application of EDTA to the soil surface effectively resulted in soil Cd and Zn being redistributed down the profile, below the plough layer, by leaching. Moreover, the application of glyphosate at a rate of 480 g ha^{-1}, in combination with EDTA, did not significantly increase uptake of metals by plants. Thus, if phytoextraction using high biomass arable crops is to be successful, additional interventions may be required to enhance plant uptake of metals.

Genetic modification has been suggested as a possible method for increasing the uptake of heavy metals by plants (Baker *et al.*, 1994; Guerinot, 2000; Karenlampi *et al.*, 2000; Williams *et al.*, 2000; Lasat, 2002). However, public acceptance of GM crops is still far from certain (Baker and Whiting, 2002). Using conventional crops, an alternative strategy involves the use of combined chemical treatments, for example, the addition of chelates such as EDTA and an acid solution (Blaylock *et al.*, 1997; Elless and Blaylock 2000). Use of combined soil chemical treatments aims to increase the solubility of metals and enhance the transfer of metal ions across root membranes. Furthermore, the use of herbicide has been suggested as a possible agent for increasing the transport of metals between roots and shoots (Section 5.1.3). A pot experiment was therefore conducted to examine the influence of herbicide and acid on the uptake of Cd and Zn by plants, when applied in combination with EDTA.

6.1.1 Acid treatments

Increases in metal solubility following reductions in soil pH have been widely reported (Section 1.2). However, effecting a significant change in soil may require substantial applications of mineral acid. For example, an estimated buffer capacity for organic soil can be calculated from Rowell (1997) who suggests that 10 t $CaCO_3$ ha^{-1} is required to effect a 1 pH unit change. This equates to 80 mmol H^+ kg^{-1} pH^{-1} and so a 5 mmol kg^{-1} treatment to organic soil would reduce soil pH by < 0.1 pH units. However, some evidence suggests that the application of even a dilute acid treatment in combination with chelates may induce a significant increase in metal uptake by plants. For example, Ensley *et al.* (1999) suggested that slight reductions in soil pH may still have additive effects to metal uptake by plants when combined with chelate applications. Blaylock *et al.* (1997) demonstrated an increase in the concentration of Pb within the shoots of *B. juncea* from 28 mg kg^{-1} to 785 mg kg^{-1}, following the application of EDTA at 5 mmol kg^{-1}. The combined application of EDTA and acetic acid, also applied at a rate of 5 mmol kg^{-1}, increased the concentrations of Pb within the plant tissue to 1471 mg kg^{-1}.

Thus, the type of mineral acid applied may also be important. Pearson (2001) found that applications of HCl were more effective at enhancing uptake of Cd by *Z. mays* (cv. "Hudson") than HNO_3 or H_2SO_4, using soil collected from the Stoke Bardolph field site. This may have been due to increased chloro-complexation following the application of HCl, combined with a pH reduction, as pH reduction was relatively uniform for all acid types applied. However, Pearson (2001) amended soil pH prior to plant establishment and observed substantial plant mortality when pH was reduced by c. 1 pH unit. Lowering soil pH below a threshold of approximately 5.5 is known to increase Al toxicity (Blaylock *et al.*, 2000). Therefore observation of plant mortality in a soil with a pH of 5.2 (Pearson, 2001) is consistent with earlier studies. Pearson (2001) observed Cd uptake of 10.6 and 16.0 mg Cd kg^{-1} in *Z. mays* tissue following application of HCl at 360 mmol kg^{-1}, with and without the additional treatment of EDTA at 20 mmol kg^{-1}. This HCl treatment reduced soil pH by just 0.7 pH units which suggests that the buffer capacity of the heavily sludged soil at Stoke Bardolph is much greater than suggested by Rowell (1997) for organic soil. A large organic

matter content and very substantial extractable phosphate concentration are likely to be the main constituents responsible for this large buffer capacity. The buffer capacity for the test soil was estimated at 505 mmol H^+ kg^{-1} pH^{-1} using data from Pearson (2001).

In the current study an acid treatment of 100 mmol HCl kg^{-1}, estimated to lower soil pH by 0.2 pH units, was applied to established plants in an effort to avoid the plant mortality observed by Pearson (2001). HCl was applied in combination with both rates of EDTA (2 and 10 mmol kg^{-1}; Chapter 5) and herbicide as described below.

6.1.2 Herbicide treatments

The ability of herbicide applications to enhance metal uptake by plants has been demonstrated in previous work, as discussed in Section 5.3.5. Although applications of glyphosate in the field experiments described in Chapter 5 did not enhance metal uptake by *Z. mays*, this may have been due to the relatively low application rates employed. The action of glyphosate involves specific targeting of the shikimic acid pathway, preventing formation of secondary compounds and arresting protein synthesis (Caseley and Coupland, 1985). Glyphosate is rapidly translocated from the foliage to the roots, inducing significant and progressive reductions in root biomass within 2 – 3 days of application (Fernandez *et al* 1994). It can be assumed that the progressive destruction of roots following glyphosate application will initially include the rupturing of root membranes. As root membranes are known to provide a barrier to diffusion (Marschner, 1995), the progressive destruction of root structure may cause an initial increase in metal ion influx across root membranes to occur.

Work by Miteva *et al.* (1995) demonstrated a significant increase in the concentrations of Cd, Zn and Cu of spring barley following applications of 2, 4-dichlorophenoxyacetic acid (2, 4-D) at a rate of 900 g ha^{-1}. 2, 4-D is a growth hormone, which kills plants by inducing uncontrolled growth and increasing membrane permeability to cations, the size of stomatal apertures and the rate of protein synthesis (Cobb, 1992). Changes to membrane permeability or increases to transpiration following application of 2,4-D, may explain an increase in metal uptake

by plants. In the current study, the effects of both glyphosate and 2, 4-D were compared, applied as either single treatments or in combinations with EDTA and HCl. Glyphosate was applied at the rate of 1440 g ha^{-1}, while 2, 4-D was applied at (1410 g ha^{-1}). These application rates equate to 3 L ha^{-1}, which is slightly lower than recommended for normal agricultural practise (Anon, 2001) and were used to try and induce root damage without causing rapid death.

6.1.3 Experimental hypothesis

The experimental hypothesis being examined was that applications of HCl and glyphosate in combination with EDTA would significantly increase the uptake of Cd by *Z. mays*.

6.2 Materials and methods

6.2.1 Experimental details

Soil was collected from the field site and prepared as described in Section 2.1. Plastic columns with a height of 30 cm, and a diameter of 9 cm (minimum) and 10.7 cm (maximum) were loosely packed with moist soil, equivalent to a dry weight of 928 g. Each column was weighed regularly and watered to maintain a constant moisture content of 78 % (dry weight basis), which is close to field capacity. It was assumed that a large soil moisture content would enhance the effect of chemical treatments. This watering regime was suspended three days prior to chemical treatment application to avoid over-watering and leaching of the chemical treatments applied. *Z. mays* (cv. "Hudson") was planted at a density of four plants per column and thinned to leave one plant, approximately one week prior to treatment. Chemical treatments were applied after five weeks of growth, with harvest occurring one week later. With four replicates of each treatment, the pots were arranged in a fully randomised block design. Glasshouse conditions were as described in Section 3.2. At harvest, the shoots were cut ≈ 1 cm above the soil surface, dried at 80^0C for 48 h and the entire shoot biomass was digested in concentrated HNO_3 (Section 2.2). Analysis for Cd, Zn and Cu was conducted using F-AAS as described in Section 2.2.

6.2.2 Chemical treatments

Eighteen treatments were applied (Table 6.1) consisting of two rates of EDTA (2 and 10 mmol kg^{-1}), two types of herbicide, and HCl (100 mmol kg^{-1}). The two types of herbicides were: Glyphosate, a broad-spectrum herbicide containing 480 g L^{-1} isoproylamine salt of glyphosate and polyoxyethylene amine surfactant, and 2, 4-D, a translocated phenoxy herbicide containing 470 g L^{-1} dimethylamine salt. The EDTA treatments were made up to 30 mL solution and the HCl treatment to 200 mL solution. All treatments consisted of 230 mL solution in total, using deionised water for controls. Deionised water was applied first, followed by HCl and then EDTA. All treatments were slowly watered into columns using a measuring cylinder.

Herbicide was applied to crop canopies using a Cooper Pegler Series 2000 CP15 sprayer, 48 h after applying EDTA and or HCl. Columns to be treated with a specific herbicide were moved to a separate glasshouse compartment and were left overnight before being returned to their original glasshouse compartment, to avoid cross contamination with non-herbicide treated columns. The two herbicides were applied in separate glasshouse compartments to avoid cross contamination.

Table 6.1. Description of chemical treatments applied in a pot experiment to examine the effects of EDTA, herbicide and HCl on metal uptake by *Z. mays*.

Treatment Number	Control (water only)	EDTA (2 mmol kg^{-1})	EDTA (10 mmol kg^{-1})	Glyphosate (1440 g ha^{-1})	2,4-D (1410 g ha^{-1})	HCl (100 mmol kg^{-1})
1	√					
2		√				
3			√			
4				√		
5		√		√		
6			√	√		
7					√	
8		√			√	
9			√		√	
10						√
11		√				√
12			√			√
13				√		√
14		√		√		√
15			√	√		√
16					√	√
17		√			√	√
18			√		√	√

6.2.3 Soil measurements

A sample of the soil used in the experimental columns was retained for analysis of pH, total and EDTA-extractable Cd, Zn, Pb, Cu and Ni, $CaCl_2$ extractable Cd, phosphate extractable in bicarbonate, loss on ignition and $CaCO_3$ content using the procedures described in Section 2.2. Soil solution samples were collected using micro-porous polymer rhizo-samplers (Knight *et al.*, 1998) over a 24 h period commencing 24 h after application of EDTA and HCl treatments. A 2 mL aliquot of soil solution was added to 30 mL polythene bottles containing 5 mL of 0.1 M HNO_3 and stored at 0 - 4 ^{0}C prior to analysis. Analysis of total Cd and Zn was carried out using F-AAS and GF-AAS. Soil from columns was retained following harvest of plant material for pH determination. Soil pH was measured following plant harvest for all columns.

6.2.4 Statistical analysis

An analysis of variance (ANOVA) was undertaken using GenStat package (5th Edition) to establish treatment effects.

6.3 Results and Discussion

6.3.1 Soil characteristics

The soil characteristics presented in Tables 6.2 and 6.3 are consistent with those obtained for the pot experiments reported in Section 3.3. Mean soil pH was reduced by 0.21 pH units following treatment with HCl alone and 0.18 units when the acid treatment was combined with 10 mmol EDTA kg^{-1}. This pH reduction is consistent with the soil buffering capacity calculated using data from Pearson (2001; Section 6.1.1). The soil solution measurements for Cd are shown in Figure 6.2 and demonstrate a significant increase in metal solubility following application of EDTA ($P<0.01$). However, application of HCl had no significant effect on Cd solubility (Fig. 6.2), which is also consistent with the impact of a small reduction in soil pH (Fig 6.1). The Cd concentration in soil solution calculated using the equation from Figure 6.1 for pH 6.19 is 0.0032 mg L^{-1}, whereas pH 5.98 is 0.0056 mg L^{-1}. The actual measured

concentration was 0.006 mg L^{-1} (Fig 6.2). This calculation is also consistent with the Cd concentration in solution of 0.0224 mg L^{-1} measured for Stoke Bardolph soil where pH was 5.0 (Andy Tye, *pers. comm.*).

Table 6.2. Total, EDTA-extractable and $CaCl_2$-extractable metal contents for the soil used in the pot experiment. Values are means (n = 3). Values in parenthesis are standard error of the mean.

	Total soil metal content (mg kg^{-1})	EDTA extractable metal content (mg kg^{-1})	$CaCl_2$ extractable metal content (mg kg^{-1})
Cd	35.0 (0.80)	22.7 (0.95)	20.0 (3.61)
Zn	2388 (66.4)	1067 (120)	-
Cu	968 (33.7)	594 (38.4)	-
Pb	652 (10.2)	270 (13.5)	-
Ni	404 (6.22)	275 (27.4)	-

Table 6.3. Soil characteristics for soil used in the pot experiment. Values are means (n = 3). Values in parenthesis are standard error of the mean.

	pH	[a]LOI (%)	[b]Available P (mg kg^{-1})
Mean	6.19 (0.05)	40.0 (6.50)	214 (26.3)

[a] LOI, Loss on ignition. [b] Bicarbonate-extractable phosphate.

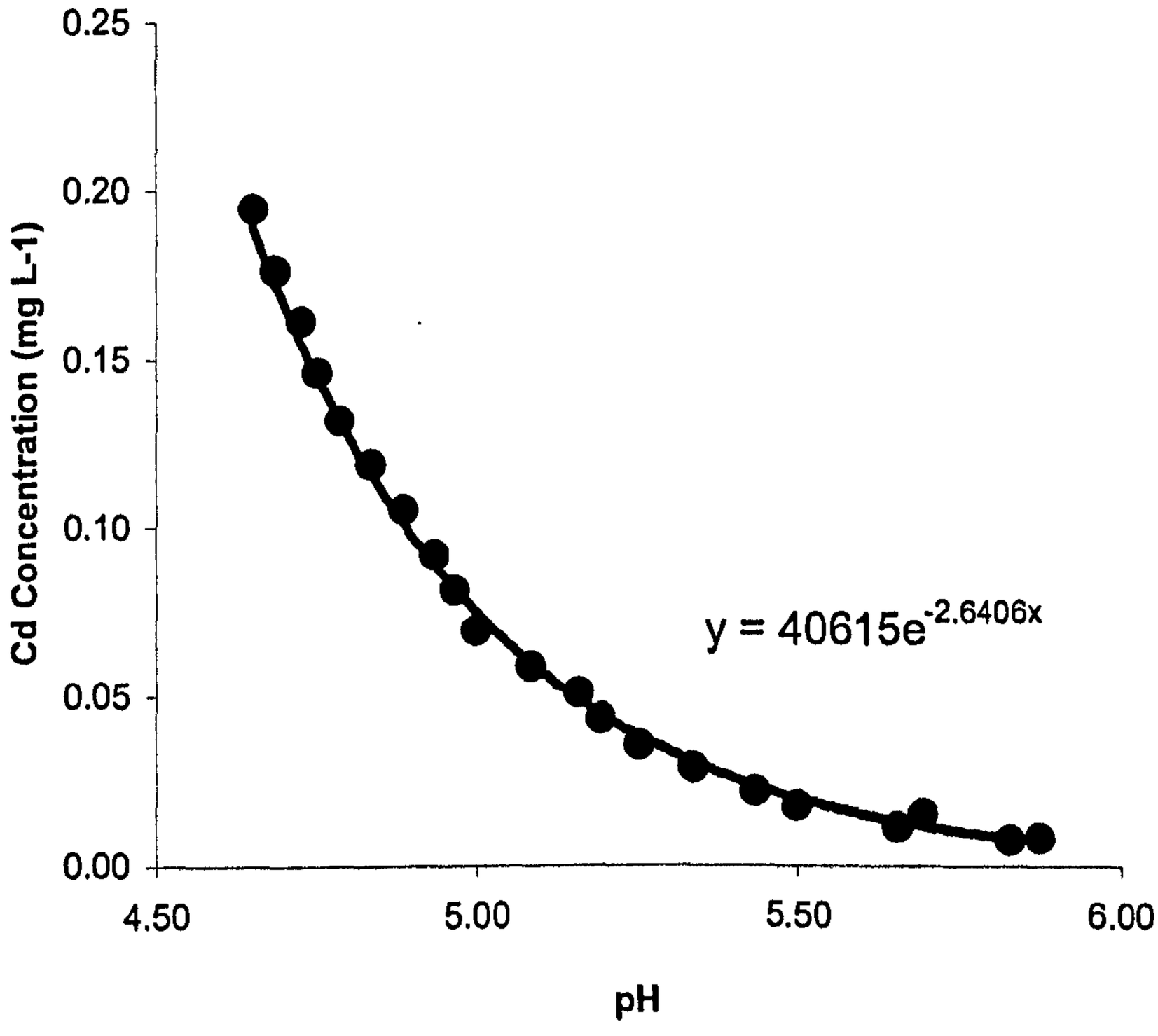


Figure 6.1. Cd concentrations in soil solution, extracted using electrolyte solutions with concentrations of 0.1 M HCl and 0.1 M $CaCl_2$. (From Pearson, 2001).

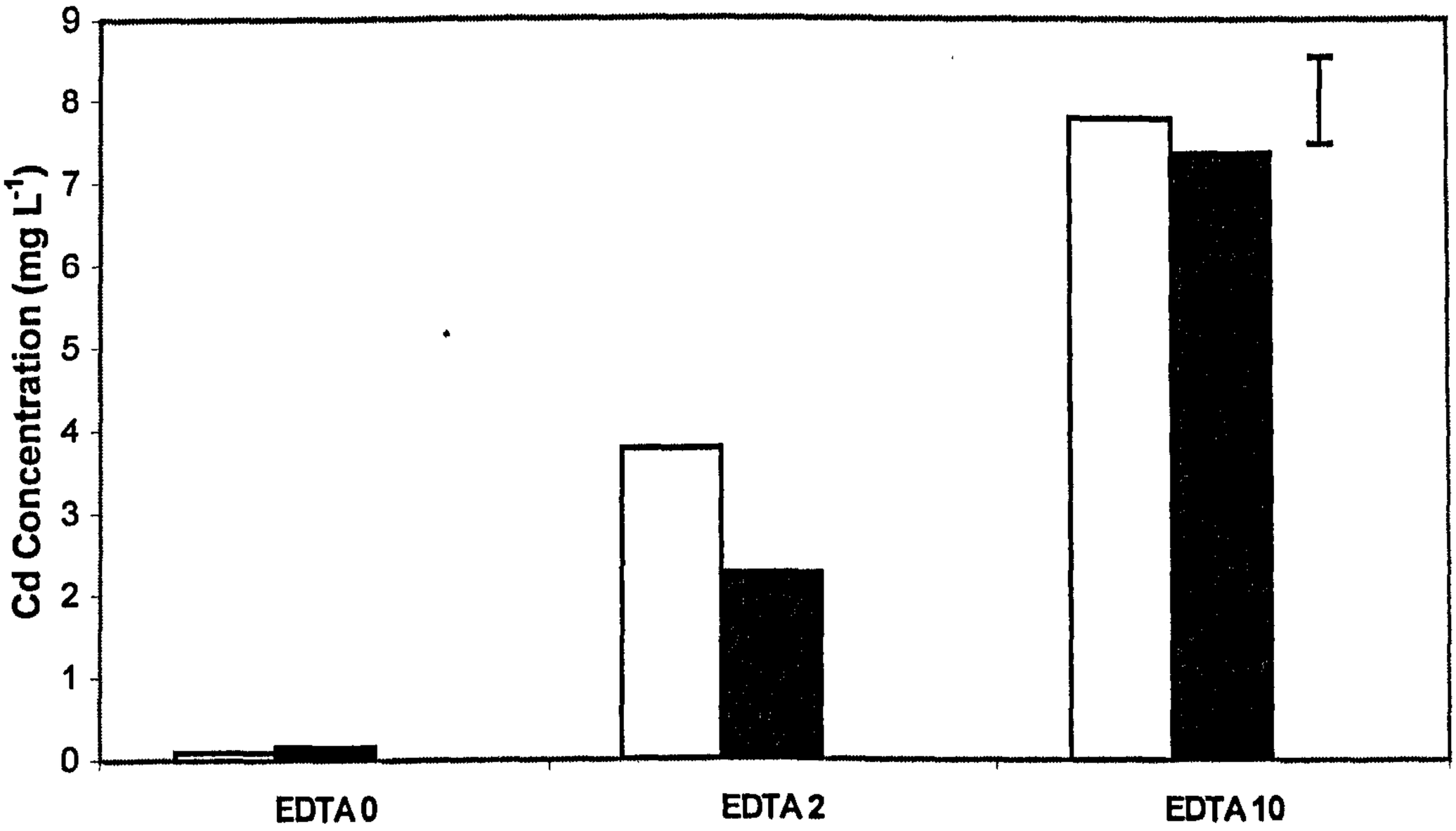


Figure 6.2. Cd concentrations in soil solution collected from columns of *Z. mays* plants over a 24 h period commencing 24 h after treatment with EDTA (0, 2 and 10 mmol kg^{-1}) with (■) or without (□) HCl (100 mmol kg^{-1}). The EDTA and HCl treatments were applied after five weeks of growth. Vertical bars show the SED value.

6.3.2 Uptake of heavy metals following treatment with and without EDTA

Figures 6.3 - 6.5 show the Cd, Zn and Cu concentrations in *Z. mays*. Tissue concentrations of Cd, Zn and Cu in the absence of EDTA applications were lower than observed in earlier studies reported in this thesis, i.e. the pot experiment reported in Chapter 3 and the field experiment reported in Chapter 5. Similarly, the addition of EDTA at both application rates (2 and 10 mmol kg^{-1}) resulted in a smaller increase in metal uptake than observed in the earlier sections. However, the increase in metal uptake following EDTA application alone, in the current section was not statistically significant. It is possible this is due, in part, to variation in uptake traits between *Z. mays* varieties. For example, Cd uptake may vary widely between cultivars (Grant *et al.*, 1999). However, as the rate of EDTA application in the pot experiment reported in Chapter 3 was 20 mmol kg^{-1}, a greater degree of metal uptake would be expected compared to applying 2 and 10 mmol kg^{-1} in the current section. Variations in plant metal uptake from different experiments may be expected, due to conditions such as; temperature and light levels, leading to differences in plant development. However, these differences do not detract from the experimental aims, which were to examine the effects on plant metal uptake resulting from the application of multiple chemical treatments.

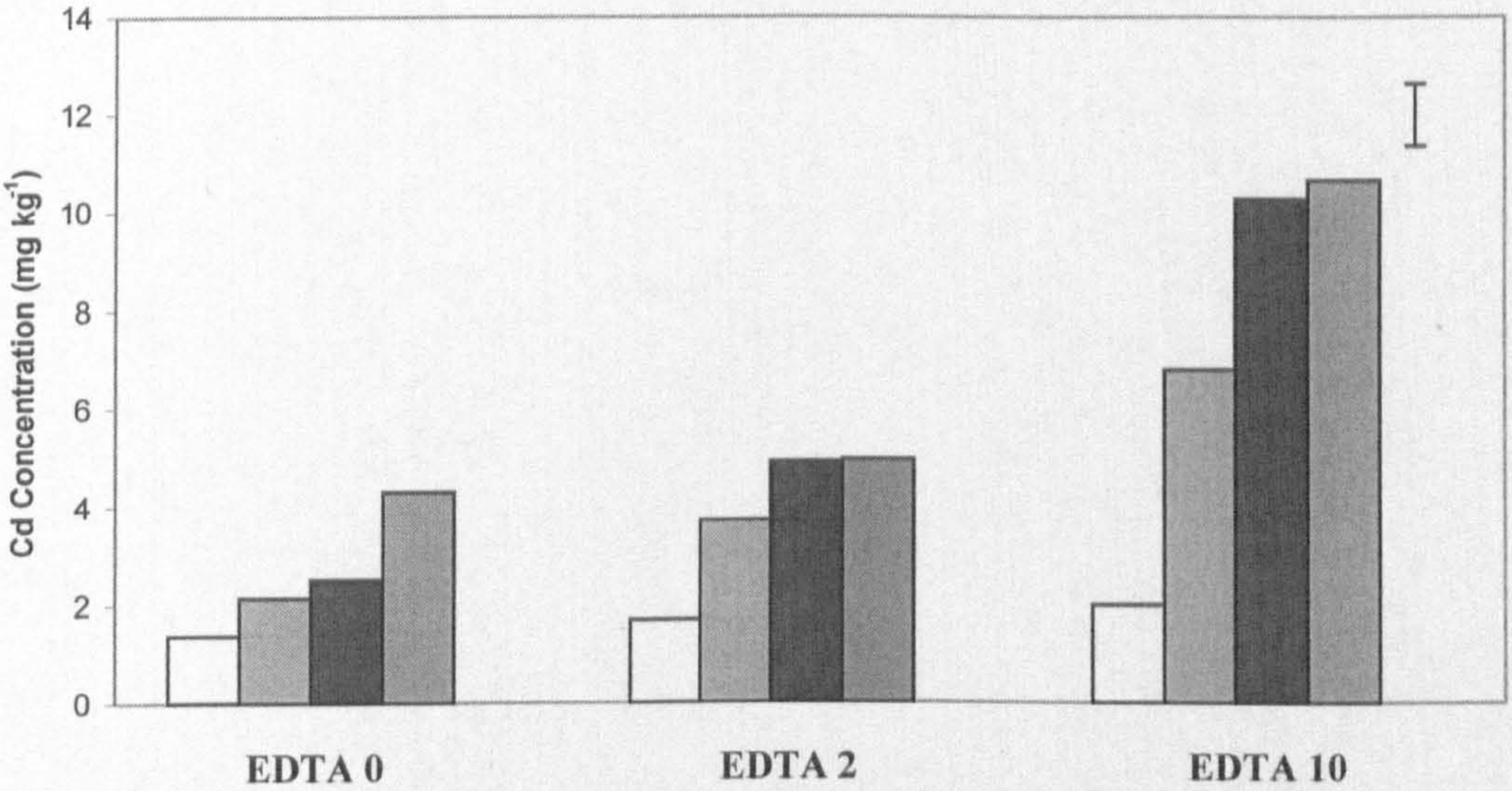


Figure 6.3. Cd concentration in the shoots of *Z. mays* grown for five weeks and then treated with EDTA (0, 2 and 10 mmol kg^{-1}) and or HCl (100 mmol kg^{-1}). Selected plants were also treated with glyphosate (3 L ha^{-1}). Key: No glyphosate or HCl (□); glyphosate (3 L ha^{-1}; ▩); HCl (100 mmol kg^{-1}; ■) and glyphosate and HCl treatments combined (▧). Vertical bars show the SED value.

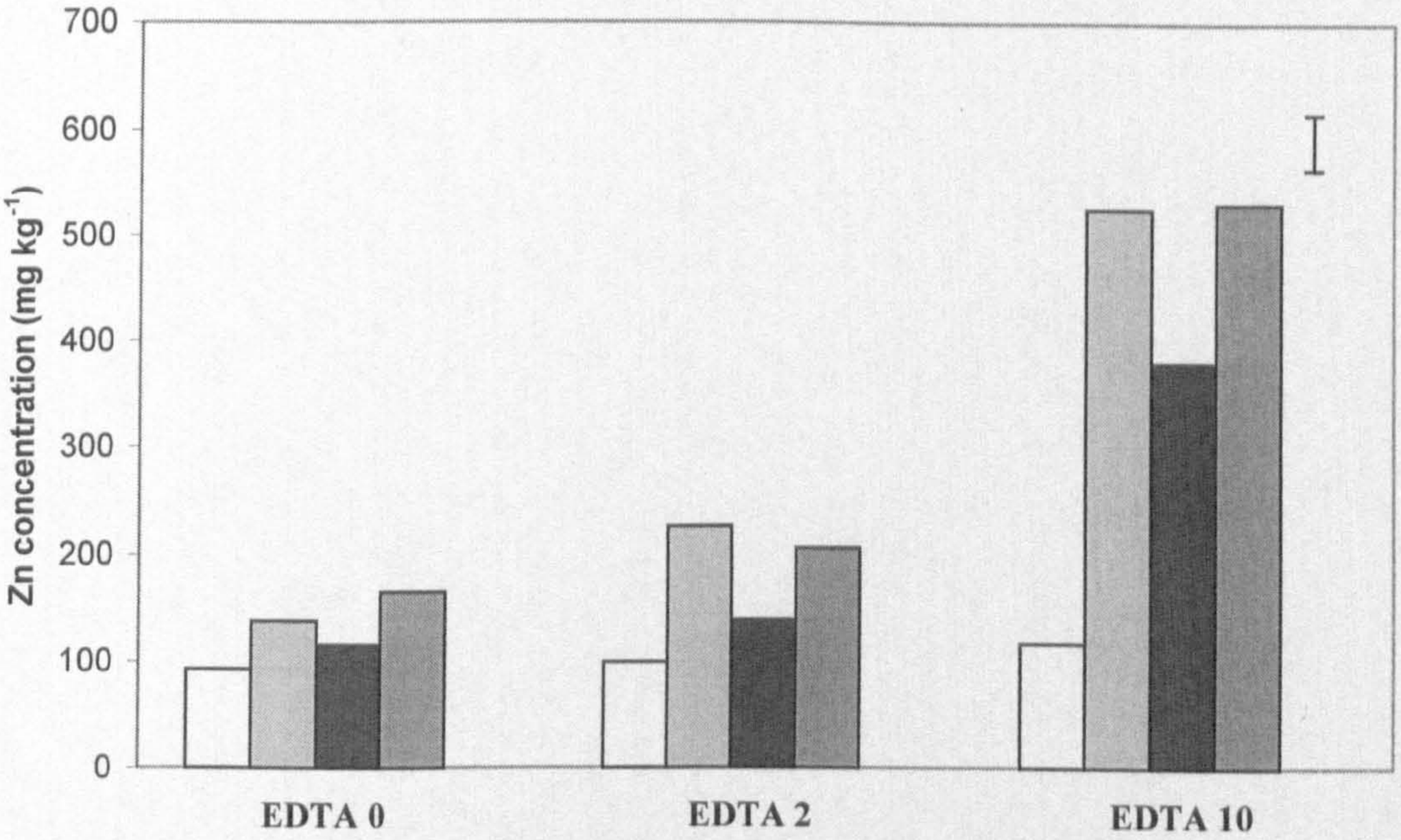


Figure 6.4. Zn concentration in the shoots of *Z. mays* grown for five weeks and then treated with EDTA (0, 2 and 10 mmol kg^{-1}) and or HCl (100 mmol kg^{-1}). Selected plants were also treated with glyphosate (3 L ha^{-1}). Key: No glyphosate or HCl (□); glyphosate (3 L ha^{-1}; ▒); HCl (100 mmol kg^{-1}; ■) and glyphosate and HCl treatments combined (▨). Vertical bars show the SED value.

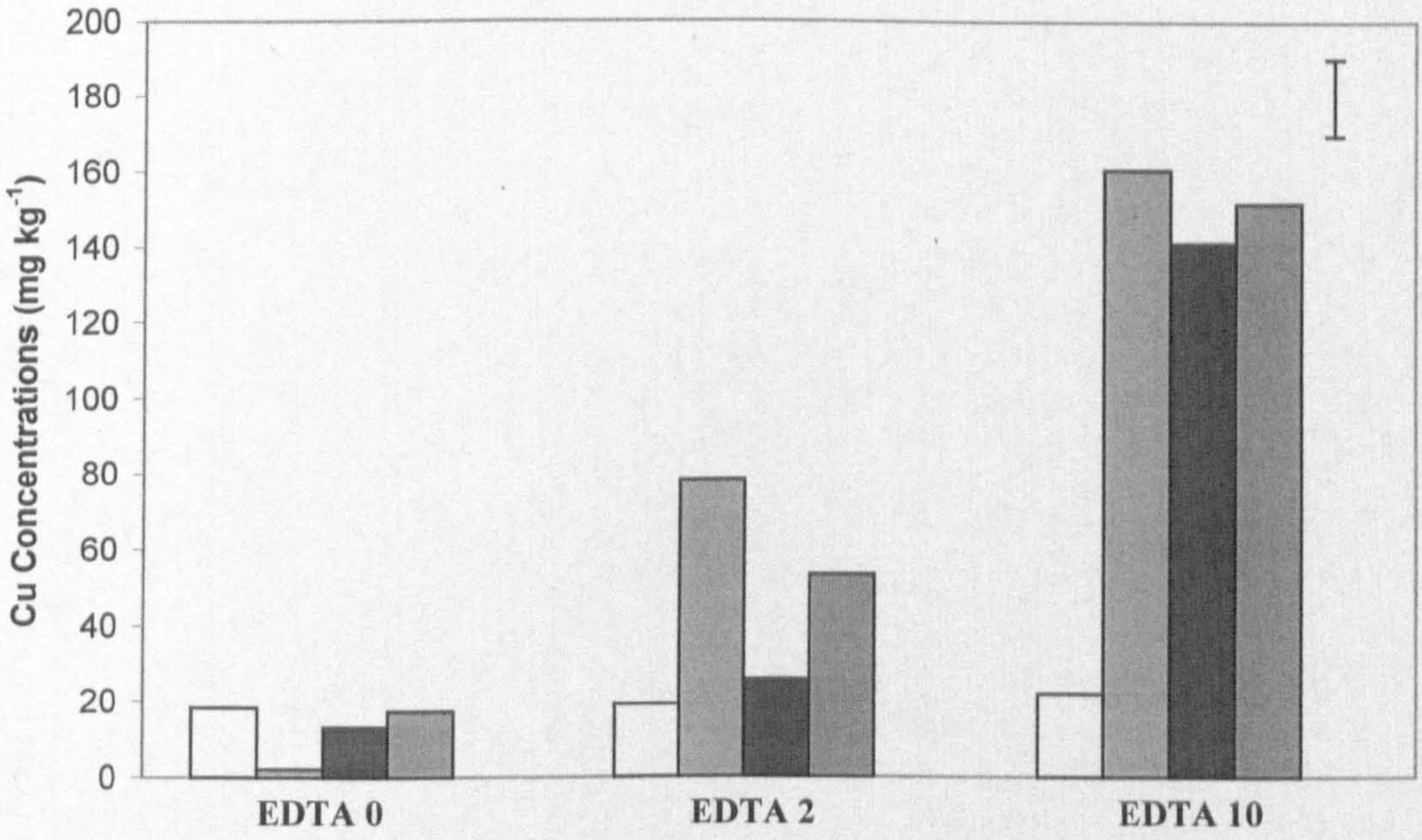


Figure 6.5. Cu concentration in the shoots of *Z. mays* grown for five weeks and then treated with EDTA (0, 2 and 10 mmol kg^{-1}) and or HCl (100 mmol kg^{-1}). Selected plants were also treated with glyphosate (3 L ha^{-1}). Key: No glyphosate or HCl (□); glyphosate (3 L ha^{-1}; ▒); HCl (100 mmol kg^{-1}; ■) and glyphosate and HCl treatments combined (▨). Vertical bars show the SED value.

6.3.3 Uptake of heavy metals following treatment with herbicide and HCl: Separately and when combined with EDTA

Cd, Zn and Cu concentrations in *Z. mays* plants following treatment with EDTA, HCl and glyphosate are shown in Figures 6.3, 6.4 and 6.5 respectively. Application of 2, 4-D, when applied as a single treatment or when combined with other agents, had no statistically significant effect on Cd, Zn or Cu concentration within the plants; therefore the results have not been presented.

Glyphosate treatment

The application of glyphosate alone did not increase the plant uptake of metals. However, application of EDTA at either 2 or 10 mmol kg^{-1} in combination with glyphosate treatment significantly increased Zn and Cu concentrations ($P<0.01$). But, only the larger dose of EDTA (i.e. EDTA at 10 mmol kg^{-1}) produced a significant increase in plant Cd concentration ($P<0.01$), when EDTA was applied in combination with glyphosate. As no statistically significant increase in metal uptake by *Z. mays* was observed in the field experiment following glyphosate application (Chapter 5), the effect observed in this section may be dependant on dose. Increases in metal uptake by plants following glyphosate treatment are consistent with the work by Ensley *et al.* (1999) and Mathis and Kayser (2001) discussed in Section 5.3.5. However, neither of these studies offers any explanation for the observations obtained.

Root damage following glyphosate treatment is likely to involve damage to membrane structure (Section 6.1.2). In addition, water loss from leaves has been shown to continue following glyphosate application (Lascano. *pers. comm*; cited by Fernadez *et al.*, 1994). This study measured loss of soil water through dead wheat plants as a result of glyphosate treatment, with plants covered in aluminium foil to prevent evaporation from dead shoots. They concluded that water loss occurred through evaporation from the dead shoots, because a better hydraulic convection between dead roots and the shoots existed via a capillary rise effect; described as a "wick" effect (Lascano. *pers. comm*; cited by Fernadez *et al.*, 1994). Therefore, it is likely that above certain threshold concentrations, glyphosate will induce sufficient root damage to facilitate an increase in metal ion influx.

As glyphosate treatment will ultimately lead to plant death, the relative timescale for metal uptake and harvest are important. However, if the observed increase in metal uptake following the combined glyphosate and EDTA treatment occurs rapidly, and is followed by harvest, this treatment combination could be utilised by phytoextraction.

Glyphosate is also known to form stable complexes with divalent metal ions (Glass, 1984; Duke *et al.*, 1985) and therefore its effectiveness is reduced if metal ions are applied in combination with the herbicide (Nilsson, 1985). However, the addition of EDTA to glyphosate solution increases the phytotoxicity of the herbicide as the EDTA complex is formed in preference to metal glyphosate complexation (Mervosh and Balke, 1991). For example, the ($\log_{10}$) stability constant for Ca-EDTA is 10.59, whereas for Ca-glyphosate it is 3.25 (Shea and Tupy, 1984). Therefore application of EDTA may increase the effectiveness of glyphosate in addition to increasing metal solubility.

Despite a significant increase in metal concentration following applications of EDTA and glyphosate, for example a 628 % increase in tissue Cu concentration, metal concentrations were still far below the level required for successful remediation of the test soil. For example, to reduce the soil Cd concentration below levels prescribed by the 1989 Sludge Regulations (SI, 1989a) within 10 years, would require a shoot Cd concentration of 1545 mg kg^{-1}, assuming a constant soil → plant transfer factor throughout the remediation period (Section 5.3.7). However, in the current study the maximum shoot Cd concentration observed in *Z. mays* was only 10.6 mg kg^{-1}.

2, 4-D treatment

It was hypothesised that the significant increase in Cd, Zn, Pb and Cu uptake in spring barley reported by Miteva *et al.* (1995) resulted from either changes in membrane permeability or increases in stomatal apertures, which are known symptoms of injury following applications of 2, 4-D (Cobb, 1992). However, Miteva *et al.* (1995) also reported a decrease in plant uptake of important nutrients such as Ca, N, P and K; although they suggested this was caused by the toxicity effect of increased metal uptake, without offering an explanation for how this increase occurred. Results from

the current study are inconsistent with those of Miteva *et al.* (1995) as no significant increases in Cd, Zn or Cu uptake were apparent (data not presented). Further work is therefore required to test how the action of 2-4, D influences metal uptake by plants. It is likely however, as is the case with glyphosate, that the dose of the herbicide and the relative timescale of the plant response are important factors.

HCl treatment

The application of HCl as a single treatment did not significantly increase plant uptake of metal. Furthermore, the application of HCl in combination with 2 mmol EDTA kg^{-1} also did not significantly increase tissue Zn or Cu concentrations. However, application of HCl in combination with 10 mmol EDTA kg^{-1} produced a significant increase in uptake of Zn and Cu ($P<0.01$). Application of both rates of EDTA with HCl significantly increased Cd concentrations in *Z. mays* ($P<0.01$). Previous work, for example by Blaylock *et al.* (1997) and Ensley *et al.* (1999), referred to the combined effect of increasing metal uptake when acid is applied to plants in combination with EDTA (Section 6.1.1). However, the pH reduction induced by HCl application in the present study was small and soil solution samples collected 24 h after treatment, provided no evidence of significant changes in metal solubility compared to columns treated with EDTA alone (Section 6.3.1). Soil pH was not measured until after plant harvest, one week after chemical application, and may not reflect the pH of soil pore water immediately following HCl treatment. However, no increase in metal solubility was observed in soil solution just 24 h after treatment (Fig. 6.2). For this reason, the enhancement of metal uptake by plants following treatment with HCl may have resulted from other factors. For example, it may be possible that the HCl treatment also induced an increase in root membrane permeability to metal ions.

In the case of Zn and Cu, the combination of EDTA and glyphosate enhanced metal uptake to a greater extent than the EDTA and HCl combined treatment. The difference in Zn uptake between EDTA and glyphosate and EDTA and HCl was significant for the 10 mmol EDTA kg^{-1} treatment ($P<0.01$). However, in the case of Cd, the increase in plant uptake was greater following the EDTA and HCl treatment compared to the EDTA and glyphosate combined treatment. Again this effect was statistically

significant for the application of 10 mmol EDTA kg^{-1} treatment ($P<0.01$). It is possible this observation is due to chloro-complexation of Cd, which would be only a minor factor for Zn and Cu.

It is interesting to note that treatment with 100 mmol HCl kg^{-1} solution in the present study, only decreased soil pH by 0.21 units, but produced a larger increase in plant Cd uptake (compared to control plants) than the increase observed in plant Cd by Pearson (2001). Pearson (2001) applied a treatment of 360 mmol HCl kg^{-1}, for example, prior to plant establishment, which decreased soil pH by 0.7 units. The application of acid prior to plant establishment may not have resulted in any direct damage to root membranes during the irrigating process. Thus it is likely that the increase in tissue Cd reported by Pearson (2001) was solely due to an increase in soil metal solubility. Consequently inducing some degree of root damage may be a part of the efficacy of mineral acid treatments applied to growing crops. The application of a dilute acid solution to established plants may also be advantageous in comparison to producing a large pH reduction prior to plant establishment for other reasons. Firstly the use of a lower volume of acid would be more cost effective, and secondly, a slight pH reduction would be easier to ameliorate later, as soil pH would need to be managed in the long term in compliance with normal agricultural practise and legislation.

Further investigation of the mode of action of acid applied to the rooting system and the subsequent enhancement of metal uptake would be useful. However, it is also important to observe that, despite significant increases in metal uptake by *Z. mays* following this treatment, tissue metal concentrations were still far lower than required for successful remediation.

Combined application of HCl and glyphosate

The combination of HCl and glyphosate significantly increased tissue Cd concentrations compared to control plants ($P<0.01$), although a similar trend was not observed for Zn or Cu. Application of EDTA at 10 mmol kg^{-1} with glyphosate and HCl significantly increased the Cd, Zn and Cu concentration of tissue compared to control plants. But, this effect was not observed following application with EDTA at 2

mmol kg^{-1}, when combined with glyphosate and HCl. Furthermore, no additional increase in metal uptake by *Z. mays* was observed for the combined EDTA, glyphosate and HCl treatments, compared to other treatment combinations. However, different treatment combinations produced the greatest degree of uptake by *Z. mays* for different metals. For example, the EDTA and glyphosate application produced the greatest increase in tissue Zn and Cu concentrations, whereas the EDTA and HCl application produced the greatest increase in tissue Cd concentrations. Thus use of the combined EDTA, glyphosate, and HCl application may be advantageous, if phytoextraction was intended to remove multiple metals.

6.4 Conclusions

The use of combined chemical treatments, such as the application of chelates and dilute acids, have been suggested as a possible strategy for enhancing metal uptake by arable crops. Some herbicides have been proposed as possible metal 'inducing' agents, although very little explanation for this process has been presented in the literature. The current study aimed to test metal uptake by *Z. mays* following combined chemical treatments; including EDTA at 2 and 10 mmol kg^{-1}, HCl at 100 mmol kg^{-1} and two forms of herbicide (glyphosate and 2, 4-D).

No significant increase in plant metal uptake was observed following application of 2, 4-D, which is not consistent with the only previous study using this agent to enhance metal uptake by plants. However, application of both glyphosate and HCl did cause some statistically significant increases in metal uptake by *Z. mays*, when combined with EDTA. It has been suggested that glyphosate application caused damage to root membrane integrity, thus increasing metal influx. HCl application to soil might normally be expected to increase solubility, although this was not shown in the current study following application of 100 mmol kg^{-1}. Despite this, metal off-take by *Z. mays* did increase significantly following HCl application, when combined with EDTA. It is therefore possible that application of a dilute acid, to an established crop, also produced root damage and increased metal influx in a similar way to glyphosate.

Different treatment combinations produced the greatest degree of metal uptake by *Z. mays* for different metals. For example, the tissue Zn and Cu concentrations were greatest following application of glyphosate when combined with EDTA, whereas the tissue Cd concentration was greatest following the application of HCl when combined with EDTA. No additional increases in *Z. mays* tissue metal concentrations were observed when glyphosate, HCl and EDTA were combined, when compared to the other treatment combinations. However, despite producing some significant increases in tissue metal concentrations, these concentrations were still far below those required for successful phytoextraction.

CHAPTER 7: CHEMICALLY ENHANCED PHYTOEXTRACTION OF CADMIUM USING SHORT ROTATION WILLOW COPPICE

7.1 Introduction

The potential for using willow for phytoextraction was discussed in detail in Section 1.3.3. Willow is a promising crop for soil remediation for several reasons. When grown as an energy crop, the majority of accumulated Cd and Zn can be recovered during the combustion process (Narodoslawsky and Obernberger, 1996). This creates a financial return for the crop *per se*, which could be used to offset the cost of phytoextraction (Section 1.3.3). However, experiments to examine metal uptake by willow have so far provided disappointing results; despite producing a large biomass, metal accumulation is usually low (Eriksson and Ledin, 1999; Pulford *et al.*, 2002). Thus, many authors have suggested that the use of willow will be restricted to remediating marginally contaminated sites (Greger and Landberg, 1995; Eriksson and Ledin, 1999; Pulford *et al.*, 2002; Section 1.3.3).

The principles of chemically-enhanced phytoextraction were discussed in detail in Section 1.3.2 and experiments conducted in the current study, to examine this soil remediation strategy using arable crops were described in Chapter 5. However, to date, few studies have examined the possible use of solubilizing agents to enhance metal uptake by short rotation coppice (SRC) species, although Robinson *et al.* (2000) demonstrated a significant increase in leaf Cd content in *Populus deltoides x P. yunnanensis* two weeks after applying EDTA application relative to control plants. However, at the end of the experiment, approximately six weeks after EDTA application, no significant difference in leaf Cd concentration was found between treated and control plants. Robinson *et al.* (2000) suggested that a surge in leaf Cd uptake occurred shortly after chelate application. The subsequent decline in leaf Cd may have resulted from phloem movement to sinks, such as newly formed roots and leaves (Scholz, 1989). Furthermore, knowledge of Cd transport in willow is limited. Although Robinson *et al.* (2000) described a pot-based experiment in which total soil Cd was amended by adding Cd in nitrate form, their work nevertheless provides evidence that EDTA may be used to enhance the leaf Cd content in (SRC) species.

Kayser *et al.* (2000) demonstrated significant increases in tissue Cd and Zn concentrations in field-grown *Salix viminalis* plants following application of Nitrilotriacetate (NTA) compared to control plants. However, in this case, the degree of soil contamination was relatively low (Section 1.3.2). Schmidt and Kaupenjohann (2002) found a 2.2-fold increase in Cd and Zn uptake by *S. viminalis* following application of ammonium sulphate in a pot experiment using soil with a low Cd concentration (<2.5 mg kg^{-1}), although, soil pH changes resulting from the application of ammonium sulphate were not examined.

The aim of the work reported in this chapter was to test the efficacy of phytoextraction using field-grown SRC willow grown in soil treated with sewage sludge. The chemical treatments applied, consisted of EDTA and HCl, applied either individually or in combination.

7.1.1 EDTA treatment

EDTA is the most widely studied solubilizing agent in phytoextraction research, as outlined in Section 1.3.2 and Chapter 5. An application rate of 2 mmol EDTA kg^{-1} of soil was selected for use with *Salix* to enable comparison with the *Z. mays* system described in Chapter 5. Although application of EDTA to arable crops may induce root damage (Vassil *et al.*, 1998), this is not problematic as the crop is harvested shortly after chelate treatment. In any case, root damage is likely to facilitate a greater influx of metal in arable crops (Vassil *et al.*, 1998; Section 1.3.2; Chapter 6). *Salix,* however, is a long-lived crop, with coppicing usually taking place at 4 or 5-year intervals for up to 24 years (Ledin, 1996). Any root damage may therefore reduce long-term crop yields and for this reason multiple treatments of low dose EDTA were also tested.

7.1.2 HCl treatment

The use of soil acidification to influence metal solubility was discussed in Section 1.2.2. In addition, work carried out in the current study to examine the combined use of acidification and chelate treatment to enhance metal uptake by *Z. mays* was

discussed in Chapter 6. In an effort to increase metal solubility, HCl was applied to selected plots of *S. caprea x cineria x viminalis* in one of two experiments (Experiment 1) carried out during the current investigation. A solution containing 100 mmol HCl kg^{-1} was shown to reduce soil pH by 0.2 pH units in a pot experiment (Chapter 6). In an effort to avoid extensive root damage and a consequent yield reduction resulting from the application of such an acidic solution, a more dilute HCl solution containing 10 mmol HCl kg^{-1} was used in this field investigation.

7.1.3 Assessment of natural mycorrhizal associations

The potential role of mycorrhizal fungi within phytoextraction systems was considered in Section 1.3.3. Current evidence for beneficial effects of mycorrhizal associations on the uptake of metals by plants is mixed, and most studies have relied on pot-based experiments using soils spiked with metals. Although trees are almost invariably mycorrhizal under normal conditions, the ability of mycorrhizal fungi to survive in metal-contaminated soils is less clear (Gildon and Tinker, 1981). However, some previous work has demonstrated significant decreases in arbuscular mycorrhizal colonisation on soils amended with sewage sludge (Val *et al.*, 1999). There is also little evidence regarding the ability of mycorrhizal fungi to tolerate chemical inputs designed to increase metal solubility within phytoextraction regimes (Pawlowska *et al.*, 2000).

Although some *Salix* species are colonised by both endo- and ecto-mycorrhizal fungi, ecto-mycorrhizas predominate (Harley and Harley, 1987). For this reason, visual assessments were made to provide evidence of ecto-mycorrhizal colonisation of the roots of both *Salix* species grown within this study, with and without application of EDTA. The field boundaries at the experimental field site contain *S. alba* trees which may have provided a source of mycorrhizal inoculation.

7.1.4 Experimental hypothesis

The experimental hypothesis being examined was that uptake of Cd by SRC willow will be significantly enhanced following application of the solubilizing agents, EDTA and HCl, thus producing a viable soil remediation strategy for soils treated with processed sewage sludge.

7.2 Materials and methods

7.2.1 Crop establishment

A field trial involving two species of willow was established in April 2000 at the Stoke Bardolph field site (Section 2.1). The two willow species were *S. caprea* x *cineria* x *viminalis* 'Calodendron' and *S.* x *dasyclados* 'Loden ' SW890129 (Plate 7.1). These species were selected for their ability to accumulate relatively high concentrations of Cd and Zn and their suitability for planting on soil treated with sewage sludge. *S. caprea x cineria x viminalis* achieved amongst the greatest uptake of Cd and Zn in an earlier screening experiment involving 20 *Salix* species at the same experimental site (Riddell-Black *et al.*, 1997). Loden was recommended as a high accumulator of Cd (up to 45 mg kg^{-1}) by Svalof Weibull, a commercial willow grower in Sweden, as a result of screening 90 willow species (Nils-Ove Bertholdsson, *pers. comm.*).

Two trials were established using 250 mm unrooted cuttings, which were hand-planted within a fenced area to avoid damage by rabbits. Both trials comprised a randomised block design with four blocks providing four replicate plots. Each plot measured 6 x 3.75 m and contained 28 trees at 0.75 m spacing within and between rows (Fig. 7.1). The first trial contained only *S. caprea x cineria x viminalis*, while the second contained equal numbers of both *S. caprea x cineria x viminalis* and *S. dasyclados* (Plate 7.1), with each species located in separate plots arranged in a randomised design. Plots were separated by a gap equivalent to one missing row of trees. Two spare plots were established for each *Salix* species but not included in the block design.

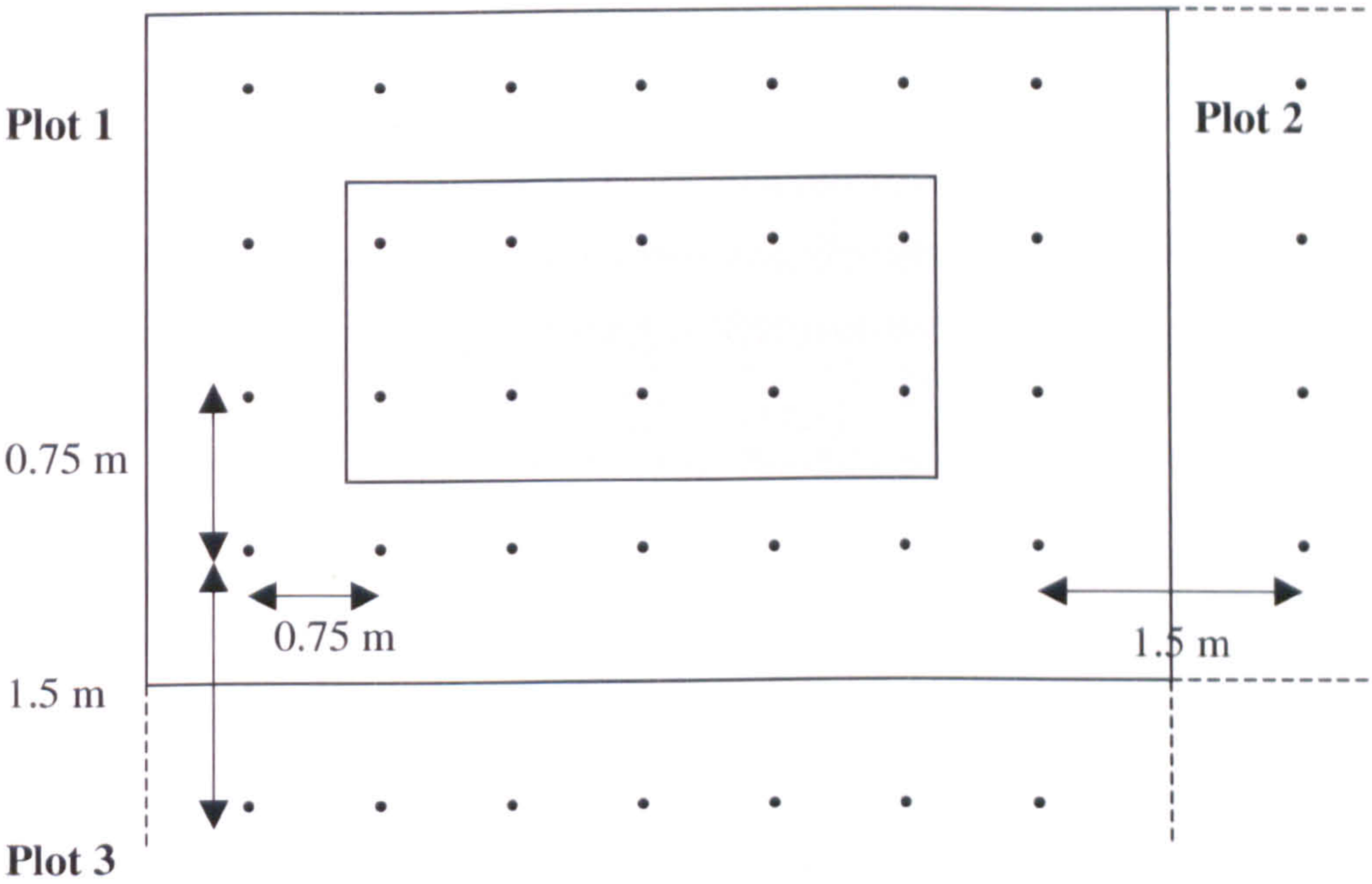


Figure 7.1. Design and spacing for willow plots within Experiments 1 and 2. Trees are denoted by, (•). Shaded area indicates the area treated with solubilizing agents. One row was left unplanted between plots.

Plate 7.1. Willow plantation established at the Stoke Bardolph field site, A: *S. caprea x cineria x viminalis* plot, B: *S. dasyclados* plot.

Mn and Fe solution were applied following the appearance of leaf chlorosis. Foliar treatments of Mn "Jett" (1.5 L ha^{-1}) and Fe solution "Ferrosol" (3 L ha^{-1}) were applied twice during June and July 2000. Fe solution was also applied once during May 2001 following the re-appearance of chlorosis. Weeding by hand was carried out regularly and no cutting back or coppicing of the trees was conducted during the first year of growth.

7.2.2 Soil chemical treatments: General details

Chemical treatments commenced during May 2001, 14 months after planting the cuttings. All chemical treatments were applied to the soil surface using irrigation piping (Section 5.2). The ten central trees in each plot were treated, leaving one untreated guard row around the edge of each plot. The first experiment involving only *S. caprea x cineria x viminalis* examined the effect of single and multiple applications of EDTA, with and without treatment with HCl. In all treatments, 100 L of solution were applied to each plot, equivalent to 2.22 cm of rainfall. EDTA treatments were made up in 50 L solution; 50 L of water was also added to improve the penetration of the treatment through the soil profile. The second experiment incorporated both *Salix* species and the single EDTA application.

7.2.3 Experiment 1: Treatment details

The treatments were: C, Control (no treatment); H, HCl applied at a rate of 10 mmol kg^{-1} of soil; ES, EDTA applied at a rate of 2 mmol kg^{-1} as a single dose; ES+H, combination of treatments H and ES; EM, EDTA applied at a rate of 0.5 mmol kg^{-1} as multiple doses (four applications at four week intervals); EM+H, combination of treatments H and EM. Formulations for the EDTA and HCl treatments were calculated for the 0 - 20 cm soil horizon. All HCl treatments were applied on 22 and 23 May 2001, with the first multiple dose of EDTA being applied on 24 May 2001. For plots receiving multiple EDTA treatments, each application was made at approximately four week intervals, with the final treatment being applied on 14 August 2001. Single dose treatments of EDTA were applied on 15 and 16 August 2001. Control plots were treated with 100 L of water. Trees in all the plots were

harvested three weeks after the final treatment. 100 L of water was applied to all plots prior to the third treatment of the multiple dose EDTA treatment as irrigation is assumed to increase the depth of treatment effectiveness. Irrigation prior to the other chemical treatments was prevented by logistical constraints.

7.2.4 Experiment 2: Treatment details

Experiment 2 tested the effect of a single application of 2 mmol EDTA kg^{-1} on both *S. caprea x cineria x viminalis* and *S. dasyclados*. 100 L of irrigation water was applied to all plots on 22 August 2001, with chemical treatments being applied on 23 August 2001. Control plots were treated with 100 L water. The trees were harvested three weeks after final chemical treatment. Root samples were collected at three random locations from the surface soil horizon (0 - 20 cm) for each plot in Blocks 1 to 3 and washed gently to remove adhered soil. Visual assessments using a stereo-microscope (x 10 magnification) were made to establish the presence or absence of ecto-mycorrhizal associations following root washing.

7.2.5 Harvest details: Experiments 1 and 2

Plant material was harvested for analysis of metal content at various stages during the experiment. One complete stem was sampled from five randomly located trees for both species in February 2001, after almost one year of growth and prior to any chemical application. Leaf samples from each plot were collected on 30 May 2001 and at weekly intervals thereafter for Experiment 1 only. The trees were segregated vertically into five strata and one leaf was sampled from each. Samples were collected from five alternate trees in each plot for both multiple EDTA treatments (i.e. those with and without co-treatment with HCl). Leaf samples were also collected for both *Salix* species during a drought period in July 2001. Ten leaves were sampled both from the lower third of the canopy, where the leaves were chlorotic, and from the upper third of the canopy, where the leaves were healthy, from five randomly located trees within the spare plots. A drought period was assumed to reduce the effectiveness of the soil chemical treatments by restricting the depth of penetration, and to limit metal uptake by reducing transpiration.

The main harvest of Experiments 1 and 2 was conducted as follows; four alternate trees from all treated plots were harvested 15 cm above ground-level, ignoring trees already used for weekly leaf sampling. All leaves were removed, mixed and sub-sampled by taking a grab-sample. One complete stem was selected at random from each harvested tree, shredded using an Al-CO Kober Silent Power 3500 plant shredder and sub-sampled, by taking a grab-sample. To measure the metal concentration of the bark and wood fractions, one further stem was selected for each sampled tree; a 10 cm length of stem was removed 50 cm from the base and the bark and wood were separated. Fresh weights were determined for each complete tree and all associated sub-samples. Duplicate sub-samples were dried at 80 ^{0}C, milled and digested in concentrated HNO_3. Total metal analysis for Cd, Zn, Cu, Pb and Ni was conducted using F-AAS as described in Section 2.2.

7.2.6 Soil measurements

Four soil cores (0 - 20 cm depth: 4 cm diameter) were collected in February 2001 from all experimental plots for soil analysis. Soil cores from each plot were mixed to give a combined overall sample, which was analysed to determine soil pH. Samples for all plots within each block were then pooled for further analysis of total and EDTA extractable Cd, Zn, Pb, Cu and Ni, $CaCl_2$ extractable Cd, bicarbonate extractable phosphate and loss on ignition using the procedures described in Section 2.2. Soil cores (0 - 20 cm depth) were also collected from five equally spaced locations throughout the willow plantation prior to the application of chemical treatments to determine soil moisture content. Further soil cores were collected for soil moisture determination during a period of drought in July 2001; four cores were taken from the 0 - 20, 40 - 60 and 80 - 100 cm horizons at equally spaced distances within the willow plantation and from the same depths at two locations situated at least 10 m outside the *Salix* plots on cultivated grassland. Soil cores (0 - 20 cm depth) were also collected from plots receiving HCl as an individual treatment immediately after the final harvest, to determine effects on soil pH measurements.

7.2.7 The effect of washing samples

The effect of washing sub-samples of leaves or stems was investigated by harvesting one spare plot of each willow species, to establish whether surface contamination influenced the measured values for metal concentration. Preparation for analysis was as described above, with the exception that the sub-samples were halved; one half was washed thoroughly in deionised water, while the other half was not washed prior to analysis using the procedures described above. The analyses were conducted in triplicate.

7.2.8 Statistical analysis

An analysis of variance (ANOVA) was undertaken using GenStat package (5th Edition) to establish treatment effects.

7.3 Results and Discussion

7.3.1 Soil characteristics

The influence of soil characteristics at the experimental site on metal uptake by plants were discussed in Section 3.3, while the general soil factors affecting metal uptake by plants were discussed in Section 1.2. Soil characteristics for plots within Experiments 1 and 2 are presented in Tables 7.1 and 7.2. The soil metal concentrations presented are means for all blocks within each experiment. In Experiment 2, there was substantial variation in metal concentration between blocks. For example, total soil Cd concentration was 43 mg kg^{-1} in Block 1, and 31 mg kg^{-1} in Block 2. Variation in heavy metal concentration within the soil profile at the field site was discussed in Section 4.3.

Table 7.3 shows the moisture content of soil collected prior to the application of each chemical treatment. Soil moisture in the 0 - 20 cm horizon for Experiment 1 plots following chemical treatment was close to or greater than field capacity, which was expected to maximise the effectiveness of the chemical treatments. Soil moisture

content for the Experiment 2 plots was much lower than for Experiment 1 plots. This may be explained by the larger biomass produced by the *S. dasyclados* trees growing in Experiment 2 plots, which is likely to have depleted soil moisture content to a greater extent than in Experiment 1, which contained only *S. caprea x cineria x viminalis.*

Table 7.1. Total, EDTA-extractable and $CaCl_2$-extractable metal content at the Stoke Bardolph field site in Experiments 1 and 2. Values are means (n = 4). Values in parenthesis show standard error of the mean.

		Total soil metal content ($mg\ kg^{-1}$)	EDTA extractable metal content ($mg\ kg^{-1}$)	$CaCl_2$ extractable metal content ($mg\ kg^{-1}$)
Experiment 1	Cd	44.3 (0.92)	28.7 (1.18)	21.7 (1.71)
	Zn	2460 (7.10)	1160 (44.0)	-
	Cu	1020 (20.3)	712 (35.5)	-
	Pb	718 (12.8)	288 (2.56)	-
	Ni	473 (15.4)	380 (4.77)	-
Experiment 2	Cd	37.4 (2.39)	24.0 (2.00)	20.9 (0.93)
	Zn	2130 (156)	1050 (75.5)	-
	Cu	878 (64.4)	640 (59.5)	-
	Pb	618 (35.2)	273 (6.18)	-
	Ni	433 (29.0)	309 (22.6)	-

Table 7.2. Soil characteristics at the Stoke Bardolph field site in Experiments 1 and 2. Values are means (n = 4). Values in parenthesis show standard error of the mean.

	pH	[a]LOI (%)	[b]Available P (mg kg^{-1})
Experiment 1	6.40 (0.03)	35.0 (0.50)	224 (10.6)
Experiment 2	6.40 (0.01)	32.0 (1.00)	225 (2.12)

[a]LOI, loss on ignition. [b]Bicarbonate-extractable phosphate.

Table 7.3. Moisture content of soil sampled prior to application of chemical treatments in Experiments 1 and 2 and calculated moisture content immediately following treatments. Values in parenthesis show standard error of the mean.

Experiment number	Treatment month (2001)	Initial soil moisture content as % of field capacity	Calculated soil moisture content as percentage of field capacity following chemical treatments
1	Late-May	98.6 (1.86)	109[1] (1.86)
1	Mid-June	97.3 (2.78)	108[1] (2.73)
1	Mid-July	85.5 (2.95)	96.6 (2.95)
1	Mid-August	86.8 (2.55)	97.9 (2.55)
2	Late-August	57.8 (2.48)	68.9 (2.43)

[1]Moisture content greater than 100% as calculation based on surface 0 - 20 cm soil profile.

7.3.2 Plant growth and biomass production

Plant growth was generally good, with an overall establishment of over 95 % being achieved. Leaf chlorosis was evident three and four months after planting and also during the spring of the second season. Fe and Mn deficiencies were considered to be the most likely cause as these may be induced by the presence of high soil Zn concentrations (Section 5.3.2). Foliar applications of Fe and Mn solution removed the occurrence of chlorosis, thereby supporting this hypothesis.

In normal SRC production, trees are harvested at 3 - 4 year intervals after leaf fall during winter or early spring (DTI, 1994). However, the limited duration of the current investigation made it necessary to harvest after 18 months of growth. Sampling was carried out during August for Experiment 1 and September for Experiment 2, earlier than for normal SRC production, to enable determination of leaf biomass and metal content.

The estimates of yield obtained in this study are based on the planting density of trees within each plot of 17793 trees ha^{-1}. Most guides recommend a planting density of 10000 willow trees ha^{-1} (DTI, 1994; ADAS, 1995; Bardos *et al.*, 2001) although, research by Willebrand (1992) demonstrated that planting densities of up to 40000 trees ha^{-1} produced significantly greater yields than the 'recommended' density of 10000 trees ha^{-1}, even when natural losses were taken into account. Similarly, the 'Welsh *Salix* Project' recommended a planting density of up to 20000 trees ha^{-1} (Rebecca Heaton, *pers. comm.*). The density of 17793 trees ha^{-1} adopted in the present study is therefore well within the range recommended in previous studies.

The mean biomass for *S. caprea x cineria x viminalis* stems was 10.0 and 11.8 t ha^{-1} respectively in Experiments 1 and 2. Mean biomass for *S. dasyclados* stems was 25.4 t ha^{-1} in experiment 2. Reported values for the stem biomass produced by *Salix* species range from 10 to 30 t ha^{-1} y^{-1} (Section 1.3.3), although Pulford *et al.* (2001) reported a biomass of only 3.8 t ha^{-1} y^{-1} for *S. caprea x cineria x viminalis* grown at the same experimental site as the present study with a planting density of only 7000 trees ha^{-1}.

The annual biomass obtained from the current study therefore compares favourably with those reported by other researchers.

Leaf biomass production in *S. caprea x cineria x viminalis* was 1.8 and 2.0 t ha^{-1} in Experiments 1 and 2 respectively and 6.1 t ha^{-1} in *S. dasyclados*. Few studies have reported leaf biomass production values for *Salix*, although Klang-Westin and Eriksson (2001) recorded values of 1.1 and 1.6 t ha^{-1} y^{-1} for *S. viminalis* and 3 and 8 t ha^{-1} y^{-1} for stem biomass. *S. viminalis* produced 5 t ha^{-1} y^{-1} of leaf biomass and 12 t ha^{-1} y^{-1} of stem biomass (Kurth Perttu, *pers. comm.*; cited by Greger and Landberg, 1995).

7.3.3 Uptake of heavy metals in the absence of treatment with solubilizing agents

The concentrations of heavy metals in the leaves and stems were measured, with and without sample washing prior to analysis, for both *Salix* species. Although few significant differences were detected, Zn concentration in washed and unwashed stems of *S. caprea x cineria x viminalis* were respectively 360 and 387 mg kg^{-1} ($P<0.01$). However, even where significant differences were observed between washed and unwashed samples, the trend was inconsistent. Thus, washed samples contained a slightly larger metal content than unwashed samples in some cases but not in others. No significant differences were detected for the Cd content of leaves or stems, irrespective of whether these were washed or unwashed, for either *Salix* species. There is therefore no evidence that surface contamination biased the values obtained for unwashed samples.

Mean Cd concentrations in the leaves, stems, bark and wood of *S. caprea x cineria x viminalis* in Experiment 1 are shown in Figure 7.2. The corresponding values for the Cd leaf and stem fractions of both *Salix* species in Experiment 2 are shown in Table 7.4. Pb concentrations in the leaf material and Ni and Pb concentrations in stem material of both *Salix* species were below detection limits for the F-AAS method in both experiments. No significant differences in heavy metal uptake by *S caprea x cineria x viminalis* plants were detected between those trees grown in Experiments 1 or 2. No significant differences in metal uptake were recorded for the leaf or stem

fractions in Experiment 2, between *Salix* species. Significant differences were detected for the Cd and Cu concentrations of bark and the Zn concentration of wood between the two *Salix* species in Experiment 2 ($P<0.05$). For example, the Cd concentration in wood of *S. caprea x cineria x viminalis* was 15.82 mg kg^{-1}, whereas the corresponding value for *S. dasyclados* was 19.0 mg kg^{-1}. These differences can be attributed to the large variation in metal concentration in *Salix* stems reported between different *Salix* species. For example Riddell-Black (1994), Landberg and Greger (1994) and Landberg and Greger (1996) all reported variability in metal uptake between willow species (Section 1.3.3).

Heavy metal concentrations in the stems of *S. caprea x cineria x viminalis* were greater in February 2001 than in August or September 2001. For example, Cd concentration was 16.0 and 19.5 mg kg^{-1} for *S. caprea x cineria x viminalis* and *S. dasyclados* respectively in February 2001, whereas the corresponding Cd concentrations in September 2001 were 8.4 and 8.2 mg kg^{-1} respectively. This result is consistent with a dilution effect resulting from increases in biomass during plant growth. A similar effect was demonstrated by Pulford *et al.* (2002), although these workers recorded much lower stem Cd concentrations for *S. caprea x cineria x viminalis* (1.8 mg kg^{-1}) and *S. dasyclados* (4.2 mg kg^{-1}) than in the current study, after 21 months of growth at the same site. However, it is likely that a different population of *S. dasyclados* was grown in the previous study; moreover, leaf metal concentrations were not reported. In contrast, Cd concentrations in *S. dasyclados* were reported by Nils-Ove Bertholdsson (*pers. comm.*) to be up to 45 mg kg^{-1} at a site in Sweden. However, this result was attributed to an unusually high Cd solubility, resulting from the acidic soil pH of 5.5. Comparison of metal uptake by *Salix* in different studies is therefore difficult, not only because of differences in soil and environmental characteristics, but also because of differences in metal uptake by the differing *Salix* species and populations examined by different workers.

Leaf samples collected during the drought period in July 2001 contained significantly different concentrations of Cd and Zn depending on where within the canopy they were collected. Leaves sampled from the bottom third of the canopy had significantly greater Cd and Zn concentrations than those from the top third of the canopy (Table

7.7; P<0.01). Interestingly, leaves sampled from the bottom third of the canopy were chlorotic, a typical symptom of drought stress in *Salix*. This result raises interesting questions about the transport of Cd and Zn within *Salix,* for example, whether leaf Cd and Zn concentrations may increase prior to senescence. Although there appears to be no published work reporting such a process, preliminary results from studies in Austria suggest that this may occur when certain *Salix* species are grown on mine spoil soil (Walter Wenzel, *pers. comm.*). Alternatively, shading in the lower canopy may have limited phloem transport of Cd to sink sites, resulting in greater leaf Cd concentrations. An investigation by Sander and Ericsson (1998) attempted to assess the vertical distribution of metal within *Salix* stems. However, modelling this variability is complex and many parameters such as changes in metal distribution during the year and the life cycle have not yet been addressed. Luyssaert *et al.* (2001) studied the variability of Cd in the leaves of *S. fragilis,* but only modelled the foliar concentrations for a single tree. More work is therefore required to investigate not only the distribution and variability of metal concentrations in leaf and stem fractions, but also the influence of *in vivo* metal transport.

The loss of leaves during periods of drought has important implications for phytoextraction, especially if chlorotic leaves have a higher metal content than healthy leaves. If one third of leaves were lost during the drought period discussed above, this would account for 10 % of the potential Cd off-take in *S. caprea x cineria x viminalis* and 18 % of the potential Cd off-take for *S. dasyclados*.

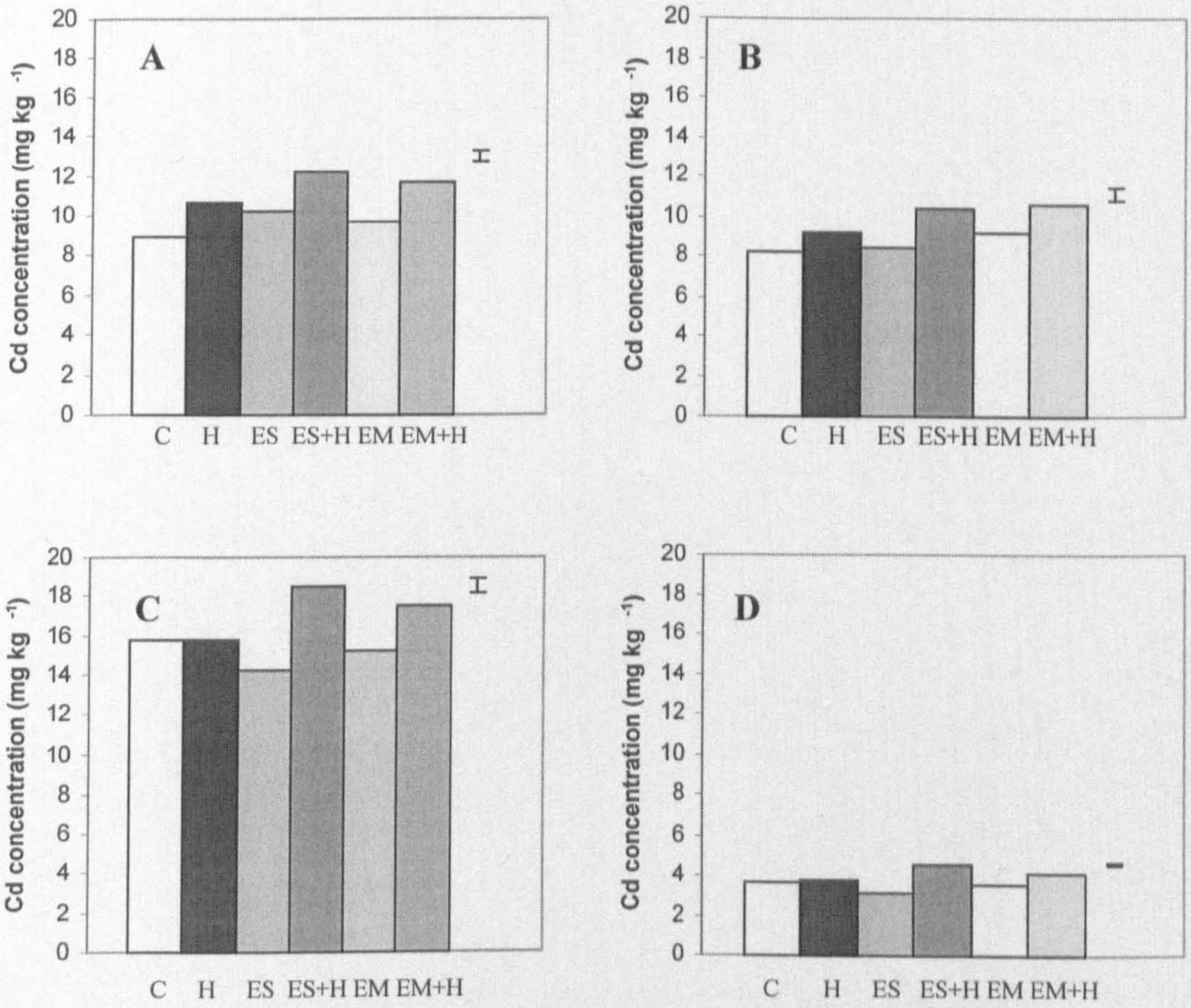


Figure 7.2. Experiment 1, Cd concentration for A; leaf, B; stem, C; bark and D; wood fractions of *S. caprea x cinereria x viminalis* trees following application of various chemical treatments. Treatments included: Control (no treatment; C); HCl applied at a rate of 10 mmol kg^{-1} in late May 2001 (H); EDTA applied at a rate of 2 mmol kg^{-1} as a single dose during mid-August 2001 (ES); combination of treatments H and ES (ES+H); EDTA applied at a rate of 0.5 mmol kg^{-1} in multiple doses (four applications applied at four week intervals between late May and mid-August 2001; EM); combination of treatments H and EM (EM+H). Vertical bars show SED values.

Table 7.4. Mean heavy metal concentrations in the leaves and stems of Sc (*S. caprea x cineria x viminalis)* and Sd (*S. dasyclados)* grown at the Stoke Bardolph field site with and without treatment with 2 mmol kg^{-1} EDTA after 17 months of growth in Experiment 2 (n = 4). Values in parenthesis show standard error of the mean. No statistically significant differences were detected between *Salix* species or between treated and untreated trees. Ni concentrations in stems were below detection for F-AAS.

	Leaves (mg kg^{-1})				Stems (mg kg^{-1})		
	Cd	**Zn**	**Cu**	**Ni**	**Cd**	**Zn**	**Cu**
Sc: No treatment	8.70 (0.17)	404 (7.81)	9.10 (0.18)	10.6 (2.27)	8.50 (0.80)	121 (6.00)	5.90 (0.62)
Sc: + EDTA	9.20 (1.36)	419 (49.6)	9.70 (0.87)	11.4 (3.24)	9.30 (0.27)	133 (4.50)	7.20 (0.43)
Sd: No treatment	14.4 (1.28)	533 (42.9)	8.50 (1.42)	8.4 (3.37)	8.20 (0.51)	97.8 (4.38)	4.13 (0.50)
Sd: + EDTA	13.0 (3.05)	516 (77.9)	7.00 (0.84)	9.9 (3.54)	8.90 (0.56)	119 (10.50)	5.70 (0.74)

Table 7.5. Heavy metal concentrations of leaves sampled from the upper and lower thirds of the canopy of *Salix* trees grown at the Stoke Bardolph field site during a drought period in July 2000. Values within columns and species denoted by ** are significantly different (P<0.01). Between element differences were not stated.

Salix species	Sample location	Cd	Zn	Cu	Ni
			(mg kg^{-1})		
S. caprea x cineria x viminalis	Upper third	8.3 (1.03) **	253.5 (27.20) **	10.3 (0.47)	8.3 (0.97)
	Lower third	22.5 (0.85) **	760.6 (42.50) **	13.0 (1.86)	9.6 (4.77)
S. dasyclados	Upper third	11.0 (0.74) **	348.7 (65.80) **	8.2 (0.67)	9.2 (1.24)
	Lower third	34.2 (2.85) **	978.1 (96.10) **	10.8 (0.53)	12.7 (1.67)

7.3.4 Uptake of heavy metals following application of EDTA

Tissue metal concentrations in *S. caprea x cineria x viminalis* trees were greater following both single and multiple EDTA treatments than in control trees, although the differences were not statistically significant. Cd concentrations in the stem, wood and bark fractions tended to be greater following the multiple dose EDTA treatment than the single dose in Experiment 1, whereas leaf Cd concentration tended to be greater following the single dose EDTA treatment (Fig. 7.2). However, these differences were not statistically significant. Therefore, combining the multiple and single dose treatments of EDTA, may enable the overall leaf and stem Cd off-take to be maximised. Increased redistribution of Cd via the phloem may help explain the increase in stem, wood and bark concentrations of Cd relative to that in the leaves following multiple EDTA treatments, as Cd may have been progressively transported from source leaves to sink organs distributed throughout the plant (Scholz, 1989). To date, only one other field experiment has attempted to enhance metal uptake by *Salix* using chemical interventions (Kayser *et al.*, 2000), although their data are not directly comparable to those obtained in this study because soil with a very low total Cd concentration was used. Nevertheless, injecting NTA into the rhizosphere significantly increased plant Cd uptake in their study although concentrations were still lower than required for successful phytoextraction.

Soil moisture content in the 0 - 20 cm soil horizon in Experiment 1 was greater than or close to field capacity following application of the chemical treatments. However, most roots in *Salix* are located in the surface 60 cm of the soil profile, although they can extend to depths below 1.3 m (Dobson, 1995; Crow, 2001). Therefore, closely matching soil water use by *Salix* within the soil profile, and at greater depths, may help enhance the effectiveness of the chemical treatments. Soil moisture content determined within the *Salix* plots during the drought period (Section 7.2.6), showed greater moisture depletion in the 40 - 60 cm horizon within the *Salix* plots. Soil moisture content as a percentage of field capacity was 47, 35 and 35 % respectively for 0 - 20, 40 - 60 and 80 - 100 cm horizons from within the *Salix* plots. The corresponding values outside the experimental area were 48, 46 and 31 % respectively. Further irrigation to increase soil moisture content at greater depths and

injecting chemical inputs further down the soil profile may have increased their effectiveness. However, leaching of metals has been demonstrated following application of EDTA even at relatively low rates (Section 5.3.6), an effect which would be increased by application of excess irrigation. It is possible that burying a physical barrier in the soil profile, thereby preventing the roots from extending vertically below a defined depth, would increase the uptake of contaminants by confining roots to the contaminated soil zone, within the surface horizons.

A slight, but statistically insignificant, decrease in stem biomass was observed following both EDTA treatments. Weekly sampling of leaves from trees receiving the multiple dose of EDTA showed no significant change in leaf Cd concentration. Although a marked increase in leaf Cd concentration was observed in leaves sampled in the week following the second and third EDTA applications, this increase was not statistically significant and was not observed following either the first or the fourth EDTA applications (data not presented). No significant increase in metal uptake was observed for either of the *Salix* species examined in Experiment 2 following treatment (Table 7.4).

7.3.5 Uptake of heavy metals following application of HCl

The HCl treatment applied in Experiment 1 significantly affected Cd concentrations in the leaf and wood of *S. caprea x cineria x viminalis* ($P < 0.05$). Foliar Cd concentrations following the combined single dose of EDTA with HCl were significantly greater than in the control plots and plots receiving only the multiple dose EDTA treatment ($P<0.05$). A similar increase was observed in the Cd concentration of wood relative to all other plots ($P<0.05$). No other significant effects were recorded following application of HCl, although stem and bark Cd concentrations and Zn concentrations in all fractions were greatest in the combined single dose of EDTA with HCl treatments.

The possible role of HCl in enhancing metal uptake was discussed in detail in Chapter 6. The HCl treatment applied in this study involved a dilute solution which induced no detectable change in bulk soil pH. However, several factors may have contributed to

the significant increase in Cd uptake following HCl application. For example: i) a slight decrease in soil pH within the rhizosphere may have increased Cd solubility; ii) uptake of $CdCl_2$ may have been increased by chloro-complexation following HCl application and iii) it is also possible that damage to the roots following application of HCl to soil may have increased metal influx (Section 6.3). Further investigations into the use of HCl, in the presence or absence of EDTA, would be useful to establish the potential of chemically-enhanced phytoextraction using *Salix*. Any damage to roots induced by HCl applications may lead to a potentially important long-term reduction in biomass, and should also be investigated.

7.3.6 Natural colonisation by mycorrhiza

Ecto-mycorrhizas were not found on the roots of either *S. caprea x cineria x vulgaris* or *S. dasyclados* sampled from Experiment 2. However this was on trees assessed after 18 months growth. It is possible that mycorrhizal colonisation of the experimental plots, will occur over the long term, and future assessments would be useful. The effect of EDTA on mycorrhizal survival could not therefore be determined as no mycorrhizas were present on the roots of *Salix* either in the presence or absence of chelate application. However, the potential beneficial effect of mycorrhizas within phytoextraction systems has been postulated (Ernst, 2000). Thus, future work will be needed to assess the survival of mycorrhizal fungi on soils contaminated with heavy metals, including any consequential effects resulting from the application of chelating compounds.

7.3.7 Estimating the phytoextraction potential for Cd and Zn at the Stoke Bardolph field site using S. caprea x cineria x viminalis *and* S. dasyclados

The estimated times required to reduce soil total Cd and Zn concentrations to the maximum permitted by the 1989 Sludge Regulations (SI, 1989) are shown in Tables 7.6 and 7.7. In these calculations, various assumptions were made as described in Section 4.3.6. For example, it was assumed that a constant soil → plant transfer factor persisted throughout the remediation period and that the labile phase was constantly replenished throughout the same period.

The total Cd content for *S. dasyclados* was approximately three fold greater than that for *S. caprea x cineria x viminalis*, an effect which can be attributed mainly to its greater biomass production. Despite this increase, the time required for remediation would still be c. 700 years, although this represents an improvement of 1724 years compared to *S. caprea x cineria x viminalis*. Total yield for Zn followed a similar trend to that for Cd, with *S. dasyclados* removing approximately three times as much as *S. caprea x cineria x viminalis*. The remediation time for Zn would exceed 1700 years for *S. dasyclados* and 5800 years for *S. caprea x cineria x viminalis* for the Stoke Bardolph field site.

The estimated costs associated with SRC production for biofuel and phytoextraction are shown in Table 7.8. Treatment with either EDTA or HCl represents the single greatest cost; thus application of 10 mmol kg^{-1} HCl would cost over £11500 ha^{-1} for a single treatment. Although the use of SRC for biofuel may be profitable (Table 7.9) producing approximately £ 630 y^{-1} profit over a 6 year period, it is still likely that Government subsidies would be necessary for its widespread commercial adoption (Mick Bates, *pers. comm.*). Clearly phytoextraction costs are much greater than the estimated profit from SRC production (Tables 7.8 and 7.9) and, given the very large time periods required for successful phytoextraction of the experimental site using willow, the cost would be prohibitive. The excavation and burial of contaminated soil from the Stoke Bardolph field site to a depth of 20 cm was estimated to cost up to £220000 ha^{-1} (Section 4.3.6). Consequently, phytoextraction using SRC is unlikely to

provide a realistic option unless a dramatic increase in tissue metal concentrations can be achieved.

To reduce the total soil Cd concentration at Stoke Bardolph below 3 mg kg^{-1} over a 24 year period, the typical length of SRC production, by growing *S. dasyclados* would require a weighted stem and leaf Cd concentration of 297 mg kg^{-1}, approximately 30 times greater than the maximum Cd uptake observed in this *Salix* species during the current study.

Table 7.6. Removal of soil Cd by *S. caprea x cineria x viminalis* and *S. dasyclados* following treatment with 10 mmol HCl kg^{-1} after 14 months of growth, and the number of years required to reduce total soil Cd below 3 mg kg^{-1} (maximum permitted Cd concentration prescribed by the 1989 Sludge Regulations (SI, 1989).

Crop	Weighted Cd concentration in plant (leaf and stem)[1,2] (mg kg^{-1})	Above-ground biomass (leaves and stem) (t ha^{-1})	Removal (kg ha^{-1})	No. years to target[3]
S. caprea x cineria x viminalis	9.41	13.07	0.12	2424
S. dasyclados	10.72	31.53	0.33	700

[1]Weighted tissue concentration for *S. dasyclados* following HCl treatment was estimated based on the assumption that the tissue Cd concentration would be similar to that measured in *S. caprea x cineria x viminalis*.

[2]If the harvest is to include both leaf and stem fractions, this should occur earlier than in normal coppice practice, i.e. during August or September.

[3]Calculation based on soil contamination to 20 cm depth, a bulk density of 1.25 g cm^{-3} and a constant soil → plant transfer factor throughout the remediation period.

Table 7.7. Removal of soil Zn by *S. caprea x cineria x viminalis* following treatment with 10 mmol HCl kg^{-1} after 14 months of growth and *S. dasyclados* following treatment with 2 mmol EDTA kg^{-1} after 17 months of growth. The number of years required to reduce total soil Zn below 300 mg kg^{-1} (maximum permitted Zn concentration prescribed by the 1989 Sludge Regulations (SI, 1989) is also shown.

Crop	Weighted Cd concentration in plant (leaf and stem)[1] (mg kg^{-1})	Above-ground biomass (leaves and stem) (t ha^{-1})	Removal (kg ha^{-1})	No. years to target[2]
S. caprea x cineria x viminalis	169	13.07	2.21	5862
S. dasyclados	198	30.71	6.09	1719

[1]If the harvest is to include both leaf and stem fractions, this should occur earlier than in normal coppice practice, i.e. during August or September.

[2]Calculation based on soil contamination to 20 cm depth, a bulk density of 1.25 g cm^{-3} and a constant soil → plant transfer factor during the remediation period.

Table 7.8. Potential annual costs and income associated with short rotation coppice for biofuel production and phytoextraction.

Costs	£ ($) ha^{-1}	Income	£ ($) ha^{-1}
Fencing	157	Set aside[2]	
Pre-planting spraying	77	Year 1 to 5	220 (309)
Cultivations	70	Year 6 onwards	200 (281)
Planting	200		
Cuttings	800		
Post-planting spraying	84		
Cut back	35		
Initial sign-up fee	125		
Total establishment costs[2,3]	**1548 (2180)**		
Running costs[3]	200	Grant (year 1 only) [2]	1000 (1408)
Annual membership fee[2]	10		
Total running costs	**210 (295)**		
Harvest cost[2]	350	Wood fuel (£44 t ha^{-1}) [2]	880 (1238)
Delivery cost[2]	240	Assume 20 t ha^{-1} y^{-1}	
Landfill cost[4,5] (£54 t^{-1})	17[5]		
Total harvest and disposal costs	**607 (855)**		
Pump and piping ha^{-1}	7000		
Labour treatment^{-1}	408		
Total irrigation costs[6]	**7408 (10435)**		
Solubilising agents ha^{-1}, [7]			
EDTA @ 2 mmol kg^{-1}	29712 (41855)		
HCl @ 10 mmol kg^{-1}	11507 (16210)		

[1] Currency conversion, £ sterling to US $ @ 1.4087 on 20/5/2002.
[2] Nix, 2001.
[3] Bardos *et al.*, 2001.
[4] Tom Diggle, Waste Recycling Group PLC. *pers. comm.*. Price for vegetable waste contaminated with heavy metals.
[5] Assumes ash comprises 10 % of initial biomass and 16 % of the final ash is contaminated with metal, therefore requiring storage (Narodoslawsky and Obernberger, 1996).
[6] Based on piping at 1.5 m spacing at £4 m^{-2} calculated for ¼ ha. i.e. piping moved to treat one ha (Nix, 2001) and 10 man-days required for chemical treatment per ha with no abstraction charges.
[7] EDTA £16 kg^{-1} for minimum of 50 kg; HCl £4.64 l^{-1} (Fisher Scientific 2002).

Table 7.9. Management costs and income associated with short rotation coppice for biofuel production over an example six-year period assuming an annual biomass of 20 t ha^{-1}. Phytoextraction costs are not included in this summary.

Year	0	1 & 2	3	4 & 5	6	Total over 6 years
Income (£ ha^{-1} y^{-1})						
Grant	1000	0	0	0	0	1000
Set Aside	220	220	220	220	200	1520
Sales	0	0	2640	0	2640	5280
Total	1220	440	2860	440	2840	7800
Outgoings (£ ha^{-1} y^{-1})						
Establishment cost	1548	0	0	0	0	1548
Harvest cost	0	0	607	0	607	1214
Running costs	0	210	210	210	210	1260
Total	1548	420	817	420	817	4022
Overall total (£ ha^{-1})	**-328**	**20**	**2043**	**20**	**2023**	**3778**
Average income y^{-1}						630

7.4 Conclusions

The chemical enhancement of metal uptake by two SRC species was tested using two field trials grown on soil heavily contaminated with heavy metals. Two willow species, *S. caprea x cineria x viminalis* and *S. dasyclados,* were grown between April 2000 and October 2001; chemical treatments were made using several different application regimes between May 2001 and August 2001. The widely researched chelate, EDTA, was applied as a single and multiple dose treatment, with and without the prior application of HCl.

No statistically significant increases in metal uptake by either willow species were induced by any of the EDTA regimes applied. However, the Cd concentrations of both leaves and wood were increased significantly relative to the control plants in *S. caprea x cineria x viminalis* following a single application of EDTA provided this was combined with HCl treatment. As no change in bulk soil pH was detected following

the treatment with HCl, consequent changes in the soil solubility of heavy metals can be assumed to be low. It is possible that the application of dilute acid to the soil caused some damage to root integrity, thus causing a greater influx of Cd when coupled with the EDTA treatment. Further investigation of the role of acidification is required, not only in terms of its effects on soil pH and metal solubility, but also with regard to disrupting root membranes.

Although the production of willow for biofuel may be profitable, estimated costs suggest that phytoextraction using willow is unlikely to be cheaper than alternative remediation strategies unless the degree of metal uptake by willow species can be substantially increased. Estimates of the time required to remediate the surface 20 cm soil horizon to Cd concentrations within current legislative thresholds suggest that periods of c. 700 and 2500 years would be required for *S. dasyclados* and *S. caprea x cineria x viminalis* respectively to reduce Cd to below 3 mg kg^{-1}, as prescribed by the 1989 Sludge Regulations. It should be recognised that the solubility of metals at the Stoke Bardolph field site, with its unusually high organic matter and extractable phosphate contents, is difficult to enhance by means of chemical inputs. However, significant increases in metal uptake by *S. caprea x cineria x viminalis* were observed following the combined application of HCl and EDTA, and further increases may be possible. Research to assess the effectiveness of chemical enhancement of metal uptake by willow is still at a very early stage, particularly when compared to equivalent studies of arable crops. Further work is therefore required to fully evaluate the efficacy of phytoextraction involving SRC species.

CHAPTER 8: FINAL DISCUSSION

8.1 Introduction

Phytoextraction has been suggested as a low cost and *in situ* alternative to other remediation methods (Matso, 1995; Boyajian and Carreira, 1997; Raskin *et al.*, 1997) and research into this subject has grown extensively in recent years (McGrath *et al.*, 2001; Section 1.3). Despite this, phytoextraction has not been widely used by the commercial sector or thoroughly tested in field trials. Consequently, few previous estimates for the remediation timescale and costs of phytoextraction have been made, and those that have are often over-simplistic. The present study aimed to test the efficacy of phytoextraction by means of pot and field trials on soil contaminated with a range of metals, including Cd, derived from long-term disposal of sewage sludge. Sewage sludge is a particular concern as the volumes produced in the UK are rising and a larger proportion will be disposed of onto agricultural land in future (DETR, 2000a; Section 1.1.6). Furthermore, legislation controlling disposal of sludge onto land and the concentrations of metals permitted in soils are being tightened (ENV.E3/LM 2000; DEFRA, 2002b).

Three main strategies of phytoextraction have emerged from recent research: i) the use of hyperaccumulator plants; ii) chemically-enhanced metal uptake using arable crops; and iii) the use of short rotation coppice (SRC; Section 1.3). In the present study, pot and field based experiments were conducted to test each of these strategies for use at a sewage sludge processing site under arable cultivation located at Stoke Bardolph, Nottingham, England.

8.2 A comparison of the three main crop systems used for phytoextraction

Field scale studies encompassing all of the main phytoextraction systems were conducted (Chapters 4, 5 and 7) and chemical treatments were applied to enhance metal uptake by arable and SRC species. Based on data derived from these field experiments, estimates were made for the time required to reduce total soil Cd concentration below 3 mg kg^{-1}, the maximum concentration prescribed by the 1989 Sludge Regulations (SI, 1989). For these calculations, it was assumed that a constant soil → plant transfer factor existed throughout the remediation period. This approach may be more realistic than simply dividing the initial plant uptake concentration by the total soil metal mass, as employed by most previous estimates (Baker *et al.*, 1994; Brown *et al.*, 1994; McGrath *et al.*, 2000; Section 1.3.1). The full calculation is explained in detail in Section 4.3.6. The total soil Cd concentration varied for the three crop types assessed: concentrations were 35.3 mg kg^{-1} for *Z. mays* plots, 37.4 mg kg^{-1} for *S. dasyclados* plots and 54.2 for *T. caerulescens* plots. Therefore, calculations of plant uptake were based on a nominal soil Cd concentration of 35.3 mg kg^{-1} and the assumption that the soil plant transfer factor was constant. It was also assumed that the plant-available Cd pool was continuously replenished from less labile forms of Cd throughout the remediation period. Detailed estimates of the costs associated with phytoextraction were also made.

8.2.1 Hyperaccumulator plants

The Ganges population of *T. caerulescens* was chosen for use in this study, as it is the best-known example of a Cd hyperaccumulator (Lombi *et al.*, 2000). However, observed tissue Cd concentrations in Ganges were lower than reported in other studies. The mean Cd concentration in Ganges was 265 mg kg^{-1} at Stoke Bardolph whereas other workers have found 1000 mg kg^{-1} in a wild population (Robinson *et al.*, 1998) and 516 mg kg^{-1} in a sandy sewage sludged soil (Lombi *et al.*, 2000). A stringent liming policy and the large available phosphate and organic matter contents in soil at the Stoke Bardolph field site, which restrict Cd solubility, provide a plausible explanation for this difference. It is therefore likely that the rate at which Ganges accumulated Cd was either limited by the low concentration of Cd^{2+} in the soil

solution or by the rate at which solution Cd was replenished from the labile pool. Consequently the Cd uptake capacity of Ganges was probably not the limiting factor. This is an interesting result, as much research in recent years has focused on enhancing the overall uptake capacity of plants, for example, by using genetic modification (Guerinot, 2000; Karenlampi *et al.*, 2000; Lasat, 2002). However, given the low Cd solubility at Stoke Bardolph, genetic modification approaches may not significantly increase the overall Cd uptake by Ganges. For example, over-expressing the genes responsible for producing metal transporter proteins (Section 1.3.1) might increase the rate of metal transfer, but may not significantly increase overall metal uptake due to low availability in soil at the Stoke Bardolph site. Alternative approaches attempting to increase the soil pool of metal accessible to roots such as inducing proliferation of fine lateral roots may be more suitable (Section 4.3.3).

It is interesting to compare the level of Cd uptake observed in the current study with that achieved by Lombi *et al.*, (2000) who explored the limits for Cd assimilation by the Ganges population of *T. caerulescens*. Lombi *et al.* (2000) grew Ganges in solution culture containing 56 mg Cd L^{-1} and measured 14,000 mg kg^{-1} in plant tissue to give a concentration factor of 250 L kg^{-1}. The average concentration in field-grown Ganges in this study was 265 mg kg^{-1}. In section 6.3.1 the average concentration of Cd in the soil pore water in pots of untreated soil from Stoke Bardolph at field capacity was found to be 0.006 mg L^{-1}. This would suggest a concentration factor for Stoke Bardolph of 44166 L kg^{-1}, which is substantially greater, then the value suggested from Lombi *et al.* (2000). In order to achieve the solution Cd concentration used by Lombi *et al.* (2000) in their solution culture experiment, the pH of the Stoke Bardolph soil would have to be considerably lower. From the equation fitted to Figure 6.1 (Section 6.3.1.) it is possibly to estimate that a soil pH value of 4.2 would be required to produce a concentration of 56 mg Cd L^{-1}. Clearly an adjustment of this magnitude would be completely impractical and would have severe implications for metal toxicity to *T. caerulescens* from Al, Mn, and Cu (Section 4.3.4).

To reduce soil Cd concentration to below 3 mg kg^{-1} at the Stoke Bardolph field site, without enhancing Cd solubility in the soil, would take c. 502 years, assuming a planting density of 50 plants m^{-2} with one crop grown per year (Table 8.1). Table 8.1

clearly shows the importance of planting density, biomass and the number of crops grown per year on Cd uptake by Ganges. However, due to the length of the growing season, planting more than one crop per year would be dependant on the extremely expensive procedure of using pre-germinated seedlings (Table 8.1). If a planting density of 90 plants m^{-2} was used and if two crops were grown each year, the overall remediation time could be reduced to c. 139 years (Table 8.1). However, to successfully reduce soil Cd concentration below 3 mg kg^{-1} in 24 years, (the normal growing period for short rotation coppice, used here for comparison) a tissue Cd concentration of 3430 mg kg^{-1} would be required (Table 8.2). Increasing the planting density to 90 plants m^{-2} and the number of crops grown each year to two, would reduce the required tissue Cd concentration to reduce the soil Cd concentration below 3 mg kg^{-1} in 24 years, to 955 mg kg^{-1} (Table 8.2). However, this concentration is still 3.5 times greater than the observed Cd concentration found for field-grown plants in unaltered soil, from this study.

There are other potential problems with successful husbandry of *T. caerulescens* as a field-grown crop, especially in relation to insect and fungal damage. Despite the claim that hyperaccumulation occurs to prevent herbivory, it was found that there was substantial damage from flea beetle attack and a high plant mortality rate was observed after 7-10 weeks growth.

Remediation of the 0 - 20 cm soil profile of the Stoke Bardolph field site was estimated to cost between £120000 and £220000 ha^{-1} using simple excavation and disposal methods. Therefore, phytoextraction growing two crops of Ganges each year is unlikely to be cost effective (Tables 8.1 and 8.2). However, growing only one crop per year would be cheaper than the excavation and burial example, providing tissue metal concentrations required to achieve remediation targets in 24 years were possible (Table 8.2).

8.2.2 Chemically-enhanced metal uptake using arable crops

Apart from this study there are very few field-scale trials of phytoextraction using arable crops and chemical enhancement (Section 5.3.4). Unfortunately, observed

metal uptake by the arable crops tested in this study was low, even after the application of chemical inputs, such as EDTA, glyphosate and KCl, to enhance plant Cd uptake. Pot studies showed that the application of combined chemical treatments, such as EDTA, HCl and glyphosate, significantly increased Cd uptake by *Z. mays*. However the concentrations were still much lower than required for successful remediation.

It is estimated that to reduce the soil Cd concentration below 3 mg kg^{-1} at the Stoke Bardolph field site using *Z. mays* would take over 2600 years, if a concentration of 10 mmol EDTA kg^{-1} were applied (Table 8.1). As EDTA is expensive, c. £30000 for a single treatment of 2 mmol kg^{-1} ha^{-1} treatment (Section 5.3.7) the associated remediation costs using *Z. mays* are prohibitive. Furthermore, downward migration of metal was observed through the soil profile following EDTA application (Section 5.3.6). For example, the soil Cd concentration in the 0 - 10 cm profile was reduced from 32 to 25.5 mg kg^{-1} seven months after application of 10 mmol EDTA kg^{-1}, and only 1 % of this reduction could be accounted for by *Z. mays* Cd off-take. This is the first measurement of metal leaching in field soils following chemically-enhanced phytoextraction and highlights one of the main difficulties for achieving successful phytoextraction: enhancing plant metal uptake by increasing soil solubility without inducing metal leaching. Furthermore, an EDTA extraction experiment (Section 3.3.1) demonstrated that the efficiency of chemically-enhanced phytoextraction where successive plant cropping and application of EDTA were employed would be substantially reduced once the labile Cd pool was removed. This suggests that the estimated time required for soil remediation using chemically-enhanced phytoextraction (Table 8.1) would be increased still further if the requirement existed to remediate beyond removal of the labile pool. Moreover, soil remediation by simply removing the plant-available metal fraction may not be acceptable to regulatory authorities, as legislative guidelines currently rely on total metal thresholds (Section 3.3).

To successfully reduce soil Cd concentration below 3 mg kg^{-1} in 24 years, a tissue Cd concentration of 690 mg kg^{-1} would be required in *Z. mays* (Table 8.2). This is approximately 90 times greater than the maximum observed in the field experiments.

Given that the estimated costs for chemically-enhanced phytoextraction using *Z. mays* are large (Tables 8.1 and 8.2) and greatly exceed the estimated cost for remediation using the excavation and burial technique, it appears unlikely that chemically-enhanced metal uptake by *Z. mays* can be adapted to provide a viable phytoextraction strategy for the Stoke Bardolph field site.

One possible advantage associated with the use of arable crops lies in the fact that adverse health effects arising from chemical treatments delivered shortly before harvest may not matter to the same extent as in the case of hyperaccumulators grown in pre-treated soil. Damage to the growing plant is part of the mechanism for enhancing uptake. However, the evidence from field-measurement of metal leaching down the soil profile suggests that it is possible to enhance metal solubility over a considerable time period in the field without achieving a proportional increase in metal uptake. This contrasts with the use of hyperaccumulators where the greatest scope for improvement appears to lie specifically with increasing divalent metal activity in the pore water without inducing toxicity from other metals. Thus, future work with large biomass crops should probably focus on the development of agents which can induce greater transfer, of complexed metal forms, from the soil solution into roots and thereafter to shoots. It is known that root-to-shoot transport of metals in chelated forms occurs (Collins *et al.*, 2002) and so the critical step may be the initial uptake from the soil solution. Measurements in Section 5.3.4 did not suggest the accumulation of large root metal concentrations.

One possibly solution to the risk of leaching is to use impermeable membranes as a base for re-stacked topsoil, possibly with re-circulated leachate, and so utilize phytoextraction as an on-site rather than *in-situ* technique.

8.2.3 Short rotation coppice

Metal concentrations in leaf and wood fractions of *Salix caprea x cineria x viminalis* increased significantly following combined applications of both EDTA and HCl. However, Cd concentrations were still low compared to the levels required for viable phytoextraction. Application of EDTA alone, at 2 mmol kg^{-1}, did not significantly

increase tissue Cd concentrations. However, *S. dasyclados* achieved a greater off-take of Cd relative to *S. caprea x cineria x viminalis*, although this was largely due to the production of a larger biomass. Stem biomasses for *S. caprea x cineria x viminalis* and *S. dasyclados* were 11.8 and 25.4 t ha^{-1} respectively; leaf biomasses were 2.0 and 6.1 t ha^{-1} respectively. Unlike either hyperaccumulators or high biomass arable crops, a market for short rotation coppice used in phytoextraction exists in the form of biofuel. However, calculations discussed in Section 7.3.7 suggest that any financial advantage is negligible when the additional phytoextraction costs are considered in comparison to normal short rotation coppice expenses.

To reduce the soil Cd concentration below 3 mg kg^{-1} using *S. dasyclados* would take 684 years and cost an estimated £ 7.9 m ha^{-1}, if an annual application of 10 mmol HCl kg^{-1} was applied (Table 8.1). Although this is a substantial improvement on the use of *Z. mays*, the remediation time is clearly not realistic. To reach the same soil Cd target in 24 years, would require an initial weighted Cd concentration for both leaf and stem fractions of 274 mg kg^{-1}, approximately 25 times greater than the value observed in the present study (Table 8.2). One major limitation to the use of SRC, which will be difficult to overcome, is that the use of large concentrations of solubilizing and enhancing agents, which can be deployed for annual crops, is probably non-viable. Any treatment, which is intended to damage roots, will clearly threaten the long-term survival of the willow trees. However, in comparison to the other phytoextraction strategies, metal uptake by willow, especially following chemical enhancement, is still at a very early research stage, therefore further optimisation and enhancement of metal uptake by willow is likely.

Table 8.1. Cd removal, number of years required to reduce soil Cd to below 3 mg kg^{-1} (maximum permitted concentration prescribed by the 1989 Sludge Regulations; SI, 1989) and the estimated cost for representative crops using the three main phytoextraction systems: i) hyperaccumulation; ii) chemically-enhanced uptake using large biomass arable crops; and iii) short rotation willow coppice.

Crop type	Chemical treatment	Removal (kg ha^{-1})	Years to target[1]	Overall cost of remediation (£ m ha^{-1})
Z. mays	10 mmol EDTA kg^{-1}	0.09	2680	341.20
S. dasyclados	10 mmol HCl kg^{-1} [1, 2]	0.33	684	7.90
T. caerulescens (Ganges) [3] 50 plants m^{-2} 1 crop y^{-1}	None	0.66	502	0.46
T. caerulescens (Ganges) [3] 50 plants m^{-2} 2 crops y^{-1}	None	1.33	279	5.67
T. caerulescens (Ganges) [3] 90 plants m^{-2} 1 crop y^{-1}	None	1.20	251	0.23
T. caerulescens (Ganges) [3] 90 plants m^{-2} 2 crops y^{-1}	None	2.40	139	5.09

[1] The calculation is based on soil contamination to a 20 cm depth, a bulk density of 1.25 g cm^{-3} and a constant soil → plant transfer factor. Values are derived from the field experiments discussed in Chapters 4, 5 and 7. Total soil Cd concentrations for the different crop types varied; the concentrations on different plots were 35.3 mg kg^{-1} for *Z. mays*, 37.4 mg kg^{-1} for *S. dasyclados* and 54.2 mg kg^{-1} for *T. caerulescens*. However, the calculations presented in this table were based on a total soil Cd concentration of 35.3 mg kg^{-1}. The plant uptake concentrations for *S. dasyclados* and *T. caerulescens* were therefore adapted, based on the assumption that the soil → plant transfer factor was constant.

[2] Cd removal by *S. dasyclados* following HCl application was estimated based on the assumption that an increase in plant tissue Cd concentration would be comparable to that measured for *S. caprea x cineria x viminalis*.

[3] The single crop of *T. caerulescens* is assumed to be planted by direct drilling, whereas growing two crops per season are assumed to be pre-germinated seedlings. This greatly affects the planting costs. Values for plants grown at the density of 90 plants m^{-2} are estimated, and assume a linear response in biomass production based on a planting density of 50 plants m^{-2}.

Table 8.2. The shoot Cd concentration required to reduce total soil Cd below the 3 mg kg^{-1} maximum permitted by the 1989 Sludge Regulations (SI, 1989) within 24 years, i.e. the normal period of growth for short rotation willow coppice and the estimated cost for representative crops using the three main phytoextraction systems: i) hyperaccumulation; ii) chemically-enhanced uptake using large biomass arable crops; and iii) short rotation willow coppice.

Crop type	Chemical treatment [1]	Biomass ($t\ ha^{-1}$)	Required concentration in shoot ($mg\ kg^{-1}$) [2]	Overall cost of remediation (£ $m\ ha^{-1}$)
Z. mays	10 mmol EDTA kg^{-1}	12.5	690	3.50
S. dasyclados	10 mmol HCl kg^{-1} [3]	31.53 [4]	274 [4]	0.27
T. caerulescens (Ganges) [5] 50 plants m^{-2} 1 crop y^{-1}	None	2.51	3435	0.02
T. caerulescens (Ganges) [5] 50 plants m^{-2} 2 crops y^{-1}	None	5.02	1720	0.48
T. caerulescens (Ganges) [5] 90 plants m^{-2} 1 crop y^{-1}	None	4.51	1915	0.02
T. caerulescens (Ganges) [5] 90 plants m^{-2} 2 crops y^{-1}	None	9.02	955	0.87

[1] Chemical treatment details correspond to those applied to the field experiments and enable estimate costs to be calculated. However, it should be noted that the chemical treatments outlined did not produce the plant metal uptake concentrations shown.

[2] The calculation is based on soil contamination to a 20 cm depth, a bulk density of 1.25 g cm^{-3} and a constant soil → plant transfer factor. Values are derived from the field experiments discussed in Chapters 4, 5 and 7. Total soil Cd levels for the different crop types varied; the concentration for *Z. mays* was 35.3 mg kg^{-1}, for *S. dasyclados* was 37.4 mg kg^{-1} and for *T. caerulescens* was 54.2 mg kg^{-1}. However, the estimate presented by this table was based on a total soil Cd concentration of 35.3 mg kg^{-1}. The plant uptake concentrations for *S. dasyclados* and *T. caerulescens* were therefore adapted, based on the assumption that the soil → plant transfer factor was constant.

[3] Cd removal by *S. dasyclados* following HCl application was an estimate based on the assumption that an increase in plant tissue Cd concentration would be comparable to that measured for *S. caprea x cineria x viminalis*.

[4] Values are weighted for biomass and Cd concentration for both leaf and stem fractions.

[5] The single crop of *T. caerulescens* is assumed to be planted by direct drilling, whereas growing two crops per season are assumed to be pre-germinated seedlings. This greatly affects the planting costs.

8.3 Future work

The results from this study demonstrate, at least for heavily contaminated soil derived from the long-term disposal of sewage sludge, that phytoextraction does not yet provide a realistic remediation tool. Furthermore, many problems will need to be overcome if phytoextraction is to become both feasible and cost effective. The scope for future scientific work into phytoextraction is therefore considerable, but increasing the number of field scale experiments will be important, particularly for helping make the step between theory and practical remediation. Specific areas of potential future work are outlined below.

The primary goal for improving uptake by hyperaccumulators is increasing the activity of free divalent metal ion in the soil solution without inducing toxicity of other metals at the same time. The planting density and biomass production of hyperaccumulator plants are also clearly important in influencing overall metal off-take (Tables 8.1 and 8.2). Therefore, optimising the planting density and researching possible methods for enhancing biomass, such as fertilisation, would be useful. This study and one of the other few field experiments in which the hyperaccumulator *T. caerulescens* was grown (Kayser *et al.*, 2000) both demonstrated substantial plant mortality. Therefore, an improved understanding of the pests and diseases which may affect hyperaccumulator plants would also be helpful. As Cd uptake by the Ganges population of *T. caerulescens* was limited at the Stoke Bardolph field site by the low concentration of Cd in solution, work to increase the size of the soil Cd pool accessed by plants is also required. Increasing root density and proliferation of fine lateral roots may provide progress on this matter.

The main problem to overcome in terms of the commercial production of arable or SRC species for phytoextraction is how to economically increase total metal solubility in the rhizosphere without increasing the downward migration of metal through the soil profile. Coupled with this problem is the need to induce some degree of root membrane damage to increase metal influx and increase the transport of metal from roots to shoots. None of these problems have yet been resolved. Rooting depth is also likely to be important, as soil contamination in agricultural soil is primarily located in

the surface 0 - 20 cm of the soil profile. However, the roots of SRC species are predominantly located in the surface 0 - 60 cm soil horizon and can extend to below 1.3 m (Dobson, 1995; Crow, 2001). Therefore, the use of a physical barrier to restrict vertical root development may help increase metal uptake by SRC species in the surface soil horizons. Similarly, to overcome metal leaching, the use of an impermeable barrier, possibly with re-circulation of leachate, may be necessary. Combination of phytoextraction with other remediation techniques such as soil washing may be a fruitful partnership to pursue.

Given that very little previous research effort has been directed at predicting the time required for soil remediation using phytoextraction, modelling metal uptake by a range of potentially viable phytoextraction species over a range of contaminated sites would be beneficial. This would help identify those contaminated sites where phytoextraction may offer a viable soil remediation tool, thus focusing research effort on a narrower remediation goal.

CHAPTER 9: REFERENCES

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APPENDIX

Example of an ANOVA table from a pot experiment described in Section 3.2.4. This consisted of a factorial experiment testing the response of applications of 20 mmol EDTA kg^{-1} and 25 mmol KCl kg^{-1} to seven plant species (Section 3.2.4). Plant species were: *Thlaspi caerulescens*, Ganges and Prayon populations; *Zea mays*; *Brassica juncea*; *Linum usitatissimum*; *Brassica napus* and *Beta vulgaris*.

Source of Variation	d. f.	s.s.	m.s.	v.r.	F pr.
Block	4	330.6	82.7	0.66	
Plant[1]	5	13839.7	2767.9	22.01	<0.01
App[2]	3	8876.4	2958.8	23.53	<0.01
Plant. App	15	7435.4	495.7	3.94	<0.01
Residual	87	10941.0	125.8		
Total	114	40584.0			

[1] 'Plant' refers to factor: plant species used

[2] 'App' refers to factor: chemical application used