The Phytoremediation Potential of *Salix*: Studies of the Interaction of Heavy Metals and Willows

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Summary

The work reported in this thesis comprised part of the BIORENEW project, a study of the remediation of industrially degraded land with biomass fuel crops, which involved collaboration between seven European research groups. This work examined willows and their interaction with heavy metals, as the genus *Salix* features many species known to colonise contaminated soils. It aimed to ascertain their biomass production, metal tolerance and metal accumulation under various conditions, and the effect of their growth on soil heavy metal distribution.

Stem and leaf samples were regularly taken from trees growing on a sludge-amended soil, to gauge the important seasonal and vertical distributions of metals in field-grown willows. Tissue samples were collected over two and a quarter years, a time period which encompassed a growing season of 5-year-old trees, a harvest, a period of re-growth and a growing season of 2-year-old trees. Samples taken from different heights revealed a trend of increasing metal concentrations with height in the bark and wood tissues, and a decreasing trend with height in leaves. This sampling height effect was more pronounced for less translocatable elements such as Cu and Pb, and markedly less so for the more readily translocated elements Zn and Cd. The observations in wood and bark are likely to be a result of upward translocation of metals in xylem and phloem and decreasing stem girth with height, while the leaf patterns are possibly due to increased biomass of leaves with height, and greater deposition of metals in lower leaves.

Younger, post-harvest tissue samples displayed a tendency to have greater Cu, Zn and Cd concentrations than those in the 5-year-old, pre-harvest trees; re-growth following the harvest of the trees led to uptake being high relative to biomass production. Metal concentrations in leafed wood samples frequently revealed a growth dilution effect between March and June. Leaf concentrations fell towards the end of the growing season, a probable combination of the effects of growth dilution and back-translocation prior to leaf abscission. Concentrations of metals rose during this period in the wood; this stem fraction received back-translocated foliar metals. Additionally, there may have been a concurrent net flux of metals from the bark, either to the wood or the roots: concentrations fell from July or September to November for all elements in the leafed bark fraction.

The effect of tree growth on the heavy metal distribution in soils was examined through analysis of samples taken from field trials. At two sites, selective extraction provided evidence for depletion of extractable metals through biomass crop growth, when compared to concentrations in adjacent unplanted areas. Plant concentrations demonstrated that metal sequestration in stems could only account for a fraction of the observed depletions; therefore redistribution of metals amongst the soil solid phases was considered likely. Sequential extractions of metals from samples collected at a third site demonstrated some evidence for this: where trees were growing or were recently harvested, extractable concentrations frequently fell significantly in the period from March to June, with a corresponding rise in the residual fraction. Resin samplers did not record any significant increases in leached metals during the experimental period.

Soil solution extractions revealed significantly higher metal concentrations in planted soils than in unplanted control soils. This is likely to be due to increased complexation of heavy metals by soluble chelating agents exuded by plant roots. Significant increases in the soil solution Pb concentrations six months after tree harvest was interpreted as an effect of root degradation. Coupled with this, microbial respiration in harvested soils displayed significantly increased rates.

The effect of willow growth on the metal distribution of a contaminated steelworks waste was considered in a nine month pot trial. Selective extractions approximately targeted metals bound on exchange sites and in the organic/Fe oxide pool, and repeatedly indicated depletion of metals in these fractions as a result of tree growth. These reductions were not apparent in samples taken from control pots; this observation also indicated that leaching of metals was not significant in this trial. The results from sequential extractions suggest the metal reductions can mainly be explained by redistribution to the carbonate-bound fraction, and other more resistant pools.

To assess whether extraction of metals by willows from soil could be enhanced, the effects of two soil amendments on the biomass production and metal uptake of willows were gauged in a pot trial. The citric acid regime resulted in positive yield increments in willow aerial tissues. This frequently corresponded with significant increases in the metal quantities achieved in the biomass. The fertilisation and enhanced metal uptake observed is thought to have arisen as a consequence of microbial activity following citric acid degradation. In contrast, the $(NH_4)_2SO_4$ treatment led to significant yield reductions; this may have been due to a combination of lowered pH and elevated tissue metal concentrations.

As the *Salix* genus has a clone specific capacity for heavy metal uptake, hydroponic experiments were carried out with the goal of developing a rapid test to distinguish willow varieties by their biomass production in conditions of metal exposure. The important factors of nutrition, pH and levels of exposure to toxic metals were assessed. The effects of the treatments on the biomass production and heavy metal uptake of two willow clones, Germany and Q83, were gauged. Initially, it was ensured that the performance of the two willow varieties was distinguishable, and not compounded by nutrient limitations; a suitable nutrient strength (1/4 strength Hoaglands solution) was selected from a tested range. Significant differences in biomass production, relative to the control, were effected after 4 to 6 weeks. Greater quantities of metals were translocated to leaves in the more nutritious regimes. In the lowest nutrient strength (1/16 strength Hoaglands solution), Q83 demonstrated a breakdown in the root sequestration of metals: concentrations and quantities of Cu, Pb and Cr in leaves vastly increased in week 6. The biomass parameters showed clear evidence for Germany being less susceptible to metal toxicity than Q83; the superiority of Germany was also displayed in the larger quantities of metals accumulated.

Following this, the optimum pH and P nutrition regimes which allowed maximum biomass production of the metal-exposed trees, were identified for the rapid test. The biomass was highest in a regime of pH 5.5 compared to in pH 3.5 and 7.5 treatments. The former caused reduced biomass production, possibly through metal toxicity, while the latter caused more marked reductions, an effect likely to have been caused by nutrition alteration: hydroponic solutions are prone to macronutrient precipitation in conditions of high pH. Hence the nutrient medium in subsequent experiments was altered to provide N as NH_4^+ as well as NO_3^- to improve buffering and maintain the pH around the optimum level. To improve the practicality of supplying P and increase tree replicate biomass, Pb was excluded from the metal cocktail and P was supplied for 7 days per week in all experiments thereafter. These adjustments succeeded in increasing the biomass of metal-exposed trees, and improving the differentiation of clones, in the rapid screening test.

Metal interaction experiments confirmed Germany to be superior to Q83 in terms of biomass production and metal uptake. A significant finding of a Cu-Ni experiment was the difference in metal toxicity response of the clones at elevated ratios of one metal to another. Relative to control values, the biomass of Germany showed a greater reduction in conditions of elevated exposure to Ni, while elevated Cu exposure caused more marked reductions in Q83. No differences in varietal response were apparent in a Zn-Cd interaction experiment: Germany consistently performed better than Q83. The clones displayed differences in the partitioning of Zn, however: Germany sequestered greater quantities in the leaves than Q83 did, while the reverse was true in the stem fraction.

Finally, it was assessed whether results from the rapid screening test for 18 willow varieties could be extrapolated to the field. This could potentially allow identification of willows suitable for planting in metal-contaminated substrates, without necessitating long-term field trials. Therefore the hydroponic data and independent field data for the clones were statistically compared. Significant correlations between the two datasets were established. Hence, relative performances of clones in the rapid screening test broadly corresponded with those in the field. Despite the influence of outliers (willow varieties which did not have comparable performances in the NFT and field), distinct groupings in the results were apparent. Certain *Salix viminalis* clones, as well as the basket willow Black Maul, proved to the least metal resistant. An intermediate group included Jorunn, Coles, Ulv and Q83, while the hardiest clones were Germany, Dasyclados, Candida and Spaethii.

The least resistant clones achieved high concentrations of Cu and Ni in the bark of field-grown trees, and featured low biomass production in the glasshouse and field. The most resistant clones proved to have superior biomass production in the glasshouse and field, and achieved high metal concentrations in the wood of field-grown trees. These clones constitute a suitable group for planting at contaminated sites where soil polishing is feasible. The intermediate group accumulated concentrations of contaminant metals in the wood of field-grown trees that were comparatively low, while their performance in the rapid screening test was reasonable. These varieties may be suitable for planting where site phytostabilisation is desirable, but metal phytoextraction is not.

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Dedication

This tome is dedicated to my parents, the beloved Crumblies (not that I ever expect them to get past the title page). They have always been hugely supportive in terms of encouragement and finance. Enormous thanks to them. Here is their son at the height of his intellectual powers, spending a morning alternately swearing and wretchedly gawping at a faulty pump.

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Author's Declaration

I hereby declare that the work reported in this thesis has been performed by myself, and that it has not been accepted in any previous application for a degree. All sources of information have been specifically acknowledged by reference to the authors.

[redacted]

Conor Watson

List of Abbreviations

Acetic acid	HAc
Atomic Absorption Spectrometry	AAS
European Community Bureau of Reference	BCR
Biomass yield	Bio
European Commission contract ENV4-CT97-0610: Bioremediation and Economic Renewal of Industrially Degraded Land by Biomass Fuel Crops	BIORENEW
Citric acid	CIT
Dicyandiamide	DCD
Deionised water	DIW
Dissolved Organic Carbon	DOC
Diethylenetriaminepentaacetic acid	DTPA
Endomycorrhizal fungus/fungi	EDM
Ethylenediaminetetraacetic acid	EDTA
Ectomycorrhizal fungus/fungi	EMF
Inductively Coupled Plasma Mass Spectrometry	ICP-MS
Inductively Coupled Plasma-Optical Emission Spectroscopy	ICP-OES
Infra Red Gas Analyser	IRGA
Low Density Polyethylene	LDPE
Modified Hoaglands Solution	MHS
Mycorrhizal	MYC
National Accreditation of Measurement and Sampling	NAMAS
Nutrient Film Technique	NFT
Ammonium acetate	NH_4OAc
Hydroxylammonium chloride	NH_3OHCl
Nitrilotriacetate	NTA
Principal Components Analysis	PCA
Polyvinyl chloride	PVC
Short Rotation Coppice	SRC
Treatment: Control Ratio	TCR
United Kingdom Accreditation Service	UKAS
Visual Assessment	VA
Water Research Centre, Medmenham	WRc
1/4 strength Modified Hoaglands Solution plus metals	1/4 + M
1/8 strength Modified Hoaglands Solution plus metals	1/8 + M
1/16 strength Modified Hoaglands Solution plus metals	1/16 + M

Chapter 1 Introduction

1.1 Sources of Heavy Metals in Soil Environments

There are two inputs of metals to the environment: natural (volcanic and weathering processes) and anthropogenic sources. The latter can dwarf the former: for example, more than one hundred times more lead can be input to ecosystems from anthropogenic sources than by natural processes (Ross, 1994a). Igneous and metamorphic rocks are the most common natural source of trace metals in soils. The easily weathered minerals augite, hornblende and olivine contribute significant amounts of nickel, copper and zinc to their soils. Natural atmospheric input of Pb and Ni by volcanic activity is relatively high.

Heavy metals are therefore ubiquitous in soil parent materials. Adding to this, there are at least five sources of anthropogenic heavy metal contamination in soil-plant systems (Alloway, 1995a):

- Metalliferous mining and smelting can lead to multi-element contamination through the weathering of heaps, and dust created by smelting. An estimated 4000 km^2 of agricultural land in the U.K. have been contaminated by one or more metals as a result of metalliferous mining. Mine tailings are estimated to account for around 50 % of the global input of Cu into the soil environment (Merrington, 1995).
- Incineration plants and industries involved in the manufacture of metal commodities, can emit metal aerosols such as those of Cd and Pb. Magazines and papers are notable sources of Pb and Zn, while discarded batteries and some plastics contain Cd at relatively high levels. Meneses *et al.* (1999) recorded increased soil concentrations of heavy metals near an old municipal solid waste incinerator.
- Fossil fuel combustion leads to further atmospheric deposition of heavy metals, including Pb, Cd, Cr and Zn (Alloway, 1995a).
- Agricultural amendments such as pesticides and inorganic fertilisers (containing heavy metal impurities) and manures/sewage sludges are also important sources, and it is not uncommon for their application rates to soils to be controlled as a way of limiting the metal loadings. Cadmium, Zn and Pb are the three main metals concentrated in sludge; significant amounts of Cr and Cu can be added too (Ross, 1994a).

- Disposal of municipal and industrial waste can lead to several heavy metals including Cd, Cu, Pb and Zn being dispersed into soil (Alloway, 1995a).

1.2 Behaviour of Heavy Metals in the Soil-Plant System

A good understanding of soil properties and conditions which influence metal reactions and transformations is required in assessing the behaviour of heavy metals in soil. Soil factors principally affecting metal solubility are pH, soluble organic matter, the type and amount of minerals present and redox potential.

The fate of heavy metals in soils also depends on the initial chemical form of the metal and the types of plants in the soil-plant system. The complexity of soil metal reactions and transformations explains why it is difficult to predict soil metal mobility, retention and bioavailability.

1.2.1 Processes controlling metal retention and mobility

Ross (1994b) outlined the following processes governing the movement and retention of heavy metals in soils:

- Cation exchange.
- Specific adsorption.
- Precipitation and co-precipitation
- Dissolution and solubility of minerals and complexes.
- Chelation.
- Uptake by plants.
- Immobilisation by soil microorganisms.
- Leaching.

Cation exchange is a reversible process which depends on the density of the negative charges on the surfaces of soil particles. Specific adsorption, or chemisorption, involves exchange of cations and anions with surface ligands to form partly covalent bonds with lattice ions of minerals. Adsorption/desorption affects the partitioning of trace elements between the solid and aqueous phase of the soil (Banuelos and Ajwa, 1999). When physico-chemical conditions and concentrations of appropriate ions permit, insoluble precipitates of heavy metals form. In co-precipitation, mixed solids, commonly Fe/Al and Fe/Mn oxides, form, in which isomorphous substitution has occurred to fix metal cations. Solid phase and soluble organic compounds are involved in metal complexation. Humic acid, an example of the former, forms insoluble chelate complexes, whereas low molecular weight organic ligands (such as amino acids and polysaccharides) can form soluble complexes and prevent adsorption or precipitation (Alloway, 1995b). Retention processes are generally much more important than leaching processes.

Cadmium, Cu, Pb, and Zn are restricted to one oxidation state in natural environments, the divalent cation, while Cr has more complex behaviour (Banuelos and Ajwa, 1999) as it can exist in several oxidation states, principally Cr (III) and Cr (VI). Cation exchange sites are important in retaining Cd^{2+} and Zn^{2+}. Less abundant soil solution ions such as Cu^{2+} must compete for cation exchange sites with more abundant Ca^{2+} and Mg^{2+}; retention on the solid phase of the soil is chiefly by chemisorption onto oxides and chelation by organic matter (Ross, 1994b). Adsorbed forms of Cu, are more stable than Cu precipitates under most conditions. However, precipitation is important in controlling the amount of Pb^{2+} in the soil solution. For example, chloropyromorphite is the least soluble of the lead phosphate minerals and could control the solubility of Pb in soils of high P status (Alloway, 1995b).

1.2.2 Processes controlling metal bioavailability to plants

Studies of metal uptake by plants have, in most cases, not taken metal bioavailability into consideration, and so the performance of the same species may differ considerably at different locations. Soil heavy metal concentration is not the only factor to be considered; metal toxicity and mobility is also affected by their physicochemical form and association with soil constituents, or speciation (Charlatchka and Cambier, 2000).

Alloway (1995b) listed the factors affecting the amount of metal absorbed by a plant as those regulating:

- the concentration and speciation of the metal in the soil solution
- the movement of the metal from the bulk soil to the root surface
- transport of the metal from the root surface into the root, and

- translocation from the root to the shoot.

Concentrations of metals in plant tissue are an integrated result of all factors which have affected uptake to the time of sampling (Riddell-Black, 1993). Cadmium and Zn have greater transfer coefficients (metal concentration in plant tissue above ground/ total concentration in soil) than Cu, Cr and Pb due to their relatively high mobility in the soil profile and within the plant. Plant growth can lead to increased soil organic matter, soil pH changes and improved soil structure (hence improved aeration and drainage), all of which affect heavy metal mobility and the size of the bioavailable heavy metal fraction. Metal toxicity responses in plants may be determined by soil parameters which can modify metal bioavailability to such an extent that tolerance becomes unnecessary (Eltrop *et al.*, 1991).

The pH of the soil is the most important factor controlling the solubility and bioavailability of heavy metals, affecting competitive proton sorption, precipitation-dissolution reactions and pH-dependent metal complexation by humic materials (Banuelos and Ajwa, 1999). Heavy metal mobility decreases with rising pH due to their precipitation in hydroxides, carbonates, organic complexes and their increased adsorption onto clay minerals and organic matter. Despite acidification increasing their solubility, plant uptake of heavy metals may decrease in these conditions due to increased competition with H^+ ions around the root.

Changing soil conditions affect metal mobility; alternate aerobic and anaerobic conditions lead to changes in pH and redox potential. Charlatchka and Cambier (2000) demonstrated the influence of reducing conditions on metal solubility of a polluted soil. Increased waterlogging led to reductive dissolution of ferric and manganic oxides and organic acid formation. This, in turn, lowered the pH which further enhanced metal solubility. These effects are limited by denitrification which consumes protons and organic compounds. In conditions of prolonged flooding, metals may be fixed by formation of precipitates such as sulphides, and readsorption of the metals onto these precipitates.

Uptake of metals may be strongly affected by ionic interactions. For example, phosphorus interferes with the uptake and translocation of metal ions such as zinc and lead. Thus the nutritional status of the soil has a considerable effect on the bioavailability of toxic metals to plants.

1.2.3 *Effects of soil amendments on heavy metal distribution and bioavailability*

The origin of the metal can affect its bioavailability. For example, Cd from inorganic sources such as mining or smelting is more readily accumulated in plants than that from sludge amendments, due to the latter increasing the adsorptive capacity of the soil (Alloway, 1995c). Therefore, the sludge itself is often the major factor determining the bioavailability of sludge-born metals rather than the soil it is amending (Sidle and Kardos 1977). Walter and Cuevas (1999) reported pH increases in a sludged soil led to a high proportion of Cd being precipitated. Hasselgren (1999), however, observed sludge application to slightly decrease pH due to the nitrification of ammonia in the applied sludge; this effect was more marked in the deeper soil layers as the buffer capacity of the organic topsoil counteracted the effect of nitrification.

Following termination of sludge application, soil is expected to revert to a new equilibrium with respect to heavy metal inputs. Metals added to soil are frequently in soluble fractions, but revert to less soluble oxide and residual fractions with time (Shuman, 1991). McGrath and Cegarra (1992) investigated the effects of sludge application on metal concentrations and found they were increased in at least one of the four metal pools extracted, in the first ten years of a 40-year experiment. After this time, the chemical forms of the metals changed little. Walter and Cuevas (1999) also found sludge applications to have changed the metal distribution in soil 5 years after application. Metals quantities increased, but this occurred in the more resistant fractions (organically bound and precipitated pools). Berti *et al.* (1997) also observed sludge amended soils to contain greater proportions of metals in the less available fractions compared to untreated soils.

As the chemical activities of the sludge-borne heavy metals approach a new balance, the plant bioavailability of the soil-borne metals will also be readjusted. Therefore, the plant availability of metals in previously sludge-treated soils is expected to reach a constant level in the long term. The yield increase due to sludge application does not necessarily entail an increase in accumulated metal: de Villarroel *et al.* (1993) reported no increase in Zn taken up by Swiss chard on sludge-treated soil. The rate of Zn desorption from the soil was suggested for this.

Fertilisers can alter soil pH and surface charge or directly react with heavy metal ions in soil. Phosphorus excesses can reduce Cd uptake by plants, while P application can enhance

Cd uptake through increased root growth and hence access to residual Cd (Mench, 1998). Tu *et al.* (2000) investigated the effects of NPK fertilisers on the distribution of Pb and Cd. Urea application increased soil pH, thus decreasing the exchangeable metal pool, while the P application may have caused a reduction in this pool via enhancement of hydroxide adsorption of metals. Metal concentrations in this fraction increased following K application, which may have been due to competitive exchange between K and heavy metal ions on soil surfaces.

1.2.4 Sources and behaviour of six heavy metals in the soil-plant system

Agricultural amendments represent the main input of Cd to soils, whereas atmospheric deposition is the principal flux of several heavy metals to soils, including Pb and Zn. In soils, heavy metals are often concentrated in surface horizons as a result of this atmospheric deposition, as well as cycling through vegetation and adsorption by organic matter (Banuelos and Ajwa, 1999).

1.2.4.1 Cadmium

Cadmium has no essential biological function and is highly toxic to plants and animals. The soil background levels of 0.06-1.1 mg kg^{-1} can be considerably increased by atmospheric deposition from the metallurgical industry and waste incineration, sludge application and impurities from phosphatic fertilisers (Alloway, 1995c). It is usually concentrated in surface horizons of soils where it is retained by the organic matter. However, unlike strongly adsorbed metals such as Cu and Pb, it can move down the soil profile depending on soil and site factors. In the soil solution, the Cd^{2+} free ion is the principal and most phytotoxic species, but organic and inorganic complexes also exist, such as $Cd(OH)_3^-$. In the majority of polluted soils, adsorption processes rather than precipitation appears to control the distribution of Cd between soluble and soil-bound forms (Alloway, 1995c). Raising soil pH decreases solution Cd concentrations due to increased hydrolysis, adsorption density and pH-dependent negative charge.

Observed linear relationships between Cd concentrations in plant tissue and soil show that total soil Cd is one of the major factors affecting plant concentrations. Relative excesses of Cu, Ni and P can reduce uptake by plants. Conversely, Pb is preferentially adsorbed by soil solid phases, leaving more Cd in solution and thus having a synergistic effect on Cd uptake. It is readily translocated to shoots after root absorption and can also be effectively absorbed into foliage and translocated.

1.2.4.2 Chromium

The essentiality of Cr to plants has not been demonstrated (McGrath, 1995). Disposal of fly ash onto land and urban sewage sludge can introduce considerable quantities of Cr into soils. The most stable and common forms of Cr are Cr (III) and the phytotoxic Cr (VI). Phytotoxicity of Cr to plants is decreased by reducing the strongly oxidising Cr (VI) species to Cr (III), which is much less mobile and absorbs to particles more strongly. This reaction occurs in the presence of organic matter such as in sewage sludge. Above pH 4, its solubility decreases, and above pH 5.5 it is completely precipitated (usually in oxides and hydroxides) or organically complexed. Uptake by roots and transport to aerial tissue, therefore, is minimal at a near-neutral pH. Small plant-available concentrations are reflected in low plant concentrations, and foliar concentrations show little relationship with overall soil Cr concentrations (McGrath, 1995). Regardless of the Cr form taken up, most Cr remains in the roots.

1.2.4.3 Copper

An essential plant micronutrient, Cu is both an activator and a component of some enzyme systems. Higher Cu concentrations are found in the soil surface, where the soil may receive inputs from smelter deposition, fertilisers, sludge, fungicides and manures from poultry and swine fed Cu-containing compounds. The distribution of copper between the soil solution and solid phase is principally controlled by specific adsorption (Baker and Senft, 1995). Its associations with organic matter are the strongest, followed by those with Fe and Mn oxides, soil silicate clays and other minerals. There are two forms of available ions: $[Cu(H_2O)_6]^{2+}$ in acid soils, and $Cu(OH)_2^0$ in neutral and alkali soils.

The COO- groups present in the solid and liquid phase organic matter form stable ligands with Cu; solid phase ligand formation is considered responsible for Cu deficiencies in organic soils and for decreases in Cu toxicity to plants from the addition of a high organic matter source to a high Cu-containing substrate (Baker and Senft, 1995). As soil organic matter is the dominant factor controlling Cu retention, the rate of decomposition of sludge organic matter therefore becomes a prime consideration.

Rates of absorption of Cu into plants are among the lowest of the essential elements. Higher activities of Zn^{2+} or Cu^{2+} in the soil solution is antagonistic to uptake of either ion, as it is thought that both ions are absorbed the same way. Ions of Ca, K and NH_4 also reduce Cu absorption, probably via differential complexing and other surface effects.

1.2.4.4 Lead

Lead is one of the most persistent heavy metals: it has an estimated half-life in most soils of more than 1000 years (Merrington, 1995). Uncontaminated soil concentrations are generally <20 mg kg^{-1}; low level contamination has raised concentrations overall to 30-100 mg kg^{-1}.The soil is often considered as a sink for anthropogenic Pb, the major sources of which are mining and smelting, manures, sewage sludge, pesticides and vehicle exhausts (Davies, 1995). Like Cu, it accumulates naturally in the surface soil horizons, with the organic fraction being largely responsible for the fixation, although the precipitated pool of Pb can be considerable. In the soil solution, it occurs as cationic and neutral inorganic species, with some organic complexation existing. It is accumulated at the endodermis, which acts as a partial barrier to translocation to the shoot (Davies, 1995). Overall, its solubility, mobility and hence bioavailability are low.

1.2.4.5 Nickel

More than 80 % of Ni emissions are anthropogenic in origin, and land disposal of fly ash and urban sewage sludge can be a major source of Ni in soils. Nickel is mobile in the soil profile relative to Cu, but less mobile than Zn and Cd. Its soil chemistry is based on the divalent Ni^{2+}, which is increasingly soluble at lower pH. Of secondary importance in determining its distribution between the solid and solution phase are clay content and the amount of hydrous Fe and Mn oxides. Concentrations of Ni in plants generally reflect soil concentrations, although they are more directly related to the concentration of soluble Ni ions and the rate of replenishment of the mobile pool (McGrath, 1995).

1.2.4.6 Zinc

Atmospheric fall-out from the burning of fossil fuels and the smelting of non-ferrous metals, sewage sludge, manure and agrochemical impurities, are anthropogenic sources of soil Zn. As a component of a large number of enzymes, it is involved in carbohydrate and protein metabolism. It is considered, with Cd, to be a very mobile and bioavailable metal. It is adsorbed principally onto clay minerals, hydrated metal oxides and organic matter. The cation can form inorganic complexes with Cl, P, SO_4, NO_3 and OH. Fulvic acids and low molecular weight organic acids mainly form soluble Zn complexes and chelates. Zinc-humate complexes are not formed at low pH due to the insolubility of humic acids.

Zinc is predominantly absorbed as a divalent cation, the main species in the soil solution below pH 7.7 (Kiekens, 1995), although soluble complexes and organic Zn chelates can be absorbed by the roots. Zinc-phosphorus interaction is one of the best known interactions in soil chemistry and plant nutrition: high P may decrease Zn availability and uptake. It is also thought Zn interactions with Fe, Cu, N and Ca may decrease Zn availability to plants. Generally, increases in soil Zn concentrations cause increases in plant tissue concentrations.

1.2.5 Summary

The movement and retention of heavy metals in soils are governed by various processes, some of which are more important to different metal ions than others. Cation exchange and specific adsorption are the major mechanisms controlling Ni, Cd and Zn movement between the soil solution and solid phase, whereas organic complexation of Cr, Cu and Pb is substantial. Soils may also contain a sizeable precipitated pool of Pb. Processes affecting heavy metal retention, mobility and plant uptake and translocation control the bioavailability of a metal, differences in which can explain contrasting performances of the same species at different locations. Soil parameters such as redox potential, nutritional status and soluble organic matter can modify metal bioavailability hugely, but the pH of the soil is the single most important factor as metal precipitation and adsorption increases with rising pH. The origin of the metal also affects its bioavailability.

1.3 Toxicity of Heavy Metals to Rhizosphere Organisms

Numerous studies have demonstrated the toxicity of metal ions to plants, resulting in reduced or altered growth patterns. In metal-contaminated soils, inhibition of microbial processes and selection of metal-tolerant microbes is evident.

1.3.1 Plant uptake of heavy metals

Plants can solubilise metals by exuding protons from roots to acidify the rhizosphere. Root deposition of organic matter (such as exudates and sloughed-off cells) stimulates soil biochemical activity, which can also mobilise adsorbed metals by inducing rhizosphere acidification and organic-metal complexation (Alloway, 1995b). Plants produce their own metal-chelating compounds (phytosiderophores) such as avenic acids, possibly in response to Zn deficiencies (Salt *et al.*, 1998), which mobilise Cu and Zn in the rhizosphere.

Heavy metal absorption by plants occurs via the root or leaf stomata. Root uptake can be either passive (such as for Pb) or active (as in uptake of Cu and Zn). Uptake involves a reversible exchange of cations or an irreversible uptake of metals bound to macromolecules. Within the root, the endodermis represents a barrier to inward diffusion of some metal ions to the xylem - such membrane exclusion will result in accumulation of ions in the free space of the root, and no participation in biological reactions. Metal passage from the root to the shoot through xylem occurs via ion exchange or organo-metallic complexes. Upward transport of elements can be highly selective; consequently, the solution reaching the ultimate destination of xylem in the leaves can have a very different composition to the one absorbed by the roots (Epstein, 1972). Particularly in the roots, the cell wall can be an important accumulator of metals, while in the leaves and stems the cell vacuole is the main place for deposition (Fargasova, 1998).

Foliar absorption of heavy metals depends on many factors, including plant species, nutritional status, thickness of cuticle and the age of the leaf (Alloway, 1995b). Some metals such as Pb do not penetrate the cuticle of higher plants but rather adhere to the leaf surface. Within leaves, metals are incorporated into proteins or are translocated in the phloem.

Relative differences in metal ion uptake between plant species is genetically controlled and may be due to various factors including the surface area of the roots and leaves, root cation exchange capacity, root exudates and the rate of evapotranspiration (Alloway, 1995b).

The common biological feature of heavy metals is that they are toxic to most plants in large concentrations and metabolised to the extent where the metal has damaging effects on the plant. This can arise through the blocking of functional groups of important molecules such as polynucleotides, the displacement/ substitution of essential metal ions from biomolecules, the denaturation or inactivation of enzymes, and the disruption of cell/organelle membranes (Ross and Kaye, 1994). The combination of these effects required to produce visible or noticeable toxicity symptoms in the plant will depend on the plant species and ecotype, as well as the soil and environmental conditions (Ross and Kaye, 1994). Such symptoms include inhibition of photosynthesis and respiration, alteration of plant-water relations, and the increased permeability of the root plasma membrane. Table 1.3.1 gives typical concentrations of some heavy metals in soils, the soil solution and plants.

Table 1.3.1 Typical concentrations of some heavy metals in soils, the soil solution and plants*

Element	Normal range in soil (total), $\mu g\ g^{-1}$ dry wt	Concentration in soil considered toxic (total), $\mu g\ g^{-1}$ dry wt	Average, $\mu g\ g^{-1}$ dry wt	Soil solution concentration considered toxic, $mg\ l^{-1}$	Normal range in plant material, $\mu g\ g^{-1}$ fresh wt	Concentration in contaminated plants, $\mu g\ g^{-1}$
Zn	10 - 300	70 - 400	50	< 0.005	8 - 400	100 - 400
Cd	0.01 - 7	3 - 8	0.06	0.001	0.2 - 0.8	5 - 30
Cu	2 - 100	60 - 125	30	0.03 - 0.3	4 - 15	20 - 100
Ni	10 - 1000	100	40	0.05	0.02 - 5	10 - 100
Pb	2 - 200	100 - 400	10	0.001	0.1 - 10	30 - 300
Cr	5 - 1000	75 - 100	100	0.001	0.03 - 15	5 - 30

*Adapted from Ross (1994a)

1.3.2 Effects of heavy metals on trees

Heavy metal contaminated soil may prove to thwart successful seed germination and subsequent seedling establishment (Lepp, 1977). Numerous dose-response experiments, exposing young trees to metals, have been carried out to establish the effects of heavy metals on tree roots. These have focused on the stimulation and depression of growth parameters such as root elongation, biomass production, and the disturbance to the mineral nutrition of tree roots. These parameters ultimately have an effect on the above-ground tree morphology. Biomass production of shoots has been found to be diminished by metal toxicity more severely than the roots, resulting in an increase in the root/shoot ratio (Kahle, 1993).

Numerous studies have focused on the impact of individual heavy metals on trees. Different metals, however, can frequently be found in high concentrations at the same location. Carlson and Bazzaz (1977) grew seedlings of American sycamore on soil treated with various concentrations of Pb, Cd or both, and noticed an important synergistic Pb-Cd interaction which affected root, new stem and foliage growth. In general, at the higher treatment concentrations each growth parameter was reduced more by the combined treatment. Burton *et al.* (1986) questioned the relevance of studies assessing the effects of single heavy metals on trees, to a situation where more than one metal contaminates the substrate, each of which can influence plant growth and the pattern of uptake of other metals in a different way. This occurs often due to the natural associations between contaminating metals in a substrate, such as Cu and Ni. To investigate any interactive effects of Cd, Cu and Ni on trees, they grew Sitka spruce seedlings in nutrient solutions containing combinations of these metals. The results indicated an additive toxic effect of Cd and Cu when both elements were present at concentrations which individually reduce yields.

Generally, tree species cannot exclude trace metals in the rhizosphere from their roots. The form of heavy metal complexes may have a strong bearing on their toxicity to tree roots; organic forms of Pb have been found to be considerably more toxic to spruce seedlings than $PbCl_2$ (Kahle, 1993). Different metals may inhibit root growth through different processes, and there may be different toxicity effects within different regions of the root. Generally, an increase in metal supply has been found to inhibit root dry matter production, with combined effects of metals usually having a more detrimental effect than separately applied metals (Kahle, 1993). Inhibition of root elongation and root browning are also symptoms of metal toxicity (Punz and Sieghardt, 1993).

The architecture of the root system may also be markedly changed by metal toxicity; the growth of the primary root was reported to have been significantly reduced in beech by Cd and Pb exposure, with a corresponding increase in the number of lateral roots (Kahle, 1993). However, some experiments have found root branching to be reduced, such as in Sitka spruce seedlings exposed to Cd, Cu and Pb (Kahle, 1993).

Water and nutrient uptake are also affected by toxic heavy metals. Ion competition and displacement of the nutrient cations can occur at uptake sites. Decreased fresh to dry weight ratios of roots with increasing metal supply have been recorded in roots, with increasing metal supply (Kahle, 1993). This partly arises from reduced water uptake due to arrested root development, but may also be due to increased resistance to water flow arising from enhanced root suberization or lignification as a result of metal toxicity. Plant cell membranes are generally considered the primary sites of metal injury, and enzymes involved in nitrogen metabolism have been reported to be disturbed by several heavy metals in tree species (Kahle, 1993).

1.3.3 Effects of heavy metals on soil microbial processes

Heavy metals can detrimentally affect soil microbial populations and processes, such as the decomposition of organic matter and the mineralisation of nitrogen. Lepp and Eardley (1978) noted possible damaging effects of long term application of heavy metal contaminated sewage sludge, including inhibition of soil processes such as nitrification, and production of foliage with high metal content, which could reduce the decomposition rate and hence cause a reduction in cycling rates of all nutrients. The abnormally large organic matter accumulations on the soil surface of an oak woodland ecosystem was attributed, by Martin and Bullock (1994), to an inhibition of decomposition closely related to aerially deposited Cd and Zn.

The symbiotic N-fixing bacteria species *Rhizobia* may be sensitive and show toxic effects of Zn (Kiekens, 1995). Wheeler *et al.* (2001) reported nodulation and nitrogenase activity of alder seedlings in symbiosis with N-fixing *Frankia* bacteria to be reduced markedly by elevated Ni concentrations. Soil biological activity such as enzyme activity and nitrification may be much more sensitive to Cu inputs than higher plant toxicity (Baker and Senft, 1995). McCarty *et al.* (1999) correlated various soil biological activities (such as respiration and phosphatase activity) with soil extractable heavy metal content, and observed no apparent relationships in diversely contaminated soils. However, Reynolds *et al.* (1999) described decreasing microbial species diversity with increasing metal content in metal-contaminated soils. In contaminated amenity soils, bacteria isolated were considerably more resistant to Zn and Cu than those isolated from an uncontaminated garden soil, indicating a selection of tolerant species (Bridges, 1989).

1.3.4 Effects of heavy metals on mycorrhizal fungi

Soil fungi may vary in sensitivity to different heavy metals. Using *in vitro* cultures, Colpaert and van Assche (1987) compared the metal tolerance of some common mycorrhizal (MYC) fungi of pine and birch trees collected from heavy metal contaminated sites, with strains of the same species from non-polluted soils. Most of the strains from polluted soils were strongly tolerant relative to those from uncontaminated sites. They suggested that MYC fungi are strongly selected for metal tolerance in polluted soils. Bell *et al.* (1988) reported a reduced active mycorrhizal root tip count in soil samples collected from a soil naturally enriched in Cu, Pb and Zn relative to a control site. This was attributed to either heavy-metal inhibition of mycorrhizal development, or a reduction in root biomass associated with soil heavy-metal enrichment.

Natural MYC fungi were reported by Berry (1985) to not associate as readily with loblolly pine seedling roots grown on sewage sludge-amended soil, as those grown in fertiliser-amended soil. Toxicity of the sludge-borne heavy metals to the MYC fungi was considered an important factor in this. Harris and Jurgensen (1977) sampled fungi-inoculated willow and poplar roots from trees grown in Cu mine tailings and found no MYC associations had developed. Elevated quantities of Cu may have inhibited fungal infection. Dixon and Buschena (1988) observed that heavy metals, particularly Cd and Ni, reduced MYC fungi colonisation rates of pine and spruce seedlings. Chappelka *et al.* (1991) investigated the effects of Pb on the MYC fungi colonisation of pot-grown loblolly pine. The fungitoxicity of Pb to certain strains of MYC fungi, and alterations in MYC fungi species composition as a result of increasing concentrations of soil-applied Pb, was demonstrated.

1.3.5 Summary

Plants solubilise heavy metals in soil through rhizosphere acidification and production of metal-chelating compounds. Metal absorption is passive or active and occurs via the root or leaf stomata. Heavy metals are toxic to most plants in large concentrations through effects such as the inactivation of enzymes and the disruption of cell membranes. More than one heavy metal can contaminate a substrate, and each might impinge upon plant growth in a different way, or synergistically interact to affect plant biomass production. Various dose-response experiments on tree seedlings have shown heavy metals to detrimentally affect root elongation, biomass production and water and nutrient uptake.

The biomass production of shoots is usually more diminished by metal toxicity, resulting in an increase in the root/shoot ratio.

Soil microbial processes such as organic matter decomposition and nitrification can be inhibited by elevated concentrations of heavy metals, resulting in reduced cycling rates for all nutrients in a soil. Some microbial processes may be more sensitive to certain heavy metals than the toxic response of higher plants. Mycorrhizal fungi vary in sensitivity to heavy metals- the fungitoxicity of metals to selected strains has been demonstrated. There may be strong selection for metal tolerance in polluted soils, leading to an alteration in the mycorrhizal fungi species composition.

1.4 Counteracting Strategies of Plants to Heavy Metal Toxicity

Baker (1981) classified plants colonising metalliferous soils on the basis of three different mechanisms enabling them to tolerate metal toxicity through internal detoxification rather than suppressed uptake. 'Accumulators' can concentrate metals in plant parts from low or high background levels, with root uptake and transport to the shoot more or less in balance. 'Excluders' maintain low shoot metal levels over a wide range of external concentrations through differential uptake and transport between root and shoot, up to a critical point where the mechanism breaks down and unrestricted transport ensues. 'Indicator' plants take up and accumulate metals in proportion to the metal levels in the soil.

These metal uptake and accumulation mechanisms may differ for each metal and operate independently. The partition ratios between shoot and root of metal concentrations in the tissue are consistently different between species. For example, the leaf/root ratio for Zn in *Thlaspi alpestre* of 2.54 suggests accumulation is the detoxifying mechanism, whereas a value of 0.32 for the same metal in *Armeria maritima* points to exclusion (Baker, 1981). Baker concluded his view of metal tolerance as a syndrome of adaptations at the cellular and biochemical level in which the three mechanisms differ in their sites of detoxification. But he pointed out that a species may act as an 'accumulator', an 'indicator' and an 'excluder' over different ranges of soil metal concentrations.

The most clear-cut separation between tolerant and non-tolerant plants is in their ability to establish, survive and reproduce in metal-contaminated substrates (Baker and Walker, 1989). Problems in interpreting these tolerance measurements arise in substrates contaminated by more than one metal; multiple tolerance can arise. Also, co-tolerance, or tolerance to metals which are not present in the immediate environment of the plant, can occur (Baker and Walker, 1989).

The success of all species that grow in contaminated substrates cannot always be explained by metal-specific tolerances that have evolved at the ecotype level. Tolerance may be a coincidence of adaptation to other stress factors such as drought and low nutrient availability. In some cases, no differences in tolerance may be detected between some species that grow in contaminated soils when compared with the same species from an unpolluted soil in reciprocal growth experiments. This is because metal stress resistance has both genotypic and phenotypic components. The former may only be altered through selection, while the latter comprises physiological adaptations within boundaries defined by the genotype. Therefore, metal tolerance is plastic: it can be induced or "lost" (Baker and Walker, 1989).

Examining the mechanisms providing plants protection against excessive heavy metal uptake, six distinct processes have been documented:

- Heavy metals can be chelated by two major classes of metal-binding polypeptides known in plants: metallothioneins and phytochelatins. The former are gene-encoded, the latter enzymatically synthesised, and both are low molecular weight polypeptides (Salt *et al.*, 1998). Production of these proteins has been demonstrated in culture experiments to be inducable through exposure to Cd, Pb, Cu and Zn (Ross and Kaye, 1994). Subsequently, the metal ion can be compartmentalised in the cell vacuole by these, or complexed in the cytoplasm.

- Exudation of organic ligands can lead to chelation of metals in the rhizosphere, following changes in root cells as a result of metal toxicity (Salt *et al.*, 1998).

- Detoxicification of heavy metals in plant tissues may be achieved through precipitation. For example, intra- and extracellular precipitation of Pb as sulphides and phosphates is likely to play a role in Pb detoxicification (Salt *et al.*, 1998).

- Cellular resistance to elevated concentrations of a metal may rely on enhanced plasma membrane resistance to the metal or repair of the metal-induced membrane damage.

- There is limited evidence to show the toxicity of Cr can be reduced in plants through biotransformation; the element is chemically reduced and/or incorporated into organic compounds (Salt *et al.*, 1998).

- Active metal efflux was reported as a process for controlling metal accumulation by Ross and Kaye (1994), especially Al.

These mechanisms may be complemented to a certain degree by mycorrhizal symbiosis (see section 1.5.3), fine root turnover, root redistribution to uncontaminated zones of soil, and selective metal uptake by roots whereby toxic metal ions are excluded (Kahle, 1993). Resistance mechanisms differ for separate pollutants, and tolerance to one metal is generally regarded as independent of tolerance to others.

1.5 Metal Tolerance In Trees

Metal-tolerant plant species in contaminated environments are favoured by their ability to survive, or competitively exclude non-tolerant species. The long generation time of trees prevents a rapid selection of tolerant genotypes, the production of which is random or induced by the pollutant (Dickinson *et al.*, 1991a). Therefore tree species are generally not able to adapt to high heavy metal concentrations in their rhizosphere, resulting in the evolution of only a few metal-tolerant ecotypes of tree species (Kahle, 1993). A characteristic feature of metalliferous soils in Europe is the absence of woody and tree species (Turner, 1994). However, the lack of reported toxicity symptoms in trees indicate their tolerance mechanisms allow higher heavy metal concentrations to be withstood than in agricultural crops (Riddell-Black, 1993). Trees that are not especially selected for metal tolerance can generally survive in metal-contaminated soil, albeit usually with a much reduced growth rate (Dickinson *et al.*, 1992). Restricted location of metals in roots and low uptake into foliage is the most common resistance trait (Dickinson and Lepp, 1997).

True tolerance requires the development of one or more precise physiological mechanisms with a genetic basis (Dickinson *et al.*, 1991a). However, there is much interest in the ability of trees to acclimate to pollution; the genetic stability of tolerance is questionable following experiments proving that it can be induced or lost in trees. The ability to acclimatise to fluctuating stresses may be very important in survival of tree species.

Dickinson *et al.* (1992) described tolerance and survival of plants on metal-contaminated soils as arising from "an orchestrated multiplicity of physiological and biochemical responses, including both avoidance and true resistance mechanisms". This section will outline the importance of both the wide genome of a tree species and facultative tolerance (or phenotypic plasticity) of individual trees to survival in a heavy metal-contaminated soil. It also considers various indices of metal tolerance studied in trees, and counteractive strategies of trees to metal toxicity.

1.5.1 Indices of metal tolerance in trees

Turner *et al.* (1991) investigated the tolerance of sycamore trees from Pb and Cu contaminated and control sites, to elevated Pb and Cu concentrations, using various indices. Different methods provided conflicting assessments of tolerance. They found pollen germination to be a useful index, while cell suspension cultures proved to give a more consistent measure of tolerance and cell growth compared to callus cultures. Overall, there was no clear evidence of increased Pb or Cu tolerance of sycamore seedlings from the contaminated sites. However, the results suggested there may be increased tolerance of some trees at the contaminated sites which is not reflected in the tolerance of seedlings: Pb concentrations in the flowers at the contaminated site were about seven times greater than those at the control site.

Dickinson *et al.* (1991b) took sycamore samples from an area in the vicinity of a Cu/Cd alloying plant. Growth studies on seedlings failed to show any evidence for differences in tolerance between trees from the contaminated site and a control site. Shake flask cultures from shoot callus samples did, however, reveal notable differences between those derived from mature trees at the contaminated and control sites; the former were considerably more tolerant of Cu. The authors conceded that, while it may be unrealistic to extrapolate the results of cell culture experiments to adult plants, the deduction that pollution stress has caused an alteration of gene expression which has provided an adaptation in the mature plant, is a valid one.

1.5.2 Studies using tree seedlings

Most investigations into the metal tolerance of trees have been based on the response of tree seedlings to elevated metal levels. Turner and Dickinson (1993) questioned whether studies on seedlings accurately reflect the responses of older trees to metals. Mature trees differ from seedlings in a number of ways, including carbon allocation, canopy structure and the fraction of photosynthetically inactive tissue, which increases with age (Turner, 1994). Seedlings are much more sensitive to adverse conditions, leading to false indications of the accumulation capacities of mature plants (Riddell-Black, 1993). Nevertheless, it has transpired through various studies that the adaptations of trees to their soil environment is of considerable importance.

Borgegard and Rydin (1989) considered the effects of heavy metals on root development in birch trees which had colonised soil covering copper mine tailings. Roots had penetrated into the spoil across the site, displaying an example of a species, not especially selected for metal tolerance, also not being excluded from a substrate containing high heavy metal concentrations. Thus some tree species on contaminated sites may have an innate tolerance, but trees lacking this may not be completely excluded.

Experiments comparing the growth of tree seedlings from trees in metal-polluted and uncontaminated areas have failed to demonstrate the existence of tolerance traits (Dickinson *et al.*, 1991a), implying that the adaptation of individual mature plants may be the most significant factor which determines the ability to survive pollution. This view was consolidated by Turner and Dickinson (1993). Growth of seedlings of sycamore collected at metal-contaminated and control sites, in both metal-amended nutrient solutions and reciprocal transplant experiments in soils, were compared. Metal tolerance was not detected. Even in contaminated soils, most seedlings survived for at least three years despite impaired growth. They suggested that some low level of innate tolerance may exist in the large genome of the trees, but facultative tolerance is very important, such as the observed proliferation of fine roots in uncontaminated zones of the soil. Root growth was 75% greater in pots containing a lower amount of contaminated soil.

Watmough and Dickinson (1995) summarised this as a strategy of avoiding toxicity, removing selection pressure and negating the necessity for the evolution of tolerance. In a heavy metal-contaminated, sycamore-dominated woodland, metal stress was withstood due to the variability in the large genome of trees, and the avoidance of the most toxic areas by individuals. Phenotypic plasticity permitted avoidance of the surface layers of soils where aerially deposited metal contaminants are usually retained.

Screening sixteen willow clones for resistance to copper in solution culture, Punshon *et al.* (1995a) found differences in metal uptake patterns and root production. Considerable variability in copper resistance was recorded between willow species and hybrids. The degree to which observed resistance was genetically determined or induced by the environment was unclear. Eltrop *et al.* (1991) investigated the soil factors determining the degree and type of heavy metal tolerance of natural tree populations on lead-mining sites. A calcium-dependent mechanism of lead tolerance was suggested for *Salix caprea*, whereas the status of plant phosphate nutrition was considered to be the major factor governing lead tolerance in silver birch.

1.5.3 The effect of mycorrhizal symbiosis

The absence of mycorrhizal (MYC) fungi may account for poor establishment of planted trees on some soils, including metal-contaminated soils. Ordinarily, MYC reduction of metal toxicity to plants is greater in acidic soils, when heavy metals are more mobile than in more neutral conditions. The heavy metal tolerance of plants may vary with MYC status. The amelioration of metal toxicity to plants by MYC fungi has been well documented, but the exact mechanisms for different species are unclear. MYC fungi can counteract metal toxicity in an indirect way, by enhancing uptake of nutrients such as phosphorus, a benefit which may overrule a negative effect of toxic metal exposure.

Direct detoxification of the metals occurs through passive binding to the fungal cell walls or the polysaccharide slime surrounding the fungal hyphae, or by intracellular sequestration in the fungus. A change in the metal binding capacity of the plant cell wall as a result of MYC fungi colonisation may also act to increase plant resistance to a metal (Pierzynski *et al.*, 1992). Denny and Wilkins (1987) investigated the amelioration of Zn toxicity to birch by a MYC fungus frequently found on metalliferous spoils. Their results revealed Zn adsorption to the hyphae surface, cell walls and polysaccharide slime as the fungal mycelium colonised fresh soil. A similar Zn sequestration mechanism has been described in associations between MYC fungi and Scots pine roots by Campbell and Jones (1995).

Factors such as pH, initial soil concentration of heavy metal, host-symbiont compatibility and metal-induced inhibition of root colonisation by the fungi all affect the ability of the fungi to reduce metal toxicity in plants (Shetty *et al.*, 1994). Fungal tissues have a limited metal accumulation capacity, and so fast-growing fungi with a rapid turnover of tissue would be able to accumulate more metals than slow growing species (Colpaert and van Assche, 1987). The amount of fungal biomass produced may greatly determine the ability of a species to protect the host from heavy metal toxicity; those producing little biomass do not retain much metal and may even increase metal uptake in plants compared to noninoculated plants (Colpaert and van Tichelen, 1996). The saturation of fungal hyphae metal binding sites causes the breakdown of the sequestration mechanism, and the subsequent inundation of the tree with the metal. Ernst (1985) reported that MYC fungi, in the long term, could not prevent translocation of metals to their pine and birch hosts in a highly contaminated soil. Dixon and Buschena (1988) also found the ameliorating effect of MYC fungi on pine and spruce seedlings had a saturation point: in conditions of high metal contamination, metal concentrations in seedling foliage were similar in inoculated and control replicates.

While there is little evidence of genetically based metal tolerance in trees, the shorter lifestyle of a fungus may facilitate genetic change in the root-fungus complex within the lifetime of an individual plant (Wilkinson and Dickinson, 1995). This may mean that plant responses to soil conditions attributed to phenotypic plasticity may actually be due to genetic changes which are confined to the MYC fungi communities rather than the plant.

1.5.4 Summary

Various mechanisms exist which provide plant protection against the effects of excessive metal uptake. These adaptations lead to internal detoxicification of the metal rather than suppressed metal uptake, and include chelation and compartmentalisation of the metal within the plant, precipitation and enhanced membrane resistance. These mechanisms are complemented by root avoidance and rapid root turnover. The long generation time of trees prevents a rapid selection of tolerant genotypes, leading to the evolution of only a few metal-tolerant ecotypes. However, the wide genome of trees and facultative tolerance, such as in the redistribution of roots to less contaminated zones of soil, allows survival of trees not selected for metal tolerance on polluted soils, albeit with a reduced growth rate. The acclimation of trees to metal stress has been studied using various indices. Pollen germination has proved to be a useful index, and cell suspension cultures are more worthwhile than variable callus cultures. Seedling growth is the most commonly used index, despite their greater sensitivity to adverse conditions than mature trees. The adaptation of individual mature plants may be the most significant factor in tree survival in metal-contaminated soil, although soil fertility can be important (such as the role of Ca and P in Pb tolerance), and metal tolerance may be increased via colonisation by mycorrhizal fungi, the hyphae of which can sequester metal ions.

1.6 Phytoremediation of Heavy Metal Contaminated Land

Decontamination of heavy-metal contaminated soils using metal immobilisation or extraction by physicochemical techniques may be hindered by cost and difficulty. Many of these methods are generally only appropriate for small areas where rapid, complete decontamination is required, and often render the soil nearly devoid of biological activity and have an adverse effect on soil structure and fertility. Microbial remediation often requires significant engineering costs to produce an environment in which the organisms can grow, such as in the provision of energy sources and in creating the appropriate redox and water potential. Also, bringing the organisms into contact with the contaminated soil may require physical disruption of the site, threatening site destabilisation and increased mobilisation of the contaminant (Stomp *et al.*, 1993). Consequently, the low-technology, in situ approach of phytoremediation is attractive as it offers site restoration and partial decontamination (Baker *et al.*, 1991). Also, the biological activity and physical structure of soils are maintained, the technique is potentially cheap and visually unobtrusive, and there is the possibility of biorecovery of metals (Baker *et al.*, 1994).

Where high metal contamination is combined with poor soil conditions such as macronutrient deficiency, a complete disappearance of natural vegetation can result, increasing the likelihood of dispersal of metals to the surrounding environment through wind and water erosion (Vangronsveld *et al.*, 1995). But with proper maintenance, metal-tolerant plants introduced onto contaminated soils can last for considerable periods of time, in contrast to costly and often temporary physico-chemical remediation techniques (Smith and Bradshaw, 1972).

Phytoremediation is defined as the use of green plants to remove pollutants from the environment or to render them harmless (Salt *et al.*, 1998). There have been five main areas of research distinguished within phytoremediation:

- Phytoextraction- the use of pollutant-accumulating plants to remove metals or organics from the soil by concentrating them in the harvestable plant parts.
- Phytodegradation- plants and associated microbes degrade organic pollutants.
- Rhizofiltration- plant roots adsorb and absorb metals from waste streams.
- Phytostabilisation- the plants reduce the bioavailability of pollutants in the environment.
- Phytovolatilisation or plant volatilisation of pollutants.

The development of this technique is being driven by the prohibitively high cost of available soil remediation methods.

1.6.1 Metal phytoextraction using hyperaccumulators

Much interest has focused on plant species capable of accumulating unusually high concentrations of potentially phytotoxic elements such as Cu, Ni and Zn from metalliferous soils. Plants able to accumulate concentrations of metals more than 100 times larger than normal species are termed hyperaccumulators, and have strongly expressed metal sequestration mechanisms, and sometimes greater internal requirements, for specific metals (Shen *et al.*, 1997). Studies have shown some species to be capable of mobilising metals from less soluble soil fractions in comparison to non-hyperaccumulating species (McGrath *et al.*, 1997). Hyperaccumulators have been recorded in almost four hundred taxa, representing more than seventy families (see Table 1.6.1). Hyperaccumulator metal concentrations in shoots normally exceed those in roots, and it has been suggested that metal hyperaccumulation has the ecological role of providing protection against fungal and insect attack (Salt *et al.*, 1998).

Table 1.6.1 Numbers of metal hyperaccumulator plants*

Metal	Concentration criterion (% in leaf dry matter)	Number of taxa	Number of families represented
Cadmium	> 0.01	2	1
Cobalt	> 0.1	26	12
Copper	> 0.1	31	11
Lead	> 0.1	13	3
Manganese	> 1.0	8	5
Nickel	> 0.1	281	36
Zinc	> 1.0	13	5

*From Raskin and Ensley, 2000.

Such plants in Europe are species endemic to areas of natural mineralisation and mine spoils. Examples of such plants include species of *Thlaspi* (Brassicaceae family) from calamine soils, which can accumulate > 3 % Zn, 0.5 % Pb and 0.1 % Cd in their shoots (Baker *et al.*, 1991), whereas *Serbertia acuminata*, which grows on serpentine soils, can accumulate up to 2.5 % Ni in its sap. The feasibility of using metal-accumulating plants to decontaminate polluted soils has been investigated by many authors. Baker *et al.* (1991) detected enhanced levels of heavy metals in the edible parts of two crop species tested, although most were present at trace concentrations only. The levels accumulated did not pose a significant health risk, but also suggested that it would take centuries for normal cropping to reduce the soil concentrations of these elements to background values. The Zn and Ni hyperaccumulator species tested were shown to accumulate metals from low as well as high background concentrations, and accumulation was not as metal-specific as predicted, having good implications for use in situations of multiple metal contamination such as sludge-amended soil.

Exploitation of metal uptake into plant biomass as a method of soil decontamination is limited by plant productivity and the concentrations of metals achieved (Baker *et al.*, 1991). For instance, *Thlaspi caerulescens* is a known Zn hyperaccumulator, but use in the field is limited because individual plants are very small and slow growing (Ebbs and Kochian, 1997). Chaney *et al.* (1997) opined that the metal hyperaccumulator phenotype is much more important than high-yield ability in terms of phytoextraction. Baker *et al.* (1994) pointed out that, although many of the European hyperaccumulator plants are of small biomass, their growth potential can be enhanced considerably by soil fertilisation. The ideal plant species to remediate a heavy metal contaminated soil would be a high biomass crop that can both tolerate and accumulate the contaminants of interest (Ebbs and Kochian, 1997). Such a combination may not be possible - there may be a trade-off between extreme hyperaccumulation and lower biomass, and vice versa.

The cropping of contaminated land with hyperaccumulating plants may result in a potentially hazardous biomass. Harvested plants may be used in electricity production in biomass-fired plants; it is hoped that combustion technology such as circulating fluidised bed boilers could reduce air pollution, and economic quantities of metals could be recovered from the residual ash (Banuelos and Ajwa, 1999).

1.6.2 Enhanced Metal Phytoextraction Using Non-Hyperaccumulators

Phytoextraction of metals can be of a long-term, continuous nature, or induced through the assistance of chelates. Continuous phytoextraction using hyperaccumulators has the major drawbacks of the plants having slow growth rates and low biomass. A dearth of reliable reports of plants capable of naturally accumulating the most environmentally important toxic metals such as Pb and Cd led to the approach of enhancing metal accumulation by high biomass crop plants through the addition of chelating and acidifying agents to soil.

Huang *et al.* (1997) investigated the potential of adding chelates to Pb-contaminated soils to increase Pb accumulation in plants, as there are two major limitations to Pb phytoextraction: the low Pb bioavailability in soil and the poor translocation of Pb from roots to shoots. Ethylenediaminetetraacetic acid (EDTA) proved to be the most effective chelate in increasing Pb desorption from the soil into the soil solution. The chelate also greatly increased the translocation of Pb from roots to shoots through prevention of cell wall retention. Corn and pea shoot Pb concentrations were greatly increased by this soil treatment. The importance of the timing of the chelate addition was recognised, to avoid chelate-induced metal movement into the groundwater. Similarly, Cooper *et al.* (1999) acknowledged the need for metal-leaching preventative measures, such as application of chelate solutions to meet plant water needs and tile drains to capture leachate.

Application of chelating agents may have detrimental effects on treated plants: Cooper *et al.* (1999) reported Cu and Zn concentrations of various herbaceous plants to increase with the application of chelates such as nitrilotriacetate (NTA) and EDTA, but these increases were frequently confounded by the reduction of dry weight of the plant. The forms of the metals in the amended soil are equally important: Anderson *et al.* (1999) found citric acid did not induce enhanced uptake of Zn, Cd and Pb by field-grown grasses in a soil in which the heavy metals were primarily associated with carbonate and oxide phases rather than organic matter.

Schremmer *et al.* (1999) grew *Salix viminalis* 'Jorr' on two heavy metal contaminated soils fertilised with $(NH_4)_2SO_4$ and a nitrification inhibitor. Plant growth was not significantly different to the control. Heavy metal contents in leaves were increased by soil acidification in a relatively neutral contaminated soil (pH 6.4). The nitrification inhibitor reduced metal leaching from an acid soil (pH 4.8). Keller *et al.* (1999) amended 3 contaminated soils with NH_4Cl and found it to increase Zn uptake by *Salix aurita* and to decrease extractable Zn concentrations. However, plant uptake only accounted for about half of this decrease; much of the mobilised Zn was interpreted as being readsorbed by the soil.

Salt *et al.* (1998) noted the potential of manipulating metal resistance mechanisms in non-hyperaccumulating plants to improve phytoextraction. Root processes may possibly be genetically manipulated in the future to enhance metal uptake, which would represent a major long-term advance in phytoextraction. Improved metal resistance alone may not be sufficient for successful phytoextraction, which also depends on metal bioavailability, root uptake and shoot accumulation. The authors highlighted the need to have a multi-disciplinary approach to phytoremediation, such as in utilising the knowledge of environmental and agricultural engineering, to improve the efficiency of plant cultivation, amendment application and disposal of metal-enriched biomass.

1.7 Phytoremediation Using Trees

The use of trees in reclamation of contaminated land is low-cost, sustainable and ecologically sound (Dickinson, 2000). At sites that cannot be economically capped or cleared, where there is no time pressure on the renovation, phytostabilisation may be a favourable remediation option (Riddell-Black, 1994). Ideally, repeated harvests of fast-growing, high biomass coppice woodland soil could decontaminate metal polluted soils and eventually release them to crops (Duncan *et al.*, 1995). However, it is important to consider the interaction of environmental factors on tree growth, and the effects of tree growth itself on soil contaminants.

1.7.1 Phytostabilisation of mining wastes

Considering the objectives of vegetation establishment on mining wastes, Johnson *et al.* (1992) outlined the following goals:

- Long-term stability of the land surface to ensure no surface erosion by water or wind.
- Reduction of leaching throughputs to lessen transfer of toxic elements to watercourses and groundwaters.
- Development of a vegetated landscape in harmony with the surrounding environment.
- Benefits in an aesthetic, productivity, or nature conservation context.

The physical and hydraulic conditions of a site are of primary importance to tree establishment. For example, mine spoil heaps are not homogenous in composition or permeability. Layers of anomalously fine material containing metal concentrations up to 100 times greater than those in bulk tailings have been observed at historic metalliferous mine sites in the U.K. (Merrington, 1995). Therefore the vertical and horizontal extent of the contamination, the properties affecting rooting, and the nutrient distribution can vary considerably in a spoil, and this heterogeneity can result in wide variations in tree survival.

A study of the relationship of tree growth with the chemical, physical, nutritional and hydrological properties of minespoil was carried out by Bending and Moffat (1999). Establishing trees on minespoil is problematic due to compaction, infertility, acidity, salinity and poor water-holding capacity. They reported restricted rooting to occur as a result of shallow water tables and high bulk density. The N deficiency and poor water-holding capacity of some sites were suitably improved by organic amendments. While the addition of organic amendments such as soil or sewage sludge to spoil may aid revegetation, the roots may not extend readily from a fertile soil layer into the underlying spoil, and it may increase the weed problem in some young woodland areas. Johnson *et al.* (1977) described cessation of root development at the interface of Pb/Zn spoil and organic amendments.

Trees have the most massive root systems of all plants, leading to improved and stable soil/spoil structure. Many tree species can grow on land of marginal quality in terms of fertility and structure. The planting of trees can provide adequate vegetation cover for erosion protection, and can transpire considerable amounts of water compared to non-woody plant species, reducing the downward migration of contaminants. Trees, of course, can also produce biomass for energy and/or chemical use, and often require little or no maintenance costs. Tree plantations therefore serve to clean up hazardous waste, stabilise the site and produce wood (Stomp *et al.*, 1993). Jobling and Stevens (1980) reported that a number of pioneer tree species, including several willow varieties, proved to have a high survival and growth rate in a variety of colliery spoil and climatic conditions. Besides improving amenity, the trees provided habitats for wildlife, improved prospects of soil formation due to a build-up of litter following leaf-fall, and stabilised the surface spoil on steeper slopes.

Taking saplings or cuttings of selected clones of birch and willow from spoil heaps and testing growth on restored opencast coal sites, Good *et al.* (1985) found many to achieve consistently higher mean survival than unselected controls over the range of sites. Generally, willow achieved higher shoot growth increments than birches, and the greatest gains in survival occurred on the poorest growth medium (where topsoil was not used), whereas there was little advantage in their use on fertile sites with good drainage. It was recognised that the low growth rate, with high survival, on these nutrient deficient sites is preferable to low survival coupled with a continued fertiliser application requirement to sustain growth.

Phytostabilisation can also be considered as a strategy for targeting specific contaminants: it was identified by Chaney *et al.* (1997) as most promising when applied to Pb (through formation of insoluble chloropyromorphites) and Cr, which can be reduced from Cr (VI) to the insoluble Cr (III) by the technique.

1.7.2 Phytoextraction

The principal characteristics of a phytoextraction species outlined by Punshon *et al.* (1995b) include:

- An ability to grow on nutrient-poor soil.
- A deep root system.
- An economically viable secondary use, such as biofuel.
- A metal resistance trait.
- Fast growth.

High metal content in agricultural crops is not desirable and is potentially dangerous. A higher metal content in trees is acceptable, as long as normal physiological activity is not affected (Labrecque *et al.*, 1995). While no commercially important trees are known to hyperaccumulate metals (Riddell-Black, 1993), heavy metal availability to trees can be increased relative to availability to non-woody plants through increased wet and dry deposition to leaf surfaces and soil, altered cuticular surface characteristics leading to greater availability of foliar-deposited metals, and soil acidification induced by tree growth causing increased metal mobilisation in soils.

Trees support the largest diversity of soil microbes, through symbiotic relationships, of all plants, including bacteria, mycorrhizal fungi and blue-green algae, and so indirectly participate in remediation of a metal-contaminated site through root-enhanced microbial activity and detoxification (Stomp *et al.*, 1993).

Screening procedures offer a rapid method of searching for tree species displaying metal tolerance, but may be of limited value when seeking such a specific characteristic (Riddell-Black, 1993). Plant breeding may lead to development of metal tolerance in some species, but there are major barriers caused by the lack of knowledge concerning the quantitative limits of tolerance, the point at which no further adaptation can be made by a species, the interactive effects of contaminants and the determination of toxicity responses by factors such as the nutritional status of the soil and plant. Also, the reliability of tolerance traits through many generations is unknown (Riddell-Black, 1993).

Dickinson (2000) reported several factors besides heavy metals to be important in the performance of trees grown on metal-contaminated sites. Weed competition and subsequent neglect may influence survival, and inadequate soils (leading to poor fertility and /or water shortage) can also have a major effect. Cultivation will aerate the soil and promote the activities of soil microbes, and hence the breakdown of organic matter.

Bridges (1989) reported soil Pb concentrations below trees in amenity soils to be lower than in adjacent open spaces, indicating redistribution by the trees.

The next sections consider the heavy metal uptake by trees grown on contaminated substrates, and the phytoremediation potential of willow trees in the context of the above five priorities.

1.7.3 Summary

Phytoremediation represents a potentially cheap method of restoring a contaminated site which maintains, and may improve, the physical structure and biological activity of the soil. Partial decontamination of the site is an added advantage. Continuous phytoextraction through the use of metal-hyperaccumulating plants is limited by their low productivity, although fertilisation may improve this. Induced phytoextraction by adding metal-chelating compounds to soil can greatly enhance metal uptake by some plants, but this is costly and increases the likelihood of chelate-induced metal leaching. Many tree species can grow on marginal land in terms of fertility and structure, leading to improved and stable structure, erosion protection, reduction of downward migration of contaminants through transpiration, and production of biomass for fuel. Numerous species of trees have successfully been established on formerly mined land. No commercially important trees are known to hyperaccumulate metals, but it is hoped that repeated harvests of high biomass trees coupled with the enhanced microbial detoxicification of contaminants could substantially deplete soil metal concentrations.

1.8 Heavy Metal Uptake by Trees Grown on Contaminated Substrates

Bioavailability of metals to trees, and subsequent metal accumulation in tree tissues, can vary hugely according to the source of metal contamination, and site conditions.

1.8.1 Heavy metal uptake by trees grown on sludge-amended sites

Lepp and Eardley (1978) examined the effects of metal-contaminated sludge on the growth and metal content of sycamore seedlings over 50 days. The beneficial effects of sludge on tree growth far outweighed any impact of the sludge-borne metals on growth processes over the experiment's duration. Sludge application effectively raised the pH of the growing medium, resulting in sub-optimal uptake conditions for uptake of Pb, Zn and Cu. The increased root Cu concentrations, however, were attributed to the increase in concentrations of soluble organic matter. They acknowledged that elements present in various quantities in sludge will cause complex interactions in initial metal uptake, and in subsequent metal redistribution in the tree. Metal burdens were not excessive and showed no significant relationship to soil metal levels.

Similarly, Labrecque *et al.* (1995) found the amounts of metals brought into soil by sludge doses did not induce phytotoxicity symptoms or decrease biomass in two willow species. Hasselgren (1999) found stem biomass production of three willow clones to be enhanced by sludge application rate; it also led to more uniform growth and a greater shoot number than in control plots. Concentrations of metals in plant tissues were not influenced by application, but generally decreased with stem and stand age.

Berry (1985) grew loblolly pine seedlings in plots amended with five different sewage sludges and monitored the growth and heavy metal accumulation of the trees. For some sludges, toxicity of heavy metals to the trees was considered to be a factor in poor seedling growth relative to an inorganic fertiliser control, although sludge application rate did not affect seed germination or seedling survival. They found Cd and Zn concentrations in the foliage to generally vary with the concentrations in a particular sludge. Cadmium and Zn uptake was calculated to be about 1 % of the amount applied, a degree of uptake not large enough to significantly decontaminate nursery fields.

Analysing heavy metal concentrations in various trees (maple, ash, pine, birch and cottonwood) grown on sludge-amended mine spoil, Morin (1981) found first year tree samples indicated rather high metal accumulation patterns within the tree species; third year harvest samples did not exhibit any significant increases in concentrations. This did not necessarily mean a decline in metal assimilation over time. It was attributed to additional biomass production diluting the concentration levels, and accumulation of the metals in the litter layer.

1.8.2 Heavy metal uptake by trees grown on unsludged substrates

Scanlon and Duggan (1979) measured concentrations of heavy metals in plant foliage in eight woody plant species grown on fly ash, and compared them with those of trees grown on soil. Foliage concentrations of Cd, Cu and Zn were higher in fly ash-grown trees than those grown in soil. Similarly, Borgegard and Rydin (1989) analysed tissue concentrations of heavy metals in birch trees which had colonised soil covering mine tailings. They found leaf concentrations of Zn, Pb and Cd to exceed values for leaf concentrations of trees growing in uncontaminated soils by about one order of magnitude. Fernandes and Henriques (1989) compared the Zn, Cu and Pb concentrations in leaves and fruits of holm-oak trees at the outskirts of a pyrites mining area with those in suitable controls. The trees in substrate affected by the mining showed pronounced stunting, reduced leaf size and extensive necrotic and chlorotic spotting, and had concentrations more than 50 times higher for Cu, and 20 times higher for Pb and Zn.

This is not always the case: the low uptake of metals by trees planted on slag at the Lanarkshire Steelworks was attributed by Salt *et al.* (1995) to the high pH of the waste and the chemical form of the slag's metal contaminants, rendering phytoextraction unfeasible at this site.

Sampling stems and leaves of *Salix*, *Silene* and *Populus* species growing on mine spoil, Dinelli and Lomini (1996) observed that metal concentrations were generally higher in the early vegetative growth stage, due to a relatively higher nutrient uptake compared to growth rate. This was followed by a period of vigorous growth which diluted the concentrations until the flowering stage, in which the minimum values for almost all elements were obtained. Senescence of trees usually produced an increase in metal levels due to concentration caused by loss of fluids.

Seasonal variations of the foliar metal concentrations in woody plants have been confirmed by other studies. A two year study of the metal content of birch leaves by Ehlin (1982) showed that Cu and Ni contents decreased at the beginning of the growth period, probably a dilution effect resulting from increased dry weight of the leaves. A marked accumulation of Zn in sycamore, beech and horse chestnut, and hazel leaves at the end of the growing season has been noted by Ross (1994c). This was interpreted as metal "shunting" occurring in the plant tissues prior to senescence, or seasonal variation in soil metal availability. Riddell-Black (1994) reported consistent increases in foliar heavy metal concentrations shortly before senescence in four willow species grown on a metal-contaminated substrate. Hasselgren (1999) interpreted a tendency of increased willow leaf Cu content in autumn as a possible detoxification effect in connection with defoliation.

1.8.3 Summary

Concentrations of heavy metals in trees grown in contaminated substrates normally greatly exceed those grown in an uncontaminated control with possible resultant toxicity symptoms, unless the soil parameters keep the bioavailability of the metal low (such as high pH or elevated organic matter concentrations following sludge application). Heavy metal concentrations in aerial tree parts show seasonal variations. Concentrations are high in the early vegetative growth stage, which are diluted by increased biomass production. Similarly, trees generally show this effect of biomass production diluting heavy metal concentrations as their age increases in years.

1.9 Heavy Metal Compartmentation Within Trees

Lepp (1977) described how trees may act as temporary or long-term sinks for atmospheric metals. Temporary retention of heavy metals can occur in the leaves, and bark surfaces may function in a similar manner. An extensive canopy of trees represents a large surface area where metal-bearing particulates can be trapped and retained. The metal can embed in the cuticular waxes, or be solubilised and subsequently taken up foliarly, with possible exportation via phloem (Zn, for example, is freely phloem mobile, whereas Pb is not). From the leaf compartment, metals are mostly returned to the decomposition cycle through leaching or following leaf abscission (Lepp, 1995). Heath *et al.* (1999) reported metal entrapment by tree foliage in birch, oak and pine shelterbelts, and subsequent soil enrichment.

Conifers shed their needles throughout the year, so the metal input to underlying soil is more diffuse than that from deciduous species, which feature a simultaneous senescence of all foliage at the end of the growing season. Leaves from deciduous species only accumulate metals on a yearly basis, whereas roots and stem tissue accumulate metals year after year.

Wood and bark are important sinks for biologically available metals, with additional sink tissue being formed each growing season. These perennial tissues are slow to enter the decomposition cycle; accumulated metals can therefore be immobilised in a metabolically inactive compartment for a considerable period of time (Lepp, 1995), if the contaminated trees are not re-used for other purposes which accelerate the return of the heavy metals to the environment, such as in combustion. While metal concentrations in wood are frequently lower than in roots and bark, the fraction may represent a much more significant proportion of the total amount of metal in a tree (Dickinson and Lepp, 1997).

1.9.1 Metal distribution studies

Trees differ in their ability to translocate various heavy metals from the root to the shoot. In sycamore seedlings grown in sludge-amended soil, Lepp and Eardley (1978) found levels of metals in the stems and leaves to be an order of magnitude less than corresponding root levels. Morin (1981) found root materials of several tree species grown on sludge-amended spoil to exhibit the highest concentrations of Cd, Cu, Ni and Zn. The major effect elemental interactions can have on the relative accumulation of the leaf and stem material was stressed; the stability and relative charge of the translocating ionic species will influence the degree of interaction with the exchange sites of the xylem.

Turner and Dickinson (1993) found, in sycamore trees grown in contaminated soil, most of the Pb not retained in the roots to be translocated to the stem, while most of the Zn not retained in the roots was translocated to the leaves. McGregor *et al.* (1995) analysed tissue of sycamore, birch and willow trees which had naturally established on sites contaminated by waste from an explosives factory and a chromium processing works. Chromium, Pb and Cu were found to have chiefly accumulated in the tree roots; Zn concentrations were highest in bark.

Numerous studies have shown accumulation to occur in actively growing tissues such as shoots and young leaves: Drew *et al.* (1987) grew poplar clones in sludge-amended soil and found Zn and Cd concentrations to be the highest in foliage. In four willow species grown on sludge-amended soil, foliage concentrations were greater than those in the stem for all varieties and metals (Riddell-Black, 1994). Of the above-ground biomass metal concentrations determined in sludge-amended willow plots by Hasselgren (1999), Cu, Pb and Cr were mainly in the stems, while Zn, Cd and Ni were in the leaves.

Foliar metal concentrations in trees vary seasonally (see Section 1.8.2). Within a tree, compartmentation of metals between roots and shoots, and partitioning of the metals within the stem can vary. This variation may not only be evident between species, but also within a single genus. Punshon *et al.* (1995b) found various willow clones to compartmentalise metals in woody tissues much more markedly than others, and especially Cd. Riddell-Black *et al.* (1997) split 20 willow varieties grown on sludge-amended soil into two groups: those which accumulated Cu and Ni in above ground biomass (which suffered reduced yield) and those which did not. Bark concentrations of all heavy metals determined were found to be consistently greater than the concentrations in wood of the same clone. Korcak (1989), however, reported little difference between bark and wood Cd concentrations in several fruit tree species.

Within the stem, elements can be radially transported. Rapid lateral movement of water from xylem to phloem in three year old *Salix* trees has been demonstrated by Epstein (1972), while Hagemeyer and Hubner (1999) reported a conceivable redistribution of Pb in stems of spruce trees, possibly via the axial xylem sap stream or in rays.

Background concentrations of Cu and Zn were found to vary between tree compartments in eight *Salix* species studied by Nissen and Lepp (1997). The species did not show a common uptake pattern for the two metals. A general trend of exclusion of Cu and concentration of Zn in shoot tissue relative to soil concentrations, however, was evident. The low concentrations of Cu reported point to exclusion from the shoot system, reflecting the low mobility of Cu following root or foliar uptake. Considerably higher leaf and bark concentrations of Zn reflected the mobility of the element after uptake, whereas low concentrations in wood displayed a low retention of Zn in the xylem tissues. It was acknowledged that the partitioning of metals between tissues may change as the soil metal levels increase.

Sander and Ericsson (1998) found concentrations of Zn, Cu, Ni and Cd in stems of *Salix viminalis* to increase significantly with height, thought principally to be a consequence of increasing bark proportions. As the stem narrows towards the shoot top, the proportion of bark increases, which generally contains a greater concentration of plant nutrients than wood. Nickel increased most from the lowest to the highest sampling level, followed by Cu, Zn, and Cd. Different elemental reallocation patterns during leaf senescence may have led to the different concentration gradients in the shoots after leaf fall. The effect of nutrient availability on the allocation patterns of the elements was also questioned.

1.9.2 Summary

Deciduous leaves can only accumulate metals for one year before senescence and leaf fall, whereas stem and root tissues accumulate metals year after year. Root concentrations of heavy metals of trees grown in contaminated substrates are often found to be the highest; Cr, Pb and Cu are known to accumulate in the root. Accumulated metal not retained in the root may be translocated to the stem or leaves. Actively growing tissue such as leaves act as the site of accumulation for the more translocatable heavy metals Cd and Zn. Within tree stems, wood and bark are important metabolically inactive sinks for metals. Clones of the same genus have been shown to compartmentalise metals in woody tissues much more markedly than others. Nutrient and heavy metal concentrations are generally greater in bark than wood. The increase of bark concentrations with stem height consequently leads to an increase in heavy metal concentrations, an effect more marked for the less translocatable elements Cu and Ni than the more mobile Cd and Zn.

1.10 The Phytoremediation Potential of *Salix*

The genus *Salix* is of the *Salicaceae* plant family. There are four hundred species of willow, with more than two hundred listed hybrids (Newsholme, 1992). Eighteen species of willow are regarded as native to the British isles. The majority of the genus *Salix* grow in lowland wetland habitats and have evolved a number of varieties and hybrids (Sommerville, 1992). The large number of species and hybrids of *Salix* suggest a wide genetic variability within the genus. While there are creeping forms such as *Salix repens* and willow bushes (for example *S. aurita*), most species are multi-stemmed small trees such as *S. caprea* and *S. cinerea*. A few species are single trunk trees which reach to over 20 m in height, such as *Salix alba*. In almost all, trunks or branches touching the ground can take root, and shoots grow rigorously from a coppiced stool (Sommerville, 1992). Most species favour neutral ground with a good supply of nutrients.

The genus features many species of high productivity and invasive growth strategies (Punshon *et al.*, 1995b). Many species, such as *Salix caprea* and *S. cinerea*, and the hybrid *S. viminalis*, are known to colonise edaphically extreme soils (Dickinson *et al.*, 1994). Mang (1992) detailed the importance of willow in vegetation colonising heavy metal contaminated dried silt removed from a port, and drew attention to the suitability of selected clones for planting in polluted areas. The soil consolidation provided by the enmeshment of their spreading roots is a feature which can be exploited in the reclamation of land. The demand of the perennial root system of willows for water lowers the risk of contaminant leaching (Sander and Ericsson 1998), and most species are able to tolerate long drought periods. More than 150 potentially mycorrhizal fungal taxa have been recorded with willows in Britain.

Willow can be frequently harvested and coppicing can yield 10-15 dt ha^{-1} yr^{-1} (Riddell-Black, 1993). Bushy *Salix* species with erect stems, rapid growth and good rooting ability are the most suitable for biomass coppice, with *S. viminalis* being one of the most widely used species so far (Ahman and Larsson, 1994). Possible end-product uses of *Salix* biomass include fuel for direct burning as chips, raw material for the production of paper, chipboard and charcoal, a source of viscose for the textile industry, and the production of briquettes, ethanol and ruminant livestock feed supplement (McElroy and Dawson, 1986).

1.10.1 Hydroponic and field studies of Salix

In addition to high biomass productivity, *Salix* trees also feature an effective nutrient uptake, high evapotranspiration and a pronounced clone specific capacity for heavy metal uptake. Use as wood fuel could allow possible heavy metal recovery through the scrubbing of smoke gases and proper handling of ashes (Perttu and Kowalik, 1997). Punshon *et al.* (1995a) stated that willows which survive in metal-contaminated soil with minimal uptake of metals into aerial tissues, would be most appropriately used where food chain transfer of metals is to be avoided, whereas clones accumulating relatively high amounts of metal are desirable if soil remediation is to be achieved through metal removal by tree harvesting.

Landberg and Greger (1994) tested various clones for Cd and Zn accumulation and tolerance in solution culture. The huge variability of the genus is exemplified by the results: some clones were tolerant to both of the metals, others just to one; tolerant clones could feature a relatively high or low net uptake of metal, with the net transport to the shoots varying between 1 and 72 % of the total metal uptake. Greger (1999) found Cd uptake capacity of 70 *Salix* genotypes could differ by as much as 43 times between clones with the highest and lowest values. The better varieties were reported to have capacities about 5 times higher than for *Thlaspi caerulescens* and *Alysum murale*, due to high biomass production and transport of Cd to the shoot. Felix (1997) reported *Salix viminalis* to display the highest metal-accumulating ability of the various plants tested: it achieved a transfer coefficient of 3.4 for Cd in a field trial on a contaminated soil. However, the calculated 77 years to decontaminate the soil used in this study to acceptable Cd concentrations were not practicable, highlighting the limitations of yields and/or metal uptake rates to phytoextraction as a remediation tool.

Riddell-Black (1993) calculated the stem concentrations of metal necessary to reduce soil metal concentrations in a hypothetical highly polluted soil, to target concentrations over 30 years (the expected productive life of willows grown on short rotation). Although it was shown that the decontamination of highly contaminated land cannot be achieved in the short term due to the stem concentrations necessary being impossible, it was stated that renovation of less polluted land is still conceivable in a reasonable timescale, even with low metal uptake, due to the frequency of harvest.

The success of *Salix* as a phytoextracting plant depends on its biomass production, metal accumulation capacity and the site of metal accumulation in the plant (Riddell-Black, 1994). Uptake of heavy metals by four varieties of *Salix* used in woodchip production for energy was measured. The trees were grown for three years on soil that had received sewage sludge for over 50 years. The main benefit from *Salix* growth on this metal-contaminated site was reported to be site stabilisation, as the metal uptake by the harvested biomass did not look like a realistic method of renovating the site. However, she stated the growth of *Salix* may be useful if depletion of the bioavailable metal in a soil occurs.

There is encouraging evidence of this occurring from other studies: about 30 % of the bioavailable Cd was reported to be removed by *Salix* in a 90-day pot trial (Greger, 1999).

Measuring exchangeable Cd concentrations in 8 soils taken from *Salix* stands and nearby reference soils, Eriksson and Ledin (1999) found concentrations to be 30-40 % lower in the planted soils. Total Cd concentrations were not significantly reduced, but the exchangeable Cd pool was reduced. Uptake occurred throughout the soil profile to 65 cm. However, Alriksson *et al.* (1999) investigated the effects of five tree species' growth (including willow) over 6 years on Cd soil concentrations, and found high biomass Cd uptake was not related to a corresponding depletion of the soluble Cd pool. This pool increased due to decreasing pH.

The bark and wood concentrations of heavy metals in a further 20 willow varieties were determined by Riddell-Black *et al.* (1997). Overall, the concentrations in the three year old trees suggested certain clones have potential to take up significant quantities of metals. Stem Cd concentrations in this study were up to an order of magnitude greater than soil concentrations. Punshon *et al.* (1995a) also found various *Salix* clones to compartmentalise Cd in woody tissues much more markedly than others, representing a beneficial trait for long-term removal of the contaminant from the soil. Ostman (1994) calculated the annual Cd uptake by willow (exceeding that supplied by fertilisers and air deposition) to be around 3-4% of the plant available Cd in Swedish soils, and suggested that a rotation cycle of around 20 - 25 years would reduce the Cd concentrations to natural levels. Commercially grown *Salix* has been shown to accumulate 20-30 g Cd per hectare per year (Goransson and Philippot, 1994).

The general trend of exclusion of Cu and accumulation of Zn in shoot tissue of eight *Salix* species, identified by Nissen and Lepp (1997), indicates a low potential for the depletion of soil Cu through repeated harvests, whereas there is some potential for Zn concentration in combustion residues. Dickinson *et al.* (1994) carried out a series of experiments to investigate the establishment and growth of a number of *Salix* clones placed in metal-contaminated soils, and the uptake and partitioning of the metals within the plant. Translocation from the roots to the shoots was greatest for Zn (stem concentrations were as high as 0.8 % in plants grown in mining spoil), importantly making removal of the metal during harvest feasible. McGregor *et al.* (1995) noted that changes in Zn concentrations within tree tissues in different parts of the growing season suggested an optimum harvest time would be over winter, fitting in well with usual coppicing practice.

Labrecque *et al.* (1994) estimated the bioaccumulation of various metals by *Salix discolor* and *S. viminalis* with respect to metal application in sludge. About 50-80 % of the total quantity of bioaccumulated metals were found in the roots and stem-branch biomass, representing an immobilisation of metals relative to that accumulated in leaves, which is returned to the soil at the end of each growing season. The metal transfer coefficients for Cd and Zn were considerably greater than those for Ni, Cu and Pb (Labrecque *et al.*, 1995). Increased metal application to the trees did not necessarily lead to increased tissue metal concentrations; Cu, Ni and Pb plant concentrations were less dependent on soil concentrations, whereas those for Cd and Zn were more so, pointing to higher soil solution solubilities of these metals and species preference for them.

Landberg and Greger (1996) gauged the tolerance and accumulation of Cd, Cu and Zn of stems of different *Salix* clones grown on metal polluted and unpolluted areas. They found no differences between the polluted and control areas in the tolerance of *Salix* to the metals or in concentrations of the heavy metals in the collected stems. However, growth of clones from the polluted area was generally stimulated at a low metal concentration. The variation in accumulation and tolerance to heavy metals was wider within the species than between the species. Clones from the polluted area had higher metal accumulations in their roots, and a lower translocation of metals to the shoots, probably a mechanism to protect the shoot.

Punshon and Dickinson (1999) also reported considerable variation between and within clones when they investigated the resistance of *Salix* to Zn, Cd, Cu and Ni in hydroponic experiments. Resistance was not species-specific, rather clone- or hybrid-specific. The considerable intra-clonal variation was demonstrated by the survival of a proportion of cuttings of some of the most sensitive clones in the high metal regimes. To put this into context, the large variation of the growth of *Salix* in uncontaminated soils was acknowledged. This variation causes difficulties in phytoremediation screening programs, but may be essential in allowing provision of a remediation technique for a site through selective planting.

1.10.2 Summary

The genus *Salix* features many species known to colonise inhospitable soils. Soil is consolidated by the tree's spreading roots, highlighting the suitability of willows for phytostabilisation of polluted sites. The genus has a clone specific capacity for heavy metal uptake. Although the concentrations of metals accumulated in willow tissue indicate that decontamination of highly contaminated soils is impossible in the short term, remediation of less polluted soil may still be possible, if the bioavailable pool of heavy metals is depleted, due to the frequency of harvest. The huge variability of the genus is apparent when considering the contrasting amounts of metals accumulated by various species. Considerable within-species variability in metal accumulation is also evident, making results difficult to interpret, but some species seem to display high capacities to accumulate heavy metals within woody tissues, particularly Cd and Zn. This points to potential significant removal of some contaminants from soil. Willows surviving in metal-contaminated soil with minimal translocation of metals to the shoot could phytostabilise a site where food chain transfer of metals is to be avoided, while clones accumulating relatively large quantities of metals should be planted if phytoextraction of metals through repeated harvesting is to be achieved.

1.11 Aims and Objectives

The work reported in this thesis comprised part of the BIORENEW project (European Commission contract ENV4-CT97-0610: Bioremediation and Economic Renewal of Industrially Degraded Land by Biomass Fuel Crops), a study which involved collaboration between seven European research groups. Glasgow University cooperated closely with the University of Hohenheim and WRc (Water Research Centre, Medmenham). The work described in this thesis examined willows and their interaction with heavy metals, and had several aims:

- To thoroughly examine the compartmentation of metals within willow tissues, as well as the effects of season and sampling height on the metal concentrations measured, through the regular collection and analysis of stem and leaf samples taken from trees growing on contaminated substrates.

- To establish the effects of willow growth on soil heavy metal distribution, via selective and sequential extraction of field and pot trial soil samples. If depletions were observed, the work aimed to quantify the contribution of metal sequestration in the above-ground biomass to this through analysis of vegetation samples.

- To assess whether extraction of metals from soil by crops could be enhanced by soil amendments in a pot trial.

- To utilise hydroponic apparatus to assess important factors affecting the metal tolerance of willows, including nutrition, pH and levels of exposure to toxic metals.

- To develop a hydroponic test which could distinguish willow varieties according to their responses to metal exposure.

- To ascertain whether a rapid screening test of willow varieties' metal tolerance could be developed, via the establishment of significant correlations between data gathered for willow varieties' performances in hydroponic experiments, and the same clones in independent field trials.

Chapter 2 Materials and Methods

All glass and plasticware used had been washed overnight in 2 % Decon 90, rinsed in tap water repeatedly, washed in 2 % HNO_3, rinsed in deionised water (DIW) and dried prior to use. All reagents used were analytical grade.

2.1 Field Studies of Willows Grown on Contaminated Substrates

2.1.1 Sample Site Descriptions

Vegetation and soil samples were collected from three willow stands which were established on sacrificial dumping grounds of sewage sludge in Yorkshire and Berkshire. There was considerable variation in the range of contamination and age of the willow plantations at the different sites.

2.1.1.1 Spofforth, Yorkshire (SE 370526)

This 2.5 hectare site comprises five blocks of roughly equal size, each of which contain the following varieties (stocked at a rate of 10,000 trees ha^{-1}) in separate strips: *Salix burjatica* ('Germany'), *S. triandra x viminalis* ('Q83'), *S. dasyclados*, *S. viminalis* ('Q683') and *S. viminalis* ('Bowles Hybrid'). The five year old trees were harvested in December 1998; subsequent samples were taken in 1999 and 2000 from trees which regrew from the original stools. The pH (determined by potentiometry) and total soil concentrations (analysed using inductively coupled plasma-optical emission spectroscopy [ICP-OES] except Cd, which was analysed using atomic absorption spectrometry [AAS]) from samples taken in July 1994, are presented in Table 2.1.1.1 below. The site has an organic carbon content of 1.92-2.93 %, and is highly calcareous, with a lime content of 52-56 % (Schmidt, personal communication).

Table 2.1.1.1 Heavy metal concentration ranges and pH in Spofforth soil, July 1994*

Determinand	Zn	Cd	Cu	Ni	Pb	Cr	pH
Concentration range, mg kg^{-1}	339-2490	1.03-3.61	42.1-217	14.3-27.9	165-475	20.5-127	7.1-7.8

*Data from Yorkshire Environmental Lab Services, Rotheram (NAMAS accredited)

2.1.1.2 Slough, Berkshire (SU 945793)

This 0.2 ha site area of land has a large accumulation of sediment from the sludge. It was cleared of weeds and levelled in 1998 specifically for the growth of 56 willow varieties and 13 *Phalaris* varieties as part of the BIORENEW project. The trees were planted at a stocking rate of 15,000 ha^{-1}, while the *Phalaris* was planted at a seed density of 20 kg ha^{-1}. Typical total soil concentrations are presented below (Table 2.1.1.2). The table does not display a range, but the liquid nature of the sludge applied ensured a relatively uniform dispersal of the metal contaminants (Riddell-Black, pers. comm.). While less calcareous (2 % lime) and hence more acidic than Spofforth (pH 6.2), the site is highly organic, having an organic carbon content of 9.90 % (Schmidt, pers. comm.).

Table 2.1.1.2 Slough soil metal concentrations*

Determinand	Zn	Cd	Cu	Ni	Pb	Cr
Concentration (mg kg^{-1}), 0-25 cm depth	2135	182	877	171	415	599
25-50 cm depth	437	30.6	164	64.4	83.9	149

*Determined at WRc laboratories, Medmenham (UKAS accredited)

2.1.1.3 Rodley, Yorkshire (SE 233363)

The 2.5 ha site was planted with 5 willow varieties (Bowles Hybrid, Q683, Gigantea, Q699 and Mullatin) in 1993 at a stocking rate of 20,000 trees ha^{-1}. The trees were harvested in

1997. Total soil concentrations and pH of samples taken in July 1994 (Table 2.1.1.3) were determined as in Section 2.1.1.1.

*Table 2.1.1.3**   *pH and total metal concentrations in Rodley soil*

Determinand	Zn	Cd	Cu	Ni	Pb	Cr	pH
Concentration range, mg kg^{-1}	77.4-257	0.19-1.91	17.7-71.1	15.9-20.1	< 50-212	57.9-490	5.4-5.8

*Data from Yorkshire Environmental Lab Services, Rotheram (NAMAS accredited).

2.1.2 Vegetation sampling protocol: sampling height studies

In June 1998, five trees of *Salix burjatica* 'Germany' were cut from each of the five blocks at Spofforth. Leaf samples (enough to provide several grams of oven-dry material) were taken from the following zones along the stem: 0.5-1.5m, 1.5-2.5 m, and 2.5-3.5 m. Stems were sampled at metre intervals (0.5-0.6 m, 1.5-1.6 m, and 2.5-2.6 m) and separated into wood and bark. Tissue samples were washed with DIW, dried at < 40° C to a constant mass, and ground in a Christy Lab mill grinder. The grinder was thoroughly cleaned between each sample batch to avoid cross-contamination. All field vegetation samples were analysed at the Water Research Centre, Medmenham (WRc). These samples were determined for Zn, Cd, Cu, Ni, Pb and Cr concentrations; data are presented in Chapter 3. Analytical apparatus at WRc are described in Section 2.5.

2.1.3 Vegetation sampling protocol: seasonal studies

Triplicate tree samples were taken from Germany and Q683 strips at Spofforth, from each of the five blocks. Samples were taken of leaves, and stem samples were taken from the midpoint of the leafed and non-leafed portion of the shoot. Portions of stems were sampled in approximately 10 cm lengths and separated into wood and bark. All samples were prepared for analysis as in Section 2.1.2.

Analyses were carried out on July and September 1998 samples from all fractions for the following determinands: Zn, Cd, Cu, Ni, Pb and Cr. The last 3 determinands were frequently found to be at or below the detection limit. For the purpose of time series sample analysis, the subsequent analyses (November 1998, June and November 1999, and March, June, September 2000) were limited to the determinands Zn, Cd and Cu. Results are displayed in Chapter 3.

2.1.4 Soil microbial respiration determination

In June 1999, triplicate soil samples (0-25 cm depth) were collected with a soil corer from areas in a block at Spofforth which were unplanted, unharvested under willow growth and harvested under willow growth. They were stored at 4° C in a dark coldroom before sample preparation. Soils were sieved through a 5.6 mm mesh prior to being adjusted to 50% field capacity and incubated at 25° C for 10 days in sealed, but not airtight, plastic bags. Respiration rate of 150 g subsamples were determined in an Infra Red Gas Analyser (IRGA) CO_2 respiration apparatus (The Analytical Development Co. Ltd, Type 225-Mk3). Carbon dioxide evolution was recorded for a minimum of 12 hours with a Rikadenki chart recorder, and mean respiration rates were calculated from when a steady rate of evolution had been reached (see Chapter 4).

The estimated respiration rate in mg CO_2-C kg soil^{-1} hour^{-1} was calculated following:

$$\frac{[(((\text{Estimated } CO_2 \text{ in air} * \text{flow rate})/1000) * 60) \quad * \quad ((1000/\text{dry wt. soil})/1000)]}{1.865}$$

where the estimated CO_2 in air, ppm, is (the mean peak height of run * 20)/dry wt. soil, the constant 1.865 is to convert ml CO_2 into mg CO_2-C, and the flow rate and peak heights are recorded by the IRGA apparatus.

2.1.4.1 Gravimetric determination of field capacity

A dry ceramic dish and a filter paper (Whatman number 1, 185 mm) were weighed on a Sartorius four-figure weighing balance. Approximately 50 g of field-moist soil was placed into a weighed funnel containing a wet filter paper. This was stood on a glass jar and saturated with 100 ml DIW in three portions. The soil was left to drain freely for three hours, after which the funnel, paper and saturated soil were weighed prior to being placed in the ceramic dish. The dish was dried overnight at 105° C and then reweighed.

Field capacity was calculated according to the equation:

$$\frac{[(\text{Wt. saturated soil + paper + funnel}) - (\text{wt. funnel + wet paper}) - (\text{wt. oven dry soil})] * 100}{\text{Weight of oven dry soil}}$$

2.1.5 Soil sampling and heavy metal extraction procedures

2.1.5.1 Spofforth

Triplicate samples of soil were taken from Spofforth areas described in Section 2.1.4 at two depths, 0-25 cm and 25- 50 cm. Samples were collected in December 1998, March 1999 and June 1999. Sequential extraction analysis of the samples was carried out using the European Community Bureau of Reference (BCR) method (Ure *et al.*, 1993). Table 2.1.5.1 outlines the extractants and their target phases.

Table 2.1.5.1 BCR 3-stage sequential extraction scheme*

1	0.11 M Acetic Acid (HAc)	Soil solution, carbonates, exchangeable metals
2	0.1 M Hydroxylammonium Chloride ($OHNH_3Cl$) at pH 2	Iron/manganese oxyhydroxides
3	8.8 M hydrogen peroxide then M Ammonium Acetate (NH_4OAc) at pH 2	Organic matter and sulphides
(Residual)	Aqua Regia	Remaining non-silicate bound metals

*From Davidson *et al.*, 1998

Minor modifications were made to the procedure: the mechanical end-over-end shaker rotated at 60 rpm rather than 30 rpm, and the soil was treated with the various extractants in a 200 ml MSE centrifuge bottle rather than a 100 ml tube. Extractable soil concentrations of Zn, Cd, Cu, Ni, Pb and Cr were determined at Glasgow (Section 2.4); data are shown in Chapter 4.

2.1.5.2 Slough

Triplicate soil samples (0-25 cm depth) were collected from areas under growing plants of five *Salix* varieties (Jorunn, Germany, Q83, Candida and Bowles Hybrid) and three *Phalaris* varieties ('Palaton' [V1], 'SWRF8701' [V2] and 'Vantage' [V4]). Samples were also taken from unplanted areas. Glass jars containing air-dried, sieved (<2 mm) 5 g subsamples were rotated for 16 hours on an end-over-end shaker with 50 ml of an extractant: M ammonium acetate (NH_4OAc), pH 6 or 0.025 M ammonium ethylenediaminetetraacetic acid (NH_4EDTA), pH 4.6, prior to filtration through a Whatman 50 filter paper (150 mm) and determination of metal concentrations (see Chapter 4).

2.1.5.3 Rodley

At Rodley, triplicate soil samples were taken from areas under growing willows, and in unplanted areas, at two depths (0-25 cm and 25-50 cm). They were extracted and analysed as in Section 2.1.5.2. Results are shown in Chapter 4.

2.1.6 Soil Solution Extraction Procedure

Soil samples were collected from Spofforth as described in Section 2.1.4, and from Rodley as outlined in Section 2.1.5.3. Approximately 500 g of field-moist soil (sieved < 5.6 mm) was placed in perforated soil cylinder (containing a wet-strengthened filter paper at its base) before being adjusted to 50 % field capacity with DIW. The soil was left to equilibrate overnight with plastic wrapped over the top of the cylinder to prevent drying. The cylinders were centrifuged at 3000 rpm in a Jouan KR422 centrifuge for 20 minutes. Extracted solution was filtered through a hardened ashless filter paper (Whatman 541) and refrigerated prior to Inductively Coupled Plasma Mass Spectrometry (ICP-MS) determination of heavy metals (Section 2.5). Results are given in Chapter 4.

2.2 Pot Studies of Willows Grown on Contaminated Substrates

2.2.1 Sample Site Descriptions

2.2.1.1 Stoke-Bardolph, Nottinghamshire (SK 636419)

This 0.6 ha site was previously used for the long term recycling of sewage sludge: it received sludge for more than 50 years. The site was used in a screening trial of 20 willow varieties from 1995 until 1997 (Riddell-Black *et al.*, 1997), and has since been used to grow maize. Total soil heavy metal concentrations are presented in Table 2.2.1.1. Its pH is 6.3 and it is considerably calcareous (7.7 % lime) and highly organic, containing 10.2 % organic carbon (Pulford, pers. comm.).

*Table 2.2.1.1** *Properties of Stoke-Bardolph soil*

Metal	Zn	Cd	Cu	Ni	Pb	Cr
Concentration (mg kg^{-1})	2000	30	300	500	700	1500

*From Riddell-Black *et al.* (1997)

This soil was also prepared as an in-house reference material for soil analyses carried out at Glasgow (see Section 2.6).

2.2.1.2 Steelworks Waste Tip

The site in central Scotland covers approximately one square kilometre. Industrial waste generated by an iron and steelworks (iron oxide-rich filter cakes, and slags) was disposed there between 1947 and 1984, since when vegetation has naturally re-established. The waste is highly calcareous, with a lime content of 19.1 %, and its organic carbon content is 1.11 % (Schmidt, pers. comm). Its contaminant concentrations and pH are highly variable across the site; Table 2.2.1.2 gives average measurements of these determinands.

*Table 2.2.1.2** *Steelworks waste pH and total metal concentrations*

Determinand	Zn	Cd	Cu	Ni	Pb	Cr	pH
Concentration (mg kg^{-1})	2042	< 1	125	77	584	116	10.6

*Data supplied by Hepple (pers. comm.).

2.2.2 Soil Manipulation Pot Trial

The Stoke-Bardolph soil was sieved (< 5.6 mm), and four plant varieties were grown prior to transplantation:

- *Salix burjatica* 'Germany'
- *Salix triandra x viminalis* 'Q83'
- *Phalaris* 'SWRF8701'
- Barley, *Hordeum vulgare* cv. 'Triumph'.

Salix plants were grown for 4 weeks as in Section 2.3.1, and *Phalaris* and barley plants were grown from seed in compost for 2 weeks.

There were 3 soil treatments:

- No treatment control;
- 1.83 g ammonium sulphate plus 0.07 g dicyandiamide (DCD) per kg soil (Schremmer *et al.*, 1999);
- 1.00 g citric acid (CIT) per kg soil (Blaylock *et al.*, 1997).

Soil amendments were mixed thoroughly into the soil prior to 2.5 kg being placed in each pot. All treatments were replicated five times. The pots were put in a randomised block in a greenhouse. The glasshouse temperature was stabilised at a minimum of 20° C between the hours of 07:00 to 19:00, and 8° C between 19:00 and 07:00. The base of each pot was regularly watered every 2 or 3 days with 200 ml DIW. The experiment was carried out in summer (photoperiod approximately fourteen hours), therefore no supplementary lighting was provided.

Plant aerial tissues were harvested after 4 weeks. Samples were prepared as in Section 2.1.2 and weighed. Metal concentrations were determined as in Section 2.5. Data are given in Chapter 6.

2.2.3 Willow Growth Pot Trial

Samples of the steelworks waste were homogenised by rolling pooled samples in a barrel after removal of stones. Forty 9-inch diameter pots were filled with the waste.

Cuttings approximately 15 cm in length of *Salix burjatica* 'Germany' and *S. triandra x viminalis* 'Q83' were rooted in aerated troughs containing DIW for 35 days, followed by 1/8 strength MHS for 20 days. The trees were kept in a greenhouse in Glasgow maintained at 20° C by thermostat heaters, under 400 W metal halide lights (Sunlight Systems) providing a photoperiod of 14 hours.

Eight trees of each of the varieties were transplanted to the pots. A further eight trees of each variety had their roots dipped in a fungal inoculum "Mycor VAM Cocktail" (Symbio) containing *Glomus* sp., *G. etunicatum*, *G. clarum* and *Enterophosphora columbiana* immediately before planting. The remaining eight pots were kept as controls; four had samples of the inoculum placed in them. The pots were placed in a randomised block and irrigated every two or three days with approximately 500 ml of DIW, for 9 months.

Aerial plant tissues were prepared as in Section 2.1.2, weighed and analysed as described in Section 2.4. A soil corer was used to remove a few grams of substrate from the central base of each pot at the beginning and end of the 9 month experiment. Air-dried subsamples of each of these were digested in aqua regia for pseudototal metal determination (Section 2.4.1). A further two subsamples from each pot were selectively extracted by the procedure described in Section 2.1.5.2.

A suitable 4-step sequential extraction procedure was kindly carried out on subsamples of the pot trial by contacts at the University of Hohenheim. The series involved extraction of 4 g samples with a total of 50 ml M NH_4OAc (pH 6.0), 50 ml NH_4OAc with an appropriate amount of HCl to remove carbonate-bound metals, 90 ml 0.1 M $OHNH_3Cl$ and M NH_4OAc (pH 6.0), and 100 ml 0.025 M NH_4-EDTA (pH 4.6). Results from this experiment are displayed in Chapter 5.

2.3 Hydroponic Studies

Glasshouse plant experiments are frequently conducted hydroponically to counter soil heterogeneity and provide a homogenous nutrient medium to the experimental plants. Solutions can be stagnant, or circulated through the plants' root environment. The nutrient film technique (NFT) involves circulating a thin film of nutrient solution through the roots of plants supported (usually in pots) in a gently angled trough.

Each tank and channel of the NFT systems described below was flushed with 5 % HNO_3 for 24 hours, followed by DIW for 24 hours. Leaks were ensured to be minimal. The tanks, channels and channel pipes were emptied of all solution and thoroughly dried prior to the start of the experiment.

2.3.1 Willow Cutting Establishment

Black plastic pots (80 x 70 x 70 mm), with a lid made from the base of a larger 100 x 90 x 90 mm pot were used to root cuttings. The lid had a 20 mm hole drilled into it with a wood borer, for insertion of the cutting. Several holes around 5 mm in diameter were punctured in the base of the pot, and grooves were cut into the bottom 1 cm of the side of each pot using a hot needle and spatula to allow uninhibited root emergence. Stems of willow clones (AF Hill & Son, Worcestershire) were cut to 100 mm lengths and placed in pots filled with LBS perlite. These were kept in troughs containing DIW, filled to 6 cm depth. The cuttings were grown in DIW for two weeks, and 1/16 strength modified Hoaglands solution (MHS) for 2 weeks (see Section 2.3.5.1). After 4 weeks, uniform rooted cuttings with similar heights and number of stems were selected for placement in the NFT system.

2.3.2 Multi-tank Nutrient Film Technique (NFT) Set-Up at WRc

At WRc, the multiple-tank NFT system consisted of 14 individual units. Channels were constructed from 1m x 12 cm x 7 cm PVC guttering (Jewsons) and were arranged on a bench with 20 cm spacing between each channel. The channel solution inlets were elevated by 10 cm over the outlets to promote solution flow.

The channel inlet was fitted with a PVC stopped end and sealed with silicone. A 15mm tubing adapter (Sandvik) was attached to this. The outlet of the channel was connected to a square gutter down pipe (65mm x 50mm) which emptied into a 10 litre reservoir tank via an angled gutter joint. Solutions were pumped from the tank in 15 mm tubing (Tricoflex A5) by a small 2-way pump (Eheim) which was controlled from a central switching box. To facilitate cleaning of the tubes, two valves (Sandvik) were incorporated into this tubing either side of a connector (Sandvik). A unit from the system is displayed in Figure 2.3.2. All channels were covered in black-and-white LDPE plastic sheeting penetrated by the sapling stems, with the white surface outermost, to prevent algal colonisation and minimise evaporation losses, and secured with waterproof tape.

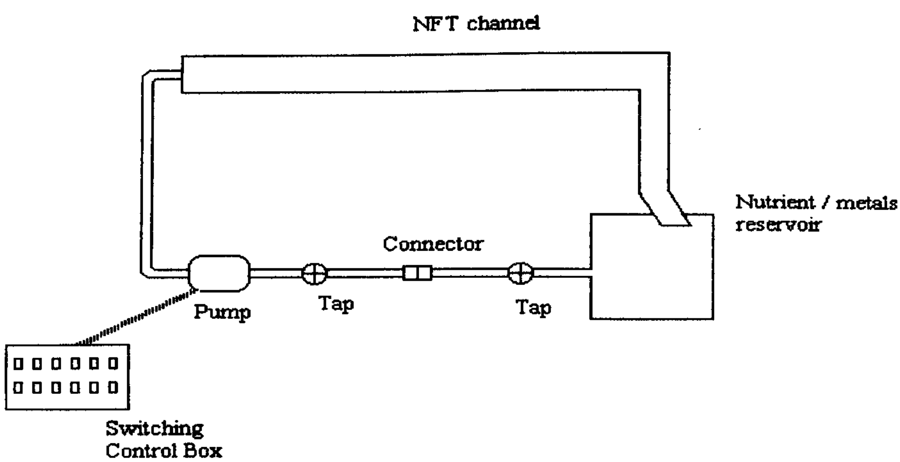

Figure 2.3.2 A single unit from the WRc multi-tank NFT system (courtesy of Val Woods, WRc)

2.3.3 Multi-tank NFT Set-Up at Glasgow

The system comprised of 12 channels (130 x 15 x 7.5 cm "Aqua Troughs", Sunlight Systems). The same pots were used as at WRc, filled with Silvapel perlite. Each channel drained into a funnel and 5 litre reservoir (see Plate 2.3.3). Solution was circulated to the top of each channel with a Mini-Jet MN404 pump (Sunlight Systems). Each channel was wrapped in LDPE black and white plastic sheeting. Temperature and light were controlled as described in Section 2.2.3.

Plate 2.3.3 Outlets of Glasgow multi-tank NFT channels

2.3.4 Central Reservoir NFT Set-Up at WRc

The NFT apparatus (Figure 2.3.4) consisted of three alternating plastic reservoir tanks (100 litre) linked to 14 PVC guttering channels by a centrifugal Stuart 16MKIII pump (dimensions and elevation as in Section 2.3.2). The channels were arranged on a bench with 10 cm spacing between each channel. A hole was bored in the inlet end of each channel to accept a 5 mm plastic tube. A hole was made in the base of the outlet guttering to accommodate a piece of tubing (20 mm diameter). The outlet tube from each channel descended to a single plastic drainpipe (6 cm diameter) which was again angled to create a flow of the solution towards a collecting tube and ultimately the reservoir tanks. Two valves were incorporated into this return tubing. There were three 100 l tanks connected into the system via valves. Two were used as media reservoirs; the third tank was filled with DIW and was used only to clean the system. Two of these systems were constructed to allow simultaneous testing of metal-exposed and control trees.

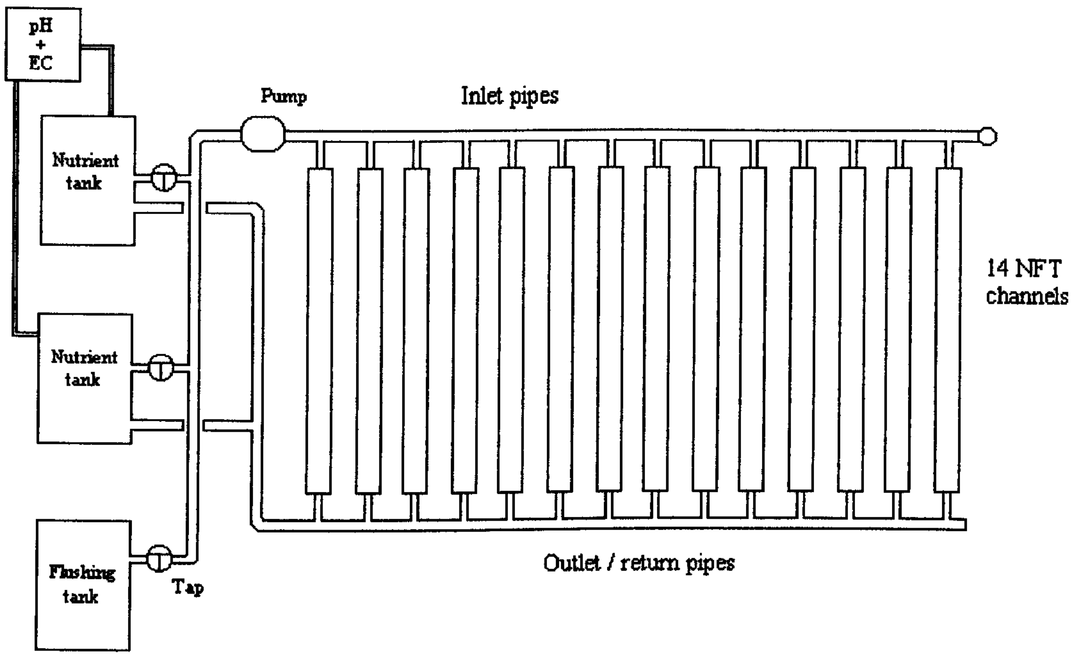

Figure 2.3.4 Central reservoir NFT design at WRc (courtesy of Val Woods). The pH and conductivity meters were not used in experiments

2.3.5 Background Nutrient Solution Strength Experiment

2.3.5.1 Solution Preparation

The following quantities of stock solution were made up to 20 l to make 1/4-strength modified Hoaglands solution (MHS):

- 25 ml M $Ca(NO_3)_2.4H_2O$
- 25 ml M KNO_3
- 25 ml 0.02 M Fe-EDTA
- 10 ml M $MgSO_4.7H_2O$
- 10 ml micronutrient solution (containing 2.86 g H_3BO_3, 0.11 g $ZnCl_2$, 0.06 g $CuCl_2.2H_2O$, 1.81 g $MnCl_2.4H_2O$ and 0.03 g $Na_2MoO_4.2H_2O$ made up to 1 litre)
- 5 ml M KH_2PO_4

The KH_2PO_4 was added during or after the dilution to the final volume to avoid problems of poorly soluble phosphates precipitating out of the stock solution. This recipe was adjusted accordingly to provide 1/8 and 1/16 strength MHS. The pH of the nutrient solutions was adjusted to 5.5 using 0.1 M NaOH and an Orion 420 pH meter. 0.1 M stock solutions of Zn, Cd, Pb, Cr, Cu and Ni were made using the following metal salts: $ZnSO_4.7H_2O$, $3CdSO_4.8H_2O$, $Pb(NO_3)_2$, $CrCl_3.6H_2O$, $CuSO_4.5H_2O$ and $NiSO_4.6H_2O$. Appropriate quantities of the stocks were added to the nutrient solutions to provide a 200 μM Zn solution, and 10 μM Cd, Pb, Cr, Cu and Ni solutions. All solutions were stored in containers within black bags to prevent algal colonisation.

2.3.5.2 Experimental Design

Cuttings of *Salix burjatica* 'Germany' and *S. triandra x viminalis* 'Q83' 10 cm long were rooted as described in Section 2.3.1. Six pots were randomly assigned to each of twelve channels in the WRc multi-tank system. The twelve channels were randomly assigned one of the four experiment treatments, each replicated three times:

- Control (1/4 strength MHS)
- 1/4 strength MHS plus metal cocktail
- 1/8 strength MHS plus metal cocktail
- 1/16 strength MHS plus metal cocktail

2.3.5.3 Solution rotation and tree sampling regime

Each tank was filled with 7 litres of appropriate solution. The saplings were exposed to a metal-amended MHS, with P omitted to avoid Pb phosphate precipitation problems, for five days, followed by two days exposure to a phosphorus-containing MHS not amended with metals. Control trees were also deprived of P for 5 days of the week. The tanks and channel pipes were drained of all liquid and thoroughly dried between the switch of solutions.

A tree was removed from each channel every week for 6 weeks. The sample heights were recorded, along with the number of shoots and a description of the leaves and roots. Samples were photographed in groups of treatments, and also across the range of treatments, to display any marked differences in growth.

The samples were then separated into leaf and root portions, weighed and prepared for analysis as in Section 2.1.2. Zinc, Cd, Cu, Ni, Pb and Cr were determined in the tissue fractions as in Section 2.5. Data are portrayed in Chapter 7.

2.3.6 Metal Ratio Experiments

2.3.6.1 Experimental Design

In the WRc multi-tank system, two blocks of six channels were used, one for each variety ('Q83' and 'Germany'). Treatments were randomly assigned to a channel within each block, and 6 pots were assigned to each channel as in Section 2.3.5.2. The six treatments used were:

- Control (no metal)
- 100 μM Cu and 10 μM Ni
- 50 μM Cu and 10 μM Ni
- 10 μM Cu and 10 μM Ni
- 10 μM Cu and 50 μM Ni
- 10 μM Cu and 100 μM Ni

A second experiment was run with the same design as before using the following treatments:

- Control (no metal)
- 200 μM Zn and 10 μM Cd
- 100 μM Zn and 10 μM Cd
- 50 μM Zn and 10 μM Cd
- 10 μM Zn and 10 μM Cd
- 10 μM Zn and 100 μM Cd

In both cases the background solution was 1/4 strength MHS. Each channel was supplied with 10 l of treatment solution (set at pH 5.5 as in Section 2.3.5.1) which was replaced each week.

2.3.6.2 Sampling Regime

In both experiments, three trees from each channel were sampled at the end of weeks 3 and 6 of the experiment. Samples heights were recorded prior to separation into leaf, stem and root fractions. Samples were prepared as in Section 2.1.2, weighed and analysed for the relevant determinands (see Section 2.5). Results are presented in Chapter 8.

2.3.7 Phosphorus Nutrition Experiment

In the Glasgow multi-tank system, six trees were randomly assigned to a channel. The background solution was 1/4 strength MHS, and Pb was excluded from the metal cocktail. The following range of treatments were randomly assigned to a channel containing either *Salix burjatica* (Germany) or *S. triandra x viminalis* (Q83):

- 5 days metal exposure, 2 days P provision
- Control (2 days P provision, no metals)
- 5 days metal exposure, 7 days P provision
- Control (7 days P provision, no metals)

Control solutions providing 7 days P were changed twice a week. Samples were taken as in Section 2.3.6.2, and prepared as in Section 2.1.2 prior to weighing (data shown in Chapter 8).

2.3.8 pH Experiment

The Glasgow multi-tank system was used in this experiment. The channels contained one of the following metal treatments (in a background solution of 1/4 strength MHS) or a corresponding control at the same pH:

- *Salix burjatica* (Germany), pH 3.5
- *S. triandra x viminalis* (Q83), pH 3.5
- Germany, pH 5.5
- Q83, pH 5.5
- Germany, pH 7.5
- Q83, pH 7.5

The pH of the solutions were adjusted using 10 % HNO_3 or M NaOH and a Mettler Delta 320 pH meter. Solutions were changed twice weekly, and the sampling regime followed that outlined in Section 2.3.7. Results are given in Chapter 8.

2.3.9 Multiple Willow Clone Screening Experiment

2.3.9.1 Solution Preparation

Contrasting with the previous experiments described, the composition of the background nutrient solution (1/4 strength MHS) was altered to provide N as NH_4^+ as well as NO_3^-. In the metal-exposed replicates, the metal concentrations were doubled after the first half of the six-week experiment. Changes to the original metal/nutrient solution recipe are displayed in Table 2.3.9.1. Entries in bold highlight a change in the solution recipe.

Table 2.3.9.1 Composition of 20 l of the nutrient/metal solution in initial and subsequent NFT runs.

Initial tests	Subsequent tests	Initial tests	Subsequent tests
25 ml M $Ca(NO_3)_2.4H_2O$	**20 ml M $Ca(NO_3)_2.4H_2O$**	200 μM Zn	**100 μM Zn for first 3 weeks of experiment, then 200 μM Zn for final 3 weeks**
25 ml M KNO_3	25 ml M KNO_3	10 μM Cd	**5 μM Cd then 10 μM Cd**
25 ml 0.02 M Fe-EDTA	25 ml 0.02 M Fe-EDTA	10 μM Cu	**5 μM Cu then 10 μM Cu**
10 ml M $MgSO_4.7H_2O$	10 ml M $MgSO_4.7H_2O$	10 μM Ni	**5 μM Ni then 10 μM Ni**
10 ml micronutrient solution*	10 ml micronutrient solution*	10 μM Cr	**5 μM then 10 μM Cr**
5 ml M KH_2PO_4	**10 ml M $NH_4H_2PO_4$**	10 μM Pb	**No Pb applied**

*containing (H_3BO_3, $ZnCl_2$, $CuCl_2.2H_2O$, $MnCl_2.4H_2O$, $Na_2MoO_4.2H_2O$ and $MnCl_2.4H_2O$)

2.3.9.2 Experimental Design

The two central reservoir NFT systems at WRc were used to screen seven varieties at one time. One system was used as the control with the trees exposed to 1/4 strength MHS only, while in the other the trees were exposed to the 1/4 strength MHS plus metal cocktail. Duplicate channels of each variety were assigned randomly in both the control and treatment systems. Each channel contained six trees. The volume and frequency of solution rotation was consistent in all NFT experiments (140 l fresh solution per system per week).

Experiments produced data for a total of eighteen clones: *Salix burjatica* 'Germany', *S. triandra x viminalis* 'Q83', *S. caprea x viminalis* 'Coles', *S. aurita x cinerea x viminalis* 'Delamere', *S. viminalis* 'Jorunn', *S. viminalis* 'Ulv', *S. viminalis* 'Orm', *S. viminalis* 'Q683', *S. triandra* 'Black Maul', *S. viminalis* 'Bowles Hybrid', *S. caprea x cinerea x viminalis* 'Calodendron', *S. candida*, *S. dasyclados*, *S. aquatica* 'Gigantea', *S. viminalis* 'Jor', *S. viminalis* 'Lysta 699', *S. spaethii*, and *S. viminalis* 'Tora'. Q83 was the reference clone for each NFT run.

2.3.9.3 Sampling Regime

This followed the protocol outlined in Section 2.3.7; additionally, stem lengths were measured at weekly intervals, allowing the following glasshouse data to be obtained for each clone: root biomass, leaf biomass, stem biomass and the height increase. Results are shown in Chapter 9.

2.4 Heavy Metal Analysis at Glasgow

Samples were analysed using a Perkin-Elmer 1100B AAS apparatus. Within each sample batch, blanks were checked regularly (at least every six samples) to check for contamination from reagents, glassware or any step of the analytical procedure. Blank correction was applied where appropriate. A standard solution was also checked regularly during routine AAS analysis (at least every 12 samples) to check for instrumental drift. A standard solution was also prepared from a separate stock solution to assess the accuracy of the stock solution normally used.

2.4.1 Soil Analysis

Approximately 0.25 g subsamples, ground with a mortar and pestle, were weighed on a Mettler AE 200 4-figure balance, and digested for a minimum of 4 hours (after an overnight cold digestion) at 125° C in boiling tubes in a Tecator digestion block. Samples were digested in batches of 40, with a minimum of 2 blanks per batch. Samples were digested in 10 ml aqua regia (consisting of 3 parts 6 M HCl to one part concentrated HNO_3). Following this, the aqua regia was filtered, with DIW washings, through a Whatman no. 50 (150 mm) filter paper and made up to 50 ml in a volumetric flask. Conversion factors from air-dried to oven-dried at 105° C had been calculated for each substrate; all results were reported on an oven-dried basis.

2.4.2 Plant Analysis

All plant tissue samples were dried to a constant mass at < 40° C, ground (< 2 mm) using a Fritsch laboratory cutting mill "Pulverisette 19". Approximately one gram of material was accurately weighed using a 4-figure balance and placed into a digestion tube. Ten ml of concentrated HNO_3 was added to each tube and left overnight prior to being heated at 125° C in a digesting block for 4 hours. Each batch of 40 contained at least two blanks and two samples of certified reference material (Section 2.6). The digest was transferred with washings and filtered through a Whatman 50 paper (150mm) prior to being made up to 100 ml with DIW.

2.5 Heavy Metal Analysis at WRc

Dried, ground and sieved plant samples (Section 2.1.2) were digested by boiling in concentrated nitric acid. The digest was made up to volume and the metals (Zn, Cd, Cu, Ni, Pb and Cr) were determined by AAS using a Thermo Jarrell Ash Video 12E Spectrometer. Concentrations were reported on a dry matter basis, after moisture content was determined gravimetrically by drying a known weight of sample to a constant mass at 105° C. Where concentrations of Cr, Pb and Ni were very low, Graphite Furnace AAS apparatus was utilised in their determination, using a Perkin-Elmer 5000 Zeeman Atomic Spectrometer. Metal concentrations in soil solution extracts (Section 2.1.6) were quantified by ICP-MS, using a Perkin-Elmer Elan 6000 Atomic Spectrometer.

2.6 Analytical Quality Control

2.6.1 Glasgow

Total metal concentrations were determined in 40 replicates of each of the substrates below. Subsequently, these were used as internal reference materials in soil and spoil analyses. Their metal ranges are displayed in Tables 2.6.1 and 2.6.2. The materials were collected in March 1998, homogenised by rolling in a barrel, air-dried and sieved <2 mm.

Table 2.6.1 Total metal concentrations in Newton colliery spoil (mg kg^{-1})

Metal	Pb	Cr	Cu	Ni	Zn	Cd
Mean	244	42	125	54.6	1020	5.44
Std. Error	13.9	0.56	11.5	1.09	150	0.83
95 % C.I.	(216, 273)	(41.0, 43.3)	(102, 149)	(52.4, 56.8)	(713, 1327)	(3.74, 7.14)

Table 2.6.2 Total metal concentrations in Stoke-Bardolph soil (mg kg^{-1})

Metal	Pb	Cr	Cu	Ni	Zn	Cd
Mean	627	1733	1087	423	2052	45.5
Std. Error	18.9	27.4	17.0	11.7	42.0	0.80
95 % C.I.	(588, 665)	(1677, 1789)	(1052, 1122)	(399, 447)	(1966, 2138)	(43.9, 47.2)

Batches of vegetation analysis carried out at Glasgow contained samples of the certified reference material SRM 1573a (tomato leaves).

2.6.2 BIORENEW Ring Tests

As part of the BIORENEW project's analytical quality assurance, samples of the in-house reference materials were sent to WRc for an additional check on analytical consistency. Also, twice during the project, five ring test materials were sent to Glasgow for determination of Cu and Cd concentrations to assess the precision and accuracy of the results, to ensure results produced by all the partners within the project were comparable, and to gauge the analytical procedures' quality. The performance targets were 5 %

precision and 10 % bias. These materials were soil, a soil digest (in 20 % aqua regia), plant tissue material, a plant tissue digest (in 10 % HNO_3) and a standard solution (in 0.5 M HNO_3). They were prepared and analysed within routine batches, in triplicate. Glasgow's results were satisfactory in both BIORENEW ring tests.

2.7 Statistical Analysis

The statistical package Minitab 13 for Windows was used to compare treatment means using analysis of variance; Fisher's Least Significant Difference test subsequently identified any significantly different means at 95 % confidence level. This was applied to data presented in Chapters 3, 4, 6, 7 and 8. In Chapter 5, within each treatment (control, Q83, Germany), a paired t test was used to compare the data from February and November following an initial ANOVA.

The Principal Components Analysis program CANOCO Version 4.0 for Windows was used to visually express the relative importance of various measurements taken from willow clones grown in the NFT experiments and at Stoke Bardolph in a field trial (Riddell-Black *et al.*, 1997), and any groupings of the clones according to these parameters. When assessing the linear relationship between greenhouse and field variables, Minitab 13 for Windows was used to test the significance of the Pearson point correlation.

Chapter 3 Heavy Metal Compartmentation in Field-Grown Willows: Effects of Sampling Height, Harvest and Season

3.1 Introduction

In considering the phytoextraction of heavy metals by willow from contaminated soils, the compartmentation of translocated metals is highly important. For example, extracted metals are mainly returned to the soil from leaves, through leaching or following leaf abscission (Lepp, 1995). Metal concentrations in wood are frequently lower than in roots and bark, but the fraction represents a very significant proportion of the total amount of metal in a tree due to its biomass (Dickinson and Lepp, 1997).

The effects of season, and the location on the tree where the samples are taken from, on tissue metal concentrations are significant. Previous seasonal studies have been limited to foliar metal concentrations, while the importance of sampling height has been demonstrated by Sander and Ericsson (1998), but the considerably different stem fractions of wood and bark were not distinguished.

Therefore, this chapter had two main aims:

- To comprehensively study the compartmentation of metals in the above-ground willow fractions in trees grown at a contaminated site.
- To thoroughly study the effects of sampling height and season on metal concentrations in leaves, wood and bark through regularly collected vegetation samples from field-grown willows.

The Spofforth site was used for all vegetation sampling. The sampling regime is detailed in Sections 2.1.2 and 2.1.3, and the analysis methodology in Section 2.5.

3.2 Sampling Height Effects

Previous work examining metal concentrations in stem samples at different sampling heights observed an increasing trend in metal contents with increasing height, and attributed it to increasing proportions of bark in a stem (Sander and Ericsson, 1998). A batch of stem portions collected in June 1998 were separated into bark and wood fractions to provide a rigorous examination of this factor, while leaf samples collected from different tree heights were also analysed.

Table 2.1.1.1 exemplifies the considerable variability in metal concentrations in the soil across the Spofforth site. As metal concentrations in tissues of plants grown on contaminated soils can be positively correlated with those in soil, this variability often translated into variable herbage concentrations. This variability reduced the clarity of the trends when the data were pooled for Germany replicates taken from the site's 5 blocks. This is exemplified by the frequently large standard errors contained in Table 3.2. However, the table consistently displays a trend of increasing concentrations with height in the bark and wood samples, and a decreasing trend with height in leaf samples. These trends were significant for Cu, Ni, Pb and Cr, particularly in the bark and leaf fractions, but not for Zn and Cd.

Table 3.2 Mean concentrations (mg kg^{-1}) of metals (standard error in brackets) in Germany fractions sampled at 3 heights, June 1998. "nd" denotes a figure at or below the detection limit. For each metal and tree fraction, means (n=5) lacking a letter, or with a letter in common, are not significantly different (p > 0.05) after a Fisher LSD test

	Zn	Cd	Cu	Ni	Pb	Cr
Bark						
0.5-0.6 m	385 (42.5)	2.23 (0.26)	3.77 (0.13) a	0.87 (0.07) a	1.42 (0.08) a	0.50 (0.07) a
1.5-1.6 m	487 (41.6)	2.58 (0.32)	4.91 (0.19) a	0.96 (0.09) a	2.30 (0.12) a	0.55 (0.06) a
2.5-2.6 m	490 (60.4)	2.63 (0.27)	8.19 (0.92) b	1.58 (0.18) b	2.65 (0.42) b	0.83 (0.12) b
Wood						
0.5-0.6 m	48.8 (5.43)	0.44 (0.05)	8.29 (0.56) a	nd	nd	nd
1.5-1.6 m	73.1 (11.5)	0.50 (0.04)	12.7 (1.27) b	nd	nd	nd
2.5-2.6 m	90.3 (22.8)	0.58 (0.07)	18.9 (2.05) c	nd	nd	nd
Leaves						
0.5-1.5 m	1183 (188)	3.21 (0.41)	8.71 (0.50)	2.39 (0.20) a	1.60 (0.14)	0.60 (0.07) a
1.5-2.5 m	1079 (198)	3.45 (0.48)	8.65 (0.09)	1.88 (0.15) b	1.40 (0.10)	0.40 (0.06) b
2.5-3.5 m	1246 (187)	2.88 (0.31)	8.38 (0.34)	1.58 (0.08) b	nd	0.32 (0.03) b

Zn concentrations were the greatest, followed by Cu, Cd, Ni, Pb and Cr. Plant uptake and transport of Cr to aerial tissues is minimal at a near-neutral pH (McGrath, 1995), explaining the very low tissue concentrations found. Leaf concentrations of Zn, Cd and Ni were greater than in the stem fractions. Lead concentrations appear to be highest in the bark, while the greatest Cu concentrations were in the wood. These trends have been observed in other studies: of the above-ground biomass metal concentrations in willows grown on sludge-amended soil, Cu, Pb and Cr were mainly in the stems, while Zn, Cd and Ni were in the leaves (Hasselgren, 1999). Turner and Dickinson (1993) noted that most of the Pb not retained in the roots of sycamore trees grown in Pb-contaminated soil was translocated to the stem.

Importantly, the sampling height effect on Cu concentrations in bark and wood was sufficiently strong to display a marked trend in the pooled data. The Ni and Cr bark data revealed a similar, although less marked, trend of increasing bark concentrations with increasing sample height across the suite of samples. Interestingly, these significant increases with sampling height seem to be paired with corresponding significant decreases in leaf concentrations of Ni and Cr with sampling height.

The leaf data displayed no trends for the readily translocated Zn and Cd (concentrations are similar at all sampling heights), and only a very slight trend of decreasing concentrations with height for the essential micronutrient Cu. This was also reported by Guha and Mitchell (1966): Ni, Cr and Pb concentrations in beech, sycamore and horse chestnut leaves were considerably lower at the top than the base of the tree; while Zn and Cu showed only a slight reduction in concentrations with height.

This indicates that a greater proportion of the translocated Zn, Cu and Cd was deposited in the leaves, offsetting the trend of decreasing foliar concentrations with increasing height observed for Ni, Cr and Pb. There may have also been a physiological mechanism in which Ni, Cr and Pb were moved in greater quantities into the lower leaves. Alternatively, there may have been a growth dilution effect whereby leaves at the top of the stem have greater biomass, thus decreasing the foliar concentrations of Ni, Cr and Pb but not affecting the essential micronutrients Zn and Cu, nor the readily translocated Cd.

Figure 3.2.1 shows concentrations of metals in bark of a typical sample clearly revealing an effect of increasing concentrations with increasing height; metal which is translocated from the root is transported to the top of the stem. Figures 3.2.2 and 3.2.3 respectively display concentrations of Cu and Zn in the wood and bark stem fractions. The elements contrast in that greater concentrations of Cu are found in the wood, whereas Zn is present in greater concentrations in the bark. It is likely that, in the wood fraction, stem girth has a considerable effect: increasing thickness towards the bottom of the stem may impose a dilution effect on the metal concentrations.

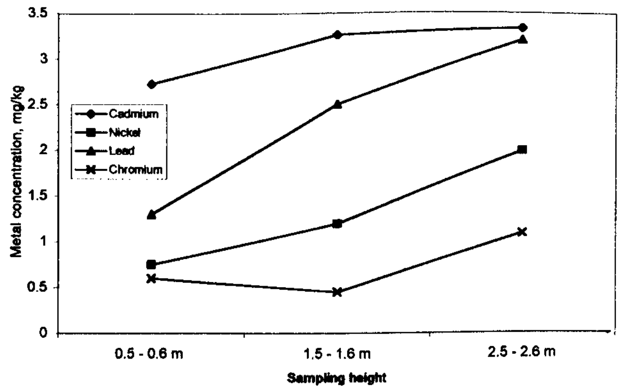

Figure 3.2.1 Metal concentrations (mg kg^{-1}) in bark at 3 sampling heights within a typical Germany tree sample, June 1998

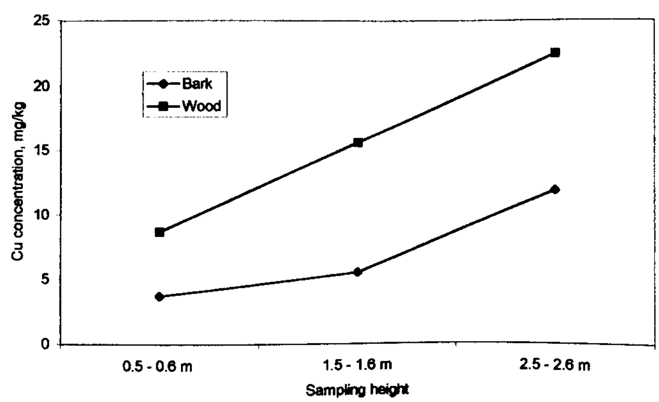

Figure 3.2.2 Cu concentrations in wood and bark (mg kg^{-1}) at 3 sampling heights within a typical Germany tree sample, June 1998

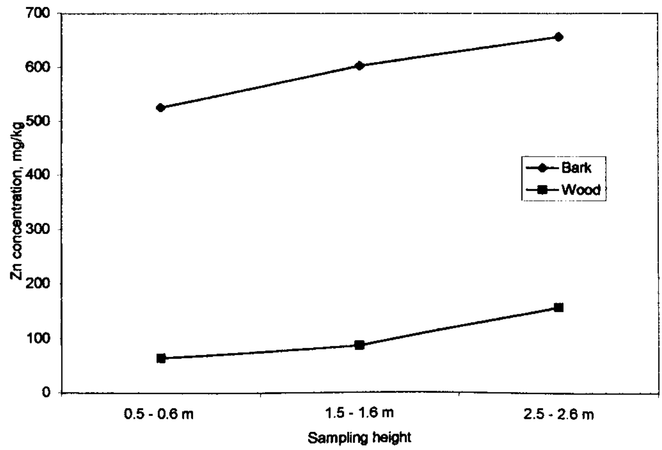

Figure 3.2.3 Zn concentrations in wood and bark (mg kg^{-1}) at 3 sampling heights within a typical Germany tree sample, June 1998

It is obvious from Figures 3.2.1 to 3.2.3 that the sampling height effect is more pronounced for less translocatable elements such as Cu and Pb, and markedly less pronounced for more readily translocated elements such as Zn and Cd.

In stems of *Salix viminalis*, Sander and Ericsson (1998) reported Ni and Cu to increase most from the lowest to the highest sampling point, followed by Zn and Cd. This corresponds with the observations made at Spofforth. The trends observed by these authors were thought principally to be a consequence of increasing bark proportions; data presented here indicate concentrations increase within separate stem fractions, which is likely to be a result of upward translocation of metals in xylem and phloem and decreasing stem girth with height. Transfer of metals between wood and bark is discussed in Section 3.4. Sampling height thus has a major impact on results; stem samples for the seasonal study of metal compartmentation were therefore standardised by being taken at the midpoint of the leafed and non-leafed stem (Section 3.4).

3.3 Compartmentation of Metals in Spofforth Willows

As values for the contents of Ni, Pb and Cr were frequently close to or below the detection limit in wood samples (Table 3.2), this study was limited to the three most abundant heavy metals in the Spofforth willow tissues: Zn, Cd and Cu. Partitioning of metals between root and shoot, and within the stem, can vary considerably in different willow varieties (Punshon *et al.*, 1995b). Despite this, statistical analysis using analysis of variance revealed interesting results when the data for samples of all 5 varieties collected in July 1998 were pooled, and the distribution of the metals throughout the five fractions (Tables 3.3.1 to 3.3.3) examined.

Table 3.3.1 Mean metal concentrations (mg kg^{-1}) of Zn in 5 willow varieties, July 1998. Means (n=25) without a letter in common are significantly different (p < 0.05) after a Fisher LSD test

Leaves	543 a
Leafed Bark	285 b
Non-Leafed Bark	274 b
Leafed Wood	35.2 c
Non-Leafed Wood	28.3 c

Table 3.3.2 Mean metal concentrations (mg kg^{-1}) of Cd in 5 willow varieties, July 1998. Letters apply as in Figure 3.3.1

Leaves	2.03 a
Leafed Bark	1.57 b
Non-Leafed Bark	1.56 b
Leafed Wood	0.32 c
Non-Leafed Wood	0.28 c

Table 3.3.3 Mean metal concentrations (mg kg^{-1}) of Cu in 5 willow varieties, July 1998. Letters apply as in Figure 3.3.1

Leaves	10.1 a
Leafed Wood	7.66 b
Unleafed Wood	4.84 c
Leafed Bark	4.74 c
Unleafed Bark	3.46 d

Leaf concentrations of all three metals were significantly greater than in the stem fractions. Bark concentrations of Zn and Cd were significantly greater than those in wood; the reverse was true for Cu. Nissen and Lepp (1997) determined Cu and Zn concentrations in shoot and leaf tissue of eight *Salix* species. Copper concentrations were in the order leaves > wood > bark, while Zn concentrations ranked leaves > bark > wood, corresponding with the trends observed in Spofforth samples.

The results are supported further by other studies. Riddell-Black (1994) harvested willow varieties grown on sludge-amended soil, and reported greater foliage concentrations of Zn, Cd and Cu than those in the stem. Zinc and Cd concentrations were higher in leaves than in wood in field-grown sycamore trees (Turner and Dickinson, 1993), and in poplar clones grown in sludge-amended soil (Drew *et al.* 1987).

Where bark and wood metal concentrations are similar, the trees are generally exposed to less metal contamination, such as the Cd concentrations in several fruit tree species grown in a 6-year trial (Korcak, 1989); elevated bark levels tend to signify pollution (Dickinson and Lepp, 1997). Willow bark concentrations of Zn and Cd were found to be consistently greater than the concentrations in wood of the same clone by Riddell-Black *et al.* (1997), and concentrations of metals in willows grown on sludge-amended soil were normally higher in the bark than in the wood (Hasselgren, 1999). Therefore the Zn and Cd concentrations in Spofforth samples point to uptake from a contaminated substrate.

Additionally, the metals display sampling height effects; the leafed wood and bark contain greater concentrations than the corresponding non-leafed fractions further down the stem. This is most marked for the less easily translocated Cu, and hence concentrations are significantly greater in the leafed stem fractions.

3.4 Effects of Season and Harvest

Concentrations of metals in samples taken from the five *Salix* varieties in July 1998 were compared. Germany consistently achieved the greatest wood and bark concentrations, and Q683 the lowest, while this clone also displayed the lowest foliar concentrations. Of the four varieties grown on a sludge amended soil by Riddell-Black (1994), Q683 generally achieved lower stem concentrations of metals than Q83, Bowles' Hybrid and Dasyclados.

Therefore, for the analysis of time series samples, it was decided to concentrate on the two varieties Germany and Q683, and the determinands Zn, Cd and Cu. Trees were harvested in December 1998, and subsequently re-grew from the coppiced stools. Bark and wood could not be distinguished in June 1999, so only leaf data are presented for this date. Conversely, senescence was advanced or completed in November 1998 and 1999, so no time series leaf data are presented for these dates. Small samples of leaves and litter were collected at November 1999, however (Section 3.4.4.1). No Q683 leaves could be sampled in March 2000. Table 3.4 summarises the vegetation fractions collected on each of the sampling dates. Where no significant differences were recorded between metal concentrations of any of the time series sampling points, the data have not been presented.

Table 3.4 Germany and Q683 samples collected during nine sampling dates at Spofforth, 1998 - 2000

July 1998	September 1998	November 1998	December 1998	June 1999	November 1999	March 2000	June 2000	September 2000
Leaves	Leaves	No leaves	*Harvest*	Leaves	No leaves	Leaves (Germany only)	Leaves	Leaves
Leafed Wood	Leafed Wood	Leafed Wood	*Harvest*	No leafed Wood	Leafed Wood	Leafed Wood	Leafed Wood	Leafed Wood
Non-Leafed Wood	Non-Leafed Wood	Non-Leafed Wood	*Harvest*	No non-leafed wood	Non-Leafed Wood	Non-Leafed Wood	Non-Leafed Wood	Non-Leafed Wood
Leafed Bark	Leafed Bark	Leafed Bark	*Harvest*	No leafed bark	Leafed Bark	Leafed Bark	Leafed Bark	Leafed Bark
Non-Leafed Bark	Non-Leafed Bark	Non-Leafed Bark	*Harvest*	No non-leafed bark	Non-Leafed Bark	Non-Leafed Bark	Non-Leafed Bark	Non-Leafed Bark

3.4.1 Copper

Leaf concentrations (Figure 3.4.1.1) in both varieties showed decreases towards the end of the growing season in 1998 and 2000; this was statistically significant during 2000 for Q683. Interestingly, the concentrations in the 5 year old, pre-harvest trees are greater in Q683 than in Germany, whereas the reverse is true in all other fractions. Growth dilution is evident from March to June 2000 in Germany leaves, the concentrations displaying a significant decrease in this clone.

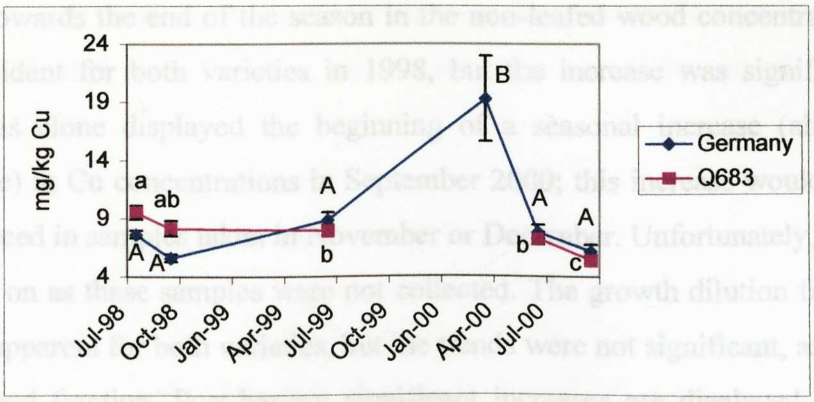

Figure 3.4.1.1 *Cu concentrations in leaves (mg kg^{-1}) at 6 sampling dates: July 1998 to September 2000. Within each variety, means (n=5) displaying no letters, or with a letter in common, are not significantly different after a Fisher LSD test (p> 0.05). Capitals refer to Germany; lower case letters to Q683*

The leafed wood data (Figure 3.4.1.2) display a gentle decrease in concentrations towards the end of the growing season. This was not significant from September to November 1998, but it was significant from March to September 2000 in both varieties. Concentrations in samples taken in November 1999, after the trees had regrown following the December 1998 harvest, were significantly greater in both varieties. This is possibly due to the rate of metal uptake in the younger trees being high relative to biomass production. This phenomenon is observed elsewhere in this section and is referred to as a "post-harvest increase". As in the leaf fraction, a growth dilution effect from March to June 2000 was evident, significant in Germany.

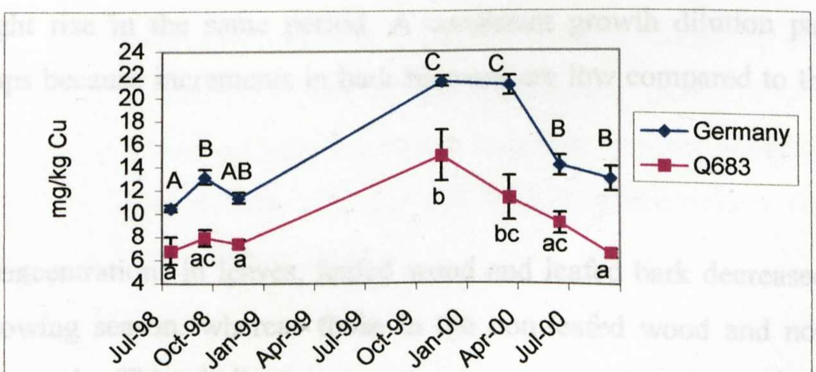

Figure 3.4.1.2 *Cu concentrations in leafed wood (mg kg^{-1}) at 7 sampling dates: July 1998 to September 2000. Letters apply as in Figure 3.4.1.1*

An increase towards the end of the season in the non-leafed wood concentrations (Figure 3.4.1.3) is evident for both varieties in 1998, but the increase was significant only in Germany. This clone displayed the beginning of a seasonal increase (although not a significant one) in Cu concentrations in September 2000; this increase would probably be more pronounced in samples taken in November or December. Unfortunately, this can only be speculated on as these samples were not collected. The growth dilution from March to June 2000 is apparent for both varieties, but the trends were not significant, as they were in the leafed wood fraction. Post-harvest significant increases are displayed in November 1999 for Q683 relative to November 1998, and in Germany relative to September 1998.

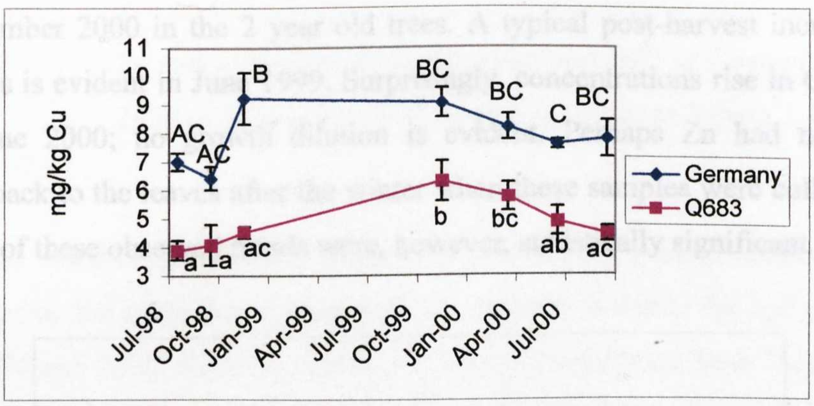

Figure 3.4.1.3 *Cu concentrations in non-leafed wood (mg kg^{-1}) at 7 sampling dates: July 1998 to September 2000. Letters apply as in Figure 3.4.1.1*

The leafed and non-leafed bark concentrations did not reveal any statistically significant trends. They contrasted in that the former displayed a marginal, non-significant seasonal decrease from July 1998 and June 2000 to the end of the respective season, while the latter showed a slight rise in the same period. A consistent growth dilution pattern did not emerge, perhaps because increments in bark biomass are low compared to those in leaves and wood.

Overall, Cu concentrations in leaves, leafed wood and leafed bark decreased towards the end of the growing season, whereas those in the non-leafed wood and non-leafed bark fractions increased. This indicates a pre-senescence migration of this essential micronutrient from leaves and bark into wood. This trend is particularly marked in the 1998 samples; it is not clear in the 2000 samples as November samples were not collected and analysed. Copper does not move from old leaves to new growing points (Baker and Senft, 1995), but it does appear to have been redistributed into stems towards the later stages of the growing season. Post-harvest increases in concentration are more marked in the leaves and leafed stem fractions, as are decreases caused by growth dilution from March to June 2000.

3.4.2 Zinc

The Zn data (Figures 3.4.2.1 to 3.4.2.3) also reveal comparable trends to those for Cu. The leaf concentrations (Figure 3.4.2.1) show a decreasing trend in both varieties towards the end of the growing season from July to September 1998 in the 5 year old trees, and from July to September 2000 in the 2 year old trees. A typical post-harvest increase in foliar concentrations is evident in June 1999. Surprisingly, concentrations rise in Germany from March to June 2000; no growth dilution is evident. Perhaps Zn had not been fully translocated back to the leaves after the winter when these samples were collected in early spring. None of these observed trends were, however, statistically significant.

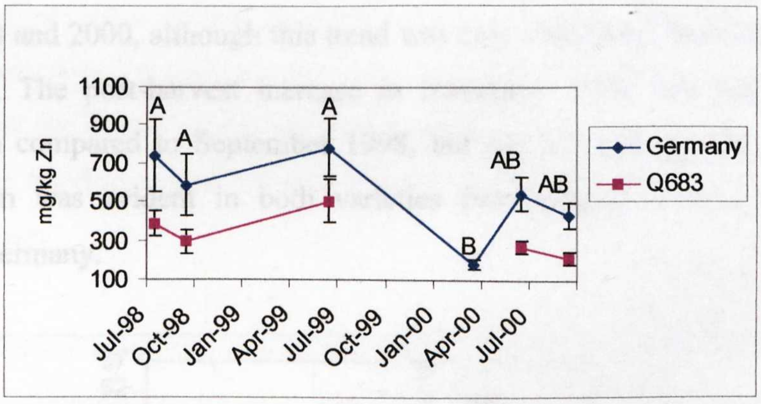

Figure 3.4.2.1 *Zn concentrations in leaves (mg kg^{-1}) at 6 sampling dates: July 1998 to September 2000. Within each variety, means (n=5) displaying no letters, or with a letter in common, are not significantly different after a Fisher LSD test (p> 0.05). Capitals refer to Germany; lower case letters to Q683*

This decreasing trend in leaf concentrations during a growing season seems to be matched by a corresponding rise from July in the concentrations of leafed wood (Figure 3.4.2.2). This phenomenon was significant in Germany from July to September 1998, and from July and September to November 1998 in Q683. The trend was evident, but not significant, in Germany from June to September 2000. This fraction also displayed a post-harvest increase in Germany. The expected growth dilution trends from March to June 2000 were evident in both clones, but only significant in Germany.

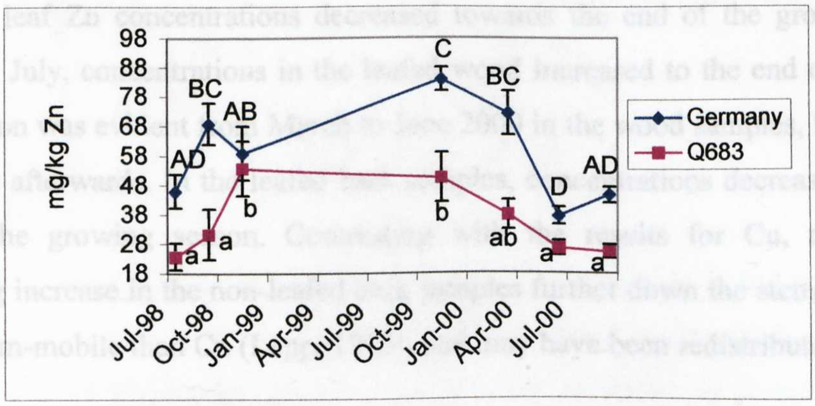

Figure 3.4.2.2 *Zn concentrations in leafed wood (mg kg^{-1}) at 7 sampling dates: July 1998 to September 2000. Letters apply as in Figure 3.4.2.1*

The non-leafed wood concentrations (Figure 3.4.2.3) display a trend similar to the leafed fraction further up the stem; concentrations rose gradually towards the end of the growing seasons in 1998 and 2000, although this trend was only significant from July to November 1998 in Q683. The post-harvest increase in November 1999 was only significant in Germany when compared to September 1998, but not November 1998, concentrations. Growth dilution was evident in both varieties from March to June 2000, but only significant in Germany.

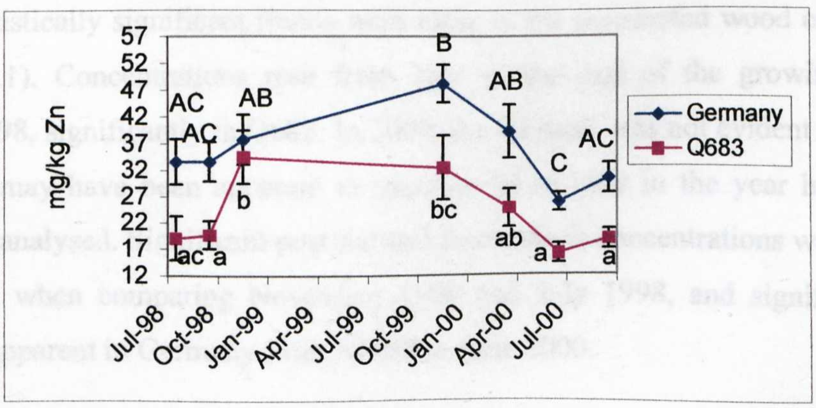

Figure 3.4.2.3 *Zn concentrations in non-leafed wood (mg kg^{-1}) at 7 sampling dates: July 1998 to September 2000. Letters apply as in Figure 3.4.2.1*

As for Cu, no significant differences were recorded for the Zn concentrations of the leafed and non-leafed bark fractions. However, both fractions displayed non-significant trends similar to those observed in the leaves; concentrations fell towards the end of the growing season. The fractions did not display marked post-harvest rises in concentrations, nor growth dilutions from March to June 2000.

In summary, leaf Zn concentrations decreased towards the end of the growing season. From June or July, concentrations in the leafed wood increased to the end of the season. Growth dilution was evident from March to June 2000 in the wood samples, followed by a clear increase afterwards. In the leafed bark samples, concentrations decreased slightly to the end of the growing season. Contrasting with the results for Cu, there was no corresponding increase in the non-leafed bark samples further down the stem; this element is more phloem-mobile than Cu (Lepp, 1995), and may have been redistributed via phloem to the roots.

3.4.3 Cadmium

Similar trends are apparent for Cd in the tree fractions (Figures 3.4.3.1 to 3.4.3.3). Leaf concentrations displayed a decreasing trend towards the end of the growing season, but data were considerably variable and no significant differences were recorded. Similarly, variable data for the leafed wood fraction prevented any significant differences being recorded, but the trend observed was one of concentrations marginally rising to the end of the growing season.

However, statistically significant trends were clear in the non-leafed wood concentrations (Figure 3.4.3.1). Concentrations rose from July to the end of the growing season in November 1998, significantly in Q683. In 2000, the increase was not evident in September samples, but may have been apparent in samples taken later in the year had they been collected and analysed. Significant post-harvest increases in concentrations were evident in both varieties when comparing November 1999 and July 1998, and significant growth dilution was apparent in Germany from March to June 2000.

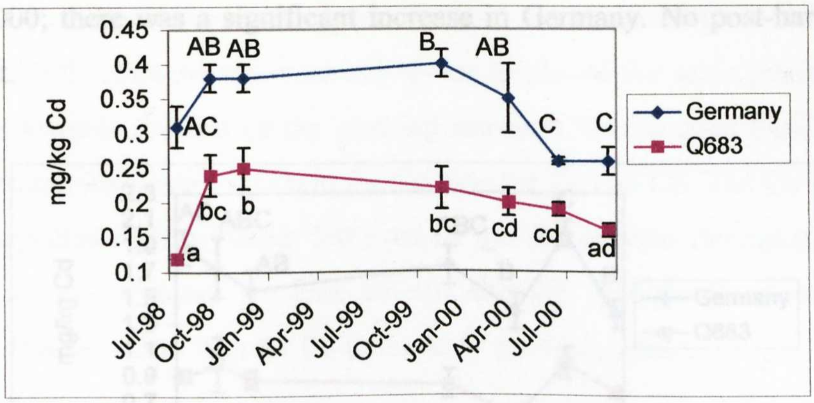

Figure 3.4.3.1 *Cd concentrations in non-leafed wood (mg kg^{-1}) at 7 sampling dates: July 1998 to September 2000. Within each variety, means (n=5) displaying no letters, or with a letter in common, are not significantly different after a Fisher LSD test (p> 0.05). Capitals refer to Germany; lower case letters to Q683*

Similar to the trend observed in the leaf concentrations, leafed bark concentrations (Figure 3.4.3.2) showed clear decreases towards the end of the growing season from July to November in 1998, and to September in 2000, although only significantly in Q683 in 1998. No significant post-harvest increases were observed in either variety, nor was there any evidence of growth dilution between March and June 2000. There was actually a significant increase in concentrations during this time in Q683.

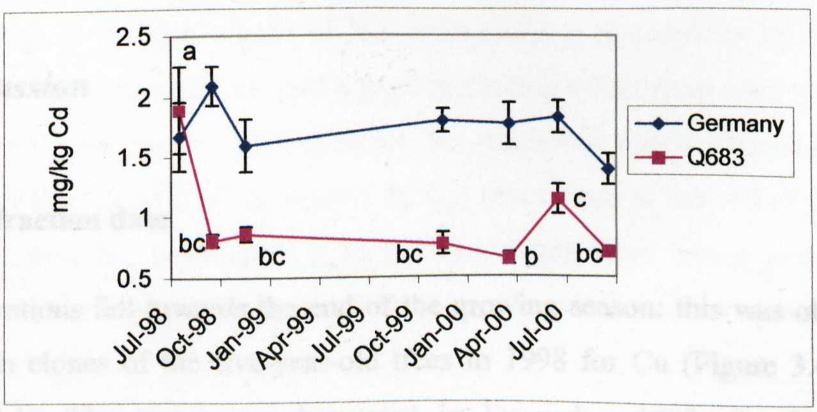

Figure 3.4.3.2 *Cd concentrations in leafed bark (mg kg^{-1}) at 7 sampling dates: July 1998 to September 2000. Letters apply as in Figure 3.4.3.1*

These trends were also apparent in the non-leafed bark data (Figure 3.4.3.3): the trends of decreasing concentrations towards the end of the growing season were apparent, but were significant only in Germany in 2000. No growth dilution was evident from March to

September 2000; there was a significant increase in Germany. No post-harvest increases were apparent.

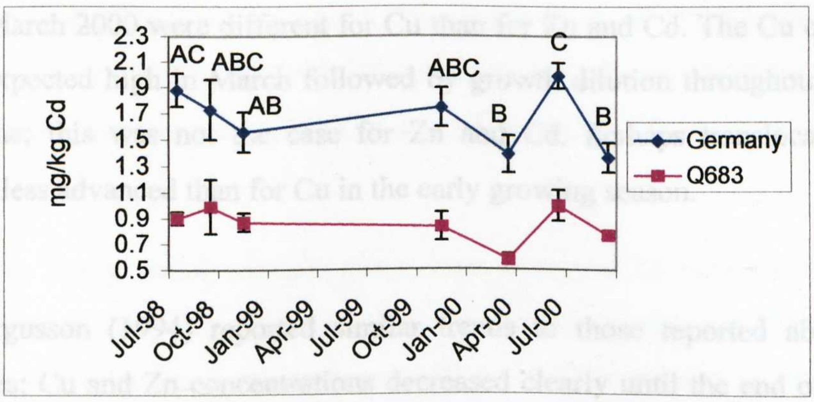

Figure 3.4.3.3 Cd concentrations in non-leafed bark (mg kg^{-1}) at 7 sampling dates: July 1998 to September 2000. Letters apply as in Figure 3.4.3.1

Generally speaking, Cd leaf concentrations of the clones, and particularly Germany, displayed a fall towards the end of the growing season and a post-harvest increase. There was a rise in concentrations towards the end of the growing season evident in the non-leafed wood fractions in 1998. Germany 1999 and 2000 samples displayed the expected post-harvest rise in concentrations and growth dilution from March to June, respectively. The bark fractions displayed a decrease in concentrations from July to November 1998, and from June to September 2000.

3.4.4 Discussion

3.4.4.1 Leaf fraction data

Leaf concentrations fell towards the end of the growing season: this was observed in the leaves of both clones of the five-year-old trees in 1998 for Cu (Figure 3.4.1.1) and Zn (Figure 3.4.2.1). The trees were harvested in December 1998; the June 1999 leaf concentrations were therefore those in 6 month old trees. The concentrations showed a marked increase in both clones from the pre-harvest levels for Zn, and in Germany for Cu. This was probably due to the uptake of metals being high relative to the biomass production.

A drop of metal concentrations was apparent in both clones for all three metals from June to September 2000. Thus the two-year old leaves displayed the same pattern as the five-year-old trees towards the end of the growing season. Concentration trends in Germany leaves from March 2000 were different for Cu than for Zn and Cd. The Cu concentrations showed the expected high in March followed by growth dilution throughout the growing season to June; this was not the case for Zn and Cd. Perhaps translocation of these elements was less advanced than for Cu in the early growing season.

Kim and Fergusson (1994) reported similar trends to those reported above in horse chestnut leaves: Cu and Zn concentrations decreased clearly until the end of the growing season, and Cd displayed a tendency to do so. The decrease was thought to have mainly arisen due to growth dilution, but they acknowledged a possible loss of metal ions from the leaves. Copper concentrations were highest in the new leaves, matching the observation made in this study. Ehlin (1982) reported a decrease in birch leaf Cu contents at the beginning of the growth period, attributed to a growth dilution effect. Dinelli and Lombini (1996) observed Cu concentrations in willow tissues grown on mine spoil to be higher in the early vegetative growth stage, followed by a period of vigorous growth which diluted the concentrations.

Guha and Mitchell (1966) measured trace element concentrations in leaves of sycamore, beech and horse-chestnut throughout the growing season. They identified the important effect of change in the dry weight of leaves in causing fluctuations in concentrations. Copper and Zn concentrations showed a growth dilution effect from May until June, then slowly decreased until the end of September; the minimum concentrations and quantities were found towards the end of the season, before senescence at the end of October. They suggested this decrease represented a back translocation from leaves prior to leaf fall. Further evidence for a slight tendency of back translocation in October was reported for essential elements such as Mn, Mg and Co.

Perennial species in temperate climates display a typical remobilisation of nutrients from the leaves to the woody plant parts prior to leaf drop (Marschner, 1995). Senescence of leaves is associated with higher rates of nutrient export from leaves than import; remobilisation of Cu and Zn is closely related to this phenomenon. Leaf concentrations from May to October in sycamore in Guha and Mitchell's study provide evidence for this: Cu concentrations dropped from 15.8 to 7.1 mg kg^{-1} and Zn from 39 to 33 mg kg^{-1}. At

Spofforth, Cu concentrations were similarly reduced by over 50 % from March to September 2000 (a combination of the effects of growth dilution and back translocation), while Zn concentrations from July to September 2000 displayed a similarly marginal decrease, not as marked as for Cu. A May to September decrease of Cu concentrations was observed in birch leaves by Ehlin (1982). Concentrations of Cu and Zn from April to June decreased in *Salix* grown on mine spoil (Dinelli and Lombini, 1996).

However, foliar heavy metal concentrations were observed to increase shortly before senescence in four *Salix* species grown on sludge-amended soil (Riddell-Black, 1994). In a similar trial, a slight tendency of increased willow leaf Cu content in relation to stem content in autumn was interpreted as a possible detoxification effect in connection with defoliation (Hasselgren, 1999). Increases in Zn concentrations in sycamore, beech, horse chestnut and hazel leaves at the end of the growing season have been recorded (Ross, 1994c). This may have been metal "shunting" occurring in the plant tissues prior to senescence, or seasonal variation in soil metal availability.

Perhaps the different trends arose at Spofforth due to the relatively non-toxic levels of contamination at this site: metal “shunting” to the leaves may occur only when metal concentrations in stems reach sublethal concentrations. Although Section 3.3 outlines how the bark concentrations of Zn and Cd exceed the wood concentrations, pointing to uptake from a contaminated substrate, concentrations in leaves and stems of Cd, for example, are considerably higher in the willow tissues in the study by Riddell-Black (1994). Indeed, the majority of metal concentrations in all the fractions from Spofforth fall in the normal range of plant concentrations detailed in Table 1.3.1; only in some cases do the bark concentrations of Zn, and the wood concentrations of Cu, fall into the range indicative of contaminated plants. Spofforth leaf concentrations of Cu, and particularly Zn, fall into this range, but the lack of evidence for increases in leaf concentrations of these metals towards the end of the growing season suggests this is just a result of translocation (which is partly offset by back-translocation), rather than a detoxification mechanism.

Dinelli and Lombini (1996) reported senescence of leaves to usually produce an increase in metal concentrations due to loss of fluids. This is further exemplified by the considerably greater concentrations of metals in the litter collected from Spofforth when compared to the concentrations of leaves (both reported on a fresh weight basis) remaining on the trees (Table 3.4.4.1): loss of fluid is probably the main factor in the considerably elevated levels.

Consistent increases in N and P concentrations of decomposing litters from four tree species grown on an Indian mine spoil have been observed by Singh *et al.* (1999).

Table 3.4.4.1 *Metal concentrations (mg kg^{-1}) in Spofforth leaves and litter samples collected in November 1999*

Metal	Copper	Zinc	Cadmium
Leaf	6.49	310	1.60
Litter	9.57	1190	3.52

Therefore, not insignificant quantities of metals would eventually be returned to the soil via leaf degradation at this site. Decreasing pH in decaying leaf litter can occur due to the leaching of acidic material from the vacuoles (Bernhard-Reversat, 1999). Considerable amounts of soluble organic matter can be released too, which can lead to chelation and mobilisation of metals in the soil profile, as demonstrated by Pohlman and McColl (1988). However, the next section considers the reallocation of some of the metals to stems prior to leaf fall.

3.4.4.2 Wood fraction data

Concentrations of all metals rose from July to November 1998 in both clones of the 5 year-old trees in the non-leafed and leafed wood fractions. Post-harvest rises in concentrations were seen in November 1999 for Cu (Figures 3.4.1.2 and 3.4.1.3) and Zn (Figures 3.4.2.2 and 3.4.2.3), for at least one of the clones. Concentrations of all three metals fell from March 2000 to June 2000 in the wood fractions, displaying a probable growth dilution effect. From June to September 2000, Zn concentrations display a rise (Figures 3.4.2.2 and 3.4.2.3). This is not clear in the Cd and Cu data, although concentrations in Germany are clearly beginning to level off. It is likely that the rise displayed in the 1998 trees would be evident if samples had been taken in November or December 2000.

Younger, post-harvest trees at Spofforth (November 1999) displayed a tendency to have greater concentrations than in the 5-year-old, pre-harvest trees. This was marked in the leaf fraction, such as for Zn (Figure 3.4.2.1), and in the leafed wood fraction, particularly for Cu (Figure 3.4.1.2). Metal concentrations have previously been observed to decrease with tree age (Morin, 1981; Sander and Ericsson, 1998; Hasselgren, 1999). Biomass production was attributed to the dilution of metal concentration levels; at Spofforth, the harvest of trees led to uptake being high relative to biomass production. Subsequent years' growth would presumably decrease these initially high concentrations.

Metals can be redistributed from plant leaves via the xylem and phloem, usually in anionic or organic complexes (Alloway, 1995b). The hypothesis, that significant quantities of heavy metals are translocated from Spofforth leaves into wood prior to leaf-fall, has been reported for N, P and K in 12 willow species by Grigal *et al.* (1976). They observed a decrease in leaf concentrations of these elements between June and December, with an increase in concentrations of these elements in stems at the time of leaf abscission. Their data indicated a flux of nutrients into the above-ground woody plant parts towards the end of the growing season to be utilised in early-season growth the next spring, when concentrations are "recharged".

The flux described for metals at the fertile Spofforth site are not drastic; reallocation of nutrients from leaf to stem during senescence is thought to be more effective when their availability is low. As willow leaves are often shed green, the amount of nutrients translocated in this way is thought to be low (Sander and Ericsson, 1998). Also, these authors pointed out how reallocation patterns during leaf senescence can be different, which could explain how the concentration shifts are not the same at Spofforth: they seem more pronounced for the micronutrients Cu and Zn than for the non-essential Cd.

3.4.4.3 Bark fraction data

In contrast to xylem (wood), transport of elements in phloem (bark) is bidirectional (Marschner, 1995). Concentrations fell slightly from July or September 1998 to November 1998 for all elements in the leafed bark fraction. This is also observed in the non-leafed bark concentrations of Cd (Figure 3.4.3.3). Perhaps illustrating the lower phloem mobility of Cu relative to Zn, Cu concentrations rose in the non-leafed bark fraction. From June to September 2000, concentrations in bark fell. The bark data did not reveal an effect of growth dilution from March to June 2000. The effects of this phenomenon are much stronger in leaves and wood than in bark, so the Cd bark concentration rises witnessed during this period are probably due to increased deposition of the translocated metal.

Root-absorbed metals are distributed to the shoot via xylem (Lepp, 1995). Rapid lateral movement of water from xylem to phloem in three year old willow trees has been demonstrated, as has lateral movement of radioactive K from the xylem into phloem (Epstein, 1972). Xylem to bark movement of water and nutrients occurs in a slight transpiration stream through rays; it is also likely to occur through cell walls (Zimmermann, 1971). Transfer of elements from phloem to xylem can occur if an adequate concentration gradient exists (Marschner, 1995). During senescence, large molecules are broken down and nutrients are returned to the stem via phloem; assimilates are translocated in rays from the phloem through the cambium into the wood (Zimmermann, 1964). This may explain the apparent decrease in bark metal concentrations, and concurrent increase in wood concentrations, towards the end of the growing season.

Hagemeyer and Hubner (1999) measured radial distributions of Pb in stems of 6-year-old spruce trees grown in Pb-amended soil and found that peak concentrations, and comparatively large quantities, were in inner, older rings. Thus they reported a conceivable remobilisation and redistribution of Pb; a considerable proportion of metals in trees are exchangeably bound and can be mobilised, such as via the axial xylem sap stream. Radial transport of elements is also possible in wood through rays or pits.

3.5 Summary

Tissue samples of two willow species grown on a heavy metal-contaminated site were regularly collected over two and a quarter years, a time period which encompassed a growing season of 5-year-old trees, a harvest, a period of re-growth and a growing season of 2-year-old trees. Samples taken from different heights revealed a trend of increasing metal concentrations with height in the bark and wood tissues, and a decreasing trend with height in leaves. This sampling height effect was more pronounced for less translocatable elements such as Cu and Pb, and markedly less so for more readily translocated elements such as Zn and Cd. The observations in wood and bark are likely to be a result of upward translocation of metals in xylem and phloem and decreasing stem girth with height, while the leaf patterns are possibly due to increased biomass of leaves with height, and greater deposition of metals in lower leaves.

Leaf concentrations of Cu, Zn and Cd were significantly greater than in the stem fractions. Bark concentrations of Zn and Cd were significantly greater than those in wood; the reverse was true for Cu. Younger, post-harvest tissue samples (June and November 1999) displayed a tendency to have greater concentrations than those in the 5-year-old, pre-harvest trees. This was particularly marked in the leaves and leafed wood fractions; re-growth following the harvest of the trees led to uptake being high relative to biomass production. Metal concentrations in leafed wood samples from 2000 frequently revealed a growth dilution effect between March and June.

Leaf concentrations fell towards the end of the growing season, a probable combination of the effects of growth dilution and back-translocation prior to leaf abscission. Concentrations of metals rose during this period in the non-leafed and leafed wood fractions; this stem fraction was probably the site of deposition of back-translocated foliar metals. Additionally, there may have been a concurrent net flux of metals from the bark, either to the wood or the roots: concentrations fell from July or September to November 1998 for all elements in the leafed bark fraction. This was also observed in the non-leafed bark concentrations of Cd. Perhaps illustrating the lower phloem mobility of Cu relative to Zn, Cu concentrations rose in the non-leafed bark fraction in this part of the growth season.

Chapter 4 Field Studies of the Effects of Tree Growth and Harvest on Soil Heavy Metal Distribution and Soil Microbial Respiration

4.1 Introduction

The work presented in this chapter aimed to:

- assess the effects of the growth of biomass crops in the field, and their harvest, on soil heavy metal distribution.
- ascertain whether the growth and harvest of willows affected soil microbial respiration.

Description of the sites where the soils were obtained and the soil characteristics are outlined in Section 2.1. The soil sampling regime and procedures for heavy metal extraction from the soils and soil solutions are also given in Section 2.1, as is the methodology for determining the soil respiration. Analytical techniques for all results are described in Sections 2.5 and 2.6.

All of the soils discussed in this chapter had been amended with sewage sludge for a number of years (improving soil fertility but with the side effect of increased soil metal concentrations), prior to being planted with *Phalaris* and/or willow varieties. Using total concentrations to assess soil contamination assumes all forms of metals have the same impact on the environment, which is untenable (Tessier *et al.*, 1979). Therefore studies of metal fractionation by sequential extraction are relevant to such soil studies. Sequential extraction of soil samples may provide information on the origin of the metals. While high levels in the exchangeable, easily reducible and acid soluble pools may indicate anthropogenic pollution (Tack and Verloo, 1995), metals added to soil revert to less soluble oxide and residual fractions with time (Shuman, 1991). Walter and Cuevas (1999) found sludge applications to have changed the metal distribution in soil 5 years after application. The metal concentrations were increased, but in the more resistant fractions (organically bound and precipitated pools). Sludge amended soils studied by Berti *et al.*

(1997) contained greater proportions of metals in the less available fractions compared to untreated soils.

Data are presented from selective extractions carried out on soils obtained at Slough (Section 4.2), Rodley (Section 4.3) and Spofforth (Section 4.4).

4.2 Selective extraction of Slough samples

Soil samples were taken from areas under growing plants of five willow varieties and three *Phalaris* varieties. Samples (all 0-25 cm depth) were also taken from unplanted areas around the margins of the areas where biomass crops were grown. The metal concentrations extracted by M ammonium acetate, pH 6 (NH_4OAc) at Slough (Table 4.2.1) display a consistent trend of higher concentrations in the unplanted soil than in the planted soils. This was significant for the Ni results for soil samples where four of the five willow varieties grew, and where two of the three *Phalaris* varieties were planted.

Table 4.2.1 Mean NH_4OAc-extractable metals (mg kg^{-1}) in Slough soil. For each element, means (n=3) lacking a letter, or with a letter in common, are not significantly different ($p > 0.05$) after a Fisher LSD test

	Zn	Cd	Cu	Ni	Pb	Cr
UNPLANTED	87.1	20.9	22.0	17.1 a	2.37	0.25
Candida	72.7	18.7	21.7	14.3 bc	1.75	0.24
Germany	79.4	20.0	18.6	14.0 bc	1.87	0.24
Bowles	82.5	20.3	19.3	14.9 bc	1.87	0.24
Q83	79.9	20.8	20.0	14.8 ab	1.69	0.20
Jorunn	74.1	19.2	18.7	13.4 bc	1.73	0.17
V1	72.5	18.0	18.6	14.4 bc	1.54	0.20
V2	93.0	22.6	21.2	17.9 ac	1.77	0.24
V4	67.7	17.1	18.0	12.8 b	1.60	0.24

Many more significant differences are evident in the results for the 0.025 M ammonium ethylenediamine tetraacetic acid, pH 4.6 (NH_4EDTA) extractable heavy metals, except for Cr (Table 4.2.2). These results clearly demonstrate the evident depletion the growth of the various biomass crop varieties has effected in the NH_4EDTA extractable concentrations: relative to the unplanted area, concentrations in soil samples taken from below at least six of the eight plant varieties were significantly lower.

Table 4.2.2 Mean NH_4EDTA extractable metals (mg kg^{-1}) in Slough soil. Letters apply as in Table 4.2.1

	Zn	Cd	Cu	Ni	Pb	Cr
UNPLANTED	2325 a	161 a	791 a	112 a	306 a	15.1
Candida	2007 ab	122 bc	648 bc	88.1 bc	252 b	12.3
Germany	1777 b	127 bc	604 bc	88.6 bc	239 b	12.0
Bowles	1843 bc	129 bc	639 bc	87.9 bc	249 b	12.5
Q83	1783 b	135 ab	606 bc	92.0 ac	255 b	14.7
Jorunn	1744 b	126 bc	543 b	76.5 b	246 b	12.2
V1	1677 b	123 bc	565 b	86.7 bc	241 b	12.4
V2	2260 ac	159 ac	725 ac	107 ac	291 ab	17.0
V4	1819 bc	121 b	597 bc	84.9 bc	241 b	13.1

In most cases, growth of the *Phalaris* variety V2 and the willow clone Q83 did not result in significantly lower extractable metal concentrations than in the unplanted areas. It is not known whether this was due to elevated soil contaminant concentrations (or hotspots) in the areas where these crops were planted, or inferior growth and metal uptake of the varieties, or a combination of these potential factors. Chapter 9 displays graphs illustrating the inferiority of Q83 relative to Candida, Germany and Jorunn, in terms of its biomass production in glasshouse trials and in two field trials (Slough and Stoke-Bardolph). The root activity of plants can alter the extractable metal concentrations in the soil through redistribution of the metals and uptake of metals into the plant biomass; the more productive clones are likely to have caused the observed changes via the former.

Indeed, the depletions cannot be attributed to plant uptake: Table 4.2.3 displays the stem Ni concentrations (supplied by WRc, a Partner in the BIORENEW project team) of four willow and two *Phalaris* varieties grown at the site. Alongside these values, the table lists the required stem concentrations for the observed depletion in soil NH_4EDTA and NH_4OAc extractable concentrations to have been caused entirely by plant uptake and sequestration of Ni in the stem. The calculation was based on the assumptions that the soil has a bulk density of 1.3 and that the plants produced an oven-dry yield of ten tonnes per hectare and exploited the top 50 cm of soil. By extrapolating the soil extractable metal concentrations to quantities of metal per hectare, the expected stem concentrations to have effected the depletions could be calculated.

Table 4.2.3 Required and actual heavy metal stem concentrations (mg kg^{-1}) in four willow and two Phalaris *varieties to have caused the depletions in soil extractable Ni concentrations*

	Actual stem Ni concentration (mg kg^{-1})	Required stem Ni concentration to cause NH_4EDTA-extractable Ni depletion	Required stem Ni concentration to cause NH_4OAc-extractable Ni depletion	Actual stem Ni concentration as %ge of required stem Ni concentration to cause NH_4EDTA-extractable Ni depletion	Actual stem Ni concentration as %ge of required stem Ni concentration to cause NH_4OAc-extractable Ni depletion
Candida	7.1	15535	1820	0.05	0.39
Germany	3.9	15210	2015	0.03	0.19
Bowles	4.9	15665	1430	0.03	0.34
Jorunn	4.6	23075	2405	0.02	0.19
V1	40.6	16445	1755	0.25	2.31
V4	37.8	17615	2795	0.21	1.35

Evidently, uptake by the biomass crops accounted for only a small fraction of the observed depletions. Therefore the decreases could be attributed to leaching, or redistribution to a less extractable soil phase. Consideration to this question is given in Sections 4.3, 4.4 and Chapter 5. Significant quantities of metals may also have been sequestered in the tree roots; this factor is considered in Chapter 7.

The metal concentrations extracted by NH_4EDTA were considerably higher than those displaced by the NH_4OAc extractant. This is to be expected in such an organic soil: Schwarz *et al.* (1999) used 0.025 M NH_4EDTA (pH 4.6) to release metals bound to organic matter, while M NH_4OAc (pH 6) was used to target easily mobilisable metals. However, NH_4OAc has been criticised as an extractant for exchangeable metals as it may also attack carbonates below pH 8.2 (Sheppard and Stephenson, 1997) and oxide coatings (Shuman, 1991). Therefore simple salts such as KNO_3 have been preferred in some studies when targeting this fraction. Despite this, distinctions should be drawn between functional (for example, "plant-available species") and operational (such as NH_4OAc extractable metal) definitions of speciation (Ure *et al.*, 1993). The targeting of the former is fraught with error due to non-specificity of the extractants.

4.3 Analysis of Rodley samples

Soil samples were collected for both heavy metal and soil solution extraction from areas under growing willows, and in unplanted areas. The substrate was less heavily contaminated than at Slough, and was considerably more heterogeneous.

4.3.1 Selective Extractions

The Rodley results demonstrate a similarly clear trend to those obtained from Slough samples. Tables 4.3.1.1 and 4.3.1.2 display the consistent trend of extractable metal concentrations being higher in the unplanted discard areas than where trees have grown, except at 25-50 cm depth for NH_4OAc extractable Ni and Pb, and NH_4EDTA extractable Pb and Zn. Due to the considerable substrate variability, the standard errors were relatively large, and so no significant differences were detected by a series of t-tests.

Table 4.3.1.1 NH_4OAc extractable metal concentrations (n=3, standard error in brackets) in Rodley soil, mg kg^{-1}

	Zn	Cd	Cu	Ni	Pb	Cr
Tree 0-25 cm	6.39 (0.68)	0.46 (0.03)	0.66 (0.07)	0.56 (0.05)	7.77 (3.06)	0.44 (0.15)
Unplanted 0-25 cm	24.4 (11.7)	1.02 (0.39)	2.49 (1.36)	1.16 (0.52)	8.17 (2.94)	0.68 (0.24)
Tree 25-50 cm	3.71 (0.81)	0.17 (0.03)	0.67 (0.12)	0.75 (0.09)	1.50 (0.29)	0.17 (0.03)
Unplanted 25-50 cm	4.17 (1.48)	0.37 (0.12)	1.40 (0.25)	0.57 (0.22)	0.70 (0.06)	0.17 (0.07)

Table 4.3.1.2 NH_4EDTA extractable metal concentrations (n=3, standard error in brackets) in Rodley soil, mg kg^{-1}

	Zn	Cd	Cu	Ni	Pb	Cr
Tree 0-25 cm	41.0 (5.05)	0.71 (0.10)	23.0 (2.17)	2.39 (0.25)	70.6 (14.1)	12.7 (2.42)
Unplanted 0-25 cm	220 (99.3)	3.06 (1.45)	151 (73.5)	11.6 (4.97)	328 (116)	29.8 (8.07)
Tree 25-50 cm	26.4 (4.02)	0.30 (0.04)	16.1 (1.72)	1.51 (0.11)	29.7 (3.53)	4.01 (1.53)
Unplanted 25-50 cm	25.7 (8.24)	0.60 (0.17)	18.5 (0.94)	3.52 (1.73)	19.6 (1.43)	6.47 (2.18)

The results for Cd are presented to illustrate the typical trends observed in the data obtained from the Rodley samples. Figures 4.3.1.1 and 4.3.1.2 respectively show lower concentrations of NH_4OAc and NH_4EDTA extractable Cd, in the areas under growing willows relative to the unplanted area. This trend is apparent at both soil depths.

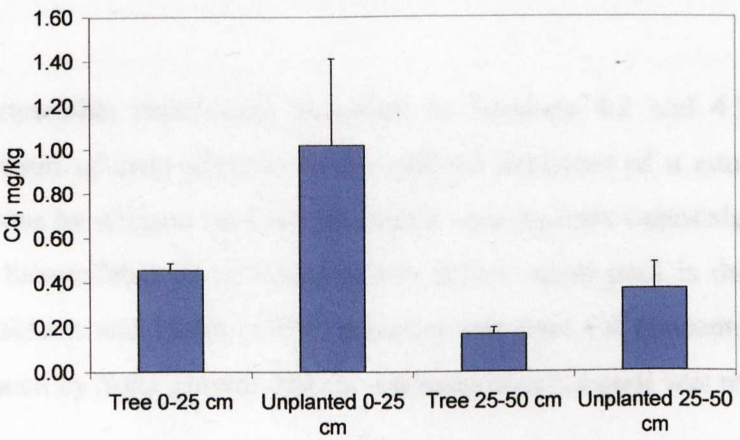

Figure 4.3.1.1 *NH_4OAc-extractable Cd (mg kg^{-1}) in Rodley soil (n=3)*

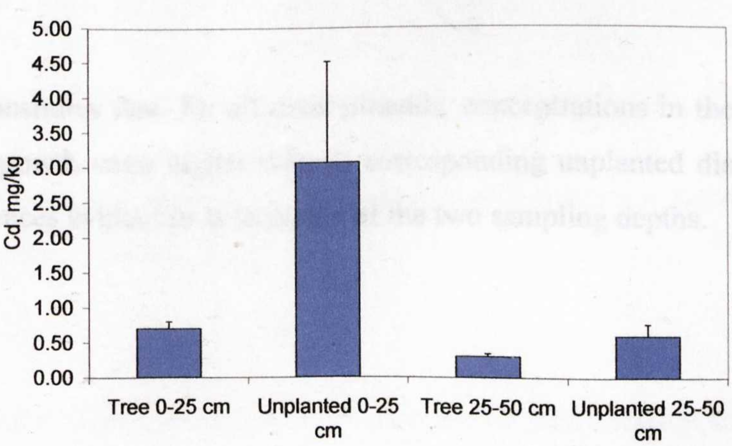

Figure 4.3.1.2 *NH_4EDTA extractable Cd (mg kg^{-1}) in Rodley soil (n=3)*

The observed depletions in extractable heavy metals following crop growth at both Slough and Rodley are supported by other studies: Eriksson and Ledin (1999) observed concentrations of exchangeable Cd (extracted by either 0.01 M $CaCl_2$ or M NH_4NO_3) in *Salix* plot soils to be significantly lower than in nearby reference areas, concluding that cultivation of the trees reduced the amount of plant-available Cd in the soil. Over the eight sites, concentrations were generally about 30-40 % lower in *Salix* soils than in the reference fields. Bridges (1989) reported soil Pb concentrations below trees in soils in Wales to be lower than in adjacent open spaces, indicating redistribution by the trees. In this study, this was observed for both extractants, but only in the upper soil (0 – 25 cm depth).

Interestingly, the soil solution data for the same element (Section 4.3.2) clearly display higher concentrations in both soil depths under tree growth relative to the unplanted soil. Possible relationships between observed extractable metal concentration depletion under growing trees and increased soil solution concentrations are considered in the next section.

Therefore the extractable metal data presented in Sections 4.2 and 4.3.1 demonstrate depletions as a result of crop growth. While marked depletion of a contaminated site's metal concentrations by willows may not take place on a realistic timescale, their growth is beneficial if the bioavailable or environmentally active metal pool is depleted (Riddell-Black, 1994). Eriksson and Ledin (1999) reported that total Cd concentrations were not significantly reduced by *Salix* growth, but the exchangeable Cd pool was reduced.

4.3.2 Soil Solution Extractions

Table 4.3.2 demonstrates that, for all determinands, concentrations in the soil solution in soils under tree growth were higher than in corresponding unplanted discard areas, with significant differences evident in at least one of the two sampling depths.

Table 4.3.2 Soil solution metal concentrations in Rodley soil ($\mu g\ l^{-1}$). For each determinand and soil depth, means (n=3) without a letter are not significantly different ($p > 0.05$) after a Fisher LSD test. Letters in capitals refer to 0-25 cm soil depth; lower case letters to 25-50 cm depth. Significantly different means are denoted by one ($p < 0.05$), two ($p < 0.01$) or three ($p < 0.001$) asterisks

	Zn	Cd	Cu	Ni	Pb	Cr
Tree 0-25 cm	2137 A	15.1 A	63.2 A	89.7 A	54.3 A	86.0 A
Unplanted 0-25 cm	1057 B***	6.47 B***	42.1 B*	59.5 B**	34.3 B*	78.6 B***
Tree 25-50 cm	1240 a	4.71 a	48.3	46.4 a	16.4	41.4 a
Unplanted 25-50 cm	175 b*	1.39 b**	33.8	23.1 b**	13.0	37.2 b**

Figure 4.3.2 illustrates a typical trend, for Cd. Concentrations are significantly greater under tree growth than in the unplanted area, at both depths. Examining the NH_4OAc and NH_4EDTA extractable concentrations of this element from the same areas (Figures 4.3.1.1 and 4.3.1.2), it appears that these pools are depleted, and a fraction of the mobilised metal is brought into solution by tree growth, from where it is ultimately taken up by the tree or leached. But this fraction is likely to be small, and the largest fraction of mobilised metal is likely to be redistributed to other soil pools. This question is addressed in Section 4.4.1.

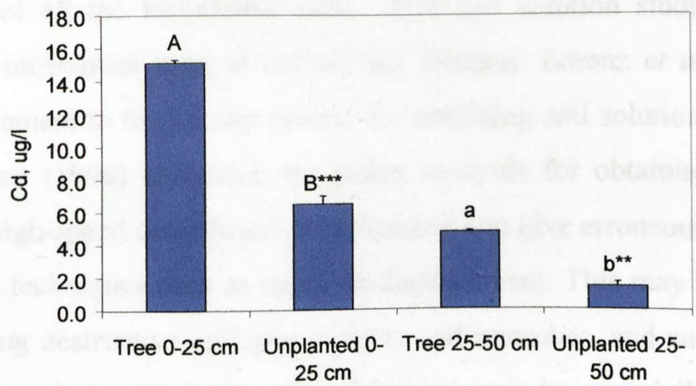

Figure 4.3.2 Cd concentrations ($\mu g\ l^{-1}$) in Rodley soil solutions. Letters and asterisks apply as in Table 4.3.2

No discernible trends were apparent in the extractable Cr concentration results, but similar patterns emerged for Zn, Pb, Ni and Cu. However, Romkens *et al*. (1999) reported copper activities in soil solution to be two orders of magnitude lower in pots where *Agrostis* *capillaris* was grown, than in bare pots. In their experiment, they hypothesised that plant growth lowered Cu solubility indirectly by raising the pH, solution Ca and dissolved organic carbon (DOC) concentrations of a contaminated acidic sandy soil, leading to increased complexation of Cu. They stressed their results were only valid for Cu-contaminated, non-calcareous sandy soils. Plant growth had a very different effect on the Cu concentrations of the Rodley soil, one that is considerably organic and has a pH close to neutral. Nielsen (1976) reported that copper concentrations in soil solution samples of a copper-amended calcareous peat increased in the latter part of the growth period due to increases in the content of organic complexing agents. In pots where no plants were grown, soil solution Cu concentrations decreased and attained an almost constant value after 35 days.

Therefore soil properties have a strong bearing on the effect of plant growth on heavy metal concentrations in soil solutions. Furthermore, results reported in pot studies may have been exaggerated relative to a field environment, where less of the soil profile is influenced by root activity. Care should be taken in the interpretation of soil solution analyses also, as extraction technique and sample storage can bias results.

Dahlgren (1993) compared the effects of five different soil solution extraction procedures on solute chemistry. Solute concentrations were demonstrated to be highly dependent on the extraction method: centrifugation, as was used in this study, led to the highest solute concentrations of all the techniques used. Thus soil solution studies are operationally defined by the techniques used to extract the solution. Lorenz *et al.* (1997) considered solution displacement to be a better system for obtaining soil solution than centrifugation. Ross and Bartlett (1990) examined extraction methods for obtaining soil solutions and suggested that high-speed centrifugation techniques can give erroneous results compared to other extraction techniques such as miscible displacement. This may have been due to the technique causing destructive collapse of roots and microbes, and subsequent leaching of ions and organic substances; near-surface horizons may be especially susceptible to this. Despite these limitations, the soil solution data for Rodley produced consistent trends, and are compared with the results obtained for Spofforth using the same technique (Section 4.4.2).

4.4 Spofforth

The Spofforth short rotation coppice (SRC) site was coppiced in December 1998. Sequential extractions of soil samples collected at this time, and afterwards in March and June 1999, were carried out to assess the metal fractionation, and any mobilisation and redistribution of metals due to the growth of trees and/or the harvesting of the trees and subsequent root degradation. Soil solutions were also extracted in June 1999, and further soil samples at this date were collected to determine the effects of tree harvest and growth on microbial respiration.

4.4.1 Sequential extraction results

Total concentrations in the Spofforth soil samples ranked Zn = Pb > Cu > Cr = Ni > Cd. Zinc, Pb and Cu results clearly showed greater concentrations in the 0-25 cm samples across the range of extractants. Results for Cr, Ni and Cd did not display markedly different concentrations between the 0-25 cm and 25-50 cm depths.

The frequently large standard errors in the data presented below exemplify the variability of the Spofforth substrate. Furthermore, precision is generally low when extractable concentrations approach the detection limit (Tessier *et al.*, 1979), as was the case for certain determinands displaced by the less rigorous extractants.

Therefore conclusions about metal mobilisation under areas where trees were growing or were harvested, cannot be drawn with certainty. However, the temporal trends were not entirely biased by substrate variability, as significant differences were rarely observed in the unplanted (control) soil metal concentration data, and were much more frequently recorded in the harvested ("cut") and unharvested ("uncut") regimes. These significant differences were recorded much less frequently between December and March than in March to June. The absence of many significant differences due to the growth or harvest of trees in the former period is interpreted as being due to slower biological activity (resulting from slower root growth or degradation) during the colder months, which represent a "dormant spell" for willows and a period of reduced soil microbial activity.

Figures 4.4.1.1 displays the metal distribution of the unplanted soils (0-25 cm depth) at the beginning of the six month experimental time period; the metal extracted by the four solutions in the sequence are presented as percentages of the total. The metal distributions in the unplanted soils were even over the three sampling times of the experiment.

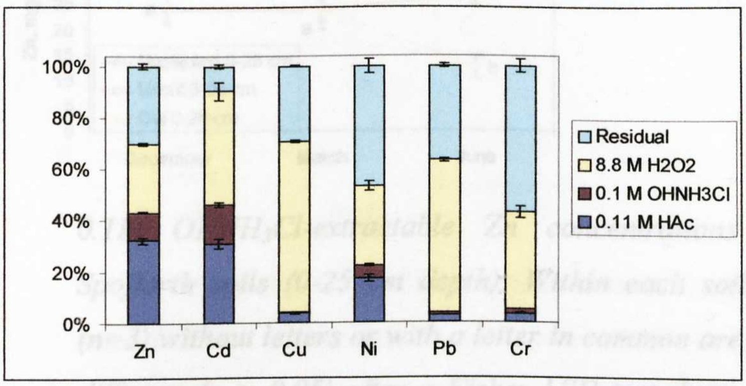

Figure 4.4.1.1 *Metal fractions in unplanted Spofforth soil samples (n=3) collected in December 1998 (0–25 cm depth), expressed as percentages of the total*

Broadly speaking, the BCR procedure according to Davidson *et al.* (1998) divides metals into acid soluble/exchangeable, reducible and oxidisable fractions (see Table 2.1.5.1), which are extracted sequentially by 0.11 M acetic acid (HAc), 0.1 M hydroxylammonium chloride, pH 2 ($OHNH_3Cl$) and 8.8 M hydrogen peroxide (H_2O_2). The $OHNH_3Cl$ extractable fraction was consistently the smallest across the range of determinands. Therefore the reducible metal pool is relatively small compared to the acid soluble/exchangeable and oxidisable pools. The oxidisable pool represented the greatest fraction of Pb, Cu and Cd in the soil. Zinc concentrations were fairly evenly distributed throughout the four fractions while the greatest concentrations of Ni and Cr were in the residual (aqua regia extractable) fraction. Other authors have reported that Cr is mainly in the residual fraction in sludge-amended soils (McGrath and Cegarra, 1992, Berti *et al.*, 1997, Walter and Cuevas, 1999). Due to an experimental error, no Cd data for March can be presented, therefore graphs displaying results for this element over the three sampling times have been excluded from this section.

Zinc and Cd were the only two metals of which a considerable portion was $OHNH_3Cl$ extractable (10–15 %). Figure 4.4.1.2 displays the concentrations of Zn displaced by the extractant from samples (0–25 cm depth) collected in December, March and June. Concentrations in the harvested regime fell significantly from March to June.

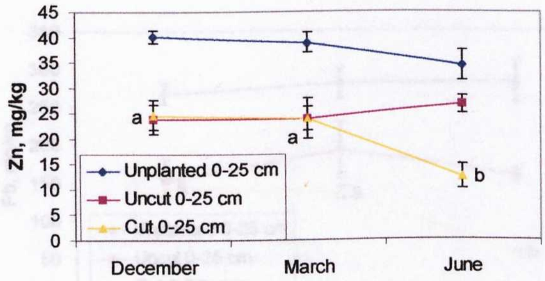

Figure 4.4.1.2 *0.1M $OHNH_3Cl$-extractable Zn concentrations (mg kg^{-1}) in Spofforth soils (0-25 cm depth). Within each soil regime, means (n=3) without letters or with a letter in common are not significantly different ($p > 0.05$) after a Fisher LSD test. Letters in upper case refer to the unharvested or "uncut" treatment; lower case letters apply to the harvested or "cut" regime*

The depletion of Zn concentrations from March to June in soil where the trees have been harvested can be observed when the metals extracted by each solution are presented as percentages of the total (Figure 4.4.1.3): the 0.1 M $OHNH_3Cl$ fraction falls from March to June.

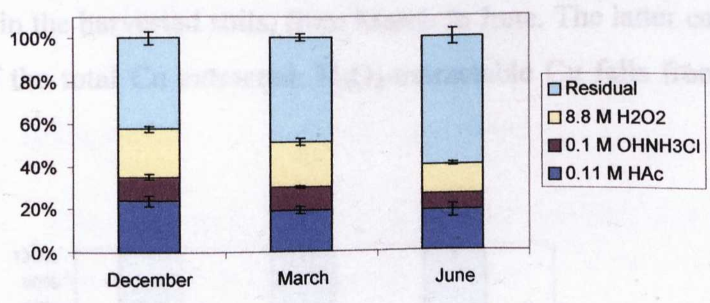

Figure 4.4.1.3 *Zinc fractions in harvested Spofforth soil (0-25 cm depth) expressed as percentages of the total (n=3)*

Figure 4.4.1.2 also clearly displays that, in the upper soil depth (0-25 cm), the extractable Zn concentrations were greater in the unplanted soil than in the soil where trees are/have been growing (unharvested and harvested soils). This trend was also clear in the results for the largest pools (8.8 M H_2O_2-extractable) of Pb (Figure 4.4.1.4) and Cu (Figure 4.4.1.5). These trends were not apparent for Cr, Ni and Cd.

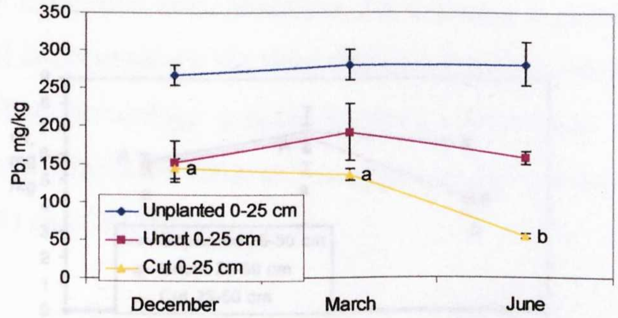

Figure 4.4.1.4 *8.8M H_2O_2-extractable Pb concentrations (mg kg^{-1}) in Spofforth soils (0-25 cm depth). Letters apply as in Figure 4.4.1.2*

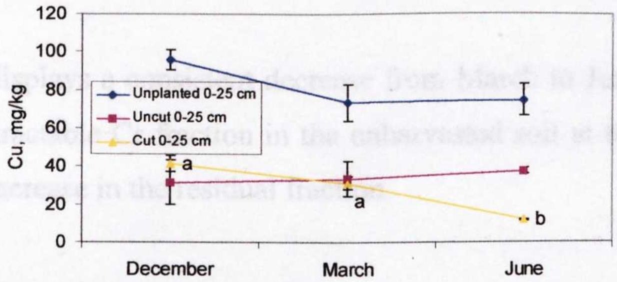

Figure 4.4.1.5 *8.8M H_2O_2-extractable Cu concentrations (mg kg^{-1}) in Spofforth soils (0-25 cm depth). Letters apply as in Figure 4.4.1.2*

Figures 4.4.1.4 and 4.4.1.5 also display significant decreases in H_2O_2-extractable Pb and Cu concentrations in the harvested soils, from March to June. The latter can be observed in the percentages of the total Cu extracted: H_2O_2-extractable Cu falls from March to June (Figure 4.4.1.6).

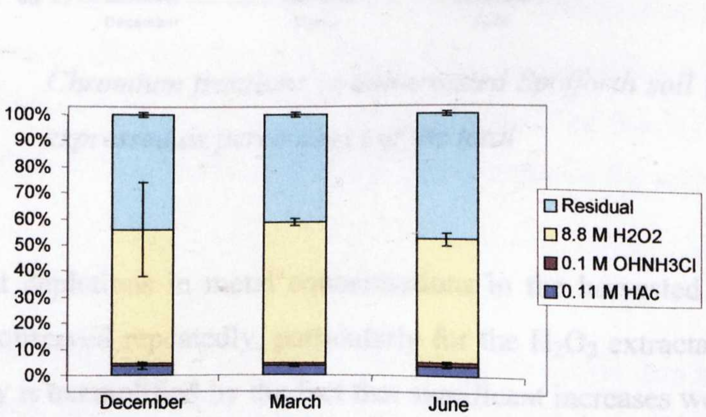

Figure 4.4.1.6 *Copper fractions in harvested Spofforth soil (0-25 cm depth) expressed as percentages of the total*

In samples collected from the 25–50 cm depth, this trend was observed in both the harvested and unharvested H_2O_2-extractable Cr concentrations (Figure 4.4.1.7): significant decreases were recorded from March to June.

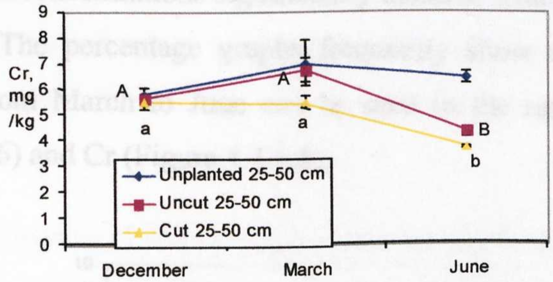

Figure 4.4.1.7 *8.8 M H_2O_2-extractable Cr concentrations (mg kg^{-1}) in Spofforth soil (25-50 cm depth). Letters apply as in Figure 4.4.1.2*

Figure 4.4.1.8 displays a consistent decrease from March to June in the proportion of the 8.8 M H_2O_2-extractable Cr fraction in the unharvested soil at this sampling depth, with a corresponding increase in the residual fraction.

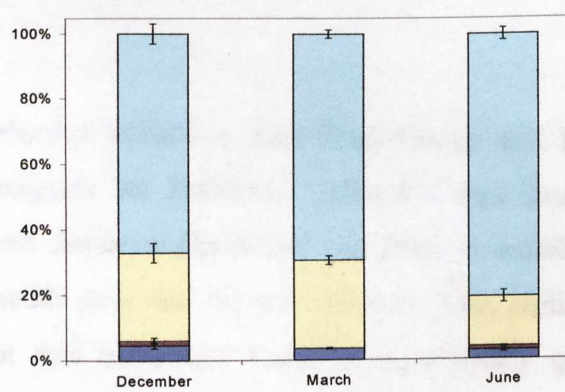

Figure 4.4.1.8 *Chromium fractions in unharvested Spofforth soil (25-50 cm depth) expressed as percentages of the total*

Overall, significant depletions in metal concentrations in the harvested and unharvested soil regimes were observed repeatedly, particularly for the H_2O_2 extractant. However, the substrate variability is exemplified by the fact that significant increases were only recorded in the aqua regia extractable, or residual, fraction for Ni and Cr. McGrath and Cegarra (1992) displayed evidence of Ni moving into less extractable fractions after long time periods in a sludge-amended soil.

Given that data presented in Sections 4.2, 4.4.2 and Chapter 5 demonstrate that plant uptake and leaching represent a relatively small fraction of the fate of mobilised metal, the residual fraction is presumably mainly to where mobilised metals are redistributed following root exudation or degradation. An example is provided in Figure 4.4.1.9, where the residual Ni concentrations significantly increase from March to June in the unharvested regime soil. The percentage graphs frequently show this fraction to increase in size: increments from March to June can be seen in the results for Zn (Figure 4.4.1.3), Cu (Figure 4.4.1.6) and Cr (Figure 4.4.1.8).

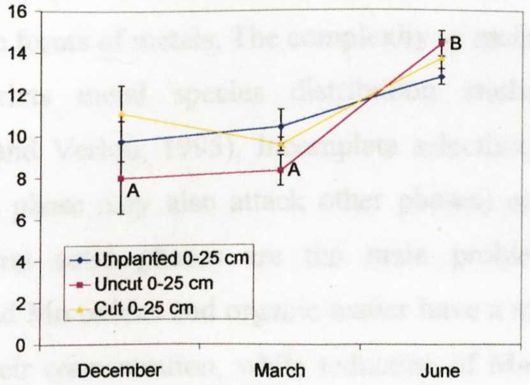

Figure 4.4.1.9 *Aqua regia extractable concentrations of Ni (mg kg^{-1}) in Spofforth soil (0-25 cm depth). Letters apply as in Figure 4.4.1.2*

Therefore the selective extraction data from Slough and Rodley (Sections 4.2 and 4.3 respectively), alongside the sequential extraction data from Spofforth, reveal that tree growth and harvest displaces significant quantities of metals from exchange sites in soil. The mobilised metals pass into the soil solution; data, particularly from Rodley (Section 4.3.2), show that this movement leads to significantly greater concentrations in soil solutions where trees are planted. This increase is likely to represent a small portion of the mobilised metals, as is metal uptake by the growing plants (Table 4.2.3). Therefore most of the mobilised metal pool is deposited on residual sites, evidence for which is presented in this section.

The lack of definitive, consistent trends in the sequential extraction data is likely to be due to both substrate variability and the pitfalls of sequential extraction as an analytical tool in examining changes in soil chemistry. Arrouays *et al.* (2000) detailed how the spatial variability of a soil, or microheterogeneity, may bias the monitoring of temporal changes in its metal contents, unless a sufficient number of samples are taken. The number of samples taken per sampling visit could have been increased, but this would have lengthened an already large amount of time spent on routine analysis. Also, Davidson *et al.* (1998) used the procedure on duplicate samples of industrially contaminated soil, and reported generally good reproducibility (to within 10 %) of concentrations of extracted metals. A more thorough and controlled pot experiment, with a greater number of replicates, was carried out to examine the effects of tree growth on substrate extractable metal concentrations (Chapter 5).

McGrath and Cegarra (1992) stressed that sequential extractions must not be regarded as extracting exclusive forms of metals. The complexity of metal reactions and kinetics in soil environments restricts metal species distribution studies to operationally defined procedures (Tack and Verloo, 1995). Incomplete selectivity of the reagents (extractants targeting one solid phase may also attack other phases) and redistribution of mobilised metals to remaining solid phases are the main problems in sequential extraction procedures. Iron and Mn oxides and organic matter have a metal-scavenging action far out of proportion to their concentration, while reduction of Mn and Fe oxides by NH_3OHCl can be hindered by occluding organic matter (Sheppard and Stephenson, 1997), possibly causing the oxidisable fraction to have an artificially high value for a metal, and the reducible values to be too low. Furthermore, H_2O_2 does not destroy all forms of organic matter, while metals mobilised by this extractant may precipitate as hydroxides (Shuman, 1991). Metals may be mobilised and lost when DIW rinses are discarded, as they were in the BCR procedure used in this study. Predictions of the mobility of soil contaminants based on selective extraction are therefore tenuous at best. Acknowledgement must be made of the possibility that the results displayed above may be biased by these analytical pitfalls.

Despite these limitations sequential extraction is a useful analytical tool when a standardised, rigorously tested procedure such as the BCR protocol is implemented in conjunction with other techniques such as selective extraction and soil solution extraction. Alternative techniques have their drawbacks too. The use of ion-specific electrodes is limited by low sensitivity and interferences. Ion exchange resins, on the other hand, are insensitive to interference by electrochemically active substances such as humic acids. But this technique is only suitable for the speciation of metals that form relatively stable organic complexes because of on-column reactions of labile complexes (Tack and Verloo, 1995).

4.4.2 Soil solution extractions

Table 4.4.2 displays the mean data for the metals present in soil solutions extracted from Spofforth samples in June (0-25 cm depth). In contrast to the sequential extraction data, only Pb concentrations were significantly different in the three soil regimes. The significantly higher concentrations in the soil solutions where trees had been harvested, was possibly a result of enhanced organic matter degradation (such as root decay) following harvest, and subsequent release of soluble, organically complexed Pb.

Resin samplers installed by the University of Hohenheim as part of the BIORENEW project detected no significant increases in leached Cu and Zn after trees were cut (Schmidt, pers. comm.). Copper was also found to be more detectable than Zn. Both these observations correspond with the results below, which display no significant increase in solution Cu and Zn concentrations in the harvested and unharvested soils compared to an unplanted control.

Table 4.4.2 Mean heavy metal concentrations, $\mu g\ l^{-1}$, in June soil solution samples (standard error in brackets). For each metal, means (n=3) without letters or with a letter in common are not significantly different (p > 0.05) after a Fisher LSD test

	Zn	Cd	Cu	Ni	Pb	Cr
Unplanted	52.8 (6.5)	7.40 (3.50)	156 (35.0)	12.2 (1.22)	8.13 a (1.03)	5.63 (0.09)
Uncut	45.4 (1.36)	4.75 (0.25)	112 (3.18)	12.2 (1.68)	10.7 a (1.00)	8.17 (0.79)
Cut	47.7 (1.93)	4.55 (0.85)	128 (8.96)	13.2 (1.76)	24.0 b (3.55)	7.87 (0.84)

Also, the soil's high organic matter and lime content (Section 2.1.1.1) may have masked any clear shifts in metal distribution from the solid phases of the soil into the soil solution as a result of tree harvest or root activity. These soil properties may account for the results contrasting with those for soil solutions extracted from Rodley (Section 4.3.2). At Rodley, significant differences in soil solution concentrations between unplanted soils and soils where trees were growing, were recorded for all metals in the 0-25 cm depth. At Spofforth, no significant differences between concentrations in the unplanted and unharvested regimes were recorded. Rodley was evidently a more contaminated site; concentrations of all determinands were between 4 and 20 times greater in the Rodley soil solutions, apart from Cu, which was approximately 4 times greater in the Spofforth soil solutions.

4.4.3 Soil respiration

Soil microbes are important in the solubilisation and immobilisation of trace elements, with subsequent effects on the availability of metal ions for absorption by plants (Banuelos and Ajwa, 1999).) The cycling and availability of metals are affected by microbial organic matter breakdown, exudation of chelating agents such organic acids during periods of activity, cell lysis and indirect transformations resulting from pH or redox potential changes (Stevenson, 1991). Therefore an assessment of their activity through a respiration bioassay was pertinent to the study of the effects of tree growth and harvest.

Table 4.4.3 displays the mean respiration rates of soil samples taken in June from unplanted, unharvested and harvested areas. The results clearly show the respiration rate to be significantly greater ($p < 0.001$) in the cut area than in the uncut and unplanted area.

Table 4.4.3 Mean respiration rate of soils, mg CO_2-C/kg dry soil/h (standard error in brackets). Means (n=6) without a letter in common are significantly different ($p < 0.001$) after a Fisher LSD test

Unplanted	0.41 (0.02) a
Unharvested	0.41 (0.02) a
Harvested	0.56 (0.02) b

Therefore root degradation, and the associated stimulation of microbial activity, was significant six months after tree harvest. The expected differences between microbial activity in unplanted and unharvested soils may not have arisen due to the unplanted soil featuring considerable root activity from weeds, the growth conditions of which were nearly optimal in June.

4.5 Summary

Extractions of heavy metals from three sludge-amended soils provided a study of the effects of tree growth on the distribution of metals between the soil solid phases. All observed trends were affected by the inevitable heterogeneous contaminant distribution in soils. However, several worthwhile observations could be made. At two sites, selective extraction provided evidence for depletion of extractable metals through biomass crop growth, when compared to concentrations in adjacent unplanted areas. Plant concentrations demonstrated that plant uptake could only account for a small fraction of the observed depletions; therefore redistribution of metals amongst the soil solid phases was considered likely.

Sequential extractions of metals from samples collected at a third site demonstrated this: where trees were growing or were recently harvested, extractable concentrations frequently fell significantly in the period from March to June, with a corresponding rise in the residual fraction. Resin samplers did not record any significant increases in leached metals during the experimental period.

Soil solution extraction at one site revealed significantly higher metal concentrations in soils affected by root exudation than in unplanted control soils. This is likely to be due to increased complexation of heavy metals by soluble chelating agents exuded by plant roots. At a different site, willow harvest significantly increased the concentrations of Pb in soil solution six months after tree harvest, which was interpreted as an effect of root degradation. Coupled with this, microbial respiration in this soil regime displayed significantly increased rates.

Chapter 5 Pot Study of the Effect of Tree Growth on Heavy Metal Distribution in Soil Fractions

5.1 Introduction

Plate 5.1 Willow-planted and unplanted control pots, containing the steelworks waste substrate, arranged in a randomised block

Numerous studies have focused on the uptake of metals by plants grown on contaminated substrates, but comparatively few have considered the effect of the growth of the plant itself on the distribution of metals between soil fractions, nor the fraction of soil from which plants extract heavy metals. Following the trends identified in Chapter 4, the aims of this study were:

- to further assess whether willow growth resulted in significant depletions and redistributions of extractable heavy metal concentrations in a contaminated substrate
- to establish whether colonisation of willow roots by mycorrhizal (MYC) fungi reduces metal translocation to the plant shoot.

The study comprised a pot experiment which was intended to produce results less variable than those reported in Chapter 4 via an increased number of replicates. Section 2.2.3 gives the experimental set-up, while the chemical properties of the steelworks waste tip substrate used in this experiment are detailed in Section 2.2.1.2.

5.2 Failure of Fungal Colonisation of Willow Roots

Unfortunately, the MYC fungi did not infect the tree roots: no MYC roots could be identified in root samples (Wheeler, pers. comm.). Therefore no data referring to metal sequestration/transfer by fungal hyphae in soil-willow root systems can be reported. Generally, low nutrient availability favours MYC development: high availability reduces C allocation to the roots and hence the fungus (Colpaert and van Tichelen, 1996). The steelworks waste is not a particularly fertile substrate, and so it is unlikely that its nutrient status inhibited the symbiosis. Heavy-metal inhibition of MYC development has been reported in plants grown in contaminated substrates, for example by Bell *et al.* (1988) and Berry (1985). The fungal species in the inoculum may not be metal-tolerant; however, even in more highly contaminated substrates than the steelworks waste, fungitoxicity of heavy metals usually leads to a reduced number of MYC roots rather than total fungal absence.

It may have been more suitable to use ectomycorrhizal fungi in this experiment, as their associations contain a much greater number of species than endomycorrhizal symbioses. While willow roots can be both endo- and ectomycorrhizal, the former was chosen as cross-infection of non-mycorrhizal replicates was less likely. An explanation for the lack of MYC roots in this pot trial may lie in the incompatibility of the host and symbiont. However, endomycorrhizal associations generally lack host specificity (Colpaert and van Tichelen, 1996).

Therefore the high pH of the substrate (Section 2.2.1.2) is the most likely factor in inhibiting the symbiosis. Bell *et al.* (1988) reported that MYC associations in tree roots develop most extensively in acid soils. In field environments, fungi in general dominate the soil microbial biomass in conditions of low pH; soil bacteria outcompete fungi in high pH conditions, and rhizosphere bacteria may suppress mycorrhizal infection (Marschner, 1995).

5.3 Selective Extraction Results

The extent of the vertical and horizontal contamination of a spoil (and its nutrient distribution) can vary considerably: Merrington (1995) observed layers of material at mine sites containing metal concentrations up to 100 times greater than those in bulk tailings. Thus the monitoring of temporal changes in metal contents of substrates can be hidden by spatial variation, which can occur over very small areas, causing 'microheterogeneity' (Arrouays *et al.*, 2000). The steelworks spoil substrate used in this experiment is highly variable in its metal concentrations (Hepple, pers. comm.).

However, the fact that the trees were not separated into MYC and non-MYC groups provided a greater number of replicates (n = 16 for each variety) for the study of the effect of tree growth on extractable metal pools. The considerable variability of this substrate necessitated a large number of replicates; an insufficient number of samples may result in a bias in measurements which may hide temporal changes (Arrouays *et al.*, 2000).

Despite this variability, significant decreases of the extractable metal concentrations using NH_4EDTA or NH_4OAc (see Section 2.2.3) from the beginning to the end of the 9 month trial were recorded (Figures 5.3.1 to 5.3.6). These reductions occurred where trees had grown, but similar significant decreases were not observed in unplanted control pots.

These depletions were not consistently observed for all metals extracted by both reagents, nor were they consistently found as a result of the growth of both of the willow clones. However, obvious trends can be identified in the results for Zn (Figures 5.3.1 and 5.3.2) and Ni (Figures 5.3.3 and 5.3.4). Extractable concentrations for these elements display significant decreases in pots where at least one of the clones has grown.

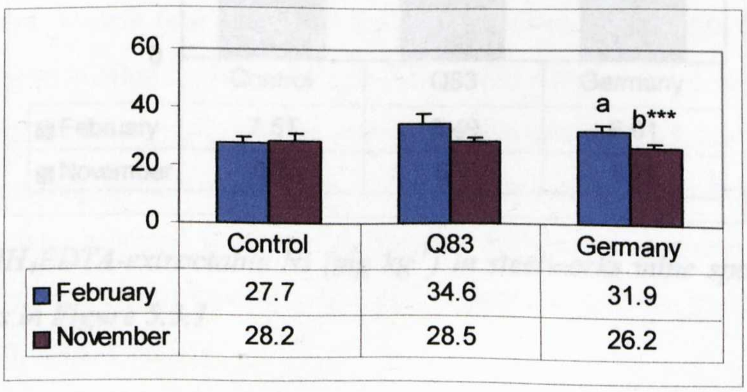

Figure 5.3.1 *NH_4OAc-extractable Zn (mg kg^{-1}) in steelworks mine spoil. Within each treatment, means (n=16) without letters are not significantly different after a paired t-test. Means without a letter in common are significantly different (* denotes $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$)*

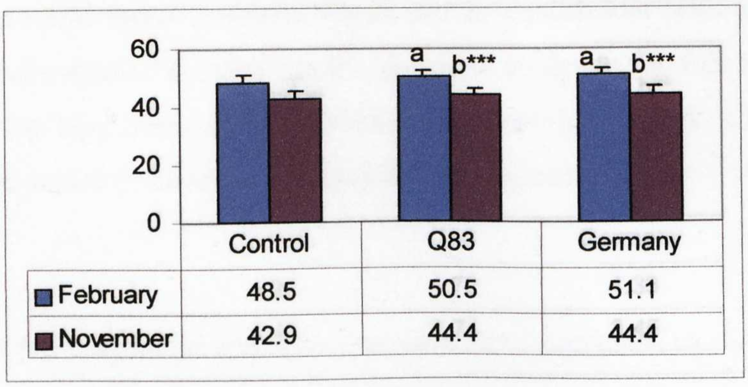

Figure 5.3.2 *NH_4EDTA-extractable Zn (mg kg^{-1}) in steelworks mine spoil. Letters apply as in Figure 5.3.1*

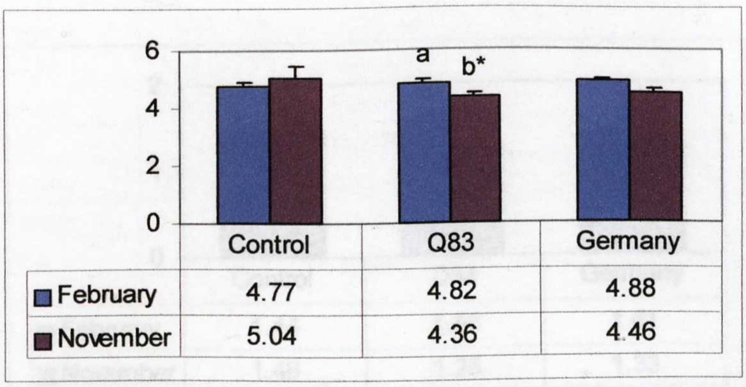

Figure 5.3.3 *NH_4OAc-extractable Ni (mg kg^{-1}) in steelworks mine spoil. Letters apply as in Figure 5.3.1*

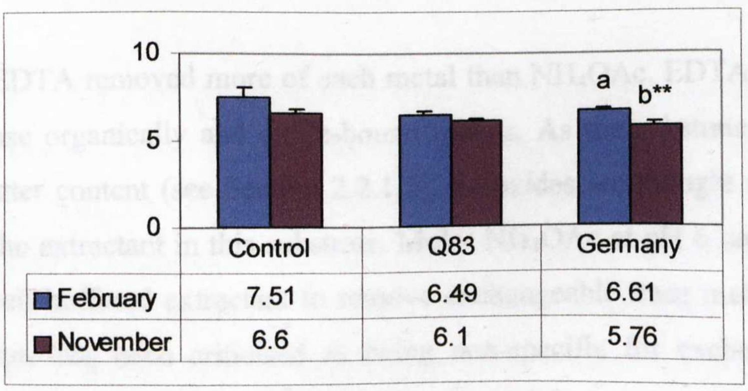

Figure 5.3.4 *NH_4EDTA-extractable Ni (mg kg^{-1}) in steelworks mine spoil. Letters apply as in Figure 5.3.1*

Similar reductions, without corresponding decreases in control pots, were also apparent in results from one of the two extractants for Cu (Figure 5.3.5), and Cd (Figure 5.3.6). No significant differences were observed in the results for Pb and Cr.

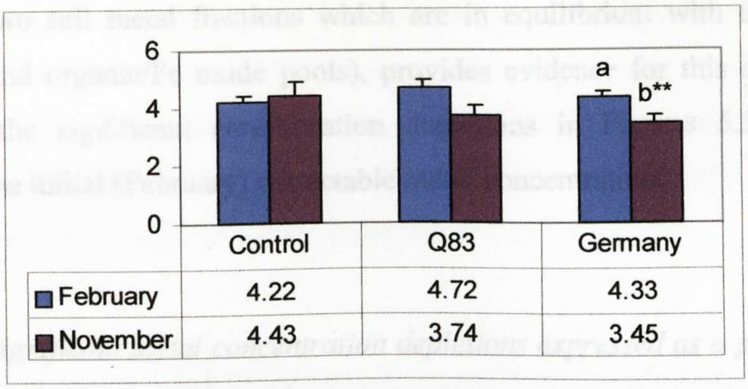

Figure 5.3.5 *NH_4OAc-extractable Cu (mg kg^{-1}) in steelworks mine spoil. Letters apply as in Figure 5.3.1*

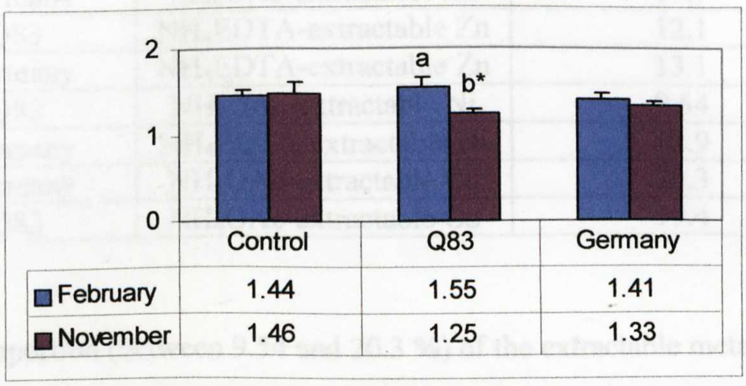

Figure 5.3.6 *NH_4OAc-extractable Cd (mg kg^{-1}) in steelworks mine spoil. Letters apply as in Figure 5.3.1*

The extractant EDTA removed more of each metal than NH_4OAc. EDTA is a complexant which can release organically and oxide-bound metals. As the substrate has a relatively low organic matter content (see Section 2.2.1.2), Fe oxides are thought to have been the chief target of the extractant in this substrate. Molar NH_4OAc at pH 6 has been described as a suitable, well-buffered extractant to remove exchangeable trace metal ions (Beckett, 1989), but acetate has been criticised as being non-specific for exchangeable ions by Shuman (1991), who reported it may also dissolve carbonates or oxide coatings. These criticisms highlight the major problem of metal extraction, namely the selectivity of the reagents used (Shuman, 1991).

Despite this, the observation of operationally defined extractable metal depletion in substrate under growing trees is not compromised. The growth of willows may be useful if reduction of the bioavailable metal in a soil occurs (Riddell-Black, 1994). The significant depletion of NH_4OAc and NH_4EDTA extractable metal concentrations, which broadly correspond to two soil metal fractions which are in equilibrium with the soil solution (exchangeable and organic/Fe oxide pools), provides evidence for this occurring. Table 5.3.1 presents the significant concentration depletions in Figures 5.3.1 to 5.3.6 as percentages of the initial (February) extractable metal concentrations.

Table 5.3.1 Significant metal concentration depletions expressed as a percentage of the initial extractable metal concentrations

Willow variety	Metal	%ge of metal depleted by plant growth
Germany	NH_4OAc-extractable Zn	17.9
Q83	NH_4EDTA-extractable Zn	12.1
Germany	NH_4EDTA-extractable Zn	13.1
Q83	NH_4OAc-extractable Ni	9.54
Germany	NH_4EDTA-extractable Ni	12.9
Germany	NH_4OAc-extractable Cu	20.3
Q83	NH_4OAc-extractable Cd	19.4

A significant proportion (between 9.54 and 20.3 %) of the extractable metals were depleted as a result of tree growth. Decreases in the organically-bound metal pool may not be a direct effect of tree growth: root exudation may have stimulated microbe activity, which in turn led to enhanced mineralisation of organic matter.

The results in Table 5.3.1 are comparable to those of other studies: Greger and Landberg (1999) reported that a high-Cd accumulating clone of *Salix viminalis* removed up to 35% of exchangeable Cd (M NH_4OAc at pH 5.2) in a 90-day pot trial. Using isotopic methods, Morel *et al.* (1999) demonstrated that the hyperaccumulators *Thlaspi caerulescens* and *Alysum murale* markedly decreased NH_4NO_3 and DTPA-extractable Zn, Cd and Ni. However, it is worth noting that long term studies have revealed that tree growth may not necessarily decrease exchangeable metal pools: Alriksson *et al.* (1999) investigated the effects of the growth of five tree species (including willow) over 6 years on Cd concentrations in soil, and found high biomass Cd uptake was not related to a corresponding depletion of the soluble (NH_4NO_3 extractable) Cd pool, which in fact increased due to decreasing pH.

From a phytoremediation perspective, it is relevant to assess the importance of metal sequestration in the above-ground biomass in contributing to the significant depletions listed above. Table 5.3.2 expresses plant uptake of the metals into aerial tree parts (based on pooled measurements of stem and leaf biomass multiplied by their metal concentrations) as a percentage of each soil depletion.

Table 5.3.2 Percentages of observed significant soil concentration depletions attributed to sequestration in above-ground willow biomass

Willow variety	Metal	Plant uptake as %ge of soil depletion
Germany	NH_4OAc-extractable Zn	19.5
Q83	NH_4EDTA-extractable Zn	21.9
Germany	NH_4EDTA-extractable Zn	16.8
Q83	NH_4OAc-extractable Ni	3.69
Germany	NH_4EDTA-extractable Ni	2.16
Germany	NH_4OAc-extractable Cu	3.71
Q83	NH_4OAc-extractable Cd	4.77

These percentages are not insignificant; a considerable fraction (approximately 20 %) of the observed Zn depletion can be attributed to plant uptake and transport to the aerial tree parts. This was markedly greater than the fraction of Cd taken into the leaves and stems, which was in turn greater than the fractions of Cu and Ni. The fractions of metals sequestered in the tree roots is likely to have been considerable too; unfortunately no measurements of this were carried out due to the analytical difficulties of distinguishing metals fixed in, and on, the root. Other factors which may have contributed to the depletions (discussed below) are leaching and the redistribution of metals to other soil pools.

Plant-induced metal leaching is a possible explanation for the diminished extractable metal concentrations. Marseille *et al.* (2000) grew different vegetal species (maize, rape and rye grass) on metal-contaminated sediments, and found concentrations of leached heavy metal were higher in the planted pots than in control pots. The increased metal mobility was mainly attributed to increased labile organic carbon due to root exudation and consequent microbial activity. Zhu *et al.* (1999) examined the impact of plants on heavy metal leaching from a contaminated topsoil/mine tailings mixture. The presence of tall fescue and big bluestem increased short-term Cd and Zn leaching, mainly due to complexation of metals following organic compound exudation by plants and microbial solubilisation, but presence of a subsoil (allowing adsorption and precipitation of the metals) acted as a sink for the mobilised metals. They argued that initial enhanced metal leaching does not outweigh the long-term benefits of planting on derelict sites, such as reduced wind erosion and runoff, and improved soil structure.

The absence of any significant reductions recorded in the control pots, which received the same irrigation regime as the planted pots, indicates leaching was minimal in this highly calcareous substrate (Section 2.2.1.2). Also, in natural systems, increased water demand through evapotranspiration will reduce leaching relative to areas where willows have not been planted.

To fully account for the observed decreases in these results, metal redistribution from the exchangeable pool to a less available phase, in addition to metal sequestration in plant roots and shoots, is probable. Data shown in Section 5.4 examine the relative distribution of heavy metals in pools extracted sequentially.

5.4 Sequential Extraction Results

A team at the University of Hohenheim comprised one of the BIORENEW project's seven partners (Section 1.11). This research group conducted many routine soil analyses and sequential extractions. Analyses kindly carried out on subsamples from this experiment (Section 2.2.3) provided results which were again affected by the substantial variability of the substrate. However, not all temporal trends were obscured by this heterogeneity and the data provided further evidence of depletion, and also redistribution, of extractable metals during the experiment, particularly for Zn. Figure 5.4.1 displays the Zn extracted by NH_3OHCl and NH_4EDTA as a percentage of the aqua regia extractable Zn; this was relatively unchanged in the unplanted control pots over the duration of the experiment. In contrast, there appeared to be depletion with time of these pools in pots where Germany or Q83 were grown.

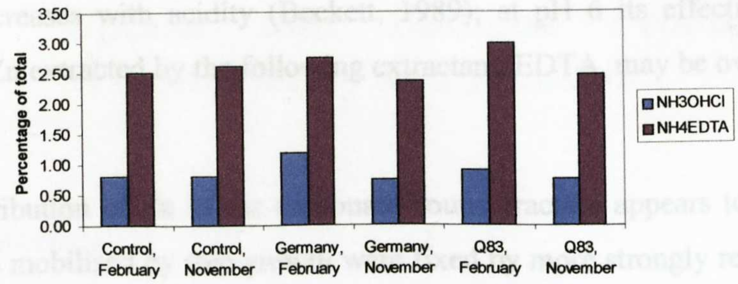

Figure 5.4.1 *Temporal changes in NH_3OHCl–extractable and NH_4EDTA-extractable Zn pools, expressed as a percentage of the pseudototal (aqua regia extractable) Zn*

Furthermore, a suitable 4-step sequential extraction procedure was applied to subsamples of the pot trial. The series involved extraction with NH_4OAc to target the easily mobilisable fraction, NH_4OAc with HCl to remove carbonate-bound metals, NH_3OHCl to extract metals occluded in Mn oxides, and the aforementioned NH_4EDTA was the final reagent in the sequence.

Figure 5.4.2 displays the distribution in Zn pools in pots where Q83 was grown. The data revealed a decrease in the NH_4OAc and EDTA-extractable Zn percentages in pots where Q83 had grown over the 9 months, with an increase in the carbonate-bound fraction of the metal.

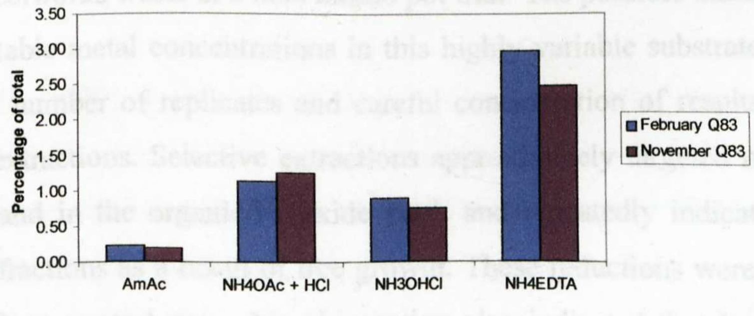

Figure 5.4.2 *Zn pools in the steelworks waste substrate under Q83, expressed as a percentage of the Zn pseudototal*

Care should be taken in interpretation of these results as they are subject to the limitations of sequential extraction detailed in Chapter 4, namely the selectivity of the extractants, and possible metal readsorption of mobilised metal onto a more resistant soil fraction.

NH_3OHCl, for example, is a mild reducing agent with an efficiency in solubilising Mn oxides which increases with acidity (Beckett, 1989); at pH 6 its effectiveness may be reduced and the Zn extracted by the following extractant, EDTA, may be overestimated.

While the redistribution of Zn to the carbonate-bound fraction appears to be slight, it is likely that metals mobilised by tree growth were fixed by more strongly resistant fractions not targeted by the range of extractants used in this study, such as the sulphides and residual organic pools. Given the calcareous nature of the substrate (Section 2.2.1.2), the carbonate-bound fraction appeared to be relatively small; perhaps the second reagent in the sequential extraction did not fully displace all carbonate-bound metals. Alternatively, much of the mobilised carbonate-metal pool might have bound to remaining soil phases during the sequential extraction, an analytical pitfall of this technique. The low percentages in Figures 5.4.1 and 5.4.2 point to a vast pool of Zn which can only be displaced by more rigorous extractants than the four described above: most of the extractable Zn is a small fraction of the aqua regia-extractable total. In the absence of evidence for leaching, redistribution to resistant metal pools appears to provide a significant contribution to the depletion of extractable metal concentrations detailed in Section 5.3.

5.5 Summary

This chapter considered the effect of willow growth on the metal distribution of a contaminated steelworks waste in a nine month pot trial. The possible masking of temporal trends in extractable metal concentrations in this highly variable substrate was countered by an increased number of replicates and careful consideration of results from selective and sequential extractions. Selective extractions approximately targeted metals bound on exchange sites and in the organic/Fe oxide pool, and repeatedly indicated depletion of metals in these fractions as a result of tree growth. These reductions were not apparent in samples taken from control pots; this observation also indicated that leaching of metals was not significant in this trial. Calculations gauging the importance of metal uptake into the above-ground biomass of the trees revealed this factor to be considerable, particularly for Zn. The results from the sequential extractions suggest the metal reductions can also be partly explained by redistribution to the carbonate-bound fraction, and other more resistant pools. Sequestration of metals in the tree roots was not quantified due to analytical difficulties in distinguishing metals fixed within, or on, roots.

Chapter 6 The Effect of Soil Additives on Metal Uptake and Biomass Production By Willow, Barley and *Phalaris*

6.1 Introduction

The use of plants in the extraction of metals from contaminated soils is subject to the limitations of the concentrations of the metals achieved in the above-ground biomass, and the amount of biomass produced by the phytoextracting plant. Compounds can be added to the soil to increase the solubility of metals around the plant roots, potentially enhancing the metal uptake by the plant. These soil amendments may also serve to fertilise the plant, thus increasing its biomass production. The aim of this work was:

- to investigate the effects of two soil amendments on the metal uptake and biomass production of three plant species grown in a contaminated soil.

The soil amendments were $(NH_4)_2SO_4$ plus dicyandiamide (DCD, a nitrification inhibitor which prevents proton release caused by nitrification of the N source), and citric acid (CIT). The modes of metal mobilisation of these amendments are, respectively, acidification (plants secrete protons to maintain an electrochemical balance in their roots with the ammonium absorbed) and chelation (a metal-chelate complex is formed).

Schremmer *et al.* (1999) reported heavy metal contents in *Salix viminalis* leaves to be increased by $(NH_4)_2SO_4$ plus DCD in a contaminated soil. This soil manipulation technique was used in this experiment to ascertain whether the effects were similar using different plant varieties in a more contaminated soil.

Metal-chelate complexes may be taken up by plants, sorbed to soil components, biologically degraded or leached. Numerous studies have focused on enhancing metal uptake using synthetic chelating agents. For example, Blaylock *et al.* (1997) reported EDTA to be the most effective chelate in enhancing metal uptake and translocation to the shoot in Indian mustard. However, Huang *et al.* (1997) acknowledged the possibility of substantial chelate-induced metal leaching. Synthetic chelates such as EDTA show a low degree of biodegradability (Kayser *et al.*, 2000). The risk of downward metal migration depends on the mobility of the complex and the length of time the chelate is active in the soil (Blaylock *et al.*, 1999); therefore, the effects of the natural chelating agent CIT, which is highly biodegradable relative to EDTA (Kulli *et al.*, 1999) on plant metal uptake and biomass production, were investigated. CIT is produced by rhizosphere bacteria and during plant decay, and has been identified in root exudates and forest litter (Stevenson, 1991). This simple organic acid is of key importance due to its wide distribution (it is one of the most abundant compounds in tree root exudates) and its formation of stable complexes with trace elements.

The amendments were tested on three plant species which could potentially be used for phytoextraction. Willow and *Phalaris* are biomass energy crops, while barley is a crop which has shown promise in previous work investigating induced phytoextraction: accumulation of Zn was enhanced by EDTA application, imparting it with a phytoextraction potential reported to be equal to that of Indian mustard (Khan *et al.*, 2000). Section 2.2.2 details the experimental design, while Section 2.2.1.1 lists the chemical properties of the soil used.

6.2 Effect of the Soil Amendments on Plant Aerial Biomass

The biomass production of barley was greater than that of *Phalaris* and willow; this is probably due to the more rapid growth of the barley over the limited time of the 4-week pot trial, rather than superior growth in a contaminated substrate. The effects of the soil amendments on the leaf and stem biomass of both willow clones are displayed in Figures 6.2.1 and 6.2.2, respectively.

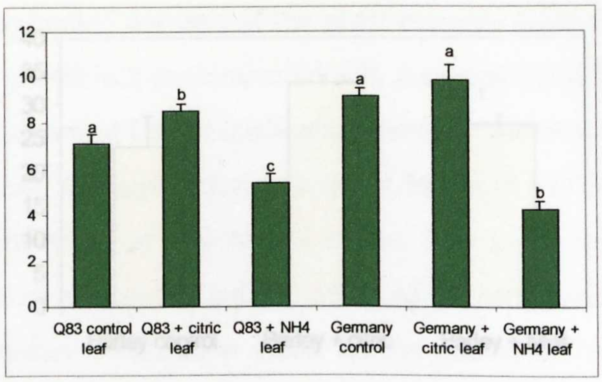

Figure 6.2.1 Dry leaf biomass (g) of two willow varieties in 3 soil manipulation regimes. Within each variety, means (n=5) without a letter, or with a letter in common, are not significantly different ($p > 0.05$) after a Fisher LSD test

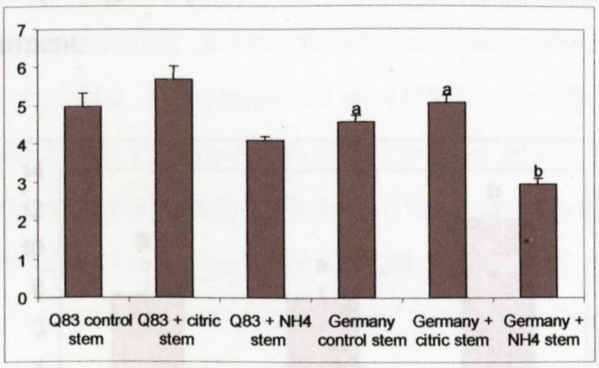

Figure 6.2.2 Dry stem biomass (g) of two willow varieties in 3 soil manipulation regimes. Letters apply as in Figure 6.2.1

A tendency of the CIT treatment to increase biomass relative to the control and to the $(NH_4)_2SO_4$ plus DCD treatment, sometimes significantly, was revealed. A yield penalty in the replicates treated with $(NH_4)_2SO_4$ plus DCD was apparent: biomass of both fractions was frequently significantly lower than in the control pots. A similar trend to those displayed in the willow biomass data was observed in barley aerial plant tissue (Figure 6.2.3), although the differences were not statistically significant.

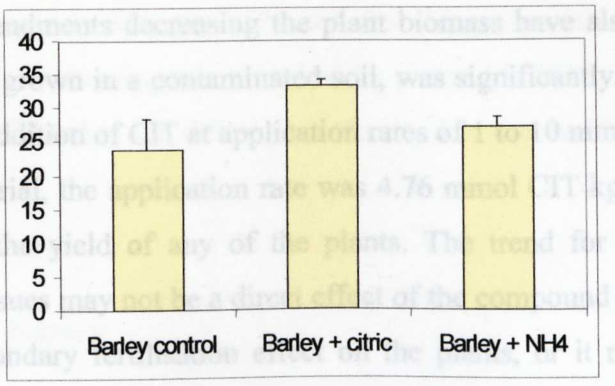

Figure 6.2.3 Dry aerial biomass (g) of barley in 3 soil manipulation regimes

This contrasted with the results for *Phalaris* (Figure 6.2.4), which displayed a significant stimulatory effect of the $(NH_4)_2SO_4$ plus DCD treatment on the aerial plant tissue biomass relative to the CIT treatment.

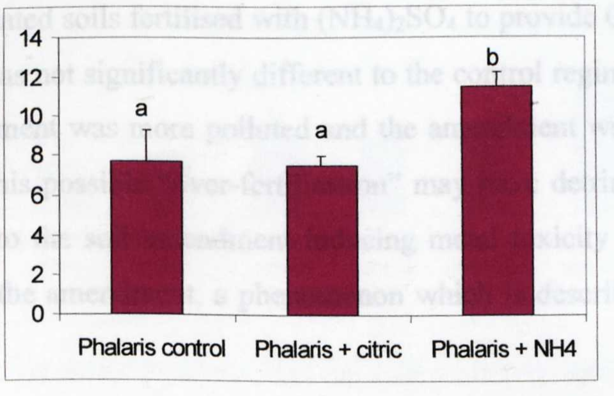

Figure 6.2.4 Dry aerial biomass (g) of Phalaris *in 3 soil manipulation regimes. Letters apply as in Figure 6.2.1*

The *Phalaris* therefore displays enhanced growth in the $(NH_4)_2SO_4$ plus DCD regime. A similar stimulatory effect of urea was displayed in an 8 week pot trial in which Kulli *et al.* (1999) grew lettuce and ryegrass on a contaminated soil. The willow results, in contrast, displayed a tendency for the $(NH_4)_2SO_4$ plus DCD to have a detrimental effect on plant biomass, while the chelating agent CIT had a fertilisation effect. The application of chelates such as nitrilotriacetate (NTA) to a contaminated soil in a 6-week trial has been demonstrated to significantly increase the dry weight of corn and sunflower shoots (Cooper *et al.*, 1999).

Examples of soil amendments decreasing the plant biomass have also been documented. Indian mustard yield, grown in a contaminated soil, was significantly reduced in a 4-week pot trial through the addition of CIT at application rates of 1 to 10 mmol kg $soil^{-1}$ (Blaylock *et al.*, 1997). In this trial, the application rate was 4.76 mmol CIT kg $soil^{-1}$ which did not significantly reduce the yield of any of the plants. The trend for CIT to increase the biomass of willow tissues may not be a direct effect of the compound itself; its degradation may have had a secondary fertilisation effect on the plants, or it may have complexed metals around the roots, permitting better growth due to reduced metal toxicity. Both of these possibilities are discussed in more detail in Section 6.3.

These results contrast with those from longer term studies. In a 4 month field trial using *Salix viminalis*, Kayser *et al.* (2000) applied an acidifying agent (sulphur) or the chelate NTA to a polluted soil, and reported no reduction in biomass production in either treatment regime. In a 17 week pot trial, Schremmer *et al.* (1999) grew *Salix viminalis* 'Jorr' on heavy metal contaminated soils fertilised with $(NH_4)_2SO_4$ to provide 0.1 g N kg $soil^{-1}$, plus DCD. Plant growth was not significantly different to the control regime. However, the soil in this 4-week experiment was more polluted and the amendment was applied to provide 0.39 g N kg $soil^{-1}$. This possible "over-fertilisation" may have detrimentally affected the willow biomass due to the soil amendment inducing metal toxicity which overcame the fertilisation effect of the amendment, a phenomenon which is described in more detail in Section 6.3.

6.3 Effect of the Soil Amendments on Metal Uptake

Several statistically significant differences were observed in the data for the metal quantities accumulated in the above-ground biomass, considerably more than in the data for metal concentrations achieved in the plant tissues. The performance of the three plant varieties were more distinguishable when comparing the quantities of metals taken up in each of the soil treatment regimes. Furthermore, quantities of metals accumulated are more important than tissue concentrations in the context of phytoremediation. For these reasons, metal quantities are focused on in this section.

Metal concentrations were generally higher in willow and *Phalaris* than in barley; as barley achieved the greatest biomass, quantities of metals were usually higher in barley than in willow and *Phalaris*. The geometry and absorbing capacities of root systems also affect the rates and amounts of metals absorbed by plants (de Villarroel *et al*., 1993); this factor may have contributed to the differences observed in the metal uptake of the 3 plant species.

6.3.1 Willow and Barley

Figures 6.3.1.1 and 6.3.1.2 display typical effects of the soil amendments on the concentrations and quantities of metals in the willow tissues. Both the CIT and $(NH_4)_2SO_4$ plus DCD treatments increased the Cu concentrations in the willow stems relative to the control, significantly in Q83. Because of the yield penalty induced in the willow by the $(NH_4)_2SO_4$ plus DCD treatment (Section 6.2), only the CIT-treated trees achieved significantly greater Cu quantities than in the control.

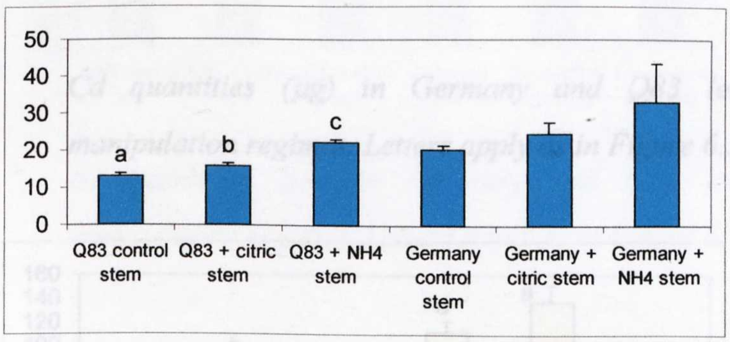

Figure 6.3.1.1 *Cu concentrations (mg kg^{-1}) in Germany and Q83 stems in 3 soil manipulation regimes. Within each variety, means (n=5) without a letter, or with a letter in common, are not significantly different (p > 0.05) after a Fisher LSD test*

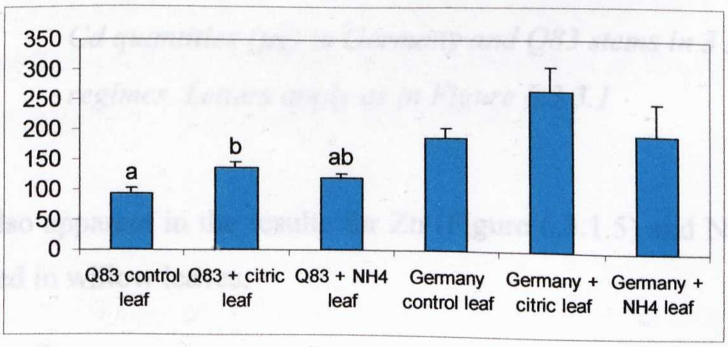

Figure 6.3.1.2 *Cu quantities (µg) in Germany and Q83 stems in 3 soil manipulation regimes. Letters apply as in Figure 6.3.3.1*

While $(NH_4)_2SO_4$ plus DCD increased Cu concentrations in the willow stems, the quantities were not significantly greater than in the control or CIT regime due to the yield penalty caused by the treatment. Indeed, the $(NH_4)_2SO_4$ plus DCD regime consistently resulted in significantly lower tissue metal quantities than in the control and/or CIT regimes. There was a tendency for quantities in the CIT treated trees to be greater than in the control trees, but not always significantly. Figures 6.3.1.3 and 6.3.1.4 display the Cd quantities in willow leaves and stems.

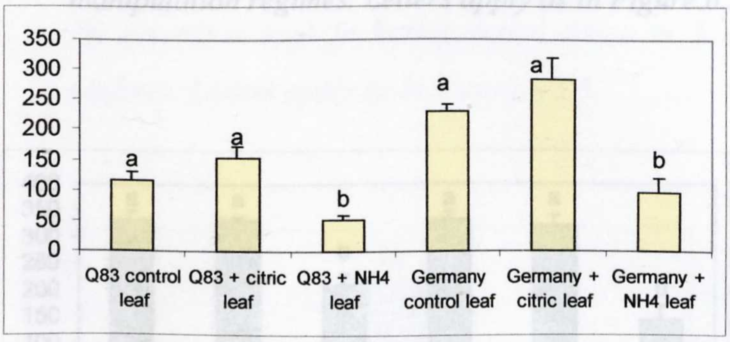

Figure 6.3.1.3 *Cd quantities (µg) in Germany and Q83 leaves in 3 soil manipulation regimes. Letters apply as in Figure 6.3.3.1*

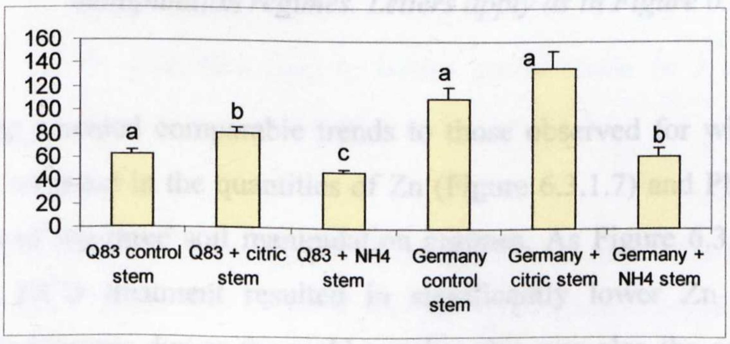

Figure 6.3.1.4 *Cd quantities (µg) in Germany and Q83 stems in 3 soil manipulation regimes. Letters apply as in Figure 6.3.3.1*

This trend was also apparent in the results for Zn (Figure 6.3.1.5) and Ni (Figure 6.3.1.6) quantities achieved in willow leaves.

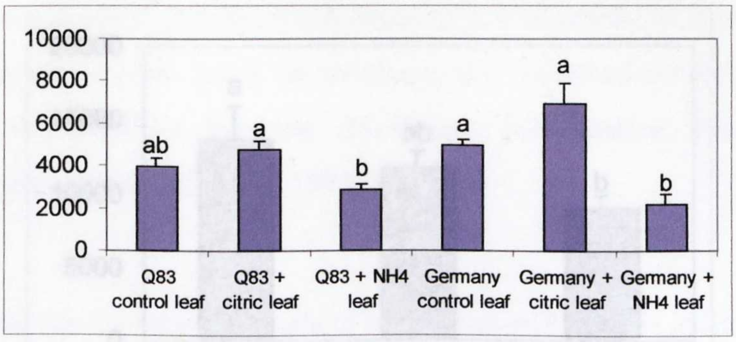

Figure 6.3.1.5 *Zn quantities (μg) in Germany and Q83 leaves in 3 soil manipulation regimes. Letters apply as in Figure 6.3.3.1*

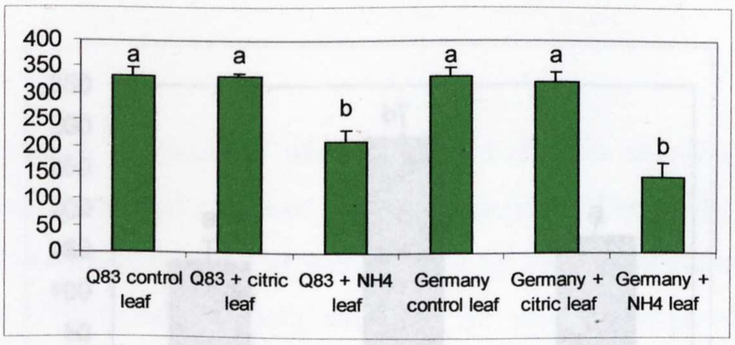

Figure 6.3.1.6 *Ni quantities (μg) in Germany and Q83 leaves in 3 soil manipulation regimes. Letters apply as in Figure 6.3.3.1*

The barley results revealed comparable trends to those observed for willow. Significant differences were recorded in the quantities of Zn (Figure 6.3.1.7) and Pb (Figure 6.3.1.8) achieved in each of the three soil manipulation regimes. As Figure 6.3.1.7 displays, the $(NH_4)_2SO_4$ plus DCD treatment resulted in significantly lower Zn quantities being sequestered in the biomass due to the yield penalty; this was also the case in the willow results (Figure 6.3.1.5). The Pb results provide a further example of the CIT regime leading to significantly greater quantities of metals being accumulated in the biomass than in the control pots, as for willow in Figures 6.3.1.2 and 6.3.1.4.

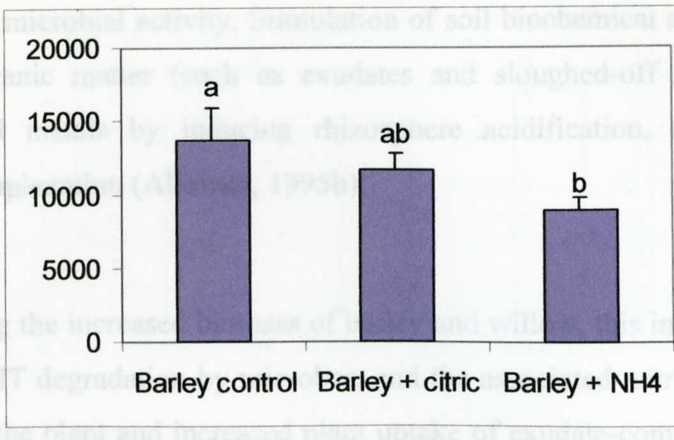

Figure 6.3.1.7 *Zn quantities (µg) in barley aerial tissue in 3 soil manipulation regimes. Letters apply as in Figure 6.3.3.1*

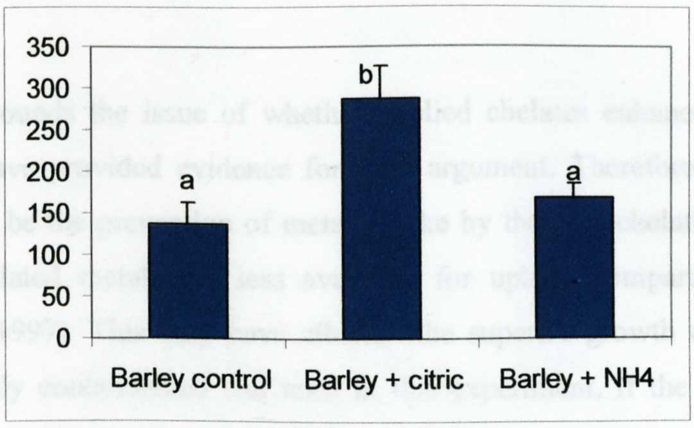

Figure 6.3.1.8 *Pb quantities (µg) in barley aerial tissue in 3 soil manipulation regimes. Letters apply as in Figure 6.3.3.1*

Overall, CIT increased metal quantities (albeit marginally) in both willow stems and barley tissues. This has been reported elsewhere: it was the most effective organic acid amendment in increasing metal availability and enhancing U accumulation in various plants (Khan *et al.*, 2000). The uptake of Zn, Cu, Ni and particularly Cd and Pb by Indian mustard was enhanced through the addition of chelates including CIT, applied at a similar rate to the one used in this experiment, by Blaylock *et al.* (1997).

The increased metal concentrations observed in the CIT regime, particularly in the willow pots (Figure 6.3.1.1), can be interpreted as either direct chelation by the chemical, leading to increased uptake by the plant, or indirect uptake induced by microbial exudates following degradation of the compound. Trees have the most massive root systems, and support the growth of the largest diversity of soil microbes, of all plants (Stomp *et al.*, 1993): hence they indirectly participate in detoxification of a metal-contaminated substrate through enhanced microbial activity. Stimulation of soil biochemical activity through root deposition of organic matter (such as exudates and sloughed-off cells) can mobilise strongly adsorbed metals by inducing rhizosphere acidification, redox changes and organic-metal complexation (Alloway, 1995b).

Therefore, viewing the increased biomass of barley and willow, this indirect explanation is more plausible; CIT degradation by microbes, and the associated nutrient cycling, has led to fertilisation of the plant and increased plant uptake of exudate-complexed metals. Kulli *et al.* (1999) reported fertilisation of lettuce and ryegrass grown on contaminated soil as an effect of NTA degradation; this chelate is similarly biodegradable to CIT (Kayser *et al.*, 2000).

Much debate surrounds the issue of whether applied chelates enhance or reduce metal uptake; studies have provided evidence for each argument. Therefore, another possible explanation could be the prevention of metal uptake by the CIT chelation; it is generally believed that chelated metals are less available for uptake compared to ionic forms (Blaylock *et al.*, 1997). This may have allowed the superior growth of the CIT treated plants in the highly contaminated soil used in this experiment. If the metals in the soil inhibited microbial degradation of the CIT, this effect may have been prolonged. This interpretation is less plausible, considering the frequently observed significant increases in metal concentrations and quantities in the plants grown in this regime.

6.3.2 Phalaris

In contrast to the results for willow and barley, the $(NH_4)_2SO_4$ plus DCD treatment in *Phalaris* frequently resulted in metal quantities that were significantly higher than those in the control regime. Such was the case for Cd, Cu and Ni (Figures 6.3.2.1, 6.3.2.2 and 6.3.2.3 respectively), which contrast with the trends for the same elements accumulated in willows reported in Section 6.3.1.

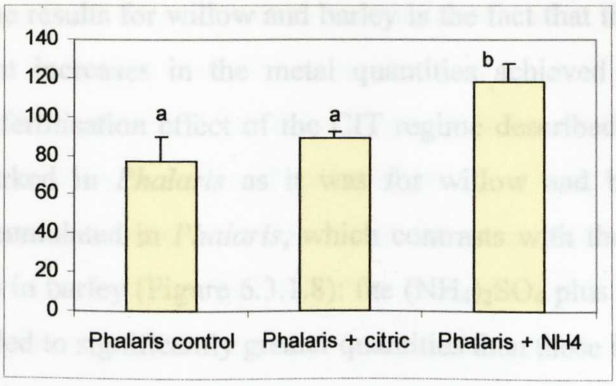

Figure 6.3.2.1 *Cd quantities (µg) in Phalaris aerial tissue in 3 soil manipulation regimes. Means (n=5) without a letter, or with a letter in common, are not significantly different ($p >$ 0.05) after a Fisher LSD test*

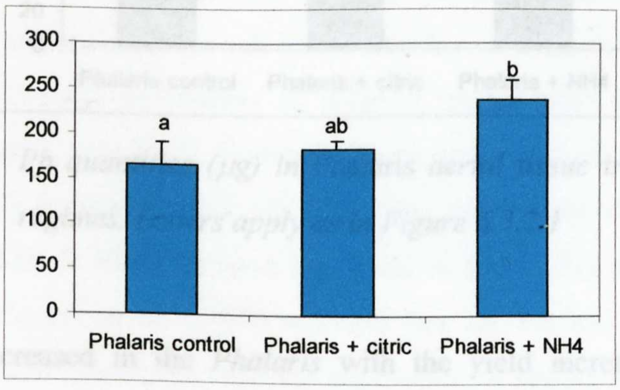

Figure 6.3.2.2 *Cu quantities (µg) in* Phalaris *aerial tissue in 3 soil manipulation regimes. Letters apply as in Figure 6.3.2.1*

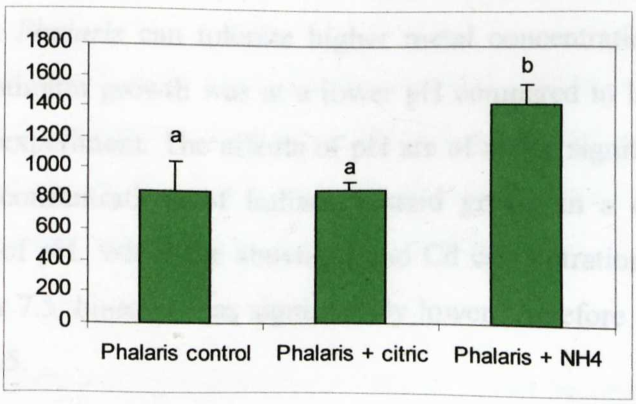

Figure 6.3.2.3 *Ni quantities (µg) in* Phalaris *aerial tissue in 3 soil manipulation regimes. Letters apply as in Figure 6.3.2.1*

Also in contrast to the results for willow and barley is the fact that the CIT regime did not effect any significant increases in the metal quantities achieved in the above-ground biomass. The likely fertilisation effect of the CIT regime described in Section 6.3.1 was therefore not as marked in *Phalaris* as it was for willow and barley. Figure 6.3.2.4 illustrates the Pb accumulated in *Phalaris*, which contrasts with the results for the same element accumulated in barley (Figure 6.3.1.8): the $(NH_4)_2SO_4$ plus DCD treatment rather than the CIT regime led to significantly greater quantities than those in the control plants.

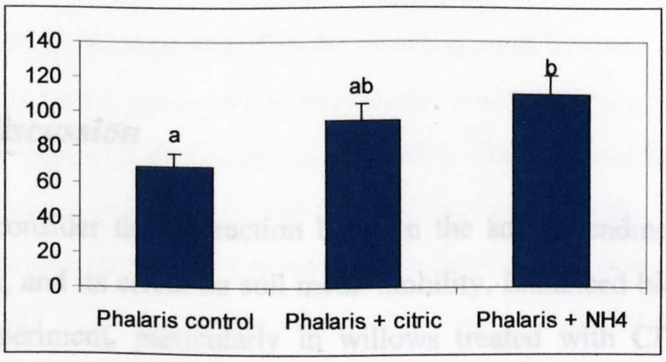

Figure 6.3.2.4 *Pb quantities (μg) in* Phalaris *aerial tissue in 3 soil manipulation regimes. Letters apply as in Figure 6.3.2.1*

Cadmium uptake increased in the *Phalaris* with the yield increments caused by the $(NH_4)_2SO_4$ plus DCD regime; this was also observed in Swiss chard grown on a polluted soil treated with N fertilisers (de Villarroel *et al*., 1993). Only the *Phalaris* did not suffer a yield penalty in the $(NH_4)_2SO_4$ plus DCD regime; its biomass was significantly increased (Figure 6.2.4). While metal concentrations were increased in willows and barley by this treatment (Figure 6.3.1.1), the biomass was significantly reduced (Section 6.2). This indicates either that *Phalaris* can tolerate higher metal concentrations than willow and barley, or that its optimum growth was at a lower pH compared to barley and willow, in the soil used in this experiment. The effects of pH are of major significance. Zaurov *et al*. (1999) studied Cd concentrations of Indian mustard grown in a contaminated soil in different conditions of pH. While the above-ground Cd concentrations were higher at pH 5.5 than at pH 6.5 or 7.5, biomass was significantly lower; therefore uptake was up to 2.5 times higher at pH 6.5.

It is likely that the increased metal uptake observed in *Phalaris* treated with $(NH_4)_2SO_4$ plus DCD was due to utilisation of the ammonium N-source by the plant and subsequent acidification of the rhizosphere, leading to enhanced metal mobility. Furthermore, N fertilisation of a sludged soil may reduce the percentage of metals in the organic complex and increase the amount in free ionic form, as observed by de Villarroel *et al.* (1993). Schremmer *et al.* (1999) reported Zn and Cd contents of *Salix viminalis* 'Jorr' leaves were not significantly increased in an acid soil (pH 4.8) fertilised with $(NH_4)_2SO_4$ plus DCD. Heavy metal contents in leaves were increased, however, by soil acidification in a relatively neutral contaminated soil (pH 6.4); the Stoke-Bardolph soil has a similar pH (Section 2.2.1.1). Keller *et al.* (1999) amended 3 contaminated soils with NH_4Cl and found it to increase Zn uptake (via decreased pH) by *Salix aurita* over 90 days.

6.3.3 General discussion

It is important to consider the interaction between the soil amendment's effect on plant biomass production, and its effect on soil metal mobility. Enhanced biomass production is evident in this experiment, particularly in willows treated with CIT, and in *Phalaris* amended with $(NH_4)_2SO_4$ plus DCD. However, the amendment's effect of increasing metal mobility (and possibly decreasing pH), can offset this fertilisation effect and even lead to biomass reduction. This is likely to have happened in the $(NH_4)_2SO_4$ plus DCD-treated willow and barley. Cooper *et al.* (1999) reported that Zn, Cu and Pb concentrations of various herbaceous plants increased with the application of chelates such as NTA in a 6-week trial, but that these increases were frequently confounded by the reduction of dry weight of the plant. Toxicity in lettuce and ryegrass was observed at the higher application rates of the NTA or urea amendments by Kulli *et al.* (1999): metal uptake overcame the fertilisation effect of the compounds.

However, longer-term field trials have displayed metal uptake in plants treated with acidifying agents and chelates to increase without a concurrent reduction in biomass production. This was observed in *Salix viminalis* grown for 4 months in a contaminated soil amended with sulphur or NTA by Kayser *et al.* (2000).

Kulli *et al.* (1999) considered the importance of letting plants become well developed and established before they are exposed to metal mobilisation agents and the possible resultant metal stress; the relative immaturity of the barley and willow used in this experiment may explain the seemingly detrimental effect of the $(NH_4)_2SO_4$ plus DCD treatment. Cooper *et al.* (1999) criticised application of chelates as a single amendment of a solid compound and instead continuously applied chelates in irrigation waters. The former approach assumes the chelate will solubilise a large portion of metal that will be rapidly taken up by the plant, which should already have produced a considerable biomass. Again, the plants in this experiment may not have been substantial enough to cope with the amount of metal solubilised by the amendment.

The potentially different trends identified in results from short and long term trials discussed above and in Section 6.2 are further highlighted by Table 6.3.3. This compares average quantities of metals sequestered in the above-ground biomass of willows grown in the pot trial (calculated on a grams per hectare basis) with quantities in the same varieties grown in a two year field trial by Pulford *et al.* (2002). The trial was carried out at the site from where the soils were collected for the pot trial, Stoke-Bardolph. While the results for Germany are comparable (the control quantities are between 1.5 and 4 times greater than the field results, likely to be due to the higher root: soil ratio in the pots), the results for Q83 are not: quantities were one to two orders of magnitude greater in the pot trial. This highlights the need for caution in extrapolating results from pot trials to the field, and how it may take more than a few weeks for obvious differences in variety performance to be detected. However, Germany was superior to Q83 in terms of metal uptake in the pot and field trials; this observation is relevant to the series of hydroponic experiments documented in Chapters 7 to 9.

Table 6.3.3 Quantities of metals in above-ground biomass (g ha^{-1}) in the pot trial and the Stoke-Bardolph field trial, calculated from the measured stem and leaf biomass yield and metal concentrations

	Zinc	*Cadmium*	*Copper*	*Nickel*
Germany control	2061	105	83.3	135
Germany CIT	2599	118	104	127
Germany $(NH_4)_2SO_4$ + DCD	700	37.1	45.6	59.5
*Germany field**	*478*	*33.5*	*58.6*	*33.4*
Q83 control	1292	48.1	50.7	106
Q83 CIT	1595	64.1	64.9	57.1
Q83 $(NH_4)_2SO_4$ + DCD	853	23.2	57.1	64.4
*Q83 field**	*33.3*	*3.1*	*4.3*	*1.7*

*Field data from Pulford *et al.* (2002)

Finally, there is also the likelihood that some of the mobilised metals were leached. Keller *et al.* (1999) reported that plant uptake only accounted for about half of the increase in extractable Zn concentrations caused by manipulation of contaminated soils with NH_4Cl, but much of the mobilised Zn was interpreted as being readsorbed by the soil. The combined effect of soil manipulation and plant growth may have caused substantial redistribution of soil metals; examples of the latter are provided in Chapters 4 and 5.

6.4 Summary

The effects of two soil amendments on the biomass production and metal uptake of three plant species were assessed in a 4 week pot trial. The citric acid regime resulted in a trend of positive yield increments in willow and barley aerial tissues. This frequently corresponded with significant increases in the metal quantities achieved in the aerial biomass of these plants. The fertilisation and enhanced metal uptake observed in willow and barley is likely to have arisen as a consequence of microbial activity following citric acid degradation. In contrast, the $(NH_4)_2SO_4$ plus DCD treatment led to significant yield reductions in these plants; this may have been due to a combination of lowered pH and elevated tissue metal concentrations in this regime. The *Phalaris* replicates were not significantly affected by the citric acid regime, but the $(NH_4)_2SO_4$ plus DCD amendment resulted in increased yields and quantities of metals achieved in the aerial tissue. In a contaminated soil amended with $(NH_4)_2SO_4$ plus DCD, *Phalaris* is therefore likely to have a lower optimum pH for growth, and possibly a better tolerance to elevated metal concentrations, than willow and barley.

Chapter 7 The Effect of NFT Background Nutrient Solution Strength on Biomass Production and Metal Uptake

7.1 Introduction

In the study of the metal resistance of plants, the use of glasshouse hydroponic methods such as the nutrient film technique (NFT) has several benefits. Principally, NFT experiments are simple, cheap and much more rapid compared to field trials, which are subject to many climatic and soil conditions, possibly rendering the influence of the heavy metals on the plants difficult to discern. In the case of willows, field testing requires at least two or three years to determine the varieties' performance. Ideally, NFT results should be comparable to field trial results; an NFT test featuring several treatments should reflect how a plant will respond to heavy metals under various field conditions and in the long term.

An NFT test was developed which allowed a thorough comparison of the performance of many willow varieties when exposed to metals in the glasshouse and field (Chapter 9). Prior to this, initial NFT experiments were necessary to thoroughly examine the effects of pH, phosphorus nutrition, and contaminant concentration (Chapter 8), and the strength of the background nutrient solution (this chapter). It was important to ensure the results were not compounded by nutrient limitations. The performances of the test varieties were therefore assessed in a range of background nutrient strengths. The work presented in this chapter had the following aims:

- to develop a test that could distinguish willow varieties according to their metal toxicity response
- to select a suitable strength of background nutrient solution for the development of the rapid screening test (Chapter 9) from a tested range

In metal resistance studies, the lack of knowledge concerning the nutritional status of the plant growth medium was acknowledged by Riddell-Black (1993). The effect of nutrient availability on the allocation patterns of heavy metals was also questioned by Sander and Ericsson (1998) in their study of field-grown willows. Absorption of non-nutrients by plant roots may arise due to the similarities of their chemical properties (such as charge and hydrated radius) to those of specific nutrient ions. For example, Ni may be absorbed instead of Cu, while Cd may be taken up rather than Zn.

Furthermore, as toxic ions and the constituents of the background solution can interact, the strength of the background solution can have a considerable effect on the indices measured and conclusions drawn in NFT experiments (Turner, 1994). For example, relatively high concentrations of P can reduce metal toxicity, while calcium is the most abundant divalent ion in the soil-plant system and inhibits the uptake of several metals, particularly Zn (Punz and Sieghardt, 1993).

This preliminary experiment tested two varieties of contrasting ability to thrive in metal-contaminated soils, *Salix burjatica* (Germany) and *S. triandra x viminalis* (Q83). Field evidence from previous trials suggests Germany can thrive in metal-contaminated soils, while Q83 cannot (Riddell-Black *et al.*, 1997). In a one-year field trial on a metal contaminated, sludge-amended soil, Germany achieved both high biomass yields and wood and bark metal concentrations of heavy metals; Q83 ranked in the lower half of the 20 varieties tested. In this experiment, the clones were grown in a variety of background nutrient strengths that were intended to represent soils of different nutritional status. Sections 2.3.5.2 and 2.3.5.3 detail the experimental set-up and sampling regime for the work presented in this chapter. Heavy metal analysis was carried out as described in Section 2.5.

7.2 Effects on Biomass Production

The experiment provided biomass data for the two clones. The following parameters were measured: the weight of the leaf and root fractions, and the height of the trees (shoot length). A comparison of the biomass data across the range of treatments was possible for each of the three sampling points (weeks 2, 4 and 6). The following data depict the effect of the metal cocktail (in three different strengths of background nutrient solution) on the mass of the leaf and root fractions, and the tree height. Clear differences in the performance of the two clones are discernible in the data.

Chlorotic leaves and brown leaf spots were consistently observed in the trees exposed to metals in the modified Hoaglands solution (MHS). In the trees exposed to metals in 1/4 strength MHS (1/4 + M treatment), this was much more marked in the upper, younger leaves. In the metal-treated trees in the weaker background nutrient solutions, 1/8 strength (1/8 + M) or 1/16 strength (1/16 + M) MHS, upper leaves were evidently chlorotic, and many lower leaves attained a chlorotic appearance, or were shed, after 4 to 6 weeks. Banuelos and Ajwa (1999) reported that chlorosis of young plant leaves and injury to older leaves may be due to Cu and Ni-induced injury to roots, causing subsequent inhibition of Fe translocation from roots to shoots. Chlorosis of older leaves may indicate N deficiency, while leaf spots can arise due to K deficiency (Punshon and Dickinson, 1999). Therefore contaminant-induced disruption to plant nutrition was observed in the metal-exposed willows.

Examining the leaf data for Germany (Figure 7.2.1) and Q83 (Figure 7.2.2), in which the four different treatment regimes were statistically compared for each sampling point, it can be seen that in week 2, neither clone displays significant differences in leaf biomass between the nutrient regimes.

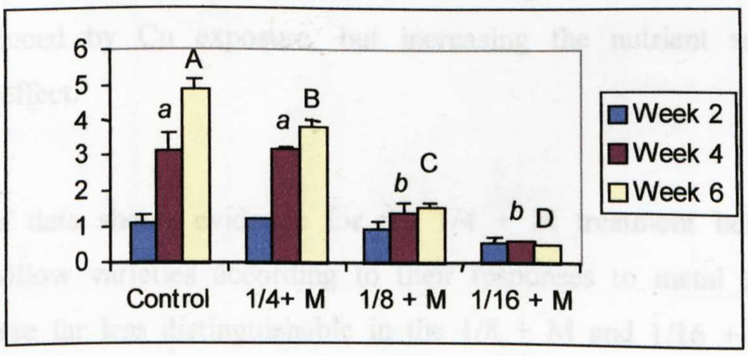

Figure 7.2.1 Dry weight (g) of leaf biomass of Germany trees exposed to four nutrient/metal regimes. Letters refer to statistical comparison across the four treatments during a sampling point: lower case letters refer to week 2, italics to week 4 and capitals to week 6. Means (n=3) without letters, and means with a letter in common, are not statistically different ($p > 0.05$) after a Fisher LSD test

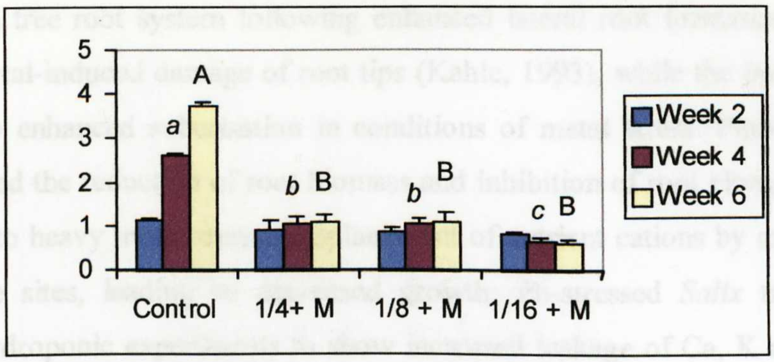

Figure 7.2.2 *Dry weight (g) of leaf biomass of Q83 trees exposed to four nutrient/metal regimes. Letters apply as in Figure 7.2.1*

A metal toxicity effect was revealed in both clones by week 4, however. In Germany, the control (1/4 strength MHS) leaf weight was significantly greater than the weight produced by trees exposed to 1/8 + M or 1/16 + M. Importantly, the Germany control leaf weight was not significantly greater than the leaf weight of the 1/4 + M regime. Whereas, in Q83, the control leaf biomass was significantly greater than in all other regimes.

By week 6, the control leaf biomass was significantly greater than in all other treatments in both clones, although the reduction in biomass relative to the control was much more marked in Q83. In Germany, each reduction in background nutrient strength resulted in a significant biomass reduction. This has previously been observed in hydroponic studies: Turner *et al.* (1991) showed that sycamore seedling shoot and root growth was significantly reduced by Cu exposure, but increasing the nutrient solution strength ameliorated this effect.

Overall, the leaf data shows evidence for the 1/4 + M treatment being suitable for distinguishing willow varieties according to their responses to metal exposure. Clone performances were far less distinguishable in the 1/8 + M and 1/16 + M regimes. No significant difference between the control and 1/4 + M treatments (two solutions with the same nutrient strength) was effected in Germany until week 6, whereas in Q83 a significant reduction had occurred by week 4.

The root samples of the metal-exposed trees were markedly browner, shorter and more branched than the numerous long white roots of the control regime. The increase in the branching of a tree root system following enhanced lateral root formation is a common response to metal-induced damage of root tips (Kahle, 1993), while the browning of roots may be due to enhanced suberisation in conditions of metal stress. Punz and Sieghardt (1993) described the reduction of root biomass and inhibition of root elongation as typical root reactions to heavy metal stress. Displacement of nutrient cations by metals can occur at root uptake sites, leading to decreased growth: Pb-stressed *Salix* trees have been observed in hydroponic experiments to show increased leakage of Ca, K and Mg (Kahle, 1993).

As in the leaf data, the root data for Germany and Q83 (Figures 7.2.3 and 7.2.4 respectively) provide further evidence for the suitability of the 1/4 + M treatment for the purposes of a metal toxicity response screening test; clone performances were clearly distinguishable. The results also further demonstrate Germany's superiority in terms of metal resistance. In Germany, the control and 1/4 + M root fractions were significantly heavier than those produced in the weaker background nutrient strengths by week 2, while no differences were observed in Q83. By week 4, the Q83 results show the control root fraction was significantly greater than in all other treatments, whereas in Germany, the root biomass of the metal-exposed trees in the top strength background nutrient solution (1/4 + M) was not significantly different to that of the control.

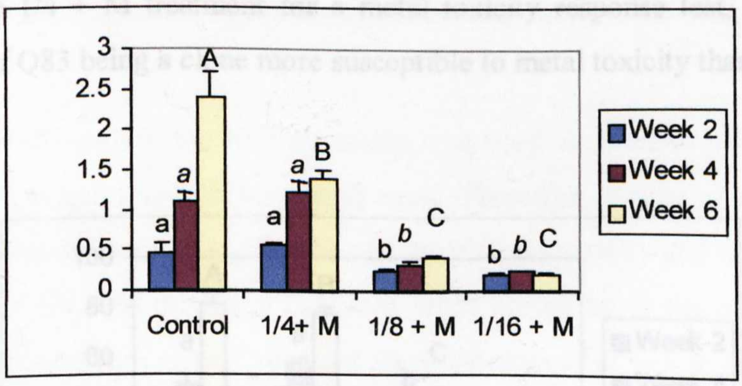

Figure 7.2.3 *Dry weight (g) of root biomass of Germany trees exposed to four nutrient/metal regimes. Letters apply as in Figure 7.2.1*

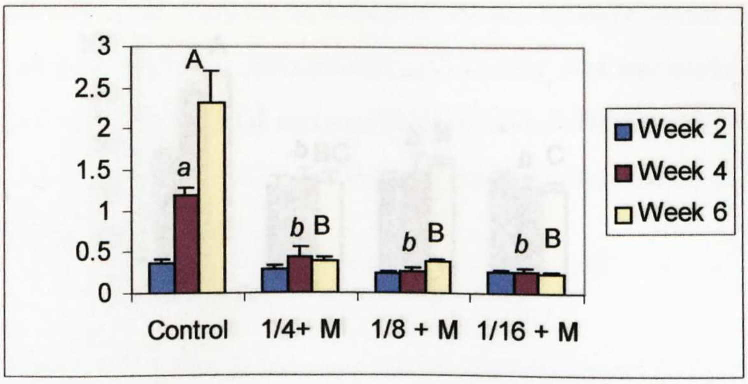

Figure 7.2.4 *Dry weight (g) of root biomass of Q83 trees exposed to four nutrient/metal regimes. Letters apply as in Figure 7.2.1*

As in the leaf fraction data, the control fraction was significantly heavier than in all of the metal-exposed regimes by week 6, but the reduction was much more marked in Q83. In Germany, the 1/4 + M fraction in week 6 was significantly heavier than in the 1/8 + M and 1/16 + M regimes. This demonstrates that Germany can still produce considerable quantities of root biomass despite being exposed to metals, at this nutrient strength. The same cannot be said of Q83: the metal-treated root biomass were similar in all treatments at the three sampling points of the experiment.

The height data for Germany (Figure 7.2.5) and Q83 (Figure 7.2.6) further point to the suitability of the 1/4 + M treatment for a metal toxicity response test, and support the observed trend of Q83 being a clone more susceptible to metal toxicity than Germany.

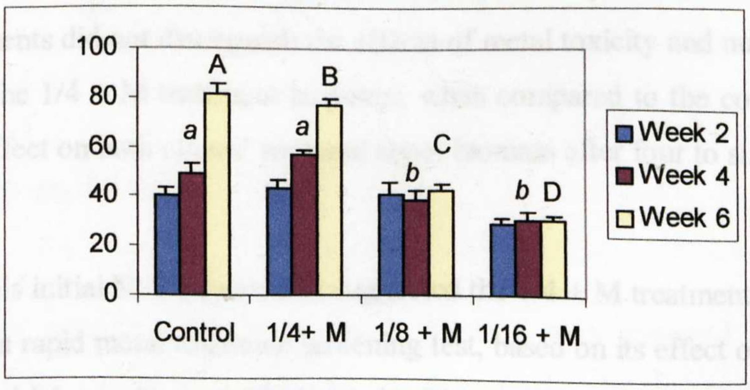

Figure 7.2.5 *Height (cm) of Germany trees exposed to four nutrient/metal regimes. Letters apply as in Figure 7.2.1.*

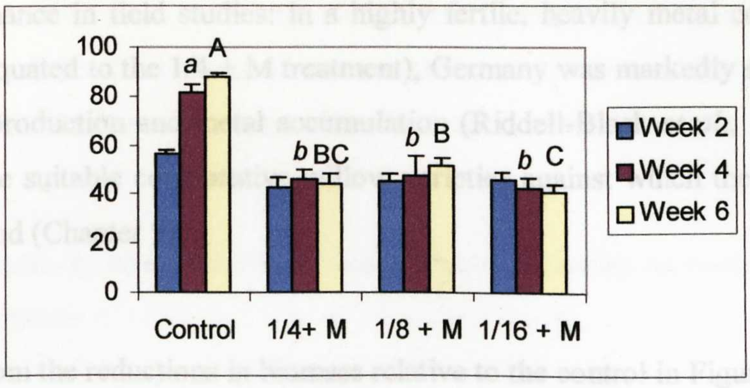

Figure 7.2.6 *Height (cm) of Q83 trees exposed to four nutrient/metal regimes. Letters apply as in Figure 7.2.1.*

Discernible differences in the performance of the clones became apparent in the 1/4 + M results within the timescale of the experiment. The data of both clones displayed no significant treatment effects after 2 weeks. After 4 weeks, the height of the control replicates was significantly greater than in all other treatments in Q83. In Germany, the control and 1/4 + M height data were not significantly different, but were both significantly greater than the 1/16 + M height. By week 6, the height of the Germany control replicates was still not significantly greater than the 1/4 + M regime trees. As displayed in the leaf data in the metal-treated trees, each reduction in background nutrient strength led to a significant reduction in height. In Q83 the pattern in week 6 was similar to that in week 4, although the 1/8 + M height was significantly greater than in the 1/16 + M regime.

As the number of channels in the NFT apparatus restricted the number of treatments, there were no control 1/8 and 1/16 MHS-exposed trees. Therefore, results of the 1/8 + M and 1/16 + M treatments did not distinguish the effects of metal toxicity and nutrient deficiency on the plants. The 1/4 + M treatment however, when compared to the control, revealed a metal toxicity effect on both clones' root and shoot biomass after four to six weeks.

The results of this initial NFT experiment suggested the 1/4 + M treatment was suitable for the purposes of a rapid metal tolerance screening test, based on its effect on the biomass of plant fractions which can be quantified simply. This treatment was modified and used in subsequent experiments (Chapters 8 and 9). Additionally, the biomass results clearly indicate that Germany is a less susceptible willow variety to metal toxicity than Q83. The relative performance of these varieties in this experiment broadly corresponded to their relative performance in field studies: in a highly fertile, heavily metal contaminated soil (which can be equated to the 1/4 + M treatment), Germany was markedly superior in terms of its biomass production and metal accumulation (Riddell-Black *et al.*, 1997). Germany and Q83 became suitable comparative willow varieties against which the performance of others were tested (Chapter 9).

It is apparent from the reductions in biomass relative to the control in Figures 7.2.1 to 7.2.6 that the height biomass parameter is the least sensitive to metal toxicity; leaf biomass production is more sensitive, and root biomass production more sensitive still. When viewing the data for the control and 1/4 + M regimes in week 6, expressed as a ratio of the metal-treated to control biomass (Figure 7.2.7), this is obvious. Portraying data this way proved to be highly effective in visualising the effect of a hydroponic treatment on a clone's biomass, and is used in conjunction with quantitative data in Chapter 8.

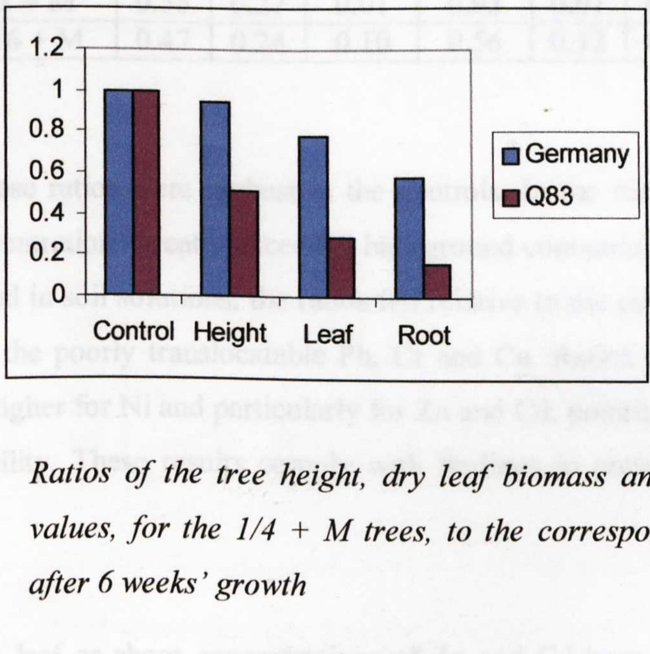

Figure 7.2.7 *Ratios of the tree height, dry leaf biomass and dry root biomass values, for the 1/4 + M trees, to the corresponding control value after 6 weeks' growth*

7.3 Effects on Metal Uptake

All leaf and root samples taken in weeks 2, 4 and 6 were digested for heavy metal determination. This provided a plethora of data useful in the comparison of the performance of the two clones in terms of the metal concentrations achieved, and the total amount of metal accumulated in the tissue when considered in conjunction with the weights of the fraction. Concentrations and quantities of metals in the roots and leaves of the clones are presented in detail later in this section. Firstly however, a good overall picture of metal translocation by the trees in the four hydroponic treatments is provided by an examination of the distribution of accumulated metals between the leaves and roots (Table 7.3.1). This illustrates the contrasting distribution of the six metals.

Table 7.3.1 Ratio of metal quantities accumulated in leaves to roots, after 6 weeks' growth

Germany	Zn	Cd	Cu	Ni	Pb	Cr
Control	1.97	1.63	1.29	1.10	0.07	0.35
1/4 + M	1.37	1.69	0.04	1.51	0.01	0.01
1/8 + M	0.65	0.32	0.02	0.78	0.02	0.03
1/16 + M	0.22	0.07	0.02	0.31	0.02	0.03

Q83	Zn	Cd	Cu	Ni	Pb	Cr
Control	1.84	2.70	1.00	0.37	0.32	0.54
1/4 + M	1.06	0.68	0.04	0.96	0.01	0.02
1/8 + M	0.55	0.27	0.01	0.93	0.01	0.01
1/16 + M	0.47	0.24	0.10	0.56	0.12	0.21

After six weeks, these ratios were highest in the controls. In the metal-treated plants, in which solution concentrations greatly exceeded background contaminant concentrations or levels typically found in soil solutions, the ratios fell relative to the control ratios. This fall was very sharp for the poorly translocatable Pb, Cr and Cu. Ratios across all treatments were considerably higher for Ni and particularly for Zn and Cd, pointing to these elements' greater translocatability. These results comply with findings in previous glasshouse and field experiments.

In soil experiments, leaf or shoot concentrations of Zn and Cd have been reported to be higher than in roots, for example in *Thlaspi caerulescens* (Knight *et al.*, 1997) and in poplar clones (Drew *et al.*, 1987). Nissen and Lepp (1997) reported a trend of exclusion of Cu and concentration of Zn in shoot and leaf tissue of eight *Salix* species, due to the elements' contrasting mobilities. Zinc and Cd are much more weakly chelated than Cu, and so greater proportions of absorbed Zn and Cd are translocated to shoots (Chaney *et al.*, 1997). Copper and Cr levels in roots of sycamore seedlings grown in sludge-amended soil were an order of magnitude higher than in leaves (Lepp and Eardley, 1978). Chromium, Pb and Cu were found to have chiefly accumulated in the roots of *Salix caprea* grown on two heavy metal contaminated sites (McGregor *et al.*, 1995). In a series of hydroponic experiments testing the metal resistance of various *Salix* varieties, Punshon and Dickinson (1999) found copper accumulation was lower in foliage than in the roots. All of these trends are apparent in the results for metal-exposed trees in Table 7.3.1.

In the Germany results, the ratios of each metal in the leaves to roots generally decreased with decreasing nutrient strength in the metal-treated plants. This is the case in the Q83 results for Zn, Cd and Ni. However, a huge increase in the 1/16 + M ratios of Cu, Pb and Cr is evident in this clone, relative to the 1/8 + M ratios. Large increases in the Q83 leaf concentrations (Figure 7.3.1) and quantities (Figure 7.3.2) of Cu, Pb and Cr in the 1/16 + M regime trees between weeks 4 and 6 may indicate a breakdown of a root sequestration mechanism following continuous metal stress, and subsequent transport of the metals to the shoot. This has been demonstrated in birch trees for Zn (Kahle, 1993). Another possibility is that the trees translocated the metals as a strategy to avoid metal stress, as most of the leaves in this regime were shed. Therefore Q83 may be more likely to translocate metals in contaminated soils of low fertility comparable to the 1/16 + M treatment.

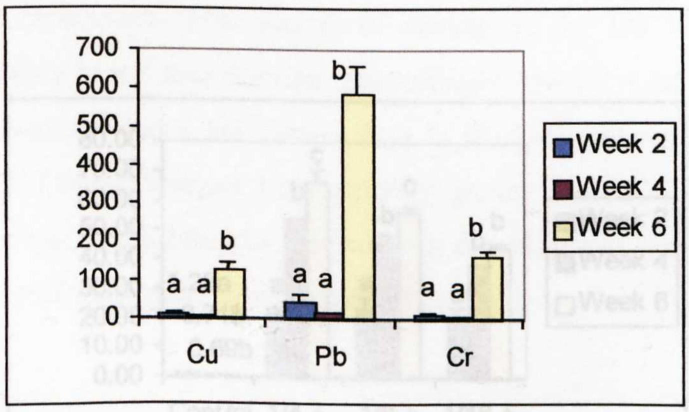

Figure 7.3.1 *Metal concentrations (mg kg^{-1}) in Q83 leaves in 1/16 + M treated trees. For each metal, means (n=3) without a letter in common are significantly different ($p < 0.05$) after a Fisher LSD test*

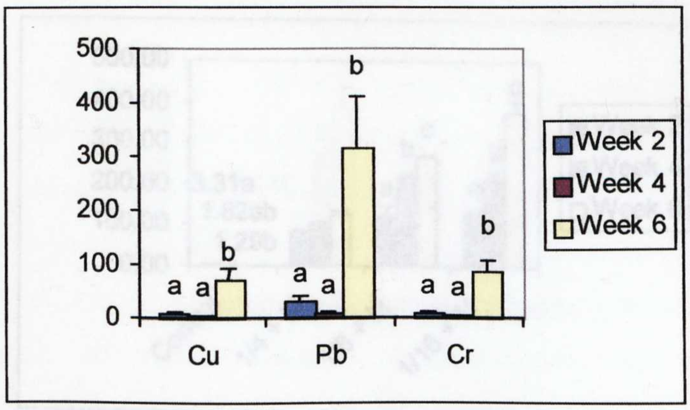

Figure 7.3.2 *Metal quantities (μg) in Q83 leaves in the 1/16 + M treated trees. Letters apply as in Figure 7.3.1*

Metal concentrations generally decreased in control tree tissues with time due to growth dilution. Concentrations generally rose with time in the roots and leaves of the metal-treated trees. Particularly clear trends were evident in the results for the translocatable elements Zn, Cd and Ni; the following graphs focus on the latter. Figures 7.3.3 and 7.3.4 show Ni concentrations in the leaves and roots of Germany replicates taken in weeks 2, 4 and 6, across the range of treatments.

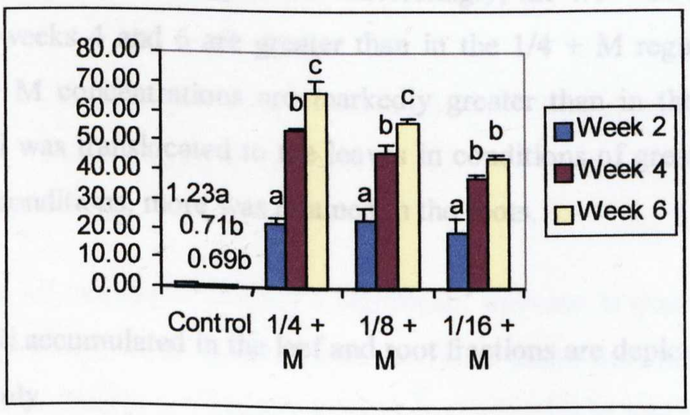

Figure 7.3.3 *Ni concentrations (mg kg^{-1}) in Germany leaves in four nutrient/metal regimes. For each regime, means (n=3) without a letter in common are significantly different ($p < 0.05$) after a Fisher LSD test*

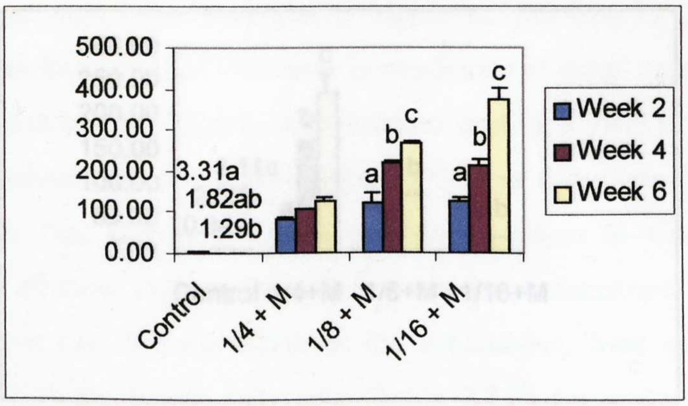

Figure 7.3.4 *Ni concentrations (mg kg^{-1}) in Germany roots in four nutrient/metal regimes. Letters apply as in Figure 7.3.3*

Concentrations in the control regime significantly decreased between weeks 2 and 6 in both fractions, pointing to growth dilution of the absorbed metals. In the metal-treated trees, concentrations rose with time in each treatment. Therefore, in conditions of constant supply of elevated concentrations of a metal, growth dilution of absorbed Ni did not occur as the concentrations continued to rise throughout the experiment. Hence metal absorption exceeded biomass production. This was more marked in the 1/8 + M and 1/16 + M treatments, particularly in the root fraction. Interestingly, the 1/8 + M and 1/16 + M root concentrations in weeks 4 and 6 are greater than in the 1/4 + M regime, but in the leaf fraction the 1/4 + M concentrations are markedly greater than in the other treatments. Therefore more Ni was translocated to the leaves in conditions of greater nutrition, while in poorer nutrient conditions, more was retained in the roots.

The quantities of Ni accumulated in the leaf and root fractions are depicted in Figures 7.3.5 and 7.3.6 respectively.

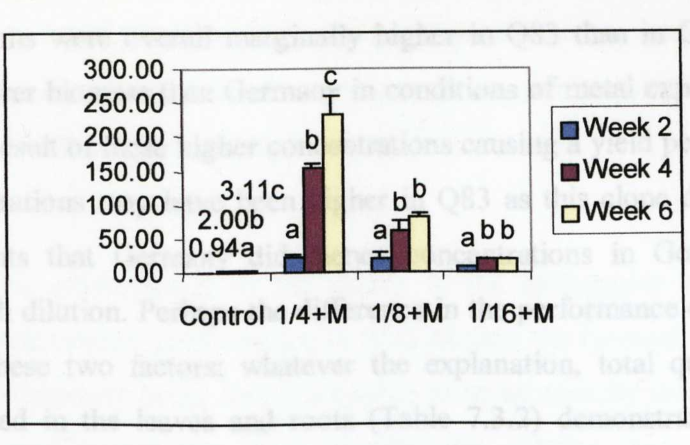

Figure 7.3.5 *Ni quantities (μg) in Germany leaves in four nutrient/metal regimes. Letters apply as in Figure 7.3.3*

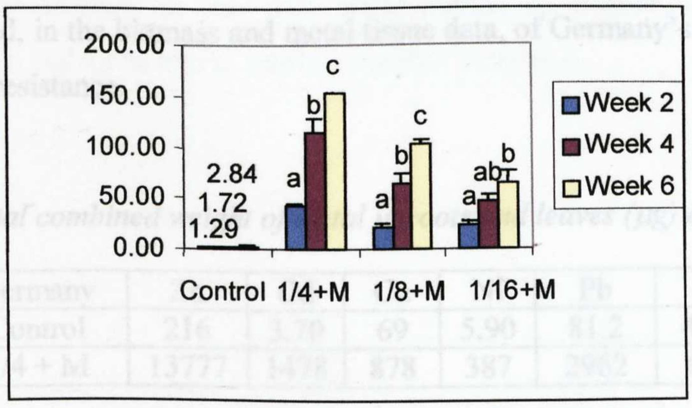

Figure 7.3.6 *Ni quantities (μg) in Germany roots in four nutrient/metal regimes. Letters apply as in Figure 7.3.3*

In the leaf fraction, all treatments display a significant increase in quantities from week 2 to 4. By week 6 however, metal quantities in the more dilute treatments appeared to be approaching a plateau. This is particularly evident in the 1/16 + M leaf fraction. In the root fraction however, Ni quantities significantly rose to week 6 in all metal treatments, suggesting that sequestration in the root occurred in all regimes, but translocation to leaves was much more marked in the 1/4 + M treatment. Quantities in the 1/4 + M trees were markedly greater than in the other metal treatments after 4 weeks. The concentrations achieved in the Germany biomass after 4 weeks in this regime did not significantly reduce the biomass yield relative to the control (see Section 7.2), and hence did not reduce the quantities of accumulated Ni. In the other metal regimes, the concentrations achieved in the biomass resulted in yield reductions relative to the 1/4 +M regime after 4 weeks; the weaker background nutrient strengths did not ameliorate the effects of metal toxicity.

Metal concentrations were overall marginally higher in Q83 than in Germany. Possibly, Q83 achieved poorer biomass than Germany in conditions of metal exposure (as shown in Section 7.2) as a result of these higher concentrations causing a yield penalty. On the other hand, the concentrations may have been higher in Q83 as this clone did not achieve the biomass increments that Germany did, hence concentrations in Germany were more affected by growth dilution. Perhaps the difference in the performance of the clones was a combination of these two factors; whatever the explanation, total quantities of all the metals accumulated in the leaves and roots (Table 7.3.2) demonstrate that, while Q83 accumulated greater quantities of metals from the relatively minor concentrations in the control solution, the greater biomass increments achieved by Germany when exposed to metals in the same strength of background nutrient solution (1/4 + M regime) led to considerably greater quantities of metals being accumulated. Therefore many examples have been provided, in the biomass and metal tissue data, of Germany's superiority to Q83 in terms of metal resistance.

Table 7.3.2 Total combined weight of metal in roots and leaves (µg) after 6 weeks

Germany	Zn	Cd	Cu	Ni	Pb	Cr
Control	216	3.70	69	5.90	81.2	4.91
1/4 + M	13777	1478	878	387	2962	688

Q83	Zn	Cd	Cu	Ni	Pb	Cr
Control	409	17.4	80.5	9.30	210	45.3
1/4 + M	4230	578	379	202	1825	270

7.4 Summary

With the goal of developing a hydroponic test to distinguish willow varieties by their response to metal exposure, tests were carried out supplying metals to willows in three different strengths of background nutrient solution, to assess the influence nutrition may have on the metal resistance of willows. The effects of the treatments on the biomass production and heavy metal uptake of two willow clones, Germany and Q83, were assessed. The results showed that, for the purposes of differentiating clone performance, the metals were best applied in the top strength nutrient solution (1/4 + M). Significant differences in biomass production, relative to the control, were effected by this regime after 4 to 6 weeks. In the lower nutrient strengths (1/8 + M and 1/16 + M), the results did not distinguish the effects of metal toxicity and nutrient deficiency on the plants; the yield reduction caused by metal uptake was not ameliorated by the nutrient supply. Root sequestration of metals occurred in all nutrient regimes, but translocation to leaves was much more marked in the highest background nutrient strength.

Overall, the relative performance of the clones in the hydroponic experiment broadly corresponded to their relative performance in field studies: the biomass parameters showed clear evidence for Germany being less susceptible to metal toxicity than Q83. In the 1/4 + M treatment, control parameters were significantly greater than the metal-exposed parameters after 4 weeks in Q83, while no significant differences were recorded until week 6 in Germany. The superiority of Germany to Q83 was also displayed in the larger quantities of metals accumulated. In the lowest nutrient strength (1/16 + M), Q83 demonstrated a breakdown in the root sequestration of metals: concentrations and quantities of Cu, Pb and Cr in leaves vastly increased in week 6.

Chapter 8 NFT Studies of the Effects of pH, P Nutrition and Metal Ratios on Willow Metal Resistance

8.1 Introduction

Initial attempts to screen the metal resistance of several willow species were marred by problems in differentiating the response of the varieties when the trees were grown in a hydroponic system in which a central reservoir provided nutrients and metals to all replicates. This contrasted with a system in which each channel of trees had its own reservoir of nutrients/metals, when the performance of the two reference clones was clearly distinguishable (Chapter 7).

Factors identified as possible contributors to this discrepancy were the more rapid alteration of pH and depletion of P in the central reservoir system. It was hoped that if the effects of these two factors on the tolerance test were understood, it would be possible to adjust the NFT test to improve the biomass of the metal-treated trees, and the differentiation of the varieties' performance. Hence, experiments succeeding the ones described in Chapter 7 were carried out in the Glasgow multi-tank NFT system and addressed the aspects of P nutrition and pH. Sections 2.3.7 and 2.3.8 describe the experimental set-up.

Burton *et al.* (1986) questioned the relevance of studies assessing the effects of single heavy metals on trees to a situation where more than one metal contaminates the substrate, each of which can influence plant growth and the pattern of uptake of other metals in a different way. This occurs often due to the natural associations between contaminating metals in a substrate, such as Cu and Ni. The interactive effect of contaminants was identified as an area requiring research in metal resistance studies by Riddell-Black (1993).

Consequently, two experiments were conducted in the NFT system to investigate the relative effects of the metals in the cocktail. Ratios of Cu to Ni were varied in the first, and Zn to Cd in the second (see Section 2.3.6 for experimental set-up). Copper and Ni are geochemically very similar. Zn and Cd are too, and are similarly bioavailable and mobile within a plant (Kiekens, 1995).

Therefore, the aims of the work described in this chapter were to further develop the NFT screening test by:

- assessing the factors of pH and P nutrition
- determining the relative effects of Cu and Ni, and Zn and Cd, on the metal toxicity response of the willow test varieties.

8.2 Experiments Examining the Factors of pH and P Nutrition

In both the pH and P nutrition experiments, the performance of the control trees after 6 weeks had by far outstripped the performance of the metal-treated trees, much more so than in the experiments described in Chapter 7. Therefore the ratios of the metal treated tree fraction to the corresponding control fraction (referred to as treatment: control ratios, or TCRs) were much smaller than in, for example, Figure 7.2.7. This may be due to natural light: these experiments were carried out in Glasgow in winter and early spring under artificial lights (see Section 2.2.3); all other NFT experiments in Chapters 7 to 9 were carried out in southern England during summer, in natural light. Despite the photoperiods being similar under the different light regimes, natural light led to considerably better growth in metal-treated willows. However, each of the biomass parameters for the week 3 samples highlighted clear trends in both experiments.

The first experiment in the Glasgow NFT apparatus examined P nutrition. Heavy metal stress to roots may be diminished by the phosphate concentration in the rhizosphere (Kahle, 1993). In the background nutrient strength experiment (Chapter 7), metals were supplied in a background nutrient solution devoid of P which was rotated with a nutrient solution containing P (minus metals), to avoid metal-phosphate precipitation problems. This pre-empted a problem mainly caused by Pb-phosphate precipitation, and resulted in P being provided to the trees for only 2 days per week. Another, undesirable, solution to the problem would be to exclude P, an essential macronutrient, from the nutrient solution (an approach taken by Cooper *et al.* in 1999 when they fed Indian mustard growing in Pb-contaminated soil).

Rather than do this, the effect of the duration of P exposure on the response of metal-exposed trees relative to control trees was tested. Lead was excluded from the metal cocktail and the metal resistance of the test clones *Salix burjatica* (Germany) and *S. triandra x viminalis* (Q83) was compared in regimes providing P for either 7 or 2 days per week. The experimental design is detailed in Section 2.3.7.

Typical graphs displaying the effect of duration of P exposure on the biomass of the metal-treated trees are given in Figures 8.2.1 and 8.2.2. They respectively show that 7 days exposure to P rather than 2 leads to increased root biomass and height of the metal-treated trees, without affecting the previously observed trend of Germany's relative superiority to Q83.

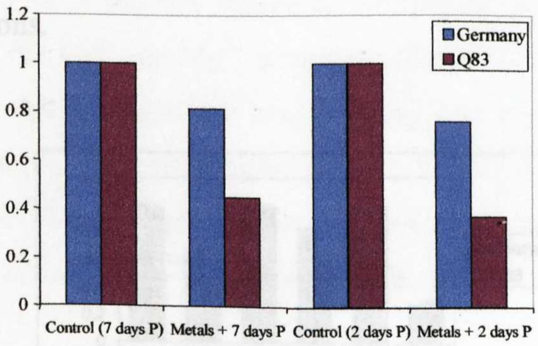

Figure 8.2.1 *Ratios of metal-treated tree root biomass to control tree root biomass, after 3 weeks' growth*

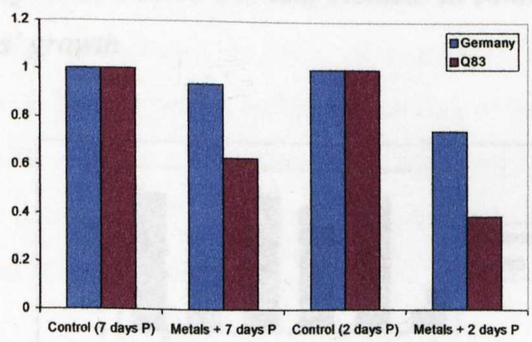

Figure 8.2.2 *Ratios of metal-treated tree to control tree height increase, after 3 weeks' growth*

Therefore, to improve both the practicality of P supply and the biomass of the metal-treated trees, Pb was excluded from the metal cocktail and P supply was increased from 2 to 7 days in all subsequent NFT experiments; quantities of P were also doubled in the MHS.

To assess the influence of pH on the metal toxicity responses of willows, the second experiment in the Glasgow NFT system compared the performance of the two reference clones in three pH regimes: 3.5, 5.5 (the pH used in Chapter 7) and 7.5. Refer to Section 2.3.8 for the experimental set-up. Figures 8.2.3 and 8.2.4 are typical graphs: the ratio of metal-treated leaf and stem biomass to the corresponding control value was largest for pH 5.5, with Germany performing better than Q83. At pH 3.5, the trend is repeated, although the biomass is reduced further in the metal-treated trees. This may have been due to increased metal uptake and hence toxicity in conditions of lower pH. Improper management of the pH of a contaminated soil may lead to metal-induced phytotoxicity in conditions of acidification (Chaney *et al.*, 1997). Increased metal uptake at lower pH has been demonstrated in hydroponic experiments too. For example, Blaylock *et al.* (1997) detailed the stimulation of Pb translocation from the roots of Indian mustard, to the shoots in more acidic conditions.

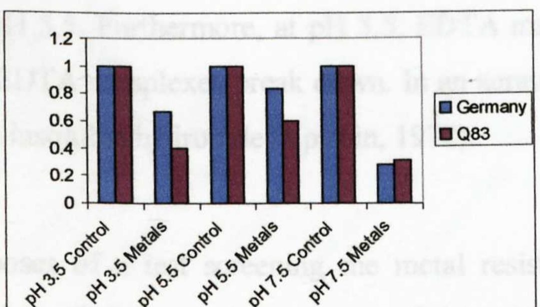

Figure 8.2.3 Ratios of metal-treated tree leaf biomass to control tree leaf biomass, after 3 weeks' growth

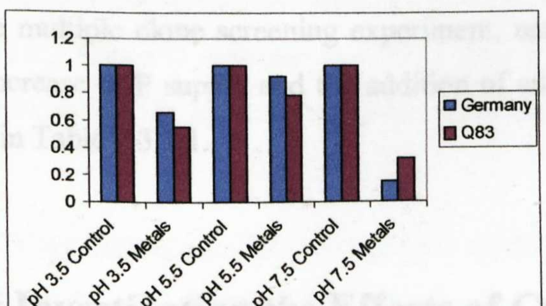

Figure 8.2.4 Ratios of metal-treated tree stem biomass to control tree stem biomass, after 3 weeks' growth

At pH 7.5, there was a very large reduction in biomass, and Q83 produced more than Germany. Given that changes in rhizosphere pH can be induced by unequal net uptake of cation and anion equivalents by plant roots (Kahle, 1993), this reduction in biomass was probably due to the pH rising rapidly in the poorly buffered Hoaglands solution. Nitrate is the nitrogen source, resulting in root secretion of anions to maintain an electrochemical balance as the macronutrient is absorbed. This led to an unusually alkaline pH, conditions in which Q83 performed marginally better than Germany. While root-induced maintenance of a high rhizosphere pH through secretion of OH^- ions, may play a role in counteracting heavy metal stress in some soils (Kahle, 1993), in hydroponic systems, increased pH leads to changes in nutrient availability which can result in yield reductions in the experimental plants.

In an NFT system, the pH can affect the solubility and speciation of macronutrients. In the concentration range of most nutrient solutions, no precipitates are formed involving K^+, NH_4^+, SO_4^{2-} or NO_3^-, but Ca^{2+} and Mg^{2+} form precipitates with HPO_4^{2-} at a higher pH (de Rijck and Schrevens, 1998), which can have a strong bearing on a clone's performance. This does not occur at pH 5.5. Furthermore, at pH 5.5, EDTA maintains Fe^{3+} in solution, while at pH 7.5 - 8, Fe-EDTA complexes break down. In an aerated solution with a pH of 8, Fe^{3+} precipitates as an insoluble hydroxide (Epstein, 1972).

Therefore, for the purposes of a test screening the metal resistance of several willow varieties, it is obvious that pH 5.5 was the optimum of those tested, and biomass of the metal-treated trees would be improved if it is maintained around this level. This was achieved by introducing ammonium as well as nitrate as the N sources; hydroponic solutions containing both have superior buffering, leading to less rapid changes in pH. The main adjustments to the multiple clone screening experiment, namely the removal of Pb from the solution, the increase of P supply and the addition of ammonium to the nutrient supply, are summarised in Table 2.3.9.1.

8.3 Experiment Investigating the Effects of Cu and Ni

8.3.1 Biomass data

Plant fraction biomass yield generally followed the trend: leaf > stem > root in both varieties across the range of treatments. The biomass of all the fractions showed that, after 6 weeks, equimolar concentrations of the metals (10 μM Cu: 10 Ni) allowed the greatest growth relative to the control. Increasing the ratio of one of the metals to the other, to five, reduced growth. Growth was reduced further by increasing the ratio of one metal to another, to ten.

Overall, Germany achieved greater leaf biomass and leaf TCRs than Q83. Figure 8.3.1.1 displays the relative amounts of leaf biomass produced by Germany and Q83 after 6 weeks, in the various regimes. This illustrates the reduction in biomass caused by increased exposure to metals, and the superiority of Germany in terms of biomass production, except at an excessively elevated exposure to Ni (100 μM). The difference in clone performance is particularly marked in the equimolar exposure to Cu and Ni. This supports previous results as it is the level of exposure used in the metal cocktail (Chapter 7).

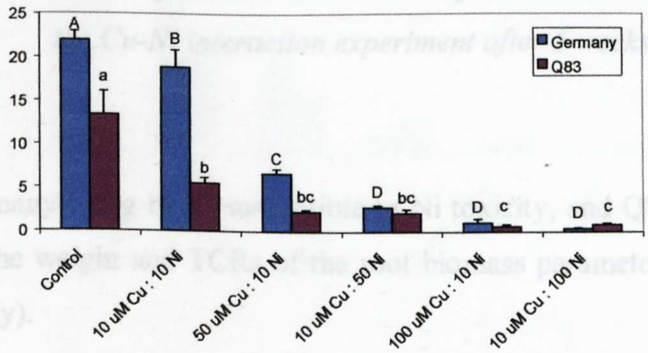

Figure 8.3.1.1 *Mean (n=3) leaf fraction biomass (g) across the range of metal treatments in the Cu-Ni interaction experiment after 6 weeks' growth. For each willow variety, means without a letter in common are significantly different ($p < 0.05$) after a Fisher LSD test. Letters in capitals refer to Germany; lower case letters refer to Q83*

When considering the TCRs, differences in the response of the two clones to elevated exposure to a metal become apparent. Figure 8.3.1.2 displays the reduction of leaf biomass relative to the control in the 50 μM Cu: 10 Ni treatment to be greater in Q83, but at 10 μM Cu: 50 Ni, the reduction is greater in Germany. Although the reduction in leaf TCRs is greater in both clones in the 10 μM Cu: 50 Ni regime than in the 50 μM Cu: 10 Ni treatment, it is far more marked in Germany. Therefore an elevated ratio of Cu appears to result in a greater reduction in Q83 biomass, and an elevated ratio of Ni results in a greater reduction of Germany biomass. Further evidence for this is provided in the TCRs for the other biomass parameters.

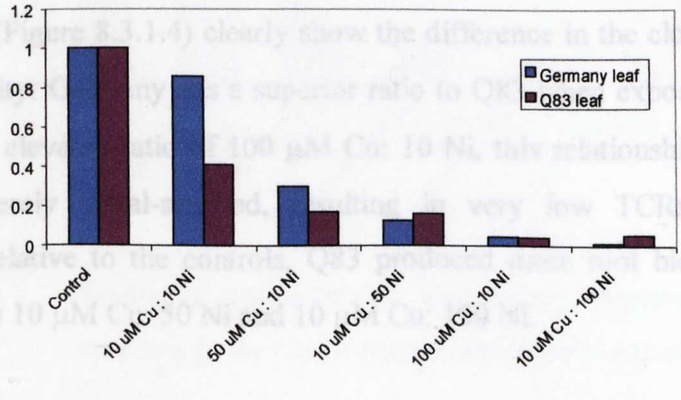

Figure 8.3.1.2 *Ratios of metal-treated tree leaf biomass to control leaf biomass in the Cu-Ni interaction experiment after 6 weeks' growth*

The trend of Germany being more susceptible to Ni toxicity, and Q83 to Cu toxicity, was also apparent in the weight and TCRs of the root biomass parameter (Figures 8.3.1.3 and 8.3.1.4 respectively).

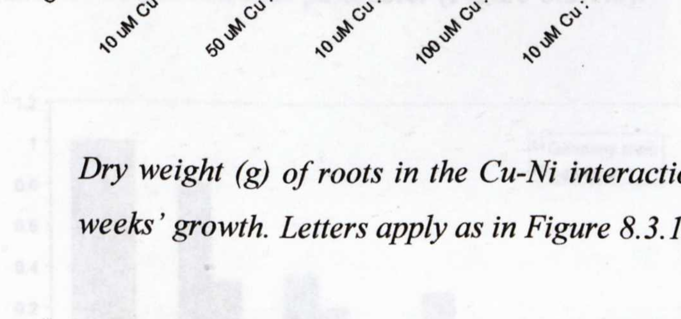

Figure 8.3.1.3 *Dry weight (g) of roots in the Cu-Ni interaction experiment after 6 weeks' growth. Letters apply as in Figure 8.3.1.1*

In all metal treatments, the root biomass of Q83 was reduced relative to the control. This was not the case in the equimolar treatment in Germany. This clone displayed a significant decrease in root biomass following exposure to 50 μM Cu: 10 Ni, which was reduced significantly further when the treatment was 10 μM Cu: 50 Ni, providing further evidence for its greater susceptibility to Ni toxicity. Both clones showed significant reductions in biomass relative to the equimolar treatment when exposed to 100 μM of one of the metals.

The root TCRs (Figure 8.3.1.4) clearly show the difference in the clones' susceptibility to Cu and Ni toxicity: Germany has a superior ratio to Q83 when exposed to 50 μM Cu: 10 Ni. At a further elevated ratio of 100 μM Cu: 10 Ni, this relationship broke down as the trees were severely metal-stressed, resulting in very low TCRs and similar clone performance. Relative to the controls, Q83 produced more root biomass than Germany when exposed to 10 μM Cu: 50 Ni and 10 μM Cu: 100 Ni.

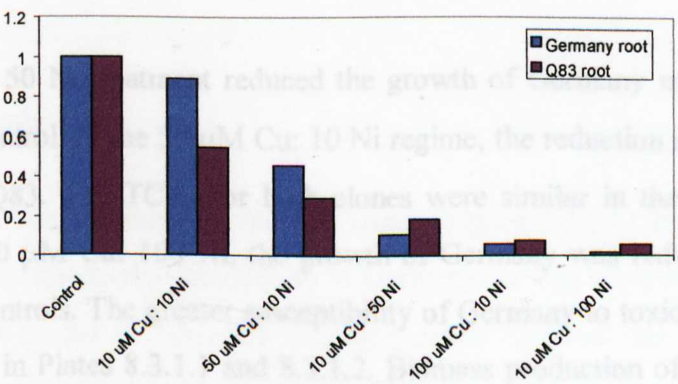

Figure 8.3.1.4 *Ratios of metal-treated tree root biomass to control root biomass in the Cu-Ni interaction experiment after 6 weeks' growth*

The different responses of the clones to exposures to elevated concentrations of Cu or Ni was a consistent observation that can also be seen in the TCRs for the stem parameter (Figure 8.3.1.5) and the height increase parameter (Figure 8.3.1.6).

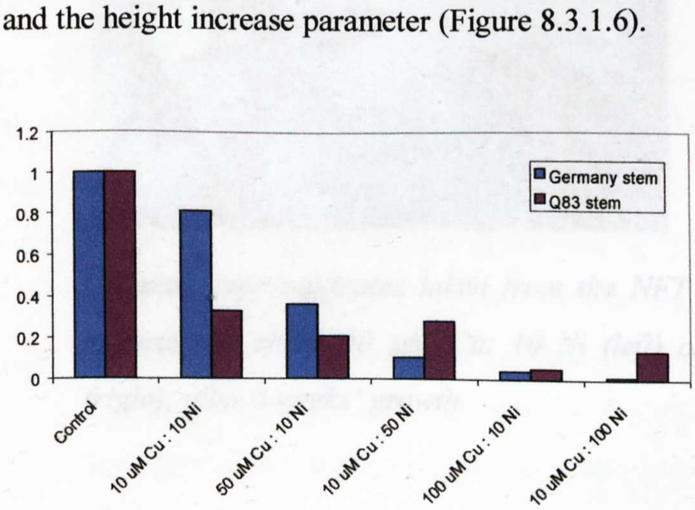

Figure 8.3.1.5 *Ratios of metal-treated tree stem biomass to control stem biomass in the Cu-Ni interaction experiment after 6 weeks' growth*

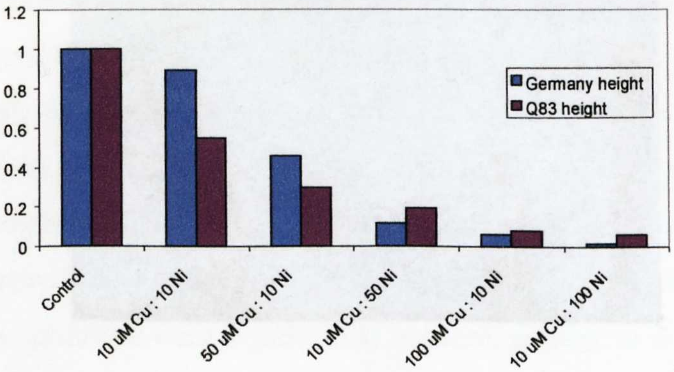

Figure 8.3.1.6 *Ratios of metal-treated tree height increase to control height increase in the Cu-Ni interaction experiment after 6 weeks' growth*

The 10 μM Cu: 50 Ni treatment reduced the growth of Germany more than it did Q83, relative to the control. In the 50 μM Cu: 10 Ni regime, the reduction relative to the control was greater in Q83. The TCRs for both clones were similar in the 100 μM Cu: 10 Ni regime; in the 10 μM Cu: 100 Ni, the growth of Germany was reduced more than Q83, relative to the controls. The greater susceptibility of Germany to toxicity of Ni than that of Cu, is displayed in Plates 8.3.1.1 and 8.3.1.2. Biomass production of the trees exposed to elevated Ni is clearly smaller than in regimes exposing the trees to elevated Cu concentrations.

Plate 8.3.1.1 *Germany tree replicates taken from the NFT apparatus following exposure to either 50 μM Cu: 10 Ni (left) or 10 μM Cu: 50 Ni (right), after 6 weeks' growth*

Plate 8.3.1.2 *Germany tree replicates taken from the NFT apparatus after exposure to either 100 μM Cu: 10 Ni (left) or 10 μM Cu: 100 Ni (right), after 6 weeks' growth*

8.3.2 Metal data

As expected, Cu concentrations in the leaf tissue (Figure 8.3.2.1) were highest in the 100 μM Cu: 10 Ni treatment, significantly so in Germany. It was observed that increasing the ratio of Ni to Cu to 5:1 or 10:1, did not significantly decrease the concentrations of Cu relative to those achieved in the equimolar treatment.

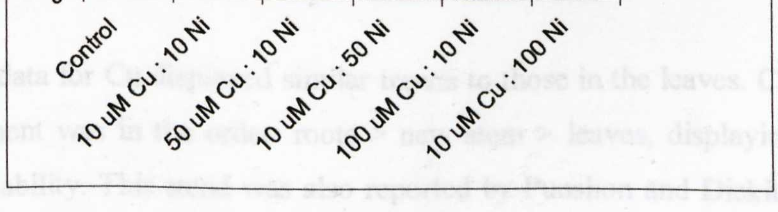

Figure 8.3.2.1 *Mean (n=3) concentrations of Cu (mg kg^{-1}) in leaves across the range of metal treatments in the Cu-Ni interaction experiment after 6 weeks' growth. For each willow variety, means without a letter in common are significantly different ($p < 0.05$) after a Fisher LSD test. Letters in capitals refer to Germany; lower case letters refer to Q83*

The greater quantities of leaf biomass achieved by Germany (Figure 8.3.1.1) resulted in greater quantities of Cu being taken into the leaves across the range of treatments (Figure 8.3.2.2). Although maximum concentrations were achieved in the 100 μM Cu: 10 Ni treatment, the yield penalties this regime caused in both clones are illustrated by the relatively low quantities of Cu taken into the leaves. The highest quantities were therefore achieved in the more resistant Germany clone in the 50 μM Cu: 10 Ni treatment. In the less resistant Q83, Cu quantities were significantly reduced, relative to the control, in all metal treatments due to the yield penalty. In the 10 μM Cu: 50 Ni regime, quantities of Cu achieved were lower than in the equimolar regime, but not significantly. Therefore a Ni:Cu ratio of 5:1 did not significantly reduce Cu accumulation in leaves. A Ni:Cu ratio of 10:1 did in both clones, however. Therefore it required a relatively high excess of Ni to significantly suppress Cu accumulation in leaves.

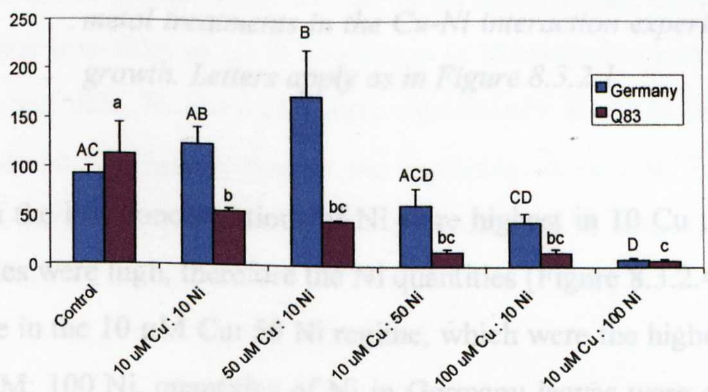

Figure 8.3.2.2 *Mean quantities of Cu (μg) in leaves across the range of metal treatments in the Cu-Ni interaction experiment after 6 weeks' growth. Letters apply as in Figure 8.3.2.1*

Stem and root data for Cu displayed similar trends to those in the leaves. Cu accumulation in this experiment was in the order: roots > new stem > leaves, displaying its relatively poor translocatability. This trend was also reported by Punshon and Dickinson (1997) for nine clones they exposed to the metal in solution culture.

Interesting differences between leaf, stem and root data were apparent in the results for Ni. Figure 8.3.2.3 displays the expected increases in leaf Ni concentrations in both clones with increasing exposure: the highest concentrations (significant in Q83) were achieved in the 10 Cu μM: 100 Ni regime. Ni concentrations in hydroponically-grown sycamore tissue were shown to be dependent on the relative concentrations of Cu and Ni in the nutrient solutions by Burton *et al.* (1986). This did not occur in the Ni leaf concentrations in this experiment: relative to the equimolar treatment, concentrations were not reduced significantly by the 50 μM Cu: 10 Ni or the 100 Cu μM: 10 Ni regime.

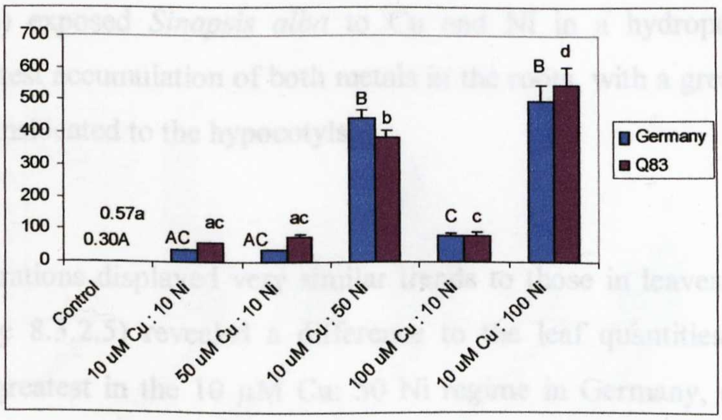

Figure 8.3.2.3 *Mean concentrations of Ni (mg kg^{-1}) in leaves across the range of metal treatments in the Cu-Ni interaction experiment after 6 weeks' growth. Letters apply as in Figure 8.3.2.1*

Again, although the leaf concentrations of Ni were highest in 10 Cu μM: 100 Ni regime, the yield penalties were high, therefore the Ni quantities (Figure 8.3.2.4) were significantly lower than those in the 10 μM Cu: 50 Ni regime, which were the highest. In all treatments except 10 Cu μM: 100 Ni, quantities of Ni in Germany leaves were greater than in Q83. There is evidence for elevated Cu concentrations suppressing uptake and translocation of Ni to leaves, supporting the observations of Burton *et al.* (1986): Ni quantities were significantly lower in the 50 μM Cu: 10 Ni and 100 Cu μM: 10 Ni treatments than in the equimolar regime.

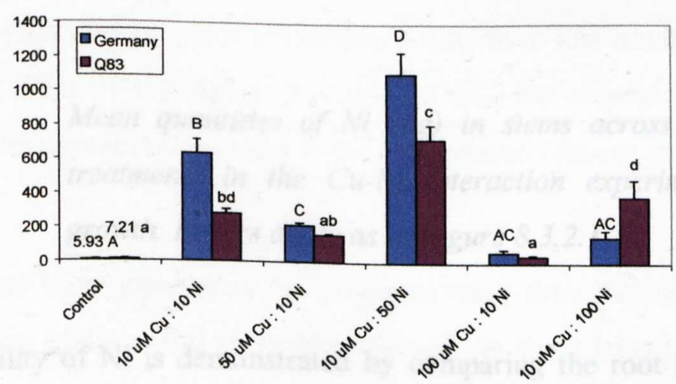

Figure 8.3.2.4 *Mean quantities of Ni (μg) in leaves across the range of metal treatments in the Cu-Ni interaction experiment after 6 weeks' growth. Letters apply as in Figure 8.3.2.1*

Ni quantities and concentrations were between five and ten times higher than Cu in the leaf tissues, across the range of treatments. This observation has since been confirmed in other studies: Kozlov *et al*. (2000) sampled birch in an industrially polluted area, and found that significant amounts of Ni, but not copper, were translocated from roots to leaves. Fargasova (1998) exposed *Sinapsis alba* to Cu and Ni in a hydroponic system, and reported the greatest accumulation of both metals in the roots, with a greater portion of Ni than Cu being translocated to the hypocotyls.

Stem Ni concentrations displayed very similar trends to those in leaves, but the Ni stem quantities (Figure 8.3.2.5) revealed a difference to the leaf quantities. While stem Ni quantities were greatest in the 10 μM Cu: 50 Ni regime in Germany, quantities in Q83 stems were greatest in the 10 μM Cu: 100 Ni regime, although not significantly. This demonstrates the lower susceptibility of Q83 to Ni toxicity than Germany, allowing it to accumulate greater quantities of Ni in conditions of excessively elevated Ni exposure. As observed in the leaf data, Ni quantities were significantly lower in the 50 μM Cu: 10 Ni and 100 Cu μM: 10 Ni regimes than in the equimolar treatment. This provides further evidence for Cu-induced suppression of Ni accumulation in tissues as demonstrated by Burton *et al.* (1986).

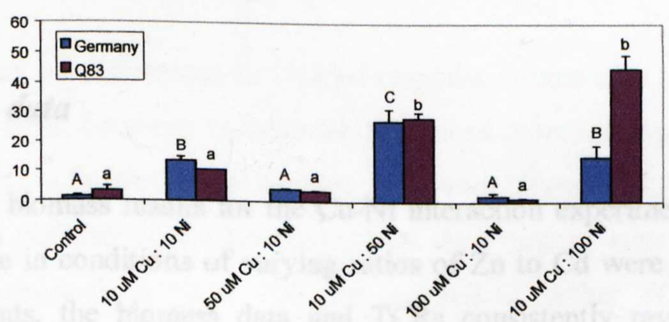

Figure 8.3.2.5 *Mean quantities of Ni (μg) in stems across the range of metal treatments in the Cu-Ni interaction experiment after 6 weeks' growth. Letters apply as in Figure 8.3.2.1*

The translocatability of Ni is demonstrated by comparing the root Ni quantities (Figure 8.3.2.6) with those in the stems and leaves. Root quantities of Ni were greatest in the 10 μM Cu: 10 Ni regime; no treatments of elevated Ni exposure caused significantly greater Ni sequestration than in this regime. But in the leaf fraction, quantities were greatest in the

10 μM Cu: 50 Ni treatment, and stem Ni quantities were greatest in either the 10 μM Cu: 50 Ni or 10 μM Cu: 100 Ni regimes. Therefore in conditions of elevated Ni exposure, Ni was translocated to the leaves and stems in significantly greater amounts than in the equimolar regime, rather than fixed in the roots in greater amounts.

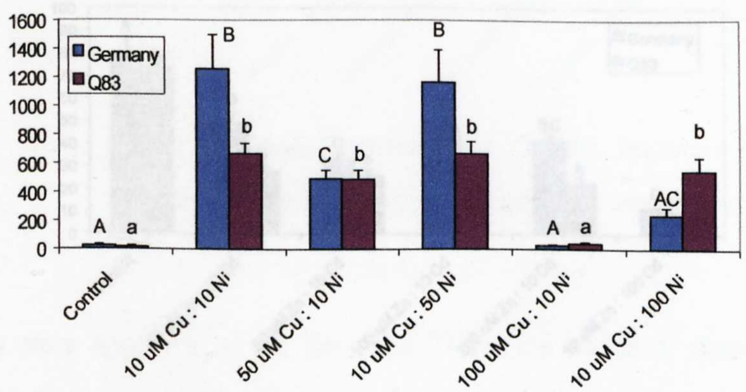

Figure 8.3.2.6 *Mean quantities of Ni (μg) in roots across the range of metal treatments in the Cu-Ni interaction experiment after 6 weeks' growth. Letters apply as in Figure 8.3.2.1*

8.4 Zn-Cd interaction experiment

8.4.1 Biomass data

In contrast to the biomass results for the Cu-Ni interaction experiment, no differences in clone performance in conditions of varying ratios of Zn to Cd were detected. Across the range of treatments, the biomass data and TCRs consistently revealed that Germany performed better than Q83 in terms of biomass production. The superiority of Germany to Q83 in the 200 μM Zn: 10 Cd regime is consistent with the observations made in Chapter 7, as these were the concentrations used in the background nutrient strength experiment.

The clearest trends were apparent in the height increase data: Figure 8.4.1.1 displays this parameter's results. All the metal treatments reduced the shoot length significantly, relative to the control. The 10 μM Zn: 100 Cd treatment consistently resulted in the poorest growth in both varieties; this caused significant reductions in both clones, relative to the equimolar regime. Cadmium toxicity is rare in plants, but usually results in leaf chlorosis, wilting and stunted growth (Alloway, 1995c); this suitably describes the trees of the 10 μM Zn: 100 Cd regime after 6 weeks. The other treatments resulted in little difference in shoot lengths across the spectrum of regimes; increased Zn exposure did not cause increased biomass reduction.

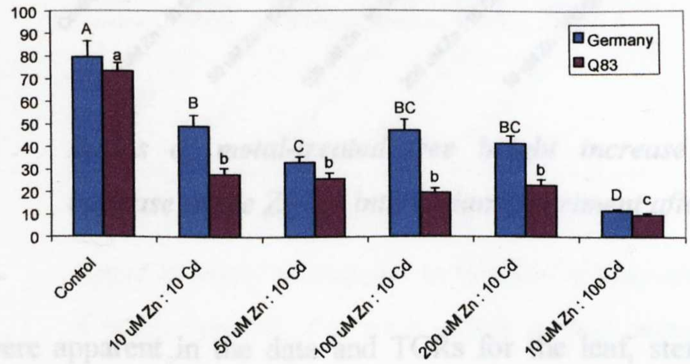

Figure 8.4.1.1 *Mean (n=3) height increases (cm) of Germany and Q83 replicates across the range of treatments in the Zn-Cd interaction experiment, after 6 weeks' growth. For each willow variety, means without a letter in common are significantly different ($p < 0.05$) after a Fisher LSD test. Letters in capitals refer to Germany; lower case letters refer to Q83*

The TCRs revealed no differences in varietal response comparable to those in the Cu-Ni interaction experiment; Germany consistently produced more biomass across the range of treatments. The TCRs for the height increase parameter (Figure 8.4.1.2) display Germany to be more metal resistant than Q83 throughout the range of exposures to Zn and Cd. Increased Cd exposure from 10 to 100 μM resulted in further TCR reductions of the biomass parameters. Conversely, it is reasonable to speculate that both varieties could tolerate higher Zn concentrations than those used in this experiment: although biomass was reduced in the metal-exposed trees, it did not appear to show increased reduction with increased Zn exposure.

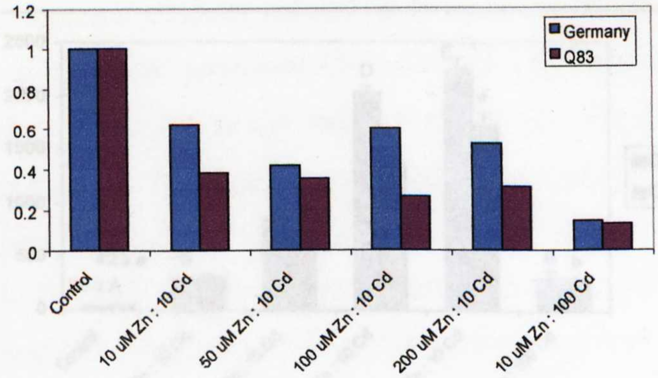

Figure 8.4.1.2 *Ratios of metal-treated tree height increase to control height increase in the Zn-Cd interaction experiment after 6 weeks' growth*

Similar trends were apparent in the data and TCRs for the leaf, stem and root biomass parameters. As no contrasting varietal performances were evident in the data (as were found in the Cu-Ni experiment) and no results contrasted to those displayed in Figures 8.4.1.1 and 8.4.1.2, the other data are not shown.

8.4.2 Metal data

Digestion of tissue samples provided data on the metal concentrations and quantities accumulated in the leaves, stems and roots of the test varieties. Data of the latter were highly variable and are not presented in this section. Overall, trends were much more discernible in the results for Zn than those for Cd. Figure 8.4.2.1 demonstrates the very clear trends evident in the leaf Zn concentration data. With each increase in Zn exposure, concentrations in the leaves significantly rose. Elevated Cd exposure did not significantly lower Zn concentrations to levels lower than in the equimolar regime.

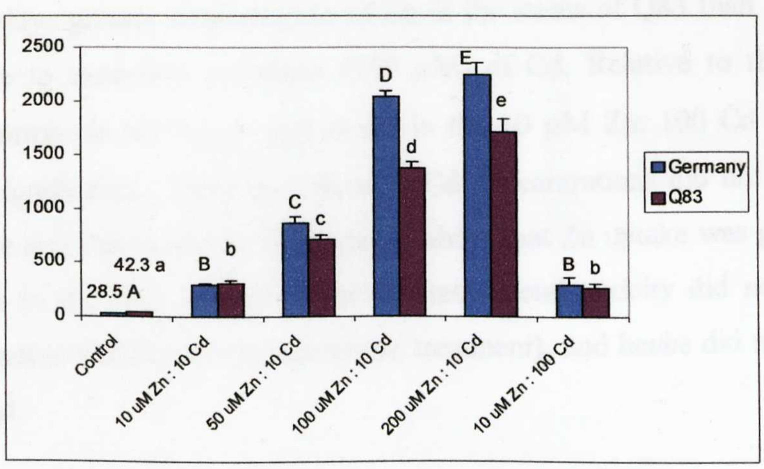

Figure 8.4.2.1 *Mean (n=3) concentrations of Zn (mg kg^{-1}) in leaves across the range of metal treatments in the Zn-Cd interaction experiment after 6 weeks' growth. For each willow variety, means without a letter in common are significantly different ($p < 0.05$) after a Fisher LSD test. Letters in capitals refer to Germany; lower case letters refer to Q83*

The metal quantity data displayed differences in the partitioning of Zn by the clones. Figure 8.4.2.2 shows greater quantities of Zn were translocated to the leaves in Germany across the range of treatments than in Q83. The trends mirror those in the concentration data: increases in Zn exposure lead to increases in Zn leaf quantities, pointing to no significant yield reduction with increased Zn exposure.

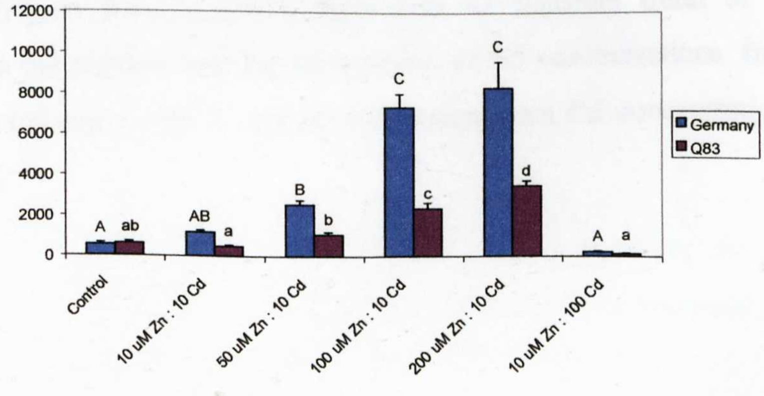

Figure 8.4.2.2 *Mean quantities of Zn (μg) in leaves across the range of metal treatments in the Zn-Cd interaction experiment after 6 weeks' growth. Letters apply as in Figure 8.4.2.1*

Figure 8.4.2.3 displays greater sequestration of Zn in the stems of Q83 than in Germany, except at exposure to excessive quantities (100 μM) of Cd. Relative to the equimolar treatment, Zn quantities in the leaves and stems in the 10 μM Zn: 100 Cd regime were reduced, but not significantly. Therefore elevated Cd concentrations did not significantly suppress Zn uptake and translocation. Both graphs show that Zn uptake was greatest when the concentrations in the NFT solution were greatest: metal toxicity did not reduce the biomass of the fraction (relative to the equimolar treatment), and hence did not reduce the quantities achieved.

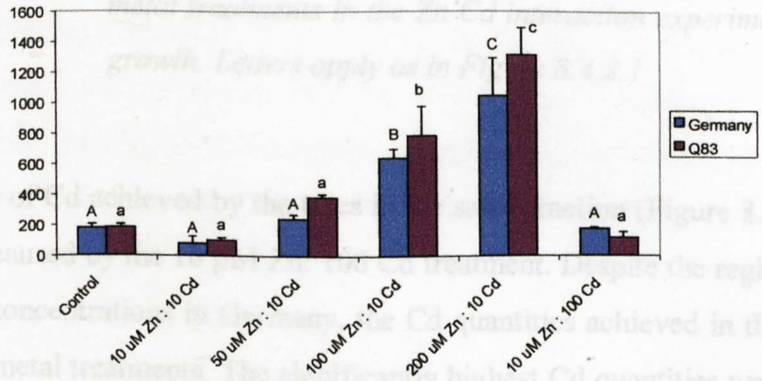

Figure 8.4.2.3 *Mean quantities of Zn (μg) in stems across the range of metal treatments in the Zn-Cd interaction experiment after 6 weeks' growth. Letters apply as in Figure 8.4.2.1*

The Cd results provided far less discernible trends than those for Zn. As the stem Cd concentrations (Figure 8.4.2.4) show, there was no apparent trend of increasing Zn concentrations in the regimes causing suppression of Cd concentrations. In Germany, the 10 μM Zn: 100 Cd regime led to statistically higher stem Cd concentrations than in the other treatments.

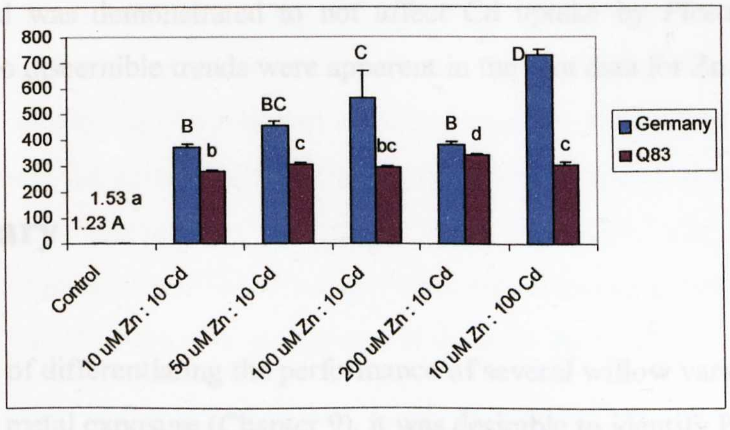

Figure 8.4.2.4 *Mean concentrations of Cd (mg kg^{-1}) in stems across the range of metal treatments in the Zn-Cd interaction experiment after 6 weeks' growth. Letters apply as in Figure 8.4.2.1*

The quantities of Cd achieved by the trees in the same fraction (Figure 8.4.2.5) display the yield penalty caused by the 10 μM Zn: 100 Cd treatment. Despite the regime leading to the highest stem concentrations in Germany, the Cd quantities achieved in this regime are the lowest of the metal treatments. The significantly highest Cd quantities were in the 200 μM Zn:10 Cd treatment for Q83, and the 100 μM Zn:10 Cd treatment for Germany.

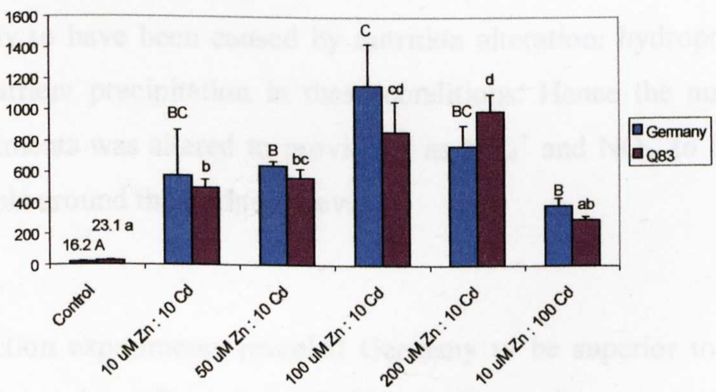

Figure 8.4.2.5 *Mean quantities of Cd (μg) in stems across the range of metal treatments in the Zn-Cd interaction experiment after 6 weeks' growth. Letters apply as in Figure 8.4.2.1*

Therefore, although Zn is reported to interfere with uptake of Cd (Banuelos and Ajwa, 1999), it has not suppressed Cd uptake and translocation to aerial plant biomass in this hydroponic experiment in which the external concentrations of both elements were relatively high. Indeed, in soils with high concentrations of Cd, Zn has either a synergistic or no effect, and was demonstrated to not affect Cd uptake by *Picea abies* seedlings (Kahle, 1993). No discernible trends were apparent in the root data for Zn or Cd.

8.5 Summary

For the purposes of differentiating the performance of several willow varieties according to their response to metal exposure (Chapter 9), it was desirable to identify P nutrition and pH regimes which allowed maximum biomass production of the metal-exposed test clones. Provision of 7 rather than 2 days P per week (as in Chapter 7) increased the biomass of the metal-treated trees, without altering the trend of Germany's superior performance to that of Q83, as identified in Chapter 7. Therefore, to improve the practicality of supplying P and increase tree replicate biomass, Pb was excluded from the metal cocktail and an increased quantity of P was supplied for 7 days per week in all subsequent experiments.

The biomass of the metal-treated trees was highest in a regime of pH 5.5 compared to biomass in pH 3.5 and 7.5 treatments. The former caused reduced biomass production, possibly through metal toxicity, while the latter caused more marked biomass reductions, a phenomenon likely to have been caused by nutrition alteration: hydroponic solutions are prone to macronutrient precipitation in these conditions. Hence the nutrient medium in subsequent experiments was altered to provide N as NH_4^+ and NO_3^- to improve buffering and maintain the pH around the optimum level.

The metal interaction experiments revealed Germany to be superior to Q83 in terms of biomass production and metal uptake, consistent with results presented in Chapter 7. A significant finding of the Cu-Ni experiment was the difference in metal toxicity response of the clones at elevated ratios of one metal to another. Relative to control values, the biomass of Germany showed a greater reduction in conditions of elevated exposure to Ni, while elevated Cu exposure caused more marked reductions in Q83. Copper-induced suppression of Ni accumulation was demonstrated at elevated Cu:Ni ratios (5:1). Nickel-induced suppression of Cu accumulation was observed, but only at excessively elevated ratios (10:1) of Ni to Cu. Copper quantities in the tree biomass ranked roots > new stem > leaves, demonstrating its low translocatability relative to Ni, quantities of which were approximately five times greater than those of Cu in the leaves.

The Zn-Cd interaction experiment showed elevated Cd concentrations reduced plant fraction biomass markedly more than a similar concentration of Zn. Zinc exposure did not cause increased reduction in biomass with increased concentrations in the medium, nor did the element suppress Cd uptake. No differences in varietal response were apparent in this experiment: Germany consistently performed better than Q83. The clones displayed differences in the partitioning of Zn, however: Germany achieved greater quantities in the leaves than Q83 did, while the reverse was true in the stem fraction.

Chapter 9 Screening of Willow Species: Comparison of NFT and Field Performance

9.1 Introduction

The development of a rapid metal resistance screening technique began with multiple-tank NFT experiments testing clone performance in different strengths of background nutrient solution amended with a metal cocktail, using two varieties of *Salix* (Germany and Q83). These results were encouraging in that the two clones' performances, relative to a control, were clearly distinguishable in the 1/4 strength MHS plus metals treatment (Chapter 7).

A further range of willow varieties were tested using this treatment, in a different NFT system which allowed a greater number of varieties to be screened simultaneously in a less laborious way: a central reservoir circulated nutrients/metals through all the channels (Section 2.3.4). This reservoir could be replenished relatively easily, in contrast to the multiple-tank system which has a separate nutrient/metal reservoir for each channel.

Initial runs in the central reservoir NFT showed that the tolerance test was not reproducible in this system. It was difficult to distinguish clone performance based on any of the biomass parameters measured. Possible reasons for this included a more rapid alteration of pH or depletion of nutrients (particularly P) in the central reservoir system. Therefore further tests were carried out to examine the influence of these factors (Chapter 8). The main findings from these experiments were that increased duration of P exposure did not affect previously observed trends, but it did increase the plant biomass, and the optimal pH for biomass production is 5.5.

The establishment of cuttings is the most sensitive period of a willow's development, and one in which the metal uptake is the least restricted (Punshon and Dickinson, 1997). Therefore tolerance test adjustments were needed to prevent large yield reductions in metal-exposed trees, and to improve clone performance differentiation in the central reservoir NFT system. The following adjustments were made (summarised in Table 2.3.9.1):

- Pb was removed from the metal cocktail to allow 7 day P exposure, and the P content of the MHS was doubled.
- The Hoaglands solution recipe was buffered with NH_4^+ to prevent rapid rising of pH from the optimum 5.5.
- Metals were added cumulatively: the metal concentrations of the cocktail were reduced by half in the first 3 weeks of the experiment, so the susceptible clones did not suffer a huge yield penalty at the beginning of the experiment.

The pH of the altered MHS was well buffered compared to in the tests described in Chapters 7 and 8, and rose much less rapidly. The moderations listed above succeeded in increasing the biomass of the metal-exposed trees and improving clone performance differentiation. Punshon and Dickinson (1997) also found that cumulative doses of Zn, Cd and Cu applied to nine willow clones in solution culture resulted in reduced phytotoxicity and increased resistance.

Finally, as toxic ions and the constituents of the background solution can interact, the strength of the background solution can have a considerable effect on the indices measured and conclusions drawn (Turner, 1994). Therefore the volume and frequency of solution rotation (10 l per channel of trees per week) were made consistent in all NFT experiments.

Two central reservoir NFT systems (see Plate 9.1) containing a suite of willow varieties in duplicate channels were utilised in this experiment, with samples being taken every 3 weeks for the measurement of four growth parameters: leaf, root and stem weight, and height increase.

Plate 9.1 Screening of willow varieties in two central reservoir NFT systems at WRc

The main aim of the work described in this chapter was:

- to establish whether data from a refined hydroponic experiment, which distinguishes the performance of willow varieties according to their biomass production in conditions of metal exposure, were statistically comparable to data from the same willow varieties grown in independent field trials.

If this was the case, performances of willows grown in a rapid NFT test could be extrapolated to the field. This could provide information on the relative performance of willow clones in heavy metal-contaminated soils, without the need for long-term field trials.

9.2 Principal Component Analysis

Both the NFT experiments and the field trials provided a plethora of data. Therefore, for the purposes of selecting datasets of willow clones grown in NFT tests for correlation with parameters of the same clones grown in field experiments, it was beneficial to obtain a visual overview of the data. Principal components analysis (PCA), despite being mathematical rather than statistical and not leading directly to tests of significance, can provide a rigid rotation of datasets to new axes (Webster, 2001). Therefore the PCA program Canoco for Windows was used to visually express the relative importance of the measurements, and any groupings of the clones according to these parameters.

9.2.1 Field data

There was thorough field data for 15 of the 18 clones listed in Section 2.3.9.2, from a trial at a heavy metal contaminated field site, Stoke-Bardolph (Riddell-Black *et al.*, 1997). The following clones were also grown at the Slough site as part of the BIORENEW project: Germany, Q83, Candida, Jorunn, and Bowles Hybrid. Data from Slough were provided by Riddell-Black (pers. comm.). This site was also sludge-amended and is comparable to Stoke-Bardolph in that it has elevated organic matter and heavy metal concentrations. Sections 2.1.1 and 2.2.1 describe the two sites.

Field data from the Stoke Bardolph trial comprised ten parameters: visual assessment scores, biomass yields and concentrations of Zn, Cd, Cu and Ni in the wood and bark. Figure 9.2.1.1 displays PCA data for 15 of the clones grown at Stoke-Bardolph, incorporating eight of the measured parameters. Biomass yield and Ni wood concentration have been excluded as data for these factors were not available for all 15 clones. Each parameter is represented by an arrow which extends either way through the graph's origin. The importance of the parameter in determining the clone's position on the graph is proportional to the length of its arrow. In this case, the most important factor is the bark concentration of Ni, while the least is the wood concentration of Cd. The grouping of the clones according to each parameter can be assessed by considering each point perpendicularly to a parameter's arrow.

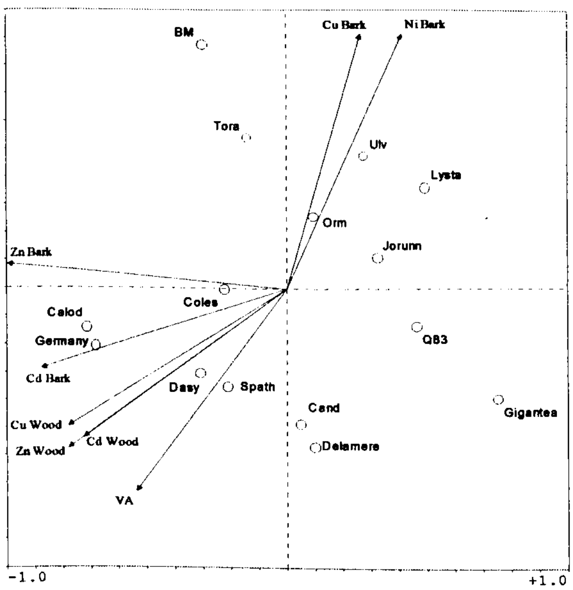

Figure 9.2.1.1 ***Principal component analysis grouping of 15 willow varieties grown at Stoke-Bardolph according to 8 measured parameters***

This graph clearly shows a grouping of the *S. viminalis* clones Tora, Ulv, Lysta 699,Orm and Jorunn, as well as the basket willow Black Maul, near the upper range of the Cu bark and Ni bark concentration arrows. If these arrows are extended through the origin, at the other end are grouped Germany, Dasyclados, Candida, Delamere, Calodendron and Spaethii, while the clones Q83, Coles and Gigantea are intermediate. The Cu and Ni bark concentration arrows run in an approximate opposite direction to the visual assessment (VA) arrow, suggesting the clones which performed well in the field had low Cu and Ni bark concentrations. This was the case: Riddell-Black *et al.* (1997) divided the trees into two distinct groups; those with bark concentrations greater than 30 mg kg^{-1} suffered reduced yield. The VA arrow also runs in the same general direction as Zn wood, Cd wood and Cu wood, indicating successful clones can accumulate these elements in the wood without undergoing a yield penalty. These observations are relevant to the NFT and field correlations, which are discussed in Section 9.3.

Figure 9.2.1.2 displays a PCA graph for all ten of the measured variables, for the 12 clones for which these data were available.

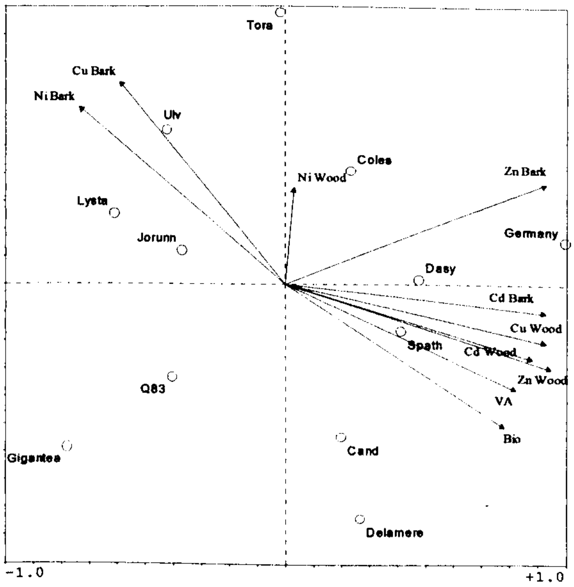

Figure 9.2.1.2 *Principal component analysis grouping of 12 willow varieties grown at Stoke-Bardolph according to 10 measured parameters*

The VA and biomass yield (Bio) arrows are very similar and run in the same direction, as expected. Zn wood and Cd wood concentration arrows are very close, highlighting these elements' broad similarity in terms of accumulation by willows. The case was the same for the Cu and Ni bark arrows, although the Zn/Cd and Cu/Ni arrows run in opposite directions, suggesting uptake of Zn and Cd had a different bearing on the groupings observed in Figure 9.2.1.2 than did uptake of Cu and Ni. Interestingly, the Ni wood arrow is the smallest, indicating this factor had about one third of the importance of the others in determining the position of the clones on the graph. Ni bark, however, was again one of the most important factors and its arrow runs in a different direction to the Ni wood arrow. Similarly, the Cu wood arrow runs in a different direction to the Cu bark arrow. In contrast to Cu and Ni, the Cd wood and bark arrows are similar in their length and spatial distribution. This confirms the observation of Riddell-Black *et al.* (1997) that wood concentrations were not necessarily good indicators of bark concentrations, and vice versa.

On this graph, the groupings observed in Figure 9.2.1.1 are still apparent: Tora, Ulv and Lysta 699 accumulated the greatest Cu and Ni bark concentrations and are consequently high on this scale but low on the VA and Bio scale; at the other end are grouped Germany, Dasyclados, Spaethii and Candida, while Coles, Q83 and Gigantea are intermediate.

Following the deductions made from Figures 9.2.1.1 and 9.2.1.2, it can be seen that several parameters were of major importance in determining the position of the clones on the graphs. Of these, the Cu and Ni bark concentration parameters were selected for correlations with NFT data. Two parameters were selected which the PCAs reveal had a different bearing to these on the positioning of the clones in the graphs: Zn and Cu wood concentrations. Finally, the VA and Bio parameters were selected for the correlations too, to assess the relationship between the biomass production of the willow varieties in the glasshouse and field trials.

9.2.2 NFT data

Section 2.3.9.2 lists the eighteen clones screened using the adjusted NFT test. The four glasshouse parameters were recorded as ratios of the metal-treated tree measurements to the corresponding control measurements. Q83 was the reference clone for each NFT run. The treatment: control ratios (TCRs) were also converted to ratios relative to this reference clone to eliminate any inter-experiment or seasonal variability in the greenhouse results, as only 7 clones were tested at one time.

Table 9.2.2 displays the TCRs, relative to Q83 values, for the four biomass parameters. It can be seen that some clones did not perform consistently in the four parameters, and had high ratios in one parameter and markedly lower ratios in others, such as Candida, Dasyclados, Delamere and Gigantea. However, the majority of the clones displayed similar ratios for each parameter, such as Spatheii, Germany, Ulv, Tora, Q683, Bowles' Hybrid, Jor, Black Maul and Lysta 699.

Table 9.2.2 Mean (n=6) Treatment Control Ratios of 15 willow varieties grown in the NFT system, relative to Q83, after 6 weeks' growth

VARIETY	HEIGHT INCREASE	STEM	ROOT	LEAF
Candida	2.02	1.33	0.83	0.83
Spatheii	1.81	1.34	1.19	1.17
Dasyclados	1.76	0.54	0.66	0.62
Germany	1.48	1.28	1.51	1.36
Jorunn	1.37	1.40	1.98	1.00
Calodendron	1.22	0.93	1.45	0.86
Ulv	1.06	1.02	1.49	1.12
Coles	1.03	0.95	1.44	1.10
Q83	1.00	1.00	1.00	1.00
Orm	0.94	0.81	1.22	0.85
Tora	0.88	0.51	0.58	0.56
Delamere	0.82	0.75	1.41	0.77
Black Maul	0.65	0.86	0.80	0.71
Lysta 699	0.63	0.67	0.78	0.67
Q683	0.56	0.49	0.45	0.62
Bowles' Hybrid	0.53	0.52	0.71	0.62
Gigantea	0.43	0.63	1.29	0.80
Jor	0.41	0.25	0.19	0.32

The biomass parameters were also converted to leaf: root, stem: root and shoot: root (incorporating combined leaf and stem totals) ratios, bringing the total number of measured parameters for the NFT willow varieties to seven. Figure 9.2.2 displays a PCA graph of the data for 15 willow varieties grown in the NFT. These were the same clones for which the extensive independent field data described in Section 9.2.1, were available.

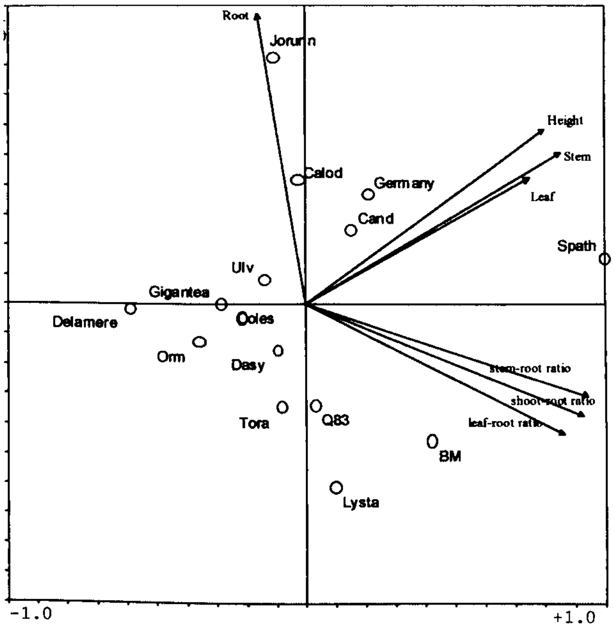

Figure 9.2.2 Principal component analysis grouping of 15 willow varieties grown in the NFT apparatus according to 7 measured parameters, after 6 weeks' growth

The spatial distributions of the above-ground biomass parameter (height, leaf and stem) arrows are similar. The longer height and stem arrows indicate a larger influence than the leaf parameter on the positioning of the clones on the graph. The root parameter arrow length also indicates this factor's importance, but the arrow occupies a different quadrant to the leaf, stem and height arrows. This suggests the root biomass parameter can give different indications regarding a clone's metal toxicity response than one based on the other biomass parameters. Such was the case: Table 9.2.2 reveals that Jorunn, for example, had a much higher root TCR than for the other measurements.

In this graph, groupings of the clones are not as discernible as for the field data in Figures 9.2.1.1 and 9.2.1.2, but some useful observations could be made. There is a grouping of Germany, Candida, Calodendron, Jorunn and Spaethii perpendicular to the stem and height parameter arrows. Extending these axes through the graph's origin, the remaining clones were difficult to classify into groups of intermediate and low performance, in comparison to the groupings according to the VA and Bio parameters in Figure 9.2.1.2, for example. As expected, the shoot: root, stem: root and leaf: root arrows were also distributed closely. However, due to the considerable differences of various clones' relative biomass production of leaves, stems and roots evident in Table 9.2.2, it proved difficult to discern any clear grouping of the clones compatible with trends identified in the previous graphs, according to these arrows.

Therefore the height increase and stem TCRs were considered the most dominant factors in determining the positioning of the clone on the PCA graph and were selected for correlations with the various data (listed in Section 9.2.1) of the same clones grown in a field trial.

9.3 Correlations

Several significant linear correlations between data for willow varieties grown in the glasshouse and in field trials were established. All NFT data in the following graphs were TCRs recorded after 6 weeks' growth. Figure 9.3.1 displays the height increase ratios of 15 clones grown in the NFT system plotted against the bark Ni concentrations of the same clones measured in the Stoke-Bardolph trial by Riddell-Black *et al.* (1997).

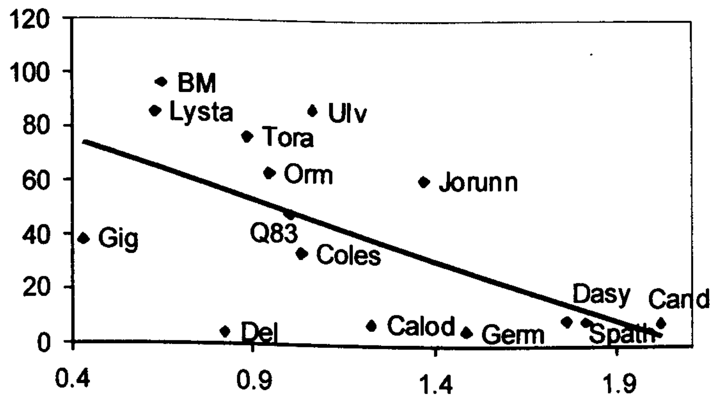

Figure 9.3.1 Negative linear correlation ($p < 0.05$, $r=0.60$) between NFT height increase ratios relative to Q83 (x axis) and Ni bark concentrations in mg kg^{-1} (y axis) for 15 clones grown at Stoke-Bardolph

This negative correlation shows that the clones which performed well in the NFT (Spaethii, Germany, Candida and Dasyclados) had accumulated lower Ni concentrations in the bark tissue in the field. A separate group of clones which had low height increase TCRs in the NFT (Black Maul and the *S. viminalis* clones Tora, Ulv, Lysta 699, and Orm) accumulated high bark Ni concentrations at Stoke-Bardolph.

A similar significant negative linear correlation was established for the same NFT parameter and the bark Cu concentrations of the field-grown clones (Figure 9.3.2), with the same groupings evident. Punshon and Dickinson (1997) reported an apparent inverse relationship between metal uptake and resistance in willows grown in solution culture; the same appears to be the case for bark-sequestered Cu and Ni in field-grown willows at Stoke-Bardolph. Greger and Landberg (1999), however, screened 150 clones of *Salix* for uptake and tolerance to Cd, Zn and Cu and reported no correlation between uptake and tolerance.

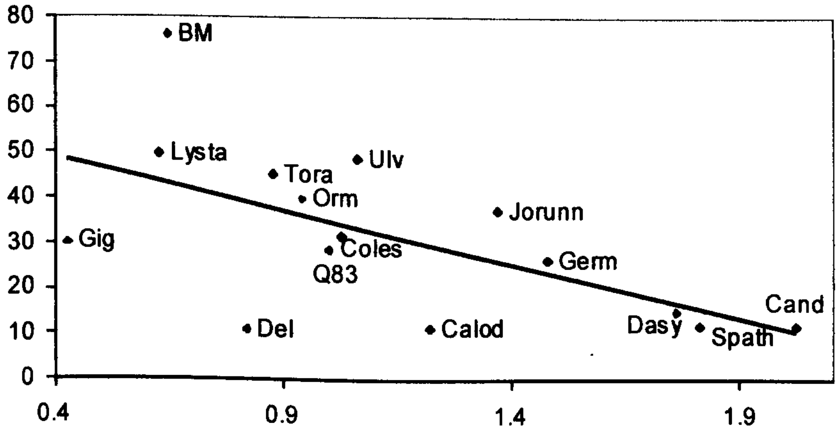

Figure 9.3.2 *Negative linear correlation ($p < 0.05$, $r=0.60$) between NFT height increase ratios relative to Q83 (x axis) and Cu bark concentrations in mg kg^{-1} (y axis) for 15 clones grown at Stoke-Bardolph*

In contrast to the bark Cu concentrations, a positive linear correlation was established between the NFT height increase parameter and the wood Cu concentrations (Figure 9.3.3). This, supplementing the data portrayed in Figure 9.2.1.2, provides additional strength to the observation of Riddell-Black *et al.* (1997) that wood concentrations of a metal do not necessarily correspond with trends shown in bark concentrations.

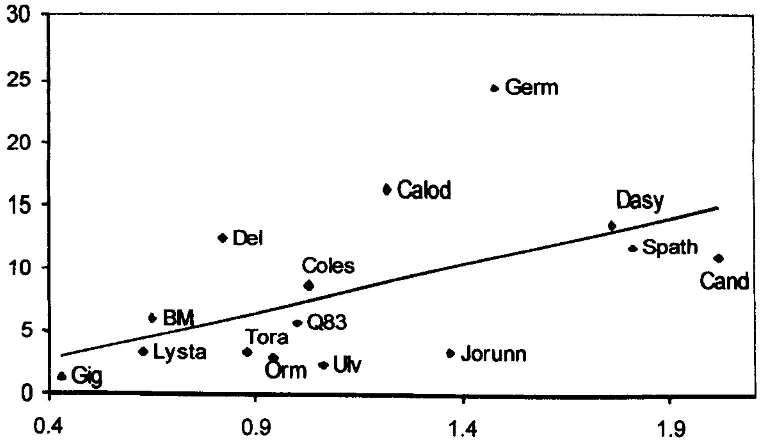

Figure 9.3.3 *Positive linear correlation ($p < 0.05$, $r=0.55$) between NFT height increase ratios relative to Q83 (x axis) and Cu wood concentrations in mg kg^{-1} (y axis) for 15 clones grown at Stoke-Bardolph*

For Zn, both wood and bark concentrations were positively correlated with this NFT parameter; Figure 9.3.4 displays a significant correlation with Zn wood concentrations. Again, a grouping of Germany, Candida, Spatheii and Dasyclados are found at the top end of the scale, which achieved relatively high metal concentrations and performed well in the NFT, while Gigantea, Lysta 699, Orm, Tora and Black Maul are at the lower end of the scale having achieved lower tissue concentrations and lower NFT height increase TCRs.

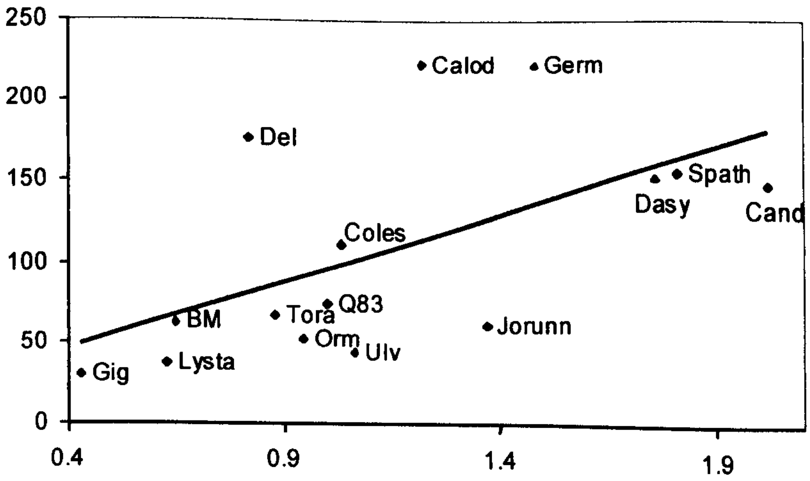

Figure 9.3.4 *Positive linear correlation ($p < 0.05$, $r=0.59$) between NFT height increase ratios relative to Q83 (x axis) and Zn wood concentrations in mg kg^{-1} (y axis) for 15 clones grown at Stoke-Bardolph*

Significant positive correlations were established between the NFT parameters and biomass measurements in the field, too. Figure 9.3.5 displays the highly significant relationship between the height increase ratios of 15 clones in the NFT and their corresponding visual assessment scores from Stoke-Bardolph.

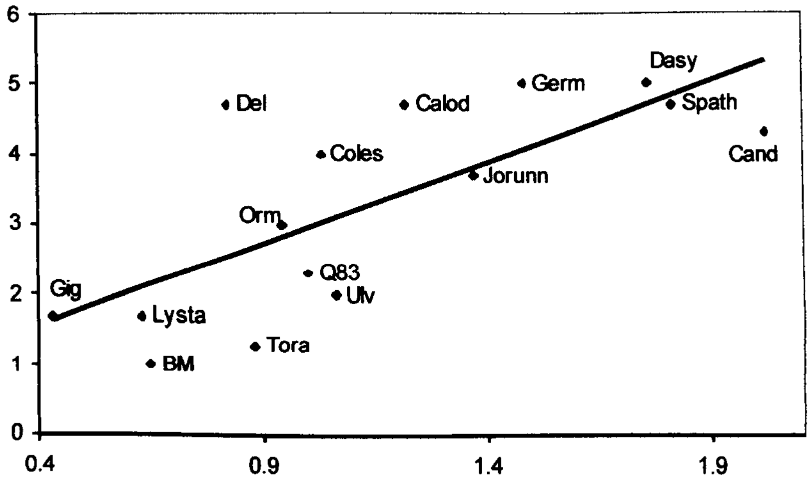

Figure 9.3.5 *Positive linear correlation ($p < 0.01$, $r=0.73$) between NFT height increase ratios relative to Q83 (x axis) and visual assessment score (y axis) for 15 clones grown at Stoke-Bardolph*

Again, the groupings of Germany, Dasyclados, Spaethii and Candida at the top end of the scale, and Gigantea, Black Maul and several of the *S. viminalis* clones at the lower end, are apparent. Figure 9.3.6 displays a similar association between the biomass yield of the field-grown clones and the height increase ratios of the NFT clones. There was no data available for Black Maul and Orm.

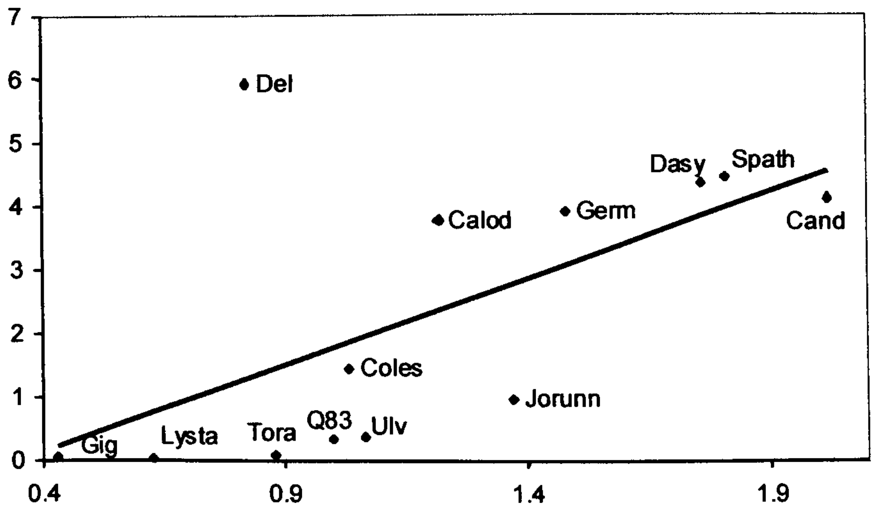

Figure 9.3.6 *Positive linear correlation ($p < 0.05$, r=0.60) between NFT height increase ratios relative to Q83 (x axis) and dry biomass yield in t ha^{-1} (y axis) for 13 clones grown at Stoke-Bardolph*

S. viminalis was the willow most adversely affected by the metal treatments in the hydroponic experiments carried out by Punshon and Dickinson (1999). This corresponds with the data provided here, as the *S. viminalis* clones achieved relatively low NFT height increase TCRs which indicates inferior biomass production in conditions of metal exposure to that of most of the other clones. Riddell-Black *et al.* (1997) reported Germany to be suitable for removing significant quantities of Cu, Zn, Ni and Cd from a sludge-amended, metal contaminated soil as it achieved high yields and wood metal concentrations. Germany was among the best clones in terms of the NFT height increase TCRs.

The hybrid *S. x calodendron* was less affected by a contaminated soil than *S. viminalis* in an experiment carried out by Punshon and Dickinson (1997). This was also the case in Figures 9.3.1 to 9.3.7: Calodendron's NFT performance is superior to those of all *S. viminalis* clones, except Jorunn. *S. caprea* and *S. cinerea* are hardy species that colonise contaminated soils (Punshon and Dickinson, 1999). Calodendron is an *S. viminalis* clone crossed with both. Perhaps this led to this hybrid displaying an overall better performance than the *S. viminalis* clones in the field and NFT.

Significant correlations were established between metal concentrations of the five clones grown at the Slough site, and the stem parameter of the same clones in the NFT system. The much higher r values in these correlations, compared to those based on Stoke-

Bardolph and NFT data, were due to the lower numbers of clones grown at Slough and hence used in the statistical comparisons. Figure 9.3.7 shows a typical significant correlation between the NFT stem parameter and leaf Cu concentrations measured in vegetation samples from the Slough site.

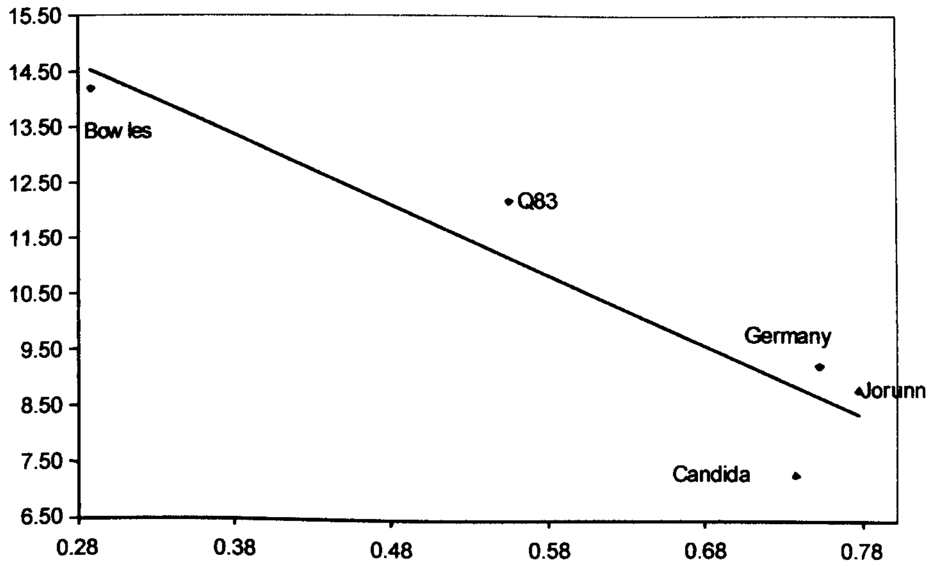

Figure 9.3.7 Negative linear correlation ($p < 0.05$, $r=0.93$) between NFT stem ratios (x axis) and leaf Cu concentrations in mg kg^{-1} (y axis) for 5 clones grown at Slough

Similar to the negative correlations established between the NFT height increase parameter and the Cu and Ni bark concentrations at Stoke-Bardolph, Candida and Germany have performed better in the NFT, and have lower leaf Cu concentrations in field-grown trees, than Q83.

The obvious groupings of certain clones in Figures 9.3.1 to 9.3.7 show that some performances in the field and NFT were clearly similar. Disappointingly, the correlations based on NFT and Stoke-Bardolph data were weakened by certain clones not following the main trend. For example, Delamere has similarly low Cu and Ni bark concentrations as Candida and high biomass production at Stoke-Bardolph, yet it is among the poorest of the clones on the basis of its height increase TCRs. Gigantea had intermediate bark concentrations of the metals at Stoke-Bardolph, yet was the poorest clone in terms of its NFT performance. However, although these outliers caused the correlations to have relatively low r values, it was possible to establish statistically significant correlations between biomass of seedlings grown in glasshouse short-term metal toxicity tests, and mature trees subjected to chronic metal stress grown in the complex environment of soil. Discrepancies between field and NFT performance, therefore, were comparatively rare, and might be explained by site specificity of the clone.

9.4 Limitations of NFT and Metal Resistance Tests

Turner (1994) suggested that hydroponic techniques may only be suitable for establishing large differences in tolerance, due to subtle differences being masked by, among other things, the ameliorating effect of the background nutrient solution. However, Punshon and Dickinson (1999) claim the strength of background solution used in this experiment (1/4-strength MHS) is sufficient to provide an adequate supply of nutrients to avoid deficiency, without ameliorating metal toxicity.

Consideration must be made of the main limitations in the development of this rapid screening test: despite their rapidity, screening procedures may not be of great value when seeking a characteristic as specific as metal resistance (Riddell-Black, 1993). Limitations arise in three main areas:

1. The possibly poor accuracy of seedlings reflecting the response of older trees to metals;

2. The high level of variation in the response of *Salix* to metals, and

3. The multiplicity of factors, other than heavy metals, that can affect tree performance in field studies.

Regarding the first point, the questionable use of seedlings in metal studies, in terms of their greater accumulation of metals than mature plants (Lehn and Bopp, 1987; Punshon and Dickinson, 1997), their greater sensitivity to adverse conditions than mature trees (Turner and Dickinson, 1993), and their difference to older trees, is well acknowledged. Turner (1994) stated that seedlings differ from mature trees in several ways, including C allocation, and the fraction of photosynthetically active tissue, which decreases with age. Therefore seedlings may be too sensitive for firm deductions in metal tolerance studies, but the adjustments to the screening test outlined in Section 9.1 at least permitted differentiation of clone performance.

Relevant to the second main limitation listed above, Punshon and Dickinson (1999) investigated the resistance of *Salix* to Zn, Cd, Cu and Ni in hydroponic experiments, and reported a high degree of variation in shoot and root growth in response to metal exposure. There was considerable variation in metal resistance that was not species-specific, rather clone- or hybrid-specific. Furthermore, they reported considerable intra-clonal variation: a proportion of the most sensitive clones survived in very high metal concentrations. Landberg and Greger (1996) measured the tolerance and accumulation of Cd, Cu and Zn of different clones of five *Salix* species and found the variation in metal accumulation and tolerance to heavy metals was wider within the species than between the species. Greger and Landberg (1999) also reported a large variation in uptake and transport of the metals among several clones.

High growth variation in response to elevated metals is therefore characteristic of most willow clones; this is also found in *Salix* grown in uncontaminated and contaminated soil trials, which puts into perspective the reproducibility problems encountered in glasshouse trials. This variation causes difficulties in phytoremediation screening programs, but allows selective planting to suit the remediation requirements of a site. Despite this variation, the replication in the NFT screening runs, as well as the applied experimental controls listed in Section 9.1, allowed for several consistent correlations between the performance of the clones in the NFT system and the field.

In reference to the third point, Dickinson (2000) reported several factors besides heavy metals to be important in the performance of trees grown on metal-contaminated sites. Weed competition and subsequent neglect influence survival at some sites; inadequate soils (featuring poor fertility and/or water shortage) can also have a major effect. Toxicity responses may also be determined by the pH of the soil (Dickinson *et al.*, 1991a). It is likely that metal toxicity was a strong factor at the very fertile, near-neutral soils in the Stoke-Bardolph and Slough trials, however.

Also, the adaptation of mature individuals may be the most significant factor in tree survival in metal-contaminated soil. Avoidance strategies allow the plant to adapt to the heterogeneous distribution of a pollutant in a contaminated soil. Trees that are not especially selected for metal tolerance can therefore survive in metal-contaminated soil, albeit usually with a reduced growth rate (Dickinson *et al.*, 1992).

Metal stress resistance may have genotypic and phenotypic components (Baker and Walker, 1989). The former may only be altered through selection, while the latter comprises physiological adaptations within boundaries defined by the genotype. Accordingly, the degree to which the Cu resistance observed in some of the 16 *Salix* clones hydroponically screened by Punshon *et al.* (1995b) was genetically determined or induced by the environment, was unclear. Thus metal tolerance is plastic: it can be induced or "lost": this phenotypic plasticity may be essential in contaminated land revegetation by trees (Punshon and Dickinson, 1999). This remains largely untested however, as the growth media used to grow the plants prior to the tolerance test may influence the plant response. In species that grow in contaminated soils, when compared with the same species from an unpolluted soil in reciprocal growth experiments, differences in tolerance may not be detected. For example, Landberg and Greger (1996) found no differences in metal tolerance and accumulation between *Salix* clones taken from polluted and control areas.

Consideration must also be made of the pitfalls of NFT metal resistance tests, and metal tolerance tests in general. As soils are such complex and heterogeneous media, they are not amenable to close experimental control. Therefore solution culture is the most important approach to investigations in plant nutrition (Epstein, 1972). Of course, the main difference is that in a nutrient solution, all of the metal applied is available to the plant, while adsorption-desorption and solubility processes govern availability in soils (Kohl, 1997). Culture solutions contain nutrient concentrations which are very high relative to those in soil solutions. Furthermore, most hydroponic studies of plant heavy metal uptake have used unrealistically high concentrations of the contaminant (Checkai *et al.*, 1987). Chaney *et al.* (1997) reported 3 μM Cd was non-toxic to solution culture maize seedlings, but still 30 times the level generally found in uncontaminated soil solutions. While plants can grow well in solutions much more dilute than those normally used in solution culture, constant withdrawal of nutrients and metals by the plant necessitates the use of concentrated solutions rather than plant requirement of high external elemental concentrations (Epstein, 1972).

Knowing that acute, short-term metal toxicity may not accurately reflect the response of a plant to chronic metal stress, Kohl (1997) developed an artificial soil system in which an ion-exchange resin is embedded in an inert sand matrix. The ions were buffered as in soils, but the growth conditions were more reproducible than in a soil system; also, the system was suitable for long-term cultivation, unlike hydroponics. Their experiment tested the response of *Armeria maritima* from metalliferous and uncontaminated soils to Zn, and found the two groups differed in their response to short-term exposure but were similarly resistant to long-term stress, pointing to the erroneous conclusions that can be made from short-term tests. As with Kohl, Checkai *et al.* (1987) used a mixed-resin hydroponic system in an experiment with tomato plants to maintain realistically low concentrations of Cd and nutrients, comparable to those in soil solutions.

Despite the possible superiority of resin experiments to hydroponic ones, biomass yield and growth rate have been recognised as useful indicators of metal resistance by Baker and Walker (1989). They acknowledged that yields are generally lower in non-tolerant plants, while seedling heights can be correlated to metal tolerance. They considered the simplicity of the method to outweigh its limitations, but advised caution in interpretation of results. Variations in response can arise due to the sensitivity of the individual and the concentration and duration of exposure to the metal; the latter has been kept as consistent as possible throughout all NFT experiments, while it was not possible to exert control over the former.

Measurements made on a variety of parameters can produce subtly different pictures of resistance (Punshon and Dickinson, 1999). But, as demonstrated in Chapter 7, the height increase parameter proved to be the most robust of the four measured and produced similar trends to those indicated by the other measurements. It was used extensively for the correlations presented in this chapter, and, despite the limitations outlined above, this chapter depicts several examples of similar performances of a group of varieties relative to others, in the glasshouse and field. While the performance of the clones tested in these experiments were quite similar, differentiation was possible and their relative performances in the glasshouse did broadly correspond with that in the field. The relative performance of Q83 and Germany were consistent throughout all NFT experiments that have been conducted in Chapters 7 to 9.

9.5 Summary

The main aim of this chapter was to ascertain whether data from the NFT screening test could be statistically correlated with data from the same clones grown independently in field trials. If this is possible, there is scope for extrapolating short-term NFT results to the field, allowing identification of willows suitable for planting in metal-contaminated substrates without necessitating long-term field trials. Adjustments to the NFT screening test succeeded in increasing the biomass of metal-treated trees, and improving the differentiation of clones, based on their biomass production in conditions of metal exposure.

Principal component analysis was used to express the relative importance of the many parameters of the willow varieties measured in both the NFT system and the field. From these, the NFT height increase and stem ratios of eighteen willow varieties, and several datasets of the same clones grown in field trials, were selected for correlations. Several significant linear associations between the NFT and field data were established. Despite the influence of outliers (willow varieties which did not have comparable performances in the NFT and field), groupings in the results became evident.

Consistently, two distinct groups in the correlations were apparent. *Salix viminalis* clones, as well as the basket willow Black Maul, proved to be less metal resistant than a group of hardier clones including Germany, Dasyclados, Candida and Spaethii. The more resistant clones proved to be superior in terms of biomass production in the glasshouse and field, and metal concentrations achieved in the wood. The less resistant clones achieved greater concentrations of Cu and Ni in the bark, and produced less biomass in the glasshouse and field.

The relative performance of some clones corresponded to those reported in previous hydroponic and pot studies. Despite some discrepancies between NFT and field performance, and the limitations of NFT tests, with sufficient replication and experimental control it was possible to establish significant correlations between the biomass of seedlings grown in short-term glasshouse metal exposure tests, and mature trees subjected to chronic metal stress grown in the complex environment of soil. Relative performances of clones in the NFT apparatus therefore broadly corresponded with those in the field.

Chapter 10 Conclusions

10.1 Introduction

The BIORENEW project was a multidisciplinary, European Commission-funded study spanning three years. The project involved collaborations between soil chemists, plant breeders, modellers and biofuel experts, and aimed to thoroughly examine several aspects of the use of biomass fuel crops in the remediation and economic renewal of industrially degraded land. The work presented in this thesis addressed one section of the project: the interaction of willows and heavy metals. This work specifically aimed to assess the metal tolerance of willow varieties and their metal accumulation under various conditions (such as in different nutrition and soil manipulation regimes), and the effect of their growth on soil heavy metal concentrations. The work comprised field, pot and hydroponic studies, and the conclusions from Chapters 3 to 9 are considered below in three categories: those resulting from field vegetation sampling, those from pot trials and soil samples collected from the field, and conclusions from the hydroponic experiments.

10.2 Field Vegetation Sampling

The collection of samples from field-grown willows, described in Chapter 3, allowed the study of two important phenomena: the vertical and seasonal distributions of metals between the tree fractions. Two major conclusions were made:

1. The importance of standardising the heights from which stem and leaf samples are collected in studies of metal uptake by field-grown mature trees was demonstrated: metal concentrations varied considerably with sampling height. Samples taken from different heights revealed a trend of increasing metal concentrations with height in the bark and wood tissues, and a decreasing trend with height in leaves. This sampling height effect was more pronounced for less translocatable elements such as Cu and Pb, and markedly less so for more readily translocated elements such as Zn and Cd.

2. Significantly, the results of the seasonal studies revealed that leaf and bark concentrations of metals fell towards the end of the growing season, with a rise in wood concentrations occurring in the same period. Therefore a back translocation of

metals, thought to be concurrent with the shunting of nutrients prior to leaf fall, is likely to occur. Trends shown in the leaf concentrations are a probable combination of the effects of growth dilution and back-translocation prior to leaf abscission.

Regarding point 1, this supports the study of stem sampling carried out by Sander and Ericsson (1998), but furthers it in that the fractions of leaf, wood and bark have been isolated and studied separately. The observations in wood and bark are likely to be a result of upward translocation of metals in xylem and phloem and decreasing stem girth with height, while the leaf patterns are possibly due to increased biomass of leaves with height, and greater deposition of metals in lower leaves.

In consideration of point 2, following current practice, the harvesting of willows takes place in winter after leaf fall. This appears to be the time in which the greatest amounts of metals are sequestered in the wood, the most significant portion of the harvestable biomass by weight. Therefore the current practice is likely to be the optimum time to harvest in terms of metal phytoextraction and recovery of metals from the biofuel ash, two of the goals of the BIORENEW project.

The technologies developed by one project member, the University of Graz, allow concentration of the metals in ash after harvested willow stems are combusted. Leaves also constitute a considerable portion of the above-ground biomass; considerable quantities of metals are translocated to this fraction. Therefore phytoextraction could be enhanced if combustion technology permitted this fraction to be used as a biofuel, and the metals recovered; considerable quantities of metals are otherwise recycled to the soil as a consequence of leaf fall.

Future studies of metal distribution in willows would be greatly aided by more frequent sampling if the problem of time consumed with routine analysis could be overcome. Root sampling would provide a complete study of metal distribution between the compartments of willow trees, but this is potentially hindered by incomplete removal of soil particles from root surfaces, which can cause considerable analytical bias. Stem analysis is destructive and necessitates separate tree samples at each sampling time. However, precision of future foliar analyses might be improved if leaf samples were collected from the same tree, to counteract variability in metal uptake between individual trees.

10.3 Pot Trials and Soil Sampling

The pertinence of examining the effects of tree growth on the soil metal distribution has been acknowledged by other authors: for example, Dickinson (2000) stressed the importance of understanding the impact of plants on metal mobility. Selective, sequential and soil solution analyses were carried out on soil samples collected from both the field (Chapter 4) and a pot trial (Chapter 5) to study this area. Additionally, the phytoextraction of metals from soils by plants is limited by the concentrations achieved in the above-ground biomass and the quantities of biomass produced. Therefore the metal uptake enhancement potential, and fertilisation effects, of two soil amendments were assessed in a pot trial (Chapter 6).

Several conclusions were reached from analyses carried out on soil samples collected in the field, and soil and vegetation analyses of pot trial samples:

1. Selective extraction of soil samples, described in Chapters 4 and 5, provided many examples of biomass crop growth effecting significant depletions in extractable soil heavy metal concentrations, when compared to concentrations in unplanted areas.

2. Plant concentrations in samples collected from a pot trial (Chapter 5) demonstrated that plant uptake and sequestration in above-ground biomass, as displayed in Table 5.3.2, could only account for a fraction of the observed significant depletions in the soil (for example, approximately 4 % of those for Cu and around 20 % of those for Zn).

3. Selective extraction results indicated a redistribution of the metals from more to less extractable pools; this redistribution is thought to constitute a major portion of the observed extractable metal depletions caused by tree growth. Sequential extractions of metals from samples collected at a field site (Chapter 4) demonstrated this: where trees were growing or were recently harvested, extractable concentrations frequently fell significantly in the period from March to June, with a corresponding rise in the residual fraction.

4. Soil solution analyses from samples collected in the field (Chapter 4) indicated water-soluble metal concentrations increase as a result of tree growth and harvest. This is probably due to complexation by soluble organic compounds following root secretion (and degradation where trees have been harvested) and increased soil microbial

activity, which was also shown to be stimulated by root growth and degradation following harvest (Chapter 4).

5. Results from resin samplers and selective extractions also indicated that tree growth did not induce significant quantities of the mobilised metals to be leached from the organic or calcareous soils used in these studies.

6. The citric acid soil amendment in the pot trial described in Chapter 6 resulted in a trend of positive yield increments in willow and barley aerial tissues. This frequently corresponded with significant increases in the metal quantities achieved in the aerial biomass of these plants. The acidifying agent $(NH_4)_2SO_4$ led to significantly lowered yield in the willow and barley, probably due to reduced soil pH and enhanced metal concentrations in the plants.

Points 1 to 4 demonstrate that biomass crop growth, directly or indirectly, mobilises soil heavy metals. Certain extractable metal pools are depleted; point 2 demonstrates how the technology of phytoextraction is applicable when removing the bioavailable fraction of translocatable contaminants such as Zn, and is less useful in the extraction of elements of relatively poor mobility, such as Cu. Soil solution metal concentrations are increased, while sequential extractions indicate that concentrations in less extractable soil pools are also increased. The findings described in point 3 should be investigated much more thoroughly in future with a rigorous sequential extraction procedure with largely increased replication. Although a research team at the University of Hohenheim developed a standardised sequential extraction procedure as part of the BIORENEW project, this was considered too laborious to be implemented on the large number of samples collected in this work; extra manpower and resources could overcome this in future studies.

Relating to point 5, perhaps leaching is a factor in less organic or more acidic soils: other authors have considered it in phytoextraction field trials. Cooper *et al.* (1999) acknowledged the need for metal-leaching preventative measures, such as tile drains to capture leachate and special irrigation practices to limit water movement out of the root zone.

Considering point 6, the $(NH_4)_2SO_4$ soil amendment should not be used where willows are growing in similarly contaminated soils. The effects of citric acid should be compared with those of other chelating agents to see if the theory proposed in Chapter 6, that the biomass increase was a result of enhanced microbial nutrient cycling following degradation of the chelate, is plausible. The aforementioned research team at the University of Hohenheim also assessed a suite of soil manipulation techniques to enhance phytoextraction as part of the BIORENEW project; the published findings of their work should provide a good background for future soil manipulation experiments.

The effects of other aspects of biomass fuel crop husbandry not addressed in this thesis should be recognised. Punshon and Dickinson (1999) stressed the need to understand the effect of tree planting on metal mobility. Cultivation, for example, will aerate the soil and promote the activities of soil microbes. Therefore the breakdown of organic matter would be enhanced, leading to the mobilisation of organically complexed metals.

10.4 Hydroponic Experiments

Hydroponic tests have many advantages in the identification of metal resistance in willow varieties, including cheapness, simplicity and rapidity. Ultimately, it was desired to develop a rapid test of willow tolerance to heavy metals by comparing the performance of many willow varieties in glasshouse tests, with the performance of the same varieties in the field. If significant correlations between the two datasets could be established, relative performances of several willow varieties in conditions of metal exposure could be extrapolated from NFT results, to the field, reducing the need for long-term field trials.

It has long been known that total decontamination of a heavily metal polluted site, through repeated willow harvests, is not feasible on a realistic timescale. However, the planting of willows constitutes a suitable remediation approach in a number of situations. Dickinson (2000) described the use of trees in reclamation as low-cost, sustainable and ecologically sound. Riddell-Black (1993) claimed that renovation of less polluted land is still possible in a reasonable timescale due to the frequency of harvest. Therefore phytoextraction is best suited for low and medium contaminated agricultural soils (Greger and Landberg, 1999). Particularly, there is potential for "soil polishing": Cd phytoextraction in moderately contaminated Swedish soils has shown promise (Ostman, 1994, Greger, 1999). However, the variability of the metal uptake trait, and the need to identify potentially suitable clones, should never be overlooked: Greger (1999) found the Cd uptake capacity of 70 *Salix* genotypes could differ by as much as 43 times between clones with the highest and lowest values. The better varieties were reported to have capacities about 5 times higher than for the hyperaccumulator *Thlaspi caerulescens*, due to high biomass production and transport of Cd to the shoot.

Therefore phytoextraction would be the main benefit from *Salix* growth on metal-contaminated land where concentrations are not sufficiently high, and the area is too large, to justify an engineering remediation strategy, and where there is no time pressure (Riddell-Black, 1994). In many contaminated sites, the introduction of trees is beneficial whether significant metal phytoextraction occurs or not, due to the soil stabilising effects of the tree roots, and the potential to harvest the trees for the purposes of biofuel or one of the many industries which utilise willows.

Overall, within the genus of *Salix*, there is potential for some clones to produce substantial quantities of biomass when growing on a contaminated site. Furthermore, some clones have a tendency to produce considerable biomass and couple this with substantial metal uptake. Therefore selection of appropriate willow varieties for growth on a contaminated soil can be made according to the site management requirements. Where soil polishing is feasible, clones which accumulate metals into the harvestable biomass would be ideal, while non-accumulating biomass producers would be beneficial where phytostabilisation is desirable, but metal removal is not.

In the development of the rapid test, many conclusions were reached about appropriate conditions for future hydroponic tests, and the suitability of various clones for planting on contaminated sites:

1. Performance of willow varieties according to their response to metal exposure was distinguishable, and not compounded by nutrient limitations; a suitable nutrient strength (1/4 strength Hoaglands) was selected from a tested range (Chapter 7). It was recognised that greater quantities of metals were translocated to leaves in the more nutritious regimes.

2. To increase the biomass of the metal-treated trees, Pb was excluded from the metal cocktail and an increased quantity of P was supplied for 7 days per week, and the

nutrient medium was altered to provide N as NH_4^+ as well as NO_3^- to improve buffering and maintain the pH around the optimum level of 5.5 (Chapter 8).

3. Chapter 9 describes how the volume of solution per tree and frequency of solution rotation were standardised (10 l per channel of trees per week), and metals were added cumulatively to avoid a large yield penalty in the susceptible clones in the first half of the experiment.

4. The most valid observation from the hydroponic research was the establishment of many significant correlations between NFT test data (particularly the height increase parameter) and independent field data, following prioritisation using Principal Component Analysis (Chapter 9).

5. From the data presented in Chapter 9, the willow varieties Candida, Spaethii, Dasyclados, Calodendron and Germany comprise a group suitable for phytoextraction: they are clones which ranked among the highest according to their biomass production in field trials and the glasshouse, and achieved high metal concentrations in the wood of the field-grown trees. The willow varieties Jorunn, Coles, Ulv and Q83 are examples of clones suitable for phytostabilisation: concentrations of contaminant metals in the wood of the field-grown trees was comparatively low, while their performance in the NFT was reasonable.

From the phytoextraction viewpoint, point 1 suggests that trees would sequester greater quantities of metals in the above-ground biomass in contaminated soils of high fertility, or if they were artificially fertilised. The experimental refinements, listed in points 1 to 3, were applied to the test and led to the observation described in point 4, and the classification of various clones listed in point 5.

Considering point 4, a rapid test has been developed which can indicate the relative performance of willow varieties in the field. It was possible to establish significant correlations between the biomass of seedlings grown in short-term glasshouse metal exposure tests, and mature trees subjected to chronic metal stress grown in the complex environment of soil. Relative performances of clones in the NFT apparatus therefore broadly corresponded with those in the field.

It should be stressed that the results have only been correlated with results from field trials in which the trees were grown in nutritious, highly organic sludge-amended soils; clone performances may be different in non-organic soils. Therefore a future requirement, besides the obvious need for a greater range of willow varieties to be tested, is for further comparison of the performances of willow varieties grown in the NFT and in a variety of field trials, each featuring different substrates, to test the applicability of the NFT test results.

Punshon and Dickinson (1999) described hydroponic screening techniques as being useful investigative tools, but pointed out that results do not accurately reflect the growth or patterns of metal uptake in the complex environment of soils. Major influences are likely to be exerted by soil organic matter, pH, mycorrhizal associations and the combinations of contaminants, weed competition and soil inadequacies. While work in this thesis addressed several of these factors individually, it would be unwise to deduce any conclusions relating to willow metal uptake based on the rapid test results, only about their relative biomass production in conditions of metal exposure. The results from the NFT test data should be compared to those from other rapid tests currently being developed. For example, the Swedish research centre Svalof Weibull AB carried out tests using willow leaf disc samples and mini-cuttings in metal-amended solutions as part of the BIORENEW project; imminent published work shall provide extensive data.

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