Screening for heavy metal tolerance in common Australian fern species

ABSTRACT

The effects of cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb) and zinc (Zn) on the growth and uptake of 10 fern species was investigated under a controlled environment in order to evaluate their suitability for phytoremediation. Fern species included Adiantum aethiopicum, Blechnum cartilagineum, Blechnum nudum, Calochlaena dubia, Dennstaedtia davallioides, Doodia aspera, Hypolepis muelleri, Nephrolepis cordifolia, Pellaea falcata and the arsenic (As) hyperaccumulating Pteris vittata. Ferns were exposed to four levels of heavy metals at concentrations of 0, 50, 100 and 500 mg kg\(^{-1}\) for a period of 20 weeks. In general, heavy metal translocation was limited, with the majority of heavy metals held in roots, suggesting an exclusion mechanism as part of the ferns’ tolerance to the applied heavy metals. Similar heavy metal-accumulation patterns were observed for all species in that accumulation generally increased with increasing levels of heavy metals applied. In most cases a sharp increase was observed between 100 and 500 mg kg\(^{-1}\) treatment levels, suggesting a breakdown in tolerance mechanisms and unrestricted heavy metals transport. This was corroborated by enhanced visual toxicity symptoms and a reduction in survival rates of ferns when exposed to 500 mg kg\(^{-1}\) treatment levels; and to a lesser extent 100 mg kg\(^{-1}\) treatment levels. Nephrolepis cordifolia and H. muelleri were identified as possible candidates in phytostabilisation of Cu-, Pb-, Ni- or Zn-contaminated soils; similarly D. davallioides appeared favourable for use in phytostabilisation of Cu- and Zn-contaminated soils. These species had high survival rates and accumulated high levels of the aforementioned heavy metals relative to the other ferns investigated. Ferns belonging to the family Blechnaceae (B. nudum, B. cartilagineum and D. aspera) and C. dubia (Family Dicksoniaceae) were least tolerant to most heavy metals, had a low survival rate and were classified as being unsuitable for phytoremediation purposes. Heavy metal tolerance was also observed in P. vittata when exposed to Cd, Cr and Cu; however, no hyperaccumulation was observed.

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2.1 INTRODUCTION

Soil pollution of heavy metals is a global problem with ramifications to human, animal and environmental health. An increase in heavy metal pollution has long been associated with population growth, fuelled by industrial advancement. Activities that have caused heavy metal contamination include mining and smelting, industrial and manufacturing emissions and application of fertilisers and sewage sludge contaminated with heavy metals (Singh 2001). Industrialised countries and developing countries are equally affected; however, in the latter there is an increased pressure to use contaminated soils for food production (Patel et al. 2005).

Cadmium and lead (Pb) contamination have been the focus of numerous studies and are considered environmentally toxic metallic pollutants (Koeppe 1977; Sanchez-Camazano et al. 1994; Liu 2003). Heavy metals including copper (Cu), chromium (Cr), nickel (Ni) and zinc (Zn) are also considered environmental pollutants and at high concentrations are often associated with the expression of phytotoxicities in plants (Chatterjee and Chatterjee 2000; Cuypers et al. 2002; Denkhaus and Salnikow 2002). Copper and Zn are essential micronutrients and are required by plants at low concentrations; beyond the critical toxicity levels of Cu > 20–30 mg kg\(^{-1}\) and Zn > 100–300 mg kg\(^{-1}\) phytotoxicity symptoms are often pronounced (Marschner 1997). There have been few studies addressing Cr toxicity in plants, however, it is known that Cr toxicity depends on its valence state, with Cr\(^{VI}\) being more toxic and mobile compared to Cr\(^{III}\) (Shanker et al. 2005).

In response to contaminated soils, a variety of physico-chemical remediation methods have been adopted, including solidification, electrokinetics and encapsulation (Mulligan et al. 2001). In many cases, these strategies have resulted in criticisms due to their high cost, energy intensiveness, site destructiveness, associated logistical problems and growing degree of public dissatisfaction (Rulkens et al. 1998). The implementation of alternative strategies that address these concerns is critical in effectively removing metallic pollutants from soil. In recent years significant progress has been made with the implementation of phytoremediation techniques as alternative technologies capable of remediation and restoring heavy metal contaminated soils.

An approach to phytoremediation is phytostabilisation, an important in situ site stabilisation technique that uses plants as a preventative barrier. The end result is a two-way barrier that
prevents the leaching of heavy metals throughout the soil profile, while minimising erosion and ecological exposure to the heavy metals. Phytostabilisation is a versatile technique and has been successfully applied in the containment of heavy metals in mine spoils, metalliferous waste and smelters (Smith and Bradshaw 1979; Pierzynski et al. 2002; Stoltz and Greger 2002).

Plants suitable for phytostabilisation need to express a degree of tolerance that enables them to survive in contaminated soils. Three mechanisms have been suggested by which plants respond to heavy metals. They relate to the heavy metal concentrations found in plants and the substrates in which they grow (Baker 1981). Plants termed ‘accumulators’ concentrate heavy metals in aboveground biomass regardless of the heavy metal concentration in which they are growing. Such properties have been found in ferns, for example P. vittata and Pytyrogramma calomelanos for As (Ma et al. 2001; Francesconi et al. 2002). Conversely, plants termed ‘excluders’ maintain low concentrations of heavy metals in aboveground biomass, compared with their substrate, up to a certain threshold before the mechanism breaks down. Last, plants termed ‘indicators’ are those in which the heavy metal concentration in the aboveground biomass reflects the substrate concentration, and are often used in mineral prospecting (Nkoane et al. 2005).

Ferns have often been associated with contaminated soils, particularly those associated with mining operations. A notable example is the Chinese brake fern (P. vittata) that was identified growing on an arsenical mine dump in Rhodesia (Wild 1974a); however, it was not until 2001 that it earned its classification as the first As hyperaccumulator (Ma et al. 2001). The silver back fern (P. calomelanos) is the only fern outside the Pteris genus that has been identified as an As hyperaccumulator (Francesconi et al. 2002). Lepp (2001) indicated that ferns are regarded as the fourth most abundant group (in terms of species richness) of plants associated with Cu-enriched substrates and also reported their dominant presence on serpentine and other ultramafic bodies. Terrestrial ferns have received less attention than vascular plants in relation to heavy metal tolerance and accumulation, and consequently there are few systematic investigations that have considered the interactions between ferns and heavy metal substrates.

Large stretches of land across Australia have been contaminated by a variety of heavy metals, mainly because of intensive agriculture and mining activities (Sproal et al. 2002; Archer and
Caldwell 2004; Pietrzak and McPhail 2004; Cooper 2005). Owing to strict quarantine regulations on import of planting material suitable for phytoremediation, a number of native Australian ferns were screened for their tolerance to a variety of heavy metals (Cd, Cr, Cu, Ni, Pb and Zn). None of these Australian species have been studied for heavy metal tolerance and accumulation. The primary objective of this study was to determine the degree of accumulation and the biomass produced when nine of the most common Australian fern species along with *P. vittata* were exposed to elevated levels of heavy metals under controlled conditions. This information could be used to select suitable species available for phytoremediation of heavy metal(s)-contaminated soils within Australia and elsewhere.

### 2.2 MATERIALS AND METHODS

#### 2.2.1 Selection of fern material

Nine Australian native fern species along with a known As hyperaccumulator *P. vittata* were screened for their potential to tolerate Cd, Cr, Cu, Ni, Pb and Zn. Species were chosen based on their hardiness, vigour and distribution pattern across Australia (McCarthy 1998), and included: *Adiantum aethiopicum*, *Blechnum cartilagineum*, *Blechnum nudum*, *Calochlaena dubia*, *Dennstaedtia davalliioides*, *Doodia aspera*, *Hypolepis muelleri*, *Nephrolepis cordifolia*, *Pellaea falcata*. Each taxon including their distribution across Australia is described in Appendix 1. With the exception of *N. cordifolia*, all ferns were procured from a specialist fern nursery (Sonters Fern Nurseries, Winmalee, NSW, Australia), and were approximately 3–4 months old. *Nephrolepis cordifolia* was propagated from samples obtained from the wild (Garigal National Park, Roseville, NSW, Australia).

#### 2.2.2 Greenhouse experiment

Potting mix (Debco® general container mix) was used in the experiment and amended with 10% coarse perlite mix for improved aeration and drainage. Approximately 800 g of air dried potting mix was weighed into each plastic pot (Ø of 14 cm; 2 L capacity) and a plastic saucer placed under each pot to collect any possible leachate, hence maintaining a closed system. Analytical reagent (AR) grade metal salts were applied as solutions at 0, 50, 100 and 500 mg kg\(^{-1}\) dry weight of potting mix. Cadmium was added as Cd(NO\(_3\))\(_2\)-4H\(_2\)O, chromium as K\(_2\)Cr\(_2\)O\(_7\), lead as Pb(NO\(_3\))\(_2\), nickel as NiSO\(_4\)-6H\(_2\)O, copper as CuSO\(_4\)-5H\(_2\)O and zinc as ZnSO\(_4\)-7H\(_2\)O. Ferns were arranged in a completely randomised experimental design and there were three replicates for each treatment. After one week of incubation, one fern (3–4 frond
stage) was transplanted into each pot (Tu and Ma 2002). Ferns were grown in a greenhouse for 20 weeks, with 11 h daily photoperiod, a photon flux of >370 µmol m\(^{-2}\) s\(^{-1}\), temperature range between 17 ºC (night) to 32 ºC (day) and relative humidity of ~65%. Ferns were watered daily with deionised water (to 60–70% water holding capacity) and no fertiliser was applied during the experimental period.

### 2.2.3 Chemical analysis

Ferns were harvested after 20 weeks and separated into roots and fronds. Harvested material was thoroughly washed using a three step washing sequence consisting of dilute 0.01 % HCl followed by tap water and lastly with deionised water (Reuter et al. 1988). Samples were then air dried, weighed and placed in a forced draft oven at approximately 80 ºC for 48–72 h. Dried samples were weighed, mechanically ground using a stainless steel grinder (<1 mm) and stored in a desiccator.

A portion of the dry fern material was digested in a mixture of nitric (HNO\(_3\)) and perchloric (HClO\(_4\)) acids and diluted to 25 mL with Barnstead E-pure\(^{®}\) water (18.2 mΩ cm\(^{-1}\); Barnstead/Thermolyne Corp., Dubuque, IA, USA) (Miller 1998). With each batch of digestions, a sample blank and two National Institute of Standards and Technology-Standard Reference Materials [NIST-SRM #1547 (peach leaves) and NIST-SRM #1575 (pine needles)] were included for quality control. Extracts were analysed for various elements using a Vista Varian 220FS flame atomic absorption spectrometer (AAS). Samples with concentrations below detection limit of flame atomic absorption spectroscopy (namely Cd, Cr, Ni and Pb) were analysed using a Vista Varian Spectra 220Z graphite furnace atomic absorption spectrometer (GF-AAS). The detection limit and wavelengths of each element are shown in Table 2.1. For all metals there was > 95% recovery and analytical precision below 10% relative standard deviation (RSD) for all samples. Standards and dilutions were prepared in 0.1 M HNO\(_3\) in order to account for any matrix effects. All glassware/plasticware was soaked in 1 M HNO\(_3\) for 24 h, rinsed in deionised water followed by Barnstead E-pure\(^{®}\) water prior to use.
Table 2.1 Detection limits and wavelengths used for atomic absorption spectrometer (AAS) and graphite furnace atomic absorption spectrometer (GF-AAS) analyses.

<table>
<thead>
<tr>
<th>Element</th>
<th>AAS Detection Limit (mg L(^{-1}))</th>
<th>GF-AAS Detection Limit (µg L(^{-1}))</th>
<th>Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>0.02</td>
<td>0.01</td>
<td>228.8</td>
</tr>
<tr>
<td>Cu</td>
<td>0.03</td>
<td>0.3</td>
<td>324.8</td>
</tr>
<tr>
<td>Pb</td>
<td>0.1</td>
<td>0.28</td>
<td>217.0</td>
</tr>
<tr>
<td>Cr</td>
<td>0.06</td>
<td>0.075</td>
<td>357.9</td>
</tr>
<tr>
<td>Ni</td>
<td>0.1</td>
<td>0.24</td>
<td>232.0</td>
</tr>
<tr>
<td>Zn</td>
<td>0.01</td>
<td>0.0075</td>
<td>213.9</td>
</tr>
</tbody>
</table>

2.2.4 Statistical analysis

Analysis of variance (two-way ANOVA) was carried out to test for significant differences with GenStat version 8.1.0.152 (Payne et al. 2005). The parameters analysed included frond and root biomass, metal concentration and metal accumulation. The raw plant data can be found in Appendix 2. Prior to ANOVA, normal probability plots and residual plots were constructed for each data set and examined for unequal variance and deviations from normality among residuals. All data were log transformed as the assumptions of constant variance and normality could not be met. An unbalanced treatment design was used as some species lost replicates during experimentation. Differences were considered significant if \(P \leq 0.05\).

2.3 RESULTS

2.3.1 Frond and root biomass

For all heavy metals, frond and root biomass differed significantly between species and the effects of Cd and Pb treatments on frond biomass were also significant (Table 2.2). In general, frond biomass was greater than root biomass for species excluding A. aethiopicum, B. cartilagineum, C. dubia and D. aspera (Figure 2.1 and 2.2). For all species, frond and root biomass was lowest when the ferns were exposed to Cd and exhibited a dose-dependence decrease with increasing Cd applied (Figure 2.1a and 2.2a). Stunted new growth and necrotic pinnae tips were observed in all but P. vittata species, which maintained uniform growth across all Cd treatment levels (Figure 2.3a and 2.3b).
Table 2.2 Results of two-way ANOVA showing probability (P) for biomass, heavy metal concentration and accumulation in ferns between populations, treatments and interaction between species and treatments.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Significance (P)</th>
<th>Cd</th>
<th>Cr</th>
<th>Cu</th>
<th>Pb</th>
<th>Ni</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fronds Species</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>0.005</td>
<td>0.564</td>
<td>0.861</td>
<td>0.038</td>
<td>0.127</td>
<td>0.143</td>
<td></td>
</tr>
<tr>
<td>Species × treatment</td>
<td>0.060</td>
<td>0.045</td>
<td>0.002</td>
<td>0.152</td>
<td>0.004</td>
<td>0.018</td>
<td></td>
</tr>
<tr>
<td>Roots Species</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>0.010</td>
<td>0.792</td>
<td>0.167</td>
<td>0.196</td>
<td>0.154</td>
<td>0.586</td>
<td></td>
</tr>
<tr>
<td>Species × treatment</td>
<td>0.044</td>
<td>0.025</td>
<td>0.001</td>
<td>0.294</td>
<td>0.009</td>
<td>0.019</td>
<td></td>
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<tr>
<td>Heavy metal Concentration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fronds Species</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
<td>0.016</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Species × treatment</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>0.010</td>
<td>0.108</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Roots Species</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Species × treatment</td>
<td>0.225</td>
<td>&lt;0.001</td>
<td>0.581</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Heavy metal accumulation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Species × treatment</td>
<td>&lt;0.001</td>
<td>0.004</td>
<td>&lt;0.001</td>
<td>&lt;0.003</td>
<td>&lt;0.001</td>
<td>0.066</td>
<td></td>
</tr>
</tbody>
</table>

Survival data indicated a higher percentage of deaths in Cd treated ferns than in those treated with other metals (Table 2.3). Among all ferns, *N. cordifolia* responded most favourably to heavy metal treatments with no reported deaths (Table 2.3) and high frond biomass across all treatments excluding Cd (Figure 2.1a and 2.3a). *D. aspera* also survived the exposure of most heavy metals excluding Pb (Table 2.2), however, lower frond biomass was recorded for *D. aspera* than for *N. cordifolia*. *Hypolepis muelleri* had the highest frond biomass across all Cr, Cu, Pb and Zn treatment levels (Figure 2.1) and a high survival rate in all heavy metals.
excluding Cd (Table 2.3). *B. nudum, B. cartilagineum* and *C. dubia* species grew least favourably (Table 2.3) with low frond biomass across most heavy metal treatments (Figure 2.1). Among these species, phytotoxicities were more pronounced and symptoms included interveinal yellowing, chlorotic, stunted growth and necrotic pinnae tips (Figure 2.3c). In general, frond biomass in ferns exposed to Zn treatments increased with increasing Zn levels (Figure 2.1f) and a high survival rate was observed among all species (Table 2.2). The only species which showed a reduction in frond biomass across all Zn treatments was *P. falcata* (Figure 2.3d).

**Table 2.3** Survival rate (%) of the 10 ferns across all treatments of various heavy metals after 20 weeks of growth.

<table>
<thead>
<tr>
<th>Survival Rate (%)</th>
<th>Cd</th>
<th>Cr</th>
<th>Cu</th>
<th>Pb</th>
<th>Ni</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adiantum aethiopicum</strong></td>
<td>67</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>89</td>
<td>100</td>
</tr>
<tr>
<td><strong>Blechnum cartilagineum</strong></td>
<td>11</td>
<td>100</td>
<td>100</td>
<td>44</td>
<td>89</td>
<td>67</td>
</tr>
<tr>
<td><strong>Blechnum nudum</strong></td>
<td>44</td>
<td>44</td>
<td>67</td>
<td>56</td>
<td>56</td>
<td>89</td>
</tr>
<tr>
<td><strong>Calochlaena dubia</strong></td>
<td>78</td>
<td>56</td>
<td>67</td>
<td>67</td>
<td>89</td>
<td>89</td>
</tr>
<tr>
<td><strong>Dennstaedtia davalliodes</strong></td>
<td>89</td>
<td>44</td>
<td>100</td>
<td>44</td>
<td>78</td>
<td>89</td>
</tr>
<tr>
<td><strong>Doodia aspera</strong></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>89</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><strong>Hypolepis muelleri</strong></td>
<td>56</td>
<td>100</td>
<td>100</td>
<td>78</td>
<td>100</td>
<td>89</td>
</tr>
<tr>
<td><strong>Nephrolepis cordifolia</strong></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><strong>Pellaea falcata</strong></td>
<td>67</td>
<td>67</td>
<td>89</td>
<td>89</td>
<td>89</td>
<td>89</td>
</tr>
<tr>
<td><strong>Pteris vittata</strong></td>
<td>78</td>
<td>67</td>
<td>67</td>
<td>67</td>
<td>78</td>
<td>89</td>
</tr>
</tbody>
</table>
Figure 2.1 Frond dry weight (g) of different fern species with various heavy metal treatments after 20 weeks of growth, (a) Cd, (b) Cr, (c) Cu, (d) Ni, (e) Pb and (f) Zn.

Log-transformed data have been back-transformed. Data points and vertical bars are least square means and ± standard errors, respectively (n=3).
Figure 2.2 Root dry weight (g) of different fern species with various heavy metal treatments after 20 weeks of growth, (a) Cd, (b) Cr, (c) Cu, (d) Ni, (e) Pb and (f) Zn.

Log-transformed data have been back-transformed. Data points and vertical bars are least square means and ± standard errors, respectively (n=3).
Chapter 2: Screening for heavy metal tolerant fern species

2.3.2 Heavy metal concentrations in the fronds and roots

Frond and root concentrations across all heavy metal treatments differed significantly between species and treatments (Table 2.2). Concentrations of Cd, Pb and Ni remained uniform across 50 and 100 mg kg\(^{-1}\) treatments and increased at the 500 mg kg\(^{-1}\) level. The highest concentration of Cd was observed in fronds of *D. davallioides* (386 mg kg\(^{-1}\) dry weight (DW)), suggesting that *D. davallioides* may be able to hyperaccumulate Cd in the aboveground biomass (Figure 2.4a). For the remaining species, the concentrations of Cd ranged from 4.1 (*N. cordifolia*) to 87.9 mg kg\(^{-1}\) DW (*P. falcata*). For Pb (Figure 2.4e), the highest concentration was observed in the fronds of *P. falcata* (62 mg kg\(^{-1}\) DW) and at both 50 and 100 mg kg\(^{-1}\) treatments, the concentration of Pb in most cases was below 6 mg kg\(^{-1}\), with the exception of *B. nudum* at the 50 mg kg\(^{-1}\) level (21 mg kg\(^{-1}\) DW). The highest Ni concentration in fronds was observed in *D. aspera* (161 mg kg\(^{-1}\) DW) and high concentrations
were also observed in *B. cartilagineum* (121 mg kg\(^{-1}\) DW). For the remaining species, the concentration of Ni ranged from 0.8 (*P. vittata*) to 68 mg kg\(^{-1}\) DW (*P. falcata*). For all species exposed to Cd, Ni and Pb treatments, the concentrations were higher in the roots than in the fronds and, in general, increased with increasing concentrations of metals. Concentrations of Cd in the roots were up to 7–fold higher than those in the fronds (Figure 2.5a), whereas concentrations of Ni and Pb were up to 36–fold higher in the roots than in the fronds (Figure 2.5d and 2.5e). Concentrations of Cr in the fronds ranged from 0.07 (*N. cordifolia*) to 33 mg kg\(^{-1}\) DW (*P. falcata*) among all species and similar concentrations were also observed in the fronds of ferns exposed to Cu, ranging from 4.12 (*C. dubia*) to 32 mg kg\(^{-1}\) DW (*D. davalliioides*) (Figure 2.4c). For all treatment levels, fronds generally contained much lower concentrations of Cr and Cu than did roots (Figure 2.5b and 2.5c) and for all species, the highest concentrations were observed at the 500 mg kg\(^{-1}\) level. Among all species, concentrations of Cr in the roots were up to 178–folds higher than those in the fronds and concentrations of Cu were up to 18–fold higher in the roots than in the fronds.

Concentrations of Zn in the fronds increased with increasing concentrations of Zn, especially between the 100 and 500 mg kg\(^{-1}\) levels (Figure 2.4f). The highest concentration of Zn was recorded in *B. nudum* (216 mg kg\(^{-1}\) DW) at the 500 mg kg\(^{-1}\) level, followed closely by *B. cartilagineum* (202 mg kg\(^{-1}\) DW). Across 50 and 100 mg kg\(^{-1}\) Zn treatments, the mean concentration of Zn in fronds ranged from 18 (*D. aspera*) to 107 mg kg\(^{-1}\) DW (*P. falcata*). The concentration of Zn in the roots followed a pattern similar to that in fronds (Figure 2.5f), except that the concentrations were up to 6–fold higher in the roots than in the fronds.
Figure 2.4 Concentrations (mg kg\(^{-1}\) DW) of Cd (a), Cr (b), Cu (c) Ni (d), Pb (e) and Zn (f) in fronds of different fern species after 20 weeks of growth.

Log-transformed data have been back-transformed. Data points and vertical bars are least square means and ± standard errors, respectively \((n=3)\).
Figure 2.5 Concentrations (mg kg\(^{-1}\)DW) of Cd (a), Cr (b), Cu (c) Ni (d), Pb (e) and Zn (f) in roots of different fern species after 20 weeks of growth.

Log-transformed data have been back-transformed. Data points and vertical bars are least square means and ± standard errors, respectively (\(n=3\)).
2.3.3 Heavy metal accumulation

Heavy metal accumulation was calculated by multiplying the total amount of biomass produced (root and frond) with the respective concentration of metals in the fronds and roots, to give an indication of the efficiency of metal accumulation for each species (per plant basis). For all 10 ferns investigated in this study, the level of heavy metal accumulation varied significantly among species and treatment levels in response to all heavy metals applied (Table 2.2). The pattern of Cd (Figure 2.6a) and Pb (Figure 2.6d) accumulation remained uniform across 50 and 100 mg kg\(^{-1}\) treatments and increased at the 500 mg kg\(^{-1}\) level. The highest accumulation of Cd was 168 µg plant\(^{-1}\) and was found in *D. davallioides*; for the remaining species, accumulation of Cd was less than 90 µg plant\(^{-1}\). Among species treated with Pb, the highest accumulation (469 µg plant\(^{-1}\)) occurred at the 500 mg kg\(^{-1}\) treatment level in *D. aspera*.

The highest accumulation of Cu was observed at the 500 mg kg\(^{-1}\) level in *H. muelleri* followed by *D. davallioides*, with values of 210 µg plant\(^{-1}\) and 195 µg plant\(^{-1}\), respectively (Figure 2.6c). The highest accumulation of Zn was observed in *A. aethiopicum* (655 µg plant\(^{-1}\)) followed by *D. davallioides* (377 µg plant\(^{-1}\)). Hypolepis muelleri recorded the highest accumulation of Zn at both the 50 and 100 mg kg\(^{-1}\) treatments, with values of 233 and 248 µg plant\(^{-1}\), respectively (Figure 2.6f). Across all Cu treatments, the accumulation of Cu in most species was below 65 µg plant\(^{-1}\) and for Zn below 240 µg plant\(^{-1}\).

In general, the level of Ni accumulation (Figure 2.6e) increased with increasing concentration of Ni. The highest level of Ni accumulation was found in *P. falcata* (196 µg plant\(^{-1}\)) followed by *B. cartilagineum* and *N. cordifolia* (both 131 µg plant\(^{-1}\)). Nickel accumulation at the 50 and 100 mg kg\(^{-1}\) treatments ranged from 3.54 (*P. falcata*) to 62 µg plant\(^{-1}\) (*D. aspera*). Accumulation of Cr (Figure 2.6b) was generally similar across all treatment levels, the highest accumulation observed in *P. vittata* (102 µg plant\(^{-1}\)) at the 500 mg kg\(^{-1}\) level.
Figure 2.6 Accumulation (µg plant⁻¹) of Cd (a), Cr (b), Cu (c), Ni (d), Pb (e) and Zn (f) in different fern species after 20 weeks of growth.

Log-transformed data have been back-transformed. Data points and vertical bars are least square means and ± standard errors, respectively (n=3).
2.3.4 Heavy metal translocation by ferns

The translocation factors (TF) is the ratio of heavy metal concentration in the shoot to the heavy metal concentration in the root, and provides an indication of internal heavy metal transportation. Translocation factors for each species are presented in Table 2.4. The data indicate that heavy metals accumulated by the ferns were largely retained in roots, as shown by mean TF values < 1. Among all species, TFs for Cu were the lowest while those of Zn were the highest.

Table 2.4 Mean heavy metal translocation factors across all treatment levels (TF; concentration ratio of heavy metal in the frond to root) of the 10 fern species after 20 weeks of growth. The degree of heavy metal accumulation in harvestable biomass for each species is ranked according to its mean frond heavy metal uptake across the 50 and 100 mg kg\(^{-1}\) levels as denoted in parentheses.

<table>
<thead>
<tr>
<th>Fern Species</th>
<th>Translocation Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cd</td>
</tr>
<tr>
<td>Adiantum aethiopicum</td>
<td>0.38 (5)</td>
</tr>
<tr>
<td>Blechnum cartilagineum</td>
<td>0.21 (6)</td>
</tr>
<tr>
<td>Blechnum nudum</td>
<td>0.60 (10)</td>
</tr>
<tr>
<td>Calochlaena dubia</td>
<td>0.53 (3)</td>
</tr>
<tr>
<td>Dennstaedtia davalliorides</td>
<td>0.88 (2)</td>
</tr>
<tr>
<td>Doodia aspera</td>
<td>0.30 (9)</td>
</tr>
<tr>
<td>Hypolepis muelleri</td>
<td>0.44 (7)</td>
</tr>
<tr>
<td>Nephrolepis cordifolia</td>
<td>0.35 (8)</td>
</tr>
<tr>
<td>Pellaea falcata</td>
<td>0.38 (1)</td>
</tr>
<tr>
<td>Pteris vittata</td>
<td>0.19 (4)</td>
</tr>
</tbody>
</table>
2.4 DISCUSSION

The results obtained from this study demonstrate the degree of physiological response of ferns to various heavy metals. The fern species investigated in this study differed widely in their ability to accumulate heavy metals. In general, significantly greater heavy metal accumulation occurred in roots than fronds suggesting a limited mobility and translocation of heavy metals once absorbed by ferns. Sequestration of heavy metals in roots enables plants to continue growing uninhibited and it is an important means of heavy metal tolerance (Ernst et al. 1992). This commonly observed phenomenon in which plants can restrict translocation of heavy metals from roots to shoots has been reported in numerous recent studies. For example, Deng et al. (2004) found greater Pb, Zn, Cu and Cd in the roots of 12 wetland species growing in metal-contaminated environments. Recently, Srivastava and Ma (2005) screened 11 fern species for Se accumulation and found higher concentrations of Se in the fronds of only three species. Weng et al. (2005) investigated the effect of Cu on four Elsholtzia ecotypes and observed stimulated root growth in ecotype E. splendens when treated with higher concentrations of applied Cu. Furthermore, the authors noted the absence of toxicity symptoms and proposed that an increase in root-to-shoot ratio with the addition of Cu could be a possible strategy leading to increased tolerance in E. splendens. In the present study A. aethiopicum, B. cartilagineum and D. aspera species exposed to Cu had a root biomass greater than frond biomass and, like E. splendens it is possible that this may be a strategy leading to Cu tolerance. These same species also had an increase in root-to-shoot ratio with increasing levels of Pb and, like Cu, it is possible that Pb sequestration in the roots increased tolerance to Pb in these species.

The growth response of ferns investigated in this study suggests the exclusion mechanism of tolerance (Baker 1981). This mechanism is effective up to a threshold value (heavy metal concentration) above which the mechanism breaks down and results in unrestricted heavy metal uptake and eventual death (Baker 1981). Indeed, this mechanism observed in the fern species investigated may be a constitutive property expressed when these ferns are exposed to high concentrations and inimical levels of heavy metals. With the exception of D. davallioiides no species were considered hyperaccumulators because concentrations of heavy metals in fronds remained below the criterion used to define hyperaccumulating species (Baker and Brooks 1989; Baker et al. 2000). Two replicates of D. davallioiides demonstrated hyperaccumulating characteristics (> 0.1% DW Cd) when treated with 500 mg kg⁻¹ of Cd;
however, severe stunting, chlorotic growth and necrotic margins were observed. Similar symptoms also occurred in this species when exposed to 50 and 100 mg kg\(^{-1}\) of Cd and across all treatments, concentrations of Cd were higher in the roots than in the fronds. These observations suggest a likely breakdown of the tolerance mechanism, which is further evident with a reduction in frond biomass of all surviving ferns (Figure 2.1a) and indicates that \textit{D. davallioides} is not a Cd hyperaccumulator. An earlier study by Tam and Singh (2004) identified \textit{N. cordifolia} as a Cu hyperaccumulator (2,324 mg kg\(^{-1}\)) growing naturally on a heavy metal-contaminated soil, however, in this study the mean concentration of Cu in fronds of \textit{N. cordifolia} was below 9 mg kg\(^{-1}\). Furthermore, a greater concentration of Cu was observed in their roots across all treatments (20–112 mg kg\(^{-1}\)).

In the present study, the degree of accumulation for all heavy metals was generally uniform across the 50 and 100 mg kg\(^{-1}\) treatments (Figure 2.6) and thereafter increased sharply when ferns were exposed to the 500 mg kg\(^{-1}\) levels. This pattern of uptake is commonly observed in excluder species and suggests a possible breakdown in the ferns’ tolerance mechanism, resulting in unrestricted uptake of heavy metals. Active transport of heavy metals may have resulted in elevated levels in the fronds of ferns treated at the 500 mg kg\(^{-1}\) level, supported by the presence of necrotic lesions and in some cases death. The species investigated have been subsequently ranked according to their heavy metal-accumulation efficiencies and hence phytoremediation potentials by taking the overall mean heavy metal accumulation in harvestable biomass at 50 and 100 mg kg\(^{-1}\) treatments (Table 2.4). The 500 mg kg\(^{-1}\) level was omitted owing to the onset of severe phytotoxicity and death of some replicates. These results indicate restricted movement of heavy metals into harvestable frond biomass as further seen with TFs < 1 (Table 2.4). This, coupled with the absence of hyperaccumulation, indicates that these ferns are unsuitable for phytoremediation of heavy metals.

However, these species may be suitable candidates for phytostabilisation of contaminated soils. Good phytostabilising plants should tolerate high concentrations of heavy metals and immobilize contaminants in the roots, with minimal translocation to harvestable biomass (Salt \textit{et al.} 1995). This enables harvestable biomass to continue growing uninhibited by heavy metals and further reduces the possibility of heavy metals passing into the food chain through the activity of herbivores (McIntyre 2003). These properties together with survival data (Table 2.3) are of paramount importance when considering species for phytostabilising
purposes. The results from this study suggest that *N. cordifolia* and *H. muelleri* may be suitable in phytostabilisation of Cu-, Pb-, Ni- or Zn-contaminated soils. With respect to each heavy metal, these species grew successfully across a wide range of concentrations, yielded higher quantities of frond biomass and accumulated higher concentrations of metals in fronds than the other investigated species. Both species are rhizomatous, thus enabling them to readily colonise large volumes of contaminated soils and further restrict the possibility of metals movement through leaching and airborne spread. *Nephrolepis cordifolia* is a particularly robust species, common throughout rainforest areas, displaying epiphytic characteristics at times and often considered a garden escape (McCarthy 1998). Similarly, *H. muelleri* is also considered robust, preferring moist shaded conditions in sclerophyll forest, but being also associated with open country (McCarthy 1998). *Dennstaedtia davallioides*, also a rhizomatous species, showed promise in phytostabilisation of soils contaminated with Cu and Zn, and expressed similar growth characteristics to those observed in *N. cordifolia* and *H. muelleri*. *D. davallioides* is associated with moist conditions in rainforest and eucalypt forests and is also regarded as a naturalised species (McCarthy 1998). The suitability of these species for phytostabilisation was further confirmed with minimal phytotoxicity symptoms observed when exposed to metal treatments up to 500 mg kg$^{-1}$.

Both *H. muelleri* and *D. davallioides* belong to the Dennstaedtiaceae family, which suggests that this fern family may be tolerant to heavy metals. Previous studies have reported heavy metal tolerance and uptake across several species within a family. For example, screening of As tolerance and accumulation in ferns by Meharg (2003) indicated that ferns in the family Preridaceae could tolerate up to 100 mg As kg$^{-1}$ in growing medium and could subsequently accumulate between 17 and 2,493 mg As kg$^{-1}$ As. Further investigation is needed to confirm if additional species belonging to the family Dennstaedtiaceae exhibit heavy metal tolerance or hyperaccumulation.

Plants expressing tolerance to heavy metals have evolved different mechanisms including chelation, trafficking and sequestration in order to survive heavy metal-rich growing conditions (Clemens 2001). Cellular and subcellular sequestration of heavy metals in roots among the ferns studied, in particularly *N. cordifolia* and *H. muelleri* may further explain their tolerance when exposed to a variety of heavy metals. Lin et al. (2003) reported that nearly 60% of the total Cu in the roots of *Helianthus annuus* L. was bound to the cell-wall
fraction and the cell wall-plasma membrane. Similarly, MacFarlane and Burchett (2000) reported higher concentrations of Cu, Zn and Pb in epidermal, cortical parenchyma and endodermal cell-wall tissues of *Avicennia marina* roots. These studies suggest that restriction of heavy metals in roots through sequestration is an important mechanism which results in a reduction of uptake and a similar mechanism may have contributed to the tolerance observed in the ferns studied. This aspect warrants further consideration to elucidate if sequestration plays a role in tolerance of heavy metals in ferns.

Among all species investigated, *C. dubia* (Dicksoniaceae) did not exhibit any favourable growth characteristics, such as tolerance to heavy metals (Table 2.3) and accumulation (Figure 2.6) for most of the heavy metals. Similarly, poor growth characteristics were observed in *B. cartilagineum* and *B. nudum*, both members of the Blechnaceae family. In both species, low survival rates (Table 2.3) and heavy metal accumulation (Figure 2.6) was observed, relative to the other species investigated. Similarly, *D. aspera* also a member of the Blechnaceae family, grew poorly in response to heavy metal treatments; however, this species had a higher survival rate than observed for both *B. cartilagineum* and *B. nudum* (Table 2.3). In the surviving replicates from all three aforementioned species, a significant degree of phototoxieties were observed, particularly with respect to Cd and Ni treatments. The symptoms included stunted new growth, necrotic frond margins, chlorosis and interveinal yellowing. These findings suggest that the aforementioned species are less suitable for phytoremediation purposes. *Pteris vittata*, which has been previously identified as an As hyperaccumulator (Ma *et al.* 2001) demonstrated relative tolerance to Cd, Cr and Cu. This species displayed higher accumulation and frond biomass production for Cd, Cr and Cu as compared to several other species investigated. These results indicate that *P. vittata* is not only tolerant to As, but could also be used in phytostabilisation of Cd-, Cr- or Cu-contaminated soils. In addition, a recent study by An *et al.* (2006) demonstrated that *P. vittata* had a high tolerance to Zn and reported concentrations of up to 271 mg kg\(^{-1}\) when exposed to 2,000 mg kg\(^{-1}\) Zn. However, when exposed to 500 mg kg\(^{-1}\) Zn, concentrations were \(\sim 70\) mg kg\(^{-1}\) which are in agreement with the results observed in the present study (73 mg kg\(^{-1}\)).
2.5 CONCLUSION

The present investigation shows that many fern species can absorb a wide range of heavy metals (Cd, Cr, Cu, Ni, Pb, and Zn) and may be used to colonise and remediate heavy metal-contaminated soils in a phytostabilisation system. The exclusion of heavy metals appeared the principal physiological response enabling the survival of these ferns when exposed to heavy metal-rich growing conditions. The accumulation response of most ferns was similar up to 100 mg kg\(^{-1}\) level and generally followed a linear increase. Thereafter, it appeared as though the exclusion mechanism enabling tolerance had deteriorated, as the onset of visual toxicity symptoms becoming more evident, coupled with a sharp increase in accumulation and a reduction in total biomass and survival rates. Moreover, restricted uptake and translocation of heavy metals was limited, with roots containing higher concentrations of metals in most cases. The growth and heavy metal-accumulation characteristics of both *N. cordifolia* and *H. muelleri* could prove useful in phytostabilisation of Cu-, Pb-, Ni- or Zn-contaminated environments, *viz.* abandoned mines, smelters, industrial and urban sites. Similarly, *D. davallioides* may also be suitable for phytostabilisation of Cu- and Zn-contaminated soils. All three species are perennials, rhizomatous and their evasive nature would ensure fast colonisation and establishment to act as a vegetation cover in order to prevent erosion and minimise the spread of heavy metals. Conversely, species belonging to the family Blechnaceae showed poor growth characteristics and would be unsuitable for such a purpose. Field-based studies of heavy metal-contaminated sites are warranted to address the scope and application of these species. Such studies should address environmental factors including temperature and light intensity and soil factors *e.g.* moisture content, metal bioavailability and multiple-metal systems.