School of Molecular and Life Sciences

Evidence for Heavy Metal Accumulation in Native Australian Plants

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

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Date: 13/08/2021

Abstract

Potentially toxic metal contaminants, or heavy metals; are ubiquitous in the environment but can be found elevated above background concentrations due to anthropogenic activities such as mining. Post-mining rehabilitation and ecological restoration attempts to return native vegetation to altered landforms created during the creation and running of the mine site. Landforms such as dry stacked tailings can contain remnant heavy metals not removed during the extraction of desired ores. Plants that are capable of growing on tailings are exposed to heavy metals, which can accumulate into plant biomass. It was proposed that plants would bioaccumulate cadmium (Cd) and lead (Pb) into their biomass, and different native plant species would interact with these heavy metals either by sequestering them into roots or having them transported to the shoots via nutrient uptake pathways. Several analytical chemistry detection instruments (ICP-MS, MP-AES, FAAS, and ASV) were employed to determine accuracy and efficiency when analysing biological samples with a complex matrix. The concentration of Cd in plants (determined using ICP-MS) ranged from 0.1–2.30 μg g^{-1}, while lead was found between 1.0 ± 0.11 μg g^{-1} to 8.7 ± 2.61 μg g^{-1}. Heavy metal dispersal in plant biomass was varied based upon substrate type, and the accumulation and storage of heavy metals was idiosyncratic among species.

Behaviour of toxic metals in native plants was further investigated in a second experiment that used metal solutions at concentrations of 25 μg mL^{-1}, 50 μg mL^{-1}, 100 μg mL^{-1} and 200 μg mL^{-1} containing As, Cd and Pb. Soils were spiked with these different solutions for an 8-week plant growth trial, with sampling of soil and water leachate undertaken to monitor changes in heavy metal concentrations over time. It was theorised that increasing concentrations of heavy metals in soils would result in concomitantly higher concentration of heavy metals accumulated into plant biomass. It was also proposed that the amount of heavy metals originally in the soil would reduce due to plant uptake and bioaccumulation. A positive correlation between spiked soil treatments and plant heavy metal concentrations was found for *Acacia saligna* (Fabaceae) and *Austrostipa scabra* (Poaceae) when exposed to As and Cd, while although *Allocasuarina huegeliana* exhibited a positive correlation with As, Cd uptake plateaued, possibly due to the species producing cluster roots that release exudates which immobilise Cd in soil. The uptake of Pb in all three species was not correlated with higher soil Pb concentration, and analysis of soil and water leachate indicated Pb was not mobile in the substrate. This study highlights the persistence of toxic heavy metals in the environment and their ability to transfer from

substrate to plant biomass. The implications of this could see heavy metals becoming a contamination issue when post-mining land is designated for agricultural uses and potentially lead to animal and human exposure through the food chain. This study also emphasises the need to monitor heavy metal concentrations in the environment and undertake preventative measures such as capping tailings with topsoil to stabilise landscapes and selecting species for re-vegetation that can tolerate and sequester heavy metals to prevent further dispersion and exposure.

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We acknowledge that Curtin University works across hundreds of traditional lands and custodial groups in Australia, and with First Nations people around the globe. We wish to pay our deepest respects to their ancestors and members of their communities, past, present, and to their emerging leaders. Our passion and commitment to work with all Australians and peoples from across the world, including our First Nations peoples are at the core of the work we do, reflective of our institutions' values and commitment to our role as leaders in the Reconciliation space in Australia.

Statement of Candidate contribution

The study presented in Chapter 1 is in preparation for submission to a peer-reviewed journal.

Schuurmans, J. E., Lewis, S. W., Wajrak, M., Cross, A. T. Determining the most effective analytical method for quantifying phytoextraction capabilities in native plants (*In preparation for submission)*

I contributed 70% which includes the conception and design, acquisition of data and methods, data conditioning and manipulation, analysis and statistical method and interpretation and discussion. ATC contributed to experimental design, sample collection, result interpretation and editing. SWL contributed with methodology development, interpretation of results and editing. MW contributed through consulting and training in electrochemistry analysis.

The study presented in Chapter 2 is in preparation for submission to a peer-reviewed journal

Schuurmans, J. E., Lewis, S. W., Wajrak, M., Cross, A. T. Australian native plants grown in arsenic, cadmium and lead spiked soil to determine accumulation capabilities and phytoremediation potential. (*In preparation for submission*)

I contributed 80% which includes the conception and design, acquisition of data and methods, data conditioning and manipulation, analysis and statistical method and interpretation and discussion. ATC contributed to experimental design, result interpretation and editing. SWL contributed to experimental design, development of working procedures and editing.

Table of Contents

Table of Figures

List of Tables

List of Abbreviations and Acronyms

A. acutivalvis	*Allocasuarina acutivalvis*
A. elegantissima	*Austrostipa elegantissima*
A. huegeliana	*Allocasuarina huegeliana*
AM	Arbuscular mycorrhizal
A. ramulosa	*Acacia ramulosa*
As	Arsenic
A. saligna	*Acacia saligna*
A. scabra	*Austrostipa scabra*
ASV	Anodic Stripping Voltammetry
Ca	Calcium
Cd	Cadmium
C.I.	Confidence interval
Cr	Chromium
CR	Cluster roots
CRM	Certified reference material
ECM	Ecto-mycorrhizal
E. loxophleba	*Eucalyptus loxophleba*
FAAS	Flame Atomic Absorption Spectroscopy
Fe	Iron
GLM	General linear model
Hg	Mercury
H. recurva	*Hakea recurva*
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
KIOP	Karara Iron Ore Project
LOD	Limit of detection
LOR	Limit of reporting
M. brevifolia	*Maireana brevifolia*
M. georgei	*Maireana georgei*
Mn	Manganese

MP-AES	Microwave Plasma Atomic Emission Spectroscopy
NF	Nitrogen fixing
NM	Non-mycorrhizal
P	Phosphorus
Pb	Lead
PDV	Portable digital voltammeter
ppb	Parts per billion
ppm	Parts per million
TSF	Tailings storage facility
µg mL^{-1}	Microgram per millilitre
µg g^{-1}	Microgram per gram
VAS	Voltammetric Analysis System
Zn	Zinc

General Introduction

Potentially toxic metal contaminants are prevalent in the environment, but are commonly found at elevated concentrations as a consequence of anthropogenic influences (Dudka & Adriano, 1997; Verbruggen *et al.*, 2009). These influences range from mining excavation, extraction processes, combustion of fossil fuels, pesticide and fertiliser use, battery, pigment and plastic manufacturing, and pharmaceutical uses that have all resulted in complications with storage, waste disposal and industrial effluent (Khan *et al.*, 2008; Wuana *et al.*, 2010; Ali *et al.*, 2013). One of the largest contributors is the mining industry, having a long legacy of metal contamination in soils and aquatic systems around the world (Marques *et al.*, 2009; Azam & Li, 2010; Franco-Hernández *et al.*, 2010; Sun *et al.*, 2010; Wuana & Okieimen, 2011). The majority of contamination from mines occurs during the handling and disposal of waste materials (Gosar, 2004; Azam & Li, 2010), including tailings which are produced as a by-product when desired minerals are extracted from the mined rock (Gosar, 2004; Zhang *et al.*, 2014; Sarwar *et al.*, 2017). Tailings contain secondary minerals, including toxicants such as heavy metals, including arsenic (As), cadmium (Cd), chromium (Cr), lead (Pb), and mercury (Hg) (Dudka & Adriano, 1997; Gosar, 2004; Tchounwou *et al.*, 2012; Hu *et al.*, 2013). Waste management of tailings results in these materials either being stored in large dam structures as liquid or paste (Azam & Li, 2010), or being de-watered and deposited onto land surrounding the mine in a technique called dry stacking (Read *et al.*, 2012; Amoah *et al.*, 2018). The edaphic conditions and biological function of soils and landforms surrounding mines can be altered chemically and physical when tailings are deposited on land (McGrath *et al.*, 2001; Wu *et al.*, 2019). Soils are also a major sink for heavy metals when released into the environment (Wuana & Okieimen, 2011), contributing further to the unfavourable conditions presented by nutrient-poor tailings upon which vegetation must grow when rehabilitation or restoration of this landforms is attempted (Raskin, 1995; Ernst, 2006; Pourrut *et al.*, 2011; Cross & Lambers, 2017; Cross *et al.*, 2018). This has resulted in a re-evaluation of how mine site operators need to be liable for, and should fund, the rehabilitation of post-mining land as a statutory requirement. At a bare minimum, industry in mining provinces such as Western Australia are required to make landforms safe, stable and non-polluting/non-contaminating (Parliament of Western Australia, 2012; Glenn *et al.*, 2014; Australia Government, 2016). This non-polluting requirement, however, is particularly challenging;

secondary heavy metals that were not extracted with the desired ore are assimilated into the substrate and do not break down, remaining persistent in the environment (Clark *et al.*, 2001; Gomes *et al.*, 2016).

Phytoextraction is the process by which plants are purposely introduced to remove heavy metals from contaminated soil or water by storing heavy metals in root or shoot tissues (Baker, 1981; Baker & Walker, 1990). Plants have evolved to have a synergistic balance of chemical uptake for nutrient supply and energy conversion; where there is a chemical imbalance due to an accumulation of toxic metals this can induce nutrient deficiencies and cause stress (Kabata-Pendias, 2000). For plants to be successful at establishing on heavy metal-contaminated land, they need to adopt strategies to tolerate heavy metal accumulation while still being able to function and grow without succumbing to toxic side-effects (Baker, 1987; Baker & Walker, 1990; Khalid *et al.*, 2017; Sarwar *et al.*, 2017). There are two primary approaches that plants have developed to cope with the uptake of heavy metals: the exclusion mechanism attempts to sequester the heavy metals to the below-ground biomass where the metals are stored in the root cell walls and vacuoles to prevent transport into shoot biomass (Menon, 1998; McGrath & Zhao, 2003; Hanikenne & Nouet, 2011; Pollard *et al.*, 2014; Khalid *et al.*, 2017), while the accumulation mechanism sees the dispersion of heavy metals in the stems and leaves of plants, allowing for greater uptake of heavy metals while the plant is growing and biomass increasing (Baker, 1981; Shaw, 1989; McGrath *et al.*, 2001; Ernst, 2006). The transportation of heavy metals from substrate to plant biomass relies heavily on soil pH (to promote metal solubility) and organic matter in the soil for adsorption or possible chelate formation (Bradl, 2005). It is also dependent on plant nutrient-acquisition strategies; for example, the presence of mycorrhizal fungi which assist plants in scavenging for macro or micronutrients can create competition with heavy metals to be up-taken by plants (Meharg & Hartley-Whitaker, 2002; Clemens, 2006; Khalid *et al.*, 2017; Sarwar *et al.*, 2017). This can be seen with arsenate (AsO_4^{3-}) being up-taken into plant biomass instead of the essential phosphate (PO_4^{3-}) (Bradl, 2005). Although heavy metals may be locked in plant tissue, they can remain a potential contamination issue, providing an entry point for heavy metals into the food chain (Ali *et al.*, 2013).

With post-mining land often being designated to either conservation, cropping or grazing (Australia Government, 2016), plants with heavy metals into their biomass will create an

exposure risk to humans, wildlife and livestock through ingestion of plant tissue through several methods (Morillo *et al.*, 2008; Ali *et al.*, 2013; Ružičková *et al.*, 2018). The predominant form of toxic metal exposure to humans is through ingestion of crop-derived food where crops are grown on contaminated agricultural land (Zhang *et al.*, 2010; Robinson *et al.*, 2015; Kumar *et al.*, 2017; Sarwar *et al.*, 2017). Humans are also at risk of biomagnification at the top of the food chain due to the consumption of dairy and meat products from livestock that has grazed and bioaccumulated heavy metals from directly ingesting plants and sediment (Thornton & Abrahams, 1983; Burger & Gochfeld, 1993; Burger, 2002; Smith *et al.*, 2009; Tsipoura *et al.*, 2011; Wuana & Okieimen, 2011). Priority metals that are of significant concern to public health are As, Cd, Pb and Hg, these are considered to be the most toxic heavy metals found in the environment (Goyer, 2004). Although, in literature they often fall under the umbrella term 'heavy metals', to describe their density and toxicity, it is actually a misnomer (Duffus, 2002; Tchounwou *et al.*, 2012; Pourret, 2018). An example of this is describing As, as a heavy metal when it is not a transitional or dense metal, making the 'heavy metal' description misleading and it being phased out in literature (Pourret, 2018). However in this text the term 'heavy metals' is used to exclusively refer to As, Cd, Pb and Hg due to their toxicity in the environment. The toxicity of metals is reliant on the concentration, exposure pathway, chemical species present and molecule structure (Tchounwou *et al.*, 2012). Other factors that influence the toxicity of heavy metals are factors such as the age, gender, genetics and health status of an exposed individual (Tchounwou *et al.*, 2012). The toxicity of heavy metals is also aggravated by their persistence in the environment (Garbisu & Alkorta, 1997; Marques *et al.*, 2009). Once heavy metals have been ingested they can cause a combination of chronic and acute effects on the neurological, gastrointestinal, haematological, cardiovascular and skeletal systems, and may be stored in teeth and bones and distributed to brain, liver and kidneys (WHO, 2010, 2011, 2019).

The potential threat of heavy metal exposure to plants, animals and humans is an issue that has been reported previously with cattle in England grazing on As contaminated land (Thornton & Abrahams, 1983; Abrahams & Thornton, 1994). Other species like birds have ingested contaminated plants and accidentally ingested sediment resulting in Pb and Cd being found to accumulate in their feathers and eggshells (Burger & Gochfeld, 1993; Burger, 2002; Tsipoura *et al.*, 2011). Currently in Western Australia one of the state's biggest exports is iron ore. In 2020 Western Australia was the largest supplier of iron ore in

the world, contributing approximately 909 million tonnes (WA Government, 2021). The iron ore mine life in Western Australia has been estimated to be stable for another 53 years (WA Government, 2021). In the Mid-West region of the state the Karara Iron Ore Project (KIOP), has had three heavy metals detected in its magnetite (Fe_3O_4) tailings; As (0.2 ± 0.01 µg g^{-1}), Cd (0.06 ± 0.01 µg g^{-1}) and Pb (5.1 ± 0.7 µg g^{-1}) (Cross & Lambers, 2017). Despite these concentrations being lower than the human health and ecological investigation levels outlines by National Environment Protection Measure (2011), they are can still have negative consequences for plant development and lead to bioaccumulation (Kabata-Pendias, 2000).The magnetite tailings have a high pH >9, contain negligible biologically-available nitrogen (N), and lack organic matter and key macronutrients to support biological soil function (Cross & Lambers, 2017; Wu *et al.*, 2019). Despite active rehabilitation and restoration of tailings being undertaken around the world, attempting to ameliorate their poor qualities and provide favourable substrates for plant growth, heavy metals are persistent and remain in the soil as competition for nutrient uptake in plants (Salt *et al.*, 1995; Hanikenne & Nouet, 2011; Hore & Luppnow, 2014; Robinson *et al.*, 2015). Toxic heavy metals can create competition by entering a plant using their nutrient channels due to being similar in mass and charge and accumulating in the roots (Menon, 1998; Hanikenne & Nouet, 2011). There is particularly a limited understanding of the phytoextraction potential for plants in alkaline substrates, where metal solubility and mobility is limited (McKenzie, 1980; Bradl, 2005).

The legacy of mine site contamination needs to be addressed to increase our understanding of potential and persistent heavy metal pollution (Dudka & Adriano, 1997; Clark *et al.*, 2001). There is also a need to understand the mobility of heavy metals and where they end up in the environment, and the potential for further exposure to higher trophic levels in the food chain or from drinking water (WHO, 2011; Ali & Khan, 2019). While there are species around the world that are proven effective hyperaccumulators of heavy metals, the number identified in Australia is limited by the vast biodiversity of species to assess (Zhang *et al.*, 2014; Reeves *et al.*, 2018). The handful of species identified in Australia so far hyperaccumulate Co, Mn, Se and Zn, and not the targeted toxic heavy metals identified in the magnetite tailings (Reeves *et al.*, 2018). Plants that hyperaccumulate heavy metals are usually only tolerant to one type of heavy metal, making it important to have a selection of native and diverse species that can survive in nutrient-poor substrates such as tailings and target a variety of problematic toxic heavy

metals (Baker & Brooks, 1989; Gardea-Torresdey *et al.*, 2005; Mok *et al.*, 2013). Restoration efforts can also benefit from understanding the behaviour and transportation of heavy metals into plants that may differ between individual species and their nutrient-acquisition strategies (Baker, 2000; Wang *et al.*, 2020).

This research aims to bridge the knowledge gap and provide insight into phytoextraction in native Australian plants. It is also a study on how different nutrient-acquisition strategies in plants interact with different heavy metals and what mechanisms are taking place for a plant to accumulate or sequester and tolerate heavy metals. The comparison of analytical instruments determines the effectiveness of emerging technology or underutilised techniques for heavy metal analysis. It also determines which analysis type is suitable for plants with can prove difficult to detect metals in due to a high matrix and interferences that occur. Developing this research can have multiple benefits including progress towards understanding how native plant species will cope in nutrient-poor substrates containing toxic metals and provide valuable knowledge for development of post-mining land.

Research aims

For the study undertaken in Chapter 1, it was hypothesised that bioaccumulation of toxic heavy metals was occurring for plants growing on alkaline magnetite tailings and that plants will have a higher concentration in the root sections when compared to the shoots. It also hypothesises that based on metal adsorption and uptake pathways of plants; there was an positive linear relationship between Fe and Cd and also Ca with Pb. Additionally, the analysis using electrochemistry and emission spectroscopy will produce concentration values for Cd and Pb that are in a similar range to inductively coupled plasma mass spectrometry (ICP-MS).

The primary objective for this chapter was to use analytical chemistry to find evidence of heavy metals in native Australian plants grown near an iron ore mine or directly in magnetite tailings. This was accomplished by:

1. Determining phytoextraction capabilities by preparing and analysing plant species grown in topsoil and tailings for their Cd and Pb concentrations.
    a. Analyse plants grown in different substrates to determine potential sources of heavy metal exposure.

b. Separate and analyse shoots and roots of plants to determine heavy metal tolerance mechanisms.
c. Determine the solubility, transportation and bioaccumulation behaviour of Cd and Pb in plants.

2. Implement a digestion technique to efficiently analyse plants and approach the difficulties of using analytical detection instruments.
    a. Determine a digestion methodology that prepares plants for analysis of toxic heavy metals.
    b. Compare spectroscopy and electrochemistry detection techniques for their accuracy, sensitivity, reliability, cost and interferences occurred.
    c. Determine the need to implement environmental monitoring of mine sites for ongoing toxic heavy metal exposure using the methods developed.

The experimentation in Chapter 2 sets up a controlled environment to quantify toxic heavy metal uptake in plants through soil spiking and continues the use of analytical chemistry's role in the determination of heavy metals in plant biomass, being used not only to determine the concentrations of these elements found in plants but also to establish their mobility and spatio-temporal distribution in soil and water. When investigating a soil spiking experiment using As, Cd and Pb it was hypothesised that native plants grown in the higher soil treatments of heavy metals would have a positive correlation and as a response would have increasing concentrations in the plant leaf tissue. This was under taken by:

1. Determining tolerance capabilities and phytoremediation potential by spiking natural topsoil with As, Cd and Pb and growing native species in the altered substrate compared to a control.
    a. Determine plants response to As, Cd and Pb by determining concentrations in above ground shoot biomass compared with individual species nutrient acquisition strategies.
    b. Observe concentration changes in soil and water leachate over an 8 week period.

c. Approach herbivory concerns and suggest monitoring implementation based on soil interactions with different plants species and their relationship with mycorrhizal fungi.

Thesis outline

Chapter 1 focused on developing analytical methods for detecting heavy metals in plants grown on alkaline mine tailings. It compares heavy metal concentrations in plants grown in different substrates, as well as the concentration results returned from different analytical instruments. The chapter discusses and compares analytical instruments based on their ability to analyse trace quantities of heavy metals for plants digested in nitric acid, creating a highly complex matrix. It also addresses interferences encountered by the instruments that distorted detection capabilities, and determines which instruments are suitable for this type of analysis and could be implemented for ongoing monitoring of heavy metal accumulation in plants.

Chapter 2 focused on the plant physiology and nutrient-acquisition strategies for determining metal accumulation and a plants response to increasing concentrations of heavy metals in the substrate. This chapter examines what is happening to the heavy metals in soils (i.e. whether they are bound to organics in soil or released in water leachate) and where they end up which includes their entry into the food chain. This is followed by a discussion on the appropriate uses for post-mining land.

Both chapters highlight the need for more comprehensive and ongoing monitoring of heavy metals in soil and plants, particularly in the context of mine sites and mined land features such as tailings storage facilities that may be utilised for post-mining land uses such as agriculture, cropping, or conservation. The methodologies developed in this thesis can assist with more appropriate design and decision making for the mining industry and in restoration projects, towards minimising the distribution of heavy metal contaminants in environments and limiting exposure pathways for plants, animals and humans.

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Chapter 1:

Determining the most effective analytical method for quantifying phytoextraction capabilities in native plants

Abstract

Alkaline magnetite (Fe_3O_4) mine tailings left from an iron ore mine in Western Australia contain heavy metals such as cadmium (Cd) and lead (Pb). Ecological restoration attempts on such chemically altered substrates means plants are potentially exposed to these heavy metals. This study undertook several analytical chemistry techniques (FAAS, MP-AES, ICP-MS and ASV) to analyse heavy metal concentrations in plants grown under glasshouse conditions in tailings, natural topsoil and tailings capped with topsoil to determine whether bioaccumulation of heavy metals was occurring. The investigation also studied how different plant species interacted with different substrates, and whether heavy metals were differently stored in plant biomass among species. Additionally, it looked at the relationship between heavy metal adsorption and uptake associated with calcium (Ca) and iron (Fe) nutrient-acquisition pathways. Results showed Cd and Pb were detected in higher concentrations for plants grown in tailings compared to topsoil. As shown in *Maireana georgei* shoots where Pb concentration was more than three times greater in in tailings (7.0 ± 4.28 µg g^{-1}) compared to those grown in topsoil (2.1 ± 0.08 µg g^{-1}). When comparing tailings capped with topsoil there was seven times less heavy metal uptake in *Maireana georgei shoots* (1.0 ± 0.11 µg g^{-1}). Cadmium was shown to be equally distributed between both the roots and shoots of plants, while Pb was shown to be preferentially sequestered in root tissues. Lead uptake in plants was also linked to nutrient-acquisition pathways for calcium (Ca) and iron (Fe), which found a positive correlation for higher lead concentrations in plant tissue. The analytical technique ASV was highly comparable, with no significant differences to ICP-MS in its detection capabilities and both were able to detect heavy metal concentrations without limitation from interferences. When requiring a portable, highly sensitive and cost efficient instrument, ASV is superior.

1.1 Introduction

The contamination of post-mining land with heavy metals occurs around the world (i.e., Karara, San Luis Potosí, Sudbury, and Yerranderie) and represents a significant environmentally-deleterious issue potentially causing a multitude of cascading ecological effects (Dudka & Adriano, 1997; Archer & Caldwell, 2004; Franco-Hernández *et al.*, 2010; Cross & Lambers, 2017). Extractive industries such as mining can devastate landscapes, not only through the process of habitat loss but also through secondary disturbances to adjacent ecosystems stemming from the deposition of mine site waste (Liu *et al.*, 2003; Gosar, 2004; Lin *et al.*, 2005). Desired ore is often a minute quantity of what is extracted from the earth compared to the volumes of waste material such as highly-processed tailings that are produced and deposited on the land (Dudka & Adriano, 1997; Gosar, 2004). Tailings are mining residues leftover from extracting mineral ores, and are generally extremely fine-grained with a powder-like texture (Wong, 2003; Jamieson, 2011; Huang *et al.*, 2012; Cross *et al.*, 2019). Tailings can be broadly divided into two groups based on chemical composition: acidic, or alkaline. Acidity is generated when tailings containing an abundance of sulphide minerals (often pyrite) is oxidised (Dold, 2014). High alkalinity (pH >9) occurs in magnetite iron ore tailings, for example, due to the addition of alkaline chemicals in the reverse flotation process when extracting iron ore (Wu *et al.*, 2019). Magnetite tailings also lack essential plant-required nutrients such as nitrogen (N) and organic carbon (C) (Cross *et al.*, 2019; Wu *et al.*, 2019). Toxic metal elements which are improperly referred to as heavy metals, (an umbrella term that describes density and toxic metals and metalloids) such as arsenic (As), cadmium (Cd) and lead (Pb) are often found in tailings as secondary minerals that were not extracted with the desired ore (Dudka & Adriano, 1997; Gosar, 2004; Cross & Lambers, 2017). The chemical and physical characteristics of tailings provide a different set of edaphic conditions to the original substrate and represent an unweathered, nutrient-poor substrate that may also contain traces of heavy metals (Clark *et al.*, 2001; Cross & Lambers, 2017).

Restoration practices on landscapes affected by metal mining have tried amendment techniques such as the integration of topsoil to ameliorate the unfavourable chemical profile of tailings, and allow native plants a chance of establishment and survival (Huang *et al.*, 2012; Cross *et al.*, 2018; Wu *et al.*, 2019). Heavy metals, however, cannot be ameliorated easily; they do not break down or degrade over time, and remain in the substrate as a persistent contaminant that establishing vegetation is then exposed to

(Dudka & Adriano, 1997; Clark *et al.*, 2001; Pourrut *et al.*, 2011; Robinson *et al.*, 2015; Ullah *et al.*, 2015). Through attempts at remediation, plants grown in metalliferous soils potentially uptake and accumulate heavy metals from soil in a process known as phytoextraction (Baker, 1981; Salt *et al.*, 1995). Attempts at finding metal tolerant plants to use in remediation are usually metallophytes species that are growing naturally near ore deposits suggesting potential tolerance abilities (van der Ent *et al.*, 2013). A study in China was able to do this by using a native Chinese Brake Fern (*Pteris vittata*) to successfully remove 3 – 704 μg g^{-1} As from the soils around an As mine site (Wei & Chen, 2006). Uptake into the plant can occur through their nutrient channels when heavy metals in soil are of a similar mass and charge to vital macro and micronutrients, i.e. Ca^{2+} and Cd^{2+} and are thus up-taken through cortical root tissue (Menon, 1998; Hanikenne & Nouet, 2011). Elements such as As, Cd and Pb commonly contained within mine tailings are known plant toxicants, and can also bioaccumulate in animals and humans having a detrimental effect on growth and development (Franco-Hernández *et al.*, 2010; Tchounwou *et al.*, 2012). There is no positive biological function for heavy metals in plant biomass, as these elements are not required for plant nutrition (McGrath *et al.*, 2001; Lambers *et al.*, 2008). Heavy metal uptake in plants can also be influenced by root architecture, plant functional traits, and the presence or absence of mycorrhizal associations (Callahan *et al.*, 2006; Clemens, 2006). Mycorrhizal fungi (arbuscular or ectomycorrhizal) have a symbiotic relationship with plants and help obtain nutrients from soil, and can sequester heavy metals in plant roots and prevent their transportation to above-ground biomass (Clemens *et al.*, 2002; Callahan *et al.*, 2006; Lambers *et al.*, 2008).

Elements such as As, Cd and Pb have been detected in tailings from several mines around the world, including Mexico, Canada and Australia (Dudka & Adriano, 1997; Archer & Caldwell, 2004; Franco-Hernández *et al.*, 2010). At the Karara Iron Ore Mine in Western Australia, unweathered magnetite tailings occur at concentrations of 0.2 ± 0.01 μg g^{-1} As, 0.06 ± 0.010 μg g^{-1}Cd, and 5.1 ± 0.70 μg g^{-1} Pb (Cross & Lambers, 2017). To be able to detect the presence of heavy metals in plants growing in or around mine tailings, biomass needs to undergo preparation to ensure the metal ions are extracted in a sample and will be detected with an analytical instrument (Havlin & Soltanpour, 1980; Zarcinas *et al.*, 1987; Zhang *et al.*, 2014; Addis & Abebaw, 2017). The capabilities of the instrument require it to detect trace quantities of heavy metals in a complicated matrix of elements while being free of any interferences (Pyle *et al.*, 1995). Flame Atomic absorption

spectrometer (FAAS) has been widely used for its detection capabilities of metals in all matrices (Barbooti, 2015). FAAS instruments run on acetylene gas, which creates a flame the sample is aspirated into and with the use of a hollow cathode lamp, measures the wavelength of light absorbed by the element (Pyle *et al.*, 1995; Barbooti, 2015). The detection of each element relies on hollow cathode lamps, so determination of an element is an individual analysis and requires samples to be re-run for a different elemental analysis (Barbooti, 2015).

Microwave plasma is an emerging technology that is quickly becoming a contender to replace FAAS instruments (Barbooti, 2015; Agilent Technologies, 2016; Fernandez de la Fuente *et al.*, 2017). Microwave plasma atomic emission spectrometers (MP-AES) eliminate the use of a naked flame and can be set up with an auto-sampler, resulting in superior efficiency and safety when compared to FAAS and its need to have an open flame with acetylene gas (Agilent Technologies, 2016). Microwave plasma works by being heated with argon gas and then stabilising and maintaining the temperature by running a constant flow of nitrogen gas, making it significantly cheaper to run than an ICP-AES analysis which relies on a constant argon gas flow (Barbooti, 2015; Agilent Technologies, 2016). MP-AES is reported as having a high correspondence with ICP-MS and an excellent sensitivity, with a wide working range and low detection limits to produce almost identical results to ICP (Karlsson *et al.*, 2015). An inductively coupled plasma mass spectrometer (ICP-MS) is the most common choice for metal analysis as its potential in analytical applications have been well established (Thompson & Walsh, 1989; Pyle *et al.*, 1995; Rehkämper *et al.*, 2001; Karlsson *et al.*, 2015). The ICP-MS instruments are considered highly stable and versatile in major, minor and trace elemental analysis within a range of complex matrices (Tirez *et al.*, 2015; Varbanova & Stefanova, 2015), and are often able to out-perform other spectroscopy instruments due to their higher plasma temperatures (Varbanova & Stefanova, 2015). The ICP-MS instrument has also shown the least amount of spectral interference compared to emission and absorbance spectroscopy when analysing complex matrices containing iron (Fe) and calcium (Ca) (Yoon *et al.*, 2005). The ICP instruments have a vast collection of literature available supporting their capabilities and are a widespread tool throughout industry and research (Thompson & Walsh, 1989; Rehkämper *et al.*, 2001).

A common disadvantage shared between FAAS, MP-AES and ICP instruments is their requirement to be used in a laboratory with gas. In contrast, electrochemical analysis can be made portable (Wajrak & Rummey, 2004). A voltammetry instrument like a PDV6000+ (B3 Electronic Design) is portable and ideal for in-situ analysis while being inexpensive to operate (Wajrak & Rummey, 2004). Voltammetry analysis has a sensitivity and limit of detection comparable to ICP and has the added benefit of being able to provide species characterisation between ions (Wang, 1985; Wajrak & Rummey, 2004). The electrochemistry technique applied in this study is anodic stripping voltammetry (ASV) and is used to detect metal ions in aqueous solutions (Bard *et al.*, 1980; Pyle *et al.*, 1995; Thomas, 2001; Modern Water, 2012). It works by accumulating a mercury film that is plated onto a glassy working electrode and applying a reducing potential to the electrode while submerged in aqueous $HgCl_2$ (Thomas, 2001; Modern Water, 2012). The aqueous sample is placed into a cell with the working electrode, along with a reference and counter electrode and the potential is reverse and ramped up in a positive direction causing the metal strip off and oxidise back into solution (Pyle *et al.*, 1995; Thomas, 2001; Modern Water, 2012). During this final stage, the current in the sample is measured and analysed to determine the concentration of metal ions (Modern Water, 2012). ASV can deduce metal detection by standard addition and has the ability to rule out interference from other elements and matrix effects (Thomas, 2001; Modern Water, 2012). The data gathered is used to make the comparisons of analysis between instruments based on accuracy, sensitivity and detection range along with cost, reliability and portability.

Environmental chemistry has the opportunity for further development and usefulness in restoration science by bridging a gap between analytical chemistry applications that can be used in determining bioaccumulation and monitoring post-mining land for flow-on effects of heavy metal exposure. Developing this research will allow for a better understanding of heavy metal transportation in plants when grown in alkaline substrates and further investigate how nutrient-acquisition strategies like the presence of mycorrhiza play a role in accumulation or sequestering. The comparison of an electrochemical technique against spectrometry can give insight to combating high matrix biological samples that give off interference when targeting trace metals. For this study, it was hypothesised that bioaccumulation of toxic metals would be evident for plants growing on alkaline magnetite tailings, and that plants would have a higher concentration of heavy metals in root biomass compared with shoot biomass. It was also theorised that based on

metal adsorption and uptake pathways of plants there would be a positive correlation between Fe and Cd uptake, and a positive correlation for Ca with Pb uptake within plants. Additionally, it was expected that electrochemical analyses would produce concentration values for Cd and Pb in a similar range to spectrometry analysis techniques.

1.2 Methods

1.2.1 Study sites

Magnetite tailings and natural topsoils were sourced from the Karara Iron Ore Project (KIOP) tailings storage facility (TSF), located in the Midwest region of Western Australia, 225 km east of Geraldton. Native plant species were chosen based on their abundance around KIOP and for their variation in nutrient-acquisition strategies and root systems. The species chosen, included *Acacia ramulosa* (*Fabaceae*), *Allocasuarina acutivalvis* (*Casuarinaceae), Eucalyptus loxophleba (Myrtaceae), Maireana georgei (Chenopodiaceae), Hakea recurva (Proteaceae) and Austrostipa scabra (Poaceae)* (Table 1.1). Seeds were sown in May 2018 and seedlings grown in a glasshouse environment at Shenton Park at UWA, Western Australia. Plants were grown in PVC tapered square pots (50 mm diameter x 120 mm height, 210 ml volume), using natural topsoil (as the control), magnetite tailings and a third substrate that was tailings amended by being capped with natural topsoil in a 3:1 ratio (water capacity maintained at 20%), the six species grown in the three substrates were replicated five times each creating 90 potential samples. The plants were harvested in November 2018, after six months of growth, and were washed in deionised water and then dried in an industrial oven for three hours at 70°C.

Above-ground biomass samples from five individuals each of *Acacia ramulosa, Austrostipa scabra, Eucalyptus loxophleba, Maireana brevifolia (Chenopodiaceae) and Maireana georgei* (Table 1.1) were also collected from undisturbed areas of the KIOP, and of *Maireana georgei, Maireana brevifolia and Austrostipa scabra* from areas of the TSF, in August 2019. A total of 40 samples were obtained and transported back to Perth to be dried and prepared for analytical analysis.

Table 1.1 Native plant species grown in topsoil, tailings and capped tailings substrates to compare heavy metal accumulation in the plants biomass sections of shoots and roots. Nutrient-acquisition strategies from Lambers *et al.* (2008), Brundrett (2009) and (Cross *et al.*, 2019).

Species name	Common name	Family	Nutrient-acquisition strategy
Acacia ramulosa	Horse mulga	Fabaceae	AM/NF
Allocasuarina acutivalvis	Sheoak	Casuarinaceae	ECM/AM/NF/CR
Austrostipa scabra	No common name	Poaceae	AM
Eucalyptus loxophleba	York gum	Myrtaceae	ECM/AM
Hakea recurva	Djarnokmurd	Proteaceae	CR
Maireana brevifolia	Small leaf bluebush	Chenopodiaceae	NM
Maireana georgei	Satiny bluebush	Chenopodiaceae	NM

Nutrient-acquisition strategies; CR = cluster root, NF = nitrogen fixing, ECM = ectomycorrhizal, AM = arbuscular mycorrhizal, NM = non-mycorrhizal

1.2.2 Preparation and Analysis of Plants

There were 72 dried plants used for heavy metal analysis, each segregated into above- and below-ground biomass before being pulverised in a grinder (Geno/Grinder 2010) using zirconium pearls. Plants were dried again, overnight at 70°C and then digested in nitric acid following procedures trialled and adapted from Havlin and Soltanpour (1980), Zarcinas *et al.* (1987), and Franco-Hernández *et al.* (2010). A successful method was developed by using 0.5 g of dry plant matter and digesting it in 10 ml of 70% concentrated nitric acid and placing it immediately on a hot plate for approximately two hours at 160°C. When plant matter was completely dissolved the digested solution was filtered and made to 50 ml using 1% nitric acid to ensure 1% of total dissolved solids in solution before undergoing metal detection analysis. Laboratory protocols DOI: dx.doi.org/10.17504/protocols.io.bwefpbbn

1.2.3 Instrument Analysis

All plants are individually digested and the samples undergo analysis with each instrument for Cd and Pb. Analysis was initially performed with a 4200 MP-AES (Agilent Technology) and a follow-up analysis of Pb was conducted with FAAS (Agilent Technology). Further analysis for Cd and Pb using ICP-MS, and Ca and Fe using ICP-OES, was conducted by ChemCentre (Bentley, Western Australia). An electrochemistry analysis was undertaken for Cd and Pb using anodic stripping voltammetry (ASV) with standard addition, which is conducted using a portable digital voltammeter (PDV6000+). The instrument works in conjunction with voltammetric analysis software (VAS). For method validation and quality control refer to Appendix I, Table A1. For limits of detection for the instruments used refer to Appendix II, Table A2.

1.2.4 Statistical analysis

Analyses were undertaken with R Studio version 1.2.1.1335 (R Core Team, 2013). General linear models (GLM) were used to assess Cd and Pb uptake in plants based on interactions between the mixed group variables where the biomass sections, shoots and roots was a within-subjects factor and the substrate types, topsoil, tailings and capped tailings was a between-subjects factor. They were also used to compare concentration readings between analytical instruments. Concentration values were log-transformed internally (Gamma family) prior to analysis. P-values were derived from the chi-squared test calculated using the ‘Anova’ function from the ‘car’ package in R Studio (Fox, 2019). The ‘emmeans’ function from the ‘CRAN’ package was used as a post-hoc test to identify differences for heavy metal concentrations based on substrate medium and plant biomass section (Lenth, 2021). It was also used to recognise significant differences for instrument detection capabilities. Analysis of Fe and Ca uptake pathways with Cd and Pb was conducted with a spearman rank correlation test.

1.3 Results

1.3.1 Cadmium analysis of plants with ICP-MS

All six plant species grown in glasshouse conditions were found to have significant relationships with Cd concentrations (Table 1.2), and them being significantly different between biomass sections and the type of substrate they were grown in (Table 1.4). For *A. ramulosa* grown in topsoil, average Cd concentrations were two times greater in the roots when compared to shoots, while roots of plants in capped tailings were 1.5 times higher in Cd than the shoots (Fig 1.1A). Average Cd concentrations in *A. acutivalvis* grown on topsoil were double of those grown on capped tailings, and the roots of plants grown on capped tailings had twice the amount of Cd compared to shoots (Fig 1.1B). In *A. scabra* the shoot biomass of plants grown in topsoil and tailings had Cd concentrations five times greater than plants grown in capped tailings (Fig 1.1C). For *E. loxophleba* the average Cd concentration in shoots of plants grown in topsoil, was tenfold the amount compared to plants grown in capped tailings, with roots of plants grown in topsoil being six times greater in Cd than plants grown in capped tailings (Fig 1.1D). The roots of *H. recurva* grown in topsoil had Cd concentrations three times greater than the shoots and root of plants grown in both capped tailings and tailings (Fig 1.1E). In *M. georgei,* plants grown in tailings had twice the amount of Cd compared to plants grown in topsoil and nearly five times the amount of Cd when compared to plants grown in capped tailings (Fig 1.1F).

Table 1.2 – GLM results for plants grown in glasshouse environments to determine significant relationships of Cd concentrations in dry plant biomass sections (roots and shoots) and type of substrate grown in (topsoil, capped tailings and tailings).

Species	Variable	Chi^2	Df	P-value
Acacia ramulosa	Substrate: Biomass	24.0	1	<0.01
Allocasuarina acutivalvis	Substrate: Biomass	22.6	1	<0.01
Austrostipa scabra	Substrate	95.9	2	<0.01
Eucalyptus loxophleba	Substrate	112.0	1	<0.01
Hakea recurva	Biomass	55.3	1	<0.01
Maireana georgei	Substrate	86.2	2	<0.01

Table 1.3 – Concentrations of Cd and Pb in dried roots and shoots of plants (mean ± SE) grown in a glasshouse in topsoil, capped tailings and tailings.

Species	Substrate	Biomass section	n	Cd (µg g^{-1})	Pb (µg g^{-1})
Acacia ramulosa	Topsoil	Shoot	5	0.06 ± 0.01	1.54 ± 0.35
		Root	5	0.12 ± 0.05	6.06 ± 3.38
	Capped	Shoot	5	0.09 ± 0.02	2.72 ± 1.02
		Root	5	0.06 ± 0.01	3.54 ± 0.81
Allocasuarina acutivalvis	Topsoil	Shoot	5	0.09 ± 0.02	1.48 ± 0.12
		Root	5	0.48 ± 0.14	4.60 ± 0.85
	Capped	Shoot	5	0.05 ± 6 $x\ 10^{-3}$	1.73 ± 0.19
		Root	4	0.23 ± 0.27	6.53 ± 0.43
	Tailings	Shoot	1	0.11	3.69
		Root	1	0.2	10.91
Austrostipa scabra	Topsoil	Shoot	5	0.31 ± 0.13	1.03 ± 0.20
		Root	5	0.52 ± 0.10	3.44 ± 1.03
	Capped	Shoot	5	0.06 ± 0.001	1.19 ± 0.07
		Root	4	0.06 ± 0.01	2.66 ± 0.45
	Tailings	Shoot	5	0.34 ± 0.28	5.85 ± 4.44
		Root	1	0.56	9.19
Eucalyptus loxophleba	Topsoil	shoot	5	0.60 ± 0.22	1.05 ± 0.08
		Root	4	0.41 ± 0.15	5.02 ± 2.60
	Capped	Shoot	5	0.06 ± 0.01	1.06 ± 0.20
		Root	5	0.07 ± 0.03	4.76 ± 0.99
Hakea recurva	Topsoil	Shoot	5	0.05 ± 4 $x\ 10^{-3}$	1.46 ± 0.22
		Root	5	0.18 ± 0.06	3.05 ± 1.31
	Capped	Shoot	5	0.05 ± 1 $x\ 10^{-3}$	1.40 ± 0.12
		Root	5	0.06 ± 0.01	4.49 ± 1.36
	Tailings	Shoot	1	0.05	1.852
Maireana georgei	Topsoil	Shoot	5	0.13 ± 0.01	2.14 ± 0.08
		Root	5	0.13 ± 0.13	5.89 ± 0.57
	Capped	Shoot	5	0.05 ± 0.01	1.03 ± 0.11
		Root	5	0.05 ± 0.01	3.50 ± 0.85
	Tailings	Shoot	5	0.30 ± 0.16	7.00 ± 4.28
		Root	5	0.22 ± 0.10	8.65 ± 2.61

Table 1.4 - Post hoc results for GLM of plants grown in glass house environment determining significant differences of Cd concentrations in dry plant biomass sections (roots and shoots) and type of substrate grown in (topsoil, capped tailings and tailings).

Species	Post hoc comparison between biomass types	Post hoc comparison between substrate types	Z-stat	P-value
Acacia ramulosa	Topsoil shoot - Topsoil root		-4.65	**<0.01**
	Capped shoot- Capped root		2.44	**0.01**
		Topsoil shoot - Capped shoot	-2.21	0.12
		Topsoil root - Capped root	4.74	**<0.01**
Allocasuarina acutivalvis	Topsoil shoot - Topsoil root		-10.41	**<0.01**
	Capped shoot- Capped root		-3.59	**<0.01**
		Topsoil shoot - Capped shoot	2.89	**0.01**
		Topsoil root - Capped root	8.95	**<0.01**
Austrostipa scabra	Topsoil shoot - Topsoil root		-2.09	**0.04**
	Capped shoot- Capped root		0.01	0.98
	Tailings shoot- Tailings root		-2.19	**0.03**
		Topsoil shoot - Capped shoot	6.73	**<0.01**
		Topsoil shoot - Tailings shoot	1.36	0.36
		Capped shoot - Tailings shoot	-5.00	**<0.01**
		Topsoil root - Capped root	8.34	**<0.01**
		Topsoil root - Tailings root	-0.10	0.98
		Capped root - Tailings root	-5.19	**<0.01**

Continued

Species	Post hoc comparison between biomass types	Post hoc comparison between substrate types	Z-stat	P-value
	Capped shoot-Capped root		0.12	0.91
		Topsoil shoot - Capped shoot	-8.92	**<0.01**
		Topsoil root - Capped root	-6.90	**<0.01**
Hakea recurva	Topsoil shoot - Topsoil root		-9.40	**<0.01**
	Capped shoot-Capped root		-1.39	0.16
		Topsoil shoot - Capped shoot	0.30	0.95
		Topsoil root - Capped root	8.31	**<0.01**
Maireana georgei	Topsoil shoot - Topsoil root		$2\ x\ 10^{-3}$	0.99
	Capped shoot-Capped root		-0.044	0.96
	Tailings shoot-Tailings root		1.45	0.15
		Topsoil shoot - Capped shoot	3.87	**<0.01**
		Topsoil shoot - Tailings shoot	-3.67	**<0.01**
		Capped shoot - Tailings shoot	-7.54	**<0.01**
		Topsoil root - Capped root	3.82	**<0.01**
		Topsoil root - Tailings root	-2.22	0.07
		Capped root - Tailings root	-6.05	**<0.01**

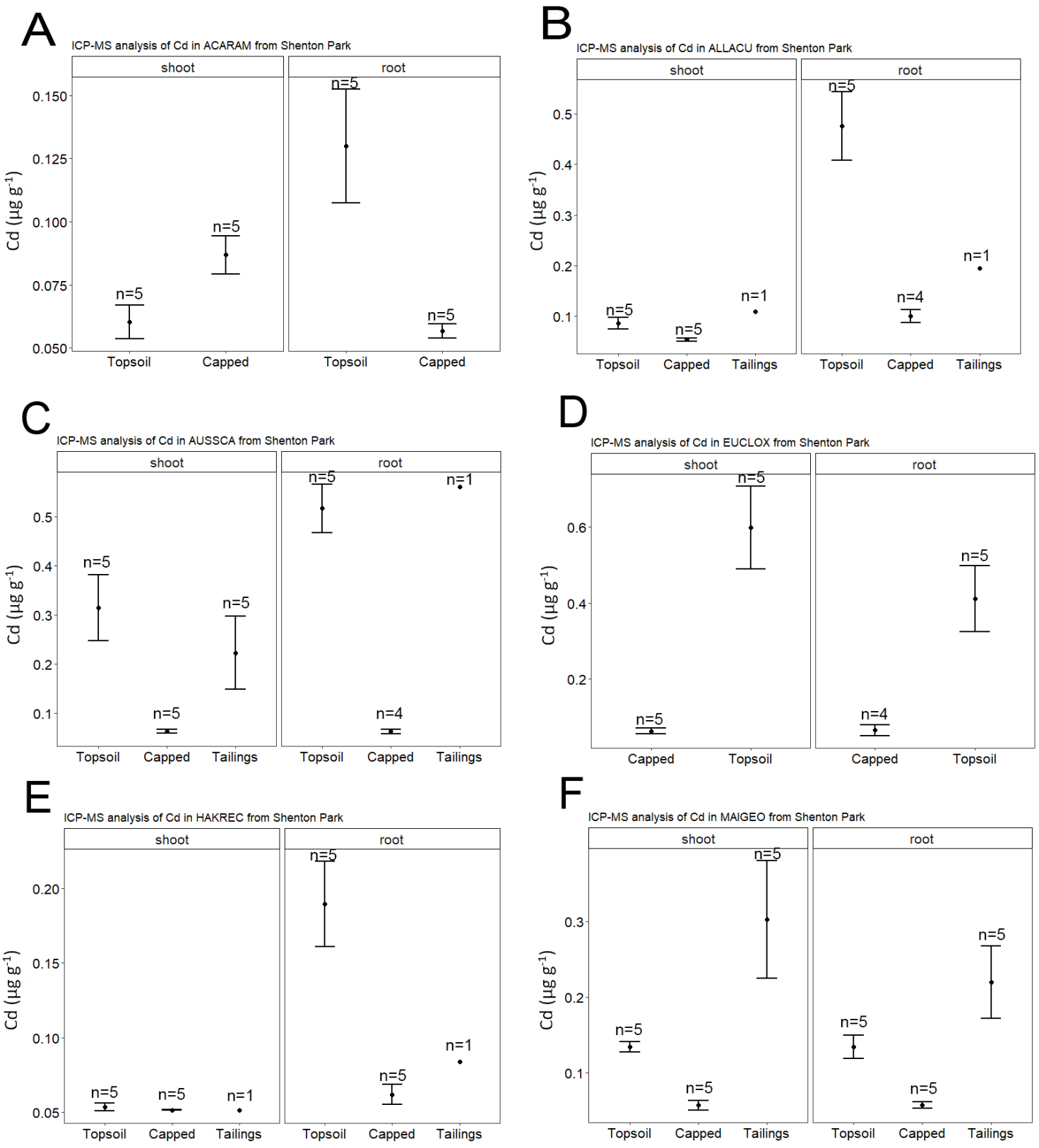

Figure 1.1 – Mean ± SE for Cd concentration in root and shoot tissues of A) *Acacia ramulosa,* B) *Allocasuarina acutivalvis*, C) *Austrostipa scabra,* D) *Eucalyptus loxophleba* E) *Hakea recurva* and F) *Maireana georgei* grown in topsoil, tailings and capped tailings, analysed by ICP-MS.

1.3.2 Lead analysis of plants with ICP-MS

All six plant species grown in glasshouse conditions were found to have significant relationships with Pb concentrations (Table 1.5), and them being significantly different between biomass sections and the type of substrate they were grown in (Table 1.6). In *A. ramulosa* the average concentration of Pb in the roots of plants grown in topsoil was four times greater than the shoots (Fig 1.2A). Average Pb concentrations were threefold higher in the roots than shoots for *A. acutivalvis* (Fig 1.2B). Concentrations of Pb in roots of *A. scabra* grown in topsoil and capped tailings had three times the concentration of the shoots, while plants grown in tailings had six times the amount of Pb, compared to shoots (Fig 1.2C). For *E. loxophleba* the roots of plants grown in topsoil and capped tailings both had Pb concentrations five times greater than the shoot sections (Fig 1.2D). In *H. recurva* grown in topsoil there was twice the amount of Pb in roots compared to the shoots, while capped tailings average Pb concentrations were threefold higher in roots than in shoots (Fig 1.2E). For *M. georgei,* Pb content was at least twofold and threefold higher in the roots and shoots, respectively, of plants grown in tailings compared with plants grown in topsoil and capped tailings (Fig 1.2F). For comparative Pb concentration values from each instrument for the plants grown in glasshouse conditions refer to Appendix III, Table A3.

Table 1.5 - GLM results for plants grown in glasshouse environments to determine significant relationships of Pb concentrations in dry plant biomass sections (roots and shoots) and type of substrate grown in (topsoil, capped tailings and tailings).

Species	Variable	Chi^2	Df	P-value
Acacia ramulosa	Biomass	14.8	1	<0.01
Allocasuarina acutivalvis	Biomass	327.0	1	<0.01
	Substrate	70.5	1	<0.01
Austrostipa scabra	Biomass	46.5	1	<0.01
Eucalyptus loxophleba	Biomass	105.4	1	<0.01
Hakea recurva	Biomass	46.3	2	<0.01
Maireana georgei	Substrate	86.6	2	<0.01

Table 1.6 - Post hoc results for GLM of plants grown in glass house environment determining significant differences of Pb concentrations in dry plant biomass sections (roots and shoots) and type of substrate grown in (topsoil, capped tailings and tailings).

Species	Post hoc comparison between biomass types	Post hoc comparison between substrate types	Z-stat	P-value
Acacia ramulosa	Topsoil shoot - Topsoil root		-4.73	**<0.01**
	Capped shoot- Capped root		-0.85	0.39
		Topsoil shoot - Capped shoot	-1.97	0.48
		Topsoil root - Capped root	1.74	0.08
Allocasuarina acutivalvis	Topsoil shoot - Topsoil root		-11.91	**<0.01**
	Capped shoot- Capped root		-13.17	**<0.01**
		Topsoil shoot - Capped shoot	-1.63	0.234
		Topsoil root - Capped root	-3.47	**<0.01**
Austrostipa scabra	Capped shoot- Capped root		-3.53	**<0.01**
	Tailings shoot- Tailings root		-2.27	**0.02**
		Topsoil shoot - Capped shoot	-0.66	0.78
		Topsoil shoot - Tailings shoot	-5.79	**<0.01**
		Capped shoot - Tailings shoot	-5.17	**<0.01**
		Topsoil root - Capped root	1.13	0.5
		Topsoil root - Tailings root	-2.63	**0.02**
		Capped root - Tailings root	-3.25	**<0.01**

Continued

Species	Post hoc comparison between biomass types	Post hoc comparison between substrate types	Z-stat	P-value
	Capped shoot-Capped root		7.56	**<0.01**
		Topsoil shoot - Capped shoot	0.06	0.95
		Topsoil root - Capped root	-0.25	0.8
Hakea recurva	Topsoil shoot - Topsoil root		-3.79	**<0.01**
	Capped shoot-Capped root		-5.98	**<0.01**
		Topsoil shoot - Capped shoot	0.21	0.97
		Topsoil root - Capped root	-1.98	0.12
Maireana georgei	Topsoil shoot - Topsoil root		-4.75	**<0.01**
	Capped shoot-Capped root		-5.76	**<0.01**
	Tailings shoot-Tailings root		-0.98	0.32
		Topsoil shoot - Capped shoot	3.46	**<0.01**
		Topsoil shoot - Tailings shoot	-5.56	**<0.01**
		Capped shoot - Tailings shoot	-9.01	**<0.01**
		Topsoil root - Capped root	2.44	**0.04**
		Topsoil root - Tailings root	-1.79	0.17
		Capped root - Tailings root	-4.23	**<0.01**

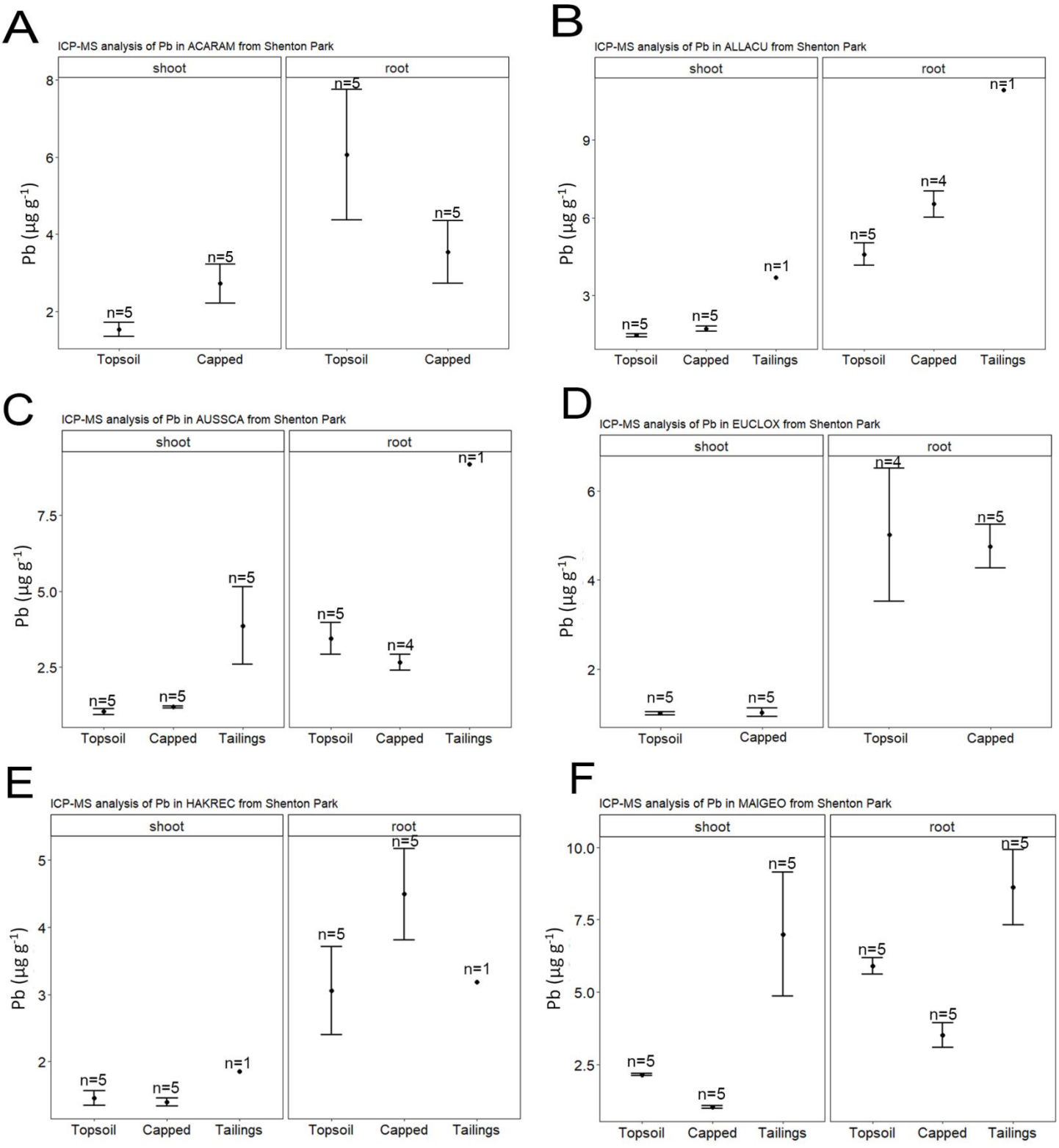

Figure 1.2 – Mean ± SE for Pb concentration in root and shoot tissues of A) *Acacia ramulosa,* B) *Allocasuarina acutivalvis*, C) *Austrostipa scabra,* D) *Eucalyptus loxophleba* E) *Hakea recurva* and F) *Maireana georgei* grown in topsoil, tailings and capped tailings, analysed by ICP-MS.

1.3.3 Analysis of Cd and Pb using ICP-MS for plants sampled at KIOP

Both Cd and Pb were detected in the above ground-biomass of plants sampled from the TSF and from adjacent undisturbed natural vegetation at the Mid West mine site. Pb concentrations was 1.5 times higher for *M.georgei* individuals occurring on TSF (3.9 ± 0.53 µg g^{-1}), compared with individuals occurring in undisturbed vegetation (2.5 ± 0.08 µg $g^{-1;}$ $chi^2 = 8.45, df = 1, P = <0.01$), while Cd concentrations did not significantly differ among plants from the two locations ($chi^2 = 1.85, df = 1, P = 0.17$). For mean concentration values detected in KIOP plants by other instruments refer to Appendix IV, Table A4 for Pb analysis and Table A5 for Cd analysis.

1.3.4 Instrument comparisons

The *M. georgei plants* were used as a data subset to undergo the ASV analysis. This data set compares Pb and Cd concentrations between instruments for glasshouse-grown plants and plants sampled from KIOP. Glasshouse-grown *M. georgei* had significant differences in Pb concentration readings between instruments ($chi^2 = 156.37, df = 3, P <0.01$). The post-hoc revealed ICP-MS readings were significantly lower from FAAS ($P <0.01$), MP-AES ($P <0.01$) and ASV ($P = 0.01$) (Fig 1.3). For *M. georgei* sampled from KIOP, there were significant differences in Pb concentration readings from the instruments ($chi^2 = 626.62, df = 3, P <0.01$). The post-hoc revealed ICP-MS readings were significantly less FAAS ($P <0.01$) and MP-AES ($P <0.01$), but not ASV ($P = 0.99$) (Fig 1.4). Only ICP-MS and ASV were able to detect Cd in *M. georgei*. When compared there was no significant difference in their concentration values for Cd ($chi^2 = 0.0, df = 1, P = 1.0$) (Fig 1.5).

1.3.5 Analysis of heavy metal uptake pathways with Fe and Ca

Due to successful replication of *M. georgei* and *A. scabra* plants grown in tailings, the species were subset and used to compare the concentrations of Ca, Cd, Fe and Pb found in their biomass. A Spearman's rho test found a significant correlation in *M.georgei* plants Ca and Pb concentrations ($r_s = 0.64, P <0.01, N = 30$), which indicated a moderate to strong positive relationship between the concentration of Pb and Ca in plant tissues (Fig 1.6).

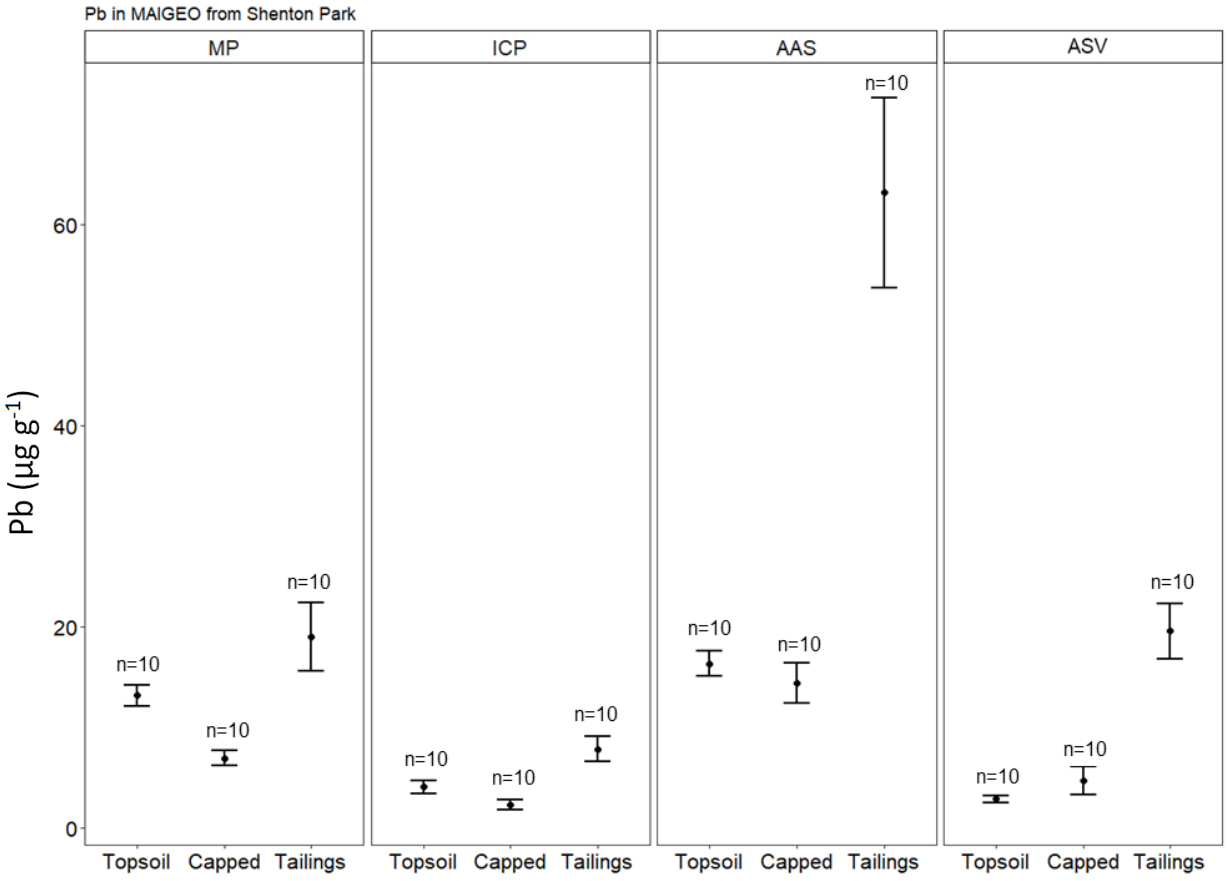

Figure 1.3 – *Maireana georgei* from Shenton Park were analysed for Pb with ICP-MS, MP-AES, FAAS and ASV. Concentration readings were compared among substrates, including topsoil, capped tailings and tailings

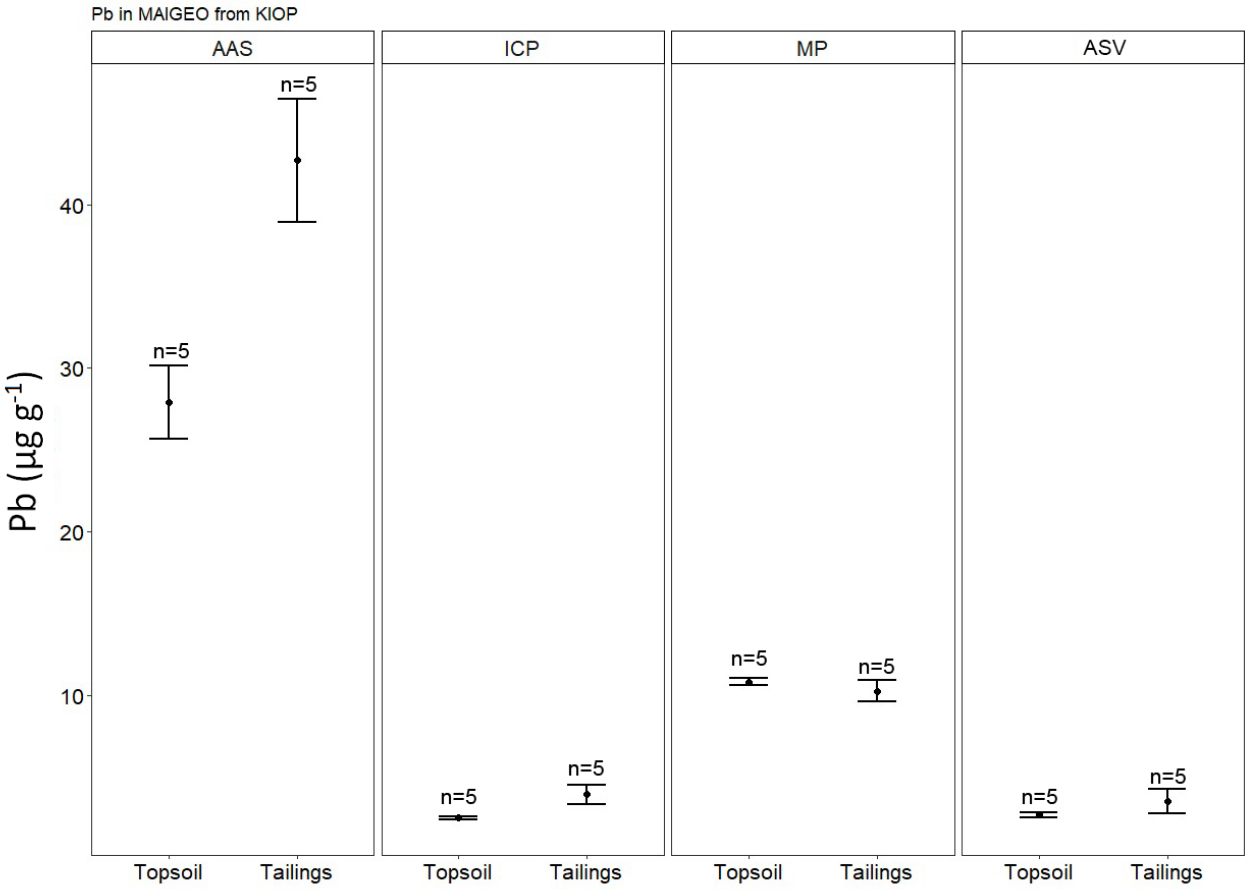

Figure 1.4– *Maireana georgei* analysed for Pb content using FAAS, ICP, MP-AES and ASV

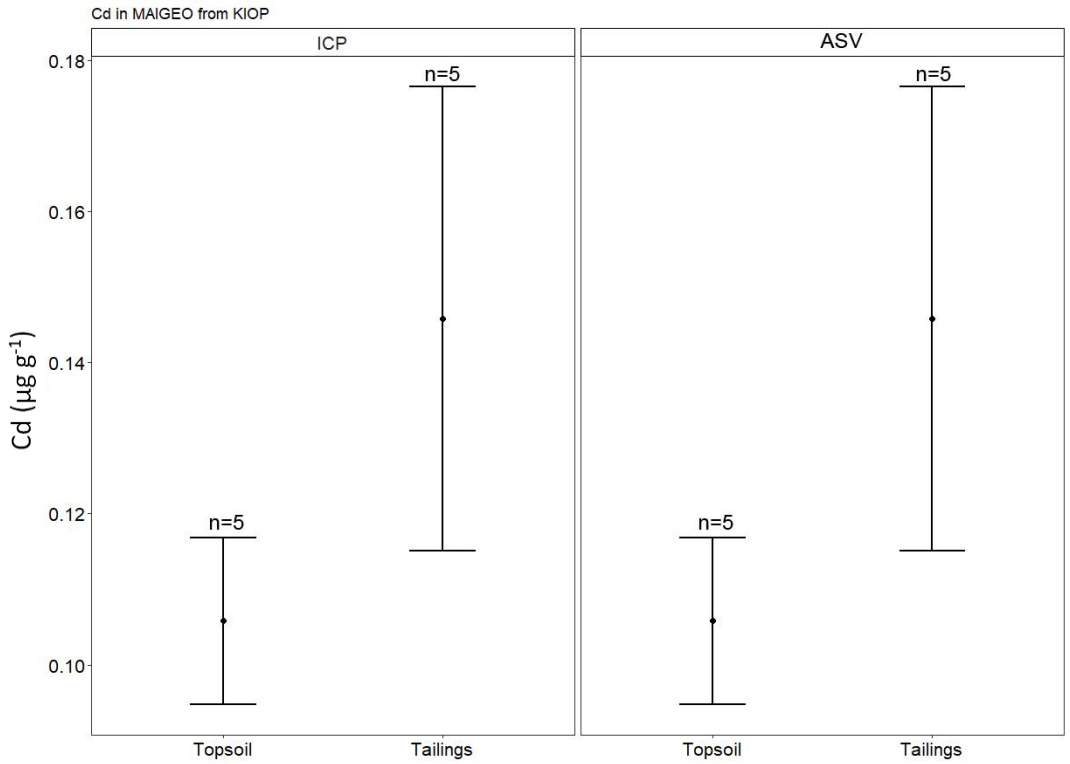

Figure 1.5 – Concentration of Cd in *Maireana georgei* analysed by ICP-MS and ASV

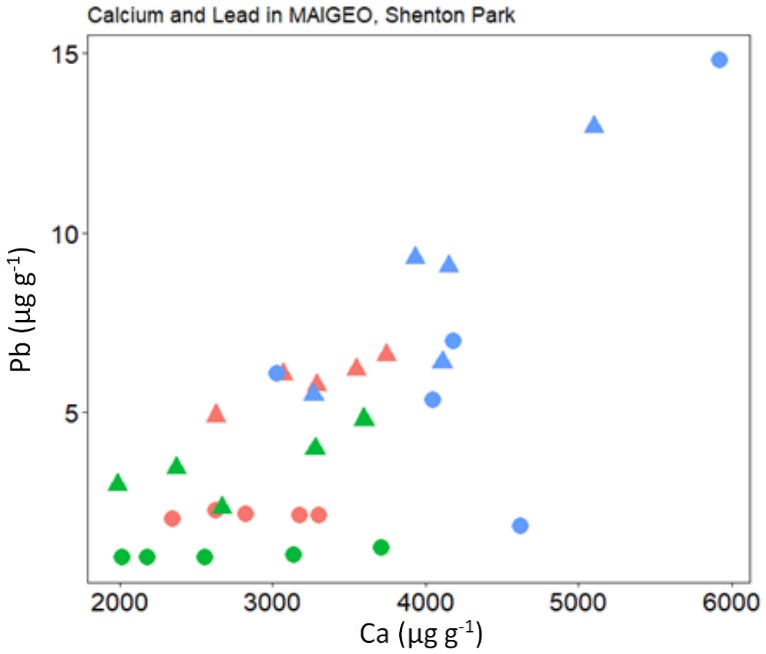

Figure 1.6 - Correlation plots for Ca and Pb in *Maireana georgei* to determine assisted uptake of lead through Ca^{2+} ion nutrient-acquisition pathways

1.4 Discussion

1.4.1 The behaviour of cadmium in plants

Cadmium in soil is either bound in an inorganic ligand or present as a free Cd^{2+} ion (Hirsch & Banin, 1990; Bradl, 2005). In high pH environments (pH >8.2) Cd solubility decreases and carbonate species such as $CdHCO_3^+$ and $CdCO_3$ become dominant in the substrate while complexation of Cd with dissolved organics is unlikely to occur due to the competition from Ca for binding sites (Bradl, 2005; Cleaver *et al.*, 2021; Huang *et al.*, 2021). Under oxidising conditions, Cd^{2+} behaves similarly to Ca^{2+} and is able to displace Ca^{2+} binding sites in soil (Christensen, 1984; Cleaver *et al.*, 2021). Soluble Cd^{2+} remains available for plants to uptake (Bradl, 2005). All plant species grown in topsoil under glasshouse conditions acquired higher concentrations of Cd in their roots than the plants grown in tailings. Natural topsoil contained 0.01 ± 0.01 µg g^{-1} of Cd, but based on pH (pH ca. 6.2), it is more likely to be in a soluble Cd^{2+} form for plants to acquire when compared to soluble Cd available in alkaline tailings. The Cd^{2+} can be up-taken through the Ca^{2+}, Fe^{2+}, Mn^{2+} and Zn^{2+} transporters (Clemens, 2006; Baxter *et al.*, 2008; Verbruggen *et al.*, 2009). Elements such as Fe, Ca, Mn and Zn are micronutrients required for plant respiration and photosynthesis (Kabata-Pendias, 2000; Lambers *et al.*, 2008), and are naturally found occurring in topsoil; and can be imported through the cortex cells of plant roots and move through to the xylem vessel where they are exported to above-ground tissues (Menon, 1998; Callahan *et al.*, 2006; Kim & Guerinot, 2007; Lambers *et al.*, 2008; Cross & Lambers, 2017). As most species in the experiment are associated with arbuscular mycorrhiza, ecto-mycorrhiza or cluster roots to sequester Cd to the roots and limit its transportation in the plant, the NM *M .georgei* does not form mycorrhizal associations to assist in the sequestration of heavy metals into roots (Lambers *et al.*, 2008), and sees an even distribution of Cd in roots and shoots (Fig 1.1F).

If Cd uptake is facilitated by these nutrient transport pathways to get to above-ground biomass in plants it may explain Cd concentrations reported for shoots of species in the present study, particularly the NM *M. georgei* which d

1.4.2 The behaviour of lead in plants

The Pb uptake mechanism varies from Cd transport, as Pb has low solubility even where it is a major pollutant in the substrate, and apparently exhibits no correlation between soil

concentration and uptake into plant biomass (Clemens, 2006; Pourrut *et al.*, 2011; van der Ent *et al.*, 2013). Pb is extremely insoluble at neutral pH, and requires an acidic environment to remain in an ionic form (Bradl, 2005). Additionally there is the potential formation of metal-chelate complexes that can assist the mobilisation of Pb through the complexion of dissolved organic matter and allowing the Pb to be in a stable soluble form that can be viable for uptake through nutrient channels or the apoplastic pathway (Raskin *et al.*, 1997; Bradl, 2005; Kumar *et al.*, 2017). Alternatively, when a plant or fungi has an excess of toxic metal elements they may sequester them through intracellular metal-binding peptides, referred to as phytochelatins, which bind the elements in a non-toxic form to reduce the amount of toxic metal left in ionic form (Scarano & Morelli, 2002). Pb behaviour in soil is also controlled, to a certain extent, by mycorrhizal transpiration, so Pb uptake varies markedly among species with different nutrient-acquisition strategies and with the presence of mycorrhizal fungi (Raskin *et al.*, 1997; Kopittke *et al.*, 2007; Kumar *et al.*, 2017). For the AM *A. scabra*, there was a strong positive correlation between shoot Pb and Fe concentrations accumulated in plant biomass, suggesting Pb enters this species through the Fe^{2+} nutrient channel. For NM *M. georgei,* Pb concentrations had a strong positive correlation with Ca concentrations accumulated in the plant suggesting it uses the nutrient uptake pathways to acquire Pb in its biomass. Concentrations of Pb were found in every sample of plant tissue in this experiment, both from glasshouse-grown plants and individuals sampled from the Mid West mine site.

Plants growing in tailings, under glasshouse conditions had higher Pb concentrations compared to plants growing in topsoil and capped tailings. When compared to plants sampled from the Mid West mine site, the same relationship for Pb accumulation was only seen in the *M. georgei species.* Species *A. ramulosa, A. acutivalvis, A. scabra, E. loxophleba* and *H. recurva* all appear to accumulate higher Pb concentrations in the root sections of the plant in the alkaline substrate. This confirms the hypothesis and coincides with previous Pb accumulation research done on Australian plant species like *Acacia heteroclite* and *Allocasuarina verticillatta* which were found to have 200 times more Pb in the roots compared to shoots and held levels as high as 71.1 mg to 92.8 mg of Pb per kilo of dry roots tissue from growing in an acidic substrate (Menon, 1998). A majority of plants used in the experiment have mycorrhizal fungi or cluster roots to assist in acquisition and sequestering of nutrients (Table 1.1). Arbuscular mycorrhizal fungi in particular, have repeatedly shown it is able to bind heavy metals in the root tissue of plants and prevent it

from travelling into the shoots of plants (Hildebrandt *et al.*, 2007; Gonzalez-Guerrero *et al.*, 2008). Species that do not have mycorrhizal associations such as *M. georgei* are not able to sequester the heavy metals in the roots and instead have an even distribution of metal in their shoots. Due to the highly leached, ancient soils found in Western Australia, there is a higher diversity of plants that do not form associations with mycorrhizal fungi, instead having specialised means of nutrient-acquisition on nutrient poor soils, potentially making them more adaptable to the edaphic conditions required to revegetate tailings (Brundrett, 2009, 2017; Zhong *et al.*, 2021). A trend that also appeared in the data is that an application of topsoil over the tailings could potentially prevent heavy metal uptake (Fig 1.1 and 1.2). Topsoil is often conserved for later use in a rehabilitation program where the aim is to restore native flora and has multiple benefits with introducing nutrients, microbes and seeds into the substrate (Australia Government, 2016). Capping methods commonly applied on TSF's can also provide stability to the landscape (i.e., stabilisation and reduction of erosion by rock armouring; (Cross *et al.*, 2021) and may assist in chemical amelioration (Pemberton, 2020). Applications of topsoil cappings over tailings appear to be advantageous and could potentially prevent the dispersal of heavy metals into plants, crops, animals and humans.

1.4.3 Analytical instruments capabilities, interferences and advantages of using voltammetry

Acid-digested plants create a high-matrix solution that can see possible interference of other elements emitting similar wavelengths when in an excited state (Havlin & Soltanpour, 1980; Agilent Technologies, 2016; Addis & Abebaw, 2017). This is specifically a concern when analysing plants that have grown in the magnetite tailings as they have higher amounts of Fe and Ca present in their environment available for uptake (Cross & Lambers, 2017). The Fe and Ca remain present in the digested sample alongside the heavy metals that are the targets for analysis (McKown *et al.*, 1978; Pyle *et al.*, 1995; Barbooti, 2015). For FAAS analysis, Pb was detected with significant signal distortion (Fig 3 & 4). It is well documented that absorption spectroscopy can suffer from interferences from other elements, like Fe and Ca in a sample containing a complex matrix (Nieman & Holler, 1998; Rubinson & Rubinson, 2000; Stafilov, 2000; Sweileh & El-Nemma, 2004; Yoon *et al.*, 2005). When FAAS analysed *M. georgei* samples grown on tailings in the glasshouse, it

produced Pb concentration readings 8 times greater than what was detected with ICP-MS (Fig 1.3 and 1.4). A possible reason for these high readings could have been due to a high concentration of Fe in the alkaline tailings (>550 µg g^{-1} (Cross & Lambers, 2017)), as well as high concentrations found in the shoots (8962 ± 4738.6 µg g^{-1}) and roots (46293 ± 6174 µg g^{-1}) of *M. georgei* grown in tailings under glasshouse conditions. Excess Fe in the digested matrix can create an interference from scattering the source beam, which occurs when particles are not entirely atomised in the flame and develop spectral overlap on the absorption bands for the target elements Cd and Pb when used in the analysis (Sweileh & El-Nemma, 2004; Arnold *et al.*, 2011). Analysis performance of the ICP-MS instrument has shown in previous studies that is has significantly less spectral overlap and signal interference in samples containing Fe (Yoon *et al.*, 2005), however avoiding this issue in FAAS analysis can be difficult. The options can include diluting the sample, which can reduce the interference from Fe, but also reduces the sensitivity of the detection method, making trace analysis of target metals difficult (Yoon *et al.*, 2005). Additionally, using a lower ordered atomic absorption line, that Fe is not picked up on but is also less sensitive to the target metal, use a standard addition method to alleviate matrix effects, or to physically removed Fe from the sample entirely (Franke *et al.*, 1978; Sweileh & El-Nemma, 2004). A secondary issue that can occur is spectral line overlap with the target metal in the analyte and the excess Fe in the matrix (Sweileh & El-Nemma, 2004). The absorption line used in the analysis of Pb was 217.0 nm and a weak absorbing line for Fe is 213.856 nm (Varian, 1989; Sweileh & El-Nemma, 2004).

High Ca concentrations is also an issue in the FAAS analysis of biological sample like plants and soil (McKown *et al.*, 1978; Varian, 1989), as the formation of Ca salts can cause interferences with light scattering if the sample is not treated with a suitable reagent, which was not done for this analysis. (Demers & Ellis, 1968; Schrenk, 1975; Baluja-Santos *et al.*, 1984; Varian, 1989; Hamilton *et al.*, 1991; Arnold *et al.*, 2011). Analysis with emission spectroscopy can have the benefit of undertaking multi-elemental detection, but it too can have issues when analysing high matrix samples like plants and have elements emitting light on similar frequencies causing interference and inaccurate readings (Barbooti, 2015; Karlsson *et al.*, 2015). The MP-AES instrument reported Pb concentration ranges within the same magnitude as ICP-MS and ASV; but comparison of concentration results indicated significant differences between the instrument readings. The MP-AES was, however, able to indicate the same pattern uptake profile for plants growing in the

different substrates. Other possibilities for not receiving a true signal reading from the MP-AES instrument is that the plant samples were digested in concentrated nitric acid. A Nitric acid matrix can consume more energy than a sample containing water during analysis. This can potentially create a cooling effect that sees a reduction in thermal conductivity on the plasma when aspirating the sample and can result in signal suppression (Grotti & Todolí, 2020). Microwave plasma has a lower operating temperatures near 6000°K (Agilent Technologies, 2021), compared to ICP-MS which can reach 10,000°K (Wilschefski & Baxter, 2019), and therefore may not fully ionise the elements in the sample and could potentially be less efficient in a complex matrix, like the plant biomass (Karlsson *et al.*, 2015).

Anodic stripping voltammetry using a PDV6000+ instrument is the only detection analysis technique that did not require the use of gas fixtures and being confined to a laboratory, making it entirely portable (Wajrak & Rummey, 2004). The results it produced analysing *M. georgei* sampled from undisturbed vegetation at the KIOP showed next to no difference when compared to ICP-MS concentrations for Pb ($P = 0.99$) and Cd ($P = 1.00$). An analysis with ASV is also subject to matric affects from samples being digested in concentrated nitric acid. To eliminate interferences samples were diluted with MilliQ water without the risk of losing detection capabilities, as ASV can detect down to parts per billion (ppb) (Wajrak & Rummey, 2004). The analysis was also conducted with the inclusion of standard addition which was able to reduce matrix effects on the signal readings. Despite this being a time consuming process, the results from ASV matching ICP-MS, makes it an ideal instrument to use as a cost effective tool for monitoring heavy metal concentrations in plants.

1.4.4 Bioaccumulation and Monitoring

Certain species of plants are able to bioaccumulate heavy metals due to growing in metalliferous soils (Ji *et al.*, 2011; Tchounwou *et al.*, 2012; Tanabe *et al.*, 2016). Species that are identified as metallophytes are often found on land near mine sites, as they are adapted to tolerate the high levels of metals naturally occurring in the area and suggest hyperaccumulator abilities (Erskine *et al.*, 2012; van der Ent *et al.*, 2013; Pollard *et al.*, 2014; Reeves *et al.*, 2018). There are several tolerance mechanisms a plant can be adapted to, ‘Accumulators’ concentrate metals in the above-ground tissue, while

‘Excluders’ sees the metal concentrations maintained in the roots until it passes a certain threshold and moves to above-ground biomass, and the ‘Indicator’ which sees the regulation of heavy metals in the plant shoots so that it compares to external concentrations it is exposed to (Baker, 1981; Baker, 2000). The average amount of Cd and Pb in alkaline tailings at KIOP was previously reported as 0.06 ± 0.01 µg g^{-1} for Cd and 5.1 ± 0.70 µg g^{-1} for Pb (Cross & Lambers, 2017). Analysis of shoot and root tissues of *A. ramulosa, A. scabra, E. loxophleba, M. brevifolia* and *M. georgei* grown on a TSF in the Mid West region of Western Australia ranged from 0.1–0.2 µg g^{-1} for Cd (Appendix IV, Table A5), and 2.1–3.9 µg g^{-1} of Pb (Appendix IV, Table A4). Although these concentrations may seem low compared to the soil health investigation levels determined by (National Environment Protection Measure, 2011) for Cd (20 µg g^{-1}) and Pb (300 µg g^{-1}), the concentrations found in plant biomass may bioaccumulate and increase further over time, as seen in the *M. georgei* grown in tailings that had a greater average concentration of Pb (7.0 ± 4.28 µg g^{-1}), than what was recorded for the tailings themselves (5.1 ± 0.70 µg g^{-1}). Even at the lower concentrations, toxic heavy metals can have a negative effect on plant physiology and become an exposure pathway risk through herbivory opening up bioaccumulation and biomagnification to other trophic levels (Burger & Gochfeld, 1993; Clark *et al.*, 2001; Burger, 2002). Heavy metals are persistent and bioaccumulate therefore monitoring the environment can help determine where heavy metals are stored, and identify which species can be used as indicators to determine if the bioaccumulated metal concentrations are at safe levels (Kabata-Pendias, 2000; Robinson *et al.*, 2015). Baseline levels of plants surveyed in from the natural topsoil surrounding KIOP contained 0.1–0.14 µg g^{-1} of Cd and 1.7–4.1 µg g^{-1} of Pb. As these concentrations are similar to plants exposed to heavy metals on the TSF, it is important to monitor distribution and negative effects exposure has to plants in surrounding areas that are be indirectly affected by mining.

1.5 Conclusion

This study provides evidence for heavy metal accumulation in plants native to the Mid West region of Western Australia, and indicates that different heavy metals can accumulate differently in different species based on substrate and plant nutrient-acquisition strategies between plant species. The concentration of Pb in plants was greater for those grown in tailings and it was shown that Pb is likely to be sequestered in

roots and not transported into above-ground biomass for plant species with mycorrhizal associations. Non-mycorrhiza species such as *M. georgei and M. brevifolia* had an even distribution of Pb in roots and shoots. The uptake of Cd was also found to occur, but it is not always sequestered to the roots in the absence of mycorrhizal fungi and can be transported into plant shoots, putting it as an increased risk for exposing animals if shoots are consumed. The acquisition of Pb into the plant also suggests that it occurs through the Ca^{2+} and nutrient transport channels. The comparison between instrument analyses indicated FAAS and MP-AES were more likely to encounter significant signal interferences when analysing at a trace level in a high matrix sample. They could not compare to the superior results produced by ICP-MS and ASV. Analysis with ASV was highly capable and matched the ICP-MS concentrations extremely well. It is an effective replacement for ICP-MS for heavy metal monitoring that operates at a fraction of the cost and has additional benefits by being portable meaning it can be taken into the field or be operated in locations outside of a laboratory. Based on the findings of this research it is recommended that further studies include monitoring to ensure the bioaccumulation in plants does not exceed human health and ecological investigation levels and those toxic heavy metals have not dispersed into surrounding areas. Further studies could also better investigate the soil chemistry of how an application of topsoil on alkaline tailings can change the solubility of toxic heavy metals. Wildlife in surrounding areas should also be monitored for exposure from plant and sediment ingestion to ensure the movement of heavy metals into higher trophic levels is limited.

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Chapter 2:

Australian native plants grown in soil spiked with arsenic, cadmium and lead to determine heavy metal accumulation capabilities and phytoremediation potential

Abstract

A higher concentration of heavy metals in the environment is often due to anthropogenic industries and their waste management. This leads to heavy metals in the environment that can contaminate sediment, water and plants. The heavy metals can undergo complex interactions with organic material and changing their solubility while also competing for uptake into plant biomass alongside essential nutrients. It was proposed that when Australian native plants; *Acacia saligna, Allocasuarina huegeliana* and *Austrostipa elegantissima* are exposed to higher treatment levels of heavy metals in spiked soil, they would uptake higher quantities of heavy metals in the plants above-ground biomass. This saw As ($0.2 \pm 2.4 \times 10^{-17}$ µg g^{-1} to 10.5 ± 5.00 µg g^{-1}), Cd (0.1 ± 0.02 µg g^{-1} to 15.8 ± 9.94 µg g^{-1}) and Pb (0.1 ± 0.02 µg g^{-1} to 4.71 ± 4.35 µg g^{-1}) levels in plant biomass ranges rise from background concentrations to correlate with the higher exposure levels in spiked soil. Additionally soil was monitored to determine if the plant uptake affected the concentrations of the soil. Water leachate was also monitored to see if heavy metals were leaving the system when plants were being watered. The analysis of heavy metals compared to plant species nutrient-acquisition strategy indicated that mycorrhiza plays a role in arsenic acquisition, with all three plant species having a positive correlation ($r_s \geq 83$) with As in leaf tissue. When exposed to cadmium (Cd) in soil, plants with arbuscular mycorrhizal fungi had a positive correlation ($r_s \geq 57$), while the plant with cluster roots had concentrations plateau, indicating a potential preventative measure to limit exposure in shoot biomass. Lead detected in leaf tissue did not correlate to concentrations in soil. From the water and soil analysis, it was also shown that heavy metal solubility changed and heavy metals remained bound in the soil instead of leaching out. With leachate levels of As (6.1 ± 0.94 µg g^{-1} to 29.0 ± 3.51 µg g^{-1}), Cd (14.9 ± 9.86 µg g-^{1} to 40.4 ± 1.08 µg g^{-1}) and Pb ($2.7 \pm$ 1,80 µg g^{-1} to 36.4 ± 2.85 µg g-^{1}) all being greater at beginning of the experiment compared to the end concentrations of As (0.05 ± 0.03 µg g^{-1} to 0.3 ± 0.08 µg g^{-1}), Cd (0.1 ± 0.08 µg g^{-1} to 4.0 ± 3.96 µg g^{-1}) and Pb (0.2 ± 0.11 µg g^{-1} to 19.5 ± 1.82 µg g^{-1}).

2.1 Introduction

The contamination of toxic heavy metals and metalloids in both terrestrial and aquatic systems is a major environmental concern (Salt *et al.*, 1995; Masindi & Muedi, 2018). Heavy metals occur naturally in soils around the world, but the concentrations of these elements are altered by anthropogenic industry and production leading to increased exposure in the environment (Masindi & Muedi, 2018). A primary route of introduction is mining operations that undertake excavation, extraction processing and the deposition of waste onto land (Franco-Hernández *et al.*, 2010; Gomes *et al.*, 2016; Sarwar *et al.*, 2017). Mining waste materials like tailings are stripped of desired ore and can contain high remnant concentrations of heavy metals (Clark *et al.*, 2001). Arsenic (As), cadmium (Cd) and lead (Pb) are often found as accessories to sought after ores (Franco-Hernández *et al.*, 2010), and have previously been detected in tailings and dust from several iron ore and gold mines within Australia (Smith *et al.*, 2003; Taylor *et al.*, 2014; Cross & Lambers, 2017). This group of metals are often labelled as 'heavy metals', which defines metals by their toxicity and a density of greater than 5 $g.L^{-1}$ (Duffus, 2002), although As does not fit the description of being a dense metal is it defined as a heavy metal from here on due to its toxic properties. The release of heavy metals into the environment can create severe contamination in sediment, ground- and surface water (Haigh, 2000). Part of restoration efforts for post-mining land is to re-introduce native plant species, and these species face the challenges of growing on a heavily-altered substrate (Australia Government, 2016; Cross et al., 2019). It is possible for plants growing in metalliferous soils to undertake phytoextraction, where toxic heavy metals are up-taken from the substrate and stored in plant biomass (Ernst, 2006; Ali et al., 2013). This can be achieved where substitution of essential nutrients occurs and is replaced with toxic heavy metals that are amassed by the plant with via protein transports (Plumlee & Ziegler, 2005). Other factors affecting the bioavailability of heavy metals for plant uptake include the plant nutrient-acquisition strategies such as associations with mycorrhizal fungi, which can sequester heavy metals into root sections of a plant and prevent transportation into above ground biomass (Baker, 2000; McGrath & Zhao, 2003). There is also the formation of organic complexes, soil pH, water solubility and the presence of manganese and iron oxides which influence heavy metal solubility and bioavailability (Masscheleyn et al., 1991; Rösner, 1998; Harvey, 2021).

The ongoing contamination of soils by heavy metals can become a potential issue when referring to the requirements for mine rehabilitation around the world, that land must be left

in a condition that is safe and non-polluting (Manero *et al.*, 2020). Mine leaseholders must also agree to rehabilitate land to a stable condition that can support its designated use; either conservation, grazing or cropping (Australia Government, 2016; Manero *et al.*, 2020). The concern for heavy metal exposure in the environment is paramount as metals like As, Cd and Pb are highly toxic even in low concentrations and do not degrade over time, only distributing and accumulating in other organisms or becoming trapped in sediment (Zhang *et al.*, 2014b; Masindi & Muedi, 2018). Heavy metals trapped in sediment or plant tissue opens a possible entry-level exposure pathway for humans through the food chain and entering higher trophic levels; via cattle ingesting edible plant tissue and sediment, and with humans consuming cattle or crop products (Kabata-Pendias, 2000; Manzano, 2015; Bicknell, 2019). The heavy metals that accumulate in human, animal and plant tissue can increase in concentrations, as consumption allows heavy metals entry into higher trophic levels meaning those at the top of the food chain experience biomagnification (Ali *et al.*, 2013). In plants, a toxic level of heavy metal exposure will reduce photosynthesis capabilities, microbial activity, and root development and once the tolerance threshold is passed it can deteriorate the plant completely and killing it (Baker, 1987; Baker & Walker, 1990; Ernst, 2006; Opaluwa *et al.*, 2012). In animals and humans As, Cd and Pb are all classified as carcinogenic and potentially fatal depending on exposure levels (WHO, 2010, 2011, 2019), further indicating they have no positive biological function. The occurrence of heavy metals left from anthropogenic activities can potentially have significant and detrimental impacts on the environment when planning the restoration or rehabilitation of mine site land that contains these metals (Ali *et al.*, 2013). Understanding accumulation, tolerance capabilities and nutrient-acquisition of plants ensure that the aspirations for ecosystem recovery and the utility of post-mining land for agricultural purposes are not compromised (Archer & Caldwell, 2004; Cross *et al.*, 2018).

This study was undertaken to better recognize the behaviour and distribution of heavy metals in the environment and their dispersal in soil, water and native plant species. We focused on three heavy metals commonly found in magnetite tailings in Western Australia (As, Cd and Pb), investigating what kind of relationship was evident between the concentrations of these elements in soil, plant tissues and leached soil water over time. It was hypothesised that as the spiked soil treatments increased the plants would respond accordingly and uptake higher amounts of heavy metal into their biomass. Plant seedlings *Acacia saligna (Fabaceae), Allocasuarina huegeliana (Casuarinaceae) and Austrostipa*

elegantissima (Poaceae) were used for this study as all three species are known to be grazed upon by cattle (Griffin *et al.*, 2011; Bicknell, 2019). *Acacia saligna* seeds can also be used in traditional bush food (Maslin *et al.*, 1998). These plant species are widely distributed throughout Western Australia and are a recommended species for revegetation of degraded land (Maslin *et al.*, 1998; Dixon, 2011; Bicknell, 2019). It was also proposed that the analysis of water leachate would indicate that heavy metals were leaching out of the soil during the experiment. The soil was also analysed at the beginning and end of the experiment as it was theorised that plants would remediate the heavy metals in the soil. This research can provide information on how different species react to heavy metals in their environment, and how soil parameters and nutrient-acquisition strategies of plants can play a role in heavy metals distribution in plant biomass. It also addresses the ramifications of using post-mining land for conservation and agriculture taking into account the entry pathway plants provide for heavy metals entering the food chain.

2.2 Methods

2.2.1 Plants and soil preparation

Seedlings of *Acacia saligna*, *Allocasuarina huegeliana* and *Austrostipa elegantissima* were sourced from Apace nursery in Fremantle, Western Australia (Table 2.1). Plants were grown in heavy metal spiked soils in a glasshouse located at Curtin University in Bentley, Western Australia, for 8 weeks. Spiked soil was prepared by using river sand as the potting medium, which was washed through a fine mesh and allowed to dry. Heavy metal stock solutions were made using the procedure from Elmer (1996), using arsenic trioxide (As_2O_3), cadmium nitrate tetrahydrate ($Cd(NO_3)_2.4H_2O$) and lead (II) nitrate ($Pb(NO_3)_2$) and were made to contain all three metals in a single solution. Solutions were made to five treatment levels; 0 µg mL^{-1}, 25 µg mL^{-1}, 50 µg mL^{-1}, 100 µg mL^{-1}, 200 µg mL^{-1}, which were determined by safe working limits in the SDS's (Sigma-Aldrich, 2020a, 2020b, 2020c), the maximum residue limits from Food Standards Australia New Zealand (2014) and (FSANZ, 2014), and from the following soil spiking technique and concentrations outlined by Intawongse and Dean (2006), Jarvis *et al.* (1976), Papazoglou (2009), and Zhang *et al.* (2014a). The control (0 µg mL^{-1}) and all other treatment levels below 200 µg mL^{-1} were additionally given potassium nitrate (KNO_3) to ensure the same exposure level of nitrates to all plant samples. The volume of solution were added to the soil to match the field

capacity of the pots and were mixed thoroughly and allowed to dry for 48 h before being used as a potting medium. The plants were potted in 140 mm pots with 1.5kg of spiked river sand and had 6 replicates per treatment per species. They were watered to 75% of the pots field capacity twice a week with tap water that had undergone a preliminary metal analysis with an MP-AES instrument (Appendix V, Table A6). At the end of the 8 week trial above-ground biomass of plants was extracted from the pots and dried in an industrial oven at 70°C over a period of 24 hours, before being analysed by ChemCentre (Bentley, Western Australia) for As, Cd and Pb with ICP-MS. For quality control and LOD refer to Appendix VI.

For collection of water leachate, plants were watered double the field capacity of the pot. Water was collected at the beginning of the experiment (Week 0), the middle (Week 4) and the end (Week 8). Water was intended to be analysed with ASV, due to its effective detection capabilities (see Chapter 1) however, due to complications with As analysis (Appendix VIII), MP-AES was used and ICP-MS later confirmed the concentration readings. Soil samples were first taken at week 0 after soil was spiked with heavy metals and had dried for 48 hours. They were sampled again after 8 weeks from the surface of the pots that grew *A. saligna*.

Table 2.1 - Native plant species assessed in the soil spiking treatments for heavy metal accumulation into above-ground biomass. Nutrient-acquisition strategies from Brundrett (2009), Cross *et al.* (2019) and Lambers *et al.* (2008).

Species	**Common name**	**Family**	**Nutrient-acquisition strategy**
Acacia saligna	Coojong or Orange Wattle	Fabaceae	AM/NF
Allocasuarina huegeliana	Rock Sheoak	Casuarinaceae	ECM/AM/NF/CR
Austrostipa elegantissima	Feather Spear Grass	Poaceae	AM

Nutrient-acquisition strategies; CR = cluster root, NF = nitrogen fixing, ECM = ectomycorrhiza, AM = arbuscular mycorrhiza, NM = non-mycorrhiza

2.2.2 Statistical analysis

Statistic software Rstudio version 1.3.1093 (R Core Team, 2013), was used to analyse heavy metal concentrations in soil, water leachate and plants. General linear models (GLM's) were used to compare concentrations of As, Cd and Pb in the soil and water samples between weeks passed. Data was log transformed within the GLM (Gamma family). P-values were derived from the chi-squared test calculated from the 'Anova' function in the 'car' package (Fox, 2019). An emmeans post-hoc test from the 'emmeans' package (Lenth, 2021), was used to identify significant differences of heavy metals to understand change in concentration in soil and water over time. For plant leaf tissue concentration of metals in plants was converted from µg $g^{-1}$to percentage of metal in dry leaf tissue. The percentage of heavy metal in dry plant tissue was run through a Spearman rank correlation analysis with the spiked soil treatments to identify positive linear relationships.

2.3 Results

2.3.1 Heavy metals in soil treatments

There was a significant relationship with As levels in soils after the 8 week experiment (Table 2.2, Fig 2.1A). Soil treated with 200 µg mL^{-1} of As in week 0, was significantly different (Table 2.3) and found to have three times more As in it when samples at 8 weeks (Table 2.4). The Cd concentrations in soil had significant differences over 8 weeks (Table 2.2, Fig 2.1B). The 200 µg mL^{-1} of Cd soil treatment was significantly different (Table 2.3), and close to 4 times higher at 8 weeks compared to week 0 (Table 2.4). The Pb concentrations in soil had a significant relationship between weeks (Table 2.2, Fig. 2.1C). The 200 µg mL^{-1} treatment of Pb (Table 2.3), was significantly higher with a concentration three times greater at week 8, compared to week 0 (Table 2.4).

Table 2.2 - GLM results to determine significant changes in As, Cd and Pb concentrations in spiked soil over 8 weeks.

Element	Variable	Chi2	Df	P-value
As	Week sampled	119.96	1	**<0.001**
Cd	Week sampled	36.92	1	**<0.001**
Pb	Week sampled	46.58	1	**<0.001**

Table 2.3 - Post hoc test results for GLM of As, Cd and Pb in spiked soil treatments when sampled at the beginning of the experiment (week 0) and at the end (week 8).

Heavy Metal	Treatment	Post hoc comparison of weeks	Z-stat	P-value
As	0 µg mL^{-1}	Week 0 - Week 8	3.00	0.08
	25 µg mL^{-1}	Week 0 - Week 8	-2.33	0.37
	50 µg mL^{-1}	Week 0 - Week 8	1.45	0.91
	100 µg mL^{-1}	Week 0 - Week 8	0.70	0.99
	200 µg mL^{-1}	Week 0 - Week 8	-10.9	**<0.01**
Cd	0 µg mL^{-1}	Week 0 - Week 8	0.78	0.99
	25 µg mL^{-1}	Week 0 - Week 8	-1.28	0.96
	50 µg mL^{-1}	Week 0 - Week 8	3.92	**<0.01**
	100 µg mL^{-1}	Week 0 - Week 8	0.34	1.00
	200 µg mL^{-1}	Week 0 - Week 8	-4.66	**<0.01**
Pb	0 µg mL^{-1}	Week 0 - Week 8	0.33	1.00
	25 µg mL^{-1}	Week 0 - Week 8	-1.53	0.88
	50 µg mL^{-1}	Week 0 - Week 8	2.75	0.16
	100 µg mL^{-1}	Week 0 - Week 8	-1.78	0.75
	200 µg mL^{-1}	Week 0 - Week 8	-6.66	**<0.01**

Table 2.4- Analysis of As, Cd and Pb concentrations (mean ± C.I.) in spiked soil used in plant growth trials of *Acacia saligna* at weeks 0 and 8 using ICP-MS

Element	Week	Treatment Levels				
		Control	**25 µg mL^{-1}**	**50 µg mL^{-1}**	**100 µg mL^{-1}**	**200 µg mL^{-1}**
As	0	1.0 ± 0.13	2.9 ± 0.24	6.0 ± 0.63	10.5 ± 2.90	9.5 ± 2.50
	8	0.7 ± 1.54	3.8 ± 0.35	5.1 ± 0.80	9.8 ± 0.30	30.7 ± 2.85
Cd	0	0.1 ± 0.01*	2.2 ± 0.30	9.0 ± 3.0	11.3 ± 1.80	8.9 ± 2.04
	8	0.1 ± 9x10^-18	3.2 ± 2.10	2.8 ± 1.35	10.2 ± 4.05	36.4 ± 30.50
Pb	0	1.8 ± 0.24	3.8 ± 0.60	10.4 ± 3.10	10.9 ± 5.30	12.3 ± 1.73
	8	1.7 ± 0.18	5.0 ± 1.2	6.4 ± 2.41	15.0 ± 2.26	40.3 ± 7.23

* denotes concentration reading was below the LOD

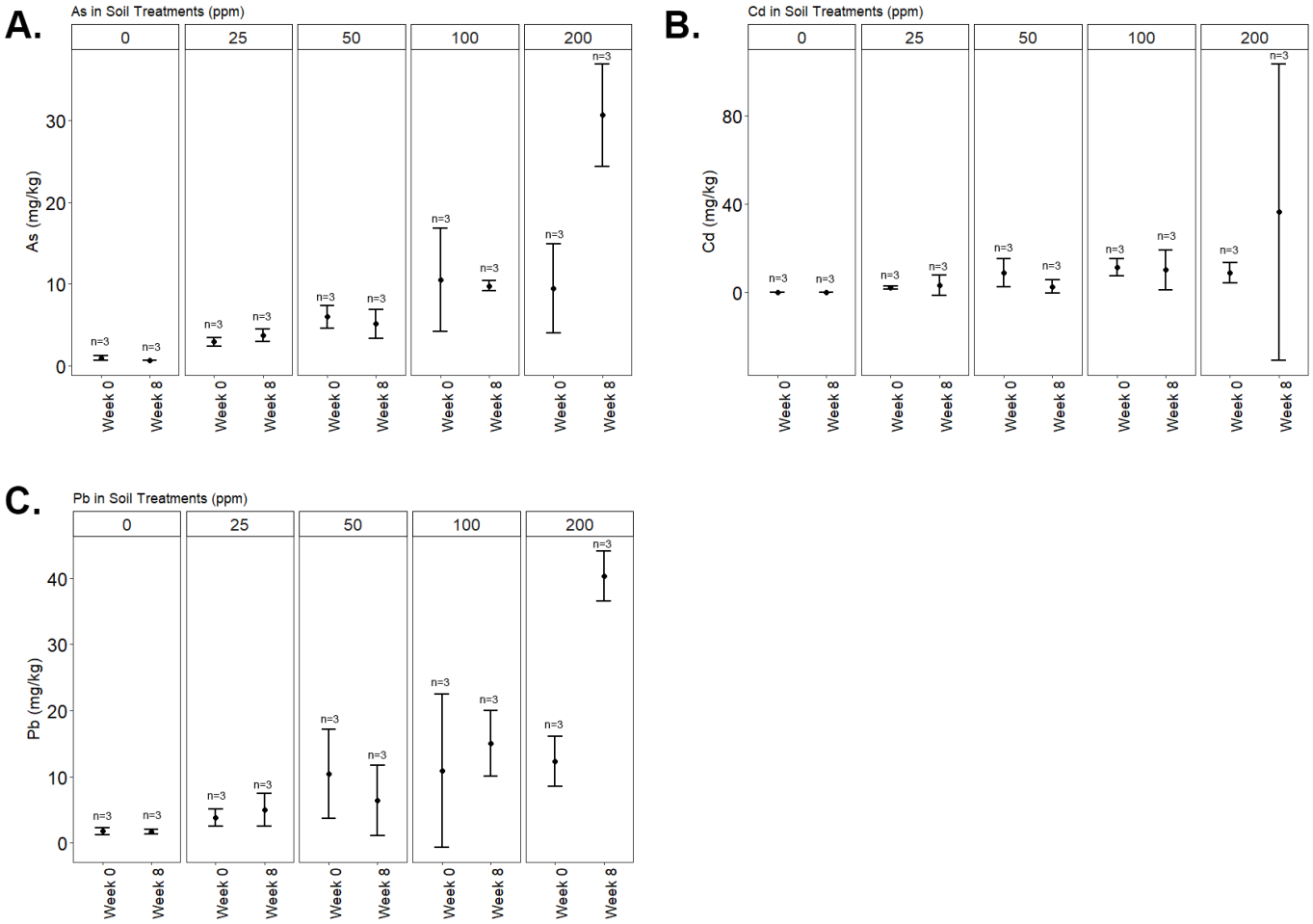

Figure 2.1- Concentrations (mean ± C.I.) of As (A), Cd (B) and Pb (C) in soils spiked with heavy metal solutions of varying concentration. Soil samples were taken after soil was spiked and dried for 48 hours (week 0), as well as from the surface of *A. saligna* pots at the end of the experiment (week 8). Analysis was undertaken by ICP-MS.

2.3.2 Heavy metals in water leachate

The As in water leachate (Fig. 2.2A) showed a significant relationship with concentrations between weeks (Table 2.5). Concentrations of As at week 0 were significantly higher than those at week 4 and 8 (Table 2.6), indicating concentration of As had diminished (Table 2.7). Analysis of Cd in water leachate (Fig. 2.2B) found a significant difference in the concentration between the weeks (Table 2.5). All Cd concentrations start the highest of week 0 and gradually decline. The Cd concentrations were not different from week 0, until they reach week 8 (Table 2.6), where concentrations have significantly reduced by ten times or more (Table 2.7). Analysis of Pb in the water leachate (Fig. 2.2C) found a significant relationship between the concentrations and the weeks sampled. At week 0 the treatments recorded the highest level of Pb in the water leachate. By week 4 only the 200 µg mL^{-1} treatment of Pb had a significantly lower concentration which had declined (Table 2.6). By week 8 the concentrations of Pb had greatly reduced in all treatments with the exception of 50 µg mL^{-1} (Table 2.7).

Table 2.5 – GLM results for determine changes in As, Cd and Pb concentrations in water leachate over 8 weeks.

Metal	Variable	Chi^2	Df	P-value
As	Week sampled	423.78	2	**<0.01**
Cd	Week sampled	69.517	2	**<0.01**
Pb	Week sampled	99.508	2	**<0.01**

Table 2.6 – Post hoc test results for GLM of As, Cd and Pb in water leachate when sampled at beginning of the experiment (week 0), middle (week 4) and end (week 8).

Heavy Metal	Treatment	Post hoc comparison of weeks	Z-stat	P-value
As	0 µg mL^{-1}	Week 0 - Week 4	4.28	**<0.01**
	25 µg mL^{-1}	Week 0 - Week 4	6.89	**<0.01**
	50 µg mL^{-1}	Week 0 - Week 4	14.7	**<0.01**
	100 µg mL^{-1}	Week 0 - Week 4	7.81	**<0.01**
	200 µg mL^{-1}	Week 0 - Week 4	9.18	**<0.01**
	0 µg mL^{-1}	Week 0 - Week 8	2.66	0.33
	25 µg mL^{-1}	Week 0 - Week 8	5.30	**<0.01**
	50 µg mL^{-1}	Week 0 - Week 8	11.60	**<0.01**
	100 µg mL^{-1}	Week 0 - Week 8	11.24	**<0.01**
	200 µg mL^{-1}	Week 0 - Week 8	16.71	**<0.01**
Cd	0 µg mL^{-1}	Week 0 - Week 4	0.51	1.00
	25 µg mL^{-1}	Week 0 - Week 4	0.67	1.00
	50 µg mL^{-1}	Week 0 - Week 4	2.98	0.16
	100 µg mL^{-1}	Week 0 - Week 4	0.57	1.00
	200 µg mL^{-1}	Week 0 - Week 4	1.83	0.89
	0 µg mL^{-1}	Week 0 - Week 8	1.76	0.91
	25 µg mL^{-1}	Week 0 - Week 8	6.24	**<0.01**
	50 µg mL^{-1}	Week 0 - Week 8	4.22	**<0.01**
	100 µg mL^{-1}	Week 0 - Week 8	3.77	**0.014**
	200 µg mL^{-1}	Week 0 - Week 8	4.97	**<0.01**
Pb	0 µg mL^{-1}	Week 0 - Week 4	1.14	0.99
	25 µg mL^{-1}	Week 0 - Week 4	2.26	0.62
	50 µg mL^{-1}	Week 0 - Week 4	0.68	1.00
	100 µg mL^{-1}	Week 0 - Week 4	2.42	0.50
	200 µg mL^{-1}	Week 0 - Week 4	7.00	**<0.01**
	0 µg mL^{-1}	Week 0 - Week 8	2.23	0.64
	25 µg mL^{-1}	Week 0 - Week 8	9.18	**<0.01**
	50 µg mL^{-1}	Week 0 - Week 8	1.27	0.99
	100 µg mL^{-1}	Week 0 - Week 8	4.97	**<0.01**
	200 µg mL^{-1}	Week 0 - Week 8	10.65	**<0.01**

Table 2.7 – Analysis of As, Cd and Pb concentrations (mean ± C.I.) in water leachate samples from spiked soil used in plant growth trials of *Acacia saligna* at weeks 0, 4 and 8, using MP-AES

		Treatment Levels				
Element	**Week**	**Control**	**25 µg mL^{-1}**	**50 µg mL^{-1}**	**100 µg mL^{-1}**	**200 µg mL^{-1}**
As	0	0.7 ± 0.13	6.1 ± 0.94	14.3 ± 0.70	10.9 ± 2.00	29.0 ± 3.51
	4	0.15 ± 0.1	0.5 ± 0.40	0.1 ± 0.04	0.04 ± 0.07	0.9 ± 0.80
	8	0.3 ± 0.08	0.1 ± 0.15	0.2 ± 0.1	0.2 ± 0.12	0.05 ± 0.03
Cd	0	0.2 ± 0.14	14.9 ± 9.86	26.2 ± 1.21	17.5 ± 1.21	40.4 ± 1.08
	4	0.1 ± 0.12	11.0 ± 0.70	6.6 ± 1.40	13.4 ± 4.04	17.3 ± 4.5
	8	0.1 ± 0.08	0.8 ± 0.90	3.7 ± 3.71	3.04 ± 1.08	4.0 ± 3.96
Pb	0	0.3 ± 0.15	27.2 ± 2.64	36.4 ± 2.85	2.7 ± 1.80	28.3 ± 12.44
	4	0.2 ± 0.16	9.0 ± 7.00	26.1 ± 5.23	0.8 ± 0.65	0.9 ± 0.80
	8	0.1 ± 0.05	0.3 ± 0.41	19.5 ± 1.82	0.2 ± 0.11	0.2 ± 0.12

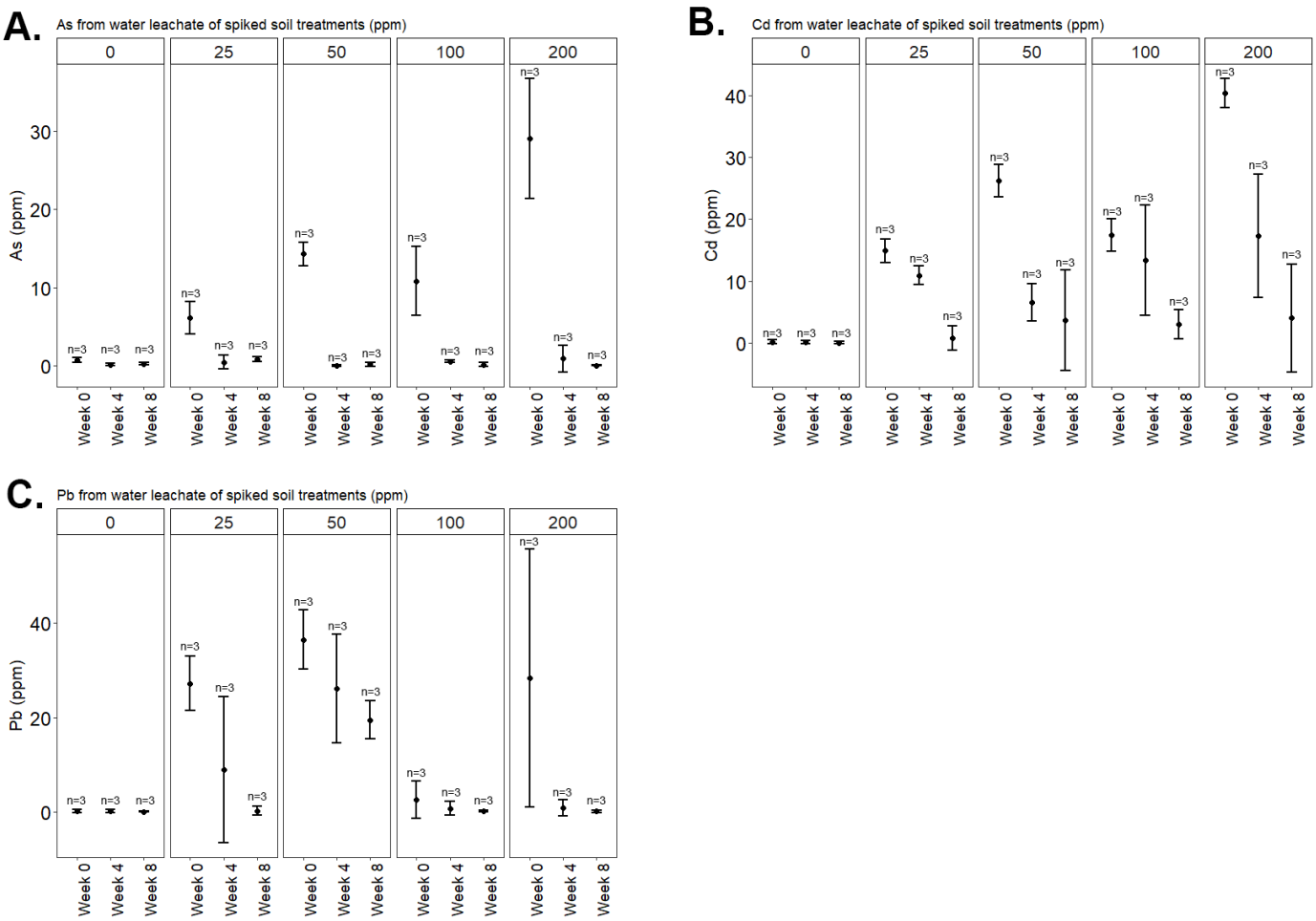

Figure 2.2- Concentrations (mean ± C.I.) of As (A), Cd (B) and Pb (C) in the water leachate from soils spiked with heavy metal solutions of varying concentration. Water was sampled after plants were watered for first time after potting (week 0), at the halfway point of the experiment (week 4), and again at the end of the experiment (week 8). Analysis was undertaken using MP-AES.

2.3.3 Analysis of arsenic in plants

Instrument analysis of As showed it was detected in every plant species for every spiked soil treatment level (Fig. 2.3, Table 2.8). There was a strong positive relationship between soil and leaf tissue As concentrations evident in all three species tested; *A. saligna* (r_s = *0.94, P <0.001, N = 30*), *A. huegeliana* (r_s = *0.91, P <0.001, N = 30*), and *A. elegantissima* (r_s = *0.83, P <0.001, N = 30*).

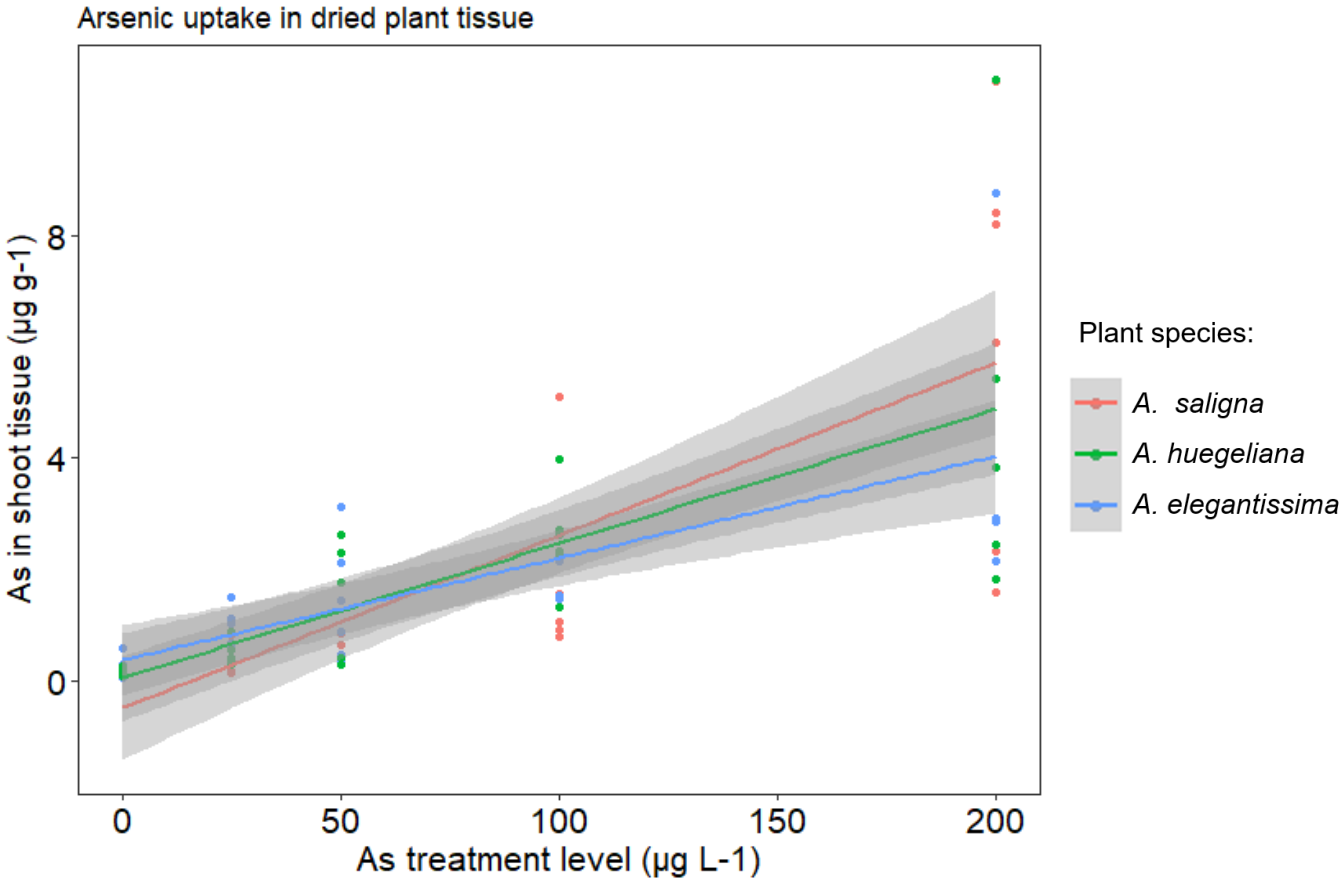

Figure 2.3 –Concentrations of As in dried leaf tissue for *Acacia saligna* (red), *Allocasuarina huegeliana* (green) and *Austrostipa elegantissima* (blue) with increasing soil As concentration from spiked soil treatments. Plants were exposed to five spiked soil treatments over an eight-week period. Analysis of As was conducted using ICP-MS

2.3.4 Analysis of cadmium in Plants

Concentrations of Cd were detected in every plant species at every treatment level (Fig. 2.4, Table 2.8). For *A. saligna* there was a significant correlation in the percentage of Cd in plants (r_s = *0.57, P <0.001, N = 30*). The percentage of Cd in plant tissue between soil treatments gradually increased for plants grown in higher spiked soil treatments. The *A.huegeliana* showed a significant correlation for Cd up-take (r_s = *0.45, P = 0.01, N = 30*), the moderate concentration of Cd in plant tissue appears to plateau when grown in higher spiked soil treatments. Analysis of *A.elegantissima* showed a significant correlation of Cd in plants tissue (r_s = *0.76, P <0.001, N = 30*). The percentage of Cd in plant tissue had a strong positive relationship what indicated plants exposed to higher treatments of spiked soil, would accumulate higher concentrations of Cd in their leaf biomass.

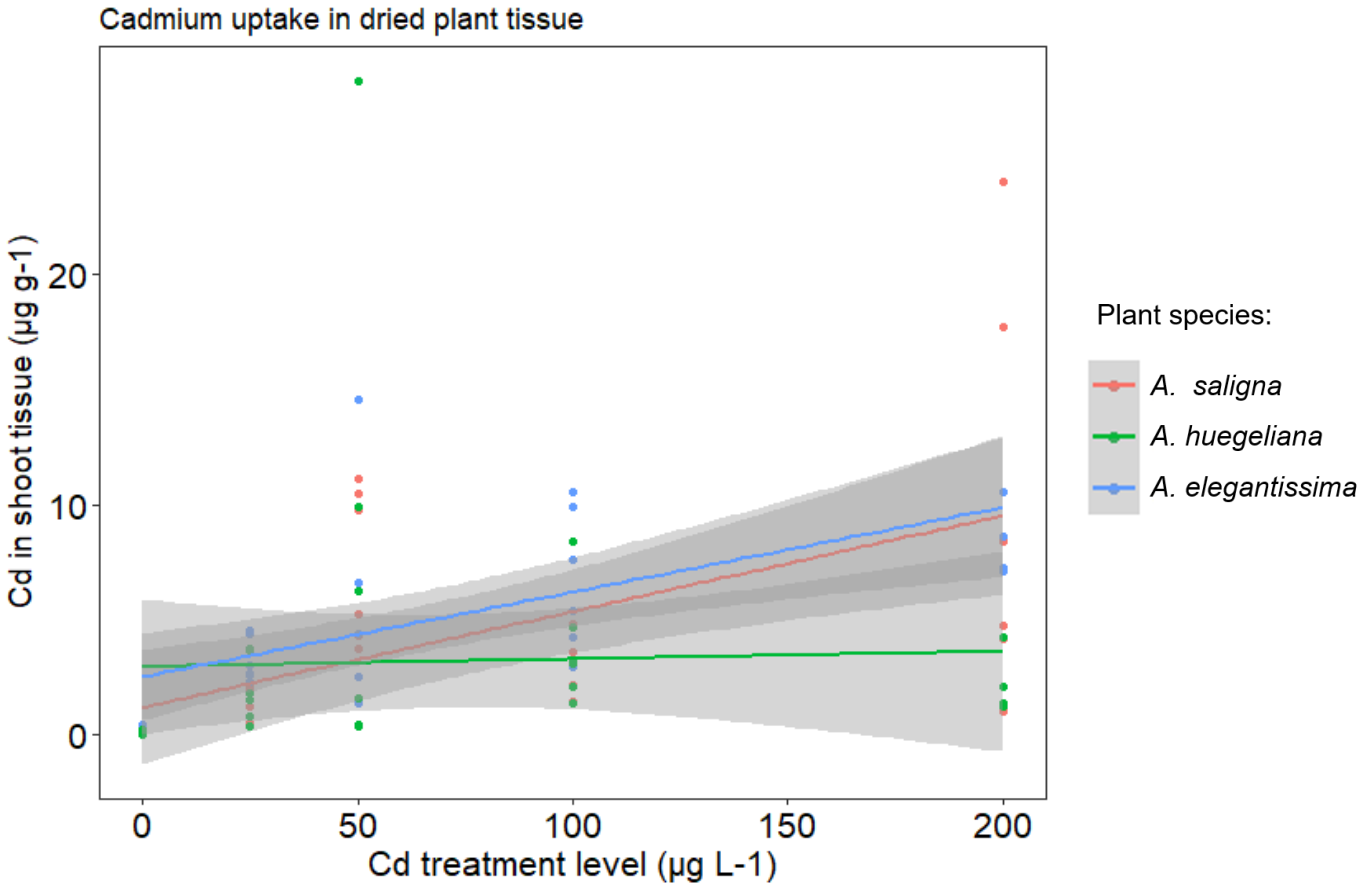

Figure 2.4 – Concentrations of Cd in dried leaf tissue for *Acacia saligna* (red), *Allocasuarina huegeliana* (green) and *Austrostipa elegantissima* (blue) with increasing soil As concentration from spiked soil treatments. Plants were exposed to five spiked soil treatments over an eight-week period. Analysis of As was conducted using ICP-MS

2.3.5 Analysis of lead in Plants

All plant species had Pb detected in the dried plant tissue (Fig. 2.5, Table 2.8). Analysis of *A. saligna, A. huegliana* and *A. elegantissima* did not indicate positive correlations of Pb in the soil with concentrations detected in leaf tissue, but does show the concentrations in all three species plateauing despite the increasing spiked soil concentrations.

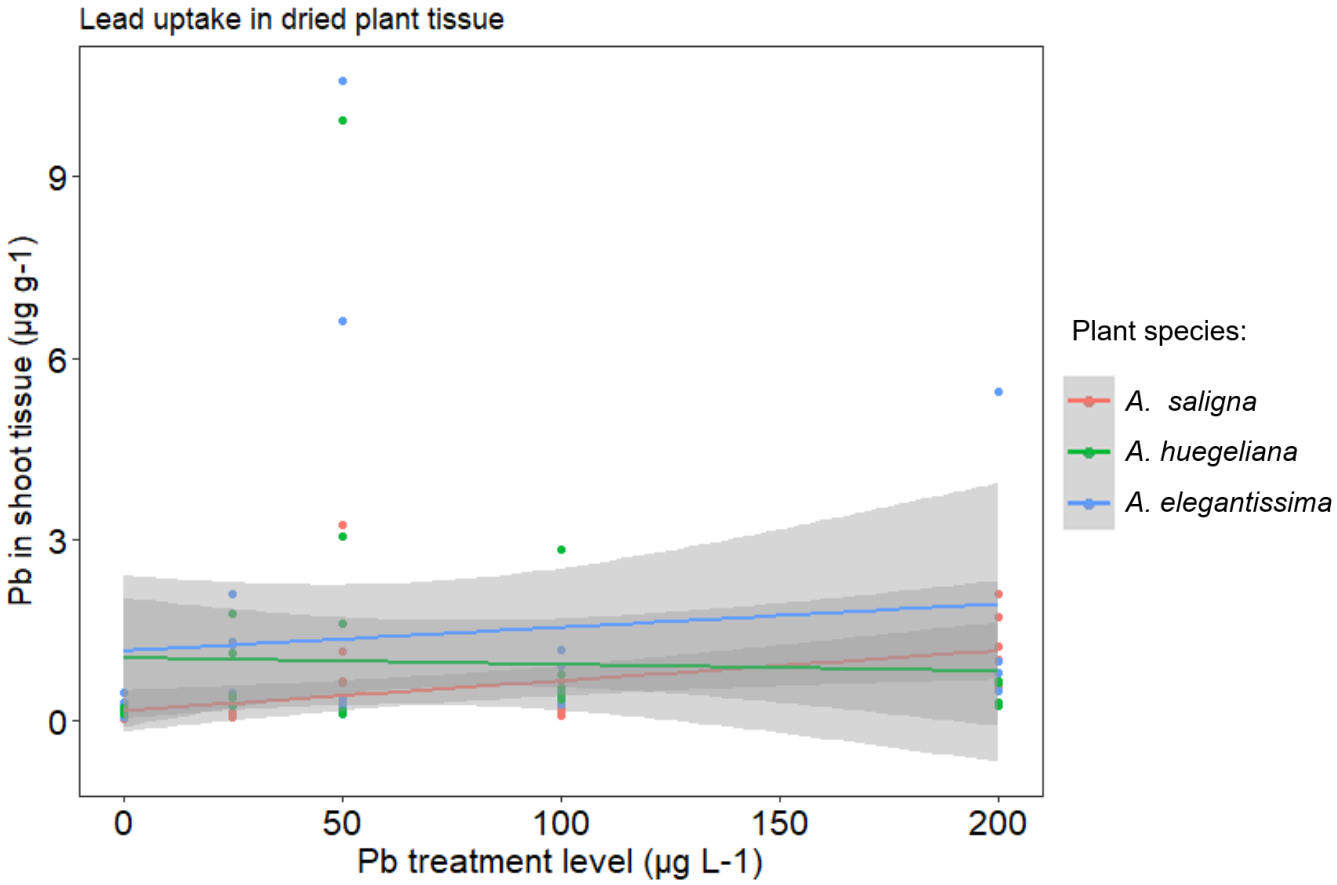

Figure 2.5 – Concentrations of Pb in dried leaf tissue for *Acacia saligna* (red), *Allocasuarina huegeliana* (green) and *Austrostipa elegantissima* (blue) with increasing soil As concentration from spiked soil treatments. Plants were exposed to five spiked soil treatments over an eight-week period. Analysis of As was conducted using ICP-MS

2.3.6 Heavy metal concentrations in plants

Table 2.8 – Concentrations of heavy metals As, Cd and Pb (mean ± C.I.) in dry leaf tissue of native plants grown in spiked soil treatments (*n* = 6 for each species) for 8 weeks. Analysis was conducted using ICP-MS

		Treatments				
Sample	**Metal (µg g^{-1})**	**0 µg mL^{-1} (control)**	**25 µg mL^{-1}**	**50 µg mL^{-1}**	**100 µg mL^{-1}**	**200 µg mL^{-1}**
Acacia saligna	As	0.2 ± 2.4 x 10^{-17}	1.3 ± 0.48	2.1 ± 0.70	4.2 ± 2.36	10.5 ± 5.00
	Cd	0.1 ± 0.02**	5.5 ± 1.32	19.6 ± 6.88	6.5 ± 2.02	15.8 ± 9.94
	Pb	0.1 ± 0.02**	0.8 ± 0.29	2.3 ± 2.89	0.8 ± 0.40	2.2 ± 1.18
Dry shoot weight (g)		3.3 ± 0.49	3.5 ± 0.74	9.8 ± 0.22	2.8 ± 0.44	1.8 ± 0.39
Allocasuarina huegeliana	As	0.5 ± 0.14	1.3 ± 0.54	2.2 ± 1.05	4.9 ± 2.00	7.7 ± 1.07*
	Cd	0.2 ± 0.19	5.4 ± 2.94	6.3 ± 5.07*	6.8 ± 2.00	3.4 ± 1.07*
	Pb	0.5 ± 0.08	2.1 ± 1.47	1.67 ± 1.53	1.5 ± 0.87	0.7 ± 0.16
Dry shoot weight (g)		3.3 ± 0.24	2.8 ± 0.43	2.2 ± 0.63	2.1 ± 0.60	1.8 ± 0.40
Austrostipa elegantissima	As	0.4 ± 0.12	1.8 ± 0.41	2.2 ± 1.21	2.9 ± 0.45	6.6 ± 6.34
	Cd	0.3 ± 0.12	6.0 ± 1.48	11.6 ± 6.74	10.1 ± 3.41	12.8 ± 3.73*
	Pb	0.5 ± 0.16	2.3 ± 0.72	4.7 ± 4.35	1.0 ± 0.40	2.2 ± 2.17*
Dry shoot weight (g)		2.1 ± 0.98	1.9 ± 0.21	1.7 ± 0.24	1.6 ± 0.17	1.4 ± 0.28

*Denotes outlier removal based on data outside of the IQR range.

**Denotes concentration reading was below the LOD

2.4 Discussion

2.4.1 Concentration changes to heavy metals in soil and water leachate

The variation of heavy metal concentrations in soil over the eight-week period (Table 2.4) was likely related to the bioavailability of heavy metals in soil changing with ambient temperature, acidification, nitrification and leaching (Hooda & Alloway, 1993). The adsorption process can also be affected by the organic matter in the soil and the formation of complex ligands such as EDTA and humic acid (Lo *et al.*, 1992). Furthermore, different metal species experience different retentions, Cd for example has increased retention that restricts its mobility when exposed to organic matter (Elliott *et al.*, 1986; Bradl, 2005). Lead mobility is reliant on a low pH, but for adsorption it can be reliant on the presence of Mn and Fe oxides and exposure to dissolved organic matter may also result in the complexion of Pb and create a low solubility (Bradl, 2005). Arsenic binding mechanisms are based on a high presence of dissolved oxygen a high pH and a high redox potential (Bradl, 2005). However in topsoil in a more acidic environment As is highly mobilised in soil tending to move downwards with leaching water (Livesey & Huang, 1981; Adriano, 2013). In previous studies As has also been shown to be lost from the first 20 cm of topsoil and found at depths >40 cm (Adriano, 2013; Alexandre, 2021). A soil spiking procedure outline by Zhang *et al.* (2014a), trialled incubation times lasting 12 weeks to ensure sufficient time for heavy metal complexes to bind to the soil, which may suggest that the allocated binding time for this experiment was not long enough initially and saw the metals binding and interacting with organic matter in the later weeks of the experiment.

Over the eight-week experimental period it was shown in the soil samples that the heavy metals were not decreasing (Table 2.7). The concentration in the water samples however was. All spiking treatments were created with water-soluble compounds that were mixed into the river sand. The first time plants are watered, the excess leachate contains remnants of these heavy metals, however at week 4 and week 8 of the experiment, the amount of heavy metals dramatically begins to decrease as these metals are left in the substrate for the duration of the experiment. The heavy metals from the treatments are likely undergoing some type of adsorption with organic material or manganese and/or iron oxides where they have changed solubility due to binding into the substrate (Bradl, 2005), making them no longer water soluble, and therefore not being detected in leachate.

2.4.3 Plants response to arsenic, cadmium and lead

The primary species of As that forms in soil and water is arsenate (AsO_4^{3-}) (Bradl, 2005). The same is said for mine tailings where the arsenic is oxidised and forms arsenate, and which is readily up-taken into metabolic pathways for many species (Meharg & Hartley-Whitaker, 2002). In lower pH ranges (6.3-8.3), the presence of chlorine, manganese and iron oxides in soil can potentially affect can the oxidation of As(III) to As(V) (Clifford & Ghurye, 2001). When As is in the environment it is reportedly more mobile in a course-grained substrate and has a high affinity for proteins and lipids making it readily accumulate into tissue (Orumwense, 1996; Bradl, 2005). All of three plant species have arbuscular mycorrhiza and all three showed an increase and uptake pattern for As (Table 2.8), with exposure ranges for *A. saligna* ($0.2 \pm 2.4 \times 10^{-17}$ µg g^{-1} to 10.5 ± 5.00 µg g^{-1}), *A. huegeliana* (0.5 ± 0.14 µg g^{-1} to 7.7 ± 1.07 µg g^{-1}) and *A. elegantissima* (0.4 ± 0.12 µg g^{-1} to 6.6 ± 6.34 µg g^{-1}) being similar (Fig 2.3). As indicated in the correlation, where there was an increase of As in the soil treatments, as in dry leaf tissue increased. This display of As behaviour in plants could be a useful tool to monitor any changes to the amount of As that is present in the environment as it appears to be responsive to concentration variations. The responsive nature may be due to As having the same valence electrons as phosphorus (P), and making arsenate (AsO_4^{3-}) in soil chemically similar to phosphate (PO_4^{3-}) (Meharg & Macnair, 1990). Plants that have arbuscular mycorrhiza are reported to have a higher ratio of P to As when compared to non-mycorrhizal species (Smith, 2008; Smith *et al.*, 2010). The presence of mycorrhizal fungi in soil assists with the uptake of phosphorus into plants through a symbiotic relationship and allows for nutrient uptake via diffusion into the roots of the plant (Smith et al., 2010). It is likely the same relationship is present for arsenate and it is given assisted uptake through the mycorrhizal hyphae in the soil and into the plant (Meharg & Macnair, 1990; Smith *et al.*, 2008).

The dominant forms of Cd in soil are often reported as being the free ion form Cd^{2+} or as an inorganic ligand complex (Emmerich *et al.*, 1982; Hirsch & Banin, 1990; Holm *et al.*, 1996). When Cd is available in the soil it readily forms stable organic compounds and chelates (Bradl, 2005). All three tested species (*A. saligna, A. huegeliana* and *A. elegantissima*) possess arbuscular mycorrhizal nutrient-acquisition strategies (Table 2.1). However, *A. huegeliana* has a mixed strategy and also forms cluster roots (Diem *et al.*, 2000). Cluster roots are densely packed short lateral roots that form as a spontaneous response to assist in nutrient acquisition (Diem *et al.*, 2000). Cluster roots work by

releasing large quantities of exudates in the form of carboxylates, usually to assist in the acquisition of phosphorus (Diem & Arahou, 1996; Lambers & Shane, 2007). Root exudate can react with Cd, possibly forming chelates which limit the availability of Cd being up-taken into plant biomass (Römer *et al.*, 2000; Lambers *et al.*, 2008). This could explain why no differences in Cd acquisition among treatment levels were reported (Table 2.8) for *A. huegeliana* (0.2 ± 0.19 µg g^{-1} to 3.4 ± 1.07 µg g^{-1}), compared to the positive linear relationships between soil and plant tissue Cd concentrations observed for *A. saligna* (0.1 ± 0.02 µg g^{-1} to 19.6 ±6.88 µg g^{-1}), and *A. elegantissima* (0.3 ± 0.12 µg g^{-1} to 12.8 ± 3.73 µg g^{-1}).

Traces of Pb can be detected throughout the natural environment (Bradl, 2005). This is evidenced by analysis of additional nursery-grown plants that were not grown in the glasshouse, but prepared immediately for a preliminary analysis to determine background concentrations of heavy metals present (Appendix I, Table 1). There were very few instances of Pb uptake differentiating based on the higher treatment levels of heavy metals in the soil (Table 2.8), between the species *A. saligna* (0.1 ± 0.12 µg g^{-1} to 2.2 ± 1.18 µg g^{-1}), *A. huegeliana* (0.5 ± 0.08 µg g^{-1} to 0.7 ± 0.16 µg g^{-1}) and *A. elegantissima* (0.5 ± 0.16 µg g^{-1} to 2.2 ± 2.17 µg g^{-1}). Multiple studies have concluded that Pb detected in sediment does not necessarily correlate to the concentrations found in plant biomass, due to very little amounts of the Pb available, being soluble (Bradl, 2005; Kopittke *et al.*, 2008; Punamiya *et al.*, 2010; Pourrut *et al.*, 2011). Bioavailability of Pb requires it in a soluble form that can be up-taken via Ca^{2+} protein channels (Raskin, 1995; Rajkumar *et al.*, 2012). This relies on Pb forming stable chelate formations and interacting with organic matter (Bradl, 2005). Mobility of lead is also highly dependent on the pH, organic matter available and the presence of manganese and iron oxides in the substrate (McKenzie, 1980). The river sand used in this experiment was rich in haematite (Fe_2O_3). The iron (Fe) likely present in the river sand can play a predominant role in the Pb adsorption in soil which inhibits mobility (McKenzie, 1980; Seregin & Ivanov, 2001; Pourrut *et al.*, 2011). This experiment analysed dry leaf tissue to address concerns of herbivory of plants, it is also likely that Pb that has been up-taken is sequestered in the roots of plants. The presence of mycorrhiza in plant roots can allow a plant to tolerate heavy metals in their tissue by restricting metal transport and sequestering them to the roots of a plant (Callahan *et al.*, 2006; Clemens, 2006; Lambers *et al.*, 2008). The arbuscular mycorrhizal associations that

all three plant species have (Table 2.1), may play a role in the absence of Pb accumulation in the leaf tissue that was witnessed (Fig. 2.5).

2.4.4 Herbivory concerns and monitoring implementations

With heavy metals bound in substrates, there is persistent contamination that is unable to break down but has the ability to transfer from one organism to another through natural interactions in the soil and water (Dube *et al.*, 2001). Monitoring plants, water and soil can indicate where these heavy metals are ending up and what is at risk of exposure. Herbivory concerns can also be addressed knowing that plants with As and Cd exposure can occur from the consumption of above-ground plant biomass, but high concentrations of lead in plants is less likely to be a occurring. Cattle have been known have high palatability for young Allocasuarina species (Bicknell, 2019). By identifying nutrient uptake strategies and metal tolerance capabilities like cluster roots and mycorrhiza that can sequester heavy metals to the roots and immobile their distribution, it becomes a useful tool in choosing which plant species should populate a landscape containing metalliferous soils that may be designated to grazing. Monitoring, however, should continue as cluster root formation, maybe a response to environmental factors and not necessarily always present (Diem *et al.*, 2000). Understanding how native species grow in contaminated substrate allows us to determine if they can be categorised as accumulators, excluders or indicators (Baker, 1981). These terms may be interchangeable depending on what heavy metals are present in the soils. All three species had a linear relationship with As uptake and can be categorised as indicators. *A. saligna* and *A. elegantissima* can be classed as indicators for Cd, whereas *A. huegeliana* is an excluder due to its Cd concentration plateauing. None of the species Pb levels correlated to the soil concentration and are all excluders for Pb. Plants that are designated as accumulators can be optimised for the potential remediation of soils from heavy metals, whereas excluders can ideally be used in rehabilitation of mining land for phytostabilisation and limit heavy metal exposure from herbivory by sequestering heavy metals to roots of plants. Additional advantages that can come from the knowledge of what plant species are metal tolerant is being able to grow plants in a contaminated substrate and gain the benefits of increasing organic matter and soil fertility, Additional benefits of identifying and utilising heavy metal tolerant plants can allow for improved soil structure, reduced erosion, and immobilise the distribution of heavy metals (Archer & Caldwell, 2004).

2.5 Conclusion

Toxic heavy metals As, Cd and Pb can be up-taken into native vegetation biomass when the metals are present in the substrate. Heavy metals undergo a myriad of chemical changes when exposed to water, oxygen and organic matter in the soil, making it difficult to assess bioaccumulation and therefore phytoextraction capabilities of plant species. It is evident however, that the nutrient-acquisition strategy for plants is a vital indicator for the heavy metal accumulation and sequestering abilities, while adsorption of heavy metals in the soil is evident with their change in solubility and absence from leachate over time. The presence of arbuscular mycorrhizal fungi was also a likely contributor for As and Cd uptake as plants were highly responsive to the different As and Cd concentrations in the soil, with the exception of the appearance of cluster roots which may have restricted Cd uptake through the root exudates forming chelates and limiting Cd mobility. The trace concentrations detection in Pb in above-ground biomass could also indicate a greater contamination issue in the roots of the plant and the sediment. An understanding of how heavy metals behaviour and disperse in soil and plants with varying nutrient-acquisition strategies can be a useful tool to not only monitor bioaccumulation phytoextraction, but to also determine suitability of plants for re-vegetation of post-mining land. The advantages of this can also determine where metal accumulates in plant tissue and if it is safe to consume sections of certain plant species. Post-mining land is often designated to the production of plant-based products or cattle grazing, potentially creating an exposure pathway to humans via consumption. Therefore industry regulators should pay attention to the longevity and bioaccumulation of heavy metals in post-mining landscapes. Further studies should be undertaken for longer periods to monitor phytoextraction effectiveness and the potential of native plants as effective remediators of heavy-metal contaminated soil. Additional studies should also be undertaken using crop species, non-mycorrhizal species, and species producing edible seeds to determine their interactions with, and accumulation of, heavy metals, and determine their suitability for use in restoration or rehabilitation of heavy metal contaminated post-mining land. Consideration should also be made for studies of post-mining land uses that take into account and support the potential for traditional Indigenous landowners to engage in customary activities such as the production of bush foods.

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General Conclusion

As evident in both chapters' plants will bioaccumulate heavy metals if they are present in their environment and due to anthropogenic activities elevated levels of heavy metals are present in the environment. Regardless of soil pH and unfavourable solubility, plants have adaptations for acquiring nutrients when there is little available in the soil and the heavy metals that may also be present come along for the ride. The aim of this study was to not only determine if heavy metals were up-taken into plants but it was used to also give insight to better recognise the behaviour and distribution patterns of heavy metals in the environment. This was done by focusing on three metals commonly found in mine tailings; As, Cd and Pb, and observing their interactions with plants in a range of soil types and conditions. This study also addresses the potential herbivory of contaminated plants that may occur either by human or animal and addresses the need for monitoring implementations as a part of post-mining regulations.

Chapter 1, which was entitled "Determining the most effective analytical method for quantifying phytoextraction capabilities in native plants", which analysed plant samples grown in magnetite tailings, topsoil and capped tailings. This study not only determined the presence of Cd and Pb in the plants and quantify their concentrations, but was also able to compare the capabilities of the analytical instruments. The MP-AES instrument is an emerging technology that was capable of detecting heavy metals within the same range as ASV and ICP-MS but suffered from significant signal interferences. Analysing with ASV accompanied with standard addition, was a highly effective technique that was able to remove matrix affects interfering with the signal reading and produced results that did not differ from the ICP-MS analysis. Despite being time consuming when having a large sample size it was able to provide a multi-elemental analysis of Cd and Pb, while being cost effective and portable if required. The instrument analyses were able to confirm the other hypotheses that roots as a point of first contact would hold much of the heavy metals it came into contact with. This also appeared to be the case a nutrient-acquisition strategies have shown to play a role in up-taking heavy metals and also with where they are stored in the plant. Species growing on alkaline tailings that did not have mycorrhiza were found to have an even distribution of heavy metals in the roots and shoots. Another theory investigated was how Pb and Cd could be getting into the plant despite the alkaline substance they were growing in. Regardless bioaccumulation was found to be occurring in a pH that Pb and Cd are not soluble in. The analysis of Fe and Ca uptake correlated with

Pb indicating that where Fe and Ca are higher, Pb in plants were higher too and it is possibly using those nutrient channels as an assisted pathway into the plant. When looking at previously results for Cd and Pb in the alkaline tailings, it appears that many of plants have a higher concentration which could be an indication of bioaccumulation.

Chapter 2, entitled “Australian native plants grown in arsenic, cadmium and lead spiked soil to determine phytoextraction capabilities and appropriate uses for post-mining land”, investigated the relationships of heavy metals in soil, water and plants. Analysis of the soil was undertaken with the hypothesis that the amount of As, Cd and Pb would be reduced over the 8 week period the spiking experiment was conducted for. Instead the results indicated that complex interactions were occurring where heavy metals were likely binding to the organic matter over time. This was also shown with the water analysis, which theorised heavy metals would leach out of the soil when the plants were watered. Instead the heavy metals were no longer in soluble form and after the first week was not leaving the soil, indicating soil adsorption had occurred. In regards to the native plants the theory was that the higher spiked soil treatments they were exposed to, the more heavy metals they would accumulate. For As accumulation this hypothesis was true, the plants responded by up-taking arsenic proportionately to what was in the spiked soil, suggesting species with arbuscular mycorrhiza could potentially be used as indicators for how much arsenic is present in a substrate. For Cd the hypothesis was correct for two of the species, but for *A. huegeliana*, Cd uptake was limited which was possibly due to the formation of cluster roots that release exudates that Cd can form complexes with and prevent uptake into the shoots of the plant. For Pb, despite being detected in the plants, the concentrations found in the shoots did not reflect the higher concentrations of the treatments in the soil, further analysis of the roots would be required to ensure if the plants were up-taking Pb at all and if it was sequestered to the roots.

This study opens to the door to the potential that remediation through phytoextraction can have for Australian native plants. The determination of phytoextraction can be developed into phytoremediation strategies for plants that are used in mine site restoration. The selection of plant species can allow for those that have tolerance and accumulation capabilities to be grown in metalliferous substrates. There is a need to implement ongoing monitoring for the bioaccumulation in plants and biomagnification through higher trophic levels. Around the world, bird life living near mine sites; have been reported for having

heavy metals in their feathers and eggs. Cattle have also been previously recorded for ingesting heavy metals found in sediment and vegetation. Further studies should be done with a focus on identifying hyperaccumulator plants species, non-mycorrhiza plant species, crop plants and those that have edible seeds that may also affect tradition land owners. Further studies should also be done with a focus on prevention, as remediation and restoration is no simple task.

The quickest way to resolve mine site waste being generated in vast quantities would be to cease mining production, yet booming economies have shown a high demand for resources that require mining and are incredibly profitable to continue with. Mining, therefore, continues to be widespread and exploited throughout Australia due to its abundance in metal ores and long mine life predictions. A long mine life in Australia means a constant source of tailings and heavy metals entering the environment. We need to start paving the way to ensure that when post-mining land contains heavy metals the issue can be addressed. The assistance of further studies can help designate an appropriate use for the land or assist in the selection of suitable plant species that can grow on metalliferous soils with minimal risk heavy metal exposure through herbivory or entry into the food chain. It's not just a practice of ethics but also a preventative measure, as being at the top of the food chain means we are exposed to the highest concentration of heavy metals.

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Appendix I - Method validation and quality control for MP-AES analysis

A pre-digestion spike was conducted with plant material of similar species collected around Curtin University, Bentley Campus. The plant material was ground and then had known concentrations of cadmium, lead added to it. The spiked plant samples were subjected to an entire nitric acid digestion procedure to identify the recovery of the spiked metal material and determine the appropriateness of the analytical method for metal detection (Table 1). Nitric acid was also digested on a hotplate with no plant material and analysed to ensure no heavy metal content in the acid contaminated the plant samples and gave untrue readings.

The MP-AES instrument was used to analyse the spiked samples and along with the manufacturer's recommendations was used to select the ideal wavelengths that could detect an accurate reading of metal concentration that was spiked and have the least amount of interference of elements with similar emission wavelengths from being in a high matrix solution. Cadmium, lead and arsenic were analysed on the wavelengths 508nm, 405nm and 197nm, respectively. Certified calibration standards of 1000 µg mL^{-1} were used to create the calibration standards and spiking solutions for cadmium, lead and arsenic when analysed with MP-AES, FAAS and voltammetry. In the analysis with FAAS and MP-AES, a certified reference material was also used from Agilent that contained 5mg.L^{-1} of Cd and Pb with a matrix of 5% HNO_3. Glassware and containers used in all procedures was soaked in 1% HNO_3 solution overnight between uses to avoid metal contamination across samples and interferences from detergent.

Table A1 - *Acacia saligna Allocasuarina fraseriana*, A*ustrostipa elegantissima, Eucalyptus todtiana Hakea lissocarphya* were collected from Curtin University campus gardens (Bentley), before being dried and spiked with a 10 µg mL^{-1} solution of Cd and Pb. Plants underwent full nitric acid digestion prior to analysis with MP-AES to determine recovery of the spiked heavy metals

Spiked species	Cd Recovery (%)	Pb Recovery (%)
Allocasuarina fraseriana	91.8	105.1
Austrostipa elegantissima	79.3	84.6
Hakea lissocarphya	122.4	102.6
Acacia saligna	88.6	90.2
Eucalyptus todtiana	72.2	80.5

Appendix II – Limits of Detection for instrument analysis

Limit of detection (LOD) was determined using a linear regression based on standard concentrations (y-axis) and the signal concentrations (x-axis) from each instrument. This was done for both the cadmium and lead (Table A2) analysis that was undertaken.

The ICP-MS analysis from ChemCentre listed a "Limit of Reporting" for calcium (0.1 µg mL^{-1}), cadmium (0.0001 µg mL^{-1}), iron (0.005 µg mL^{-1}) and lead (0.0001 µg mL^{-1}).

Ca and Fe analysis was done by ChemCentre using the ICP-OES instrument with a multi-calibration line. For Ca wavelengths 317.933nm were used for detection up to 2000 µg g^{-1} and 219.779nm for above 2000 µg g^{-1}. For the detection of Fe, 238.204nm was used for up to 200 µg g^{-1} and Fe 261.187nm for 200 µg g^{-1} to 2000 µg g^{-1}. Digested tomato leaves were also used as the certified reference material.

Table A2 - Limits of detection for instruments MP-AES, FAAS and ASV when analysing plants samples for Cd and Pb

Element	Instrument	LOD	Calibration Range	Coefficient of determination (R^2)	Linear equation
Cd	MP-AES	0.02 ppm	0.5- 4.0	0.9997	y = 2631.09x + 0.153
	ASV	0.58 ppb	0.001 – 0.02	0.9974	y = 05214x + 0.216
Pb	MP-AES	0.06 ppm	0.5- 4.0	0.9996	y = 255.11x + 0.198
	FAAS	0.20 ppm	0.25 – 4.0	0.9999	y = 0.028x + 0.0004
	ASV	0.26 ppb	0.001 – 0.02	0.9998	y = 0.811x + 0.062

Appendix III – Lead concentrations for plants grown in glasshouse conditions

Table A3 – Pb concentrations (mean ± SE) in root and shoot biomass of *Acacia ramulosa, Allocasuarina acutivalvis, Austrostipa scabra, Eucalyptus loxophleba, Hakea recurva,* and *Maireana georgei* grown in topsoil, tailings and capped tailings at the Shenton Park Field Station, analysed by ICP-MS, MP-AES, FAAS and ASV

Species	Substrate	Biomass section	ICP-MS (µg g^{-1})	MP-AES (µg g^{-1})	FAAS (µg g^{-1})	ASV (µg g^{-1})	Sample size (n)
Acacia ramulosa	Topsoil	Shoot	1.54 ± 0.35	8.36 ± 0.83	30.87 ± 4.00		5
		Root	6.06 ± 3.38	8.89 ± 0.72	51.82 ± 7.76		5
	Capped	Shoot	2.72 ± 1.02	8.75 ± 1.4**	27.21 ± 3.51		5
		Root	8.39 ± 9.77	7.36 ± 1.50**	34.75 ± 6.30		5
Allocasuarina acutivalvis	Topsoil	Shoot	1.48 ± 0.12	6.57 ± 0.59**	4.11 ± 1.21*		5
		Root	4.60 ± 0.85	8.17 ± 0.77**	8.18 ± 2.08*		5
	Capped	Shoot	1.73 ± 0.19	8.30 ± 0.32	10.17 ± 2.22		5
		Root	6.53 ± 0.43	8.89 ± 0.45**	27.93 ± 2.99		4
	Tailings	Shoot	3.69*	14.35 ± 4.01	61.84 ± 17.64		2
		Root	10.912*	47.10 ± 16.77	18.70 ± 77.61		1
Austrostipa scabra	Topsoil	Shoot	1.03 ± 0.20	5.39 ± 0.68**	3.52 ± 1.57		5
		Root	3.44 ± 1.03	7.42 ± 0.88**	11.36 ± 1.61		5
	Capped	Shoot	1.19 ± 0.07	5.35 ± 0.40**	*0.28 ± 0.25		5
		Root	2.66 ± 0.45	6.07 ± 0.28	22.87 ± 2.73		4
	Tailings	Shoot	5.85 ± 4.44	11.28 ± 2.16	54.25 ± 16.53		5
		Root	37.07 ± 20.12	5.61 ± 3.96	54.25 ± 16.53		2
Eucalyptus loxophleba	Topsoil	shoot	1.05 ± 0.08	11.71 ± 1.11	10.39 ± 2.04		5
		Root	5.02 ± 2.60	11.13 ± 0.53	30.30 ± 8.81		4
	Capped	Shoot	1.06 ± 0.20	9.34 ± 0.77	18.95 ± 1.94		5
		Root	4.76 ± 0.99	8.22 ± 0.30	55.03 ± 14.98		5
Hakea recurva	Topsoil	Shoot	1.46 ± 0.22	6.12 ± 0.31	6.52 ± 1.37**		5
		Root	3.05 ± 1.31	11.37 ± 0.73	10.64 ± 28.81		5
	Capped	Shoot	1.40 ± 0.12	8.22 ± 0.03	24.05 ± 0.82		5
		Root	4.49 ± 1.36	8.73 ± 0.42	28.51 ± 2.94		5
	Tailings	Shoot	1.852*	11.86 ± 1.27**	36.33 ± 9.07		2
Maireana georgei	Topsoil	Shoot	2.14 ± 0.08	15.91 ± 0.88	16.13 ± 4.23	2.26 ± 0.45	5
		Root	5.89 ± 0.57	10.40 ± 0.43	16.51 ± 3.021	3.42 ± 0.29	5
	Capped	Shoot	1.03 ± 0.11	6.95 ± 1.33	10.86 ± 2.90	4.06 ± 2.58	5
		Root	3.50 ± 0.85	6.82 ± 0.39	17.83 ± 6.35	5.25 ± 0.69	5
	Tailings	Shoot	7.00 ± 4.28	23.24 ± 5.20	60.62 ± 20.42	22.47 ± 3.64	5
		Root	8.65 ± 2.61	14.68 ± 2.77	65.72 ± 34.22	16.59 ± 3.38	5

* Denotes n-1 for that sample, ** denotes sample measured below LOD

Appendix IV – Heavy metals concentrations for plants growing at KIOP

For plants from KIOP, only the above ground biomass was sampled. Only species *M. georgei* was subset for analysis with ASV.

Cadmium could only be detected in plants by ICP-MS and ASV.

Table A4 - Pb concentrations (mean ± SE) in root and shoot biomass of *Acacia ramulosa, Allocasuarina acutivalvis, Austrostipa scabra, Eucalyptus loxophleba, Hakea recurva,* and *Maireana georgei* grown in topsoil, tailings and capped tailings at the KIOP analysed by ICP-MS, MP-AES, FAAS and ASV.

Species	Substrate	ICP-MS (µg g^{-1})	MP-AES (µg g^{-1})	FAAS (µg g-1µg g^{-1})	ASV (µg g^{-1})	Sample size (n)
Acacia ramulosa	Topsoil	1.65 ± 0.16	6.44 ± 0.37	11.07 ± 0.64		5
Austrostipa scabra	Topsoil	3.33 ± 0.24	7.17 ± 0.43	13.36 ± 1.03		5
	Tailings	3.47 ± 0.17	6.91 ± 0.18	16.46 ± 3.36		5
Eucalyptus loxophleba	Topsoil	4.13 ± 0.21	10.69 ± 0.83	9.28 ± 1.04		5
Maireana brevifolia	Topsoil	2.57 ± 0.47	13.90 ± 0.89	40.43 ± 3.28		5
	Tailings	2.10 ± 0.14	12.61 ± 0.45	46.44 ± 4.64		5
Maireana georgei	Topsoil	2.48 ± 0.08	10.80 ± 0.20	27.89 ± 1.98	2.67 ± 0.14	5
	Tailings	3.92 ± 0.53	10.25 ± 0.57	42.68 ± 3.36	3.48 ± 0.66	5

Table A5 - Cd concentrations (mean ± SE) in root and shoot biomass of *Acacia ramulosa, Allocasuarina acutivalvis, Austrostipa scabra, Eucalyptus loxophleba, Hakea recurva,* and *Maireana georgei* grown in topsoil, tailings and capped tailings at the KIOP, analysed by ICP-MS and ASV

Species	Substrate	ICP-MS (µg g^{-1})	ASV (µg g^{-1})	Sample size (n)
Acacia ramulosa	Topsoil	0.11 ± 0.01		5
Austrostipa scabra	Topsoil	0.09 ± 0.01		5
	Tailings	0.11 ± 0.01		5
Eucalyptus loxophleba	Topsoil	0.14 ± 0.0001		5
Maireana brevifolia	Topsoil	0.12 ± 0.03		5
	Tailings	0.16 ± 0.04		5
Maireana georgei	Topsoil	0.11 ± 0.01	0.11 ± 0.01	5
	Tailings	0.15 ± 0.03	0.15 ± 0.03	5

Appendix V - Preliminary analysis of tap water and plant biomass

Water

The plants in the experiment were watered with tap water that underwent analysis for heavy metals to determine if they contained any traces of metals that may affect the experiment. The results from MP-AES analysis found no traces of As, Cd or Pb in the water supply.

Plants

Excess plants were purchased for each species to analyse what the heavy metal content in the plants biomass would have been before exposure to the spiked soils. Analysis was undertaken by ChemCentre using an ICP-MS instrument.

Table A6 – Preliminary analysis using ICP-MS for native plant tubestock (*Acacia saligna, Austrostipa elegantissima,* and *Allocasuarina huegeliana*) to determine background concentrations of As, Cd and Pb

Species	As (µg g^{-1})	Cd (µg g^{-1})	Pb (µg g^{-1})	Sample size (n)
Acacia saligna	0.2 ± 0*	0.11 ± 0.04	0.10 ± 0.03	5
Allocasuarina huegeliana	0.66 ± 0.08*	0.03 ± 0.01	0.34 ± 0.08	5
Austrostipa elegantissima	0.26 ± 0.08	0.15 ± 0.05	0.22 ± 0.09	5

*contained samples below the limit of reporting

Appendix VI - Quality Control for spiked solutions and instrument analysis

Heavy metal stock solutions used for spiking were analysed to ensure the correct concentrations values were made for the spiking experiment. Instruments 4200 MP-AES (Agilent Technology), and PDV6000+ for the ASV analysis, were used with a 200µg mL^{-1} solution was used for analysis and diluted accordingly.

Table A7 –MP-AES and ASV analysis of a 200 µg mL^{-1} spiking solution containing As, Cd and Pb.

Instrument	As (µg mL^{-1})	Cd (µg mL^{-1})	Pb (µg mL^{-1})
MP-AES	189.4	200	199.8
ASV	-	188.7	192.6

ICP-MS was the only instrument that was not used by the author and analysis was outsourced to ChemCentre. To ensure quality control and that all instruments return similar readings they were all given the same multi-elemental CRM from Agilent which contained 5µg mL^{-1} of As, Cd and Pb.

Table A8 – Analysis results for Certified Reference Material (containing 5 µg mL^{-1} As, Cd and Pb) by MP-AES, ASV and ICP-MS

Instrument	As (µg mL^{-1})	Cd (µg mL^{-1})	Pb (µg mL^{-1})
MP-AES	4.00	4.45	4.51
ASV	-	3.26	4.76
ICP-MS	4.9	5.1	5.00

Table A9. Analysis of Standards and Blanks was conducted for every 16 samples analysed with MP-AES while analysing for arsenic (193.695 nm), cadmium (228.802 nm) and lead (405.781 nm).

Analysis check	Element	4µg mL^{-1} Standard Reading	Blank Reading
Check 1	As	3.83	0.02
	Cd	3.87	0.06
	Pb	4.02	0.01
Check 2	As	2.78	0.12
	Cd	3.78	0.0
	Pb	4.02	0.0
Check 3	As	4.01	0.0
	Cd	3.75	0.01
	Pb	4.20	0.0

Appendix VII - Limits of Detection and Limits of Reporting for heavy metal analysis

ChemCentre analysis with ICP-MS had limits of reporting for As (0.2 µg g^{-1}), Cd (0.02 µg g^{-1}) and Pb (0.005 µg g-$^{1)}$.

Analysis of water leachate was undertaken with MP-AES and analysed samples for arsenic (193.695 nm), cadmium (228.802 nm) and lead (405.781 nm).

Table A10. Limits of Detection (LOD) for As, Cd and Pb for water leachate analysis with MP-AES

Element	LOD (µg mL^{-1})	Calibration Range	R^2	Linear equation
As	0.16	0.5 – 8.0	0.9996	y = 2508x + 195
Cd	0.42	0.5 – 8.0	0.9930	y = 121473x + 38689
Pb	0.14	0.5 – 8.0	0.9998	y = 18322x - 1283

Appendix VIII - Arsenic analysis with ASV

Arsenic detection method was trialled using solid gold electrode which was plated with gold film using 40µg mL^{-1} gold standard in 0.25M CH_3COOH electrolyte solution, deposited at -1000mV for 300 seconds. The gold film was checked visually using magnifying glass and if a darker orange colour was observed that signified gold film. If gold film was not detected it was plated again.

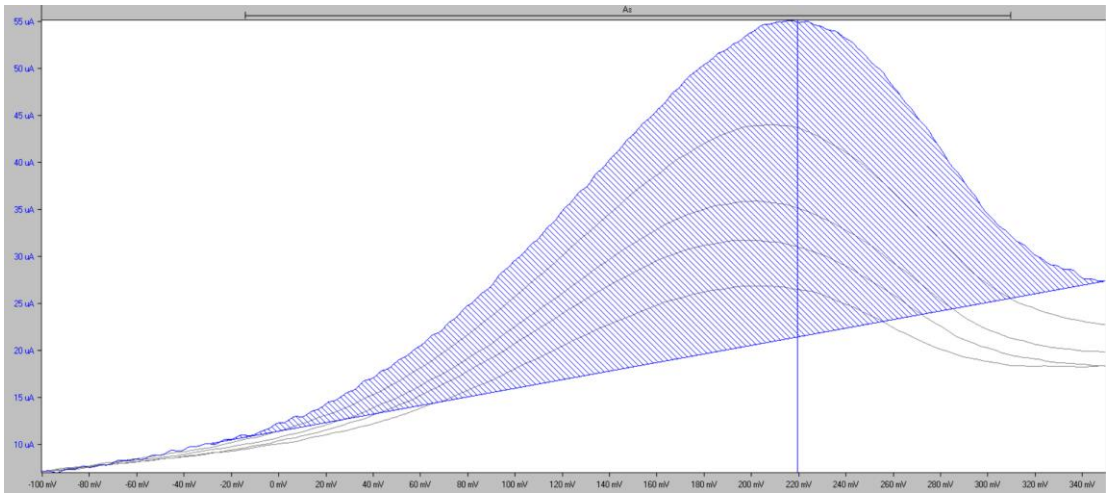

Figure A1 - A calibration for ASV was created in VAS using an ionic solution of an As standard (Sigma-Aldrich) and deposited for 120 seconds. Voltammogram displays As peaks oat concentrations 5ppb, 10ppb, 20ppb and 40ppb.

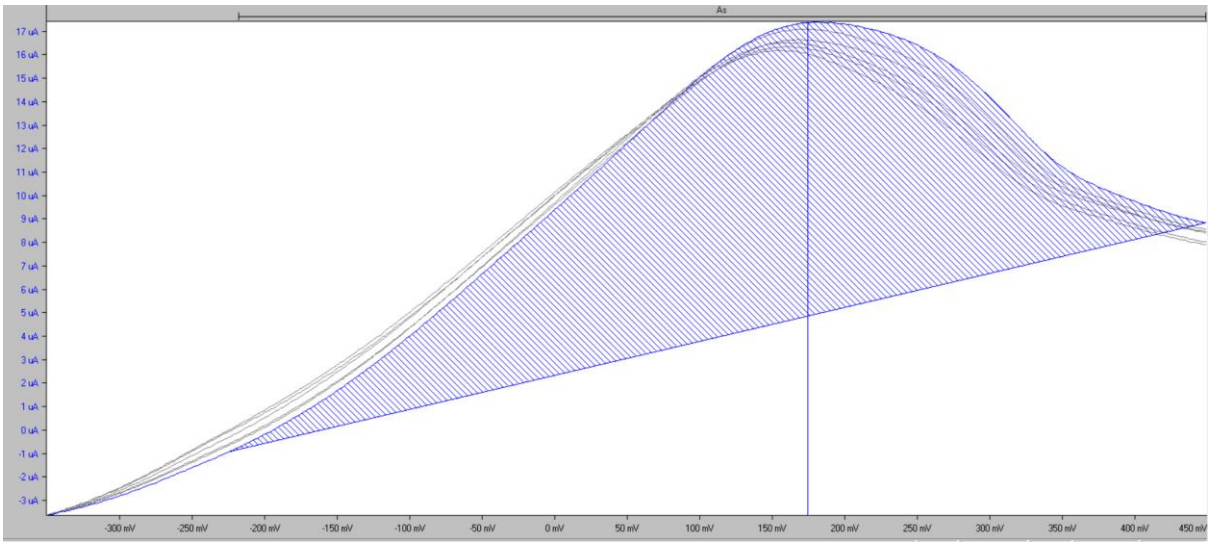

Figure A2 - Reproducibility voltammogram was created using 1ppm arsenic standard that underwent 6 runs at 120 seconds each time. The Voltammogram stacks each peak so precision and matrix interference can be examined.

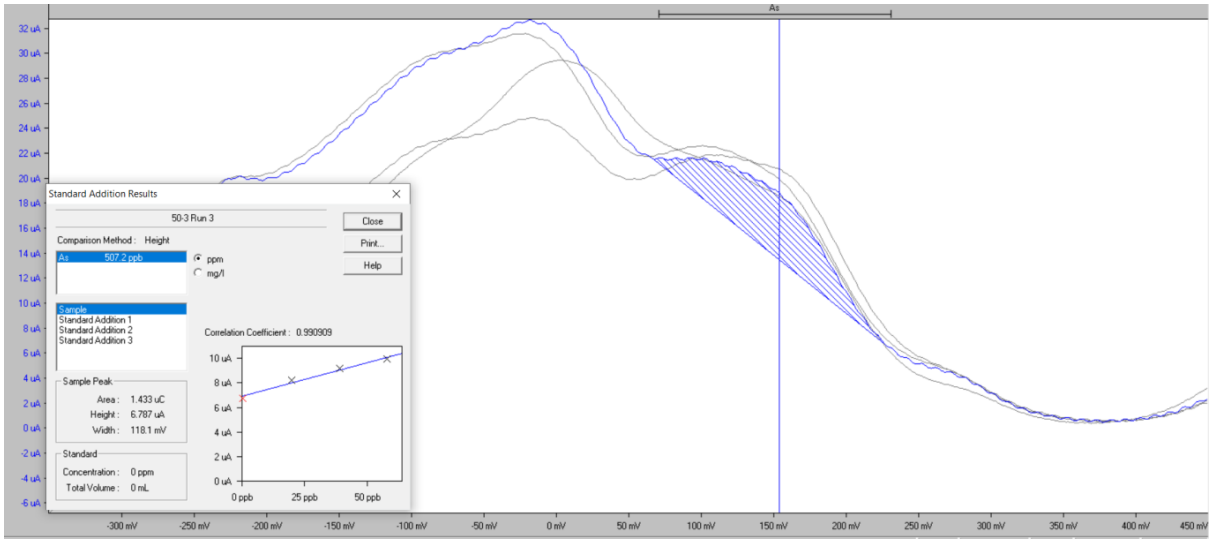

Figure A3 – ASV voltammogram for As analysis using standard addition for a water leachate sample collected from a spiked soil treatment of 50µg mL^{-1}.

Table A11 – Comparison of instruments ICP-MS, MP-AES and ASV for analysis of As in water leachate.

Instrument	**Arsenic concentration**
ASV	0.507 µg mL^{-1}
MP-AES	14.31 µg mL^{-1}
ICP-MS	14.00 µg mL^{-1}

Instrument MP-AES results for As indicated that week 0 water leachate samples had a high concentration (14.31 µg mL^{-1}); however the ASV analysis only registered a trace quantity (0.5 µg mL^{-1}). A confirmation analysis with ICP-MS also confirms the concentration is high (14 µg mL^{-1}). The analysis of As needs to be investigated further, as it is confirmed as being present in the sample yet ASV can't identify it. This may be due to the form the arsenic is in. The CRM uses arsenic trioxide which was used for the calibration and replication, but arsenic coming out of soil may have oxidise to arsenate and may require the sample to be reduced to allow detection of arsenic in the sample.