Investigating the seed germination and phytoremediation potential of New Zealand native plants in metal contaminated soils

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Abbreviations

Cd	-	cadmium
As	-	arsenic
Pb	-	lead
Cu	-	copper
Zn	-	zinc
P	-	phosphate
H_2O_2	-	hydrogen peroxide
KPO_4	-	potassium phosphate buffer
EDTA	-	ethylenediaminetetraacetic acid disodium salt
CCA	-	copper chromium arsenic
µg	-	microgram
mg	-	milligram
g	-	gram
kg	-	kilogram
µL	-	microlitre
mL	-	millilitre
L	-	litre
µM	-	micromolar
mM	-	millimolar
M	-	mol
mm	-	millimetre
Km	-	kilometre
fw	-	fresh weight
S:R	-	shoot to root ratio
NZ	-	New Zealand
UK	-	United Kingdom
USA	-	United States of America

CWG - Cadmium Working Group

NZWWA - New Zealand Waste Water Association

BPBT- Banks Peninsula Blue Tussock

GLM - generalised linear model(ling)

ROS - reactive oxygen species

PC - phytochelatin

GSH - glutathione

IAA - indole acetic acid

EC_{50} - half maximal effect concentration

NOEC- no observed effect concentration

WHC - water holding capacity

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Abstract

The unintentional accumulation of biologically, non-essential metals like cadmium (Cd) and arsenic (As) in New Zealand agricultural and urban/ industrial soils has become of some concern with regard their entry into our food chain. As conventional soil remediation technologies are often expensive and can have negative side-effects on the environment, phytoremediation is a promising "environmentally friendly" alternative with some existing successful applications in New Zealand. Additional benefits of using New Zealand native plants for phytoremediation projects include the provision of important ecological services as well as cultural and aesthetic values to the local community. The objective of this study was to determine the *in-situ* seed germination and seedling survivorship potential of New Zealand native plant species in Cd contaminated soils and investigate the presence (or absence) of physiological mechanisms conferring (or suppressing) tolerance to Cd or other metal contaminants.

An *in vitro* seed germination bioassay exposing 16 New Zealand native plant species to aqueous Cd and As solutions indicated test species appeared to have varying tolerances to Cd and As with the Rock Lily (*Arthropodium cirratum*) and Kanuka (*Kunzea ericoides*) displaying the most tolerance and sensitivity respectively. Seeds of the Rock Lily germinated when exposed to 50mM L^{-1} solutions of Cd and As (~16% and ~13% respectively) while Kanuka seeds failed to germinate when exposed to Cd concentrations >0.25mM L^{-1}. Germination rate and peroxidase activity results indicated responses differing in magnitude to Cd and As treatments therefore indicating species specific tolerances to Cd and/or As. Other test species showed a general negative dose-response trend with regard to germination rate.

Ex situ seed germination bioassay data indicated germination rates in response to Cd treated soil media and a Cd containing field soil sample. All but one of the six test species germinated and grew in the field soil sample indicating that at current average Cd content, these species would be capable of germinating and growing in New Zealand agricultural and horticultural soils. Seedling heights, root length, total chlorophyll content and peroxidase activity were all generally inhibited by exposure to increasing Cd concentrations in the *ex situ* seedling survivorship bioassay. Average Rock Lily root tissue Cd content was 48.3mg Kg^{-1} compared to 227.5mg Kg^{-1} in Kanuka suggesting the Rock Lily's tolerance may be conferred

through the ability to exclude or reduce the absorption rate of Cd. On the contrary, Kanuka may lack these same physiological mechanisms as the species suffered from low germination rates and peroxidase activity when exposed to Cd treatments.

Overall, study results suggest that tolerances to soil metal contaminants exist in New Zealand native plants with physiological de-toxification and avoidance mechanisms that confer successful *in situ* seed germination and seedling survival.

Chapter 1: Introduction

1.1 Overview

The unintentional accumulation of metals in New Zealand farmland and urban/ industrial soils through anthropogenic practices has become of particular concern in recent years as mounting evidence shows that the concentrations of metals like cadmium and arsenic are increasing in our soils (Gaw et al., 2006; McDowell et al., 2013; Taylor et al., 2010). These environmental contaminants can potentially enter into the food chain via multiple pathways causing detrimental effects to the health of many organisms across trophic levels including that of humans (Sharma and Agrawal, 2005). Given that standard soil remediation technologies such as chemical soil flushing, and excavational soil washing are often expensive and/or can cause significant disturbance to soil microorganisms and their associated ecosystem processes (Caliman et al., 2011; Dermont et al., 2008), phytoremediation is an "environmentally friendly" alternative remediation technique that uses plants to remove or reduce the effects of contaminants on the environment without disturbing existing ecosystem processes while being of low cost and requiring limited maintenance from a management perspective.

Although given the infancy of phytoremediation applications in New Zealand, there are some existing studies highlighting the success and subsequent benefits of phytoremediation projects in New Zealand. Using New Zealand native plants for the purpose of phytoremediation projects could potentially not only benefit the environment through the remediation of contaminated and currently unusable parcels of land but also provide important ecological services as well as cultural and aesthetic values to the local community. Native plant diversity has been shown to increase native invertebrate diversity (Crisp et al., 1998) thus possibly attracting other native birds, reptiles and insects back into exotic anthropogenic environments. For the purpose of the ecological restoration of current contaminated sites back to the original native vegetation, the "*in-situ*" seed germination potential of New Zealand native plant species in contaminated sites needs to be determined in-order to provide evidence for the possibility of using New Zealand native plants in phytoremediation projects.

The New Zealand native plants potentially capable of germination and survival in metal contaminated environments are likely to feature some intrinsic physiological mechanisms that confer resistance to the associated contaminants. It has been displayed in other plant species that tolerance to metals is usually achieved through the cellular exclusion of the metal(s) from the plant or alternatively the detoxifying and cellular inclusion of the metals into the plants cellular structure. Previous studies have also noted that molecular interactions that confer resistance to metal contaminants are interchangeable and enable tolerance to a broad variety of abiotic stressors. Currently there is no knowledge as to whether New Zealand native plants feature physiological mechanisms that confer resistance to heavy metal contaminants such as cadmium.

1.2 Metal contamination in New Zealand soils

1.2.1 Introduction

The accumulation of various metals in New Zealand soils due to anthropogenic practices has been noted by a variety published papers concerning the subject matter (Gaw et al., 2006; Longhurst et al., 2004). Of significant concern in the context of New Zealand soils are cadmium and arsenic, both of which are water soluble and cytotoxic (in a concentration dependent manner) (Jomova et al., 2011; Loganathan and Hedley, 1997; Rodrigues et al., 2010). There effects on New Zealand's terrestrial and aquatic environments have been widely documented (Sabti et al., 2000). The identification of sites that are potentially contaminated by excessive soil metal content is achieved through the NZ Ministry for the Environment's Harzardous Activities and Industries List (HAIL). This list details historical or current activities and/or industries that may use hazardous substances such as metals thus potentially causing environmental contamination (Ministry for the Environment, 2011). Presently there are no standards set by the NZ Ministry for the Environment that set the maximum permissible levels for soil metal contaminants which would provide guidelines to identify whether a site is considered contaminated or not. This means local authorities e.g regional councils may therefore interpret the definition of contaminated land differently (Ministry for the Environment, 2007) and whether or not a site requires soil remediation work. However non-governmental organisations like the Cadmium Working Group (CWG) which is comprised of central and local government, and fertiliser industry and primary sector representatives, have set guidelines as to what should be deemed acceptable levels of

cadmium in NZ soils. The New Zealand Water and Wastes Association (NZWWA) have also proposed their own set of guidelines analogous to those stated by the CWG applicable to a variety of metal contaminants that are present in NZ soils.

1.2.2 Cadmium contamination in New Zealand

1.2.2.1 Chemistry of cadmium in soils

As an element, cadmium is a soluble transition metal meaning that it exists as a divalent cation (Cd^{2+}) under most soil conditions. As Cd^{2+} is cationic, soil pH influences Cd^{2+} reactivity with other elements and compounds thus soil cadmium bioavailability to plants has been shown to be significantly affected by soil pH (Grant and Sheppard, 2008). Existing as Cd^{2+}, cadmium's chemical reactivity with other compounds is similar to other divalent cations like Zn^{2+} and Mg^{2+}. In a study by Guttormsen et al (1995), plant uptake of Cd from a phosphatic rock enriched sandy soil was shown to be positively correlated with increasing soil acidity. Chinese cabbage (*Brassica pekinensis* Rupr.) and carrot (*Daucus carota* L.) absorbed 23% and 46% more Cd respectively from the sampled soil at a pH of 5.5 compared to a pH of 6.5 (Guttormsen et al., 1995). Soluble soil Cd content, which is likely to be bioavailable to plants and unbound to other soil molecules, has shown to be accurately predicted by soil pH, total organic carbon and total soil Cd content in New Zealand soils (Gray and McLaren, 2006). Soil type (texture, density, organic matter content) appears to be another variable influencing Cd mobility in soils with a greater risk of Cd leaching into ground water in sandy soils than finer clay or silt soils (Loganathan and Hedley, 1997). Using a field trial in Manuwatu, New Zealand, Loganathan and Hedley (1997) showed with a variety of Cd containing P fertiliser treatments that less than 10% of the total Cd present in the ground-level applied fertiliser treatments leached through the soil profile to a depth of 120mm or more. The un-leached fraction of the applied P fertiliser cadmium is therefore potentially bio-available for uptake by plants (Loganathan and Hedley, 1997).

1.2.2.2 Contamination from applied phosphate fertilisers

Most soil cadmium content in NZ is anthropogenic from the application of phosphate fertilisers to agricultural farmland used to supplement pasture growth (McDowell et al., 2013)).Cadmium is present in phosphate fertilizers as a trace contaminant within the mined

phosphate rock from countries like Nauru, the United States of America, and Morocco. Historically, New Zealand fertiliser manufacturers received imported Cd-rich phosphate rock from Nauru for application to agricultural land however in response to evidence of increasing soil Cd content, the fertiliser industry voluntarily, created a self-imposed Cd limit in 1997 to reduce cadmium levels in phosphatic fertilisers to 280 mg Kg^{-1} P, or approximately 24 mg Kg^{-1} as superphosphate (Gaw et al., 2006). This has seen the sourcing of phosphatic rock redirected from Cd-rich Nauru phosphate rock to the lower Cd containing West Saharan phosphate rock. Collated data from plethora of global literature sources have estimated the median reactive phosphate rock trace Cd content to be 16.9 mg Kg^{-1} (Taylor et al., 2014). In New Zealand, independent audits on the fertiliser cadmium content administered by the New Zealand Fertiliser Quality Council completed between the years 2001 – 2005 stated that the average Cd content in phosphatic fertilisers was 175 mg Kg^{-1} (Cavanagh et al., 2013).

1.2.2.3 Contamination from applied biosolids

Cadmium also enters New Zealand soils through the application of nutrient fertilising biosolids which contain trace concentrations of Cd. Only biosolids derived from municipal sewerage sources are able to be applied to New Zealand land at current, with resource consent approval from local regional council being required. The NZWWA group recommends that applied grade A biosolids (treated to reduce pathogens and attractable vectors) should not contain more than 1 mg Kg^{-1} of Cd (dry-weight). Likewise, Grade B biosolids (non-treated) should not contain more than 10 mg Kg^{-1} of Cd (dry-weight) (New Zealand Water and Wastes Association, 2003). The literature review by Taylor et al (2014) cites the median Cd concentration found in sewerage (biosolids) found internationally as 3.30 mg Kg^{-1}.

1.2.2.4 Contamination from mining activities

Cadmium contamination of New Zealand's environment has also arisen through mining activities at various sites around the country. As an example, some aquatic environs such as streams and rivers that surround the Tui mine near Te Aroha, Waikato, New Zealand have experienced an increase in metal content due to the mining processes. Soluble metals like cadmium are capable of leaching from mine tailings into streams and aquifers (especially under acidic conditions) where there accumulation can result in concentration levels far

exceeding the maximum acceptable values (MAV) for inorganic determinands of health significance set by the NZ Ministry of Health (Sabti et al., 2000). Of the 30 sites where stream water samples were collected, all of which were within 4km of the Tui mine site and Te Aroha township, 11 water samples contained Cd concentrations equal to or greater than the 4 µg L^{-1} potable water Cd maximum acceptance value set by the Ministry of Health (Ministry of Health, 2008; Sabti et al., 2000).

1.2.2.5 Agricultural land contamination

In a study by McDowell et al (2013) assessing soil Cd content across New Zealand, the mean Cd content was 0.32mg Kg^{-1} (mg Kg^{-1} = ppm) for agricultural soils compared to 0.14mg Kg^{-1} for non-agricultural soils (McDowell et al., 2013). The Waikato region has been identified as a region containing high soil Cd content with agricultural soils containing on average 0.71mg Kg^{-1} and 0.11mg Kg^{-1} for non-agricultural soils (Taylor et al., 2010). As the occurrence of intensive agricultural practices increase, the accumulation of cadmium in NZ soils is likely to continue into the immediate future (Forestry, 2011; Gaw et al., 2006).

The Cadmium Working Group suggests soil Cd content should not exceed 1.8mg Kg^{-1} while the New Zealand Waste Water Association states that soil cadmium content should be capped at 1mg Kg^{-1} (Forestry, 2011; New Zealand Water and Wastes Association, 2003). Cadmium data sampled across sites from McDowell et al (2013) ranged from <0.01 to 2.70 mg Kg^{-1} suggesting that some of those sites sampled exceeded the CWG soil Cd content guidelines, and similarly Cd content ranged from 0.10 to 2.00mg Kg^{-1} in the Taylor et al (2010) study assessing Waikato agricultural soil quality with 12% of dairy, 16% of drystock and 25% of horticultural sites sampled exceeding NZWWA guidelines (Taylor et al., 2010).

1.2.2.6 Horticultural land contamination

The contamination of horticultural land from cadmium mostly occurs as a result of the application reactive phosphate rock fertilisers (Gaw et al., 2006) to supplement the growth of commercial horticultural plant species such as *Actinidia deliciosa* (common kiwifruit) and *Malus domestica* (apple tree). A study by Gaw *et al* (2006) detailed results showing that the mean Cd content of soils from horticultural sites in the Auckland, Waikato and Tasman regions were 0.4 mg Kg^{-1},1 mg Kg^{-1}, 0.5 mg Kg^{-1} respectively. Cadmium levels in 7% of

Tasman, 29% of Waikato and 16% of Auckland horticultural soil samples were equal to or exceeded the 1.0 mg Kg^{-1} threshold soil Cd concentration suggested by the NZWWA (Gaw et al., 2006). The presence of Cd in horticultural or agricultural land can have a greater effect in N.Z soils compared to other countries due to a lower soil pH which increases the propensity for plants to uptake Cd from the soil. The Auckland, Waikato and Tasman regions have mean pH values of 6.0, 6.1, and 6.2 respectively, with pH values ranging from 3.95 – 6.49 in the Waikato region for example (Gaw et al., 2006).

1.2.3 Arsenic contamination

A variety of land-use practices have resulted in anthropogenic As accumulation in New Zealand soils though elevated total soil As concentrations do occur naturally in a variety of volcanic soils around NZ due to geothermal activity, particularly around the Taupo volcanic zone of the North Island (Robinson et al., 2004). Anthropogenic soil As accumulation is likely to be found at horticultural, mining, industrial timber treatment sites as well as localised at sheep dipping sites on agricultural land (Gaw and McBride, 2010b; Sabti et al., 2000).

1.2.3.1 Chemistry of arsenic in soils

Though cadmium exists in the soil environment singularly as Cd^{2+}, arsenic is capable of taking on many different ionic forms due to complex interactions with other elemental compounds and soil organic matter within the soil profile, though most forms occurring in oxic and anoxic soils are oxyanions (negatively charged arsenic and oxygen compounds like iron arsenate – Fe_3AsO_4) (Sadiq, 1997). In the natural environment, As can also form as trivalent and pentavalent cations (Jomova et al., 2011). Arsenic also features in both organic forms (compounds comprised of As combined with carbon and/ or hydrogen) and inorganic forms (compounds comprised of As combined with elements like oxygen, sulphur or chlorine) (Jomova et al., 2011; Meharg and Hartley-Whitaker, 2002). Arsenic occurs naturally in over 200 different mineral forms, of which approximately 60% exist as arsenates, 20% as sulfides and sulfosalts, and the remaining 20% includes silicates, arsenides, arsenites, and oxides (Mandal and Suzuki, 2002). The bio-availability of arsenic depends on its chemical form and surrounding soil chemistry (i.e elemental composition and pH). Pentavalent metal arsenates like iron arsenate are relatively immobile in soil due to extremely

low solubility and less toxic than trivalent arsenides which are more soluble and mobile than arsenates also (Mandal and Suzuki, 2002).

1.2.3.2 Contamination from horticultural pesticides

In historical horticultural practices, arsenic was present in pesticides used against chewing insects of fruits and vegetables with As present in a variety of compounds including lead arsenate, calcium arsenate and copper arsenate (Mandal and Suzuki, 2002). Arsenic based insecticides have since been banned and are no longer registered for use in NZ, however one arsenic based herbicide, methylarsinic acid, is still registered for use (Gaw et al., 2006).

1.2.2.3 Contamination from livestock pesticides

The use of Arsenic based insecticides on sheep is another proven source of metal contamination in New Zealand agricultural areas. Historically, in the period between the 1840's and the early 1980's, arsenic was a chemically active component of the liquid insecticides used to kill and remove ectoparasites from the wool of sheep. The sites where these insecticides were applied are known as sheep dips and it is estimated that 50,000 of these contaminated sites exist (Gaw and McBride, 2010b). Through studies detailing field examples of soil As concentrations, it has been noted that arsenic concentrations high enough to be fatal to animals and humans have been measured in groundwater contaminated by nearby abandoned sheep dip sites (Gaw and McBride, 2010b).

1.2.3.4 Horticultural land contamination

Gaw *et al* (2006) showed that in horticultural soils from the Auckland and Waikato regions, horticultural soils (orchards) in both regions contained As concentrations three times greater than background soil As content (background As content was analysed from soil samples extracted from beneath indigenous vegetation). In the same study, results showed that As levels in 29% of the orchard samples exceeded the NZ arsenic based ecotoxicity data and soil guidelines as well as other foreign soil quality standards (Gaw et al., 2006).

1.2.3.5 Contamination from timber preservation treatments

Arsenic has historically been present in preservation treatments applied to sawn timber to prevent infestation and subsequent degradation due to saprotrophic fungi, bacteria and boring insects. Copper chromium arsenate (CCA) is the chemical used in the timber treatment process in New Zealand and the individual elements within this compound have been noted to be toxic to many organisms at varying concentrations through their migration into nearby water and soil systems thereby potentially entering the food chain (Carey et al., 1996; Katz and Salem, 2005; Robinson et al., 2006). Therefore As contamination due to CCA can be found at the site of CCA application e.g timber sawmill or the site where the products applied with CCA are used; for example, a sawn timber post being used for viticulture activities (Robinson et al., 2006). Robinson et al (2006) showed using a field trial in the Marlborough district of New Zealand that leached arsenic fractions of the CCA treatment from CCA treated timber posts into soil directly beneath the tested posts accumulated to an average concentration of 177mg kg^{-1} after a 5 year period of CCA exposure.

1.2.3.6 Contamination from mining activities

The anthropogenic entry of As into the natural environment has also occurred at the Tui mine near Te Aroha, NZ along with cadmium as previously mentioned. The acidic soil conditions of the metal ladened mine tailings has mobilised metals like arsenic thus allowing them to leach into nearby waterways and groundwater (Haffert and Craw, 2008; Sabti et al., 2000). Of the 30 sites where stream water samples were collected, all of which were within 4km of the Tui mine site and Te Aroha township, 28 water samples contained As concentrations equal to or greater than the 10 µg L^{-1} potable water As maximum acceptance value set by the Ministry of Health (Ministry of Health, 2008; Sabti et al., 2000). Another example of a mining site with elevated soil As concentrations is the McCrae mine with soil samples from the tailings area containing between 500 – 4000 mg Kg^{-1} of As which is well in excess of the 20 mg Kg^{-1} soil As content guidelines proposed by the NZWWA.

1.3 Entry of metals into the food chain

1.3.1 Absorbance of metals by plants (primary producers)

The root system of terrestrial plants serves as an anchor point to the earth's surface and also as a gateway for many essential macro and micronutrients to enter the plant for critical metabolic functions. Plants have also been documented to absorb elements with no known biological function and have been shown to be toxic, even at low concentrations (Peralta-Videa et al., 2009). Among these elements are arsenic and cadmium. The physiological sensitivity of plants to metals like Cd and As is extremely variable with some species being very sensitive to metal induced toxicity and other being very tolerant. Tolerant species are generally divided into one of two current classification groups; (hyper)accumulators of metals or excluders of metals (Ali et al., 2013).

The real problems arise though when excessive metal content of the soil is uptaken into crop plants. Field experiments testing the efficacy with which common food plants can uptake metals from contaminated soils provide evidence for whether public health concern with regard to the consumption of contaminated produce is justified (Liu et al., 2012). It is also possible for aquatic macrophytes to facilitate the entry of metals into the aquatic food chain creating an indirect (secondary consumers like fish) or direct (macrophytes like watercress or mint) pathway for metals to be consumed by humans (Robinson et al., 2004).

1.3.1.1 Absorbance of cadmium

Using a metal contaminated site in China, Liu *et al* (2012) completed experiments testing the Cd uptake in maize *(Zea mays),* tomato *(Solanum lycopersicum),* cabbage *(Brassica oleracea),* and pakchoi plants *(Brassica chinensis).* The mean background soil Cd concentration was 1.1 mg/Kg^{-1} across sites and the average Cd content of the edible part of each plant species are listed as follows:

Crop plant	**Cd concentration (mg Kg^{-1})**
Maize	0.03 ± 0.01
Tomato	0.13 ± 0.03
Cabbage	0.05 ± 0.01
Pakchoi	0.17 ± 0.07

Table 1.1: Data from Liu *et al* (2012) detailing crop plant average cadmium concentration

The Chinese National Standard Agency provides guidelines for the maximum permissible concentration (MPC) of heavy metals in the edible parts of crops (Liu et al., 2012). Classification is divided into fruit-type and leafy type vegetables with Cd limits set at 0.05 mg/Kg^{-1} for fruit-type plants and 0.20 mg Kg^{-1} for leafy type vegetables (Liu et al., 2012). According to these standards, maize and pakchoi were the plant species that contained acceptable levels of Cd (Liu et al., 2012). Gaw *et al* (2008) details the bioaccumulation of Cd in lettuce (*Lactuca sativa*) and radish (*Raphanus sativus*) growing in field extracted soils with historically varied land-uses from New Zealand. Sampled lettuce leaves contained between 1.25 and 2.10 mg Kg^{-1} and radish hypocotyls contained between 0.35 and 0.51 mg Kg^{-1} of Cd (Gaw et al., 2008). The Cd concentration in lettuce grown in four of the ten experimental soils were equivalent to or exceeded the current NZ standard set by the NZ Food Safety Authority for Cd in leafy vegetables which is 0.1 mg Kg^{-1} (Gaw et al., 2008). Sabti et al (2000) also analysed metal concentrations (including Cd) in aquatic macrophytes from the Waihou River, Waikato. At the sample site, downstream from the tailings site of the historic Tui mine, macrophyte Cd content ranged from 0.7 – 7mg L^{-1} . Uptake of Cd can also occur in aquatic macrophytes thus providing a pathway for Cd to enter the aquatic food chain. Sabti et al (2000) also analysed metal concentrations (including Cd) in aquatic macrophytes from the Waihou River, Waikato. At the sample site, downstream from the tailings site of the historic Tui mine, macrophyte Cd content ranged from 0.7 – 7mg L^{-1} .

1.3.1.2 Absorbance of arsenic

Evidence for the absorbance of various forms of As including arsenate, arsenite and other arsenic complexes exists for over 100 plant species as listed in Meharg & Hartley-Whittaker (2002). With regard to the specific interest in crop plants, Gaw *et al* (2008) examined the uptake of As into lettuce (*Lactuca sativa*) and radish (*Raphanus sativus*) shoots grown in field soil samples containing between 2 and 15 mg/Kg^{-1} of arsenic. Both lettuce and radish accumulated As from all of the soil samples tested to levels exceeding the maximum residue limit imposed by the NZ Food Safety Authority of 0.1 mg/Kg^{-1} of arsenic (Gaw et al., 2008). The maximum As concentrations found in lettuce and radish was 0.7 and 1.5 mg/Kg^{-1} respectively (Gaw et al., 2008). Some plants growing in or around aquatic areas in the Taupo Volcanic Zone (North Island, N.Z) have been shown to absorb up to 1000mg Kg^{-1} from the substrate and surrounding environment. At least one of these species is consumed by humans,

it is small leafy plant known as watercress (*Rorippa nasturtiumaquaticum*) (Robinson et al., 2004). In a pot trial using As contaminated soils obtained from Macrae's mine tailings (Otago, New Zealand), barley (*Hordeum vulgare*), rye corn (*Secale cereale*) and blue lupin (*Lupinus angustifolius*) were tested for their capacity for As uptake. Concentrations of between 20–126 mg Kg^{-1} of arsenic were detected in the shoots strongly suggesting translocation was of occurrence. A total of 53% of shoot samples exceeded the maximum tolerable dietary intake of arsenic for livestock of 50 mg Kg^{-1} recommended by the Australia New Zealand Guidelines for fresh and marine water quality (Mains et al., 2006).

1.3.2 Transfer of metals into animals (primary and secondary consumers)

Through the exemplar vectors and pathways metals like As and Cd travel to enter the food chain as described in sections 1.3.1.1 and 1.3.1.2, the consumption of metal containing plants by animals leads to the transition of metals higher up the food chain. Given the longer life-span of some of these animals compared to annual or biannual plants, contaminant metals can potentially accumulate in the tissues of animals which can lead to toxicity due to metal exposure from the consumed organisms.

Roberts et al (1994) analysed the Cd content of NZ soils and agricultural plants as well as the kidneys of grazing animals slaughtered for export. Animals were shown to accumulate Cd within the kidneys to concentrations exceeding the background pasture and soil Cd content. Older animals generally contained higher Cd kidney content than younger animals due to the increased temporal exposure to Cd in the environment (Roberts et al., 1994). Up to 20% of cattle and 28% of sheep exported between the years 1988 and 1991 had kidney Cd contents greater than the permissible level of 1mg Kg^{-1} set by the New Zealand Ministry of Health (Roberts et al., 1994).

Matri-Cid *et al* (2008) completed a 'market-basket' study in the Catalonia region of Spain assessing the average concentration of foods widely available at consumer markets (local markets, supermarkets etc.). All fruits and vegetables had low concentrations of As (<0.0001mg Kg^{-1}) however all marine fish (e.g tuna and mackerel) and bivalves had average concentrations greater than 0.001mg Kg^{-1} with red mullet containing 0.016mg Kg^{-1}, the highest As concentration recorded in the study. Cadmium concentrations were also recorded in most of the food types including fruits and vegetables but all were below 0.0001mg Kg^{-1}

Cd. Metal content was then averaged for food groups and estimates of the average metal consumption per day per unit of human body weight was calculated. The estimated daily As intake for a 70Kg male was 213.65µg while 9.97µg was the estimated average Cd intake (Marti-Cid et al., 2008).

1.4 Metal effects on plants

1.4.1 Overall effects

Metal phytotoxicity can result from alterations in a variety of physiological processes caused at the molecular level by inactivating enzymes, blocking functional groups of metabolically important molecules and disrupting their metabolic functionality, and displacing or substituting for essential elements within compounds and damaging membrane integrity. Another side-effect of metal induced toxicity is the enhanced production of reactive oxygen species (ROS) such as superoxide dismutase and free radical compounds (OH^-) (Nagajyoti et al., 2010; Rascio and Navari-Izzo, 2011) which can lead to oxidative damage such as the degradation cellular membranes due to lipid peroxidation (Nagajyoti et al., 2010). Metal contaminants in the vicinity of genetic material can lead to many forms of DNA damage and functionality disruption including DNA base modifications, inter- and intra-molecular cross-linkage of DNA and proteins, DNA strand breaks, rearrangements and de-purination. Some of this DNA disruption and damage can occur due to the metal induced production of ROS which has been shown to create a mutagenic protein known 7,8-dihydro-8-oxoguanine that can specifically miss-pair with adenine resulting in cytosine - thymine transversion mutations (Nagajyoti et al., 2010). These molecular interactions have vast consequences when considered at the organism level.

1.4.2 Cadmium effects

Numerous studies have been published detailing a negative correlation between a species total germination rate (percent germinated) and the concentration of cadmium present in test media. For example; using increasing cadmium concentrations ranging from 1000µM to 3000µM, *Sorghum bicolour* total germination was shown to decrease from 97% in control plants to 76% and 71% when exposed to 1000µM to 3000µM Cd respectively (Kuriakose and Prasad, 2008). Similar seed germination rate reductions in response to Cd exposure have

occurred in *Eruca sativa* and *Miscanthus'* along with many other species (Hsu and Chou, 1992; Ozdener and Kutbay, 2009). In relation to decreased total germination rates, the presence of Cd has been shown to inhibit seed imbibition through preventing sufficient water uptake required for germination activation thus delaying germination speed and reducing the total germination rate (Kranner and Colville, 2011). Ali *et al* (2014) discovered that low concentrations of aqueous Cd (100µM L^{-1}) lead to a stimulation of seed germination within 24 hours of exposure while 500µM L^{-1} Cd solutions caused considerable toxicity. Effects included damage to many cellular organelles such as the deformation of chloroplasts and subsequent thylakoid membranes were disorganization as well as the incomplete development of mitochondria, nuclei, endoplasmic reticulum and storage vacuoles. Grana damage and asymmetry has also been in chloroplasts exposed to toxic concentrations of cadmium (Ali et al., 2014).

The accumulation of Cd in plants has been reported to detrimentally affect enzymes involved in photosynthesis resulting in decreased photosynthetic activity through interfering with stomatal function and decreasing total chlorophyll content (Sharma and Agrawal, 2005). These effects have been noted to result in a decrease of both plant biomass and yield (Sharma and Agrawal, 2005).Cadmium has been shown to interfere with the root uptake, inter cellular translocation and utilisation of essential macro and micronutrients including Ca, Mg, P and K, as well as H_2O. A reduction in the absorption of nitrate and its translocation has been reputedly caused by Cd interference through the inhibition of nitrate reductase activity in the shoots (Nagajyoti et al., 2010; Sharma and Agrawal, 2005).

1.4.3 Arsenic effects

As previously mentioned in section 1.2.3.1, varying forms of arsenic have differing levels of toxicity to organisms. Yoon *et al* (2015) showed that arsenite (As(III)) can be more toxic than arsenate (As(V)) to plants as the NOEC (no observed effect concentration) and EC_{50} (half maximal effect concentration) values with regard to seed germination rate for all 10 species was lower for plants treated with As(III) compared to As(V).

Given the complexity of metal chelation and sequestration in plants, the exact biochemical reactions within plants upon exposure to As is not fully understood. It is thought that As(V) is potentially toxic due to its ability as a substitute for phosphate in phosphorylation reactions

including ATP synthesis (Verbruggen et al., 2009). In plants, As(V) is rapidly reduced to As(III) which probably causes toxicity due to high sulphydryl reactivity and regardless of form, also causes oxidative stress (Verbruggen et al., 2009). Symptoms of oxidative stress due to an increased concentration of reactive oxygen species (ROS) when compared to normal homeostatic conditions include lipid peroxidation and the subsequent degradation of cellular membranes (Meharg and Hartley-Whitaker, 2002; Verbruggen et al., 2009). Plant defences against arsenic induced ROS include the formation of glutathione and subsequent phytochelatin (PC) synthesis to form un-reactive As(III) – glutathione and As(III) – PC complexes respectively (Verbruggen et al., 2009).

1.5 Metal effects on human health

1.5.1 Generic effects of metals

In biological systems, oxygen free radicals are produced whenever molecular oxygen accepts electrons from divalent catonic metals like Cd^{2+}, Cu^{2+} or Pb^{2+} and many reactions can reduce oxygen to superoxide or hydrogen peroxide. These molecules can then form hydroxyl radicals (OH^-), which account for the majority of oxidative damage that occurs in biological systems (Nagajyoti et al., 2010). These hydroxyl groups can cause detrimental modifications to DNA bases through oxidation, as well as oxidise free amino acids and proteins. One of the main consequences of OH^- free radical damage to proteins is to target them for degradation by proteases (Nagajyoti et al., 2010). Metals have been noted to have extensive negative impacts on human health due to the previously mentioned biochemical processes through accumulation in tissues and organs of the body. The severity of the health symptoms caused by metal exposure is mainly dependent on various factors such as dose, individual susceptibility to specific metals and the age of the affected individuals (Jomova et al., 2011)

1.5.2 Cadmium effects

Cadmium has been noted to accumulate in the kidney, liver and intestinal mucosa with a retention time of approximately 10 years after extensive exposure to Cd sources (Peralta-Videa et al., 2009; Sharma and Agrawal, 2005). Detrimental effects of Cd to human health are known to include cancers, cardiovascular disease, as well as renal tubular damage which inhibits the reabsorption of low molecular weight proteins from the intestinal fluid back into

the blood stream (Gochfeld, 2000; Sharma and Agrawal, 2005). It is estimated that 5% of the Cd content ingested is absorbed through the intestines whereas with regard to the inhalation of Cd, for example due to occupations in Cd contaminated environs or inhalation of cigarette smoke, 25% of the total Cd inhaled is estimated to be absorbed through the lungs (Gochfeld, 2000).

1.5.3 Arsenic effects

Arsenic exists in both organic and inorganic form as trivalent and pentavalent ions with inorganic variants being of greater toxicity to organisms. The majority of As enters the body as the trivalent inorganic form via a simple diffusion mechanism whereas transport of pentavalent As across membranes requires energy expenditure (Jomova et al., 2011). Arsenic exits the body within urine, with most organic As expelled several days after entry into the body while inorganic forms are retained for a slightly longer period, although some will remain for months or even longer periods (Jomova et al., 2011). The production of reactive oxygen and reactive nitrogen species during inorganic As metabolism and the resulting morphological changes to mitochondrial structure and membrane potential has been shown to be the biochemical mechanistic cause of As toxicity and a variety of its symptoms (Jomova et al., 2011). The health symptoms caused by arsenic toxicity are vast and in general description include dermal, renal, neurological, gastrointestinal, cardiovascular and liver diseases. More specific examples include hyperpigmentation and kerotosis (skin lesions), myocardial depolarization and cardiac arrhythmia, gastrointestinal ulcers and perforations, and sensory and motor neuronal deterioration (excluding organic arsenics) (Jomova et al., 2011; Sharma and Agrawal, 2005).

1.6 Limitations of current non-organic remediation techniques of metals

To remove these contaminants from the soil, both *in situ* and *ex situ* non-organic remediation techniques are often used including chemical soil flushing, physical excavation and soil washing, solidification, and electrokinetic systems (Ali et al., 2013; Caliman et al., 2011). However, there are many drawbacks to these techniques including high costs, high levels of disturbance to existing soil biota, intensive labour (Ali et al., 2013) and with regards to chemical systems, the addition of chelating compounds that solubilise metals that may have unforeseen impacts on the environment (Caliman et al., 2011). Also some of these methods

like chemical soil flushing are not always effective in remediating a site as effective remediation is often dependent on low soil heterogeneity, low silt/clay content as well mutual exclusivity for the presence of anions or cations (Dermont et al., 2008). Add more

1.7 Overview of phytoremediation including phytoextraction and phytostabilisation

Phytoremediation uses plants to reduce the concentrations of toxic contaminants or reduce their effects on the environment (Ali et al., 2013). Within the area of phytoremediation exist further sub-disciplines including phytostabilisation, phytoextraction, phytofiltration and phytodegradation (Ali et al., 2013). Phytoextraction, also known as phytoaccumulation is one of the most useful phytoremediation techniques for the removal of metals from polluted sediments or soils (Ali et al., 2013). As the metal is sequestered within the plant, the possibility arises to remove the metal containing plant from the contaminated site to be disposed of in a more suitable containment area thus removing the contaminant metal to an area where the hazardous re-entry of the metal into the environment is prevented. Alternatively the incineration of the contaminated plant can result in the retrieval of the metal contaminant for re-use if it is of commercial value (Delplanque et al., 2013). Phytostabilisation is another useful remediation technique to prevent the impacts of contaminants on an environment.

1.7.1 Phytoextraction and physiology

1.7.1.1. Transport proteins

The intrinsic metal uptake transport proteins used for the accumulation of essential divalent cation micronutrients like zinc transport proteins (ZIP) or Ca^{2+} transporters/channels in the root epidermis can be purposefully used to extract metal contaminants from the soil into the root system (Ali et al., 2013; Clemens, 2006; Suresh and Ravishankar, 2004). In the rhizosphere, these non-essential metals like Cd may effectively compete for access to the root epidermal transport proteins used for essential micronutrients that have similar oxidation states or ionic radii to the non-essential heavy metals (Ali et al., 2013). These non-essential metals are therefore “opportunistic hitchhikers” that use the provided transport proteins reserved for essential micronutrient uptake (Clemens, 2006). Once in the root system, chelatins like glutathione (GSH), phytochelatin and metallothioneins bind to the free metal

ions, creating an inert chemical complex that can then be transported via the symplast into the stele and then be released into the xylem facilitated by membrane transport proteins (Ali et al., 2013; Suresh and Ravishankar, 2004). Upon translocation into the xylem, these chelated metal complexes are then transported up the xylem into the shoots where they are mostly stored in vacuoles, an organelle of low metabolic activity in plant cells (Ali et al., 2013).

With specific regard to Cd accumulation in plants, the IRT1 (iron transport family and a member of the ubiquitous ZIP family) protein in plasma membrane of root epidermal cells has been shown to be responsible for Cd^{2+} influx into the root cells (Clemens, 2006). Other transporters responsible for Ca^{2+}, Fe^{2+}, Mn^{2+}, and Zn^{2+} uptake have been shown to provide passage for Cd to enter the root system (Verbruggen et al., 2009) The ionic differences between As(V) and As (III) mean that the two ions are absorbed into plant roots via separate gateway systems. As(III) is mainly absorbed through members of NIP subfamily of aquaporins and its high influx into rice roots has been shown to be via a member of this protein family (Verbruggen et al., 2009). As(V) is easily incorporated into plant cells through phosphate transport proteins

The tolerance that phytoextractors show in response to levels of Cd that would induce Cd toxicity in intolerant plants is mainly due to the chelation and sequestration processes previously described. The model organism of the plant kingdom, *Arabidopsis thaliana* was shown to suffer from severe Cd^{2+} hypersensitivity when genes responsible for the synthesis of phytochelation complexes were deactivated (Ali et al., 2013; Clemens, 2006)..

1.7.1.2. Co-tolerance

Numerous publications have touted that a species metal tolerance capacity is specific to a single element and that the phenotypic tolerance to two different metals are controlled by separate physiological mechanisms. These ideas are challenged though by studies detailing that species tolerant to one metal; for example, Cu-tolerant populations of *Mimulus guttatus* can also display some evidence of tolerance to other metals (Macnair, 1993). Colzi *et al* (2014) compared a metal tolerant population of *Silene paradoxa* L. growing in a metalliferous soil containing As, Cd, Cu, and Zn, with a non-tolerant population (originating from non-metalliferous soils) to test the theory of co-tolerance. Seedlings of both the *Silene paradoxa* L. populations were grown in a pot trial using the parent metalliferous soil

homogenised with the addition of cobalt, chromium, manganese, nickel or lead, as substrate. Calculated average EC_{50} values indicated that the metalliferous population had higher tolerance for all the tested metals except for manganese when compared to the non-metalliferous population (Colzi et al., 2014).

These observed phenotypic correlations between different metal tolerances could be caused by two processes. The first is pleiotropy (or co-tolerance), where genes controlling the tolerance mechanisms for one metal, also give tolerance to another. The second is linkage disequilibrium (multiple tolerance), where populations possessing elevated frequencies of genes giving tolerance to one metal also have high frequencies of genes giving tolerance to another. Phenotypic analysis does not make it possible to distinguish between these hypotheses though as it is usually the average tolerance of populations studied, not comparison metal tolerance in clone or genotype groups (Macnair, 1993).

However there is also evidence detailing that there is limited connection between tolerance mechanism responses to different metals. For example Lee and Kang (2005) showed that *Arabidopsis* mutant plants that overexpressed *AtPCS1* (*Arabidopsis* phytochelatin synthase 1, a protein normally upregulated upon Cu exposure) displayed increased resistance to increasing Cd concentrations but no increase in resistance to increasing Cu thus suggesting that Cu detoxification mechanisms in *Arabidopsis* are different to those of Cd (Lee and Kang, 2005).

1.7.2 Phytostabilisation

Phytostabilisation, also known as phytoimmobilisation, uses selected plant species that are tolerant to metal contaminants and do not uptake and accumulate these contaminants to high concentrations for the purpose of stabilising and immobilising the metal contaminants within the soil profile (Ali et al., 2013). This technique is used to reduce the mobility and bioavailability of contaminants within the environment preventing their migration into groundwater and their entry into the food chain. Immobilisation of soil metals can occur through physical absorption by roots or chemical complexing and redox reactions (Ali et al., 2013).

1.8 Examples of New Zealand phytoremediation projects

1.8.1 Kopu wood waste disposal site (Waikato, New Zealand)

The Kopu waste wood disposal site (Waikato, NZ) contains the waste and subsequent contaminants of the now defunct Kopu sawmill. The site contains boron (B) and other hazardous timber treatment biocides and an associated leachate pond filled with contaminants (Robinson and Anderson, 2007). Kopu was revegetated in July 2000 using 10 poplar and willow clones, as well as two species of *Eucalyptus.* Since then the site has been planted with two *Populus deltoides* hybrid clones at a density of 7000 trees/ha (Robinson and Anderson, 2007). In the past, vegetation had failed to establish on the waste timber pile causing rain water to fill the leachate pond and overflow into a local stream. Since revegetation, average monthly leachate from the waste wood pile has decreased compared to pre-phytoremediation levels. Poplar leaves were shown to contain significant concentrations of Cu and chromium along with extremely large concentrations of B in the leaves. Approximately 700mg/Kg^{-1} of boron was found in poplar leaves on average which is over 28 times higher than the B concentration in the waste wood pile (40mg/Kg^{-1}) (Robinson and Anderson, 2007). Potentially these trees could be harvested thus removing B from the site and harvested material could be used to supplement B deficient orchards in other parts of NZ (Robinson and Anderson, 2007). The estimated cost of the phytoremediation project at Kopu was $200,000 over five years of management. In contrast, a local environmental authority estimated that capping the site would cost over $1.2 million (Robinson and Anderson, 2007).

1.8.2 Disused sheep dipping site (Hamilton, New Zealand

Disused sheep dipping sites are another source of environmental contamination, being distributed being throughout New Zealand with total numbers estimated to be about 50,000 sites (Gaw and McBride, 2010a). High concentrations of organophophates, organochlorines and arsenic can be found in these areas due to their presence in the insecticide dipping solution, the latter two persisting in the soil environment for an extensive period of time. Using willow clones, soil dieldrin content (an organochloride) was reduced by 20% over the period of 5 months whereas grass (non-specific) appeared to only reduce the dieldrin content by 10% with pre-trial soil dieldrin content being 20mg kg^{-1} (Robinson and Anderson, 2007). The reduction of triphenyltetrazolium chloride (TTC) to triphenylformazan (TPF) by soil

microbes is a technique used to quantify the presence of soil microbes and the associated dehydrogenase activity (Chander and Brookes, 1991; Robinson and Anderson, 2007). Soil TPF content was also shown to be increased by 600% compared to grassed areas due to the supposed reduced concentration of soil metals thus promoting soil microbial activity (Robinson and Anderson, 2007).

1.8.3 Tui mine tailings site (Te Aroha, New Zealand)

The Tui mine tailings site, also in the Waikato region, is considered to be one of New Zealands most contaminated sites due to mining activities (Robinson and Anderson, 2007). The tailings dam at the site contains high concentrations of sulphide minerals as well as lead (0.5%), cadmium (26mg/kg) and mercury (8mg/kg) that are mobile through the soil horizon due to a low ph (<3.0) thus allowing these to leach into nearby streams and aquifers. Sediment and stream water analyses have indicated that the concentration of Cd and Pb was above permissible limits set by the World Health Organisiton (Sabti et al., 2000). Studies attempting to use phytostabilising techniques with exotic and native species have been used to reduce mobile metal leachate and aeolion soil erosion both *ex* and *in situ* respectively (Morrell, 1997; Robinson and Anderson, 2007). Non-phytoaccumulating species were wanted for this project, given that an exposure pathway for the hazardous metals into the food network was unwanted. Of the five species used including *Phormium tenax*, *Hebe stricta* and *Leptospermum scoparium*, all species did not accumulate Fe, Cd or Hg concentrations within 20% of those background concentrations found in the tailings soils. Microbial activity was also significantly higher within the trial area compared to outside of it, 0.6mg kg^{-1} and 0.4mg kg^{-1} respectively (Robinson and Anderson, 2007).

1.9 Plant species selection

Several New Zealand native plants have been shown to be possible phytoextractors through various pieces of previous published research. *Pittosporum teniufolium* (Black Matipo) as well as *Coprosma robusta* (Karamu) were both recognised as phytoextractors of Cd in Hahner *et al* (2012). Both species extracted Cd from the soil and stored it in their foliage at higher concentrations than the background soil Cd levels (Hahner, 2012). Robinson & Brett (2007) showed that *Leptospermum scoparium* (Manuka) was able to sequester up to 454 mg/Kg^{-1} of Pb from a mine tailings site with high background Pb concentrations (5410mg/Kg^{-}

[1]). Manuka was also shown to uptake As from contaminated mine tailings at a site near Reefton, Westland, New Zealand. Concentrations of As between 3.5 and 3.6 mg/Kg^{-1} were detected in the sampled Manuka foliage (Craw et al., 2007). Other species selected for this study were based on several criteria including inter-species morphological diversity, phylogenetic differences and of course the limitation of seasonal availability.

1.10 Aims, objectives and hypotheses

1.11.1 Aims

At current, there are very few published studies containing content relating to the seed germination potential or the growth capabilities of New Zealand native plants growing in metal contaminated soils. Nor is there extensive knowledge and publications of the possible tolerance and response mechanisms in New Zealand native plants to abiotic stressors such as metal contaminants. However, previous studies published globally have noted that whatever molecular interactions that confer resistance to metal contaminants exist within a plant species are possibly interchangeable and enable tolerance to a broad variety of abiotic stressors rather than single specific stressors thus forming a type of abiotic stress co-tolerance.

Therefore this study seeks to form a simple base of information regarding the topic New Zealand native plants growing in metal contaminated soils and its associated ideas while complementing the existing research with regards to metal co-tolerances observed in plants. Specific objectives and hypotheses are as follows:

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1.11.2 Objectives

*Determine the "*in-situ*" (on-site) seed germination potential of New Zealand native plant species in cadmium contaminated soils

*Determine the "*in-situ"* seedling survivorship potential of New Zealand native plant species in cadmium contaminated soils

*Investigate for the presence (or absence) of physiological mechanisms conferring (or suppressing) resistance to cadmium and / or arsenic, or other metal contaminants in New Zealand native plant species

1.11.3 Hypotheses

1) The seed germination rate of the tested New Zealand native plants will show a negative dose-response upon exposure to increasing concentrations of cadmium or arsenic

2) The morphological biometrics like shoot length, root length, and biomass ratio of the tested New Zealand native plants will show a negative dose-response upon exposure to increasing concentrations of cadmium or arsenic

3) Biochemical biometrics like total shoot chlorophyll content and seed or root peroxidase activity in the tested New Zealand native plants will show a negative dose-response upon exposure to increasing concentrations of cadmium or arsenic

4) The New Zealand native plants that display a tolerance to the presence of cadmium in solution will also display tolerance to arsenic

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Plant materials

Seeds used in the research experiments were purchased from New Zealand Seeds (Rangiora, New Zealand) and stored in a refrigerator at 4°C. Seeds of the following species were purchased:

Scientific name	**Common name**
Kunzea ericoides	Manuka
Leptospermum scoparium	Kanuka
Phormium tenax	Flax
Ackama rosaefolia	Makamaka
Arthropodium cirratum	New Zealand Rock Lily
Hebe stricta	Koromiko
Festuca actae	Banks Peninsula Blue Tussock
Carex comans	Frosted Curls
Clianthus puniceus rosea	Red Kaka Beak
Clianthus puniceus albus	White Kaka Beak
Pachystegia insignis	Marlborough Rock Daisy
Pomaderris kumeraho	Golden Tainui
Clematis paniculata	Puawhananga
Heliohebe hulkeana	New Zealand Lilac
Astelia solandri	Kowharawhara/Perching Lily
Coprosma robusta	Karamu

Table 2.1: All plant species used in this study

2.1.2 Chemicals and metals used

The metal salts and the chemicals used in the completed experiments are as listed in the following table:

Name of Chemical	**Manufacturer**
$CdCl_2.xH_2O$ (cadmium chloride hydrate salt)	Aldrich, U.S.A
$Na_2HAsO_4.x7H_2O$ (disodium hydrogen arsenate heptahydrate)	J.T Baker Chemical Co, U.S.A
EDTA	BDH Chemicals, U.K
Dithizone	Sigma Chemical Co., U.S.A
Gluaicol	Sigma Chemical Co., U.S.A
Baby Bio	Bayer Garden, U.K
Acetone	Sigma Chemical Co., U.S.A
K_2PO_4	Sigma Chemical Co., U.S.A
KH_2PO_4	Sigma Chemical Co., U.S.A

Table 2.2: All metal salts and chemicals used in this study

2.1.3 Equipment used

The equipment used in the completed experiments are listed in the following table:

Name of equipment piece	Manufacturer
Plant growth chamber CAT 620	Contherm Scientific, N.Z
Thermotec 2000 laboratory drying oven	Contherm Scientific, N.Z
Hobo Pro v2 Loggers	Onset Computer Corperation, U.S.A
Hobo Pro light loggers	Onset Computer Corperation, U.S.A
QSPAR Quantum Sensor	Hansatech, U.K
Milli Q nanopure water	Millipore, U.S.A
Orion 3 Star benchtop pH meter	Thermo Fisher Scientific, U.S.A
Novospec 3 spectrophotometer	Amersham Biosciences, U.K
Centrifuge 5810R	Eppendorf, Germany
Orbital shaker SS70	Chiltern Scientific, U.K
Thermolyne Cimarec 1 Magnetic Stirrer	Thermo Fisher Scientific, U.S.A

Table 2.3: All pieces of equipment used in this study

2.2 Generic experimental conditions and design

2.2.1 Generic experimental design and methods

Stock solutions of 1.78 mol L^{-1} Cd and As were prepared using analytical grade cadmium chloride hydrate salt ($CdCl_2.xH_2O$) and disodium hydrogen arsenate heptahydrate ($Na_2HAsO_4.x7H_2O$) respectively, and nanopure water. Cadmium and arsenic solution treatments of varying concentrations (0.25, 5, 25, 50 and 100mM L^{-1}) were made from a stock solution diluted with nanopure water. Temperature and humidity data collected from the experimental areas were recorded with Hobo Pro v2 Loggers. Light intensity data were collected with Hobo Pro light loggers and QSPAR Quantum Sensory equipment. All experiments featured three experimental replicates for each tested treatment and control. One containment vessel containing media and test plant species was considered a replicate.

2.2.2 Seed Preparation

Some of the seeds of the different species used in the seed germination bioassay studies required pre-treatment of seeds before sowing to increase the probability of germination. *Coprosma robusta* seeds were immersed in tap water for a period of approximately 24 hours

before sowing to soften the hard seed coat. *Clianthus puniceus* seeds were immersed in boiled tap water (~100°c) to scarify the seed and then left to cool and soak for approximately 24 hours.

2.3 *In vitro* seed germination bioassay design and conditions

2.3.1 Experimental conditions

The seeds of different New Zealand native plant species were placed in plastic tube-shaped vessels containing 10mL of control or treatment solution along with 50µL of plant preservative mixture to prevent the colonization of fungi and bacteria on the seed coats. There were 10 seeds of a test species in each experimental replicate. The seeds were immersed in the treatment solution after they were removed directly from refrigerated storage (4°C) unless stated otherwise in section 2.1.1. Plastic vessels containing the seed samples were placed in a temperature and humidity controlled plant growth room with the parameters set as follows: no light and day/night oscillatory temperature cycle of ~ 23/18.5°C respectively. Relative humidity was monitored but not controlled, averaging 42.5% over the duration of the study. Analytical methods used: germination rate and peroxidase activity assay.

2.3.2 Effects of cadmium: *in vitro* seed germination bioassay

Following the methodology described in section 2.3.1, 16 different plant species were tested in control and cadmium treatment solution with regard to each species germination rate. The following Cd treatments were used: 0.25mM, 5mM L^{-1}, 25mM L^{-1}, and 50mM L^{-1}. Plant species used in the seed germination bioassay in solution are listed in the table below:

Scientific name	Common name
Kunzea ericoides	Manuka
Leptospermum scoparium	Kanuka
Phormium tenax	Flax
Ackama rosaefolia	Makamaka
Arthropodium cirratum	New Zealand Rock Lily
Hebe stricta	Koromiko
Festuca actae	Banks Peninsula Blue Tussock
Carex comans	Frosted Curls
Clianthus puniceus rosea	Red Kaka Beak
Clianthus puniceus albus	White Kaka Beak
Pachystegia insignis	Marlborough Rock Daisy
Pomaderris kumeraho	Golden Tainui
Clematis paniculata	Puawhananga
Heliohebe hulkeana	New Zealand Lilac
Astelia solandri	Kowharawhara/Perching Lily
Coprosma robusta	Karamu

Table 2.4: Table of all plant species used in cadmium seed germination bioassay

2.3.3 Effects of arsenic: *in vitro* seed germination bioassay

The seeds of 4 different New Zealand native plant species were tested in the arsenic *in vitro* seed germination bioassay. Three species were selected based on their apparent tolerance to Cd and the fourth species based on its apparent intolerance. The following As treatments were used: 0.25mM, 1mM L^{-1}, 5mM L^{-1}, 25mM L^{-1}, and 50mM L^{-1}. Plant species used in were as follows: *Leptospermum scoparium* (Kanuka), *Phormium tenax* (Flax), *Arthropodium cirratum* (New Zealand Rock Lily), and *Carex comans* (Frosted Curls).

2.4. Soil media bioassay design and conditions

2.4.1 Generic experimental methods

All experimental units were spatially separated forming a random experimental design to increase independence between experimental treatments and experimental replicates.

2.4.1.1 Soil preparation and experiment establishment

With regard to the *ex situ* seed germination (as well as the seedling survivorship bioassay), all soils were passed through 5mm sieve to remove larger bark or soil peds prior to experimental use and all experimental units were positioned randomly within the experiment area to reduce the effects of environmental heterogeneity on treatment groups and replicates. Treatment or control solution was added to the soil media at a 1:2 volume to weight ratio within the containment vessel using the following stepwise process: 1) addition of the total treatment or control solution volume to containment vessel, 2) adding 50% of the total required soil media to vessel, 3) mixing and homogenising of soil media and solution for ~ 1 minute, 4) addition of the test seeds or seedlings, 5) addition of the final 50% of the required soil media to vessel. The South Waikato field soil sample was homogenised with control solution as the presence of Cd and As in the soil media negated the need for Cd or As solution application.

2.4.1.2 Soil pH measurements

pH measurements were obtained by following the stepwise process described earlier in section 2.4.1.1 omitting step 4 then allowing the samples to sit for 24 hours at room temperature (~25 °c). Samples were then filtered using filter paper and pH was measured with an Orion 3 Star benchtop pH meter.

2.4.1.3 Batch Sorption experiments

A batch sorption experiment was used to assess the bioavailability of Cd in the soil matrix to germinating seeds. Tested samples included control (0mM L^{-1}), and 5mM L^{-1}, 25mM L^{-1}, and 100mM L^{-1} Cd treatments. Treatment or control solution was added to the soil media at a 1:2 volume to weight ratio within the containment vessel using the following stepwise process: 1) addition of the total treatment or control solution volume to containment vessel, 2) adding 100% of the total required soil media to vessel, 3) samples were homogenised using an

orbital shaker for approximately one hour, 4) samples were filtered three times using filter paper and then transferred to sampling containers containing 0.1% nitric acid preservative for total recoverable Cd analysis by Hill Laboratories (Hamilton, New Zealand)

2.4.2 *Ex situ* seed germination bioassay

2.4.2.1 Experimental conditions

Seeds of 6 different New Zealand native plant species that successfully germinated in the *in vitro* seed germination bioassay were then tested in the *ex situ* seed germination bioassay. Experimental establishment followed the methods and conditions described in section 2.4.1.1 with Petri-dishes as the containment vessel containing 10mL of control or cadmium solution at one of the following treatment concentrations: 5mM L^{-1}, 25mM L^{-1} and 100mM L^{-1}, and 20g of potting media or South Waikato field soil sample for the field soil sample treatment. Petri dishes were sealed using plastic film to prevent soil desiccation and 15 seeds of a test species were sown into the Petri-dish directly from refrigerated storage (4°c) unless stated otherwise in section 2.1.1. Petri-dishes containing the seed samples were placed in a temperature controlled plant growth cabinet with the parameters set as follows: 16 hours daily photoperiod at 15 - 17µmol m^{-2} s^{-1} and 25°c, and 8 hours daily dark period at 10°C. Relative humidity was monitored but not controlled with recorded relative humidity ranging from 20.1 to 94.5% and averaging 45.9%. Plant species used in the *ex situ* seed germination bioassay in soil were as follows: *Kunzea ericoides* (Kanuka), *Phormium tenax* (Flax), *Arthropodium cirratum* (New Zealand Rock Lily), *Hebe stricta* (Koromiko), *Carex comans* (Frosted Curl) and *Coprosma robusta* (Karamu). Analytical methods used: germination rate and chlorophyll content assay.

2.4.2.2 Details of South Waikato field soil sample

A field soil sample from a kiwifruit orchard in the South Waikato region of New Zealand was used a representative sample of possible *in situ* soil conditions at a metal contaminated site. Horticultural sites like kiwifruit orchards feature the highest median soil C d concentration of land uses in New Zealand due to the high phosphate fertiliser inputs applied to these high intensity crops (Taylor et al., 2014). The soil was allophonic, silt loam with a record of reactive rock phosphate fertiliser and biosolid application over previous years. Of specific

interest to this study, this soil sample contained average Cd and As concentrations of 1.7 and 8.8 mg kg^{-1} respectively. These averages are above the New Zealand wide averages for soil Cd and As content which are 0.23 and 4.0 mg kg^{-1} respectively (McDowell et al., 2013). This soil sample also contained average soil metal concentrations above the national averages noted in McDowell *et al* (2013) for the following metals: aluminium, barium, calcium, copper, magnesium, manganese, molybdenum, nickel, phosphorus, uranium, and zinc.

2.4.3 *Ex situ* seedling survivorship bioassay

2.4.3.1 Experimental conditions

Seedlings of *Arthropodium cirratum* and *Kunzea ericoides* were transplanted into ~ 315cm^2 plastic containment saucers containing 75mL of control solution or one of the following Cd treatment solutions: 5mM, and 25mM L^{-1}. Approximately 1.25mL of "Baby Bio" (Bayer Garden, U.K) nutrient solution containing nitrogen, potassium and phosphorus was added to the solution at step 2 of section 2.4.1.1 to enhance seedling growth. Ten seedlings of a test species were planted in a saucer, each containing 250g of soil media. Plastic saucers containing the seedling samples were placed in a temperature moderated glasshouse featuring halogen growth lamps during and between the months of August and December 2014. The temperature and humidity variables fell within the following ranges over the trial period: average temperature = 22.4°C, temperature range = 11.1°C to 41.0°C, average relative humidity = 52.5% and relative humidity range = 10.6 to 93.3%. Average photo-intensity equalled 215.6µmol m^{-2} s^{-1} and ranged from 0µmol m^{-2} s^{-1} (night) to 2752.8 µmol m^{-2} s^{-1} (full sunlight). The combined natural and artificial photoperiod throughout the project ranged between 16 and 17 hours. Soil moisture of each plastic containment saucer was kept between approximately 36 and 77% of total soil moisture content by mass. Plant species used in the seedling survivorship bioassay were as follows: *Kunzea ericoides* (Manuka), *Phormium tenax* (Flax), *Ackama rosaefolia* (Makamaka), *Arthropodium cirratum* (New Zealand Rock Lily), *Hebe stricta* (Koromiko), and *Coprosma robusta* (Karamu). Analytical methods used: morphological measurements, chlorophyll content assay, peroxidase activity assay and quantitative metal analysis.

2.4.3.2 Soil moisture measurements

Soil moisture was calculated using the water holding capacity (WHC) method similar to that used in Angle *et al* (2003).Mesh bottomed saucers holding 250g of potting-mix soil was saturated with tap water until the soils total water holding capacity achieved and allowed to drain for a total of 18 hours after which time the samples were weighed to determine the sample weight (organic and inorganic soil material plus the water content). To prevent evaporation from the wet soil samples, the mesh bottomed containers featured a sealed cover (Angle et al., 2003). Saturated soils were then oven dried at a temperature of 105°C for 24 hours to remove all liquid from the samples to determine the total weight of the solid organic and inorganic soil material minus the water content thus enabling the calculation of the WHC.

2.5 Analytical techniques

2.5.1 Germination rate

Assessment of the germination percentage occurred at 3 – 4 day intervals and continued for 6 weeks after the first seed of an experimental unit had germinated. Germination was defined as the emergence of the hypocotyl or radicle to length equal to or greater than 2mm. After 6 weeks of germination data was collected, selected test species seeds were harvested and stored at -20°C until peroxidase activity assays were completed at a later date.

2.5.2 Morphological measurements

Morphological measurements at the time of seedling harvest comprised of seedling height, root length, shoot and root biomass measurements. Soil bound to the seedling roots was removed by washing the roots in tap water twice, followed by blot drying before weighing and measuring. Seedling height measurements were measured with a ruler from soil level to shoot tip. Root length measurements were measured with a ruler from root collar to the longest root tip. For the fresh-weight biomass measurements, whole seedlings were partitioned into shoot and root parts by cutting the whole seedlings in half at the root collar, shoot and root biomass were then weighed separately.

2.5.3 Chlorophyll content

Leaf chlorophyll content analysis was carried by homogenising pre-weighed leaf tissue, combined from one experimental replicate, using a pestle and mortar in an ice bath with a 1.5mL homogenising solution comprised of 80% (v/v) acetone: nanopure water. The homogenate was then centrifuged for 5 minutes at 2655g and a temperature of 4°C. The supernatant obtained after centrifugation was then transferred to a cuvette and then diluted with the acetone: nanopure water solution at a 75% v/v acetone: supernatant ratio. This step of supernatant extraction and dilution was repeated three times to form 3 technical replicates from the same sample to be then spectrophotometrically analysed individually. Spectrophotometric analysis using a Novospec 3 (Amersham Biosciences) was then used to measure the absorbance of the diluted supernatant at the 645 and 663nm wavelengths. Total chlorophyll content was then calculated based on the following equation used in Porra *et al* (2002):

$$[\text{Chl } a + b] = 17.76\, E^{645} + 7.34\, E^{663}$$

Calculated total chlorophyll content (chl $a + b$) is presented as $\mu g\ g^{-1}$ (Porra, 2002).

2.5.4 Peroxidase assay

Peroxidase activity in plant tissue extracts was assayed using the gluaiacol reduction method. Pre-weighed plant tissue, approximately between 10 to 100mg fresh - weight, was homogenised using a pestle and mortar in an ice bath for approximately one minute with a 1.0mL homogenising solution comprised of 0.1M KPO_4 buffer (pH = 6.59). The homogenate was then centrifuged at 5310g for 10 minutes and at 4°C with the supernatant obtained after centrifugation being used as enzyme extract. Within a cuvette, a reaction mixture containing 2µL of gluaiacol, 5µL of 10% H_2O_2, and 10µL of the centrifuged supernatant was created with the addition of KPO_4 buffer to add up to 1mL. The solution was then left to stand for 5 minutes at room temperature (~25°C) for the gluaiacol reduction to occur. The control used in this assay was the reaction mixture without the addition of the enzyme extract. The cuvette solution was then measured for its absorbance value at the 470nm wavelength using a Novospec 3 spectrophotometer. A preliminary enzyme activity assay analysing the proportional responses of peroxidase activity to proportional increases supernatant volume within the reaction mixture was completed prior to the main experimental peroxidase assay.

This was used to ensure that the reactants within the reaction mixture were not at a saturation point thus their abundance within the reaction mixture would allow theoretically limitless reactivity between peroxidase enzymes and the reactants thus providing accurate peroxidase activity data. Enzyme activity was calculated and expressed as a unit measurement of spectrophotometric absorbance per mg (absorbance g^{-1}) of fresh weight plant-tissue.

2.5.5 Quantitative metal analysis

Quantitative analysis of the total Cd content of species used in the seedling survivorship bioassay was completed by Hill Laboratories (Hamilton, New Zealand). The root sections of harvested samples were submitted to Hill Laboratories for analysis using inductively coupled plasma mass spectrometry (ICP-MS) after nitric and hydrochloric acid micro digestion.

2.5.6 Statistical analysis

All statistical analyses were completed using R Studio version 0.98.1102. Analysis of variance (ANOVA), linear mixed effect modelling as well as binomial generalised linear modelling (GLM) and Tukey HSD post-hoc analyses were used in R Studio for assessing the statistical significance of the effects of treatments and experimental trial duration on species. Prism 6 (Graphpad Software, Inc) was used for graphing and illustrating the associated data.

Chapter 3: Results

3.1 Pre-trial results

3.1.1 pH measurements

pH measurements of soil media used in the *ex situ* seed germination and seedling survivorship bioassays were completed prior to the start of all experiments. The following table details the average pH measured for each applied treatment:

Treatment concentration (mM L^{-1})	pH
0	6.7
Field sample	6.86
5	6.14
25	5.91
100	5.69

Table 3.1: Mean pH measurements from experiment soil samples

3.1.2 Batch sorption experiment

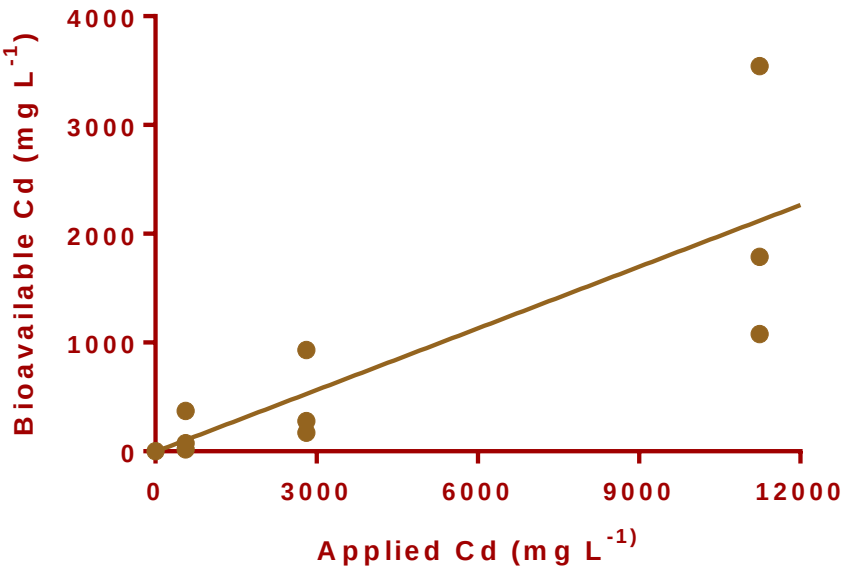

Figure 3.1 Batch sorption results detailing the concentration of the Cd treatment applied compared to the concentration of Cd bioavailable featuring linear regression (n=12)

Treatment concentration (mM L^{-1})	Percentage bioavailable
0	0.08%
5	27.50%
25	16.38%
100	19.00%

Table 3.2: Average percentage of bioavailable Cd compared to the Cd concentrations applied to experiment soil samples

Statistical analysis for the batch sorption data was not possible due to the small sample size submitted for the Cd content analysis (Figure 3.1). Table 3.2 though does provide insight into the average quantity of Cd theoretically bioavailable in the experiment soil samples, metrically expressed as the 'percentage bioavailable' compared to the concentration of Cd applied to the soil media. The average percentage of Cd bioavailable in 5mM L^{-1} treatment does appear to be an outlier given that when the most concentrated Cd treatment (100mM L^{-1}) is applied to the trial soil media only 19.00% of the Cd content is bioavailable compared to 27.50% for the 5mM L^{-1} treatment.

3.2 *in vitro* seed germination bioassay

3.2.1 Cadmium bioassay

3.2.1.1 Non-germinating species

Seeds of the following species failed to germinate in the *in vitro* seed germination bioassay under control or Cd solution treatment conditions: *Clianthus puniceus rosea* (Red Kaka Beak), *Clianthus puniceus albus* (White Kaka Beak), *Pachystegia insignis* (Marlborough Rock Daisy), *Pomaderris kumeraho* (Golden Tainui), *Clematis paniculata* (Puawhananga), *Heliohebe hulkeana* (New Zealand Lilac), *Astelia solandri* (Kowharawhara/Perching Lily) and *Ackama rosaefolia* (Makamaka).

3.2.1.2 Germination rate analysis

Statistical analysis of the germination rate involved the use of generalised linear mixed models based on a binomial function given that the foundation of the data set is binomial thus the errors are not normally distributed and the modelling system needs to be capable of including this.

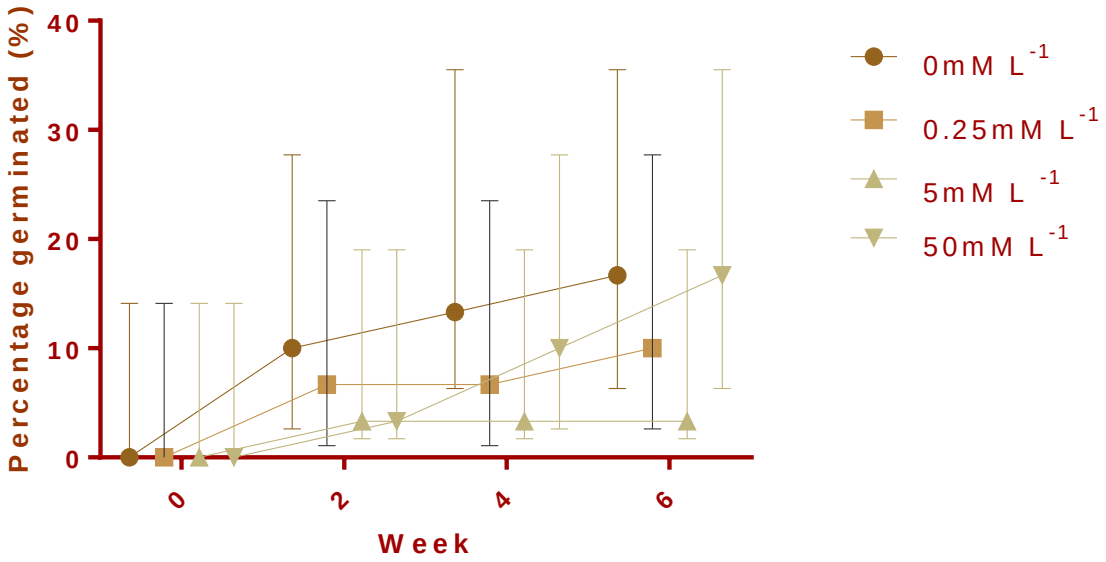

Figure 3.2: Average germination rate (%) of Rock Lily seeds (n=84) (error bars = 95% confidence interval)

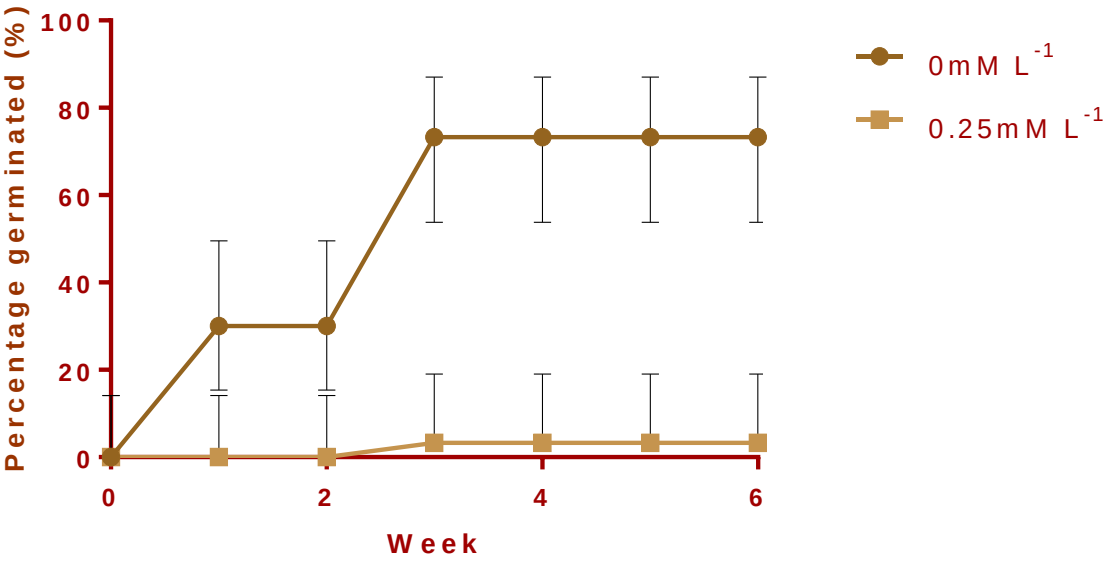

Figure 3.3: Average germination rate (%) of Kanuka seeds (n=42) (error bars = 95% confidence interval)

Using generalised linear modelling (GLM) based on a binomial function, the effects of varying aqueous Cd concentrations on the seed germination rate of different species was analysed. The average germination rate of the Rock Lily and Kanuka (Figure 3.2 and 3.3 respsectively) exposed to the control (0mM L^{-1}) treatment were considered statistically significant in difference to the other applied treatments and ANOVA analysis highlighted that the variation in the response variable due to the effects of applied treatments was significant ($p < 0.001$). The week variable was also statistically significant ($p < 0.001$) at explaining the average germination rate recorded over the 6 week trial period thus suggesting that continuous treatment exposure for the duration of the experiment effected the germination time for both species. All other parameters used in the binomial GLM and the ANOVA were calculated to be insignificant statistically ($p > 0.1$).

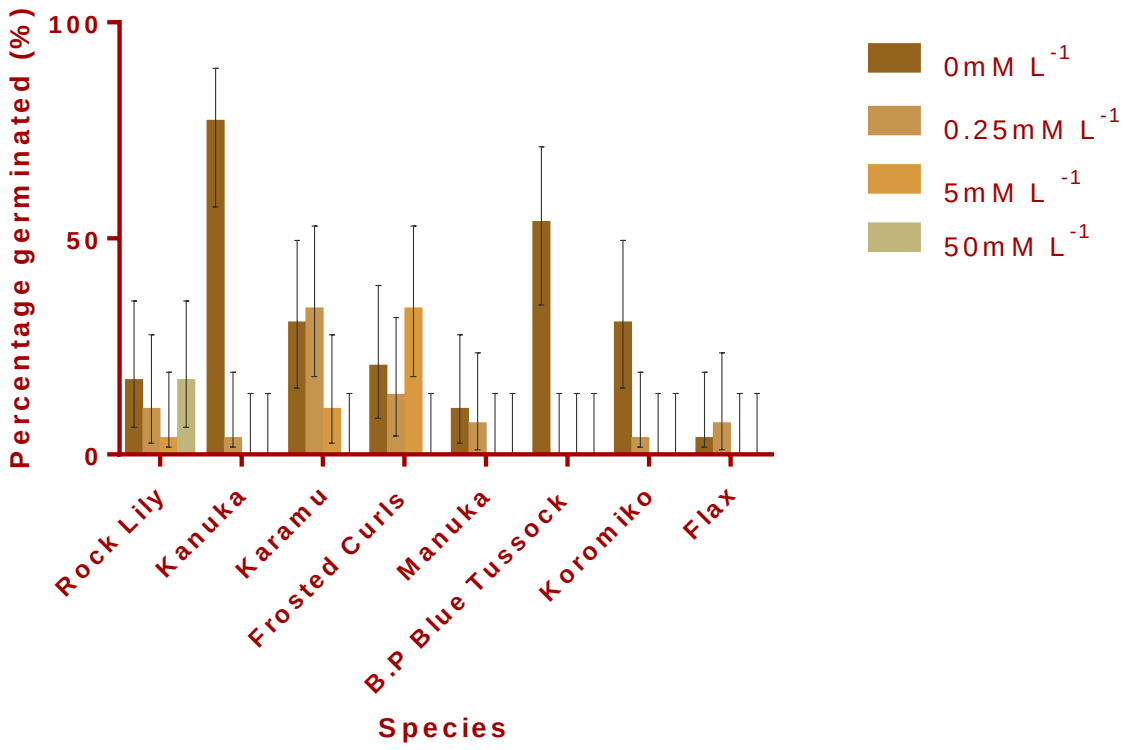

Figure 3.4: Average final germination rate (%) of test species measured at week 6 (n= 96) (errorbars = 95% confidence interval)

GLM analysis of the differences between species and treatments with regard to the average final germination rate (Figure 3.4) highlighted that the Banks Peninsula Blue Tussock was the only species with an average germination rate calculated to be significantly different to the

other test species ($p < 0.001$). Treatments 0mM L^{-1} and 50mM L^{-1} were considered statistically significant ($p < 0.001$ and 0.01 respectively) as well with regards to the difference in average final germination rate between these two treatments and the other test treatments. ANOVA analysis yielded results detailing that there was no statistical variation in average final germination rate between species ($p > 0.1$), however, the effects of treatment on the response variable was significant ($p < 0.001$). TukeyHSD results suggested no pairwise variation.

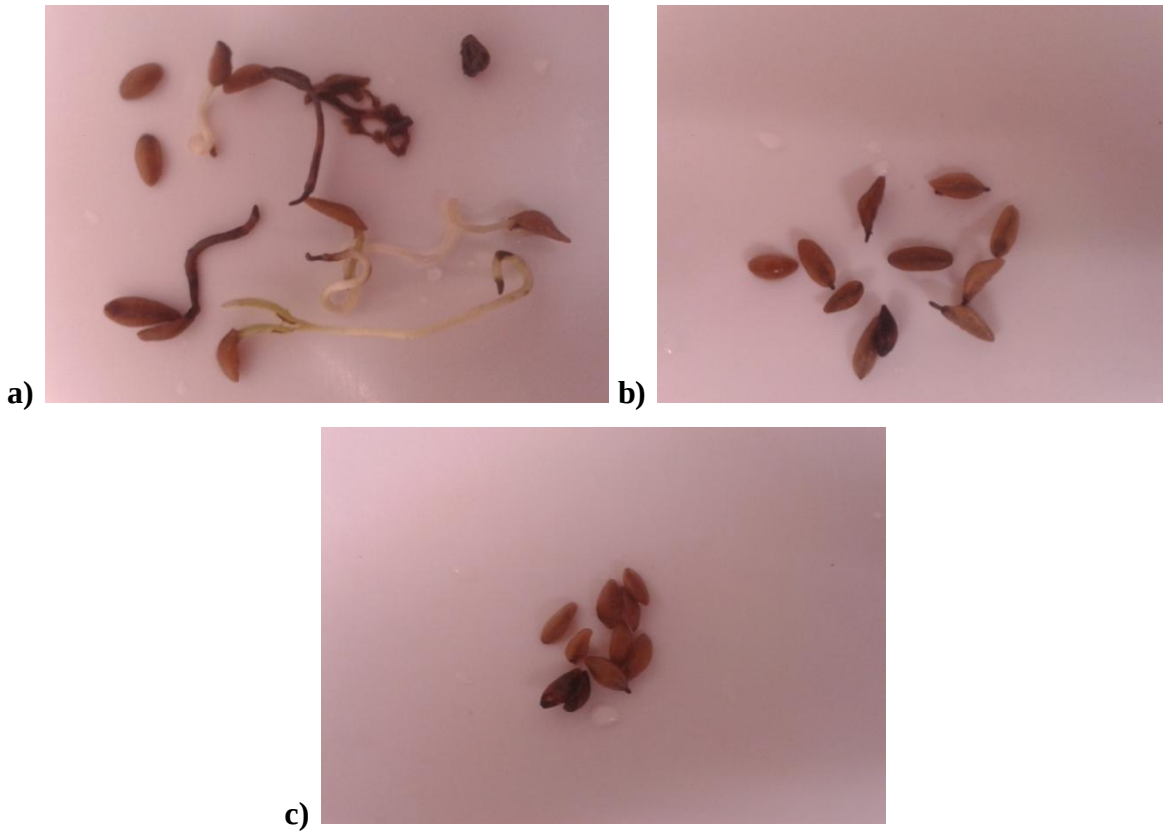

Plate 3.1: Treatment differences in seed germination rate of Karamu; a) control (0mM L^{-1} Cd), b) seeds treated with 5mM L^{-1} Cd, and c) seeds treated with 50mM L^{-1} Cd

3.2.2 Arsenic bioassay

ANOVA output of the arsenic seed germination bioassay (Figure 3.5) indicates that treatment effect is significant with $p<0.001$ but the seed germination variation between species is insignificant ($p>0.1$). All test species had germination success exposed to 50mM L^{-1} As treatment except for Kanuka. TukeyHSD results suggested no pairwise variation.

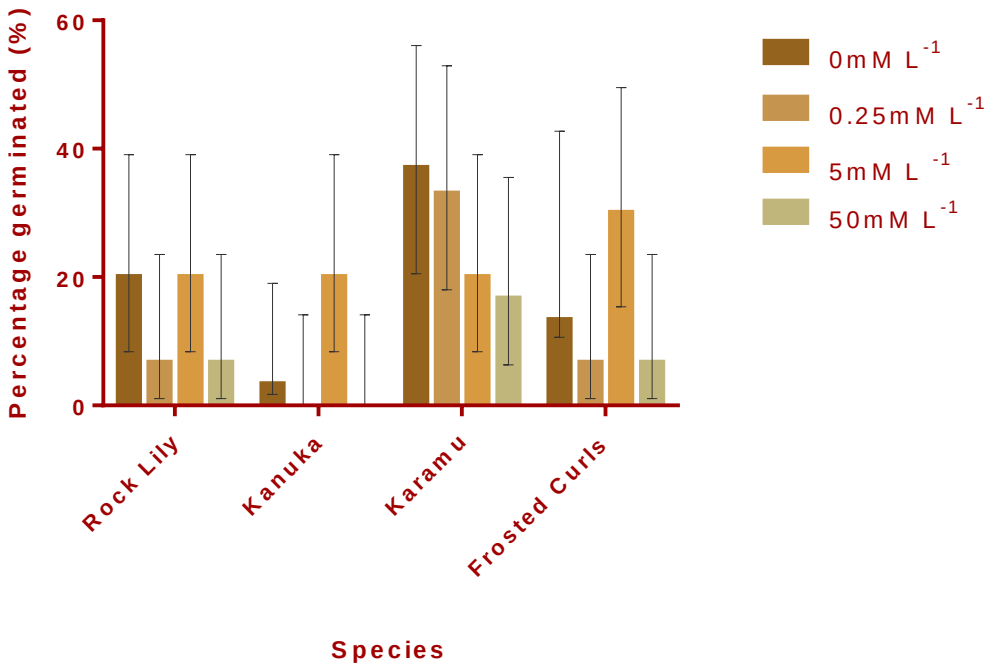

Figure 3.5: Average final germination rate (%) of test species exposed to As treatments measured at week 6 (n= 48) (errorbars = 95% confidence interval)

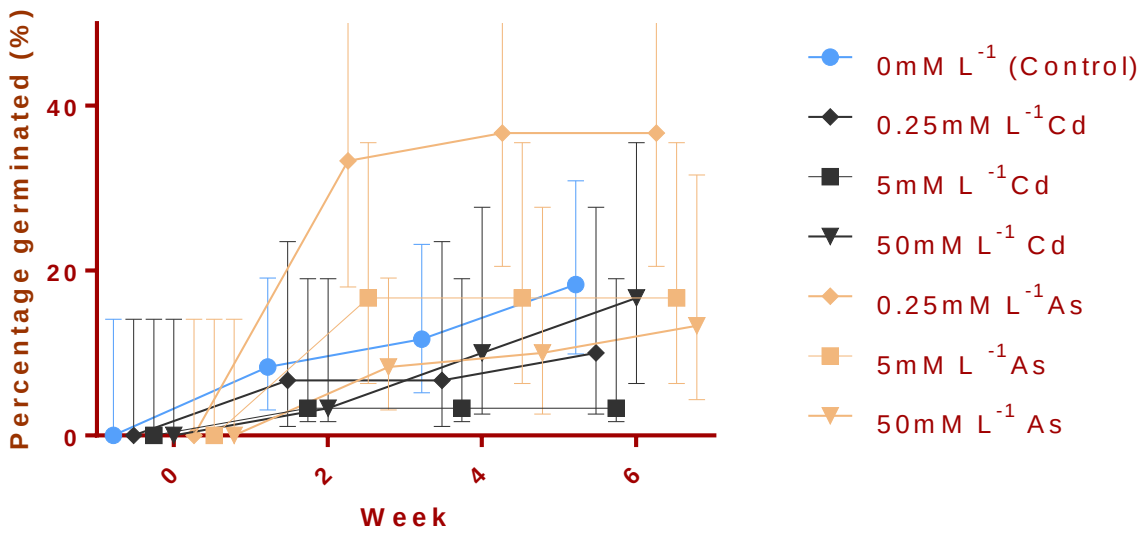

Figure 3.6: Average germination rate (%) of Rock Lily seeds exposed to As and Cd treatments (n=42) (error bars = 95% confidence interval)

ANOVA analysis results of the data presented in figure 3.6 state that both of the variables, metal type (As or Cd), and the metal treatment concentration (0mM L^{-1}, 0.25mM L^{-1}, 5mM L^{-1}, 50mM L^{-1}) as well as the combination of the two variables are considered statistically significant ($p < 0.001$ for metal type and treatment concentration, and $p < 0.01$ for the metal type : treatment interaction variable).

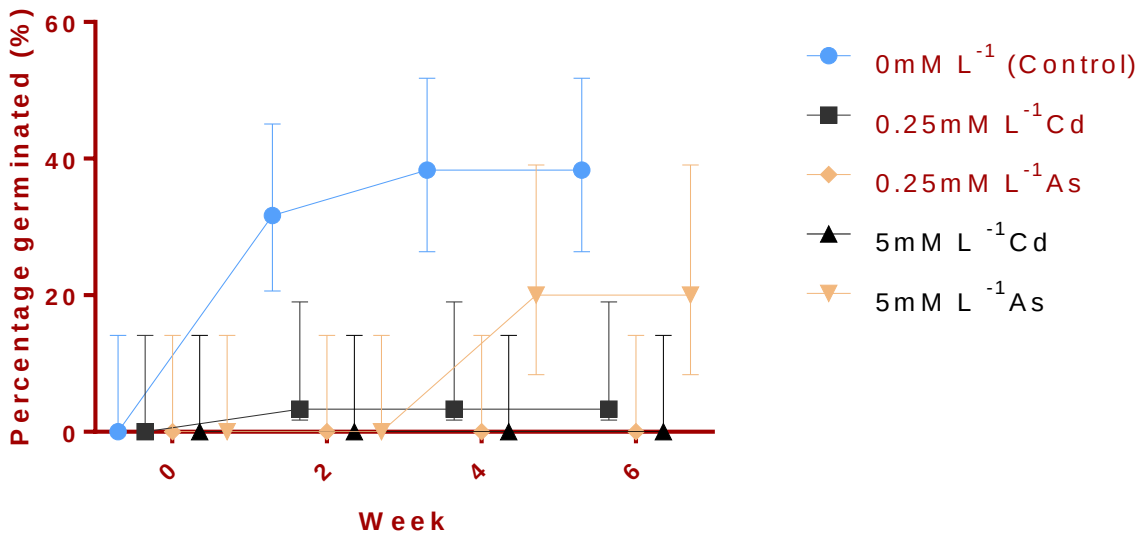

Figure 3.7: Average germination rate (%) of Kanuka seeds exposed to As and Cd treatments (n=42) (error bars = 95% confidence interval)

ANOVA analysis results of the data presented in figure 3.7 state that both of the variables, metal type (As or Cd), and the metal treatment concentration (0mM L^{-1}, 0.25mM L^{-1}, 5mM L^{-1}, and (50mM L^{-1} – not displayed)) are considered statistically significant ($p < 0.05$ for metal type and $p < 0.001$ treatment concentration variable).

3.2.3 Peroxidase assay

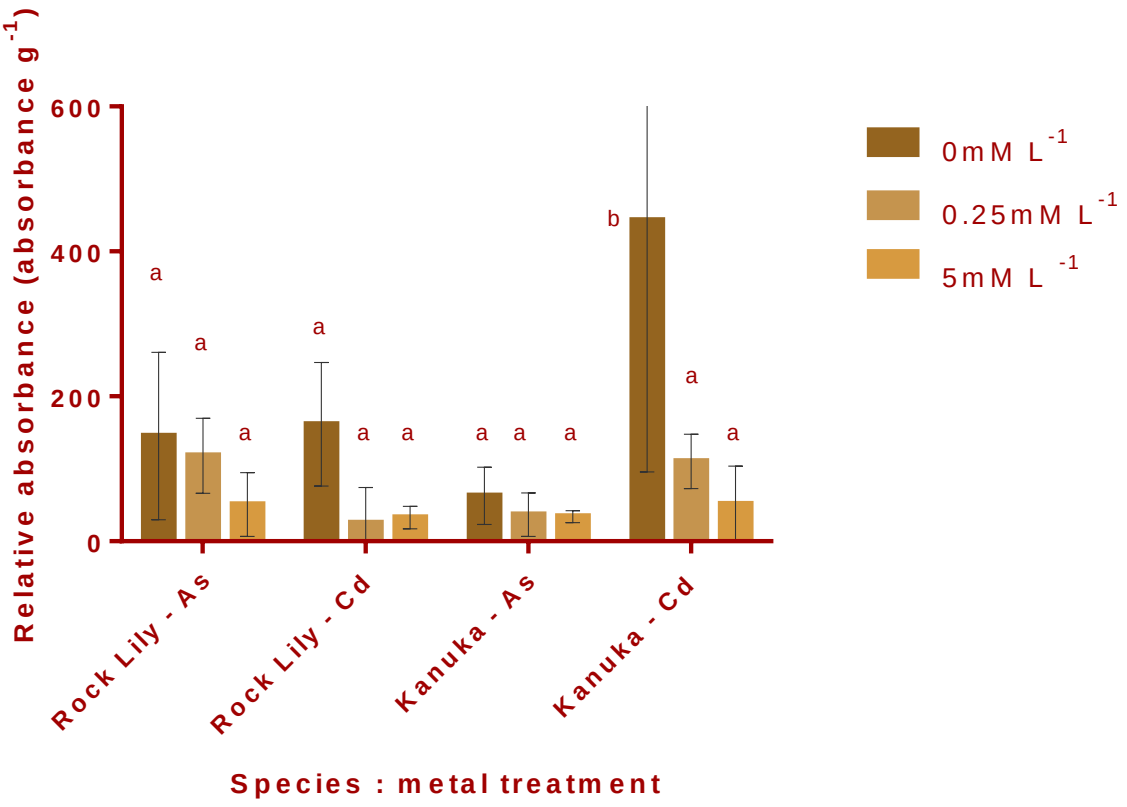

Figure 3.8: Average relative absorbance of peroxidase extracts from Rock Lily and Kanuka seeds exposed to Cd or As treatments as measured at week 6 (n=108) (error bars = standard deviation)

A peroxidase activity assay (Figure 3.8) was completed for the seeds of the Rock Lily and Kanuka after 6 weeks (experiment duration) of exposure to either Cd or As treatments of the same concentrations. Peroxidase activity was calculated as relative absorbance per gram of fresh weight plant seed which is used as a proxy for comparing peroxidase activity between species and applied treatments. ANOVA analysis results indicated that the variables; species ($p < 0.01$), metal treatment ($p < 0.001$), and the concentration of the metal treatment applied ($p < 0.001$) were all statistically significant. With regard to the individual analysis of parameters, the difference in the relative absorbance for each species metal combination (e.g Rock Lily - As) after exposure to the three different treatment concentrations was statistically insignificant ($p > 0.1$) except for seeds of both species exposed to the control (0mM L^{-1}) treatment where statistical significance was detected ($p < 0.001$) between the control and the other two treatments. In summary, these results suggest that the concentration of the type of metal (As or Cd) and the concentration of the treatment effects the peroxidase activity in the

seeds of both species with a variation in the peroxidase activity exhibited between the two species in response to the dependent variables.

3.3 *ex situ* seed germination bioassay

3.3.1 Germination rate

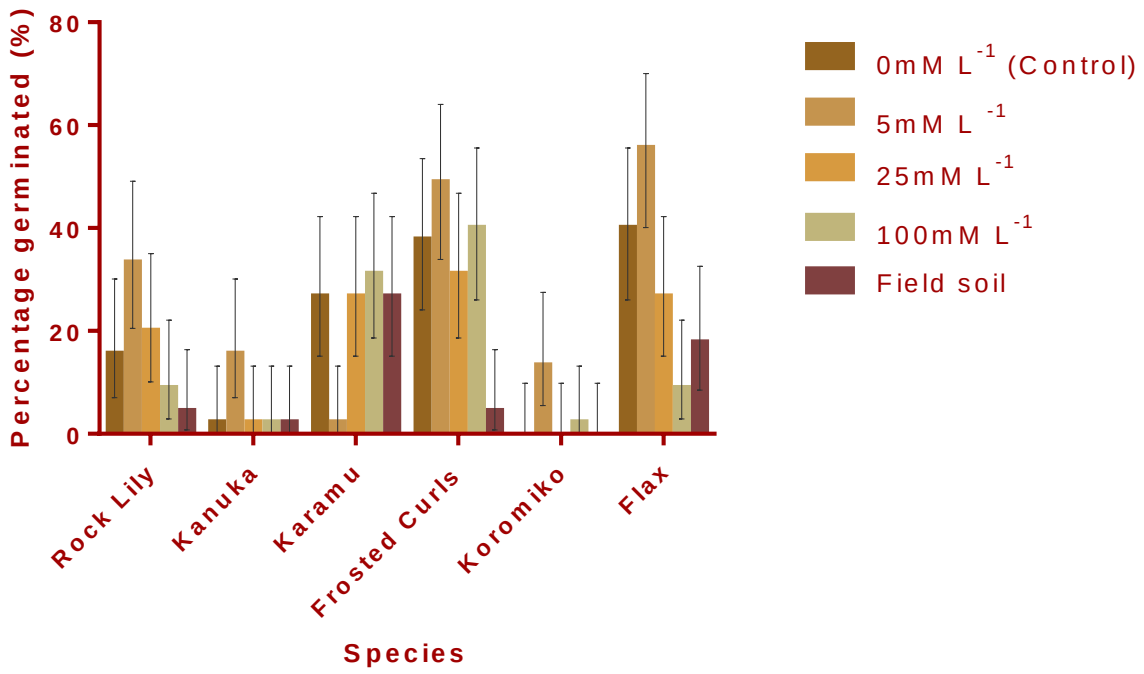

Figure 3.9: Average final germination rate (%) of test species exposed to Cd soil treatments measured at week 6 (n= 90) (errorbars = 95% confidence interval)

ANOVA analysis results of the data presented in figure 3.9 state that both of the variables, test species and the soil Cd concentration (0mM L^{-1}, 0.25mM L^{-1}, 5mM L^{-1}, 50mM L^{-1} and the field soil sample)) are both considered to be highly statistically significant ($p < 0.001$ treatment concentration variable).

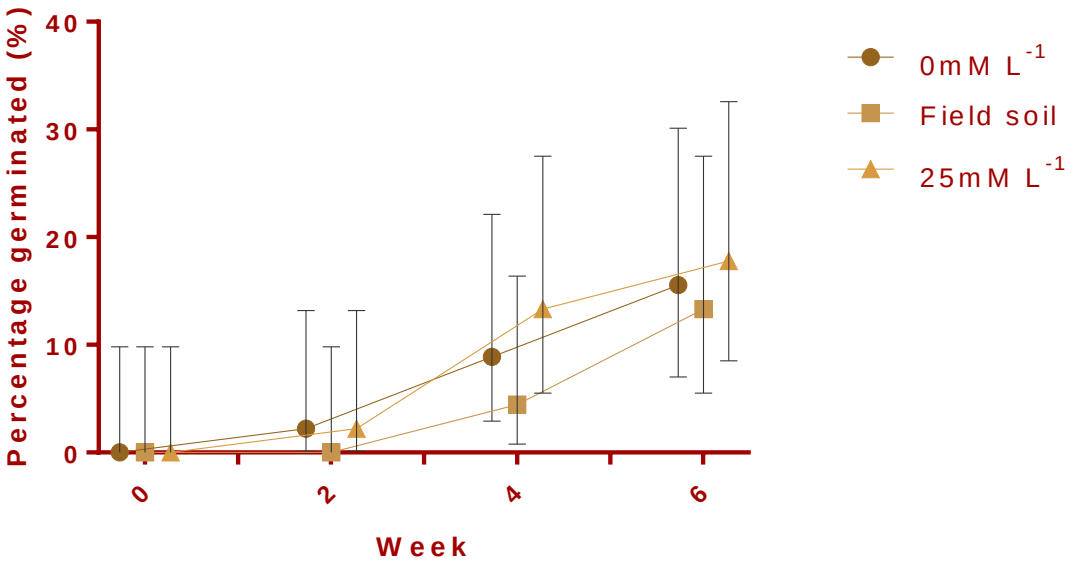

Figure 3.10: Average germination rate (%) of Rock Lily seeds exposed to Cd soil treatments (n=36) (error bars = 95% confidence interval)

ANOVA results of the data presented in figure 3.10 suggest that the week variable is highly significant ($p < 0.001$) but contrastingly, the effect of the Cd treatment concentration is statistically insignificant. This suggests that a greater proportion of the variation in the germination rate is due to the period of Cd exposure to the seeds and less of the variation is due to the applied treatment Cd concentration. (Note: 5mM L^{-1} and 50mM L^{-1} treatments analysed not displayed in figure 3.10)

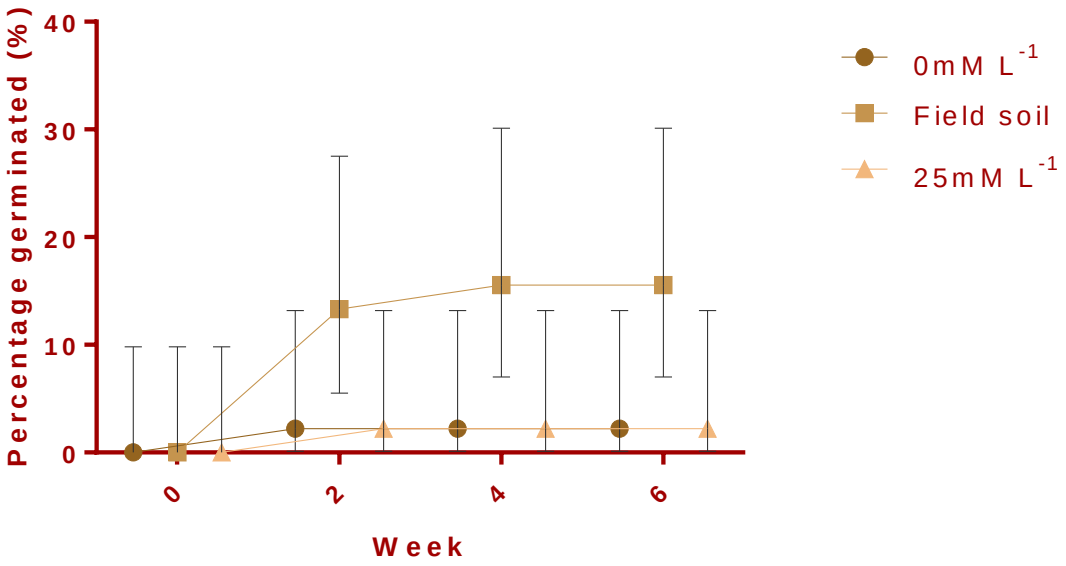

Figure 3.11: Average germination rate (%) of Kanuka seeds exposed to Cd soil treatments (n=36) (error bars = 95% confidence interval)

Contrasting to the ANOVA output with regard to the data displayed in figure 3.10, the ANOVA results from the Kanuka data displayed in figure 3.11 indicate that the period of treatment exposure (week variable) is statistically insignificant (p>0.1) while the treatment effect was considerably significant (p< 0.001).(Note: 5mM L^{-1} and 50mM L^{-1} treatments analysed not displayed in figure 3.11)

3.3.3 Chlorophyll content

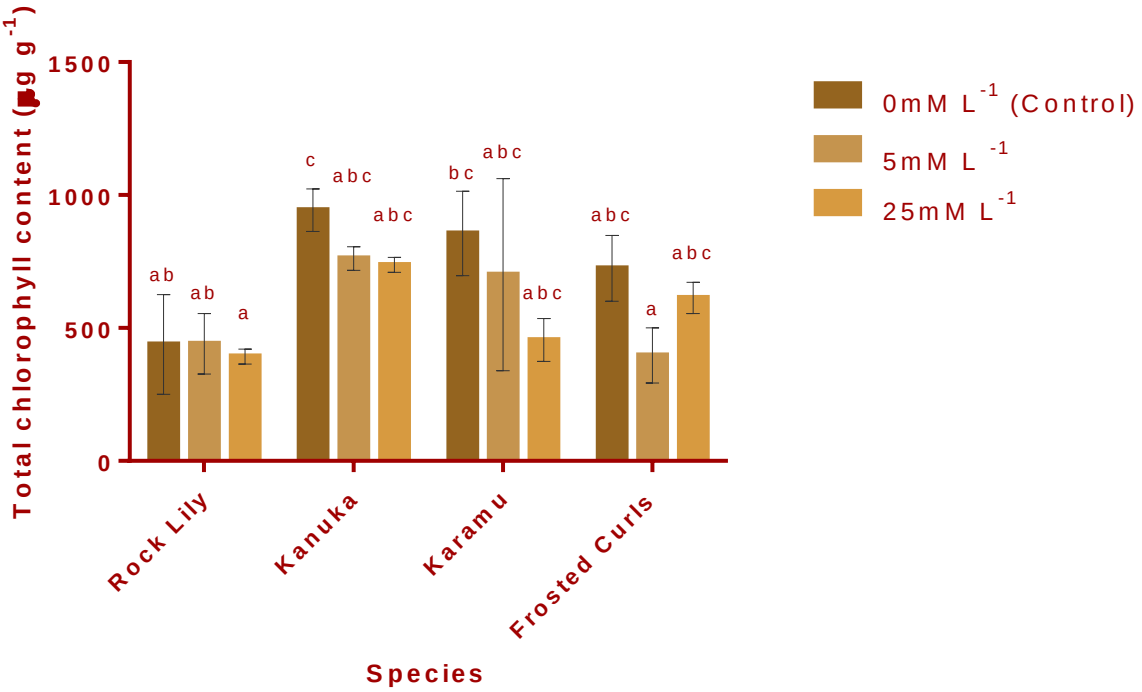

Figure 3.12: Average total chlorophyll content of test species exposed to Cd soil treatments and measured at week 6 (except pre-trial measurement) (n=108) (error bars = standard deviation)

A decrease in leaf chlorophyll content appears as the Cd concentration exposed to germinated seeds (emerged hypocotyls) suggesting that some form of chlorosis may be occurring juvenile photosynthetic tissues of the test species. ANOVA results of the data presented in figure 3.12 state that both of the variables species and Cd treatment concentration (0mM L^{-1}, 5mM L^{-1}, and 25mM L^{-1}) are considered highly statistically significant ($p < 0.001$). The interaction effect of the two variables was somewhat significant as well ($p<0.05$).

3.4 *ex* situ seedling survivorship bioassay

3.4.1 Morphological measurements

3.4.1.1 Rock Lily

The linear mixed effect modelling of the Rock lily seedling height measurements was used to assess the effects of Cd treatment and time on seedling vertical growth. Given that the measurements were conducted over the short period of six weeks (approximately six weeks

after test seedling germination), it was seen as appropriate for the RL height data to be logarithmically adjusted for the analysis in attempt to normalise the the data and satisfy statistical model assumptions.

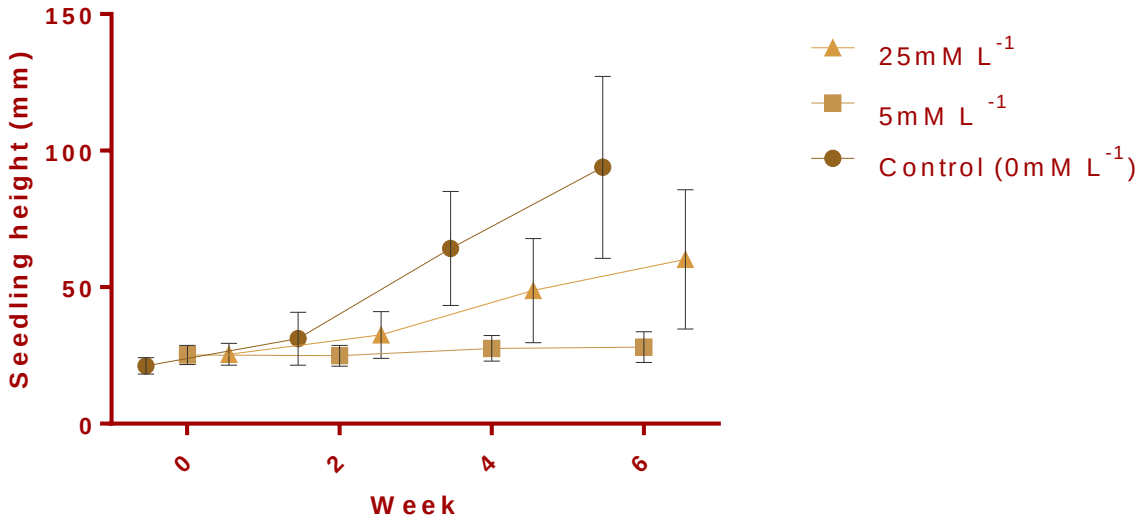

Figure 3.13: Rock Lily average seedling heights (n=360) (error bars = standard deviation)

The mean height (shoot length) of the control treatment (0mM L^{-1} Cd) Rock Lily seedlings were shown to be statistically significantly different ($p < 0.001$) to the 5mM L^{-1} and 25mM L^{-1} Cd treatments and intercept estimates based on a linear mixed effects model showed that control seedlings grew more in height over the six week period when compared to 5mM L^{-1} and 25mM L^{-1} Cd treated seedlings (Figure 3.13). Using an ANOVA analysis treatment effects were considered significant ($p < 0.001$) as well as the additional interaction effect of the week of measurement ($p < 0.001$) thus suggesting that both the concentration of the applied cadmium solution and period of time seedlings were exposed (or not exposed) to cadmium treatments affected the Rock Lily seedling height.

Rock Lily root length (Figure 3.14) followed a similar pattern to the Rock Lily height (shoot length) with regard to the effects of Cd treatments on Rock Lily size. While all treatment seedlings increased in the average root length when compared to the pre-trial measurement, seedlings treated with the 5mM L^{-1} Cd treatment experienced the least amount of root length growth on average over the 6 week period and control treatment (0mM L^{-1} Cd) seedlings had the longest root lengths on average. ANOVA analysis suggested the effect of treatment was significant as $p < 0.001$.

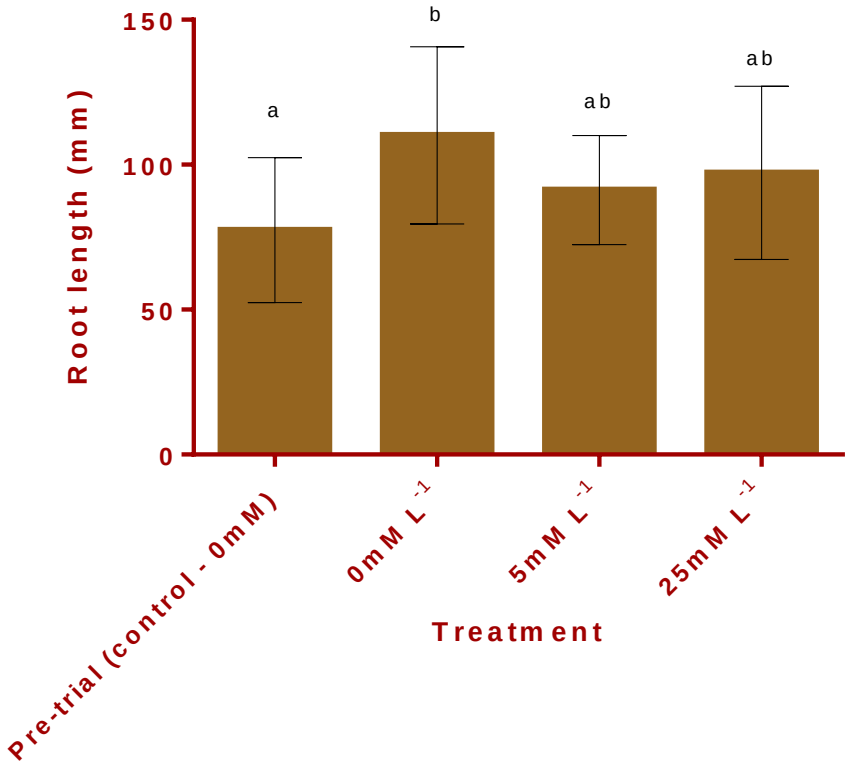

Figure 3.14: Average root length for the Rock Lily measured at week 6 (except for pre-trial measurement on week 0) (n=120) (error bars = standard deviation)

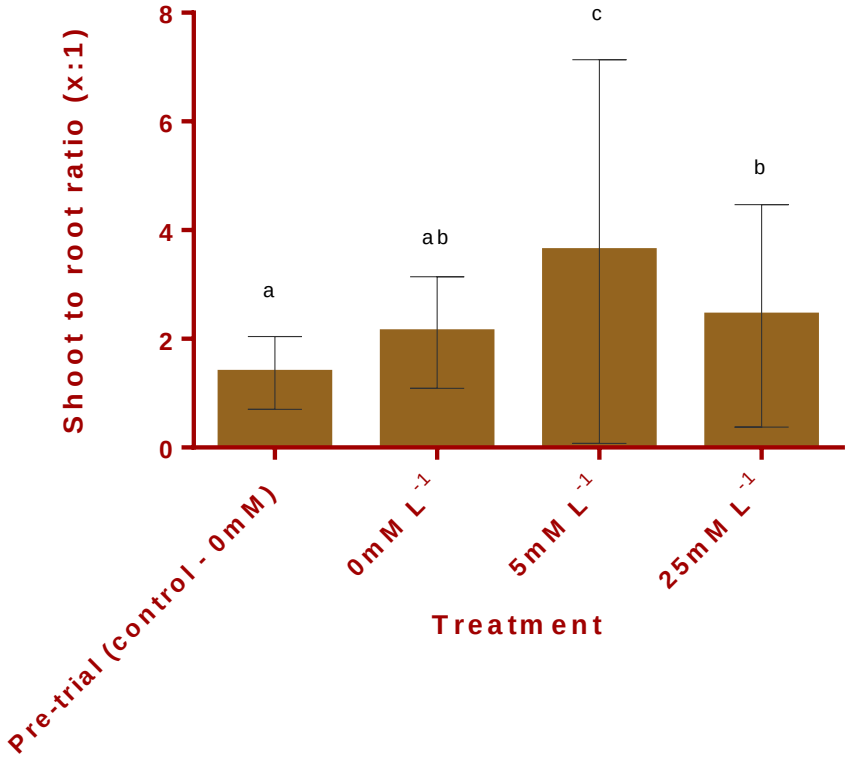

Figure 3.15: Average shoot biomass to root biomass ratio for the Rock Lily measured at week 6 (except for pre-trial measurement on week 0) (n=120) (error bars = standard deviation)

The shoot biomass to root biomass ratio (Figure 3.15) provides insight to whether the effects of cadmium have a greater effect on inhibiting (or promoting) the growth roots or the growth of shoots. The statistical significance of the average shoot biomass to root biomass of the Rock Lily seedlings was apparent after linear mixed effect modeling results showed that all treatments and measurements were somewhat statistically significant ($p < 0.1$) with regard to their contribution to causing variation in the shoot biomass to root biomass ratio in the sampled seedlings. ANOVA analysis detailed that overall variation between all treatments was statistically significant ($p < 0.001$) thus suggesting that the Cd concentration in the soil media did affect the average shoot biomass to root biomass ratio of the Rock Lily seedlings.

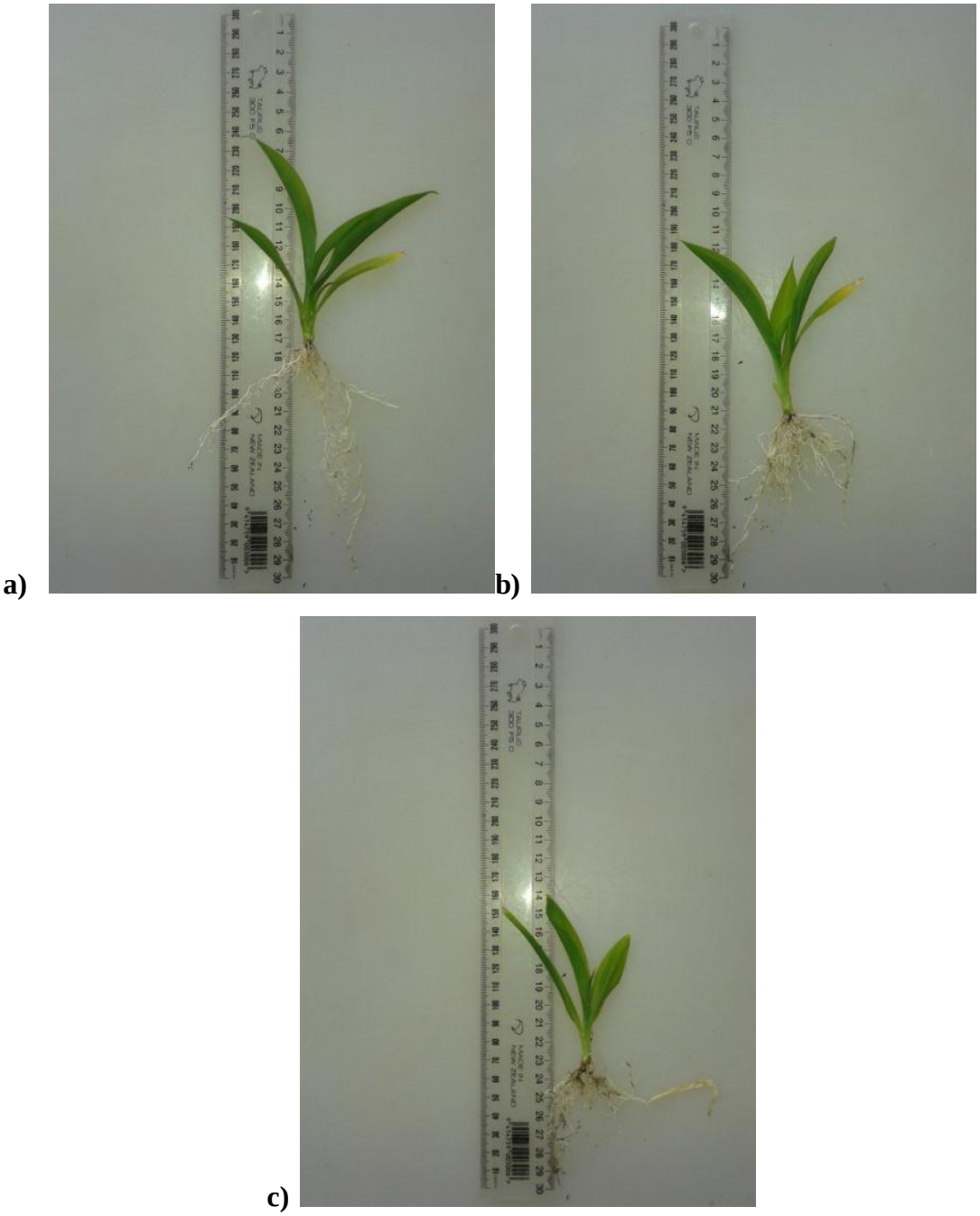

Plate 3.2: Treatment differences in seedling size of the Rock Lily; a) control (0mM L^{-1} Cd) plants, b) plants treated with 5mM L^{-1} Cd, and c) plants treated with 25mM L^{-1} Cd

Plate 3.2 highlights the evident morphological size differences between Rock Lily seedlings treated with varying concentrations of cadmium. Control (0mM L^{-1} Cd) treated seedlings showed both increased shoot length and root length in comparison with seedlings treated with 5mM L^{-1} and 25mM L^{-1} of cadmium.

3.4.1.2 Kanuka

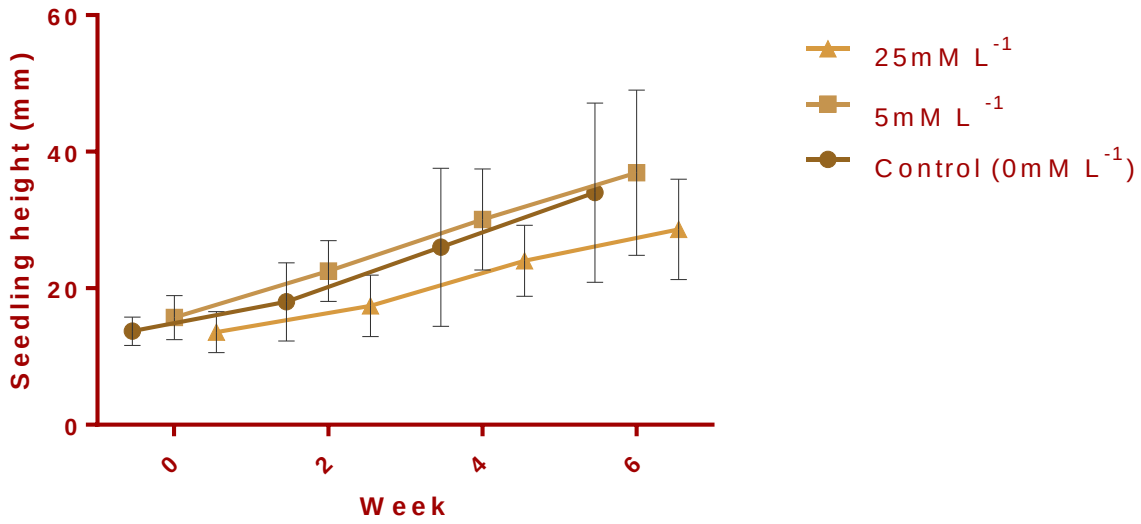

Figure 3.16: Kanuka average seedling heights (n=156) (error bars = standard deviation)

ANOVA analysis shows both treatment and week to be significant factors (both p <0.001) influencing the average seedling height of Kanuka (Figure 3.16). Linear mixed modelling shows the only measurement point at which there is no statistically significant variation between treatments occurs at week 0 (p > 0.1). Average seedling height variation due to the week of measurement and treatment effects is significant at week 2 (p < 0.1) with weeks 4 and 6 being highly significant (p < 0.001).

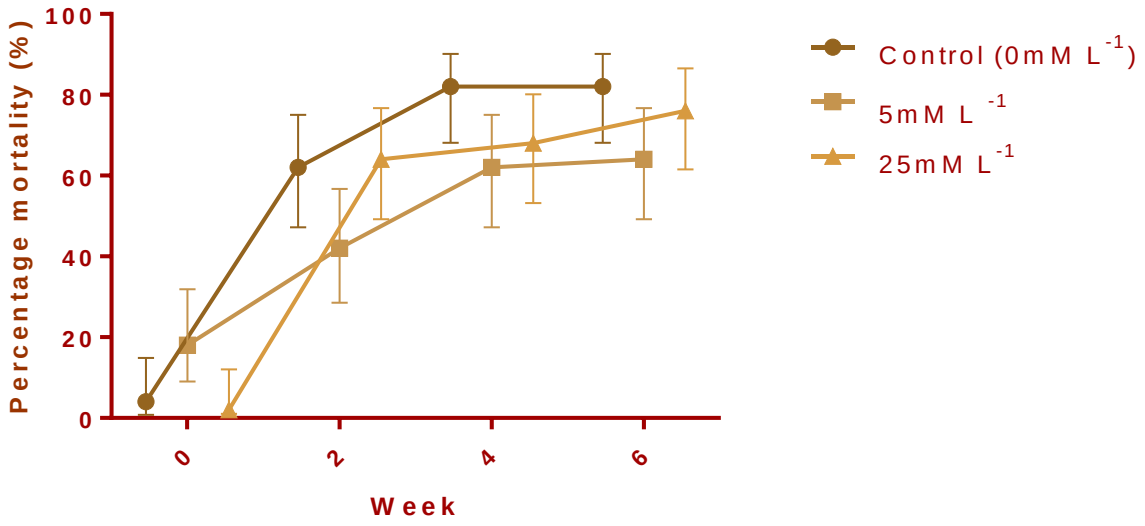

Figure 3.17: Kanuka percentage mortality rate averaged per treatment and measurement week (n=36) (errorbars = 95% confidence interval)

Meaningful statistical analysis is not possible to ascertain for the average mortality rate per treatment data of Kanuka (Figure 3.17) due to the limited sample size and the limited number of seedlings used per treatment. Any analysis of statistical output could lead to false conclusions based on the data. Visually though, figure 3.17 does display an increasing average mortality rate for Kanuka seedlings when exposed to anyone of the three treatments. The average final mortality rate for seedlings exposed to the 0mM L^{-1}, 5mM L^{-1}, and 25mM L^{-1} Cd treatments are 82%, 64% and 76% respectively.

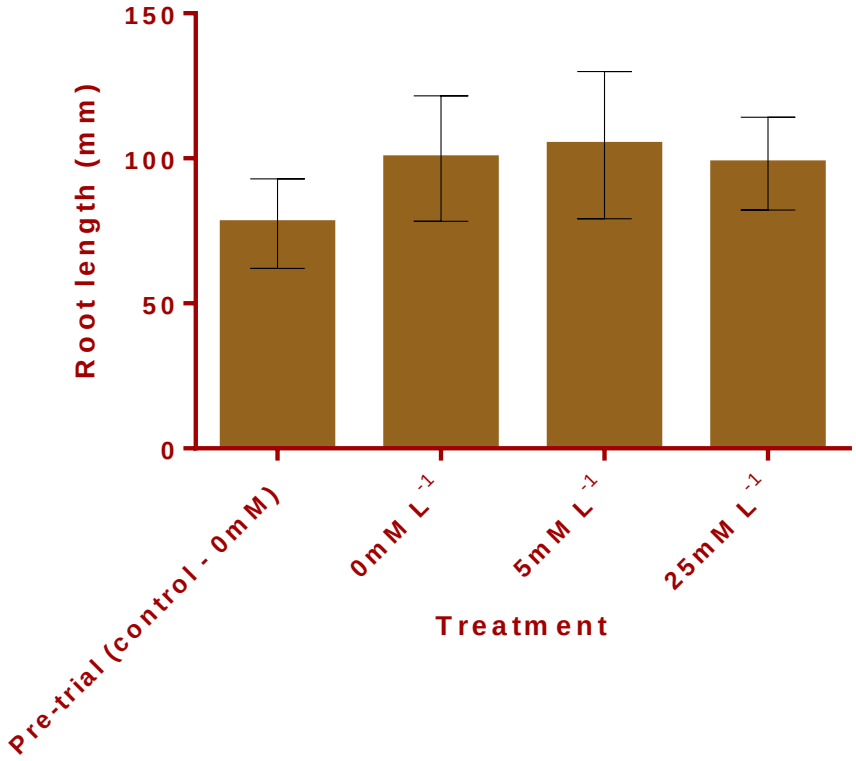

Figure 3.18: Average root length of Kanuka measured at week 6 (except for pre-trial measurement on week 0) (n=39) (error bars = standard deviation)

With regard to the average root length of Kanuka seedlings (Figure 3.18) , linear mixed effect modeling results showed that the variation in between both the pre-trial control measurement and the 5mM L^{-1} Cd treatment, and other treatments was significant ($p < 0.05$). ANOVA analysis however showed that overall variation between all treatments was not statistically significant ($p > 0.1$). TukeyHSD results suggested no pairwise variation.

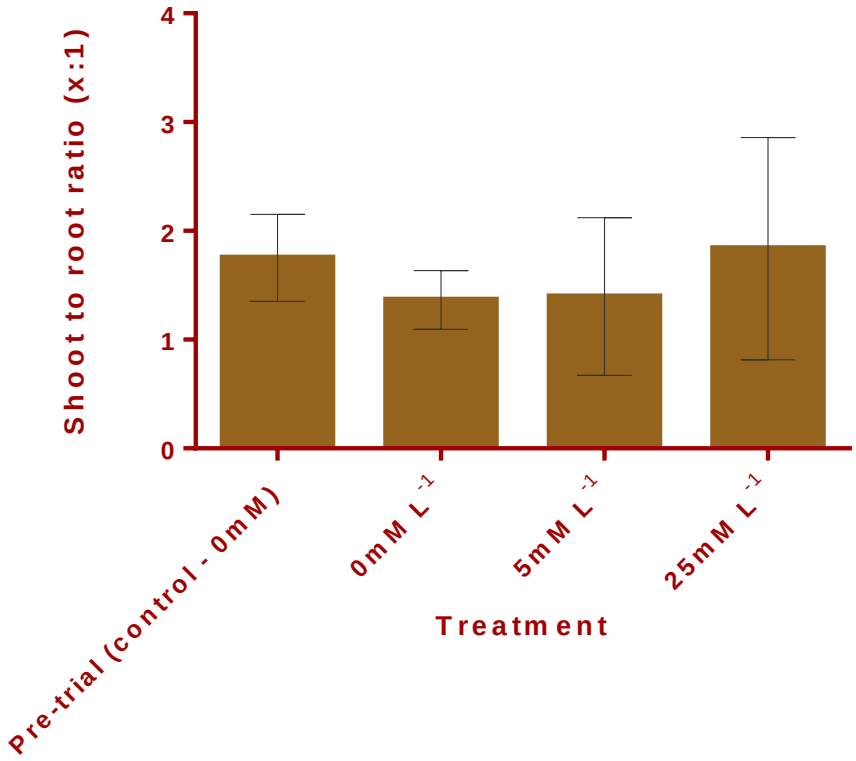

Figure 3.19: Average Kanuka shoot biomass to root biomass ratio measured at week 6 (except for pre-trial measurement on week 0) (n=49) (error bars = standard deviation)

The statistical significance of the average shoot biomass to root biomass of Kanuka seedlings (Figure 3.19) was unapparent after linear mixed effect modeling results showed that the only variation between treatments and measurements was between the pre-trial control measurement and the other treatments measured in week 6 (p <0.01). ANOVA analysis detailed that overall variation between all treatments was not statistically significant (p > 0.1) thus suggesting that the Cd concentration in the soil media did not affect the average shoot biomass to root biomass ratio. TukeyHSD results suggested no pairwise variation.

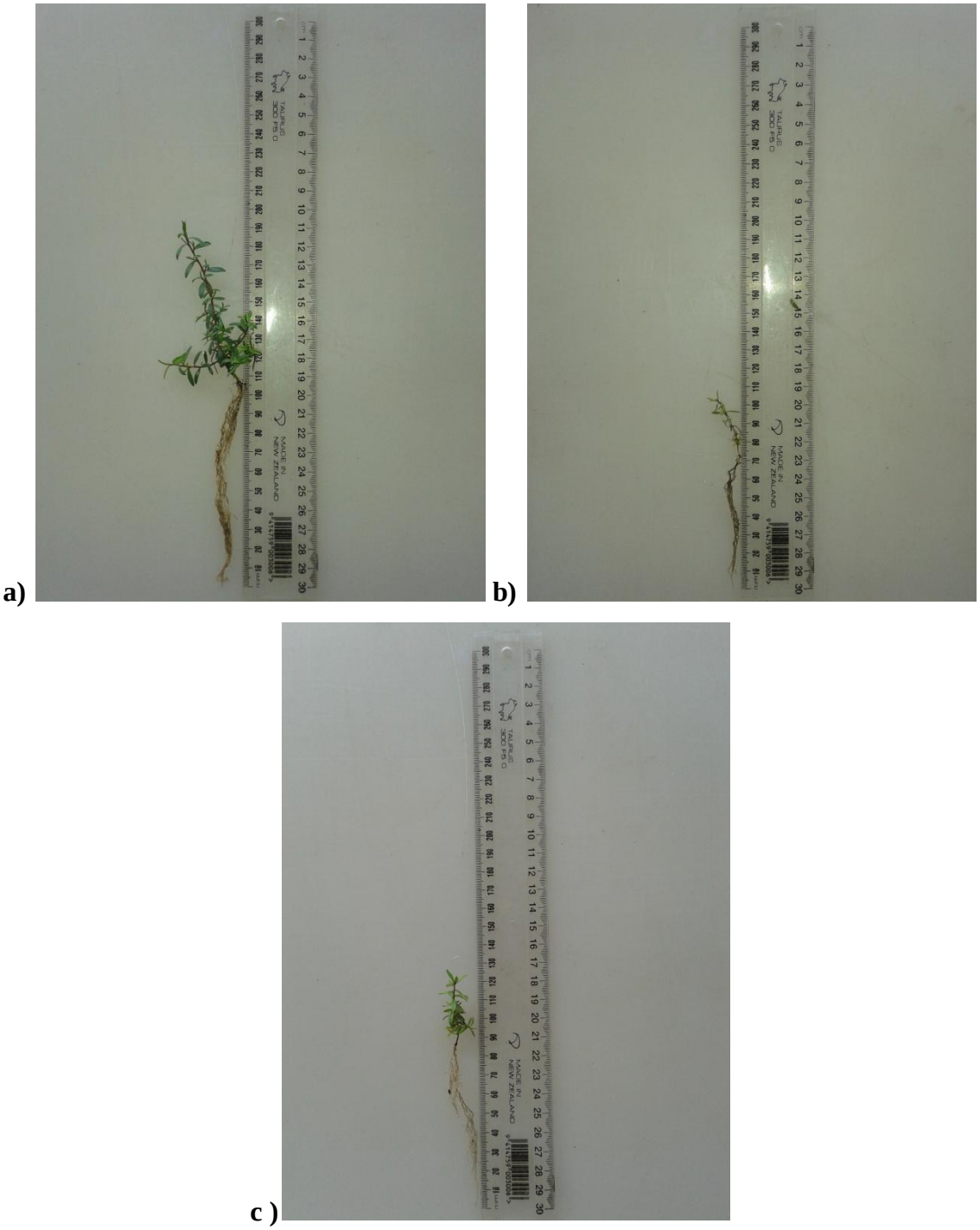

Plate 3.3: Treatment differences in seedling size of Kanuka; a) control (0mM L^{-1} Cd) plants, b) plants treated with 5mM L^{-1} Cd, and c) plants treated with 25mM L^{-1} Cd

Plate 3.2 highlights the evident morphological size differences between Kanuka seedlings treated with varying concentrations of cadmium. Control (0mM L^{-1} Cd) treated seedlings showed both apparent increased shoot length and root length in comparison with seedlings treated with 5mM L^{-1} and 25mM L^{-1} of cadmium.

3.4.2 Chlorophyll content

The average total chlorophyll content per plant (Figure 3.20) was calculated to determine any species or treatment effects with regard to Cd tolerance. The effect of species on perceived Cd tolerance appeared to be significant ($p < 0.001$) while there was no significant interaction between species and treatment ($p = 0.08985$) at the 5% level. The effects of 25mM Cd treatment was significant for Cd tolerance in both Kanuka and the Rock Lily ($p = 0.0197$ and 0.0311 respectively) in comparison to the Kanuka control treatment.

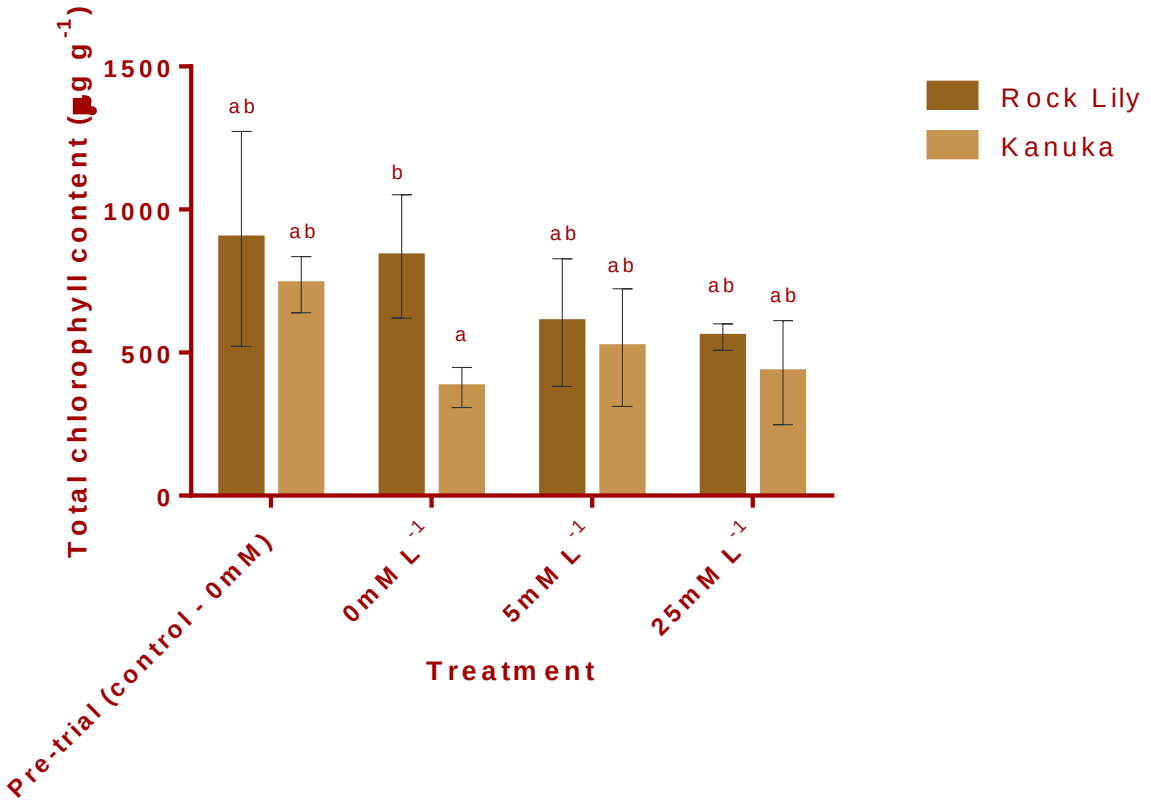

Figure 3.20: Average total chlorophyll content of Rock Lily and Kanuka seedings measured at week 6 (except pre-trial measurement) (n=72) (error bars = standard deviation)

Statistical significance of the treatment variable was $p<0.05$. A decrease in leaf chlorophyll content is also evident in photographs (Plate 3.4) detailing apparent chlorosis of the leaves of both Kanuka and the Rock Lily. Control (0mM Cd) treated Rock Lily plants as shown in plate 3.4a show no visible signs of leaf chlorosis while plants treated with 25mM Cd (plate 3.1b) show clear signs of leaf chorosis.

a)  b) 

Plate 3.4: Treatment differences in chlorosis of the leaves of the Rock Lily; a) control (0mM Cd) plants and b) plants treated with 25mM Cd

a)  b) 

Plate 3.5: Treatment differences in chlorosis of the leaves of Kanuka; a) control (0mM Cd) plants and b) plants treated with 5mM Cd

A decrease in leaf chlorophyll content in the Kanuka (Plate 3.5) is slighty less evident than seen in the Rock Lily. Control (0mM Cd) treated Kanuka plants as shown in plate 3.2a show no visible signs of leaf chlorosis while plants treated with 5mM Cd (plate 3.2b) depict slight signs of leaf chorosis.

3.4.3 Peroxidase assay

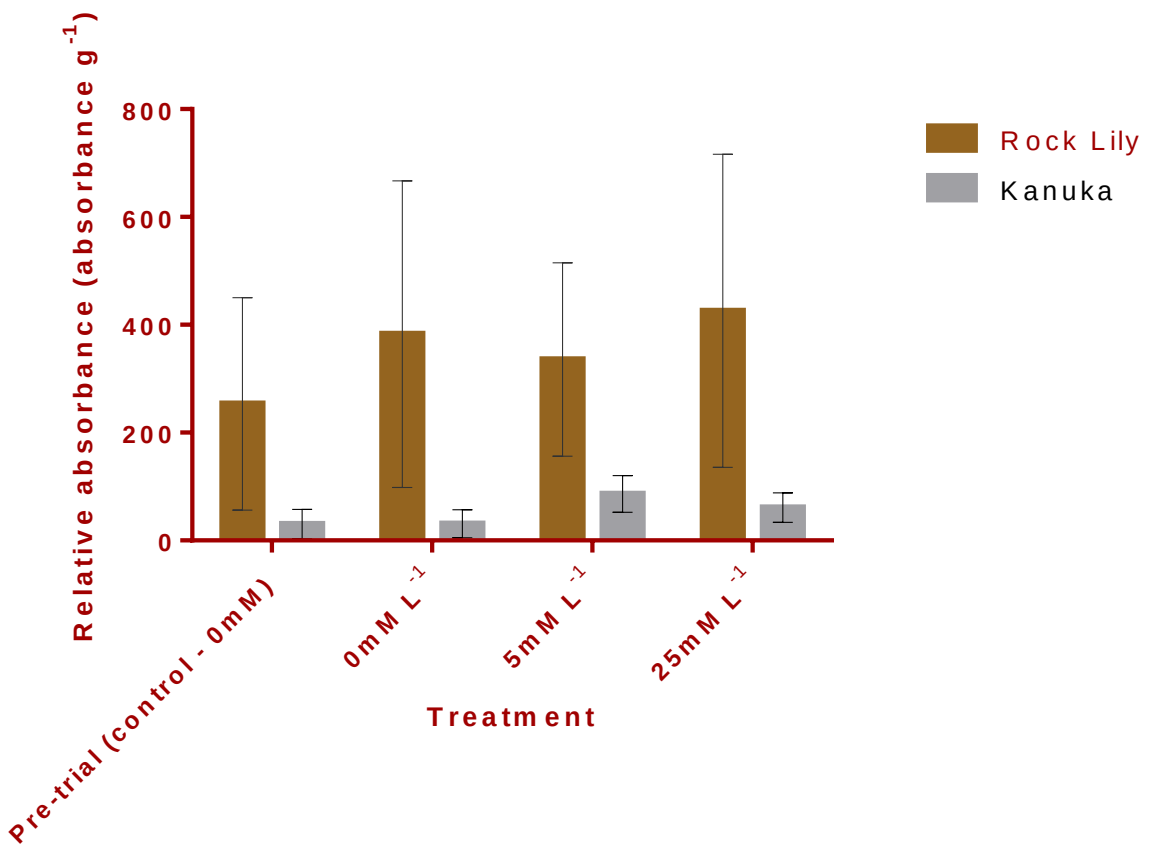

Figure 3.21: Average relative absorbance and standard deviation of peroxidase root extracts from Rock Lily and Kanuka seedings measured at week 6 (except pre-trial measurement) (n=72) (error bars = standard deviation)

Figure 3.21 clearly depicts a considerable difference in the seedling root peroxidase activity (measured in relative absorbance) of the Rock Lily in comparison to the Kanuka with the Rock Lily appearing to have significantly more root peroxidase activity than the Kanuka. This graphic depiction is supported statistically when using linear mixed effect modelling as the species variable is statistically significant ($p < 0.001$) with regard to the variation in root peroxidase activity exhibited between samples. Using species specific ANOVA analysis, the apparent variation in Kanuka root peroxidase activity between different applied treatments

appears to be significant ($p < 0.001$) while this significance in root peroxidase activity is not repeated in the Rock Lily seedlings ($p > 0.1$). TukeyHSD results suggested no pairwise variation.

3.4.4 Quantitative metal analysis

Only an extremely limited sample size was available for the quantitative metal analysis (Table 3.3) of the root system of the Rock Lily and Kanuka species therefore statistical analyses used to detect possible variations in root cadmium content due to treatment or species effects is not possible unfortunately. However the individual results of the few samples quantitatively analysed is presented below:

	0mM L^{-1}	**25mM L^{-1}**
Kanuka	1.06	270
	2.4	185
	N/A	N/A
Rock Lily	0.26	40
	0.152	28
	0.75	77

Table 3.3: Root cadmium content (mg kg^{-1}) of individual sample plants measured at week 6 (n=10)

Chapter 4: Discussion

4.1 Introduction

Determination of the *in situ* seed germination and seedling survivorship potential of New Zealand native plant species in metal contaminated sites provides evidence for considering their use in phytoremediation projects. Morphological and biochemical data from the seed germination and seedling survivorship bioassay trials provide a baseline of knowledge for this research area where at current there is minimal information. The applied biometrics give insight to the possible toxic effects of metals and the subsequent species ability to complete its life cycle starting from seed germination through to maturity and reproduction. (Ali et al., 2014). From a management perspective, phytoremediation projects using plants capable of forming self-sustaining populations are desirable due to less labour and supervisional requirements compared to short-term planted remediation projects. Also depending on the species metal tolerance mechanisms (e.g exclusion or accumulation), continuous remediation and ecosystem services may be bestowed upon a contaminated environment. Given that an environment may not be contaminated exclusively by a single metal element but possibly multiple, a species capacity to display co-tolerance to more than one metal element is advantageous.

4.2 *in vitro* seed germination bioassay

4.2.1 Germination rate

All species used in the As seed germination bioassay displayed an overall trend of a decrease in total germination as the concentration of aqueous As treatment increased. Contradictory to this trend, the total germination rate of all test species exposed to the 5mM L^{-1} As was equal to or greater than the control group except for Karamu. Mahdieh *et al* (2013) reported similar findings when exposing two wheat cultivars to increasing concentrations of As ranging from 0mg L^{-1} (control) to 30mg L^{-1} as a negative correlation was observed between response and treatment variables. Similarly low arsenic concentrations of 0 – 2.5mg L^{-1} were shown to increase the germination rate, relative shoot length and biomass in the two cultivars (Mahdieh et al., 2013). This apparent stimulation of germination through exposure to a low-dose of

arsenic is putatively caused by an overproduction of reactive oxygen species resulting in a slightly enhanced level of oxidative stress, a factor contributing to germination stimulation (Kranner and Colville, 2011).

The cadmium seed germination bioassay results in the present study were similar to the As bioassay results given that all species displayed an overall trend of a decrease in total germination as the concentration of aqueous Cd treatment increased. This supports hypothesis one as stated in section 1.11.3. The observed increase in total germination rate in response to the application of low concentrations of As was not replicated to the same extent in the Cd bioassay however as only the Frosted Curl, Karamu and Rock Lily seeds were stimulated by low concentrations of Cd (0.25mM L^{-1} and 5mM L^{-1}). Correspondingly, no germination stimulation (relative to control treatments) was detected for soybean seeds exposed to soils containing 0mg Kg^{-1} to 1600mg Kg^{-1} treatments of Cd in Luan *et al* (2008). Low concentrations of As also failed to stimulate total germination rates in Ali *et al* (2014) highlighting the discrepancies between various published results and indicating the function of complex biochemical reactions occurring in seeds exposed to metal contaminants (Luan et al., 2008). Likewise, all test species in the Cd seed germination bioassay failed to germinate in 100µM Cd solutions in results from the current study while the same solute concentration stimulated oil-seed rape germination relative to all other applied treatments in Ali *et al* (2014). The Rock Lily was the only test species to germinate when exposed to the 50mM L^{-1}Cd treatment (~ 16% final average germination rate) with an equal germination rate as displayed under control conditions (0mM L^{-1}).

Based on these results, the Rock Lily was selected as the best representative species that may feature some mechanisms of tolerance towards Cd contamination. Contrarily, Kanuka was selected as a representative species of intolerance and sensitivity to cadmium contamination based on the species high average germination rate (~76%) under control conditions (0mM L^{-1}) but its inability to germinate in aqueous Cd concentrations greater than 0.25mM L^{-1}. Both species were therefore selected for trial in the As seed germination bioassay, and the seedling survivorship bioassay due to the contrasting tolerance and intolerance to Cd thus allowing hypotheses two to four to be tested.

For the two species of particular interest in this study; Kanuka and Rock Lily, the total seed germination rate appeared to be greater in the As aqueous solution compared to Cd (Figures

3.6 and 3.7). The results of studies by Cao *et al* (2007) and Luan *et al* (2008) are somewhat contradictory to these results as As was shown to be of greater toxicity than Cd to wheat seedlings and soybean seeds respectively. Aqueous As and Cd solutions had similar root length inhibition thresholds of 0.33mM, but the inhibition rate was greater than 95% for soybean roots when the treatments concentrations were 3.3mM and 100mM for As and Cd, respectively, a 30 fold difference between the two elements (Cao et al., 2007). All of the previously mentioned studies; Cao *et al* (2007), Mahdieh *et al* (2013), and Luan *et al* (2008), use the same form of arsenic as this study which is known as sodium arsenate, which dissociates into arsenate ions (As(V)) in solution.

The average seed germination rate of the Rock Lily exposed to both Cd and As partially supports the fourth hypothesis as stated in section 1.11.3 as the species successfully germinated when exposed to 50mM L^{-1} concentration of both metal types. As shown in figure 3.6, exposed to 50mM L^{-1} concentration of As and Cd the Rock Lily seed germination rates were ~13% and ~16% respectively. The average germination rate of the seeds exposed to the control treatment was ~18.3% thus giving the impression that the metal type and the applied treatment concentration are partially limited with regard to their effect on average seed germination rate. This would suggest that the Rock Lily's seeds have the capacity to germinate in soils considerably contaminated with As and/or Cd and/or other metals. Statistically this idea is moderately contradicted though as ANOVA results state that the metal type, treatment concentration and interaction coefficient are highly significant ($p<0.01$) considering these parameters effects on average seed germination rate.

The result of Kanuka seed germination bioassay also partially oppose the predictions stated in hypothesis four as the seeds of the Kanuka (Figure 3.7) did not germinate in Cd concentrations greater than 0.25mM L^{-1} Cd (average germination rate = ~ 3.3%) but germination did occur upon seed exposure to 5mM L^{-1} of aqueous arsenic (average germination rate = ~ 20%). Kanuka appears to have greater tolerance to As than it does to Cd thus suggesting that the species may succeed to germinate in an As contaminated soil but is more probable to fail in Cd contaminated soils in comparison.

4.2.2 Peroxidase activity

Peroxidase results from both the As and Cd seed germination bioassays (Figure 3.8) visually indicate a negative correlation between increasing treatment concentration and the average seed peroxidase activity in all test species. This supports the third hypothesis created for the study. Statistically, the species, metal type, and treatment concentration variables were all very significant (p<0.001) as well as the interaction effect of all three variables. This suggests that species may have had pre-existing variations in peroxidase activity but exposure to increasing concentrations of Cd or As induced responses of differing magnitudes in the two species therefore indicating varying tolerances to Cd or As in Rock Lily and Kanuka. Evidence of reduced peroxidase activity due to Cd exposure is documented with studies indicating that cells may not be capable of removing and moderating H_2O_2 concentrations (a potentially toxic molecule if accumulated) especially if the activity of other antioxidants like catalase is compromised (Sandalio et al., 2001).

When comparing the Rock Lily seed germination and peroxidase activity results, there appears to be some correlation between these two response variables. The species has an average germination rate for all As treatments equal to 22.16% and using the same analysis, Cd treatments equal 10% germination. Correspondingly, average peroxidase activity in As treated seeds is higher (84.5 absorbance g^{-1}) than Cd treated seeds (29.0 absorbance g^{-1}) (Figure 3.8). This suggests the higher activity rate of peroxidase reduces the accumulation of ROS and the effects of oxidative stress on the test plant and contributes to conferring some metal tolerance to the species.

4.2.3 Seed coat

The varying morphologies of the various test species seed coats may contribute to a species level of tolerance and germination rate when exposed to metals like As and Cd. Evidence suggests that seed coat permeability to metals is related to morphology and metal translocation into the embryo is dependent on metal type (Kranner and Colville, 2011). In the present study, Rock Lily and Karamu displayed the most tolerance to Cd and As treatments and both species feature hard seed coat, ~2mm (spherical) and ~6mm (oval) in length respectively. In comparison; Kanuka, Manuka, Frosted Curl, and Koromiko, all feature seemingly soft seed coats with all seeds being < 1mm in size and all three species were very sensitive to the presence of Cd in solution.

The dynamics of metal – seed coat morphology interactions have been studied for maize (*Zea mays*) where results showed Cd and Pb were bound and restricted to the cells of the seed coat whereas nickel was transported across the seed coat into the endosperm and scutellum cells (Seregin and Kozhevnikova, 2005). Once inside the seed it is assumed that antioxidants are activated to mitigate the impacts of metal induced ROS but unfortunately there is very little data examining the relationships between metal exposure and antioxidative responses (Kranner and Colville, 2011). Isolated plant embryos without seed coats have been shown to be much more sensitive to metals than intact seeds, reinforcing that the seed coat is a necessary barrier to metals and other abiotic stressors (Kranner and Colville, 2011).

4.3 *ex situ* seed germination bioassay

4.3.1 Germination rate

Similarly to the laboratory based seed germination bioassay, the glasshouse equivalent of the study displayed seed germination trends analogous to the laboratory based study (Figure 3.9). Species selection for this experiment was based on germination success in the Cd seed germination bioassay with a total of six species tested. Results indicated a negative correlation between increasing treatment concentrations starting at the 5mM L^{-1} Cd treatment and the average seed germination rate in all test species except Karamu. The 5mM L^{-1} Cd treatment induced the highest germination rate of all the applied treatments in all species except Karamu thus supporting the results of the laboratory based bioassay indicating that low concentrations of metals in aqueous or soil media can effectively stimulate seed germination.

All test species except for Koromiko germinated when exposed to the South Waikato field soil treatment though the germination rate was generally lower in comparison to the other test treatments. The field soil treatment had average Cd and As concentrations of 1.7 and 8.8 mg kg^{-1} respectively which re-calculated and converted to mM Kg^{-1} equals 0.015mM Kg^{-1} Cd and 0.11mM Kg^{-1} As. The concentration of Cd used in the 5mM L^{-1} treatment used in this study expressed as mM Kg^{-1} or mg Kg^{-1} is equal to 2.5mM Kg^{-1} or 281mg Kg^{-1} respectively. Provided that the average Cd concentration for New Zealand agricultural soils is 0.32mg Kg^{-1} (McDowell et al., 2013), the results suggest that all of the test species can theoretically germinate in NZ agricultural soils containing cadmium. The presence of other elements in the

field soil sample including phosphorus, magnesium, and zinc, as well as the unsterile condition of the sample may explain why Koromiko failed to germinate when exposed to this sample but successfully germinated in the 5mM L^{-1} treatment.

4.3.2 Chlorophyll content

The chlorophyll content of the emerging hypocotyls and juvenile leaves was analysed to determine if any treatment concentration effects were disturbing the photosynthetic health and function of recently germinated seeds. ANOVA analysis showed both the species and treatment variables to be very significant ($p<0.001$) as well as the interaction effect to a lesser extent ($p<0.05$) thus suggesting that species had differing average total chlorophyll content and that treatment concentration affected chlorophyll content. The significance of the interaction effects suggests that species may have had pre-existing total chlorophyll content variations but exposure to increasing concentrations of Cd induced responses of differing magnitudes in different species. All test species displayed a decrease in the total chlorophyll content relative to the control treated seeds for each species thus suggesting that chlorosis to a certain degree may be occurring as displayed in plates 3.4 and 3.5. In addition, these results support hypothesis three. Li Chun-Xi *et al* (2006) completed research assessing the effects of As on seed germination and physiological parameters including chlorophyll content in wheat seedlings (*Triticum aestivum*) with similar results to the present study. As seeds were exposed to increasing concentrations of As solution ranging from 0 to 20mg Kg^{-1}, the total germination rate decreased ~5% relative to controls while the total chlorophyll content decreased from 1.22mg g^{-1} fw (exposed to 0mg Kg^{-1} As) to 0.82mg g^{-1} fw (exposed to 20mg Kg^{-1}) , a 32.5% decrease relative to the control (Li et al., 2007). Possible cytological explanations for the cadmium stress induced decrease in leaf chlorophyll content has been attributed to a decrease in leaf chloroplast abundance and density with no apparent changes to the variety of pigments or pigment concentrations of individual chloroplasts (Baryla et al., 2001; Sandalio et al., 2001)

4.4 *ex situ* Seedling survivorship bioassay

The most polarised results from this experiment are the two differing mortality rates of the Rock Lily and Kanuka. No seedling mortalities were recorded for the Rock Lily while the average final mortality rate for Kanuka seedlings exposed to the 0mM L^{-1}, 5mM L^{-1}, and

25mM L^{-1} Cd treatments are 82%, 64% and 76% respectively (Figure 3.17). Kanuka appeared to be far more susceptible to abiotic stressors, possibly high temperatures and/or excessive sun exposure, ultimately leading to a high mortality rate especially when compared to the Rock Lily. Unfortunately, there are no publications detailing the physiology functions like photosynthesis rates and stomatal conductance or the apparent susceptibility of Kanuka to abiotic stress with most websites describing as it as hardy and tolerant species to most climates and sunlight intensities experienced in New Zealand (Whitehead et al., 2004). In contrast to the stimulation of seed germination when exposed to low Cd treatments, Rock Lily seedlings were the most inhibited with regard to seedling height, root length, and peroxidase activity when exposed to the 5mM L^{-1} Cd treatment (Figure 3.13, 3.14, and 3.21). Inhibition of plant biometrics like shoot and root length has been shown to occur under conditions of metal stress and the subsequent interference with auxin homeostasis (Hu et al., 2013).The results from Hu *et al* (2013) detailed a decrease in hypocotyl and root length, and total chlorophyll content with a corresponding decrease in IAA (auxin) content and increase in IAA oxidase activity, an enzyme responsible for IAA catabolism (Hu et al., 2013). Shoot to root (S:R) biomass ratio provides information to whether plant growth has been partitioned in favour of photosynthetic metabolic shoot tissues or mineral and nutrient absorbing root tissues (Mokany et al., 2006). S:R biomass ratios must be analysed alongside shoot and root length data for correct interpretation. The increased S:R ratio in Rock Lily relative to the control treatment suggest that root length growth was inhibited by increasing concentrations of Cd but Cd treatments had no apparent effect Kanuka S:R ratios.

Interestingly, Kanuka seedlings exposed to the 5mM L^{-1} Cd treatment appeared to have been the least inhibited with regard to seedling height, root length, shoot to root biomass ratio, total chlorophyll content and peroxidase activity. This possibly suggests that Kanuka may be susceptible to Cd toxicity as a seed but may have greater tolerance as a growing seedling. Very few papers detail growth promotion of seedlings exposed to Cd treatments, Kumar *et al* (2008) showed peanut (*Arachis hypogaea*) seedling shoot and root growth to be promoted when exposed to100µM L^{-1} Cd when compared to all other applied Cd treatments (50 - 300µM L^{-1}). The peanut seedling peroxidase activity was suppressed however when exposed to 100µM L^{-1} Cd relative to all other applied Cd treatments, a trend also observed in the Rock Lily peroxidase activity results in the present study (Kumar et al., 2008).

Interesting results were compiled of the quantitative metal analysis of the two test species root systems. Statistical analysis of the results is impossible due to the small sample size but Kanuka appears to have absorbed 227.5mg Kg^{-1} (average of 2 test samples) from the 25mM L^{-1} (1405mg Kg^{-1}) treated soil media with 48.3mg Kg^{-1} (average of 3 test samples) absorbed by the Rock Lily. This suggests that the Rock Lilies apparent tolerance to Cd may be conferred through the physiological ability to exclude Cd from entering or ability to efflux organic acids from the root system that form Cd chelates. High molecular weight organic acids released from roots in exudates have been shown to bind to metals like Cd thus functioning as selective filters of heavy cations and reducing the flux of Cd into root cells (Morel et al., 1986). Lettuce (*Lactuca sativa*) plants exposed to increasing concentrations of Cd (ranging from 0µM L^{-1} to 100µM L^{-1}) were shown to release increasing concentrations of asparagine (a known chelation agent of Cd) in root exudates (Lea et al., 2007; Morel et al., 1986). Likewise, in a variety of plants including maize (*Zea mays*) and wheat (*Triticum aestivum*) aluminium tolerance by exclusion is mediated by the aluminium activated release of organic acid anions such as oxalate, citrate, or malate which complex with the Al^{3+} cation in the rhizosphere and prevent its entry into the root apex (Kochian et al., 2002).

A lack of physiological exclusion mechanisms may explain why Kanuka absorbed considerably more Cd from the soil media over the same six week trial period. Vast documentation exists for the physiological existence of Cd chelation and sequestration agents such as phytochellations and metallothioneins but given the high mortality rate (Figure 3.17) and decreased chlorophyll content (Figure 3.20), these may not be constitutively expressed in high concentrations or may be suppressed upon metal exposure in Kanuka. Though Kanuka did uptake greater concentrations of Cd from the soil media than Rock Lily, the species apparent toxicity to Cd discounts it from being considered as a possible hyperaccumulator. In maize leaves of plants exposed to Cd treatments, the total chlorophyll and phytochelatin oligomer content generally indicated a negative correlation with increasing Cd treatments and subsequent decreased water content (Drazkiewicz et al., 2003). Although, peroxidase activity response to varying Cd treatments was significant and peroxidase activity was shown to temporally increase from week 0 to week 6 (Figure 3.21) as shown in other species like peanut (*Arachis hypogaea*) (Kumar et al., 2008), the low root peroxidase content in comparison to the Rock Lily highlights that Kanuka may constitutively contain low concentrations of peroxidase and possibly other detoxification mechanisms thus leading to the species apparent vulnerability to experiencing Cd induced metal toxicity. In rice (*Oriza*

sativa L.) seedling leaf tissue, plants treated with a 100µM L^{-1} Cd solution appeared to have reduced peroxidase activity and correspondingly increased H_2O_2 concentrations compared to control plants (Wu et al., 2014). This suggests that the uptake of metals like Cd may disrupt the homeostasis of peroxidase function resulting in an accumulation of ROS like H_2O_2.

In the context of the Cd content of New Zealand agricultural soils, it is probable that Rock Lily or Kanuka seedlings would be capable of surviving and functioning in these soils. Although an actual field sample containing Cd was not used in this seedling survivorship bioassay, the lowest Cd treatment used in this experiment was 5mM L^{-1} or 281mg Kg^{-1} which far exceeds the 0.32mg Kg^{-1} average New Zealand agricultural soil Cd content or the maximum Waikato soil Cd content of 2.00mg Kg^{-1} (McDowell et al., 2013; Taylor et al., 2010). In relation to few published studies about New Zealand native plants uptaking non-essential metals like Cd from soils, this study only partially supports existing data. Using data from Craw *et al* (2007) and Hahner *et al* (2012); Karamu, Black Matipo and Manuka were all selected for this study with only Karamu indicating a seed germination tolerance to Cd and As solutions.

Statistical evidence did not support the theory of a Cd induced negative dose-response relationship with morphological and physiological responses in Kanuka in relation to hypotheses two and three. This may have occurred due to the fact that the cohort of Kanuka seedlings experienced a much higher mortality rate when compared to the Rock Lily which resulted in a limited number of seedlings surviving the 6 week trial period after which time destructive sampling of seedlings for biomass and root length measurements was completed. The limited number of seedlings available for destructive sampling meant that the sample size available for the statistical analyses of shoot and root biomass, root length, and root peroxidase activity was too small thus limiting the power and accuracy of the statistical methods employed. In particular, the toxicity symptoms visually apparent in the Kanuka seedlings exposed to the two cadmium treatments (5mM L^{-1} and 25mM L^{-1}) affected root growth to such an extent that the quantitative metal analysis of these root samples was forfeited due to an inability to comply with minimum sample biomass requirements (0.5g) set by Hill Laboratories.

4.5 Limitations to this study and possible improvements for further research

With regard to the seed germination bioassay, the statistical strength of the binomial generalised linear models used to model and analyse the seed germination rate of the different species in response to As and Cd treatments of varying concentrations would have been increased had the number of seeds used to each replicate been increased to approximately 50 seeds instead of the 10 seeds used per replicate as in this study (Mahdieh et al., 2013; Yoon et al., 2015). Other studies like Luan *et al* (2008) have used 10 seeds per replicate but using more seeds per test replicate would enable the binomial GLM to detect the effects of the dependent variables on the seed germination rate with greater accuracy and resolution.

The high average mortality rate per treatment apparent for Kanuka in the seedling survivorship bioassay prevented some statistical analysis due to the limited sample size and the limited number of seedlings used per treatment. The sensitivity of the Kanuka seedlings to abiotic stresses was unexpected and it can only be assumed that the stress was due to high glasshouse temperatures (temperatures up to 41°C were recorded for short periods of time) and the subsequent excessive dehydration of soil moisture content. Likewise to the seed germination bioassay data, binomial GLM would be a useful analytical method to detect independent variable effects on the average mortality rate but it would require 40 – 50 samples within each replicate ideally. However given the unexpected sensitivity of Kanuka to apparent abiotic stress meant that the extra replicates used in the study were insufficient additions to form an acceptable sample size.

4.6 Conclusion

Considering the overall results of the seed germination and seedling survivorship bioassays, experimental units (either seeds or seedlings) showed a negative dose-response relationship between the (independent) variable and the cadmium or arsenic metal treatment variable. Notable examples of this occurrence were displayed in both the Rock Lily and Kanuka seedlings upon exposure to increasing concentrations of Cd in soil media (Figures 3.13 and 3.16). Similar dose-response studies testing biometrics like seed germination rates, seedling growth, and peroxidase activity in plants have yielded similar results (Kuriakose and Prasad, 2008; Sandalio et al., 2001).

Of the 16 species tested in the *in vitro* Cd seed germination bioassay, 8 species failed to germinate entirely while 5 species failed to germinate at Cd concentrations >0.25mM L^{-1}

including Kanuka. The three most tolerant species to Cd; Rock Lily, Karamu, and Frosted Curls, and the most intolerant, Kanuka, were then tested in the As seed germination bioassay. Rock Lily and Kanuka seed germination rate and peroxidase activity results indicated responses differing in magnitude to Cd and As treatments therefore indicating species specific tolerances to Cd and/or As. Variation in seed coat morphology and protein composition may explain some of the apparent tolerances and sensitivities.

Ex situ seed germination bioassay data indicated the capacity for the test species to germinate in soil media obtained from a Cd contaminated field site in South Waikato. Results showed that all test species except for Koromiko germinated and grew in the field soil sample indicating that at current average Cd content, these species would be capable of germinating and growing in New Zealand agricultural and horticultural soils. However, the leaf total chlorophyll content of the germinated seedlings did decrease in response to increasing Cd treatment concentrations (5mM L^{-1} and 25mM L^{-1}) possibly due to a decrease in chloroplast abundance and density induced by Cd toxicity.

Seedling heights, root length, total chlorophyll content and peroxidase activity were all generally inhibited by exposure to increasing Cd concentrations in the *ex situ* seedling survivorship bioassay. Interestingly, the experimental units exposed to the 5mM L^{-1} Cd treatment contradicted the general negative dose-response trend with regard to some biometrics. Rock Lily seedling height and root length was the most inhibited by the 5mM L^{-1} Cd treatment while Kanuka seedling height, total chlorophyll content (at week 6) and peroxidase activity was promoted when exposed to the same treatment.

While acknowledging some of the negative dose-response results exhibited by the New Zealand Rock Lily, the species ability to germinate at all concentrations of As and Cd treatments in the seed germination bioassay, and apparent lack of mortality and high rate of peroxidase activity in the seedling survivorship bioassay suggest that this species may have the physiological mechanisms conferring a capacity to function and survive in a metal contaminated environment. The average Rock Lily root tissue Cd content was 48.3mg Kg^{-1} compared to 227.5mg Kg^{-1} in Kanuka suggesting the Rock Lily's tolerance may be conferred through the ability to exclude or reduce the absorption rate of Cd. On the contrary, Kanuka may lack these same mechanisms as the species suffered from low germination rates and peroxidase activity when exposed to Cd treatments. Kanuka's high seedling mortality rate in

response to Cd and control (0mM L^{-1}) treatments may suggest the species has high sensitivities to abiotic stressors other than metals as well.

4.7 Future areas of research

Field trials are often used in parallel to pot trials as results in the field can be different from those in the laboratory or greenhouse due to the uncontrollable, heterogeneity of soil contamination at field sites as well as the tendency for plant roots to intentionally avoid high metal concentration spots in the field (Ali et al., 2013; Zhivotovsky et al., 2011). For example, Kim et al (2010) compared Cd, Cu, Pb, and zinc uptake in pot trials and field trials using *Echinochloa crus-galli* (barnyard grass) and *Abutilon avicennae* (Indian mallow) plants. Virtually all plants in the field trial contained lower tissue concentrations of metals when compared to pot trial plants except for field trial barnyard grass which contained higher Cu concentrations than pot trial barnyard grass (Kim et al., 2010).

The vast majority of phytoremediation studies focus on a species response(s) to measureable parameters at the juvenile or mature stages of a species lifecycle. A small number of studies also focus on the germination stage of a species lifecycle. However, there are very few publications detailing the effects of metal contaminants on the entire lifecycle of a species including a possible subsequent generation. Parent plant to seed translocation of non-essential metals like Cd has been shown in rice (*Oriza sativa*) and Indian mustard (*Brassica juncea*) via the phloem or xylem vascular systems. This process is ultimately governed by the occurrence and flux of root to shoot translocation of metal(s) (Kranner and Colville, 2011). Research conducted at this scale would be extremely beneficial to providing evidence that phytoremediation projects could be comprised of self-sustaining species within a self-sustaining ecosystem.

If a similar research project using the experimental structure and methods of the seedling survivorship bioassay was started, it would be of interest to include Karamu and Flax as the documented results of these species in the seed germination bioassay highlight the possibility that the species may be tolerant to soil metal contaminant. Unfortunately, given the time constraints of the study and the inconclusive nature of some of the preliminary trials and results, the apparent tolerances of these two species went unrecognised and the species were not included in the seedling survivorship bioassay.

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Appendix 1

Stock solution preparation:

Cadmium stock solution:

Salt:	**g L^{-1}:**	**mol L^{-1}:**
$CdCl_2.xH_2O$	200.00	1.78

Arsenic stock solution:

Salt:	**g L^{-1}:**	**mol L^{-1}:**
$Na_2HAsO_4.x7H_2O$	133.29	1.78

Stock solutions were created fresh when required for experimental in batches of 10mL. These salts were dissolved into nanopure H_2O and then stored at room temperature (~25°C).

KPO_4 buffer preparation:

Stock salt preparation:

Salt:	**g L^{-1}:**	**mol L^{-1}:**
K_2HPO_4	174.18	1
KH_2PO_4	136.09	1

Aqueous salt solutions of K_2HPO_4 and KH_2PO_4 were created in batches of 500mL. These salts were dissolved into nanopure H_2O and then stored at room temperature (~25°C). Both salt solutions were used to create the KPO_4 buffer solution.

Buffer preparation:

For preparation of 0.1M KPO_4 buffer at 25°C with a pH of 6.59:

Aqueous salt:	**mL:**
K_2HPO_4	~37.9
KH_2PO_4	~62.1

Stock solutions were created when required for experimental use in batches of 100mL. These aqueous salt solutions were mixed together using a Thermolyne Cimarec 1 Magnetic Stirrer and the pH was reviewed using an Orion 3 Star benchtop pH meter. The 0.1M KPO_4 buffer was stored in refrigeration (~4°C).

Appendix 2

Preliminary enzyme activity assay:

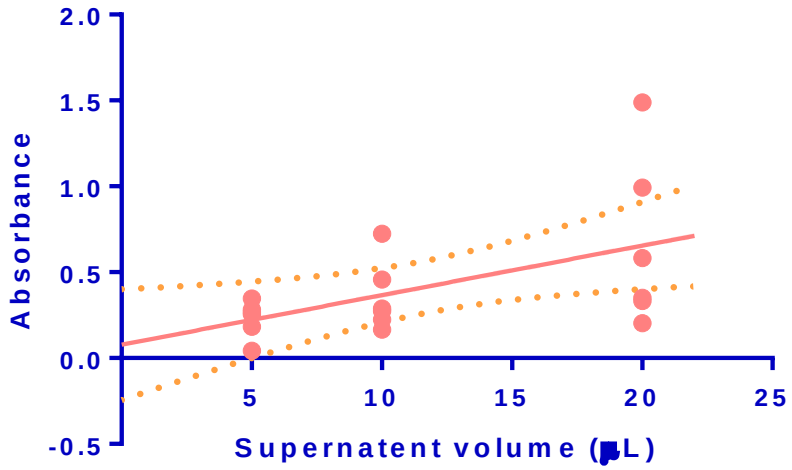

Figure A2.1: Preliminary enzyme activity assay results with fitted linear regression and 95% confidence interval band (n=18)

A preliminary enzyme activity assay analysing the proportional responses of peroxidase activity to proportional increases supernatant volume within the reaction mixture was completed prior to the experimental peroxidase assay comparing treatment effects to ensure that with regards to the experimental peroxidase assay, that reactants within the reaction mixture were not at a saturation point thus their abundance within the reaction mixture would allow theoretically limitless reactivity between peroxidase enzymes and the reactants.

Appendix 3

Seed germination bioassay data:

Week	Treatment (mM/L)	Rep	Percent germination (%)	Week	Treatment (mM/L)	Rep	Percent germination (%)
1	0	1	0.00%	4	0	1	10.00%
1	0	2	10.00%	4	0	2	20.00%
1	0	3	0.00%	4	0	3	30.00%
1	0.25	1	0.00%	4	0.25	1	10.00%
1	0.25	2	0.00%	4	0.25	2	10.00%
1	0.25	3	0.00%	4	0.25	3	10.00%
1	5	1	10.00%	4	5	1	10.00%
1	5	2	10.00%	4	5	2	0.00%
1	5	3	10.00%	4	5	3	10.00%
1	50	1	0.00%	4	50	1	0.00%
1	50	2	0.00%	4	50	2	0.00%
1	50	3	0.00%	4	50	3	0.00%
2	0	1	0.00%	5	0	1	20.00%
2	0	2	10.00%	5	0	2	20.00%
2	0	3	10.00%	5	0	3	30.00%
2	0.25	1	0.00%	5	0.25	1	10.00%
2	0.25	2	0.00%	5	0.25	2	20.00%
2	0.25	3	0.00%	5	0.25	3	20.00%
2	5	1	10.00%	5	5	1	10.00%
2	5	2	0.00%	5	5	2	10.00%
2	5	3	0.00%	5	5	3	10.00%
2	50	1	0.00%	5	50	1	0.00%
2	50	2	0.00%	5	50	2	0.00%
2	50	3	0.00%	5	50	3	0.00%
3	0	1	10.00%	6	0	1	20.00%
3	0	2	20.00%	6	0	2	40.00%
3	0	3	20.00%	6	0	3	30.00%
3	0.25	1	10.00%	6	0.25	1	30.00%
3	0.25	2	0.00%	6	0.25	2	40.00%
3	0.25	3	10.00%	6	0.25	3	30.00%
3	5	1	10.00%	6	5	1	10.00%
3	5	2	0.00%	6	5	2	10.00%
3	5	3	0.00%	6	5	3	10.00%
3	50	1	0.00%	6	50	1	0.00%
3	50	2	0.00%	6	50	2	0.00%
3	50	3	0.00%	6	50	3	0.00%

Table A3.1: Karamu (*Coprosma robusta*) *in vitro* germination data (Rep = experimental replicate, week = week of measurement)

Week	Treatment (mM/L)	Rep	Percent germination (%)	Week	Treatment (mM/L)	Rep	Percent germination (%)
1	0	1	0.00%	4	0	1	0.00%
1	0	2	0.00%	4	0	2	0.00%
1	0	3	10.00%	4	0	3	10.00%
1	0.25	1	0.00%	4	0.25	1	0.00%
1	0.25	2	0.00%	4	0.25	2	10.00%
1	0.25	3	0.00%	4	0.25	3	10.00%
1	5	1	0.00%	4	5	1	0.00%
1	5	2	0.00%	4	5	2	0.00%
1	5	3	0.00%	4	5	3	0.00%
1	50	1	0.00%	4	50	1	0.00%
1	50	2	0.00%	4	50	2	0.00%
1	50	3	0.00%	4	50	3	0.00%
2	0	1	0.00%	5	0	1	0.00%
2	0	2	0.00%	5	0	2	0.00%
2	0	3	10.00%	5	0	3	10.00%
2	0.25	1	0.00%	5	0.25	1	0.00%
2	0.25	2	10.00%	5	0.25	2	10.00%
2	0.25	3	0.00%	5	0.25	3	10.00%
2	5	1	0.00%	5	5	1	0.00%
2	5	2	0.00%	5	5	2	0.00%
2	5	3	0.00%	5	5	3	0.00%
2	50	1	0.00%	5	50	1	0.00%
2	50	2	0.00%	5	50	2	0.00%
2	50	3	0.00%	5	50	3	0.00%
3	0	1	0.00%	6	0	1	0.00%
3	0	2	0.00%	6	0	2	0.00%
3	0	3	10.00%	6	0	3	10.00%
3	0.25	1	0.00%	6	0.25	1	0.00%
3	0.25	2	10.00%	6	0.25	2	10.00%
3	0.25	3	10.00%	6	0.25	3	10.00%
3	5	1	0.00%	6	5	1	0.00%
3	5	2	0.00%	6	5	2	0.00%
3	5	3	0.00%	6	5	3	0.00%
3	50	1	0.00%	6	50	1	0.00%
3	50	2	0.00%	6	50	2	0.00%
3	50	3	0.00%	6	50	3	0.00%

Table A3.2: Flax (*Phormium tenax*) *in vitro* germination data (Rep = experimental replicate, week = week of measurement)

Week	Treatment (mM/L)	Rep	Percent germination (%)	Week	Treatment (mM/L)	Rep	Percent germination (%)
1	0	1	30.00%	4	0	1	50.00%
1	0	2	30.00%	4	0	2	30.00%
1	0	3	10.00%	4	0	3	10.00%
1	0.25	1	0.00%	4	0.25	1	10.00%
1	0.25	2	0.00%	4	0.25	2	0.00%
1	0.25	3	0.00%	4	0.25	3	0.00%
1	5	1	0.00%	4	5	1	0.00%
1	5	2	0.00%	4	5	2	0.00%
1	5	3	0.00%	4	5	3	0.00%
1	50	1	0.00%	4	50	1	0.00%
1	50	2	0.00%	4	50	2	0.00%
1	50	3	0.00%	4	50	3	0.00%
2	0	1	50.00%	5	0	1	50.00%
2	0	2	30.00%	5	0	2	30.00%
2	0	3	10.00%	5	0	3	10.00%
2	0.25	1	10.00%	5	0.25	1	10.00%
2	0.25	2	0.00%	5	0.25	2	0.00%
2	0.25	3	0.00%	5	0.25	3	0.00%
2	5	1	0.00%	5	5	1	0.00%
2	5	2	0.00%	5	5	2	0.00%
2	5	3	0.00%	5	5	3	0.00%
2	50	1	0.00%	5	50	1	0.00%
2	50	2	0.00%	5	50	2	0.00%
2	50	3	0.00%	5	50	3	0.00%
3	0	1	50.00%	6	0	1	50.00%
3	0	2	30.00%	6	0	2	30.00%
3	0	3	10.00%	6	0	3	10.00%
3	0.25	1	10.00%	6	0.25	1	10.00%
3	0.25	2	0.00%	6	0.25	2	0.00%
3	0.25	3	0.00%	6	0.25	3	0.00%
3	5	1	0.00%	6	5	1	0.00%
3	5	2	0.00%	6	5	2	0.00%
3	5	3	0.00%	6	5	3	0.00%
3	50	1	0.00%	6	50	1	0.00%
3	50	2	0.00%	6	50	2	0.00%
3	50	3	0.00%	6	50	3	0.00%

Table A3.3: Koromiko (*Hebe salicifolia*) *in vitro* germination data (Rep = experimental replicate, week = week of measurement)

Week	Treatment (mM/L)	Rep	Percent germination (%)	Week	Treatment (mM/L)	Rep	Percent germination (%)
1	0	1	10.00%	4	0	1	60.00%
1	0	2	40.00%	4	0	2	50.00%
1	0	3	0.00%	4	0	3	50.00%
1	0.25	1	0.00%	4	0.25	1	0.00%
1	0.25	2	0.00%	4	0.25	2	0.00%
1	0.25	3	0.00%	4	0.25	3	0.00%
1	5	1	0.00%	4	5	1	0.00%
1	5	2	0.00%	4	5	2	0.00%
1	5	3	0.00%	4	5	3	0.00%
1	50	1	0.00%	4	50	1	0.00%
1	50	2	0.00%	4	50	2	0.00%
1	50	3	0.00%	4	50	3	0.00%
2	0	1	50.00%	5	0	1	60.00%
2	0	2	50.00%	5	0	2	50.00%
2	0	3	20.00%	5	0	3	50.00%
2	0.25	1	0.00%	5	0.25	1	0.00%
2	0.25	2	0.00%	5	0.25	2	0.00%
2	0.25	3	0.00%	5	0.25	3	0.00%
2	5	1	60.00%	5	5	1	0.00%
2	5	2	50.00%	5	5	2	0.00%
2	5	3	50.00%	5	5	3	0.00%
2	50	1	0.00%	5	50	1	0.00%
2	50	2	0.00%	5	50	2	0.00%
2	50	3	0.00%	5	50	3	0.00%
3	0	1	60.00%	6	0	1	60.00%
3	0	2	50.00%	6	0	2	50.00%
3	0	3	50.00%	6	0	3	50.00%
3	0.25	1	0.00%	6	0.25	1	0.00%
3	0.25	2	0.00%	6	0.25	2	0.00%
3	0.25	3	0.00%	6	0.25	3	0.00%
3	5	1	0.00%	6	5	1	0.00%
3	5	2	0.00%	6	5	2	0.00%
3	5	3	0.00%	6	5	3	0.00%
3	50	1	0.00%	6	50	1	0.00%
3	50	2	0.00%	6	50	2	0.00%
3	50	3	0.00%	6	50	3	0.00%

Table A3.4: Banks Peninsula Blue Tussock (*Festuca actae*) *in vitro* germination data (Rep = experimental replicate, week = week of measurement)

Week	Treatment (mM/L)	Rep	Percent germination (%)	Week	Treatment (mM/L)	Rep	Percent germination (%)
1	0	1	0.00%	4	0	1	0.00%
1	0	2	10.00%	4	0	2	20.00%
1	0	3	20.00%	4	0	3	10.00%
1	0.25	1	0.00%	4	0.25	1	0.00%
1	0.25	2	0.00%	4	0.25	2	0.00%
1	0.25	3	20.00%	4	0.25	3	20.00%
1	5	1	0.00%	4	5	1	0.00%
1	5	2	0.00%	4	5	2	0.00%
1	5	3	0.00%	4	5	3	0.00%
1	50	1	0.00%	4	50	1	0.00%
1	50	2	0.00%	4	50	2	0.00%
1	50	3	0.00%	4	50	3	0.00%
2	0	1	0.00%	5	0	1	0.00%
2	0	2	10.00%	5	0	2	20.00%
2	0	3	20.00%	5	0	3	10.00%
2	0.25	1	0.00%	5	0.25	1	0.00%
2	0.25	2	0.00%	5	0.25	2	0.00%
2	0.25	3	20.00%	5	0.25	3	20.00%
2	5	1	0.00%	5	5	1	0.00%
2	5	2	0.00%	5	5	2	0.00%
2	5	3	0.00%	5	5	3	0.00%
2	50	1	0.00%	5	50	1	0.00%
2	50	2	0.00%	5	50	2	0.00%
2	50	3	0.00%	5	50	3	0.00%
3	0	1	0.00%	6	0	1	0.00%
3	0	2	10.00%	6	0	2	20.00%
3	0	3	20.00%	6	0	3	10.00%
3	0.25	1	0.00%	6	0.25	1	0.00%
3	0.25	2	0.00%	6	0.25	2	0.00%
3	0.25	3	20.00%	6	0.25	3	20.00%
3	5	1	0.00%	6	5	1	0.00%
3	5	2	0.00%	6	5	2	0.00%
3	5	3	0.00%	6	5	3	0.00%
3	50	1	0.00%	6	50	1	0.00%
3	50	2	0.00%	6	50	2	0.00%
3	50	3	0.00%	6	50	3	0.00%

Error! No text of specified style in document. **Manuka (*Leptospermum scoparium*) *in vitro* germination data (Rep = experimental replicate, week = week of measurement)**

Week	Treatment (mM/L)	Rep	Percent germination (%)	Week	Treatment (mM/L)	Rep	Percent germination (%)
1	0	1	0.00%	4	0	1	0.00%
1	0	2	0.00%	4	0	2	0.00%
1	0	3	0.00%	4	0	3	0.00%
1	0.25	1	0.00%	4	0.25	1	0.00%
1	0.25	2	0.00%	4	0.25	2	10.00%
1	0.25	3	0.00%	4	0.25	3	0.00%
1	5	1	0.00%	4	5	1	10.00%
1	5	2	0.00%	4	5	2	30.00%
1	5	3	0.00%	4	5	3	20.00%
1	50	1	0.00%	4	50	1	0.00%
1	50	2	0.00%	4	50	2	0.00%
1	50	3	0.00%	4	50	3	0.00%
2	0	1	0.00%	5	0	1	0.00%
2	0	2	0.00%	5	0	2	10.00%
2	0	3	0.00%	5	0	3	10.00%
2	0.25	1	0.00%	5	0.25	1	0.00%
2	0.25	2	0.00%	5	0.25	2	10.00%
2	0.25	3	0.00%	5	0.25	3	0.00%
2	5	1	0.00%	5	5	1	20.00%
2	5	2	0.00%	5	5	2	30.00%
2	5	3	0.00%	5	5	3	20.00%
2	50	1	0.00%	5	50	1	0.00%
2	50	2	0.00%	5	50	2	0.00%
2	50	3	0.00%	5	50	3	0.00%
3	0	1	0.00%	6	0	1	10.00%
3	0	2	0.00%	6	0	2	10.00%
3	0	3	0.00%	6	0	3	20.00%
3	0.25	1	0.00%	6	0.25	1	10.00%
3	0.25	2	10.00%	6	0.25	2	10.00%
3	0.25	3	0.00%	6	0.25	3	0.00%
3	5	1	10.00%	6	5	1	30.00%
3	5	2	20.00%	6	5	2	40.00%
3	5	3	10.00%	6	5	3	20.00%
3	50	1	0.00%	6	50	1	0.00%
3	50	2	0.00%	6	50	2	0.00%
3	50	3	0.00%	6	50	3	0.00%

Table A3.6: Frosted curls (*Carex comans*) *in vitro* germination data (Rep = experimental replicate, week = week of measurement)

Week	Treatment (mM/L)	Rep	Percent germination (%)	Week	Treatment (mM/L)	Rep	Percent germination (%)
1	0	1	10.00%	4	0	1	0.00%
1	0	2	20.00%	4	0	2	40.00%
1	0	3	0.00%	4	0	3	70.00%
1	Field	1	60.00%	4	Field	1	100.00%
1	Field	2	50.00%	4	Field	2	70.00%
1	Field	3	40.00%	4	Field	3	90.00%
1	0.25	1	10.00%	4	0.25	1	50.00%
1	0.25	2	0.00%	4	0.25	2	50.00%
1	0.25	3	0.00%	4	0.25	3	30.00%
1	5	1	0.00%	4	5	1	0.00%
1	5	2	0.00%	4	5	2	30.00%
1	5	3	0.00%	4	5	3	20.00%
1	50	1	0.00%	4	50	1	20.00%
1	50	2	0.00%	4	50	2	20.00%
1	50	3	0.00%	4	50	3	30.00%
2	0	1	90.00%	5	0	1	80.00%
2	0	2	40.00%	5	0	2	40.00%
2	0	3	50.00%	5	0	3	60.00%
2	Field	1	100.00%	5	Field	1	100.00%
2	Field	2	70.00%	5	Field	2	80.00%
2	Field	3	90.00%	5	Field	3	80.00%
2	0.25	1	50.00%	5	0.25	1	50.00%
2	0.25	2	50.00%	5	0.25	2	60.00%
2	0.25	3	20.00%	5	0.25	3	30.00%
2	5	1	10.00%	5	5	1	0.00%
2	5	2	20.00%	5	5	2	30.00%
2	5	3	40.00%	5	5	3	20.00%
2	50	1	0.00%	5	50	1	20.00%
2	50	2	0.00%	5	50	2	20.00%
2	50	3	10.00%	5	50	3	40.00%
3	0	1	90.00%	6	0	1	80.00%
3	0	2	50.00%	6	0	2	40.00%
3	0	3	70.00%	6	0	3	60.00%
3	Field	1	100.00%	6	Field	1	90.00%
3	Field	2	70.00%	6	Field	2	80.00%
3	Field	3	90.00%	6	Field	3	80.00%
3	0.25	1	50.00%	6	0.25	1	50.00%
3	0.25	2	50.00%	6	0.25	2	40.00%
3	0.25	3	30.00%	6	0.25	3	30.00%
3	5	1	10.00%	6	5	1	0.00%
3	5	2	30.00%	6	5	2	20.00%
3	5	3	30.00%	6	5	3	20.00%
3	50	1	20.00%	6	50	1	20.00%
3	50	2	0.00%	6	50	2	20.00%
3	50	3	20.00%	6	50	3	40.00%

Table A3.7: Flax (*Phormium tenax*) *ex situ* germination data (Rep = experimental replicate, week = week of measurement)

Week	Treatment (mM/L)	Rep	Percent germination (%)	Week	Treatment (mM/L)	Rep	Percent germination (%)
1	0	1	0%	4	0	1	20%
1	0	2	7%	4	0	2	27%
1	0	3	0%	4	0	3	13%
1	Field	1	0%	4	Field	1	7%
1	Field	2	0%	4	Field	2	0%
1	Field	3	0%	4	Field	3	0%
1	0.25	1	0%	4	0.25	1	27%
1	0.25	2	7%	4	0.25	2	33%
1	0.25	3	7%	4	0.25	3	20%
1	5	1	13%	4	5	1	33%
1	5	2	13%	4	5	2	27%
1	5	3	13%	4	5	3	33%
1	50	1	0%	4	50	1	20%
1	50	2	0%	4	50	2	40%
1	50	3	7%	4	50	3	20%
2	0	1	13%	5	0	1	27%
2	0	2	13%	5	0	2	27%
2	0	3	13%	5	0	3	27%
2	Field	1	0%	5	Field	1	7%
2	Field	2	0%	5	Field	2	0%
2	Field	3	0%	5	Field	3	0%
2	0.25	1	27%	5	0.25	1	27%
2	0.25	2	27%	5	0.25	2	33%
2	0.25	3	13%	5	0.25	3	20%
2	5	1	20%	5	5	1	33%
2	5	2	13%	5	5	2	27%
2	5	3	20%	5	5	3	33%
2	50	1	7%	5	50	1	20%
2	50	2	7%	5	50	2	47%
2	50	3	13%	5	50	3	13%
3	0	1	13%	6	0	1	27%
3	0	2	20%	6	0	2	33%
3	0	3	13%	6	0	3	27%
3	Field	1	0%	6	Field	1	7%
3	Field	2	0%	6	Field	2	7%
3	Field	3	0%	6	Field	3	7%
3	0.25	1	27%	6	0.25	1	33%
3	0.25	2	33%	6	0.25	2	40%
3	0.25	3	20%	6	0.25	3	27%
3	5	1	33%	6	5	1	33%
3	5	2	13%	6	5	2	27%
3	5	3	33%	6	5	3	33%
3	50	1	13%	6	50	1	20%
3	50	2	40%	6	50	2	53%
3	50	3	13%	6	50	3	13%

Table A3.8: Karamu (*Coprosma robusta*) *ex situ* germination data (Rep = experimental replicate, week = week of measurement)

Week	Treatment (mM/L)	Rep	Percent germination (%)	Week	Treatment (mM/L)	Rep	Percent germination (%)
1	0	1	13%	4	0	1	33%
1	0	2	13%	4	0	2	40%
1	0	3	20%	4	0	3	40%
1	Field	1	13%	4	Field	1	53%
1	Field	2	13%	4	Field	2	40%
1	Field	3	27%	4	Field	3	40%
1	0.25	1	13%	4	0.25	1	33%
1	0.25	2	7%	4	0.25	2	20%
1	0.25	3	7%	4	0.25	3	40%
1	5	1	13%	4	5	1	40%
1	5	2	0%	4	5	2	47%
1	5	3	13%	4	5	3	33%
1	50	1	0%	4	50	1	0%
1	50	2	0%	4	50	2	7%
1	50	3	0%	4	50	3	7%
2	0	1	13%	5	0	1	33%
2	0	2	13%	5	0	2	40%
2	0	3	20%	5	0	3	40%
2	Field	1	20%	5	Field	1	53%
2	Field	2	13%	5	Field	2	40%
2	Field	3	33%	5	Field	3	53%
2	0.25	1	13%	5	0.25	1	33%
2	0.25	2	7%	5	0.25	2	20%
2	0.25	3	13%	5	0.25	3	40%
2	5	1	13%	5	5	1	40%
2	5	2	0%	5	5	2	47%
2	5	3	20%	5	5	3	33%
2	50	1	0%	5	50	1	0%
2	50	2	0%	5	50	2	7%
2	50	3	0%	5	50	3	7%
3	0	1	27%	6	0	1	33%
3	0	2	40%	6	0	2	40%
3	0	3	33%	6	0	3	40%
3	Field	1	40%	6	Field	1	67%
3	Field	2	33%	6	Field	2	47%
3	Field	3	40%	6	Field	3	60%
3	0.25	1	33%	6	0.25	1	33%
3	0.25	2	20%	6	0.25	2	27%
3	0.25	3	40%	6	0.25	3	40%
3	5	1	33%	6	5	1	40%
3	5	2	20%	6	5	2	40%
3	5	3	33%	6	5	3	33%
3	50	1	0%	6	50	1	0%
3	50	2	7%	6	50	2	7%
3	50	3	7%	6	50	3	7%

Table A3.9: Frosted Curls (*Carex comans*) *ex situ* germination data (Rep = experimental replicate, week = week of measurement)

Week	Treatment (mM/L)	Rep	Percent germination (%)	Week	Treatment (mM/L)	Rep	Percent germination (%)
1	0	1	0%	4	0	1	7%
1	0	2	0%	4	0	2	0%
1	0	3	0%	4	0	3	0%
1	Field	1	0%	4	Field	1	20%
1	Field	2	33%	4	Field	2	40%
1	Field	3	0%	4	Field	3	7%
1	0.25	1	0%	4	0.25	1	0%
1	0.25	2	0%	4	0.25	2	0%
1	0.25	3	0%	4	0.25	3	0%
1	5	1	0%	4	5	1	0%
1	5	2	0%	4	5	2	0%
1	5	3	0%	4	5	3	7%
1	50	1	0%	4	50	1	0%
1	50	2	0%	4	50	2	0%
1	50	3	0%	4	50	3	0%
2	0	1	0%	5	0	1	0%
2	0	2	0%	5	0	2	0%
2	0	3	0%	5	0	3	0%
2	Field	1	20%	5	Field	1	20%
2	Field	2	40%	5	Field	2	40%
2	Field	3	0%	5	Field	3	7%
2	0.25	1	0%	5	0.25	1	0%
2	0.25	2	0%	5	0.25	2	0%
2	0.25	3	0%	5	0.25	3	0%
2	5	1	0%	5	5	1	0%
2	5	2	0%	5	5	2	0%
2	5	3	7%	5	5	3	7%
2	50	1	0%	5	50	1	0%
2	50	2	0%	5	50	2	0%
2	50	3	0%	5	50	3	0%
3	0	1	0%	6	0	1	0%
3	0	2	0%	6	0	2	0%
3	0	3	0%	6	0	3	0%
3	Field	1	20%	6	Field	1	20%
3	Field	2	40%	6	Field	2	40%
3	Field	3	0%	6	Field	3	7%
3	0.25	1	0%	6	0.25	1	0%
3	0.25	2	0%	6	0.25	2	0%
3	0.25	3	0%	6	0.25	3	0%
3	5	1	0%	6	5	1	0%
3	5	2	0%	6	5	2	0%
3	5	3	7%	6	5	3	7%
3	50	1	0%	6	50	1	0%
3	50	2	0%	6	50	2	0%
3	50	3	0%	6	50	3	0%

Table A3.10: Koromiko (*Hebe salicifolia*) *ex situ* germination data (Rep = experimental replicate, week = week of measurement)