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**Phytoextraction of Palladium and Gold
from Broken Hill Gossan**

Phytoextraction of Palladium and Gold from Broken Hill Gossan

A thesis presented in partial fulfilment of the requirements for the degree of

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at Massey University, Manawatū,

New Zealand

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Abstract

The research in this thesis was conducted as part of the Phytocat Project; a collaborative effort between University of York (UK), Yale University (USA), University of British Columbia (Canada) and Massey. The aim of the Phytocat project was to yield a target concentration of $1,000 \mu\text{g g}^{-1}$ palladium in plants, so that the plants could be used as catalysts in chemical reactions. This thesis focussed on the phytoextraction of palladium from Broken Hill gossan, a platinum group element-rich rock collected from Australia. The gossan and surrounding soil has an elevated concentration of iron, copper, nickel and precious metals.

Samples of species native to the Broken Hill gossan and the associated rhizosphere soil were collected from the field and analysed to screen natural levels of metal accumulation in plants of the area. Five native plant species were identified: *Solanum centrale* (bush tomato), *Brassica* sp, *Ptilotus obovatus* (silver tail), *Sclerolaena lanicuspis* (copper burr) and *Tetragonia moorei* (annual spinach). The copper concentration in all plant tissues had a strong relationship with copper in soil. An individual *Solanum centrale* plant recorded a copper concentration of $277 \mu\text{g g}^{-1}$ from soil with concentration of $796 \mu\text{g g}^{-1}$ suggesting that this species is a copper tolerant plant from Broken Hill. No anomalous levels of nickel were recorded in plant tissues. The average palladium concentration measured in the rhizosphere soil was 28.8 ng g^{-1} . However, the five native plant species could not concentrate palladium in their biomass. Solubility of palladium was suggested to be poor in natural environment.

To study the potential of induced hyperaccumulation to increase the palladium uptake in plants, 60 kg of gossan from the field was collected, crushed and used as a plant growth medium for controlled plant trials at Massey University. Two types of gossan rock were collected, classified by the dominant form of iron oxide mineral in the rock structure: goethite dominated (soil A) and hematite dominated (soil B). The goethite material (A) has a higher total and soluble metals concentration than the hematite material.

Initial trials focused on *Brassica juncea*. However, despite germinating, this plant grew poorly on both types of gossan. Insufficient biomass was available to induce uptake of metals, and therefore only the natural levels of metal uptake in the poorly developed plants was quantified. Total harvested aerial biomass was 5.1 g from 39 pots each containing 800 g of gossan. The mean metal concentrations in plants grown in the two soils was not significantly different ($p < 0.05$). The concentration of palladium in the plant biomass ranged from 2,130 to 2,909 ng g^{-1} . This study proposed that $1,000 \text{ ng g}^{-1}$ palladium is a suitable hyperaccumulation

threshold level and therefore *B. juncea* on the gossan was able to hyperaccumulate palladium. The average copper concentration in the biomass was $759 \mu\text{g g}^{-1}$ and it is likely that high copper solubility in the growth substrate affected plant growth performance.

A second trial used *Cannabis sativa* (Hemp) due to recorded metal tolerance of this species. Pots were re-seeded with *C. sativa*. Hemp germinated and grew well relative to *B. juncea*. Potassium cyanide solution ($50 \text{ mL of } 8 \text{ g L}^{-1}$) was applied to each pot at the point of maximum biomass to induce the solubility of precious metals and therefore to induce hyperaccumulation. Significant metal concentration values after KCN treatment were as follows: Copper ($6,726 \mu\text{g g}^{-1}$) > nickel ($184 \mu\text{g g}^{-1}$) > palladium ($62 \mu\text{g g}^{-1}$) > gold ($9 \mu\text{g g}^{-1}$). Following established criteria values, copper, palladium, and gold hyperaccumulation was observed. The mean metal concentrations of copper, nickel, and palladium from Hemp grown in soil B were greater compared to Hemp grown in soil A and control plants ($p < 0.05$). However, gold concentration between Hemp A and Hemp B was not different significantly ($p > 0.05$). These results were anomalous compared to the recorded total and soluble metal concentration of the two rocks.

This study concluded that total metal in soil is not an indication for metal concentration in plant tissues. Accumulated metal in plants is a function of the concentration of soluble metal in soil that can be readily absorbed by plants. Different characteristics of the substrate (in this case iron oxide) may influence metal uptake in plants. Iron oxide minerals were identified as plant competitors for soluble metals in soil solution. In this case, goethite adsorbs more soluble metal ions than hematite and therefore plants grown on the goethite substrate accumulated less metal relative to the hematite soil despite the goethite rocks having a greater total and soluble metal concentration. Metal tolerance was also highlighted as an important factor in the induced accumulation of palladium. Palladium is often associated with copper in soils and tolerance to copper is a key factor. In this work, *Brassica juncea* was proven less tolerant to copper than *C. sativa*. The target of $1000 \mu\text{g g}^{-1}$ palladium in plants has not yet been reached but the Broken Hill gossan is highlighted as a useful substrate for ongoing work. There is good potential to test the native copper tolerant species *Solanum centrale*, for induced metal uptake in the future.

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Chapter 1 - Introduction

In the 21st century, the application of metal-dependent technologies has transformed and evolved our daily life. Metals are the backbone to technology we now take for granted, for example mobile phones, computers and laptops. To illustrate this, in 2008 reported global production of laptops and personal computers reached up to 300 million. The reported amount of metal used for this production was 150,000 tonne of copper, 9,100 tonne of cobalt, 300 tonne of silver, 66 tonne of gold, and 24 tonne of palladium (Hunt, 2013). As the global electronics market sector grows, the corresponding increase in demand for metals is inevitable.

It is believed that global metal demands are still being supplied by conventional mining operations through open cut or underground techniques. However, many researchers argue that mining is an expensive activity and it bears environmental risks. Such risks are the huge amount of solid wastes include acid mine drainage, heavy metal exposure to contaminate the environment, enormous water and energy consumption, as well as greenhouse gas emissions (Glaister & Mudd, 2010; Hudson-Edwards, Jamieson, & Lottermoser, 2011).

Increasing environmental awareness on green technology is changing trends in worldwide metal demands. Green products and the associated technology have driven increased precious metals consumption, such as platinum group metals (PGMs). For example, the introduction of environmental regulations on emissions controls from vehicle transports started in the United States in 1975. To control vehicle emissions, catalytic converters which contain PGMs are used. The catalytic converters change hydrocarbons, NO_x, and CO into less toxic substances, such as H₂O, N₂, and CO₂ (Alonso et al., 2012; Ely et al., 2001)

Since 1990, palladium has been used to replace platinum in vehicle exhaust catalytic converters technology. Worldwide PGM production in 2007 reached to 509 tonnes. Approximately 50 percent of global palladium production is used in automotive industries, 30 percent for jewellery, and the rest for electric components, chemicals, and catalysts (Glaister & Mudd, 2010; Kabata-Pendias, 2010; Kielhorn, Melber, Keller, & Mangelsdorf, 2002).

Palladium is classified as a 'critical' element. As discussed in Hunt (2013), palladium has been identified as having high supply risk and high impact will be apparent if its supply is scarce, a notable economics perspective. Currently major palladium mining is only conducted in South

Africa where ore grade ranges between 3 and 8 gram per tonnes PGM and there is approximately 63,000 tonnes of quantified PGM reserve. Other countries also reported to contribute to global palladium production are Russia, Canada, the United States, and Zimbabwe (Glaister & Mudd, 2010; Mpinga, Eksteen, Aldrich, & Dyer, 2015).

It has been suggested that green plants have capability to accumulate metals in their biomass thereby extracting these from soil. Some plants have been identified to accumulate metals at extreme concentrations from their natural soils. Such plant species are described as accumulator and hyperaccumulator species. Following these findings, an alternative option to extract metals by using plants was proposed. This method is known as phytoextraction and it is believed that the phytoextraction method would be an economical and environmentally friendly method to recover target metals from soils. In application, phytoextraction could be divided into two classes. First is phytoremediation which is intended to clean up heavy metal contaminants from the environment. Secondly is phytomining which is targeted to extract economically important metals such nickel, palladium, and gold (Anderson, 2013).

The idea of precious metals extraction by using plants has been discussed since 1950. A field study to investigate the natural occurrence of gold uptake in plants was reported by Warren and Delavault (1950). Approximately 20 years after this publication, other researchers attempted to study gold uptake by plants in the controlled environment (Girling & Peterson, 1980; Shacklette, Lakin, Hubert, & Curtin, 1970). Their observation concluded that soluble gold can be absorbed into plant tissues. In 1999, a group of researcher tried to induce gold at high concentrations in plants. Since then, gold phytoextraction has received considerable interest. Researches interest on gold phytoextraction is driven by economic reasons; the price of gold is relatively high in international commodity markets (Anderson, 2013).

On the other hand, although palladium is a high demand metal, studies on palladium uptake in plant species is limited. Kothny (1979) is likely to be the first author to report natural palladium uptake in plants. After his publication, there were limited researches focusing on palladium uptake in plant species. Study on palladium uptake by plants from mine tailings under greenhouse conditions was reported in Walton (2002). Several field studies to monitor palladium uptake in a contaminated environment have been reported (Colombo, Monhemius, & Plant, 2008b; Nemutandani, Dutertre, Chimuka, Cukrowska, & Tutu, 2006). More recently, a group of researchers from the University of York succeeded to synthesise palladium nanoparticles in living plants (Parker et al., 2014). Based on this work, the authors suggested

further study to investigate plant species which might be suitable to be grown on waste containing palladium in order to synthesise 'green' nanoparticles.

Considering that palladium is needed in a wide variety of industry sectors and demands are increasing, it is important to secure its supply in the future. To address this challenge, the York group joined with international collaborators to conduct a research project called the Phytocat Project. The project aims to explore plant species which have capability in metal extraction from mine wastes. The project started since 2012 and is funded by the G8 Research Councils Initiative on Multilateral Research Funding. Collaborators on this project include the University of York (UoY), the University of British Columbia (UBC), and the Yale University School of Forestry and Environmental Studies (Yale) (Phytocat, 2013).

Palladium is not considered to be soluble to plants, and no natural hyperaccumulator of palladium is recognised. Therefore, induced hyperaccumulation must be used to effect palladium uptake. Extensive work by several laboratories has shown that potassium cyanide is the most effective chemical to promote gold and other precious metal solubility and thereby induce hyperaccumulation of this metal in plants. In relation to the Phytocat Project, the target of the research program is to achieve a target concentration of 1000 ppm palladium in plants (dry weight).

Linked to the Phytocat research group is a non G8 funded team from Massey University in New Zealand (the current research). To assist in meeting the aims of Phytocat, a bulk sample of platinum group metal-rich gossan from the Broken Hill mineral complex in Australia was collected in July 2014 and transported to Massey (Meech & Anderson, 2014). Three projects were conducted at Massey which contributed to the Phytocat project (1) rock analysis by microprobe and SEM techniques to discover the distribution and form of metals in the rock, (2) geochemical analysis to explore potential availability of copper, nickel, gold and palladium for plant uptake in the rock as function of chemical amendments, and (3) accumulation trials with *Brassica juncea* and *Cannabis sativa* to induce palladium and other metals into the biomass.

Aim of this thesis

The research described in this thesis was conducted to achieve the objectives of project three of the Massey University Phytocat research group. The overall aim of the research described in this thesis was to conduct plant trials on Broken Hill gossan, to quantify the palladium and other precious metal concentration that could be induced into plants. The Phytocat

programme has set a target palladium concentration in plants of 1,000 mg/kg (dry weight) for catalytic activity. The assessment of the Phytocat programme was that the Broken Hill Gossan, with a palladium concentration as high as 50 $\mu\text{g g}^{-1}$, represents perhaps the best real-life substrate for phytomining studies to achieve this target concentration in plants.

This thesis consists of seven chapters. A description for each chapter is as follows:

Chapter 1 - Introduction

Presents background justifications on palladium oriented research, for instance the Phytocat Project, and its component sub-projects which have been conducted in Massey University.

Chapter 2 – Literature review

Reviews current knowledge in relation to phytoextraction. This chapter also include an overview of natural and induced hyperaccumulator plant species, chemical solvents being used in phytoextraction studies, basic information about plant species being used on this thesis, as well as identifies the knowledge gap on palladium-plant studies.

Chapter 3 – Materials and methods

This section describes characteristics of Broken Hill, the area of study, and gossan substrates, the growth medium for plant trials. Experimental designs for greenhouse trials are also provided, as well as analysis method, quality controls, and statistical analysis.

Chapter 4– Natural metal uptake in native plants from Broken Hill

Five plant samples which naturally grown on gossan substrate were collected in order to determine the possibility of natural hyperaccumulator species. Analysis on correlation between the concentration of metal in soils and plants is detailed. Findings from this chapter could be used to investigate interesting plant species for further palladium extraction trials.

Chapter 5 – Greenhouse trial: Metal uptake in *Brassica juncea*

Initial pot trials using *B. juncea* were conducted. This section is used as investigation for natural metal uptake under greenhouse environment. From this trial, the ability of *B. juncea* to be grown on copper rich soil was examined. Moreover, this chapter tries to simulate natural metal uptake in the environment. Hence, water is being used to solubilise metals.

Chapter 6 – Greenhouse trial: Induced hyperaccumulation in *Cannabis sativa*

In contrast to Chapter 5, this section studies the use of potassium cyanide (KCN) to solubilise metals. *Cannabis sativa* is used to uptake soluble metals in the substrate. This section describes research which aimed to induce as much as possible palladium in plant's aerial portions (target 1000 $\mu\text{g g}^{-1}$).

Chapter 7 – General discussion

This final chapter is prepared to discuss interesting findings from previous chapters. Implications and conclusions from this study are addressed here.

Chapter 2 - Literature Review

2.1. Soil – Plant Interaction

2.1.1. Metal accumulation in plant species

Plants accumulate metal in their organs as a strategy to survive in a metal-rich soil environment. Baker (1981) proposed three types of soil – plant interactions: (1) metal accumulators (hyperaccumulators), (2) indicators, and (3) excluders. A hyperaccumulator is a plant which containing a high metal concentration in its aerial parts. Furthermore, hyperaccumulator plants are able to accumulate metal at an extreme concentration compared to other 'normal' plants. Metal indicator plants have an ability to extract metal from soil and metal levels in their organs reflect the metal concentration in the soil environment. On the other hands, the metal excluder plants tend to regulate constant and low metal level in their above ground parts.

Metal accumulation in plant species has been recorded since the 16th century when Andrea Cesalpino reported plants growing on ultramafic rocks in Tuscany, Italy. In the 20th century, a further deep description about plant-soil relationships was put forward by Minguzi and Vergnano who reported the unusual nickel accumulation by *Alyssum bertolonii*. They measured about 7,900 $\mu\text{g g}^{-1}$ (0.79 %) of nickel detected in dried leaves, while the soil only contained 0.42 percent of nickel (Brooks, 1998). The anomaly of high metal accumulation (hyperaccumulation) in plants is suggested as an adaptation in extreme conditions like in an ultramafic environment (Koptsik, 2014).

The term hyperaccumulation was suggested by Brooks, Lee, Reeves, and Jaffre (1977). They investigated 2,000 herbarium specimens around the world. They found some species in New Caledonia, such as *Homalium francii* Guillaumin which accumulates nickel as much as 14,500 $\mu\text{g g}^{-1}$ dry weight. According to this finding, Robert Brooks and his colleagues proposed to describe such plants as hyperaccumulators. Since then, researchers have explored other plants which possess the ability to accumulate high metal concentrations. Recently more than 400 plant species from 45 plant families have been identified as hyperaccumulators, and about 75% are nickel hyperaccumulator (Küpper & Kroneck, 2007; Sheoran, Sheoran, & Poonia, 2009).

2.1.2. Metal bioavailability

The potential of plants to uptake metal from soils is limited by metal bioavailability which is a function of solubility and the strength of binding to soil particles (Clemens, Palmgren, & Krämer, 2002; Robinson, 1997). Metal bioavailability is a condition when metals are soluble and ready to be absorbed, for this case in the plants. Furthermore, bioavailability is a dynamic process which includes three factors, such as metal availability in the soil environment, uptake process by plant species and accumulation effects in the plants (Harmsen, 2007). Accordingly, metal absorption by plants is restricted by metal solubility in the rhizosphere. In plant metal extraction technology metal solubility has a significant role (Moraghan, 1993; Moreno, Anderson, Stewart, & Robinson, 2004). Bioavailability of metals in soils depends on some aspects, such as pH, Eh, soil drainage, competition between ions, and composition of clay mineral in the soils (Brooks, 1983).

2.1.3. Metal transport

Metal accumulation in the plant tissues begins at the interface of soils and root zones. First, soils which consist of clay minerals have more available metals than those with coarse structures. The surface of clay minerals have more cations and are involved in cation exchange processes with plant roots (Brooks, 1983). In addition, soil acidity enhances metal availability to the plants. Plants actively exude organic acids through their root systems, which create an acid environment in the rhizosphere, changing metal speciation and increasing availability to plants. Chemical aspects in the soils such as nutrient, pH and redox potential (E_h) also affect metal bioavailability (Deng, Ye, & Wong, 2004). Lulofs (1993) stated that metal mobility occurred as an interaction result between Eh and pH; where Eh is responsible to solubilise metal in solution and pH enhances the mobility.

Furthermore, Deng et al. (2004) suggested that a high level of Phosphorus in soil tends to adsorb (precipitate) metals and decreases the possibility of metal uptake by the plants. Furthermore, Robinson, Bañuelos, Conesa, Evangelou, and Schulin (2009) suggested another three factors which affect metal solubility in the soils, there are metal properties, plant factors, as well as microbes in the root zones.

Roots play an important role to absorb metals from soils (Kumar, Dushenkov, Motto, & Raskin, 1995). Plant roots excrete organic acid which make metals more soluble in the soil. Plant roots exude the H^+ ion and decreases pH up to 2 units (Robinson et al., 2009). Decreasing pH changes the dissolution-precipitation equilibrium of heavy metal ions, and makes heavy metals more soluble into soil solution (Wei, Teixeira da Silva, & Zhou, 2008). According to Nascimento and Xing (2006), the exuded organic acid from plant roots is the main factor to mobilize metals from soils and is responsible for natural metal uptake in plants. Besides decreasing the pH level, organic acids also form complex binding units with metal ions and change soil characteristics. The soluble metals in the rhizosphere can then be absorbed by root cells, and transported to aerial parts through the xylem.

Transpiration rate influences metal translocation, to aboveground parts of plants. This theory has been suggested by Girling and Peterson (1980). In a research trial they employed *Hordeum vulgare* with various treatments, such as blowing, vaseline, and vaseline and inhibitor abscisic acid (ABA to manipulate transpiration). Results showed that under breeze treatment, the gold content in *H. vulgare* increased.

2.1.4. Metal sink

Leaves have been identified as the main sinks for nickel (Psaras, Constantinidis, Cotsopoulos, & Manetas, 2000). According to Bhatia et al. (2003), plant's organs have been used for metal sink. The trend for nickel concentrations in plant organs is suggested as leaf > stem > root. However, this pattern is not always the same in different plant species. For example, *Sebertia acuminata* accumulates more than 11% of nickel in latex (Baker & Brooks, 1989).

Cannabis sativa grown in hydroponic culture contained 160 mg L^{-1} CuSO_4 solution was shown to distribute copper only in leaves. No copper was been detected in stems. Moreover, the study indicated that the upper leaf epidermal cells are the main location for copper accumulation. Copper accumulation in the epidermis cells is not common. Typically plants use vacuoles to accumulate excess metals in the cells (Arru, Rognoni, Baroncini, Bonatti, & Perata, 2004).

A study about palladium uptake in higher plants and its localization in plant tissues was conducted by Ronchini et al. (2015). They grew Pea seeds (*Pisum sativum* L.) on vermiculate substrate and treated this with various concentration of palladium ($0.10 - 25 \text{ mg L}^{-1}$) in 1 L of

nutrient solution. The mean concentration of Pd in the main plant structures was 198, 2.1, 1.2, and 0.34 $\mu\text{g g}^{-1}$ in roots, leaves, stems, and pods respectively. Hence the order of Pd sinks in above-ground portions can be suggested as leaf > stem > pod. This study also assessed the range of platinum metals uptake in *Brassica napus*. From their observations, the highest PGM concentration was found in leaves and stems for platinum, followed by rhodium and palladium. The palladium concentration was high in the roots indicating its phytoavailability is relatively low (Nischkauer, Herincs, Puschenreiter, Wenzel, & Limbeck, 2013).

A study reported by Beattie and Haverkamp (2011) proposed that gold metal nanoparticles were distributed in stems and roots. The authors asserted that gold metal particles mostly can be found in the chloroplast. Following this theme, observations on natural gold uptake in *Eucalyptus* trees reported that plants can naturally uptake and distribute gold to all parts. This study concluded that the highest gold concentration can be detected in leaves (Lintern, Anand, Ryan, & Paterson, 2013).

2.2. Metals in plants and their relationship

2.2.1. Metals for plant

Plants genetically are designed to become a living pump which can absorb and accumulate metals from soil (Koptsik, 2014; Salt et al., 1995). To support their growth and development, plants require some essential metals. These metals such as Fe, Mo, Cu, Mg, Ni, and Zn (Salt et al., 1995) are essential and are required in small amounts as micronutrients. For instance, pot trials by Nicks and Chambers (1998) showed that plants need Ni to support their growth. *Streptanthus polygaloides*, a hyperaccumulator plant from California is reported to require Ni to reach optimum growth. In this study, Ni content in the biomass of *S. polygaloides* reached 7,820 $\mu\text{g g}^{-1}$ and nickel concentrations as much as 14,800 $\mu\text{g g}^{-1}$ were found in the leaf.

As essential elements, micronutrients like copper, iron, nickel are needed in the plants tissues at concentrations of less than 1000 mg kg^{-1} (Robinson et al., 2009). Table 1 indicates the acceptable concentration of essential elements in plants. On the other hand, if the concentration of essential metals exceeds the limit, it may cause toxicity to the plant tissue. Other metals such as gold and palladium are not recognised as important nutrients in a plant's metabolism.

Most hyperaccumulator plant species possess capability to excrete metal chelating compounds in the rhizosphere. The metal chelating compounds are organic acids such as oxalic, malic, malonic and citric acids. These chelating compounds make metal more soluble and available for plant uptake mechanisms (Eapen & D'Souza, 2005).

Table 2.1 Concentrations of essential metals in plants

Essential elements	Concentration ($\mu\text{g/g}$ dry weight)
Cu	4 – 15
Ni	1
Fe	140
Mn	15 – 100
Mo	1 – 10
Co	0.05 – 0.5
Zn	8 – 100

(Modified from Nagajyoti, Lee, & Sreekanth, 2010)

In addition, a plant's action to uptake a high heavy metal concentration into its above-ground biomass has been suggested as a protection mechanism to counter pathogen or predators attack (Krämer, 2010). A work by Martens and Boyd (1994) demonstrated elemental protection in a hyperaccumulator plant. The authors grew *Streptanthus polygaloides*, a nickel hyperaccumulator, in a greenhouse media amended with nickel. They used *S. polygaloides* leaves to feed three larvae of *Euchloehyantis*, *Pierisrapae*, and grasshoppers. The results demonstrated that larvae fed nickel amended-biomass cannot survive a high nickel concentration in the leaves. However, hyperaccumulation is not always successful for every organism. Boyd (1998) noted that some organisms can breach the elemental defence in hyperaccumulators, and this is described as a limitation of elemental protection systems.

2.2.2. Phytotoxicity: Heavy metal effects on vegetation

Plants need essential elements in order to support normal growth and development. Although essential metals are good, exposure to high concentrations of these metals may cause phytotoxicity effects (Rascio & Navari-Izzo, 2011). Chang suggested criteria for phytotoxicity

symptoms: (1) examined effects affect not only plant growth but also plant's physical condition, (2) appearances of physical damages in plant parts are constant, and (3) the damage not only occurred physically but also disturbs biochemical process (Ross & Kaye, 1994).

Metal phytotoxicity will occur at cellular level, and damage membranes, interrupt enzymes, and hinder the action important molecules for metabolism (Rascio & Navari-Izzo, 2011). High metal contents in plants affect aerobic cells by disturbing stable equilibrium between antioxidants and reactive oxygen species (ROS). When metal toxicity is active at cellular level, then it affects osmotic pressure and causes poor growth performance or foliar damages (Bednarova et al., 2014). Furthermore, Ross and Kaye (1994) suggested that chemical analysis in leaves can determine the level of phytotoxicity in the plants. Even though metal concentration in soil is considered as an important factor in phytotoxicity symptoms, there are additional factors which are also responsible for phytotoxicity. Such factors are:

- (1) Threshold levels of phytotoxicity in various plant species are different.
- (2) Soil characteristics affect metal translocation from root to above ground parts.
- (3) Roots' behaviour to reduce or fully isolate metal transfer into the plants.
- (4) Chemical properties in leaves also affected by pH, water availability, salinity and redox.

2.2.3. Heavy metal detoxification

Plants have a mechanism to against heavy metal toxicity and this is defined as detoxification. Plants produce phytochelatins which function for detoxification. Phytochelatins (PCs) as described in (Brooks, 1998) are "glutathione derived peptides with the general structure of $(\gamma\text{-Glu-Cys})_n\text{Gly}$, where n varies from 2 to 11". Phytochelatins are an important compound in order to hinder phytotoxicity effects in plant tissues. High level of PCs can be found in metal tolerant plants. PCs function to complex with heavy metals for detoxification purposes. When plants produce a low amount of PCs, this is suggested to inhibit plant growth or to cause death. It is suggested that heavy metal ions are responsible for the activation of phytochelatin synthesis in plants (Brooks, 1998; Cobbett, 2000).

2.3. Metals relevant to the Phytocat Project

Previous sections have described the relationship between metals in soil and plant. In the next sections, four metals that are considered relevant to the Phytocat Project will be discussed. These metals are copper, nickel, gold, and palladium.

2.3.1. Copper in plants

The range of copper concentration in the Earth's crust is suggested to be from 25 to 75 mg kg⁻¹, with a mean concentration of 55 mg kg⁻¹. Generally, the background concentration of copper in surface soils is suggested between 14 and 109 mg kg⁻¹ (Kabata-Pendias, 2010). Moreover, according to Mackie, Müller, and Kandeler (2012), the concentration of copper in uncontaminated soil is suggested to be less than 20 mg kg⁻¹, while a concentration higher than 100 mg kg⁻¹ is indicative of mineral-rich soils.

Plants need copper as an activator and cofactor of enzyme reactions. This metal is also important in structural functions in nucleic acid metabolism, electron transfer, redox reactions, respiration, antioxidative defence, and photosynthesis (Ducic & Polle, 2005; Nagajyoti et al., 2010). Moreover, Ducic and Polle (2005) advised that copper is a redox-reactive element with an electrochemical potential of -260 mV. This potential means that the copper ion can be an electron carrier between -420 mV and +800 mV. Hence, with this potential, the copper ion is very reactive and important in redox reaction as well as being a potential toxic risk for plant cells.

As a micronutrient, copper is needed at narrow range of concentration, which is between 5 and 20 mg kg⁻¹ in 'normal' plants. A copper concentration below 4 mg kg⁻¹ is classified as deficient, while higher than 20 mg kg⁻¹ can be considered as toxic (Reimann et al., 2001). Although a 'normal' plant is suggested to have a safe copper concentration from 5 – 20 mg kg⁻¹, there are species which can accumulate at extremely high copper concentration in their plant tissues. One of these species is *Ipomoea alpine* which has been reported to accumulate up to 1.23 percent copper dry tissues. This plant is recognised as a Zairean copper hyperaccumulator (Brooks, 1998).

Phytotoxicity of copper in hemp is reported at 2.5 mM CuSO_4 solution or 400 mg L^{-1} Cu^{2+} (Arru et al., 2004). Exposure to a high copper concentration may affect plant growth reduction, especially roots. Roots are plant's first organ to be affected by high copper concentrations. Copper is suggested to interfere with root system development and inhibit root hairs forming (Nair & Chung, 2015). Root deformations due to toxicity may lead to necrosis, chlorosis, and growth restriction. These symptoms can be observed in affected plants (Ducic & Polle, 2005).

When exposed to a toxic copper concentration, plants have biochemical responses to reduce metal toxicity effects, through making complex metal ions, reducing metal influx, and enhancing antioxidant production through a detoxification process. Moreover, plants control metal uptake and transport through compartmentalization and sequestration in order to maintain copper concentrations at an acceptable level in tissues (Ducic & Polle, 2005). Based on relative metal absorption rates by plants, four common trace metals can be ordered as $\text{Cu} > \text{Zn} > \text{Co} > \text{Ni}$ (Mishra & Kar, 1974).

2.3.2. Nickel in plants

The nickel concentration in soil ranges from 0.2 to 5,000 mg kg^{-1} . A range of 20 and 50 mg kg^{-1} is recommended as the world mean concentration. The nickel concentration depends on geological conditions as well as soil texture. Soil rich in clay may contain more higher nickel than coarser soils (Alloway, 2012).

Plants use nickel as important component in urease, a nickel-containing metalloenzyme which has a role to generate urea as a nitrogen source (Sirko & Brodzik, 1999). Normally, plants take up Ni^{2+} , the ionic form of nickel from soils (Mishra & Kar, 1974). Mishra and Kar (1974) asserted that soil acidity increases the amount of exchangeable nickel content, and this can be absorbed and accumulated in the plants easily. Although nickel is essential for plant metabolism, 'normal' plants need nickel in very small amount, and it is described as an 'ultra-micronutrient'. On the other hand, nickel hyperaccumulator plants are observed to suffer nickel deficiency if be grown in 'normal' soil (Küpper & Kroneck, 2007).

Plants need nickel generally at a concentration range of 0.05 and 10 mg kg^{-1} dry weight. Phytotoxicity of nickel is suggested above 100 mg kg^{-1} . However, some plants have been reported to concentrate nickel higher than this concentration. A concentration less than 10 mg kg^{-1} dry weight is to be considered as threshold toxic concentration in sensitive species.

Whereas a moderately tolerant plant has nickel critical toxicity level higher than 50 mg kg⁻¹ dry weight (Alloway, 2012).

The effects of nickel toxicity appear at a concentration of 10 to 15 µg g⁻¹ in the plant tissues (Rascio & Navari-Izzo, 2011). Mohammadzadeh, Tavakoli, Chaichi, and Motesharezadeh (2014) observed an effect of nickel on the growth of sun flower, *Helianthus annuus* L. They concluded that nickel at a concentration of 450 mg kg⁻¹ in soil decreases plant growth, and also has an effect on iron and zinc uptake, and the photosynthetic content. Although nickel has been suggested as an essential element for vascular plants, its essentiality is still in debate. Some plants need nickel as a protection mechanism against the poisonous effects of urea in plant tissues. However, other species will grow well on substrate which has no nickel (Polacco, Mazzafera, & Tezotto, 2013; Ronchini et al., 2015).

2.3.3. Gold in plants

Gold is classified as a non-essential element in plant metabolism (Lambers, Pons, & Chapin III, 2008; Wilson-Corral, Anderson, Rodriguez-Lopez, Arenas-Vargas, & Lopez-Perez, 2011). Gold is described as a rare element on the earth's crust, and its solubility is low, thus the concentration of gold in plant tissues is suggested low compared to other abundant metals, such as copper and nickel (Babula et al., 2008). Gold concentrations in wild plants are approximately less than 1 – 2 ppb (Lintern et al., 2013). The maximum gold concentration in plants which are growing on gold-rich soils is 10 ng g⁻¹ (10 ppb) dry weight (Anderson, Brooks, Stewart, & Simcock, 1998).

However Lintern et al. (2013) reported anomalous gold uptake by native *Eucalyptus* trees at Freddo Gold Prospect, Western Australia. They found that *Eucalyptus* trees which thrive above a 35 meter deep gold deposit in southern Australia contain gold in their dried leaves and twigs at a concentration of 80 and 44 ppb respectively. Transpiration mechanisms can lead to gold transport in plants (Girling & Peterson, 1980). As reported by Lintern et al. (2013), gold accumulation in *Eucalyptus* trees can happen if gold is in a water-soluble form (ionic). In addition, according to Girling and Peterson (1980), cyanogenic species are believed to promote gold uptake through the exudation of cyanide in root zones.

2.3.4. Palladium in plants

Platinum Group Metals (PGMs) is a group of six rare metals which possess similar chemical and physical characteristics, such as palladium (Pd), platinum (Pt), ruthenium (Ru), rhodium (Rh), iridium (Ir) and osmium (Os) (Ravindra, Bencs, & Van Grieken, 2004). Currently, the PGMs are widely used in the automotive industry as a catalytic converter to reduce vehicle emissions (Glaister & Mudd, 2010). In metallic form, PGMs have low reactivity and no toxic effects for living organisms (Palacios, Gómez, Moldovan, & Gómez, 2000).

However, a study of toxicity effects of palladium in endive plants *Cichoriu mendivia* var. *Crispum* was reported by Alt et al. (2002). Two days after treatment, the trial plants showed severe stress symptoms, at a level of 8.7 ng g^{-1} palladium in the plant tissues. In plant systems, palladium tends to bound with high molecular-weight proteins and also may cause chlorophyll decomposition, DNA damage and necrosis (Alt et al., 2002; Gagnon, Newkirk, & Hicks, 2006).

Kabata-Pendias (2010) proposed that from a geochemical perspective palladium is chemically reactive, thus palladium can readily have association with other minerals. The author suggested that palladium therefore has potential risks to the environment. In addition, toxicity of PGMs increased under acidic environment, especially when chloride ions are present (Colombo, Monhemius, & Plant, 2008a).

2.4. Natural hyperaccumulation vs Induced hyperaccumulation

2.4.1. Natural hyperaccumulation

A high concentration of metal accumulated by normal plants is generally found in root systems. In contrast, hyperaccumulator species have an ability to translocate a high heavy metal concentration into aboveground biomass. Thus, the metal content of hyperaccumulator plants are higher in the leaves than in the roots (Krämer, 2010). Most studies have reported that natural hyperaccumulation tends to occur for metals that are readily available for plant uptake. Sheoran, Sheoran, and Poonia (2013) mentioned that microbial activity and plants that exude cyanogenic compounds in soils will increase metal solubility and mobility.

The term hyperaccumulation was coined by Brooks et al. (1977). These authors proposed that a hyperaccumulator is a plant that concentrates greater than $1,000 \text{ } \mu\text{g g}^{-1}$ of nickel dry weight

in leaves in natural habitat. Further criterion for hyperaccumulator species have been suggested by Reeves as cited in van der Ent, Baker, Reeves, Pollard, and Schat (2012). Reeves proposed that a hyperaccumulator is a plant which can accumulate minimum $1,000 \mu\text{g g}^{-1}$ of nickel dry weight in any aerial part tissue in natural environment. In addition, van der Ent et al. (2012) proposed some additional criterions: (1) the aerial part tissues should be limited to leaves, and (2) the hyperaccumulation occurred from active metal translocation from the root systems to leaf tissue. Furthermore, the hyperaccumulation appellation has also been suggested for other metals, such as As, Cd, Co, Cr, Cu, Mn, Pb, Se, Zn (van der Ent et al., 2012). Krzciuk and Galuszka (2014) determined that about 500 plant taxa are hyperaccumulators, and about 90 percent of discovered hyperaccumulators are endemic to ultramafic ecosystems.

2.4.1.1. Copper hyperaccumulator

An observation in the DR Congo reported that copper and cobalt hyperaccumulators are abundant in the Copper Belt region (Brooks, Reeves, Morrison, & Malaisse, 1980). The first definition of a copper hyperaccumulator is a plant which accumulate nickel higher than $1,000 \mu\text{g g}^{-1}$ dry weight (Malaisse, Gregoire, Brooks, Morrison, & Reeves, 1978). They demonstrated that *Aeolanthus biformifolius* De wild. (Labiateae) from Shaba Province contained copper up to 1.37 percent dry weight. A more recent study showed that copper hyperaccumulators also can be found outside Africa, such as in China, Sri Lanka, and Indonesia (van der Ent et al., 2012).

Some attempts to review copper threshold levels have been conducted by some researchers, such as Krämer and van der Ent. According to Krämer (2010), in normal plants copper concentration of $1 - 5 \mu\text{g g}^{-1}$ is suggested as a critical deficiency level, and concentration of $20 - 30 \mu\text{g g}^{-1}$ as a critical toxicity level (Krämer, 2010). Based on these values, van der Ent et al. (2012) proposed the toxicity threshold level of Cu accumulation in dried leaves is 300 microgram per gram. Bhargava, Carmona, Bhargava, and Srivastava (2012) reported at least 34 hyperaccumulator species have been discovered. These species include three families, namely Commelinaceae, Convolvulaceae, and Crassulaceae.

2.4.1.2. Nickel hyperaccumulators

The number of reported plant species to hyperaccumulate nickel is much greater than copper because of the wide distribution of nickel-rich soils compared to copper-rich soils. As noted in van der Ent, Baker, van Balgooy, and Tjoa (2013), approximately 400 nickel hyperaccumulators have been reported as of 2012. The families of nickel hyperaccumulators are Phyllanthaceae, Rubiaceae, Salicaceae, Brassicaceae, Sapotaceae, Asteraceae, and Sapotaceae (Bhargava et al., 2012; van der Ent et al., 2013).

Reeves, Brooks, and Macfarlane (1981) described that maximum concentration of nickel in 'normal' plant species as 10 microgram per gram dry weight. For general plants, nickel concentrations in the tissues ranged from 10 to 100 microgram per gram. In contrast, nickel hyperaccumulator species *Alyssum* sp. are able to accumulate nickel to a concentration of 1,000 to 25,000 $\mu\text{g g}^{-1}$ dry matter. In addition, Brooks et al. (1977) proposed two criterion for nickel hyperaccumulation. Plants which contain a level of nickel between 100 and 1,000 $\mu\text{g g}^{-1}$ on dry weight basis are classified as strong accumulators. Plants containing nickel more than 1,000 $\mu\text{g g}^{-1}$ in dry weight are called hyperaccumulators.

2.4.1.3. Gold natural accumulation

Under natural conditions, the occurrence of gold in earth's crust in the Au (0) form is very low. Au (0) is less soluble than other base metals (Sheoran et al., 2013). Earlier researches have reported gold accumulation in wild plants. Such work was started by Lungwitz in 1900 as cited in Erdman and Olson (1985). Lungwitz found gold in samples of ash prepared from trees and tree organs. He suggested that gold accumulation in plants was sourced from dissolved gold in water.

Recently, a field study at Freddo Gold Prospect, Western Australia confirmed that *Eucalyptus* trees absorb gold solution from gold deposit about 30 meters underground. This study quantified gold concentrations in dried leaves and twigs at 80 and 44 ppb respectively, while the background gold concentration in soil was 6 ppb (Lintern et al., 2013).

The above facts demonstrate that plants are able to uptake gold. However to date, there are no known wild plant species that hyperaccumulate gold in their biomass. Anderson, Brooks,

Stewart, and Simcock (1999) mentioned that natural gold uptake in native plants grown in a gold rich habitat may not exceed 10 ng g⁻¹ in dry matter. Moreover they asserted that gold concentrations more than 10 ng g⁻¹ may be caused by dust contamination and that airborne contamination may affect gold uptake analysis. Thus, Anderson, Moreno, Geurts, et al. (2005) defined gold hyperaccumulators to be a plant that concentrates 1,000 ppb gold dry weight.

2.4.1.4. Natural accumulation of palladium

A study on natural palladium accumulation as a result of biogeochemical plant uptake was noted in Kothny (1979). In his report, Kothny reported natural palladium uptake in plants was in the range of 30 – 400 µg kg⁻¹. The highest palladium concentration was observed from *Quercus chrysolepsis*, while the concentration of palladium in soil was 140 ppb. The quotient ash:soil for palladium was 2.8.

Nemutandani et al. (2006) observed metal accumulation in *Berkheya coddii* and described its potential for phytoextraction. *Berkheya coddii* was sampled from a nickel-rich area in Mpumalanga, South Africa. Their results suggested that in natural conditions, *B. coddii* can accumulate 22 metals. Moreover, this plant was suggested to extract palladium in leaves and roots at a concentration of 710 ppb and 180 ppb respectively. The background concentration of palladium in sampling sites was 70 ppb. Therefore the author concluded that natural palladium uptake occurred in *B. coddii* with a leaves:soil ratio of 10.1.

It is likely that the highest palladium concentration in plant as natural occurrence was reported from a biogeochemical survey in the Stillwater Complex, Montana in 1986. *Pseudotsuga menziesii* was quantified to concentrate palladium at a range of 400 to 15,000 ppb ash weight (Brooks, 1992).

2.4.2. Induced hyperaccumulation

Precious metals such as platinum, palladium, and gold have low solubility in the natural environment, hence these metals are not absorbed by plants (Anderson, Meech, Veiga, & Krisnayanti, 2014). Passive metal accumulation through induced hyperaccumulation occurs when a plant is forced to absorb metals after soil amendments are applied to soil (Anderson,

2000). This process is the opposite mechanism to that which naturally occurs in metal-extractor species.

The solubility of target metals which have low solubility in soil solution can be increased by using chemicals as soil amendments. Some chemicals which are commonly being used for this method including thiosulphate (Msuya, Brooks, & Anderson, 2000), thiocyanate (Anderson, Brooks, Stewart, & Simcock, 1999), and potassium cyanide (Anderson, Moreno, & Meech, 2005). Those chemicals oxidise target metals (i.e gold and palladium) and form complex ions. From these complex ions, the target metals can be extracted from soils and accumulated in plants through transpiration mechanisms (Koptsik, 2014; Walton, 2002).

Induced hyperaccumulation was firstly reported by Huang and Cunningham (1996) who made lead soluble in soil with HEDTA and induced uptake into the aboveground parts of *Zea mays*. In this trial the trial plants were reported to accumulate lead up to 10,600 mg kg⁻¹ (1 percent). Following this success, further attempts to induce metal accumulation in non-hyperaccumulator plants were undertaken.

Induced hyperaccumulation is aimed to enrich target metals with low solubility in plants. However, the soil amendments also make other metals more available to plants. Hence, all now available metals will flood plants tissues and will promote metal toxicity symptoms, such as chlorosis and necrosis.

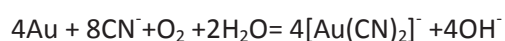
2.4.2.1. Soil Amendments

Soil amendments are the class of compounds used to increase the mobility of target elements in soil substrates. According to Robinson et al. (2003), soil amendments, as well as fertilizers, and surfactants are expected to increase metal availability in the soil solution, microbial activity, as well as plant growth. A range of chemicals are suggested to be suitable candidate for soil amendments to induce hyperaccumulation. Research by Sakambari (2015) focused on the comparison of 10 chemicals to extract metals from soils. The list of chemicals included cyanide, humic acid, thiocyanate, and hydrochloric acid.

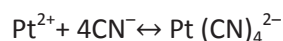
Cyanide is very reactive and will complex with many metal cations to create metal-cyanide complexes. Based on bonding strengths between cyanide and metal ions, according to Rajat, David, and George (2005), there are two types of complex of metal-cyanide: (1) strong metal-

cyanide complexes, and (2) weak metal-cyanide complexes. Strong metal-cyanide complexes, are gold cyanide ($\text{Au}(\text{CN})_2^-$), platinum cyanide ($\text{Pt}(\text{CN})_4^{2-}$), and cobalt cyanide ($\text{Co}(\text{CN})_6^{3-}$). Weak metal-cyanide complexes include copper cyanide ($\text{Cu}(\text{CN})_3^{2-}$), nickel cyanide ($\text{Ni}(\text{CN})_4^{2-}$), and cadmium cyanide ($\text{Cd}(\text{CN})_4^{2-}$).

Cyanide is noted as the best chemical to perform induced hyperaccumulation of precious metals in plants. Anderson (2013) noted that plants concentrate as much as 0.8 percent gold dry weight after cyanide amendment. Moreover, cyanide is commonly used in the mining industry because gold and cyanide form a stable complex, require less chemical treatments than heat-based processes, and lower cost (Johnson, Grimes, Leinz, & Rye, 2008). When free cyanide (CN^-) is present in gold containing substrates, a chemical reaction to form gold-cyanide complex can be written as follows (Anderson, 2005):



A small cyanide concentration up to a few μg per gram can create mobile cyanide complexes with other metals as occurs for the gold-cyanide complex. Such metals are palladium, platinum, iron, and copper. The formation of PGM – cyanide complexes has been described by Reith, Campbell, Ball, Pring, and Southam (2014), according to the following reaction:



Hence, cyanide application is expected to increase the solubility of PGMs and gold in soil solution which can subsequently be absorbed into the aerial parts of plants.

2.4.2.2. Gold induced hyperaccumulation

Naturally, gold is insoluble, and thus its bioavailability is low (Anderson, Brooks, Stewart, Simcock, & Robinson, 1999). However, gold from soils and minerals can be solubilised naturally through microbial activity and cyanogenic plants (Wilson-Corral et al., 2011).

The first attempt to solubilise gold and make it available for plant uptake has been reported by Anderson et al. (1998). The authors solubilised gold using thiosulphate and thiocyanate. After treatments, they demonstrated that the chemicals made gold more soluble and uptake could be induced into *Brassica juncea*. The results showed *B. juncea* to uptake 57 mg kg^{-1} Au dry weight (57,000 ppb). Referring to the definition of gold hyperaccumulation (1,000 ppb), the

result suggested that gold hyperaccumulation occurred in *B. juncea* after thiocyanate treatment.

Another trial of thiocyanate-induced gold hyperaccumulation was reported in Msuya et al. (2000). The study observing root crop performance to extract gold from an artificial soil containing 3.8 mg kg⁻¹ of elemental gold. Plant groups with thiocyanate amendment extracting more gold in the roots than plant groups with thiosulfate. The highest reported gold accumulation concentration was 220 mg kg⁻¹ in *Raphanus sativus* 'Salad radish' with thiocyanate treatment, and 189 mg kg⁻¹ dry weight in *Daucus carota* 'Top weight carrot' with thiosulfate amendment. This finding is higher than previously reported for *B. juncea*, with its maximum value was 57 mg kg⁻¹.

The toxicity of thiocyanates and thiosulfates is relatively low, and both are unlikely to cause significant environmental problem because their half-life is about 6 months in the environment (Msuya et al., 2000). Moreover, compared to cyanide, thiocyanate is approximately five hundred times less toxic (Anderson, Brooks, Stewart, & Simcock, 1999). In practice, soil amendment to promote induced hyperaccumulation is applied when a plant reaches its maximum growth, and usually one week before harvesting (Koptsik, 2014).

2.4.2.3. Palladium induced hyperaccumulation

Knowledge about palladium induced hyperaccumulation in plants is limited. There is some works focussed on the toxicity effects and distribution of palladium in the environment. For instance, the effect of palladium on living organisms as an impact from automotive derived contaminations has been researched (Colombo et al., 2008b).

Enhancing the solubility of platinum group metals and their uptake in plant's biomass is theoretically possible and was suggested in 1999 (Anderson, Brooks, Stewart, Simcock, et al., 1999). However, phytoextraction trials of palladium uptake appear to only have been done in 2002. This first (and only) trial to examine induced palladium hyperaccumulation in plants was reported in Walton (2002). In this trial Walton examined the potential of metal extraction from Klipfontein mine tailings (South Africa) using *Brassica juncea* and *Berkheya coddii*. The two plant species were treated with thiourea, thiocyanate, and potassium cyanide. From this work Walton concluded that 10 g L⁻¹ potassium cyanide induced more palladium uptake in

plant trials. About 7,677 ppb palladium was detected in *B. coddii*, while the palladium concentration in the soil sample was 315 ppb.

2.5. Form of palladium and gold in plants

Previous sections have described how plants have capability to accumulate metals in high concentration in their living tissues. Metal uptake mechanisms may occur naturally or through induced hyperaccumulation by using chemicals. The first report about gold structure in plants was reported by Dunn as cited in Anderson, Bhatti, Gardea-Torresdey, and Parsons (2013). Dunn asserted that a crystalline structure of gold with diameter of 0.25 μm can be found in *Picea mariana* from natural uptake.

In 2002, the first attempt to study the form of gold in living tissues of Alfalfa plants grown in a gold chloride rich media was conducted by Gardea-Torresdey et al. (2002). The Alfalfa plants were grown on agar medium growth, and Au^{3+} solutions were added at various concentrations. Their results described how plants can reduce Au^{3+} and uptake $\text{Au}(0)$ from agar solid medium. Further analysis on the $\text{Au}(0)$ forms indicated that gold nanoparticles existed in the living tissues. They reported gold nanoparticles size between 2 and 40 nm in diameter. Following this finding, many observations related to precious nanoparticles biosynthesis by using plants have been reported. For an overview of these works refer to Table 2.2.

Table 2.2 Study on precious metals uptake in plant's living tissues: nanoparticle synthesis in plant tissues

Nanoparticles	Plant	Size	Concentration	Growth medium	Key finding	Reference
Gold	<i>Medicago sativa</i>	2 – 40 nm	N/A	Agar	Au (0) nanoparticles can be synthesised in the living plant.	Gardea-Torresdey et al. (2002)
Gold	<i>Brassica juncea</i>	5 – 50 nm	760 µg/g	Soil	Possibility of Au-Cu-Ag class of alloy synthesis by using vascular plants.	Haverkamp, Marshall, and van Agterveld (2007)
Gold	<i>Sesbania drummondii</i>	6 – 20 nm	Root: 1,000 – 9,000 mg/kg Shoot: 10 – 85 mg/kg	Chloroaurate solution	First report on gold synthesis in plant	Sharma et al. (2007)
Silver, Gold	<i>B. juncea</i>	Au: 2 – 100 nm (leaves, stems) Au: 2 – 40 nm (roots) Ag: 4 – 6 nm (stems) Ag: 10 – 30 nm (roots)	N/A	Hydroponic	Au nanoparticles occurred densely around chloroplast.	Beattie and Haverkamp (2011)
Gold	<i>Eucalyptus trees</i>	8 µm	Leaves: 80 ppb Twigs: 44 ppb Bark: 4 ppb	Soil	Au particle formation in natural condition.	Lintern et al. (2013)
Gold	<i>B. juncea</i>	50 nm – 2 µm	Shoot: < 10 – 3,612 mg/kg Root: 10.2 – 2,224 mg/kg	Soil	Investigate Ag and/or Cu effect on Au synthesis	Anderson et al. (2013)
Gold, Palladium	<i>Berkheya coddii</i>	N/A	Gold: 1,600 ppb Palladium: 7,700 ppb	Soil	First trial on Pd uptake in controlled environment	Walton (2002)
Palladium	<i>Arabidopsis</i>	3 – 32 nm	N/A	Liquid culture	First Pd synthesis in living plants.	Parker et al. (2014)
Palladium	<i>Pisum sativum</i>	N/A	Stems: 0.03 – 1.2 µg/g Leaves: 2 µg/g Fruits: 0.01 – 0.34 µg/g	Vermiculite	Uptake of Pd and its spatial distribution in higher plants	Ronchini et al. (2015)

N/A: not applicable

2.6. What is Phytoextraction?

Plants possess ability to take up metals from soil. Discoveries and observations of metal accumulation in plant tissues were first reported in 1935 by Byers and in 1948 by Minguzzi and Vergnano (Lasat, 2000). The absorbed metals are transported and accumulated in plant's living tissues. These process make plant's tissues a living storage of metals. Extraction of stored valuable metals therefore becomes a real possibility when a plant reaches maturity. Accumulated metals are mainly located in aerial parts, such as leaves and shoots. The next challenge is then to harvest aerial parts and burn those materials to liberate particular metals from plant tissues. At large scale, it has been proposed that harvested plant materials of "bio-ore" may be sent to smelters and also generate power through the combustion process (Robinson, 1997).

The aim of phytoextraction is to exploit plants to uptake particular metals from soil. The term of phytoextraction is derived from phyton, a Greek word which is connected to plant, and extraction, of Latin origin meaning an action to take out with effort. According to Ernst (1996), interaction between plants and soil affects the success of phytoextraction. In practice, phytoextraction can be implemented for different purposes, such as for mining (phytomining), pollution remediation (phytoremediation), and the current approach for catalysts (phytocatalyst for the Phytocat project). For example, phytoremediation aims to reduce heavy metal pollution, such as lead, cadmium and zinc to meet acceptable value (Chang, Ko, Tsai, Wang, & Chung, 2014). On the other hand, Anderson, Moreno, and Meech (2005) demonstrated a successful field trial of gold phytomining using *Zea mays* and *Brassica juncea*.

Criteria of a plant species that is suitable for metal extraction includes the ability to accumulate and tolerate a high concentration of heavy metals in living tissues; fast growth rate; extensive root system; high biomass at harvestable parts; and easy to harvest (Yang, Feng, He, & Stoffella, 2005). Therefore it is important to choose a suitable plant species for use in phytoextraction; as Alloway and Jackson (1991) described, various maize cultivars differ significantly in metal absorption. Despite plant factors which are clearly important, soil properties also have relevant effects in phytoextraction. Such soil properties are pH, metal speciation in soil, soil carbonates, hydrous oxides, and organic matter (Alloway & Jackson, 1991).

2.6.1. Phytomining

Phytomining is an application of phytoextraction which targets valuable metals such as gold and palladium. There are three main processes in phytomining: (1) grow high-biomass and fast-growing plant species on soils which contain low-grade metals, (2) harvest above ground parts, and (3) incinerate the biomass to recover valuable metals. Plants which accumulate metals translocate soluble metals from soils to their aerial tissues (Sheoran et al., 2009). Phytomining methods can be used to recover metals from low-grade ores in mining operations (Anderson, Brooks, Stewart, & Simcock, 1999). The source of low-grade ores can be from mine tailings waste or metal-rich soils (Wilson-Corral et al., 2011). In a natural environment, metals occur in various concentrations. For instance, the concentration of gold and the platinum groups metals (PGMs) can be found from a few grams per ton to percent concentrations for the base metals like copper, nickel, and iron (Hunt et al., 2014).

Basically, phytomining can be done for all types of metals. However, less valuable metals are not suitable for phytomining operations. To generate profits, a phytomining operation tends to focus on valuable metals which can overcome production costs. Moreover, concentrations of valuable metals in natural soil substrates may vary. In order to achieve economical metal concentrations in the harvestable parts, a substrate modification is needed (Brooks, Chambers, Nicks, & Robinson, 1998). This approach is needed only if no hyperaccumulators are known. Thus, Brooks et al. (1998) suggest three main parameters to value economic feasibility of phytomining operations. Such parameters are the metal price, the highest accumulated metal in the biomass, and the plant biomass production. According to Anderson, Moreno, and Meech (2005), to gain profit, a gold phytoextraction is required to yield at least 1 kg of gold per hectare. Such a target can be achieved from minimum biomass production of 10 tonnes in 1 hectare, and gold accumulation of 100 mg/kg on their harvestable parts.

Phytomining field trials have been attempted by the US Bureau of Mines by using *Streptanthus polygaloides*, a plant species which hyperaccumulates nickel (Brooks et al., 1998). The 'crop' of nickel is grown on soil uneconomic for conventional mining, which contains 0.35% nickel. It is assumed that *S. Polygaloides* may extract a minimum 1% nickel in dry mass. Thus, the potential return of a nickel phytomining is expected about \$513 per hectare.

In 1998, a successfully phytomining field trial was demonstrated in Rustenburg, South Africa (Anderson, Brooks, Stewart, Simcock, et al., 1999). The field trial was using *Berkheya coddii*, the nickel hyperaccumulator from South Africa to extract nickel from area near the Anglo-

American Platinum Corporation (Amplats) refinery. The trial was successful to recover a pure form of nickel from plant tissues as “green-ore”. This result showed that it is a real possibility to extract pure metal from plant’s tissues.

2.6.2. Plant species for phytomining

Preceding sections have defined phytoextraction and discussed its application for valuable metals extraction. The Phytocat project targets the extraction of copper, nickel, gold, and palladium in plant’s biomass. In this final section of the Introduction, the two species *Brassica juncea* and *Cannabis sativa*, plant species which are considered fit to phytoextraction’s criteria and which are exploited in the current study, will be described.

2.6.2.1. Indian mustard (*Brassica juncea*)

Brassica juncea, also known as Indian mustard, has been cultivated since 2000 BC. This plant is an annual crop and has a good adaptation to various environmental conditions. Therefore, this species can be found from mountainous to coastal areas. *Brassica juncea* is cultivated for vegetable, oilseed, green manuring, fodder, medicinal and spice use (Hammer, Gladis, Laghetti, & Pignone, 2013).

Brassica sp. produces high biomass up to nine ton per hectare and the root reaches to depths of two meters. With these high biomass yields, this plant can be used as a cover crop. Depending on the soil environmental conditions, *Brassica* can cover up to 80 percent of the soil surface. Thus, *Brassica* is well-suited for erosion control (Chen, Clark, Kremen, Lowley, & Price, 2007). A cover crop like *Brassica* is useful to increase nitrogen and organic carbon contents in soil. *Brassica* also well-known as a non-leguminous plant which can preserve nitrogen in the soil (Smith & Brennan, 2005).

Furthermore, *B. juncea* is suggested to have some advantages, such as tolerance to heat and drought, ability to grow in a poor environment, and a need for less water than other crops (Edwards, Salisbury, Burton, Hopkins, & Batley, 2007). Based on these given characteristics, *B. juncea* is suggested to be able to thrive in metal-rich soils.

In addition, *B. juncea* has been used for gold phytomining trials, due to its fast growth rate and high biomass (Anderson et al., 1998). They reported the maximum gold accumulation in dried leaves of *B. juncea* was 57 µg per g. Induced hyperaccumulation is necessary to allow the plant to be used for gold phytomining. In order to achieve valuable results in a phytomining operation, they proposed a minimal accumulation of 17 µg gold per g dry mass in *B. juncea*.

2.6.2.2. Hemp (*Cannabis sativa*)

Cannabis sativa, alternatively known as hemp, has been suggested as the oldest cultivated crops in human civilization (Amaducci et al., 2014). Historical records note that hemp has been used in Chinese medications since 2727 BC (Boulloc, Allegret, & Arnaud, 2012). Moreover, *C. sativa* has been used for various purposes; for cannabinoids, fiber production and seed (Werf, Mathussen, & Haverkort, 1996). In the modern day, hemp has become an alternative resource in construction industries, textile production, papermaking, as well as automotive industries (Boulloc et al., 2012). However, *C. sativa* contains psychotropic compounds. Therefore the use of this plant is restricted by many government regulations.

Hemp cultivars are reported to contain delta-9-tetrahydrocannabinol (THC), the narcotic compound, and range between 2 and 56 per cent (Townshend, Boleyn, McGill, & Rowarth, 2010). Thus, hemp cultivation in New Zealand is regulated under the Misuse of Drugs Act, No. 116 (1975) Schedule 3 Part 1. Moreover, the Ministry of Health regulates that cultivation of industrial hemp in New Zealand is only permitted for varieties with a THC content less than 0.35 percent (Ministry of Health, 2010). In the current research, two varieties of hemp have been exploited and research into the THC content in the Fasamo cultivar has defined an average result of 0.09 percent and in the Ferimon cultivar 0.14 percent (Small & Marcus, 2003). According to their THC contents, Fasamo and Ferimon, as used in the current work, are acceptable for trial use.

Cannabis is a dioecious plant, this means that male and female flowers grow on separate plants (Griga & Bjelková, 2013). Generally, the male plants are less durable than the female plants. The male plants will deteriorate after flowering and produce less fiber and seeds (Boulloc et al., 2012). *Cannabis* can grow up to 5 m tall in four to six month in a good environment condition, such as enough sunlight, good soil drainage, and sufficient water and nutrients. In an arid environment, hemp matures in a much reduced timeframe, will grow to a

height of 20 cm, and has a small amount of leaves. When grown in close distance, approximately 100 plants per square meters, hemp grows tall and thin (Griga & Bjelková, 2013).

Work by Angelova, Ivanova, Delibaltova, and Ivanov (2004) suggested that fiber crops, such as hemp, flax and cotton can extract metals from contaminated soil. They found that these crops have different metal concentrations in their vegetative organs. According to their study, hemp accumulates a high concentration of heavy metals in the flowers, while cotton stores metal in the leaves, and flax in the seeds. For example, a copper concentration of $10.2 \text{ mg kg}^{-1} \text{ Cu}$ was measured in hemp flowers. In comparison, concentrations in the leaves are $3.6 \text{ mg kg}^{-1} \text{ Cu}$, and in the seeds are $8.9 \text{ mg kg}^{-1} \text{ Cu}$. Furthermore, they concluded that hemp, flax, and cotton are tolerant to heavy metals and are suitable to be planted in soil which contains a high level of heavy metals.

Cannabis sativa has previously been a research object to evaluate the potential of induced hyperaccumulation (Kos & Leštan, 2004). In this trial, Kos and Leštan found that *C. sativa* can accumulate as much as $1,750 \text{ mg kg}^{-1} \text{ Pb}$, $1,300 \text{ mg kg}^{-1} \text{ Zn}$, and $7.2 \text{ mg kg}^{-1} \text{ Cd}$, after application of the biodegradable chelator, [S,S]-stereoisomer of ethylenediamine-disuccinate. Furthermore, Kos, Greman, and Leštan (2003) implied, that *C. sativa* produces approximately 20 to 30 tonne dry biomass per hectare. The authors asserted that hemp produces a high biomass and is a good candidate for phytoextraction.

2.7. Purpose of the current study

Studies into gold phytomining have been conducted since the first gold extraction greenhouse Anderson et al. (1998) and field trials were reported (Anderson, Moreno, & Meech, 2005). Knowledge about gold induced hyperaccumulation in plant species is well established. However, very little is known about palladium uptake in plant species. The first attempt to elucidate this gap was carried out by Walton (2002). His trial confirmed that *B. coddii* can accumulate 7,700 ppb palladium from a substrate containing 315 ppb palladium. However the growth substrate on Walton's work contained low soluble copper.

The purpose of this study described in this thesis was to investigate plant growth performance on gossan soils from Broken Hill which contain a high concentration of palladium and copper, to identify potential plant species to be used for palladium phytoextraction, and to achieve a palladium uptake concentration of $1,000 \text{ } \mu\text{g g}^{-1}$ in suitable plant species.

Chapter 3 - Materials and Methods

3.1. Introduction

A general geochemical review of platinum group metals (PGM) ore bodies worldwide shows that soil-like media associated with mineralisation containing sulphide materials is not expected to be suitable for plant growth trials because of salinity, low pH level, and the potential for metal toxicity. However, a previous geochemical survey of Broken Hill, Australia suggests the presence of soils of oxidised PGM rich rock that has potential to be more suitable for plant growth trials.

In the 1990's, a 120 kg sample of suspected PGM rich rock was collected from Mulga Springs, Broken Hill with the intended purpose of using this rock as an PGM reference material. Results of analytical work reported metal contents as follows: 0.71% copper, 0.34% nickel, 0.57 gram per tonne gold (ppm), 50 gram per tonne palladium, 3 gram per tonne rhodium, 3 gram per tonne osmium, 4.4 gram per tonne iridium, and 2 gram per tonne ruthenium (Meech & Anderson, 2014). Following from this initial report, in 2014, rock, soil, and plant samples were collected from the same location by the Phytocat project for purposes of this study. Sixty kg of rock chips and pieces were collected and transported to Massey University.

3.2. Site description

Broken Hill is located in New South Wales, Australia (see Figure 3.1). Broken Hill area is an arid environment with an annual rainfall of approximately 251.3 mm. Mean number of days with rainfall more than 1 mm is about 28 days per year. The mean temperature ranges from 11.6 to 24.5 degrees Celsius (Bureau of Meteorology, 2015).

This area has been well-known since the early 20th century as one of the most mineralised ore bodies on earth, and is suggested as to be as old as 100s of millions of years. The Broken Hill Mineral complex is a sulphide deposit with a considerable quantity of the world's known zinc, silver and lead (Birch, 2007). Geological surveys on the Broken Hill area in the 1970's and 1980's suggested the prospect of significant copper, nickel and platinum group elements (PGE) associated with ultramafic rocks at the Mulga Springs and Mt Darling locations. The Mulga Springs area contains very high grade mineralisation of PGE, copper, nickel, and gold. The

highly mineralised area is located on a basal contact of an olivine rich cumulate ultramafic structure. Hence, the Broken Hill area is also believed to have notable potential for bulk tonnage of precious metals such as gold and PGM, as well as copper and nickel (Golden Cross Resources, 2013).



Figure 3.1 Gossan sampling location is marked with distance about 20 Km from Broken Hill township. The study location is situated in New South Wales, Australia as indicated in inset map.

3.3. Methods for metal analysis

Metal analysis was conducted in the laboratory of the Soil and Earth Sciences Group, Massey University. Analysis for copper and nickel was conducted by using Flame Atomic Absorption Spectrometry (FAAS), while analysis for gold and palladium was conducted using Graphite Furnace Atomic Absorption Spectrometry (GFAAS).

3.3.1. Plant sample preparation

Analysis of plant samples followed the methodology used by Anderson, Moreno, and Meech (2005). Plant samples (0.1 gram) were weighted, put into borosilicate digestion tubes and ashed in a furnace at 525 °C overnight. The resulting ashes were digested with 2 mL of nitric-hydrochloric acid (*aqua regia*) in a hot water bath for one hour. The digested samples were transferred into 10 mL sample tubes, and made to 10 mL with 2M HCl. Before analysis, samples were split into 2 aliquots each of 5 mL for separate measurement using FAAS and GFAAS.

3.3.2. Soil sample preparation

Analysis of soil samples was conducted using the digestion method described by Anderson, Moreno, and Meech (2005). Soil samples (1 gram) were weighed, and put into cation digestion tubes, followed by adding 10 mL of nitric-hydrochloric acid and left to stand overnight. The samples then were digested in a block digester at 120 °C to volume of 3 mL. About 5 mL of concentrated HCl was added and digested further to final volume of 3 mL. After digestion, the samples were made up to 25 mL with deionised water and then vortex mixed. Mix solutions were filtered through Wattman Filter Paper (42) and transferred into P35 tubes to be analysed in FAAS and GFAAS.

3.3.3. Methods for copper and nickel analyses

A GBCAvanta Sigma Flame AAS (GBC Scientific Equipment, USA) was utilised for analysing copper and nickel from soil and plant samples. Metal concentration was determined directly on samples described in section 3.3.1 and 3.3.2. Table 3.1 describes settings for FAAS, and defines the standard solutions used for this analysis see Tables 3.2 and 3.3.

Table 3.1 Methods for flame atomic absorption spectroscopy (FAAS)

Setting	Element	
	Copper	Nickel
Lamp current (mA)	3.0	4.0
Wavelength (nm)	324.7	351.5
Slit width (nm)	0.5	0.5
Flame type	Air-Acetylene	Air-Acetylene
Workhead height (mm)	12.5	15.0

Table 3.2 List of standard solutions

Metal	Standard solution	Concentration	Manufacturer
Copper	Cu (NO ₃) ₂ in HNO ₃ 0.5 mol/L	1001 ± 2 mg/L	Merck - Germany
Nickel	Ni (NO ₃) ₂ in HNO ₃ 0.5 mol/L	1001 ± 2 mg/L	Merck - Germany
Gold	H(AuCl ₄) in HCl 2 mol/L	1001 ± 5 mg/L	Merck – Germany
Palladium	Palladium atomic absorption	1011 ppm of Pd in 5.1 wt% HCl	Sigma – Aldrich, Inc. USA

Table 3.3 Series of standard concentrations

Standard	Nickel (ppm)	Copper (ppm)	Gold (ppb)	Palladium (ppb)
1	10	5	20	20
2	20	10	40	40
3	40	15	60	60
4	60	20	80	80
5	80	25	100	100

3.3.4. Methods for gold and palladium analyses

To analyse gold and palladium in plant and soil samples, graphite furnace atomic absorption spectroscopy (PerkinElmer, Inc. USA) was used. Extraction of precious metals was conducted by adding 0.1 mL of 100% potassium iodide into 5 mL of samples, followed by adding 2 mL of Methyl iso-Butyl Ketone SpS (Romil, England), and mixing the solutions. After mixing, the organic layer of MIBK remained on top of the solutions. This layer contained gold and palladium. Approximately 1 mL of the organic layer was transferred into GFAAS analysis cups. Calibration for analysis was performed using a 100 ng mL⁻¹ gold or palladium solution which was automatically diluted to prepare a standard curved. These solutions were prepared to run automatic dilution series during the analysis. Settings for GFAAS are described in Tables 3.4, 3.5 and 3.6. For the standard solutions used for this analysis see Tables 3.2 and 3.3.

Table 3.4 Methods for graphite furnace atomic absorption spectroscopy (GFAAS)

Setting	Element	
	Gold	Palladium
Wavelength (nm)	242.8	247.6
Slit width (nm)	0.7L	0.7L
Measurement signal	Peak area	Peak area

Table 3.5 Furnace program for gold test

Step #	Temperature (°C)	Ramp time	Hold time	Internal flow	Gas type
1	110	1	30	250	Normal
2	130	15	30	250	Normal
3	800	10	20	250	Normal
4	1800	0	5	0	Normal
5	2450	1	3	250	Normal

Table 3.6 Furnace program for palladium test

Step #	Temperature (°C)	Ramp time	Hold time	Internal flow	Gas type
1	110	1	30	250	Normal
2	130	15	30	250	Normal
3	900	10	20	250	Normal
4	2200	0	5	0	Normal
5	2450	1	3	250	Normal

3.4. Quality control

Certified Standard Reference Material PTM-1a and internal plant standards were used to assess measurement quality. Analytical results from each reference material are illustrated in Table 3.7. Results for copper and nickel were in a good agreement with the certified Standard Reference Material. In addition, internal plant standards were used to validate analytical accuracy for Au analysis. Results of gold analyses from two internal plant standards were 34 mg kg⁻¹ (*Brassica juncea*), and 11 mg kg⁻¹ (*Zea mays*). These values were in a good agreement to previous publication after Anderson, Moreno, and Meech (2005), with gold concentrations of 30 mg kg⁻¹ and 10 mg kg⁻¹ respectively. Blank samples also were prepared and analysed for every 10 analytical samples.

Table 3.7 Target concentration for PTM-1a

Metal	Mean concentration PTM-1a	Mean analytical result
Copper (wt %)	24.96± 0.04	23.15
Nickel (wt %)	47.44± 0.11	45.27

3.5. Data analysis

3.5.1. Data calculation

FAAS

$$[x] = \frac{[FAAS] \times IV \times DF}{WP}$$

Target metal concentration $[x]$ was expressed in $\mu\text{g g}^{-1}$ (ppm). To quantify the target metal concentration in solution samples, the concentration from the instrument (FAAS) was multiplied by initial sample volume (IV), which is 10 mL. If the sample had been diluted, this volume was multiplied by the dilution factor (DF). The total metal value was divided by plant weight (WP) from sample preparation. This formula applied to copper and nickel analysis.

GFAAS

$$[x] = \frac{[GFAAS] \times MIBK \times CF \times DF}{WP}$$

This formula was used to calculate gold and palladium concentrations. Target metal concentration $[x]$ was presented in ng g^{-1} (ppb). To quantify the target metal concentration in solution samples, the concentration from the instrument [GFAAS] was multiplied by MIBK volume, which is 2 mL. Volume was multiplied by 2, as conversion factor (CF) to bring measured volume to initial volume. If the sample had been diluted, this volume was multiplied with dilution factor (DF). The total metal value was divided by plant weight (WP) in gram.

3.5.2. Statistical analysis

Statistical analysis was performed using Minitab version 16. Data were presented as mean and standard error (SE). Analysis of variance (ANOVA) with confidence interval of 95% was used to determine whether there were significant differences between the mean values. Significance level was determined if probability level less than 5% ($p < 0.05$). The Tukey's HSD test to determine grouping of mean values was taken post-hoc if there were indications of significance different between means. Results from post-hoc test would be presented by letter. Means which share same letter are not different significantly.

In addition, to find out any relationship between two variables, a correlation analysis was conducted using EXCEL 2007. Further analysis on linear association between two variables, a coefficient of determination (R^2) analysis, was used. Values reported by this test are such that $0 \leq R^2 \leq 1$.

3.6. Total metal concentration in growth substrates

Values in Table 3.8 contrasted results from two laboratories: University of British Columbia (UBC) and Massey University (MU). Both laboratories were using different method in order to detect metal concentrations from Broken Hill rock assessed in this study (two types of gossan). Analysis at the University of British Columbia used Inductively Coupled Plasma (ICP-MS) to analyse copper, nickel and gold. While palladium, was detected using Neutron Activation method. In comparison, the Massey University laboratory measured copper and nickel using the FAAS method, and GFAAS for gold and palladium analysis.

Although different methods were employed, the two laboratories reported approximately similar concentrations for copper and nickel. The concentration of nickel in both soils had similar values. Analysis of noble metal concentration demonstrated that concentration of total palladium as presented by UBC was 1.5 fold higher than reported by Massey. In comparison, total gold concentration as reported from Massey was 4 fold higher than UBC.

Soil A was identified as a goethite dominant substrate and physically was light and porous. Where soil B was originally sourced from hematite dominant substrate and described as heavy and dense material. Comparison of total metal content from these two soil groups demonstrated that soil A contained more metals than soil B. Such metal concentrations are copper (1.4 percent), gold (3.8 ppm) and palladium (32.65 ppm). Additionally, as much as 0.6 percent of nickel was identified in soil B.

Further analysis to show differences between the two soil groups was performed using ANOVA test with 95 percent confidence interval. Results presented in Table 3.9 depict different concentrations between the two rock samples. Means with a different letter indicate significant differences. It is suggested that the total gold concentration in both soil groups was the same with $p < 0.05$. Nickel and palladium concentrations in soil A and soil B were different significantly, with p value < 0.05 .

Table 3.8 Comparison of total metal analysis

Laboratory		UBC				MU		
Method	ICP	ICP	ICP	NA	FAAS	FAAS	GFAAS	GFAAS
Element	Cu	Ni	Au	Pd	Cu	Ni	Au	Pd
Unit	ppm	ppm	ppb	ppb	ppm	ppm	ppb	ppb
Soil A	>10000	3088.5	1286.7	48000	13962.2	6354.4	3804.7	32650.2
Soil B	>10000	6800.4	369.2	28000	10890	3483.5	1565.6	16665

Laboratory: UBC (University of British Columbia), MU (Massey University)

Method: ICP (Inductively Coupled Plasma), NA (Neutron Activation), FAAS (Flame Atomic Absorption Spectroscopy), GFAAS (Graphite furnace atomic absorption spectrometry)

Table 3.9. Statistics analysis of metal concentrations for growth medium

Soil Group	N	Cu (µg/g)	Ni (µg/g)	Au (ng/g)	Pd (ng/g)
Soil A	3	13962.2 (444)a	3483.5 (218)b	3804.7 (1682)a	32650.2 (2378)a
Soil B	3	10890 (97)b	6354.4 (68.8)a	1565.6 (533)a	16665 (1892)b

Means are presented with standard error in parentheses. Mean with different letter is significantly different ($p < 0.05$).

Chapter 4 - Natural metal uptake in native plants from Broken Hill

4.1. Introduction

Mineralised soils are often associated with a unique flora. Ultramafic soils in particular have more than 400 plant species from 45 plant families that have been identified as hyperaccumulators, and about 75% are nickel hyperaccumulators (Küpper & Kroneck, 2007; Sheoran et al., 2009). A high metal concentration in plant's tissues has been proposed as an adaptation strategy to survive in mineralised environments.

Hybanthus floribundus, a native plant species to ultramafic soils found in Western Australia contained 1,600 mg nickel kg⁻¹ dry weight in its leaves and the background nickel concentration in soil was 700 mg nickel kg⁻¹. The concentration of nickel in this native species was about 2.3 fold than in soil. Spatial distribution of *H. floribundus* occur only at laterised ultramafic outcrops (Anderson, Brooks, Stewart, Simcock, et al., 1999).

Broken Hill is one of the most mineralised region on earth. Total metal analysis from soil samples collected at this location indicate a high content of copper, nickel, gold, and palladium. A community of native plant species are found growing well on this arid environment. If these plant species could thrive on mineralised soils, so there might be a plant species that is able to uptake metals at extreme concentrations in its biomass.

4.2. Aim and Methodology

The aim of this chapter is to investigate high metal uptake in five native plant species by contrasting metal concentration in plant and soil.

Plant species and soil samples were collected by Chris Anderson and John Meech during a field visit in July 2014. Fifteen soil and 28 plant samples were collected. Plants were identified as belonging to the species *Sclerolaena lanicuspis* (copper burr), *Ptilotus obovatus* (silver tail), *Solanum centrale* (bush tomato), *Brassica* sp, and *Tetragonia moorei* (annual spinach). The analytical method for soil and plant samples followed general metal analysis as described in Section 3.3.

The geographic position of sampling points was recorded and is tabulated in Table 4.1. Spatial distribution of sampling point is provided in Figure 4.1.

Correlation analysis was used in this chapter to discover the relationship of metal concentrations in soil substrate and plant biomass. Correlation interaction here will be notified based on the determination coefficient (R^2). Moreover, the ratio of metal concentration between soil and plant to determine a plant's ability to enrich metal in its biomass was analysed. The plant soil ratio is expressed as a bioaccumulation coefficient. Any ratio value greater than one, would be considered as an accumulator/hyperaccumulator.

Table 4.1 Sampling coordinates

Soil sample	Latitude	Longitude
S4	31.886179° S	141.665974° E
S5	31.886369° S	141.666007° E
S6	31.886514° S	141.665744° E
S7	31.886536° S	141.665046° E
S8	31.886600° S	141.664951° E
S9	31.886501° S	141.664750° E
S10	31.885994° S	141.665211° E
S14	31.885859° S	141.665052° E
S16	31.885808° S	141.666111° E
S19	31.885984° S	141.665359° E
SA	31.885641° S	141.665378° E
SC	31.885812° S	141.665569° E
SD	31.885827° S	141.666077° E
SE	31.885950° S	141.666691° E
SF	31.885900° S	141.665866° E

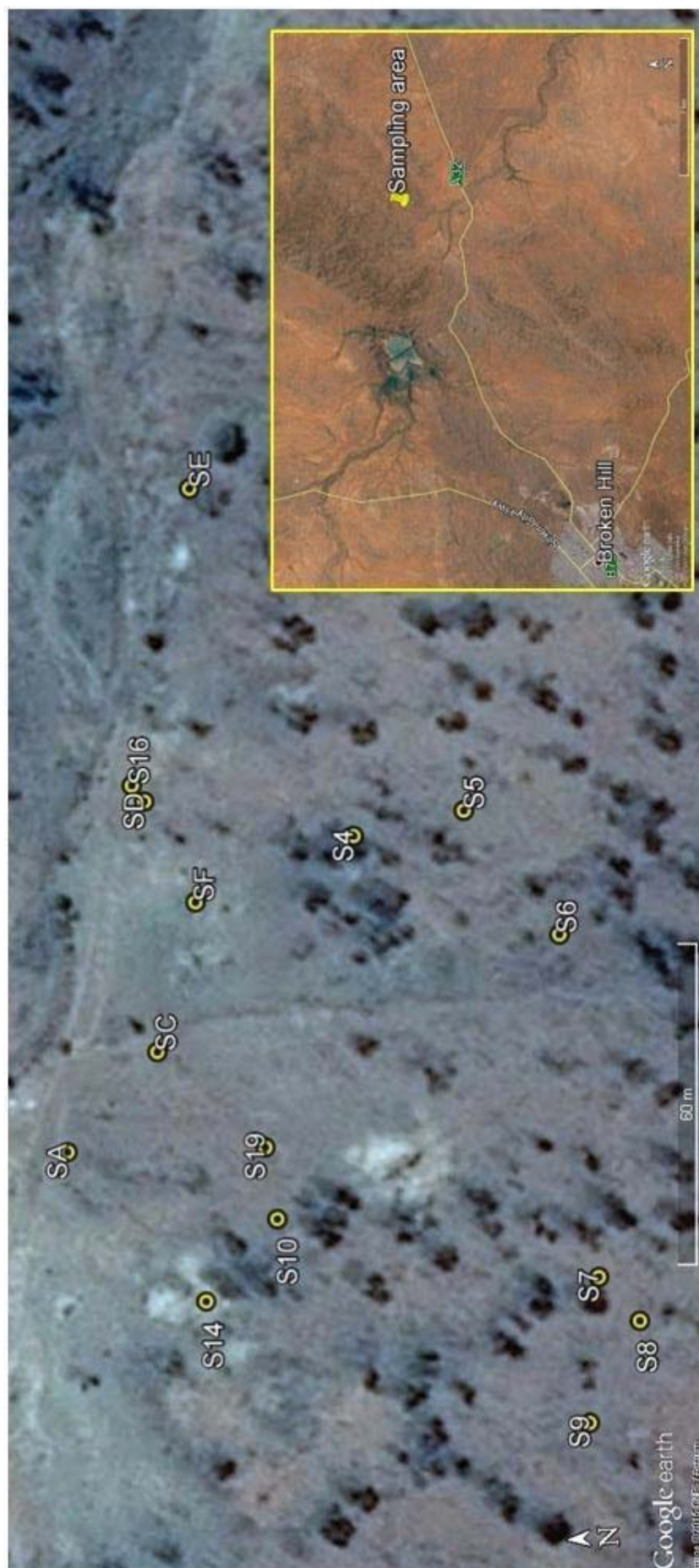


Figure 4.1. Sampling distribution of native plant species. Inset map indicated sampling area and relative distance to Broken Hill.

4.3. Metal concentration from adjacent root zone

4.3.1. Copper and nickel concentration in soil

The metal concentrations of 27 samples of gossan soil are presented in Table 4.2. These data represent background metal concentration in the rhizosphere across the area of gossan exposure. Range of copper and nickel concentrations are high compared to precious metals (Table 4.2). The big range for these base metals indicates variable distribution of copper and nickel in soils.

The highest copper concentration is $2,194 \mu\text{g g}^{-1}$ observed from sample S10 (see Figure 3.1). The mean copper concentration in soil is $378.8 \mu\text{g g}^{-1}$. Hence this outlier concentration can be expected as an anomaly at the particular area. Moreover, copper analysis from an adjacent sampling point at S14 found approximately $556.9 \mu\text{g copper g}^{-1}$. The sampling point S14 is located about 20 metres to the northwest from S10. Thus, these findings can be assumed as hotspot copper location in Broken Hill. Other sampling points reported with a copper concentration greater than the mean concentration are S4 ($796.3 \mu\text{g g}^{-1}$) and S19 ($615.3 \mu\text{g g}^{-1}$).

The nickel concentration from sampling areas also reported high with range from 25 to $1,135 \mu\text{g g}^{-1}$. The big difference between minimum and maximum concentration suggested that nickel in the environment is very diverse. The reported mean concentration of nickel is $317.7 \mu\text{g g}^{-1}$. Hence any concentration which exceeds the mean value is suggested high. The trend of mean concentration in base metals is depicted in Figure 4.1. Visually, mean nickel concentration is slightly lower than copper. ANOVA analysis as depicted in Figure 4.1 suggested that the mean concentration of copper and nickel is not significantly different ($p > 0.05$).

The highest nickel concentration was identified from S10. As described earlier, this sampling point also recorded the high copper concentration. Furthermore, other sampling points such as S4, S8, S14 and SE were discovered to contain nickel greater than the mean value with a concentration of 430.6, 970.7, 477, and $347.4 \mu\text{g g}^{-1}$ respectively. There are three sampling points which have been indicated to have a high copper concentration.

Table 4.2 Mean concentrations of plant and its substrate

Plant	N	Metal	Unit	Mean Soil	Mean Plant
<i>Ptilotus obovatus</i>	15	Cu	µg/g	378.78	28.17
	15	Ni	µg/g	317.65	4.98
	15	Au	ng/g	17.24	2.16
<i>Brassica sp</i>	5	Cu	µg/g	779.01	13.99
	5	Ni	µg/g	477.67	6.73
	5	Au	ng/g	38.96	0.42
<i>Solanum centrale</i>	4	Cu	µg/g	575.18	103.83
	4	Ni	µg/g	346.66	40.71
	4	Au	ng/g	42.94	7.70
<i>Tetragonia moorei</i>	2	Cu	µg/g	1495.25	73.81
	2	Ni	µg/g	782.55	13.1
	2	Au	ng/g	31.66	2.91
<i>Sclerolaena lanicuspis</i>	1	Cu	µg/g	796.33	71.48
	1	Ni	µg/g	430.58	27.05
	1	Au	ng/g	43.27	<DL

<DL : below detection limit

Table 4.3 Range of metal concentration in soil and plant samples

Metal	Unit	Soil	Plant
Cu	µg/g	24 – 2,194	4.8 – 277.4
Ni	µg/g	25 – 1,135	<DL – 89.5
Au	ng/g	<DL -111	<DL – 24.6
Pd	ng/g	<DL - 192	N/A

<DL : below detection limit; N/A : not applicable

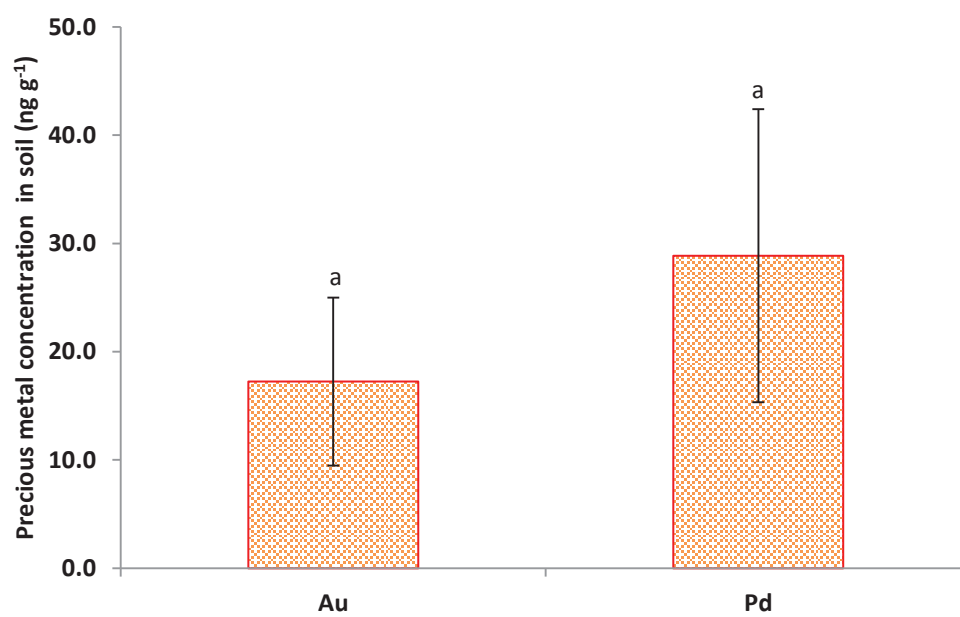
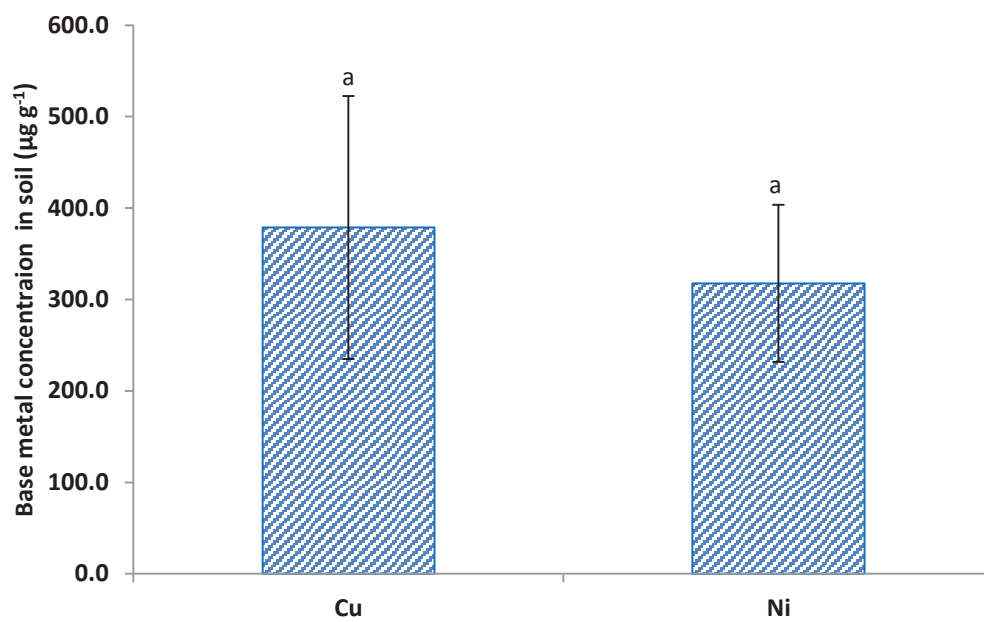


Figure 4.2. Mean metal concentrations in soil for base metals (above) and precious metals (below). Error bars represent standard error from 15 sampling data. Mean with same letter indicate significance level > 0.05 .

4.3.2. Gold and palladium concentration in soil

The gold concentration in the root zone ranges from below detection to 111 ng g^{-1} . From the range of values it is obvious that there are some sampling points with the normal background concentration of gold, such as S5, S6, S7, S8, and SD. The highest gold concentration was found at S14. In comparison, gold concentrations from S10, the adjacent sampling point to S14 was only 20 ng g^{-1} . The mean gold concentration from this area is reported at $17.2 \text{ ng gold g}^{-1}$. Hence gold concentration from S14 can be considered high. Although these two sampling points are separated by 20 metres, their concentration is about five fold different. Two other sampling points which exceeded the mean concentration are S4 (43.3 ng g^{-1}) and SF (46.3 ng g^{-1}).

The mean palladium concentration was reported at 28.8 ng g^{-1} , which is 1.7 fold higher than mean gold concentration, and an indication that palladium concentration in Broken Hill is higher than gold. However, their mean concentrations as illustrated in Figure 4.2 are not different significantly ($p > 0.05$).

The palladium concentration ranged between below detection limit and 192 ng g^{-1} . The highest palladium concentration was observed from S10 which has been identified to concentrate high copper and nickel. Besides S10, there are three sampling points with palladium concentration higher than 28.9 ng g^{-1} . These locations are at S4 (68.1 ng g^{-1}), S14 (82.3 ng g^{-1}), and S19 (41 ng g^{-1}). However, not every sampling point contains palladium. About seven from 15 sampling sites reported normal background palladium. Thus it can be assumed that a high palladium concentration can be found only at specific areas.

4.4. Metal concentration in plant tissues

4.4.1. Copper and nickel uptake

The range of copper concentrations in plant biomass was from 4.8 to $277.4 \text{ } \mu\text{g g}^{-1}$ dry weight, with an average concentration of $40.4 \text{ } \mu\text{g g}^{-1}$ dry weight. Examination of the range of copper uptake in every plant sample as illustrated in Table 4.4 indicates a decreasing trend of copper accumulation in the order *S. centrale* ($249.4 \text{ } \mu\text{g g}^{-1}$) > *P. obovatus* ($89 \text{ } \mu\text{g g}^{-1}$) > *T. moorei* ($56.6 \text{ } \mu\text{g g}^{-1}$) > *Brassica* sp ($32.7 \text{ } \mu\text{g g}^{-1}$). The widest range for copper concentration of $249.4 \text{ } \mu\text{g g}^{-1}$

dry weight was observed in *S. centrale*. The highest individual copper uptake in *S. centrale* was reported at 277.4 $\mu\text{g g}^{-1}$ dry weight that was collected from location S4. Spatial analysis could not be performed for *S. lanicuspis* because of the limited number of sample.

Besides containing a higher copper concentration, *Solanum centrale* from S4 also was discovered to have a higher nickel concentration. Approximately 89.5 $\mu\text{g nickel g}^{-1}$ dry weight was measured. In addition, the order for decreasing range of nickel concentrations was *S. centrale* (80.2 $\mu\text{g g}^{-1}$) > *T. moorei* (26.2 $\mu\text{g g}^{-1}$) > *P. obovatus* (23.1 $\mu\text{g g}^{-1}$) > *Brassica* sp (20.6 $\mu\text{g g}^{-1}$). Although some plant species were reported to uptake nickel at some particular concentrations, several samples were identified containing no nickel (i.e. below detection limit). Soil characteristics and nickel availability at a particular sampling points is assumed to affect nickel uptake in the field.

Table 4.4 Range concentration from five native plants tissues

Plant	Concentration			
	Cu($\mu\text{g/g}$)	Ni($\mu\text{g/g}$)	Au(ng/g)	Pd (ng/g)
'Normal' concentration	20	100	10	<8.7
<i>Brassica</i> sp	4.8 - 37.5	<DL - 20.6	<DL - 1.4	<DL
<i>S. centrale</i>	28 - 277.4	<DL - 89.5	<DL - 23.2	<DL
<i>S. lanicuspis</i>	71.5	27.0	<DL	<DL
<i>T. moorei</i>	45.5 - 102.1	<DL - 26.2	1.4 - 4.3	<DL
<i>P. obovatus</i>	8.4 - 89	<DL - 23.1	<DL - 24.6	<DL

<DL : below detection limit

4.4.2. Gold and palladium uptake

Soil analysis as presented in Figure 4.1 showed that the mean total palladium in the root zone is higher than gold, and they are statistically different. In contrast, Figure 4.2 illustrated that there is no plant sample concentrating palladium in the aboveground biomass. It is assumed that palladium occurs in non-soluble fractions in the rhizosphere. Therefore, no plant can

accumulate palladium in the aerial parts. In comparison, native plants were observed to accumulate gold in the aboveground biomass. Some plants are able to accumulate gold at detectable concentration.

The gold concentration in plants ranges from <DL – 24.6 ng g⁻¹ dry weight. However, some plants were reported concentrating a high level of gold in their aboveground portions. Such plants are *P. obovatus* (24.6 ng g⁻¹) and *S. centrale* (23.2 ng g⁻¹). On average, the gold concentration in plants was observed at 2.6 ng g⁻¹ from a background mean gold concentration in soil of 17.2 ng g⁻¹. High gold concentrations may reflect the presence of soluble gold in the root zone and subsequent plant uptake.

Moreover, individual plants of *P. obovatus*, and *S. centrale* were reported accumulating gold at significant concentration in their biomass, and it is expected that these plant species have an ability to extract a higher gold concentration than the other three native species. According to their mean gold uptake concentrations, this study may order the plants as follows: *S. centrale* (7.70 ng g⁻¹) > *T. moorei* (2.91 ng g⁻¹) > *P. obovatus* (2.16 ng g⁻¹) > *Brassica* sp (0.42 ng g⁻¹) > *S. lanicuspis* (0 ng g⁻¹).

4.5. Bioaccumulation coefficient

The ratio of metal concentration in plant to total metal in soil samples defines a bioaccumulation coefficient (BC) for plant species. The bioaccumulation coefficient represents a plant's enrichment ability to concentrate a particular metal into aboveground biomass (Wang, Yan, Dai, Wang, & Wu, 2012). According to Krzciuk and Galuszka (2014), plants with bioaccumulation coefficient < 1 are defined as excluders, while value greater than one is proposed as an accumulator or hyperaccumulators. Results in Table 4.5 presented BC value from this study.

4.5.1. Bioaccumulation coefficient for copper and nickel uptake

Analysis of copper bioaccumulation coefficients indicated a range of values from 0.02 to 0.2 in copper uptake (Table 4.4). The order of copper's bioaccumulation coefficient is: *S. centrale* (0.2) > *S. lanicuspis* (0.09) > *P. obovatus* (0.07) > *T. moorei* (0.05) > *Brassica* sp (0.02). It is

obvious that none of the BC values are greater than one and this is an indication that there are no copper accumulators in the collected native plants. The threshold level for copper hyperaccumulation is $300 \mu\text{g g}^{-1}$ (van der Ent et al., 2012), and the highest individual copper concentration in *S. centrale* is about $277.4 \mu\text{g g}^{-1}$ dry weight. Although this species is not considered as an accumulator, this copper concentration exceeds the toxicity level in 'normal' plants and is notable.

The order for BC in Ni is as follow: *S. centrale* (0.1) > *S. lanicuspis* (0.06) > *P. obovatus* = *T. moorei* (0.02) > *Brassica* sp (0.01). Again, this analysis indicated that there is no Ni accumulator species within the native plants, as BC values are less than one. A plant which concentrate Ni between 100 and $1,000 \mu\text{g g}^{-1}$ on dry weight basis can be suggested as a strong accumulators (Brooks et al., 1977). This study confirmed there is no such plant identified from the Broken Hill samples.

Table 4.5. Bioaccumulation coefficient

	Plant	Metal		
		Cu	Ni	Au
Bioaccumulation	<i>P. obovatus</i>	0.07	0.02	0.13
Coefficient (BC)	<i>Brassica</i> sp	0.02	0.01	0.01
	<i>S. centrale</i>	0.2	0.1	0.2
	<i>T. moorei</i>	0.05	0.02	0.09
	<i>S. lanicuspis</i>	0.09	0.06	N/A

N/A: not applicable

In general, the trend of copper and nickel uptake illustrated that the copper is more phytoavailable than nickel. This finding is depicted in Figure 4.3. ANOVA analysis confirmed that the mean of copper and nickel is different significantly at a probability of 5% (0.05). Moreover, the trend of BC of base metal uptake as presented in Figure 4.4 noted that in general, native species tend to uptake higher copper than nickel. For example copper concentration in *T. moorei* is about 5.63 greater than nickel. This ratio is similar to *P. obovatus* where the copper concentration is approximately 5.65 greater than nickel. The recorded trend of BC copper and nickel in *S. centrale* differ only slightly.

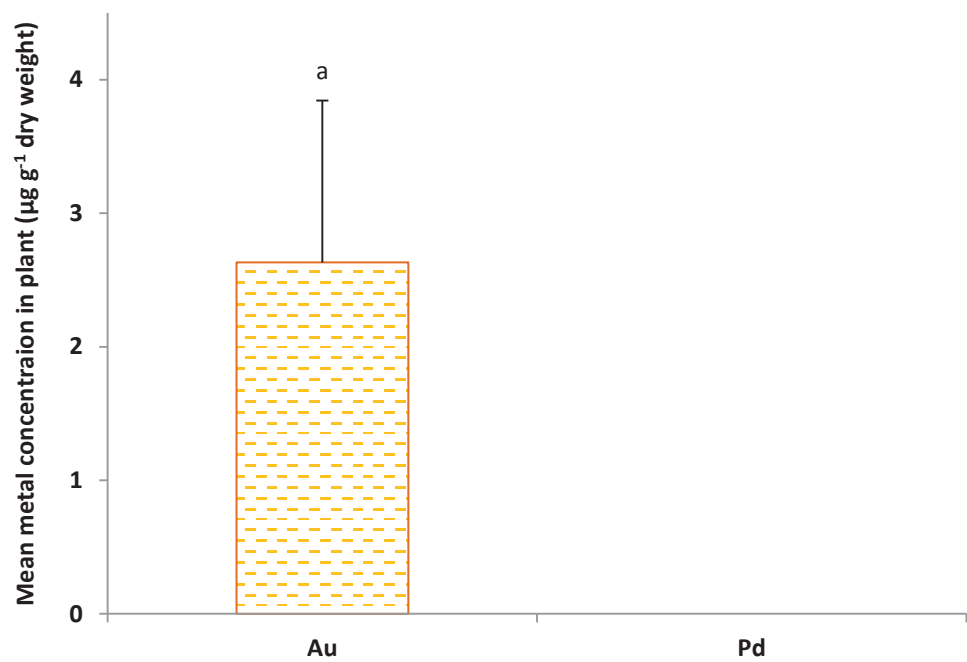
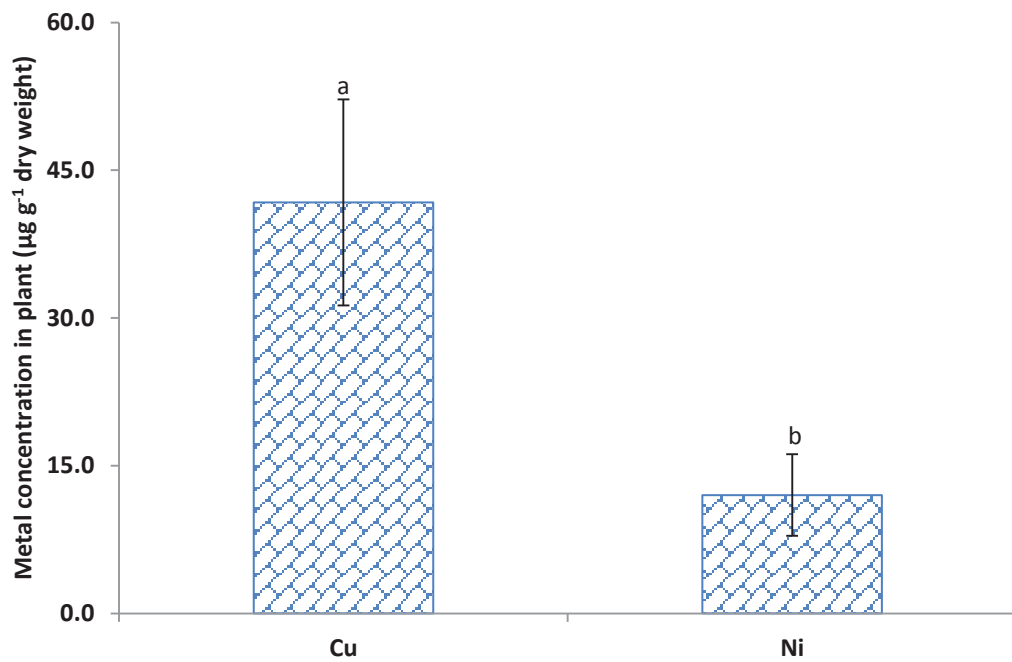


Figure 4.3. Mean concentrations from 27 samples of plant presented in $\mu\text{g g}^{-1}$ for copper and nickel (above) and in gold (below). The difference between two groups is presented with different letter ($p < 0.05$).

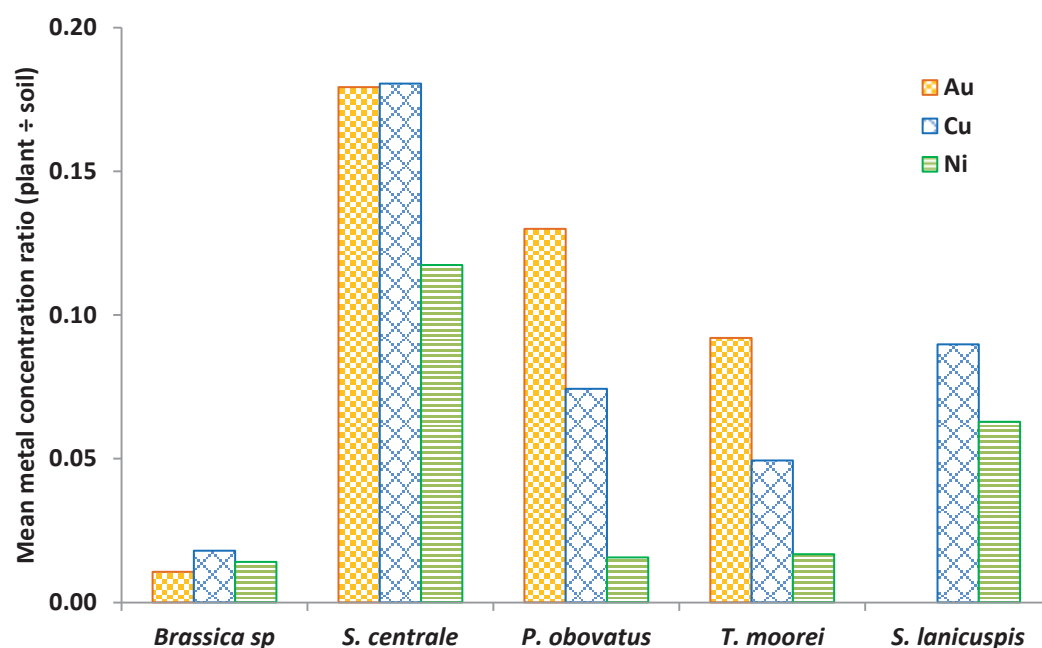


Figure 4.4. Bioaccumulation coefficients in five native plants

4.5.2. Bioaccumulation coefficient for gold and palladium

The bioaccumulation coefficient from five native species shows that in general, all native plants have the ability to concentrate gold in their biomass at various concentrations, except for *S. lanicuspis* (Figure 4.4). The BC of gold ranged from 0 to 0.2. The order of gold BC is as follows: *S. centrale* (0.2) > *P. obovatus* (0.13) > *T. moorei* (0.09) > *Brassica sp* (0.01) > *S. lanicuspis* (0).

Although the total palladium concentration in soil was higher than gold (Figure 4.1), in fact no palladium uptake has been found in plant biomass. Moreover, the bioaccumulation coefficient for precious metals here can be an indication for metal availability in adjacent root zones. If the bioaccumulation coefficient is an indication for gold availability, then soil S19 and SF could be expected as potential locations where natural gold uptake likely occurred. These two soils were a growth substrate for *S. lanicuspis*, and *P. obovatus*.

Another possibility is the native plants have a mechanism in their root system to exude organic chemicals which may react with gold, transform this into soluble form and make it easier to be

extracted by plants. It is expected that the exudates may have no affect on palladium. Therefore no palladium can be detected in the plant's biomass. In general, the trend of native plants to enrich metal in their biomass as expressed in bioaccumulation coefficients can be written as follow gold > copper > nickel > palladium.

4.6. Copper and nickel correlation analysis

Statistical analysis to see any possible correlations between metals in soil-plants is described in this section. The determination coefficients (R^2) between metal in soils and plants are presented in Table 4.6. Since some numbers of sample are limited, correlation analysis was only conducted for three species. The plants are *P. obovatus*, *Brassica* sp, and *S. centrale*.

The highest mean copper in plant was recorded for *Solanum centrale* at a concentration of $103.83 \mu\text{g g}^{-1}$ dry weight. Analysis of the determination coefficient value (R^2) proposed that *Brassica* sp still has the highest determination coefficient, $R^2 = 0.96$. The determination coefficient in other species was reported at 0.69 (*P. obovatus*), and 0.61 (*S. centrale*). These findings indicated there is strong relationship between copper in root zone and in *Brassica* sp (Figure 4.5).

The highest mean nickel concentration in plants also was reported from *S. centrale* ($40.71 \mu\text{g g}^{-1}$). To identify whether nickel in plant is affected by nickel in soil, a regression analysis was taken. Results reported that nickel in *S. centrale* and in soil have strong correlation ($R^2 = 0.895$). Investigation for the other two species found a low correlation between nickel in soil and in plant $R^2 = 0.013$ and $R^2 = 0.012$ for *Brassica* sp and *P. obovatus* respectively.

Table 4.6. Coefficient of determination

	Plant	Metal		
		Cu	Ni	Au
Coefficient	<i>P. obovatus</i>	0.69	0.012	0.067
Determination (R^2)	<i>Brassica</i> sp	0.96	0.014	0.34
	<i>S. centrale</i>	0.61	0.9	0.017
	<i>T. moorei</i>	N/A	N/A	N/A
	<i>S. lanicuspis</i>	N/A	N/A	N/A

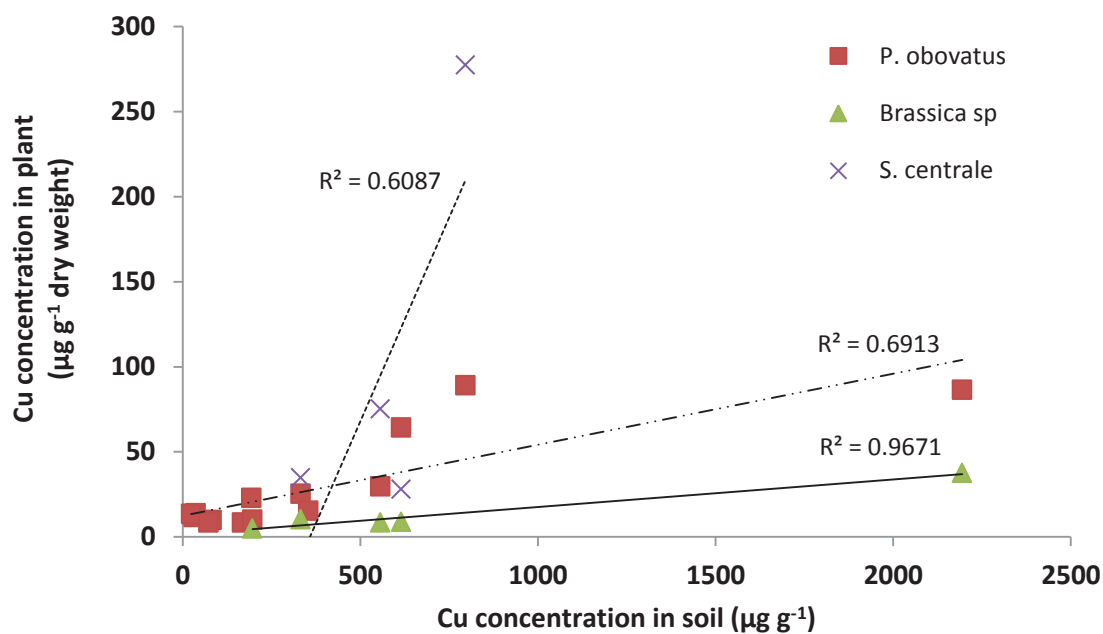


Figure 4.5. Plot for Cu uptake into plant's aerial parts as a function of Cu concentration in root zone

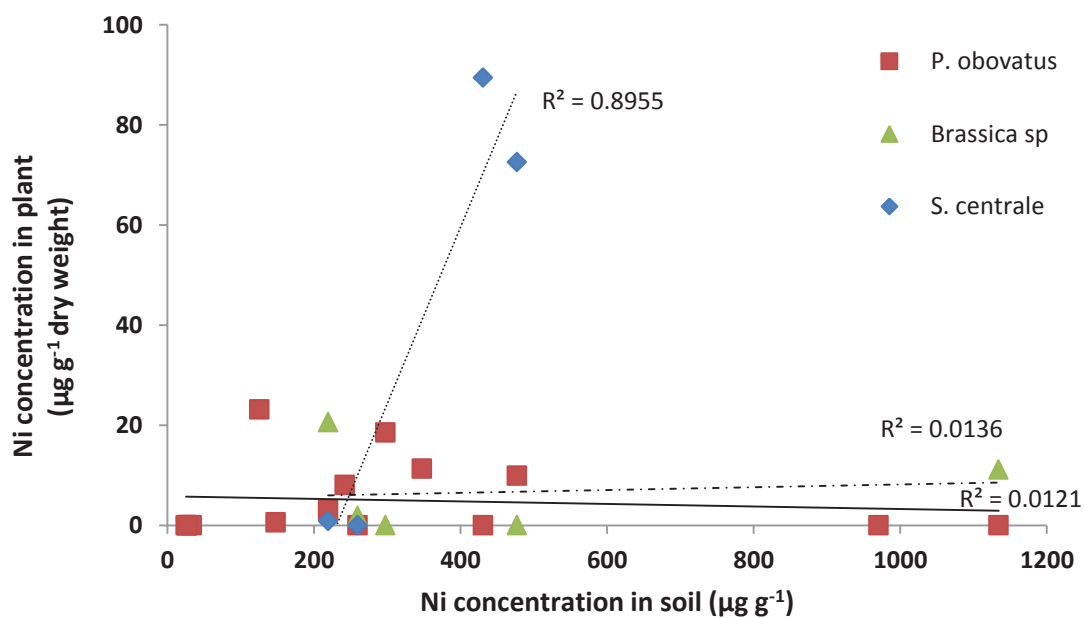


Figure 4.6. Plot for Ni uptake into plant's aerial parts as a function of Ni concentration in root zone

4.7. Metals mobility and its uptake in native plants

4.7.1. Copper nickel mobility and uptake

ANOVA analysis for mean copper and nickel concentration in soil showed that they are not statistically different (Figure 4.1). This finding is an indication that copper and nickel are present in similar amounts across the sampling location. Homogenous base metals content in the soil has been assumed as a result of physical and chemical processes that occur in arid regions.

Soil samples from Broken Hill were identified as containing a high level of iron oxide minerals. As described in Lulofs (1993), in arid areas such as Broken Hill, soil pH is suggested neutral, thus iron oxides may exist. The abundant existence of iron oxides in arid environments is likely to lead to higher base metal adsorption potential and the oxides may become primary base metals scavengers. A mechanism for metal adsorption onto iron oxide structures has been proposed by Alloway (1994). He mentioned that metal adsorption on goethite occurred in three ways: (1) surface adsorption, (2) fixation and adsorption within the particles of a mineral, and (3) diffusion into goethite particles. Therefore, it is possible that iron oxide minerals, such as goethite, are responsible for base metals distribution in Broken Hill area.

Since copper was homogeneously distributed across the sampling locations, plants are assumed to uptake copper at similar concentrations. This study found that the range of copper uptake in plants started from 4.8 to 277.4 $\mu\text{g g}^{-1}$ dry weight. For comparison, the range of copper in a 'normal' plants is suggested from 5 to 20 $\mu\text{g g}^{-1}$ (Reimann et al., 2001). The highest copper concentration was observed in *S. centrale* from location S4 with a soil copper level of 796.3 $\mu\text{g g}^{-1}$. Hence this species was proposed to uptake copper about 14 fold higher than a 'normal' plant. The bioaccumulation coefficient for *S. centrale* was 0.2. Although the BC value is less than one, the maximum copper uptake can be an indication that *S. centrale* is a copper tolerant species. A decreasing order of copper concentration in native species is as follow: *S. centrale* > *S. lanicuspis* > *P. obovatus* > *T. moorei* > *Brassica* sp.

Oxides present in the Broken Hill soil may adsorb base metals and make base metals less available in the environment. The BC value can be interpreted as an index of the available copper fractions which exists in the vicinity of the root zone. Generally speaking, although the total copper concentration in the soil was high, its bioavailability fraction was limited. Moreover, it is likely that native plant species have a strategy to control copper uptake at a safe concentration from the adjacent root zone.

Plants absorb Ni in the form $\text{Ni}(\text{H}_2\text{O})_6^{2+}$, and it is suggested that uptake will occur in a positive correlation with soil Ni concentrations, until a toxic level is reached in plant tissues (Alloway, 2012). The plot of nickel uptake as illustrated in Figure 4.6 is in agreement with this idea. A positive correlation as defined by correlation coefficients between nickel in soil and *S. centrale* was observed ($R^2 = 0.9$).

Besides concentrating a high level of copper in its aerial portions, *Solanum centrale* was also identified to uptake a high concentration of nickel. Individual nickel uptake from soil S4 was $89.5 \mu\text{g g}^{-1}$ at a background concentration of $430.6 \mu\text{g g}^{-1}$. In general, the average nickel uptake from four samples of *S. centrale* was approximately $40.71 \mu\text{g g}^{-1}$ dry weight.

In addition, observing the nickel bioaccumulation coefficient in the five plant species, there is a pattern that can be suggested. Here, the order of decreasing nickel concentration in plants was: *S. centrale* > *S. lanicuspis* > *T. moorei* > *P. obovatus* > *Brassica* sp. Based on these facts, this study assumed that *S. centrale* is a moderately tolerant nickel species from Broken Hill.

4.7.2. Precious metals mobility and uptake

Weathering processes are suggested to liberate gold from its primary ore deposit, and distribute this through solution chemistry into the environment. This gold mobility is suggested higher in a weathering environment. For example weathered host materials at a gold mine in south-eastern New South Wales were suggested to account for approximately 50 percent of the total gold that was analysed to occur in the extractable fractions (Alloway, 2012). This type of environment has been suggested to also apply to Broken Hill (Meech & Anderson, 2014).

In an arid environment, which containing high Cl^- concentrations, mobility and dispersion of gold is expected to occur as Au^{1+} or Au^{3+} – chloride complexes (Alloway, 2012). Besides physical processes, metal mobility is suggested to result from several geochemical factors which possibly interact with each other. Specifically, there are two factors which can be considered as the most important to mobilise an element: hydrogen ion concentration (pH), and the reduction oxidation potential (Eh). It is Eh that affects element mobility in solution. However, changes in pH are suggested to enhance metal mobility in soils (Lulofs, 1993).

Since the Broken Hill region is suggested as a weathering environment. Hence gold mobility and dispersion through solution form may exist. The gold which is in a bioavailable form is

likely to occur in the vicinity of the rhizosphere. Metal in soluble form is easily absorbed by plants from the rhizosphere and then can be transported into aerial mass through the xylem (Lintern et al., 2013). Therefore, some native plants may concentrate gold in their aerial biomass.

4.8. Conclusions

Copper and nickel concentration in soils. The highest total copper and nickel concentration in soil was $2,194.2 \mu\text{g g}^{-1}$ and $1,134.5 \mu\text{g g}^{-1}$ respectively and were found at sampling point S10. Average base metals concentration is suggested at $378.8 \mu\text{g g}^{-1}$ for copper and $317.7 \mu\text{g g}^{-1}$ for nickel.

Gold and palladium concentration in soils. The highest total palladium concentration from rhizosphere samples was $1,921 \text{ ng g}^{-1}$. This value was identified from soil sample S10. The reported mean concentration for total palladium was 28.9 ng g^{-1} . Analysis of soil gold concentration suggested that the highest gold concentration was $111 \text{ ng gold g}^{-1}$ observed for sampling point S14. The mean gold concentration in soil was reported at 17.2 ng g^{-1} .

Potential copper tolerance. No hyperaccumulator species for any of the four metals were recorded. However, the highest copper uptake at concentration of $277.4 \mu\text{g g}^{-1}$ was found in *Solanum central*. This result is anomalous and suggests that this species may be copper tolerant and could be the focus of further research.

Chapter 5 - Greenhouse trial: Metal uptake in *Brassica juncea*

5.1. Introduction

The previous chapter reported some measurable concentrations of total palladium from the root zone of Broken Hill soils. However, no native plant species was found to concentrate palladium in its aboveground biomass. Therefore palladium is suggested to have poor solubility in gossan environment. In order to study palladium solubility in soil and promote its uptake in plant species, a greenhouse trial using *Brassica juncea* was conducted.

The Brassicaceae family is well-known for its tolerance to heavy metals, thus some species under this family have been identified as metal hyperaccumulators, including those from the genus *Alyssum* and *Thlaspi* (Krämer, 2010; Rascio & Navari-Izzo, 2011).

Brassica juncea has been used in numerous experiments to explore metal hyperaccumulation capability in plant species (Grispen, Nelissen, & Verkleij, 2006). As described in Vamerali, Bandiera, and Mosca (2010), *B. juncea* is identified as a hyperaccumulator of Zn, Pd, Cd, Cr, Cu, as well as Caesium-137. *B. juncea* has been recommended for metal uptake trials because this species has short life, higher shoot biomass and easy to grow (Chigbo, Batty, and Bartlett, 2013). Based on available evidence, *B. juncea* was chosen for this metal uptake trials.

5.2. Aims and methodology

The aims of this chapter were (1) to examine the performance of *B. juncea* grown on gossan soil, (2) to assess any high net metal uptake in plant's biomass, (3) to induce hyperaccumulation of palladium to a target concentration of $1,000 \mu\text{g g}^{-1}$, and (4) to identify factors that may affect plant growth and its metal uptake.

The greenhouse experiments were conducted between November 2014 and March 2015 at Plant Growth Unit Massey University in Palmerston North, New Zealand. The soil used on this trial was crushed gossan rock collected from Broken Hill, Australia which contained mixture of fine ($< 0.1 \text{ mm}$) and coarse sizes ($< 4 \text{ mm}$). To provide a good growth conditions for plants and maintain substrate pH level of 7, the soil samples were mixed with dolomite and agricultural lime at rate of 5 gram per kilogram soil. The mixed substrates then transferred into 1 L pots and filled to approximate 800 grams per pot. There were 18 pots for soil A and 21 pots for soil

B seeded with *Brassica juncea*. The pots then watered every seven days prior to seeding and then irrigation was continued, as required, to maintain the water content in the trial pots.

Liquid fertilizer, Yates Thrive All Purpose Liquid Plant Food containing Nitrogen 12.4%, Potassium 6.2%, Phosphorous 2.7% and Trace Elements was applied. Prior to application, the concentrated liquid fertilizer was diluted to a concentration of 0.33% (v/v) as recommended by the manufacturer. About 10 mL of diluted fertilizer then applied to the pots every two weeks. After 55 days, plants' above-ground biomass was harvested and oven dried at 70 °C. Subsequently, plant biomass was weighed and be prepared for copper, nickel, gold, and palladium analysis. Analytical methods for plant samples refer to Section 3.3.

Metal solubility in this study represents metal concentration in water extraction solution. Comparison between metal concentration in plant and soluble metal fraction was used to determine plant's ability in metal uptake.

5.3. Growth of *Brassica juncea*

Observations during the experiment showed that *B. juncea* in both soil groups exhibited poor growth signs. Some pot trials were reseeded in order to boost plant growth. From 18 pot trials, only one pot showed good growing signs. It is likely that high solubility of metals caused metal stress in plants. Figure 5.1 and 5.2 illustrate growth progress of *B. juncea* on the 40th and 55th day after sowing. Retarded growth and chlorosis as indicated by small shoots is attributed to metal toxicity.

Growth temperature was predicted to be an additional factor to impede growth of *B. juncea*. During the trial, from December 2014 to February 2015, greenhouse temperature ranged from 14 °C to 35 °C. Heat stress was suggested to affect *B. juncea* growth. Angadi et al. (2000) advised that temperature of 35 °C may cause heat stress to *B. juncea*. Hence temperature stress was likely to affect plant growth performance. Poor physical appearance from the plant included stunted growth, thin stems, small leaves, chlorosis. The worst effect was necrosis, which was documented in this study.



Figure 5.2 *Brassica juncea* grown in soil A at day 40 after sowing



Figure 5.1 *Brassica juncea* in soil A at day 55

5.4. Biomass production

The current study confirmed that *B. juncea* grown in gossan soils has poor growth performance as side effects from toxic metal stress. The primary cause of metal stress is proposed to be copper, due to the high concentration of this metal in the gossan. Total biomass production is shown in Table 5.1. The results illustrated that *B. juncea* from soil A produced more biomass (3.2 gram), than *B. juncea* from soil B (1.9 gram). The average weight of biomass production per pot is about 0.24 gram from Soil Group A and 0.11 gram from Soil Group B. Biomass production from plant trials in soil A was 1.7 higher than in soil B.

ANOVA analysis showed that mean biomass of *B. juncea* on soil A was different statistically to *B. juncea* in soil B with p value less than 0.05. Presumably the difference is associated with copper availability in the soil substrate. Copper was less soluble in soil A than soil B (Sakambari, 2015). Hence Brassica grown on soil A would have less copper toxicity effects and suggested to yield more biomass than Brassica in soil B.

Table 5.1. Total harvested biomass (gram dry weight)

Plant	N	Total	Mean
<i>B. juncea</i> A	18	3.2	0.2 (0.03)a
<i>B. juncea</i> B	21	1.9	0.1 (0.01)b

Means are presented with standard error in parentheses. Means with different letter are significantly different ($p < 0.05$).

Since *Brassica juncea* had poor growth performance, as indicated from low biomass production, treatment to induce hyperaccumulate palladium in this plant could not be conducted. The following sections report and discuss metal uptake in *B. juncea* during water treatment as a simulation of metal solubility and metal uptake in the nature environment.

5.5. Metal uptake in *Brassica juncea*

5.5.1. Copper and Nickel uptake

Brassica juncea grown on soil A recorded an average copper concentration of $208.4 \mu\text{g g}^{-1}$ dry weight (Table 5.2). The measured copper concentration from soil A ranged between 50 and $440.2 \mu\text{g g}^{-1}$ dry weight. Brassica on both soils had the same minimum copper concentration at $50 \mu\text{g g}^{-1}$. However, Brassica in soil B concentrated more copper in its biomass. Maximum copper concentration in Brassica B was $759 \mu\text{g g}^{-1}$. In comparison, the copper concentration in Brassica B was about 1.7 fold higher than copper concentration in Brassica A. Despite the maximum concentrations and mean values between Brassica A and Brassica B being variable, they were not significantly different (Figure 5.3).

The copper concentrations in this study suggested that *B. juncea* grown on both soils concentrated copper higher than a normal plant. It has been suggested that an acceptable maximum copper concentration for normal plants is about $20 \mu\text{g g}^{-1}$. This study found that on average *B. juncea* can accumulate a copper concentration about 10.4 - 13.5 higher than found in normal plants.

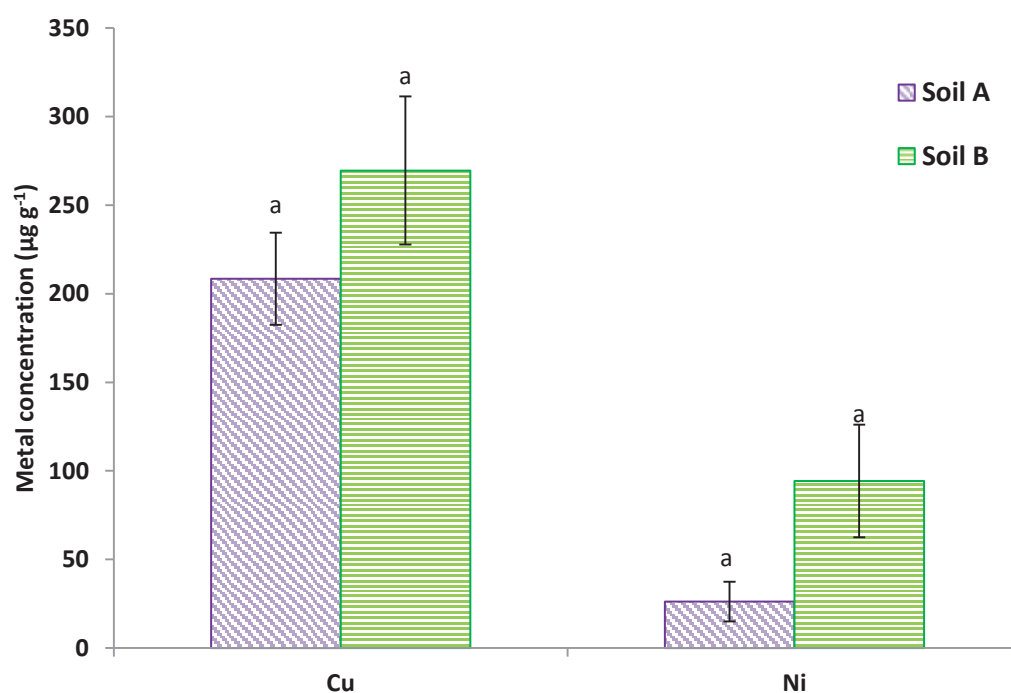
Brassica juncea grown on soil B recorded a nickel concentration of $94.3 \mu\text{g g}^{-1}$. Compared to Brassica on soil A, this mean concentration was about 3.6 fold higher for Brassica B. Moreover, Brassica on soil B recorded the higher individual nickel value. Maximum individual nickel concentration in Brassica B was $601.7 \mu\text{g g}^{-1}$, and Brassica A was $197.1 \mu\text{g g}^{-1}$.

In comparison to the normal nickel concentration in plants, this analysis suggested that on average, the nickel concentration in *B. juncea* was still within the expected concentration ($< 100 \mu\text{g g}^{-1}$). However, an individual *B. juncea* grown on soil B was identified to concentrate nickel approximately 6 fold higher than normal plants. According to Brooks et al. (1977), this maximum individual concentration can be classified as strong nickel accumulator.

Table 5.2. Mean metal concentration in aerial parts

Metal	Unit	Normal Plant	Brassica A (n = 18)		Brassica B (n = 21)	
			Mean	Range	Mean	Range
Cu	µg/g	20	208.4 (26)a	50 - 440.2	269.5 (41.8)a	50.1 - 759
Ni	µg/g	100	26.2 (11.2)a	<DL - 197.1	94.3 (31.8)a	<DL - 601.7
Au	ng/g	10	192.7 (37.5)a	35.3 - 633.1	275.7 (41.8)a	31.1 - 609.4
Pd	ng/g	8.7	726.5 (121)a	96.51 - 2130.3	1175 (197)a	191.7 - 2909.4

Means are presented with standard error in parentheses. Means with same letter are not significantly different ($p > 0.05$)

Figure 5.3. Copper and nickel concentration in *B. juncea* grown on two soil types

5.5.2. Palladium and gold uptake

The average palladium concentration in *Brassica juncea* grown on soil B was 1,175 ng g⁻¹. In comparison, Brassica A concentrated palladium to a concentration of 726.5 ng g⁻¹. The palladium concentration for Brassica B has a wider range than the palladium concentration for Brassica A. The difference between maximum and minimum concentration in Brassica B was 2,700 ng g⁻¹, while Brassica A was 2,033 ng g⁻¹. Although the mean and range of palladium was higher in Brassica B than in Brassica A, the values are not statistically different (Figure 5.4).

Brassica on soil A recorded the highest palladium concentration of 2,130 ng g⁻¹, whereas the highest palladium concentration in an individual *B. juncea* grown on soil B was 2,909 ng g⁻¹. Under natural conditions, palladium has low solubility. It is suggested that the natural palladium concentration in plants worldwide is < 10 ng g⁻¹ (Kabata-Pendias & Mukherjee, 2007). This study found that both Brassica plants under greenhouse trials concentrated palladium to a level about 200 times higher than normal plants, which is indicative of a level of hyperaccumulation. As the target of this study was to achieve a concentration of 1,000 µg g⁻¹ in plant's biomass, these results suggest that *B. juncea* has potential plant to extract a high palladium concentration in its biomass after induced hyperaccumulation.

The gold concentration in Brassica A averaged 192.7 ng g⁻¹. In contrast, the concentration in Brassica B was 275.7 ng g⁻¹, which is 1.4 higher than the reported mean gold concentration in Brassica A. Although their means were different, based on ANOVA analysis they were not different significantly ($p > 0.05$). Range concentration analysis as presented in Table 5.1 suggested that Brassica A has a wider range of gold concentrations than Brassica B. The highest individual uptake observed in Brassica A was 633.1 ng g⁻¹. While the maximum individual gold concentration in Brassica B was at 609.4 ng g⁻¹.

Anderson, Brooks, Stewart, and Simcock (1999) mentioned that natural gold uptake in native plants that grow in a gold rich habitat may not exceed 10 ng per g in dry matter. The mean concentrations found in this study are about 10 to 20 fold higher than 10 ng gold g⁻¹ in dry matter plant. Subsequently, the study demonstrated that *Brassica juncea* was able to uptake a high concentration of gold into their biomass. Despite the mean gold concentrations being higher than normal plants, the concentration was still below that recommended level for gold hyperaccumulation which is 1,000 ng g⁻¹.

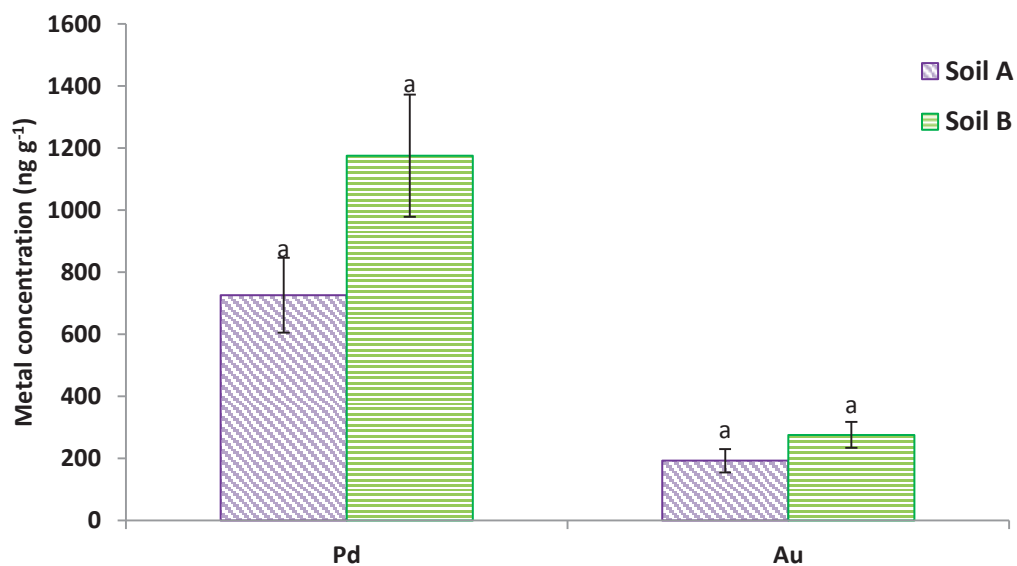


Figure 5.4. Palladium and gold mean concentration in *B. juncea* grown on two soil types

5.6. Relationship between total and soluble metal

Data for the total metal concentration from Broken Hill soil as presented in Table 5.3 demonstrate that copper, gold, and palladium is higher in soil A, whereas nickel was higher in soil B. The order of metal concentration from the highest to the lowest in soil A is: Cu (1.3%) > Ni (0.3%) > Pd (32 ppm) > Au (3 ppm). While in soil B the order as: Cu (1%) > Ni (0.6%) > Pd (17 ppm) > Au (1.6 ppm). Concentrations of metal solubility as a function of water extraction illustrated that copper, nickel, and palladium are more soluble in soil B, while gold is found to be more soluble in soil A (Figures 5.5 and 5.6). These findings suggest that total metal concentration may not dictate the concentration of metal solubility in the soil solution.

Tack, Callewaert, and Verloo (1996) described that metal solubility and its phytoavailability are affected by changes in the oxidation status of soils and sediments.

Both soil types are oxidised substrates, hence another possible factor to mobilise metal in soil solution may come from pH. The level of pH between two soils differed by 0.78 pH units, and soil B was slightly more acidic than soil A, thus this study assumed that the pH difference influenced metal solubility.

Table 5.3 Metal solubility and its uptake ratio in *Brassica juncea*

Metal	Unit	Mean total metal		Mean extractable metal (H ₂ O)		Metal uptake ratio	
		Soil A	Soil B	Soil A	Soil B	Brassica A	Brassica B
pH	-	-	-	7.23	6.45	-	-
Cu	µg/g	13,962	10,890	1.82	11.31	114.5	23.8
Ni	µg/g	3,484	6,354	0	0.9	N/A	104.8
Au	ng/g	3,805	1,566	0.16	0.09	1,204.40	3,063.80
Pd	ng/g	32,650	16,665	0.03	0.36	24,200	3,263.90
Data of extractable metal (H ₂ O) after Sakambari (2015)							

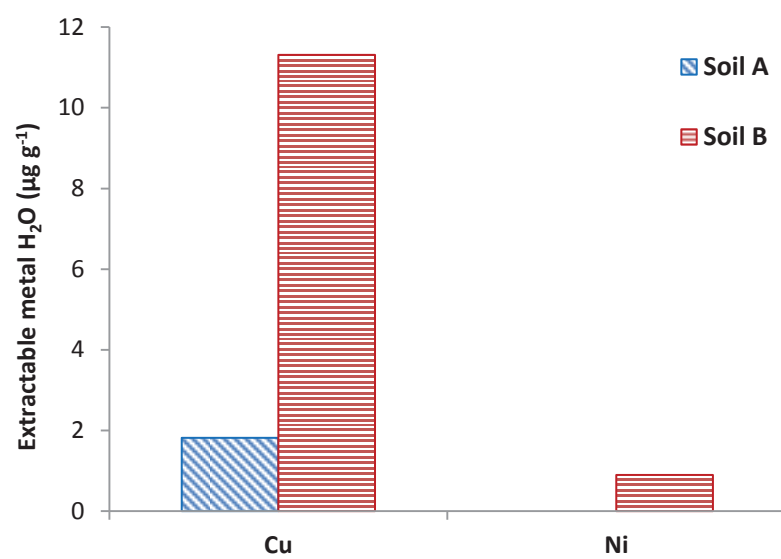


Figure 5.5. Water extractable copper and nickel from two soil types

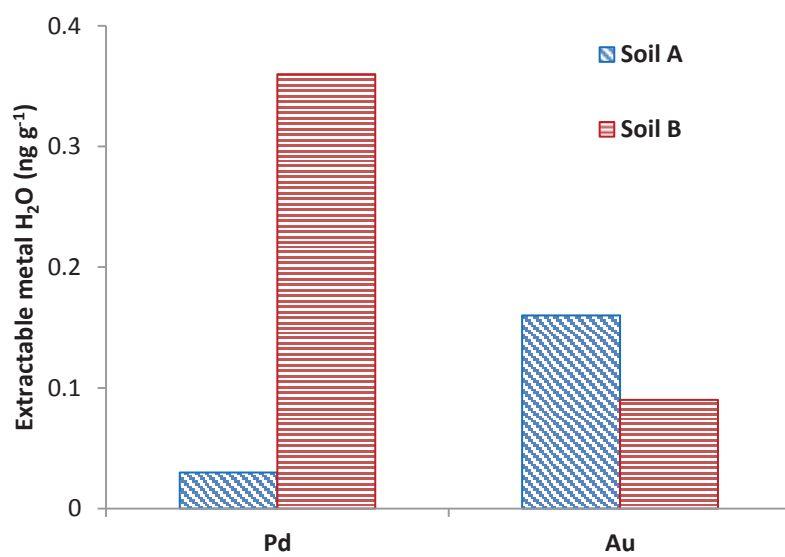


Figure 5.6. Water extractable palladium and gold from two soil types

5.7. Metal uptake ratio

The metal uptake ratio in this section is defined as comparison between metal concentration in water extraction (overnight extraction at a 10: 1 solution to soil ratio) and metal concentration in plant.

5.7.1. Uptake ratio for copper and nickel

Analysis of the copper plant – soil ratio is presented in Table 5.3, and a trend graph is shown in Figure 5.7. The reported copper concentration in the plant's dry biomass indicated that Brassica will uptake soluble copper from both soil types and concentrate this in aerial portions. Brassica grown on soil A was reported to accumulate copper to a concentration that is 114.5 times greater than the water soluble concentration in soil; the available copper concentration in soil was $1.82 \mu\text{g g}^{-1}$ whereas the concentration in Brassica was $208.4 \mu\text{g g}^{-1}$. Calculation of the copper uptake ratio in Brassica B found that this plant can uptake nearly 23.8 more copper than is water soluble in soil. This study suggested that Brassica has a greater uptake potential from soil A relative to soil B and this may be related to the mineralogy of the two soils (goethite vs haematite)

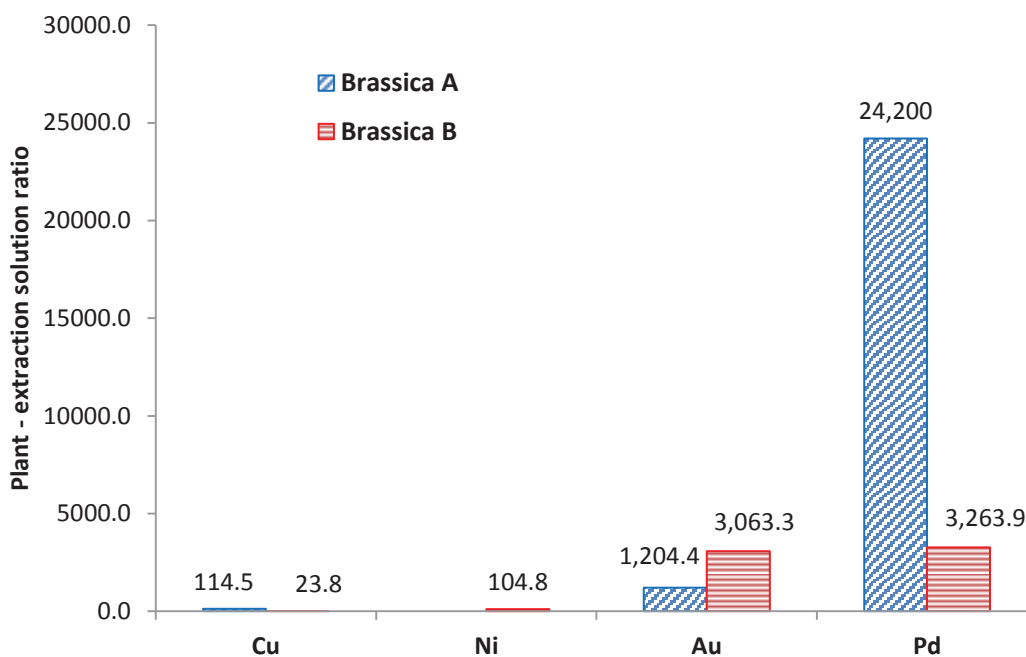


Figure 5.7. Ratio of metal uptake in *Brassica juncea* from two soil types

Nickel solubility under water extraction was low. Soluble nickel was only detected in soil B. Therefore the nickel uptake ratio analysis was only applicable to Brassica grown on soil B. Brassica B was able to concentrate nickel to a level 104.8 times higher than soil and this was manifest as a concentration of $94.3 \mu\text{g g}^{-1}$ dry weight detected in aboveground biomass. Although available nickel was undetected in soil A, Brassica A was reported to accumulate $26.2 \mu\text{g nickel g}^{-1}$ dry weight. This is an indication that soluble nickel was present in the gossan at very low concentration.

In general, the uptake ratio for copper was higher in Brassica A, and the ratio for nickel was higher in Brassica B. The trend of copper nickel uptake ratio is illustrated in Figure 5.5. However, compared to palladium and gold, the uptake ratios for copper and nickel were low.

5.7.2. Uptake ratio for palladium and gold

Brassica grown on soil A had a higher concentration of palladium in its biomass than in soil. The concentration factor for Brassica A was 24,200. Brassica B also concentrated Pd into biomass but the concentration factor of soil B was approximately 3,264. Brassica grown on the two soil types was able to extract and concentrate palladium in its biomass, with Brassica on soil A concentrating palladium 7.5 times more than Brassica B.

In comparing gold uptake in both soil types, Brassica B accumulated a higher gold concentration in its biomass than Brassica A (275.7 and 192.7 ng g^{-1} dry weight respectively). The concentration factor for Brassica B was 3,064. Despite gold being more soluble in soil A, the concentration factor for Brassica on this soil was 1,204. Brassica in the two soils demonstrated an ability to concentrate soluble gold from soil, however greater potential for uptake was found from soil B relative to soil A.

In general, concentration factors are an indicator of a plant's ability to concentrate soluble metals in its biomass. The pattern of metal uptake ratio for Brassica A can be ordered as follows: $\text{Pd} > \text{Au} > \text{Cu}$. While the order of metal concentration in Brassica B is suggested as: $\text{Pd} > \text{Au} > \text{Ni} > \text{Cu}$. These findings noted that Brassica from the two soils accumulated a palladium and gold according to the magnitude of the soluble pool of metal in soil.

Discussion

5.8. Metal solubility and plant growth

The soluble metal fraction estimated using water extractant was aimed to simulate water irrigation during the plant trials in the greenhouse (Table 5.1). The soluble fractions represent the bioavailable fraction of a metal. Plants will readily uptake the bioavailable fractions (Harmsen, 2007). The soluble metals are transported by the water and deposited in aboveground mass through transpiration. As the water is transpired the heavy metals will be left in plant tissues. The heavy metals than will be stored in the vacuole, which is an important part in the plant's cell to store waste and contaminated materials. However, plant cells have a maximum capacity to contain the dangerous materials. When the concentration exceeds a certain level, plant cells eventually succumb to necrosis.

Analysis of metal solubility as a function of water extraction shows that extractability of metals in soil A can be ordered as follows: Cu > Au > Pd > Ni. Metal solubility in soil B can be arranged from the highest to the lowest as: Cu > Ni > Pd > Au.

Data on metal concentration in Brassica from the two soil types noted that the plants concentrated metals in the order: Cu > Ni > Pd > Au. Since copper and nickel more available for plant uptake, these heavy metals were expected to inhibit plant's growth. *Brassica juncea* grown on the two types of gossan exhibited poor growth performance

5.9. Copper uptake and its effects to plant growth

Copper uptake likely occurred as a function of transpiration. Soluble copper in the rhizosphere was absorbed and transported from roots to aerial parts of *B. juncea* through the xylem. As water evaporates from plant tissues, copper will be concentrated in aerial parts, and mostly in leaves. The highest copper concentration in the two soil types would be assumed as one factor to inhibit plant growth performance. The range of copper concentration in 'normal' plants is recommended from 5 to 20 $\mu\text{g g}^{-1}$ (Reimann et al., 2001). Higher than this 'normal' copper concentration, plants would be affected by copper toxicity.

In this study, Brassica grown on gossan soils was reported to uptake copper at a concentration between 50 and 759 $\mu\text{g g}^{-1}$ dry weight. The highest copper concentration exceeded the threshold value for a copper hyperaccumulation; 300 $\mu\text{g g}^{-1}$ as recommended by van der Ent et al. (2012). Hence, evidence from this study suggests that copper would be one factor to hamper growth and development of *B. juncea* of the Broken Hill gossan.

Chigbo et al. (2013) described the effect of copper on the growth performance of *B. juncea*. The study found that copper inhibited growth of plant trials as indicated by a reduction in root biomass and shoot dry weight. Another study supported this idea and also mentioned that copper at a concentration higher than 100 – 250 mg kg^{-1} may decrease plant growth, and biomass production (Balamurugan, Thiagarajan, Manivasagaperumal, & Vijayarengan, 2011).

The current study confirmed that *B. juncea* grown in gossan soils had poor growth performance due to side effects from toxic metal stress, namely copper. Total biomass production was shown in Table 5.2. The results illustrated that *B. juncea* from soil A produced more biomass (3.2 gram), than *B. juncea* from soil B (1.9 gram). Average weight of biomass is about 0.24 gram from Soil Group A and 0.11 gram from Soil Group B. Biomass production from plant trials in soil A was 1.7 higher than in soil B.

ANOVA analysis showed that mean biomass of *B. juncea* on soil A was different statistically to *B. juncea* in soil B with p value less than 0.05. Presumably the difference has association with copper availability in soil substrate. Copper was less soluble in soil A than soil B. Hence Brassica grown on soil A would have less copper toxicity effects and is suggested to yield more biomass than Brassica in soil B.

5.10. Nickel uptake and its effects to plant growth

Analysis of the soluble nickel concentration in soil demonstrated that nickel in soil A is less available than in soil B. Low nickel availability could be an effect from the high pH of the substrate, which reduces nickel solubility (Robinson, 1997).

The concentration of nickel in normal plants is recommended at a range of 0.05 and 10 mg kg^{-1} dry weight. The threshold for nickel phytotoxicity is suggested at 100 mg kg^{-1} (DM). Higher than this concentration, a plant may experience nickel toxicity (Alloway, 2012). A phytotoxicity study of nickel was presented by Mohammadzadeh et al. (2014). They reported that a nickel

concentration of 450 mg kg^{-1} in soil may cause decreased plant growth, and damage photosynthetic content. Hence, the same effects are expected to exist for Brassica plants in this study. The observed nickel concentration in Brassica was at a toxic level, and presumably hindered plant growth and development. Despite these phytotoxicity facts, the maximum concentration of $601.7 \text{ } \mu\text{g g}^{-1}$ recorded in this study suggests that the species is a strong accumulator (Brooks et al., 1977).

5.11. Palladium uptake and its effect on plant growth

Palladium solubility in soil B (0.36 ng g^{-1}) was higher than in soil A (0.03 ng g^{-1}). Since palladium is more soluble in soil B, Brassica grown on soil B was predicted to extract more palladium in its biomass as a function of transpiration. This study demonstrated that Brassica B concentrated palladium in its biomass to a higher concentration than in soil. Reported palladium from Brassica B was $1,175 \text{ ng g}^{-1}$. While Brassica A concentrated 726 ng g^{-1} .

The ratio of palladium uptake was found higher from Brassica A (24,200) compared to Brassica B (3,264). This study found that the ratio of palladium uptake was higher than reported in Walton (2002). In his trial, Walton demonstrated that *B. juncea* can 'naturally' extract palladium at a concentration of 6.35 ppb in the aboveground parts from an artificial substrate containing 148 ppb soil. The palladium concentration ratio (plant to soil) in this study was 0.04. Compared to Walton's work, *B. juncea* on this study concentrate palladium 114 to 450 higher.

One study of toxicity effects demonstrated that endive plants *Cichoriu mendivia* var. *Crispum* experienced severe stress symptoms at a level of $8.7 \text{ ng palladium g}^{-1}$ in the plant tissues (Alt et al., 2002). It has been reported that in plant systems, palladium tends to bind with high molecular-weight proteins and also may cause chlorophyll decomposition, DNA damage and necrosis (Alt et al., 2002; Gagnon et al., 2006). Palladium concentrations in the current study in *B. juncea* were about 83 to 135 higher than the reported by Alt et al. (2002). Presumably, high palladium concentration in plant biomass contributed to affect plant growth performance.

The highest palladium concentration in *B. juncea* from both soils was 2,130 and 2,909 ng g^{-1} . Considering that palladium has low solubility in natural environments and its concentration is

assumed to be no more than 10 ng g^{-1} , *Brassica juncea* from this study is suggested to hyperaccumulate palladium in its aerial portions.

5.12. Gold uptake

The solubility of precious metals described in Table 5.1 demonstrated that gold from soil A was more soluble than gold in soil B. A concentration of 0.16 ng g^{-1} was detected from soil A. Gold solubility in soil A was about 1.7 fold higher than in soil B.

In contrast with other studies, findings from this trial pointed out that gold uptake in *B. juncea*, without induced hyperaccumulation, from Broken Hill substrate was high. For instance, the gold concentration in *Eucalyptus* trees which thrive on the gold rich Freddo Gold Prospect deposit in Australia was reported at 80 ppb in dried leaves (Lintern et al., 2013). The average gold concentration in *B. juncea* under conditions of the greenhouse trial ranged of 192 to 275.7 ng g^{-1} . This finding suggested that this concentration is about 2.4 to 3.4 higher than was reported from *Eucalyptus* trees.

Anderson, Moreno, Geurts, et al. (2005) proposed that threshold level for gold hyperaccumulator is 1,000 ppb gold in dry weight. Results from current study suggested no indication of gold hyperaccumulation in *B. juncea*.

5.13. Conclusions

Brassica juncea was assessed to hyperaccumulate copper. The reported maximum copper concentration from individual plants was between 440 and $759 \mu\text{g g}^{-1}$. The study concluded that copper uptake occurred at hyperaccumulator level.

Nickel concentration in *B. juncea* was reported high. The highest nickel concentration was reported between 197 and $601 \mu\text{g g}^{-1}$. At this value, strong nickel accumulation is proposed to occur in *B. juncea*.

Palladium uptake in aerial parts of *B. juncea* ranged from 2,130 to $2,909 \text{ ng g}^{-1}$. Based on this concentration, this study found that palladium uptake was $> 1,000 \text{ ng g}^{-1}$ and proposed that hyperaccumulation of palladium occurred.

The highest gold concentration reported between 609.4 and 633.1 ng g⁻¹. The results demonstrated no indication of hyperaccumulation. However, these concentrations were higher compared to 'normal' gold concentration in wild plants (normal concentration of 10 ng g⁻¹).

High metal solubility in the growth substrate was suggested to have affected plant growth performance. Poor growth performance, as indicated by metal stress symptoms and low biomass yield prevented the treatment of plants with amendments to induce hyperaccumulation. Natural solubility of copper, nickel, and palladium in soils is likely to have caused toxicity to plant tissues. Reported metal effects in *B. juncea* were chlorosis, and necrosis.

Besides high solubility of toxic metals, high temperature stress was expected to have influenced plant growth performance.

Based on the findings of this chapter, *Brassica juncea* was discounted as a species suitable for ongoing Phytocat trials on the Broken Hill gossan. There is no realistic chance to achieve the target palladium concentration of 1000 µg g⁻¹ in a plant species that will not grow on the gossan substrate.

Chapter 6 - Greenhouse trial: Induced hyperaccumulation in *Cannabis sativa*

6.1. Introduction

Under natural conditions, plant can absorb metals which have appreciable solubility in water, but some plant species, such as hyperaccumulators have capability to accumulate a higher level of metals in their biomass than would normally be expected. However, precious metals such as gold and palladium are less soluble than essential metals such as copper and nickel. Beside plant genetic limitations, metal absorption is also limited by rhizosphere distribution. Metals are randomly distributed in soil. This condition may hinder plant's capability to take up metals (Anderson, Brooks, Stewart, Simcock, et al., 1999; Robinson, Anderson, & Dickinson, 2015).

Induced hyperaccumulation is a method to increase metal solubility in soil and make plants accumulate more metals. This method has been demonstrated in *Zea mays* by adding HEDTA as a soil amendment (Huang & Cunningham, 1996). Work by Anderson, Moreno, and Meech (2005) demonstrated the use of cyanide to induce gold uptake in *Brassica juncea*. The work proposed that induced hyperaccumulation could be viably applied to precious metals, such as gold. There are two key points that can be suggested from this work: (1) the possibility to hyperaccumulate precious metals in non hyperaccumulator species, and (2) it is possible to induce solubility of low soluble metals by using cyanide.

Chapter 5 proved that *Brassica juncea* had poor growth performance because of heavy metal stress and an induced hyperaccumulation treatment could not be used. The current study uses industrial hemp (*Cannabis sativa* L) as a species for the induced hyperaccumulation of palladium as well as gold, copper, and nickel. *Cannabis sativa* was chosen because this species has been suggested to have potential for mineral extraction purposes (Angelova et al., 2004). Moreover, hemp can grow in extreme soil conditions and required limited exogenous fertiliser inputs (Linger, Müssig, Fischer, & Kobert, 2002).

6.2. Aims and methodology

The aims of this work were (1) to induce the hyperaccumulation of palladium to a concentration of $1,000 \mu\text{g g}^{-1}$ (as well as other available metals) with potassium cyanide (KCN), (2) to demonstrate hemp's ability to extract metals from metal-rich soils, (3) to identify metal uptake ratios in plant relative to the metal concentration in an extraction solution, and (4) to determine what factors may affect metal uptake in hemp species.

Induced hyperaccumulation trials used the same substrate as the *B. juncea* trial. Seeds of *Cannabis sativa* L. 'Fasamo' and 'Ferimon' (two cultivars) were used. Before sowing the seeds, the pots were watered frequently to provide enough moisture for the trial. Seeds were sowed in the summer, on 18 February 2015. Each pot in the two soil groups contained five seeds of *C. sativa*. Five holes about 1 cm deep were prepared and the seeds were added into the holes and covered with growth medium. About 195 hemp seeds were used for this study. Detailed seed distribution per cultivar in each soil group is described in Table 6.1.

Table 6.1. Number of Fasamo and Ferimon seeds

Soil group	Cultivar	Number of Pot	Number of seed
A	Fasamo	9	45
A	Ferimon	9	45
B	Fasamo	10	50
B	Ferimon	11	55

To prevent water logging of the pots, irrigation was maintained when the substrates were dry. During the trial, greenhouse temperature was reported ranging from 12 to 40°C. After 33 days of growth, potassium cyanide solution (50 mL of 8 g L^{-1}) was irrigated into pots of the two soil groups. Three pots in each soil type did not receive KCN treatment and were defined a treatment controls. Irrigation was stopped after KCN treatment, except for control groups. KCN caused heavy metals in the soils to become soluble and induced uptake into the Hemp. Metal toxicity symptoms such as yellow shoots, and leaves turning brownish and falling from plants were observed, although these observations were a continuation of normal

observations throughout plant growth. Three days after treatment, aerial portions (leaves and stems) from all pot trials were harvested. Plant aerial parts were placed in paper bag and then oven dried at 70 °C. After constant weight was reached, plant samples then cut and weighed for copper, nickel, gold and palladium analysis. Metal analysis followed the methods described in Section 3.3.

6.3. *Cannabis sativa* growth on Broken Hill substrate

6.3.1. Germination rate

The Broken Hill gossan is a mineralised soil which contains high level of heavy metals, such as copper and nickel. The high content of these toxic metals has potential to influence plant growth and development. Besides the growth medium factor, temperature is also suggested to have an important role in the trial. *Cannabis sativa* required growth temperature from 1 °C to 45 °C, and optimum temperature around 35 °C (van der Werf, Brouwer, Wijnhuizen, & Withagen, 1995).

During the plant growth experiment, greenhouse temperature was ranged between 12 °C and 40 °C. This good range of temperature supported germination of hemp seeds. In soil A there were 27 seeds of Fasamo and 27 seeds of Ferimon. In soil B there were 33 seeds of Fasamo and 34 seeds of Ferimon. Based on daily observations, the germination rate for Fasamo was 68.4 percent and Ferimon was 66.0 percent. All germinated seeds grew to maturity.

6.3.2. Heavy metals effects in plant growth

During the growth period of 34 days, indications of toxicity in the plant trials were observed. According to Gagnon et al. (2006), physical evidence of heavy metal toxicity is genetic mutation as heavy metal intoxication can cause false DNA transcription. Figure 6.1 shows abnormality in plant growth and development. Some plants looked stunted with abnormal size and shape of stems and leaves. This finding was observed in 4 pots from the total 39 pot trials.

Other physical effects of phytotoxicity are presented in Figure 6.2. Observations in the plant trials indicated that metal toxicity symptoms or browning leaves, yellowish stems and fallen leaves. Physical damage as found in this study are irreversible and also anticipated to affect biochemical metabolism (Ross & Kaye, 1994).

Cannabis sativa is considered to be resistant to extreme soil conditions. Plants in the current study showed 'light' toxicity effect such as in leaves tips and stems. There was no necrosis found during the experiment.

After induced hyperaccumulation was initiated, the resulting high level of cyanide in plant tissue would be expected to affect plant growth metabolism. A high level of cyanide in plant tissues may inhibit electron transport mechanism and hence signify toxic effects. Generally, all plant species have a detoxification mechanism to exclude a contaminant from plant tissue before they reach a toxicity level. However, different species express different detoxification level.

There are two enzymes where are responsible in detoxification mechanisms in plant tissues. These enzymes are sulfurtransferases and β -cyanoalanine synthase (Brice, 2005). Induced hyperaccumulation exposed plants to toxic conditions where cyanide and soluble toxic metals were present in the root zone. The effects of these factors were noticed one day after KCN treatment. Figure 6.3 compares plant growth performance between the control and treatment group, and Figure 6.4 illustrated effects of KCN treatment in male and female plants.

The addition of 50 mL of 8 gram per litre KCN in the pot trials was expected to flood the rhizosphere with soluble cyanide metal complexes. These complexes were suggested to be translocated into aerial parts through root systems. *Cannabis sativa* on the left of Figure 6.3 showed a certain degree of toxicity effects. A high concentration of metal in the aerial parts resulted withered stems, chlorosis and damage in plant's membrane. On the other hand, after three days of treatment, only a small numbers of the plants exhibited necrosis. Therefore this study recommended that *C. sativa* is a strong plant. This species can survive under metal stress conditions induced by KCN treatment of soil.



Figure 6.1. Mutation (circled) in *C. sativa* as metal toxicity effects



Figure 6.2. Chlorosis in *C. sativa* leaves



Figure 6.3. Growth appearance comparison of *C. sativa* before (right) and after (left) KCN treatment



Figure 6.4 Male (above) and female (below) plants showing effects of toxicity after KCN treatment

6.4. Metal uptake

6.4.1. Copper and nickel uptake

In this trial, *Cannabis sativa* was treated with potassium cyanide (KCN) at a concentration of 8 g L⁻¹ to make metals more soluble and ready for plant uptake. KCN was chosen due to its ability to promote solubility of precious metals. Table 6.2 summaries the concentration of extractable metal in soil and the metal concentrations in hemp aerial parts. Review of the extractable metal concentration demonstrates that metal solubility from both soil types, from highest to lowest, is as follows: Cu > Ni > Pd > Au. The highest concentration of soluble copper and nickel, were found in soil B at a concentration of 1,099, and 35.7 µg g⁻¹ respectively. As copper is more soluble in soil B, Hemp grown in soil B is likely to extract a higher copper concentration than Hemp on soil A. The average copper concentration in Hemp on soil B was 4,439 µg g⁻¹ dry weight. This concentration is 115 fold higher than control group. The highest individual copper concentration was 4,972.5 and 6,726 µg g⁻¹ dry weight on soil A and B respectively. Compared to the 'normal' copper concentration in plants, these values are 248 to 336 higher. Analysis of variance of the copper mean concentration in the three treatment groups indicated that they are significantly different ($p < 0.05$).

The mean concentration for nickel in Hemp on soil B was 101 µg g⁻¹ dry weight with a concentration range from 42.3 to 184 µg g⁻¹ dry weight. In comparison, the nickel concentration from the control plants was 16 µg g⁻¹. After KCN treatment, hemp showed ability to uptake soluble nickel from the soil substrate. The normal nickel concentration in plants is suggested to be 100 µg g⁻¹ dry weight. Compared to this value, the nickel concentration in hemp after KCN application was not high. Figure 6.5 indicates that the mean concentration between control and Hemp A was not significantly different ($p > 0.05$).

Table 6.2. Metal uptake in *Cannabis sativa* after induced hyperaccumulation treatment with KCN 8 g L⁻¹

Metal	Unit	Normal	Mean extractable				Range			
			metal (KCN)		Aerial biomass		Control		Hemp	
			Soil A	Soil B	Control (n = 10)	Hemp A (n = 23)	Hemp B (n = 26)	Control	Hemp A	Hemp B
Cu	µg/g	20	44.5	1,099	38.7 (7.9) c	3,263 (140.5) b	4,438.9 (205) a	3 - 90.7	2,236.8 - 4,972.5	2,416 - 6,726
Ni	µg/g	100	5.01	35.7	16 (4.6) b	36.3 (5.2) b	101.1 (7.3) a	<DL - 34	<DL - 80.5	42.3 - 184
Pd	ng/g	8.7	277	205.5	38.8 (25.9) c	11,632 (873.7) b	30,336.3 (2,507) a	<DL - 258.4	6,274.1 - 23,204.6	9,596 - 62,420
Au	ng/g	10	34.4	20.2	27.5 (13.3) b	5,357 (330.5) a	4,528.1 (302) a	<DL - 111.6	2,686.1 - 9,097.8	2,164 - 7,635

Mean concentrations are presented with standard error in parentheses. Means with different letter are significantly different (p < 0.05).

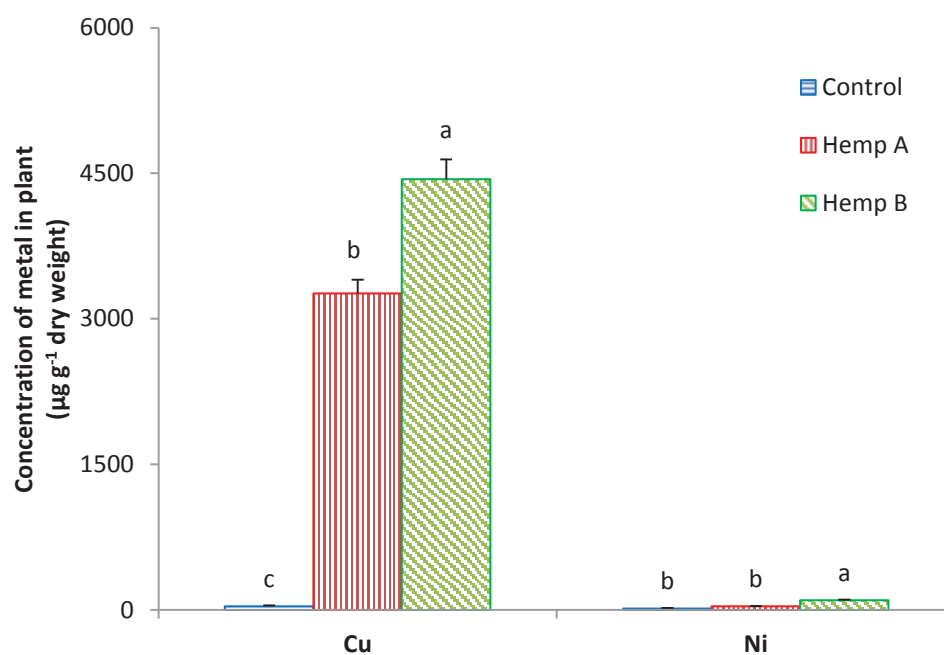


Figure 6.5. Mean concentration of Cu, and Ni in *Cannabis sativa* treated with KCN 8 g L⁻¹. Mean with different letter are different significantly (p < 0.05).

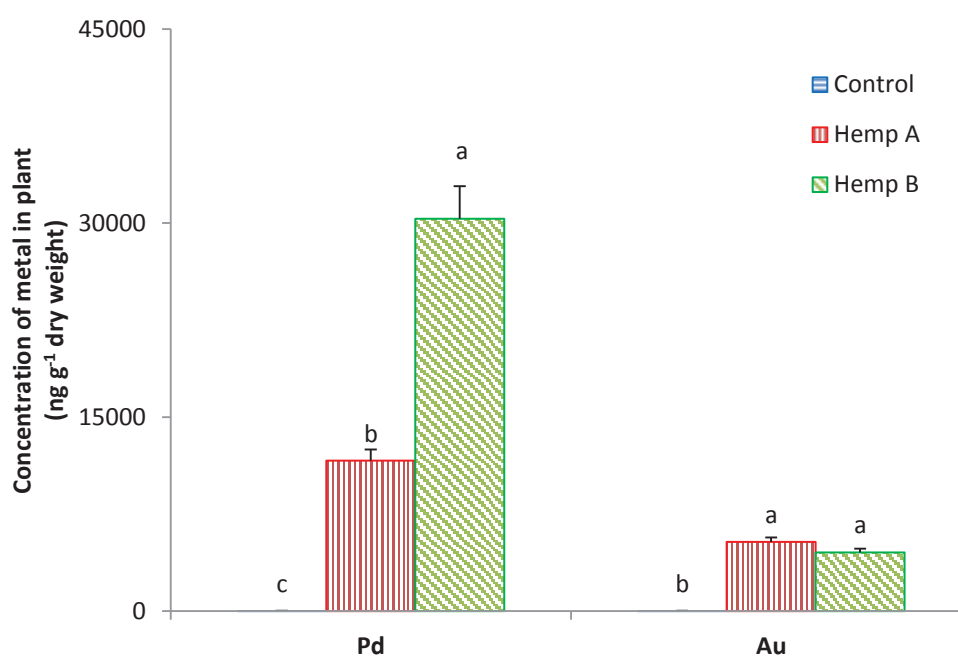


Figure 6.6. Mean concentration of Pd and Au in *C. sativa* treated with KCN 8 g L⁻¹. Mean with different letter are different significantly (p < 0.05).

6.4.2. Palladium and gold uptake

Palladium solubility (water soluble) in the two soil types was 277 and 205.5 ng g⁻¹ for soil A and soil B respectively. Based on this solubility data, Hemp grown on soil A would be expected to uptake a higher palladium concentration compared to Hemp B. However, ANOVA analysis (Figure 6.6) showed that Hemp B had the highest palladium concentration in its biomass, followed by Hemp A, and control group. This study noted that palladium uptake in three treatment groups were different statistically ($p < 0.05$).

The average palladium concentration in Hemp B was 30,336.3 ng g⁻¹, with range of concentration between 9,596 to 62,420 ng g⁻¹. Compared to the control group, mean palladium uptake in Hemp B was 780 times higher. The mean palladium concentration in Hemp A was 11,632 ng g⁻¹. The mean concentration of palladium uptake in Hemp B suggested was 2.6 fold higher than in Hemp A.

Since palladium is poorly soluble in soil, there is no natural palladium concentration in wild plants. Therefore, it could be assumed that palladium uptake in normal plants would be below 10 ng g⁻¹. Hence, the threshold level of palladium hyperaccumulation in plant would be expected at 1,000 ng g⁻¹. Results from this study indicated that hyperaccumulation of palladium occurred in Hemp growing on both soils.

Gold solubility after KCN treatment in soil A was 34.4 ng g⁻¹, which is 1.7 fold higher than in soil B. Therefore as indicated for palladium, Hemp grown on soil A could be expected to extract more gold than on soil B. Gold uptake in this induced hyperaccumulation trial suggested that gold uptake in Hemp on soil A and B were not different significantly ($p > 0.05$). However, both treatment groups were different significantly to the control group. The concentration of gold in the control group was 27.5 ng g⁻¹. Whereas the gold concentration in Hemp A and Hemp B was 5,357 and 4,528 ng g⁻¹ respectively with a range for both treatment groups between 2,164 and 9,097.8 ng g⁻¹. Under KCN treatment, the highest individual value in Hemp A was 9,099.8 ng g⁻¹, while in Hemp B was reported at 7,635 ng g⁻¹.

A threshold level for gold hyperaccumulation in plant was defined as 1,000 ng g⁻¹ (Anderson, Moreno, Geurts, et al., 2005). The findings of this trial reported that gold hyperaccumulation uptake was observed in Hemp A and Hemp B.

6.5. Metal sink in *Cannabis sativa*

6.5.1. Copper and nickel sink

Localisation of copper and nickel has previously been reported to be higher in hemp's leaves (Arru et al., 2004; Psaras et al., 2000). The copper concentration in *C. sativa* grown on gossan soils indicated that copper was higher in leaf than stem for Hemp B, but this difference was not significant. The concentration of copper in leaf Hemp B was reported at 4,774 $\mu\text{g g}^{-1}$ while stem concentrated 4,102 $\mu\text{g g}^{-1}$. The difference between leaf and stem tissue concentration was not significant for other groups (Figure 6.7).

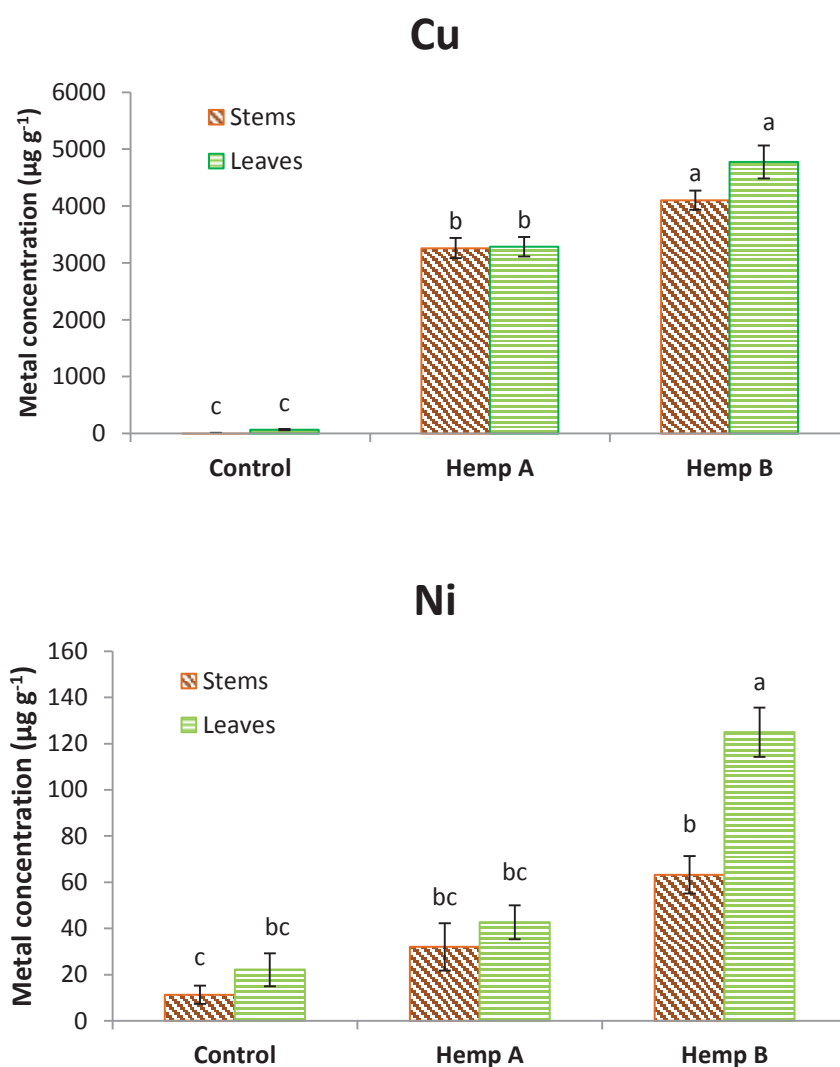


Figure 6.7. Copper and nickel sink in *C. sativa*

The trend for nickel uptake in aerial parts indicated that the nickel concentrations in leaf and stem were again significantly different growing on soil B ($p < 0.05$). The highest nickel concentration in leaf from this treatment group was $124.9 \mu\text{g g}^{-1}$. For comparison, the mean concentration of nickel in stem and leaf of Hemp A was suggested to be not significantly different ($p > 0.05$).

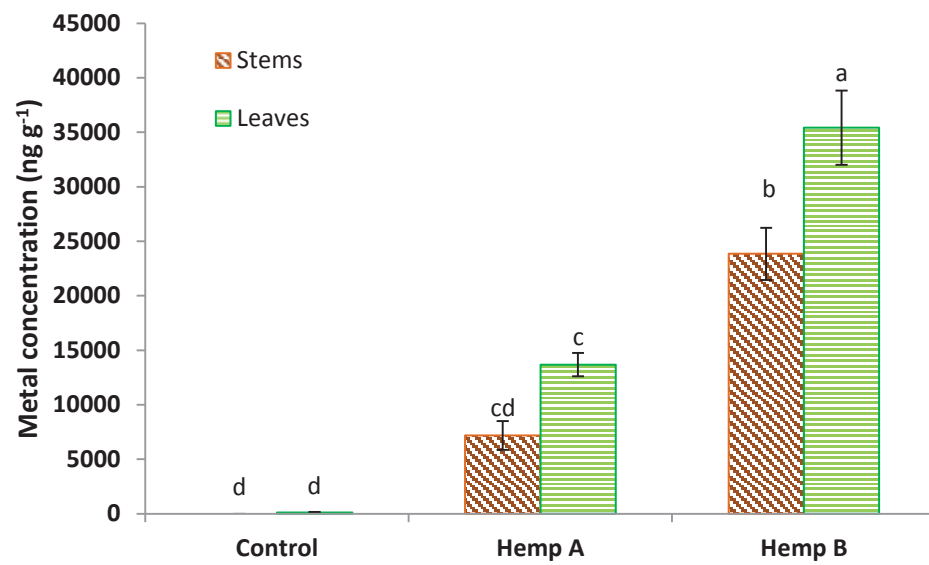
6.5.2. Palladium and gold sink

The concentration of palladium in *C. sativa* was higher in leaf than stem as illustrated in Figure 6.8, and for Hemp B this difference was significant ($p < 0.05$). The highest reported mean palladium concentration in leaf for Hemp B was $35,425 \text{ ng g}^{-1}$. This concentration was about 1.5 higher than in its stem.

Gold uptake in Hemp (Table 6.3) demonstrated that the gold concentration was localised higher in stem rather than in leaf when grown on the gossan. The reported gold concentration from stem Hemp A was $7,221 \text{ ng g}^{-1}$, while the concentration of gold in stem Hemp B was $6,111 \text{ ng g}^{-1}$. ANOVA test as illustrated in Figure 6.8 proved that the gold concentration in stems was statistically different than in leaves ($p < 0.05$). The gold concentration in leaf and stem in the control group was not significantly different.

This study therefore found that the precious metal sink in *C. sativa* is different. Hemp B localised palladium in leaf more than in the stem. However, this pattern could not be applied for gold where the metal concentration was higher in stem than leaf. This pattern could be found in Hemp A and Hemp B.

Pd



Au

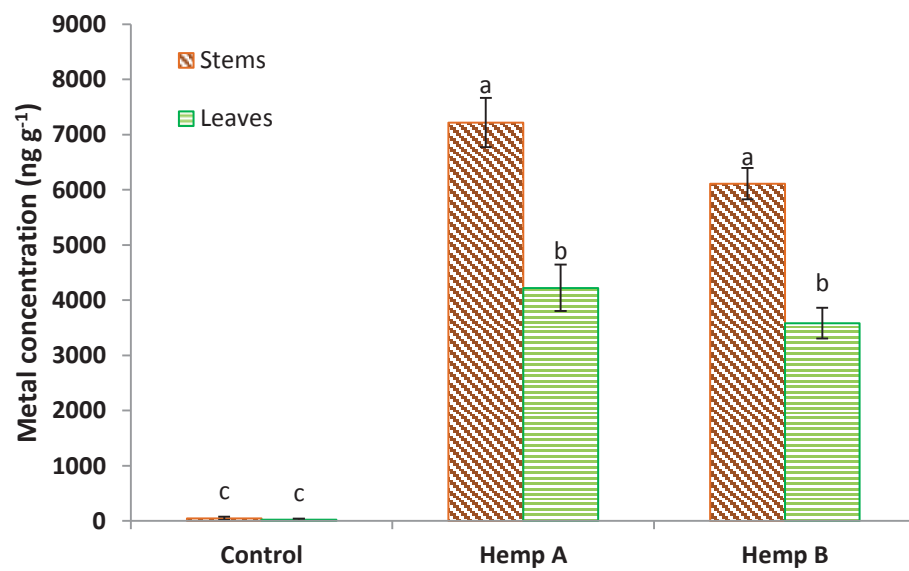


Figure 6.8. Palladium and gold sink in *C. sativa*

6.6. Biomass and metal content

Total biomass production from aerial parts of 'Fasamo' and 'Ferimon' as presented in Table 6.3 was 15.27 gram dry weight. Total yield biomass, included leaves and stems in Fasamo (n = 21) was 6.9 gram, and Ferimon (n = 28) was 8.38 gram. Detailed analysis of the dry weight plant parts showed that Ferimon produced higher biomass (5.47 gram) in its leaves compared to Fasamo (4.21 gram). Both cultivars had less biomass in stems compared to leaves. Stem weight in Fasamo and Ferimon observed at 2.68 and 2.91 gram respectively.

Considering total uptake of metals, *C. sativa* accumulated more copper, nickel and palladium in the leaves. In contrast, the gold content of stems higher than in leaves.

Metal content in the aerial parts indicated that copper was the greatest of the four tested metals for both soil types. The order of total metal content from the highest to the lowest among both Fasamo and Ferimon was as follows: Cu (58.4 mg) > Ni (1.1 mg) > Pd (0.3 mg) > Au (0.07 mg).

In order to examine significant differences in the metal content within the tissues of the two cultivars, an ANOVA test with confidence interval of 95 percent was employed. ANOVA test indicated that mean concentrations of copper in Fasamo leaf compared to other aerial parts was different significantly ($p < 0.05$). The highest copper content on Fasamo of 18.5 mg was reported from leaf. The lowest copper content in hemp plants was in Ferimon stem at 9.9 mg. The trend of metal content as depicted in Figure 6.9 showed that hemp's leaves contained higher copper than its stems.

The highest nickel content of 0.43 mg was reported for Fasamo leaves (Figure 6.10). ANOVA test for mean nickel content suggested that nickel content in Fasamo leaf was not different significantly ($p < 0.05$) compared to leaves of Ferimon, but was greater than Ni content of both stems.

Table 6.3. Biomass yield and metal mass per plant after KCN treatment

Cultivar	N	Part	Weight (gram dry weight)	Metal mass per plant (µg)				Mean metal mass per plant (µg)			
				Cu	Ni	Au	Pd	Cu	Ni	Au	Pd
Fasamo	21	Leaf	4.21	18,583	439.7	17.96	126.49	885 (135) a	20.9 (4.5) a	0.86 (0.1) a	6 (1.1) a
	21	Stem	2.68	10,294.9	172.32	18.23	54.31	490.2 (93.4) bc	8.2 (2) bc	0.87 (0.15) a	2.6 (0.6) bc
Ferimon	28	Leaf	5.47	19,636	386.26	18.54	116.83	701.3 (80.1) ab	13.8 (2.2) ab	0.66 (0.09) a	4.2 (0.6) ab
	28	Stem	2.91	9,935.9	90.077	17.34	41.31	354.9 (66.3) c	3.2 (0.8) c	0.62 (0.12) a	1.5 (0.3) c
Total			15.27	58,5	1,1	72.08	338.9				

Mean concentrations are presented with standard error in parentheses. Means with different letter are significantly different (p < 0.05).

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Table 6.4. Metal concentration in stem and leaf of *C. sativa* after induced hyperaccumulation treatment

Metal	Unit	Stem				Leaf			
		Control		Hemp A		Hemp B		Control	
Cu	µg/g	5.7 (3.5) c		3,260 (178) b		4,102 (170) a		67.5 (13.3) c	
Ni	µg/g	11.3 (3.98) c		32 (10.2) bc		63.2 (8.06) b		22.1 (7.09) bc	
Pd	ng/g	<DL		7,184 (1,325) cd		23,843 (2,404) b		98 (67.8) d	
Au	ng/g	45.4 (30.5) c		7,221 (448) a		6,111 (284) a		23.7 (17.8) c	

Mean concentrations are presented with standard error in parentheses. Means with different letter are significantly different (p < 0.05).

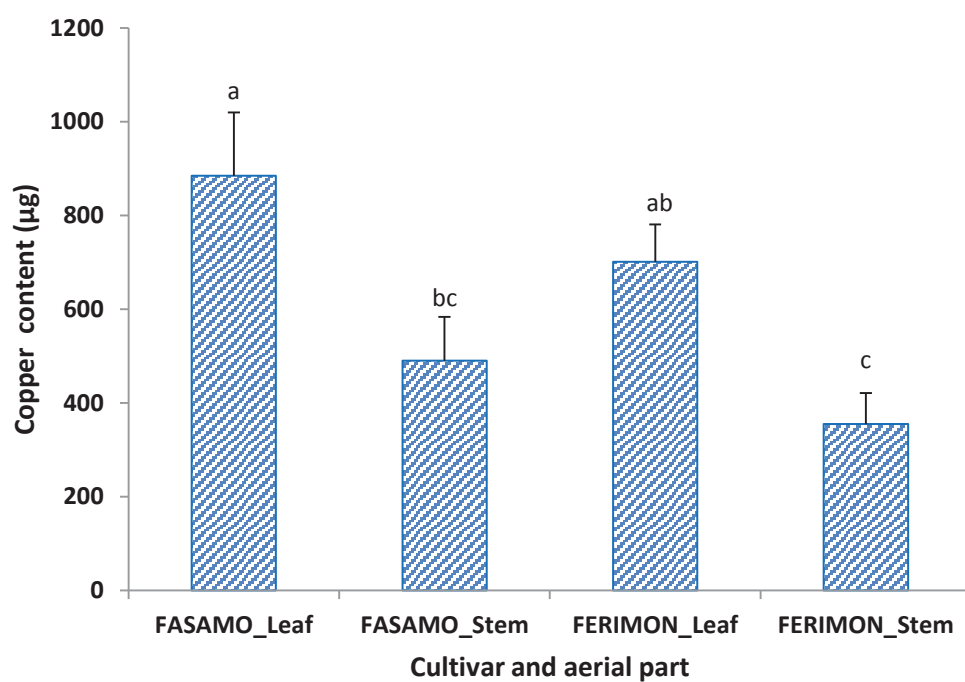


Figure 6.9. Copper content in Fasamo and Ferimon aerial parts after induced trial

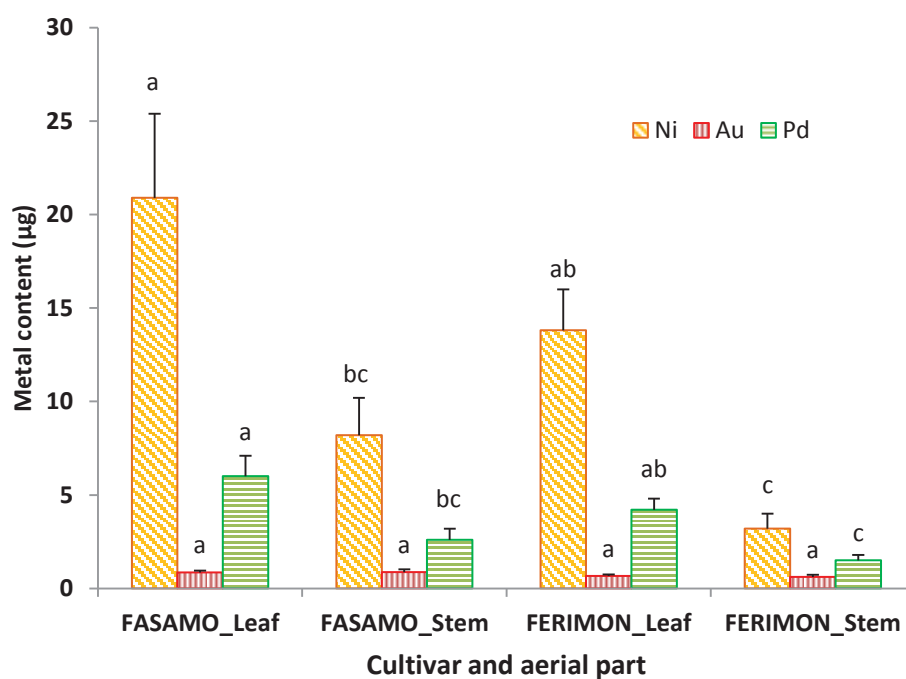


Figure 6.10. Ni, Au and Pd content in Fasamo and Ferimon aerial parts after induced trial

Besides containing a higher copper and nickel in its leaf, Fasamo also contained a higher mass of palladium in leaves. Palladium content was reported at 0.1 mg, and its mean was significantly different ($p < 0.05$) compared to the other parameter observations. The lowest palladium content of 41.31 μg was found in Ferimon stem (Figure 6.10).

While the gold concentration was higher in stems as a function of soil different (refer section 6.5), analysis of the gold content in leaves and stems from Fasamo and Ferimon showed no significant difference ($p > 0.05$). Therefore gold uptake and its content in hemp's aerial parts was not affected by cultivar differences.

These results suggested that the highest high metal content of copper, nickel, and palladium was found in Fasamo leaves. In contrast, the lowest metal content was found in Ferimon stems. Metal content in both cultivars was observed to have the same pattern, and was ordered as follows: $\text{Cu} > \text{Ni} > \text{Pd} > \text{Au}$.

6.7. Metal uptake ratio

The metal uptake ratio in this analysis is defined as comparison between metal concentration in KCN extraction solution and metal concentration in plant. The metal uptake ratio as presented in Table 6.5 showed that there was an ordering of metal plant – soil ratios. The order for uptake ratio from highest to lowest for Hemp growing on soil A was as follows: $\text{Au} > \text{Pd} > \text{Cu} > \text{Ni}$. For Hemp on soil B the order was: $\text{Au} > \text{Pd} > \text{Cu} > \text{Ni}$. The highest uptake ratio occurred for gold followed by palladium for both soils. The copper uptake ratio was reported very low for Hemp on soil B, while nickel was found as the lowest in Hemp B (Figure 6.11).

Hemp grown on soil B was observed to have a gold concentration that was 224 times higher than in soil. The measured soluble gold in soil B was 20.2 ng g^{-1} and its concentration in plant biomass was 4,528 ng g^{-1} . A higher palladium uptake ratio was also reported for Hemp B. *C. sativa* from soil B recorded a palladium of 30,336 ng g^{-1} from an extractable palladium concentration in soil of 205.5 ng g^{-1} . The ratio of palladium concentration in plant to soil was 147.6.

Table 6.5. Percent extractable metal and metal uptake ratio in Hemp from two soil types

Metal	Unit	Mean total metal		Mean extractable metal (KCN)		Percent extractable		Metal uptake ratio	
		Soil A	Soil B	Soil A	Soil B	Soil A	Soil B	Hemp A	Hemp B
Cu	µg/g	13,962	10,890	445	1,099	3.2%	10.1%	7.3	4
Ni	µg/g	3,484	6,354	5.01	35.7	0.1%	1.0%	7.2	2.8
Pd	ng/g	3,805	1,566	277	205.5	0.9%	1.3%	42	147.6
Au	ng/g	32,650	16,665	34.4	20.2	0.8%	1.2%	155.7	224.2

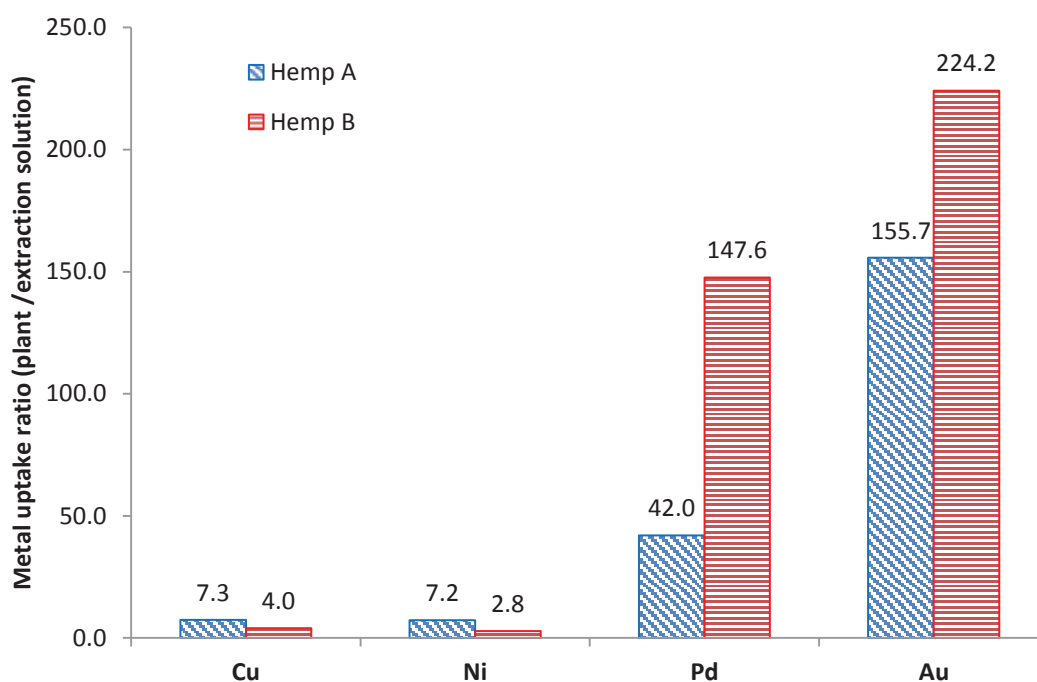


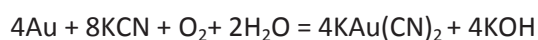
Figure 6.11. Metal uptake ratio under induced hyperaccumulation trial in *C. sativa*

Discussion

6.8. Metal availability

Metal extraction by plants requires interaction of soil condition, available metal in soil, and rhizosphere. According to Gobran, Wenzel, and Lombi (2001), the rhizosphere is an area of millimetres in distance surrounding roots. In this zone, interactions between metals and roots exist. Metals and rhizosphere interactions lead to affect on metal-soil bonds. Exudated organic chemicals from plant root may alter metal ion stability and make metals more soluble in soils. In soluble form, metals are easily absorbed and transported into aerial parts.

Potassium cyanide (KCN) has been used to solubilise precious metals in soils. Once metals are in soluble form, Hemp can absorb and concentrate them in plant biomass. In the induced hyperaccumulation trial, cyanide anions (CN⁻) and metal cations formed metal-cyanide complex (Sakambari, 2015). According to Johnson (2015), reaction of KCN and gold in this work can be expressed as follows:



Johnson asserted that gold ionization occurred where an oxidant exists. Subsequently, every gold ion then coordinated with two CN⁻ forms a soluble metal-cyanide complex. In this soluble form the cyanometallic complexes can be absorbed by plants through the rhizosphere, and can be translocated to aerial parts through transpiration mechanisms.

Sakambari (2015) reported that mineral differences in soil A and soil B determined the capability of KCN in metal extraction. Under KCN treatment, the order of extractable metal concentration in both soils was as follows: Cu > Ni > Pd > Au. KCN was able to solubilise precious metals in both soils. This confirmed the finding of Johnson (2015). Besides that, KCN was also determined to bond with copper and nickel with a higher concentration. This results were in agreement with Wilson-Corral, Anderson, and Rodriguez-Lopez (2012).

Metal extractable data can be used to determine the relative performance of the hemp trial to uptake precious metal in the different soil types. Both soil substrates have different precious metal availability. The total concentration of gold and palladium was higher in soil A. Therefore hemp on soil A could be anticipated to accumulate a higher concentration of gold and palladium in the aboveground mass.

Percent extractable values were calculated to analyse the percentage of available metals in soil after KCN treatment, relative to total metal concentrations. The percent of extractable metals show that soil B has more soluble metals than soil A (Table 6.4). Metals such as copper, nickel, gold, and palladium are high in soil B. This result implies that KCN has a good performance to leach copper, nickel, gold, and palladium. Moreover, KCN is suggested to give best performance for solubilising gold and palladium. As a generalisation, percentages of extractable metals in those noble metals are high, compared to other metals.

6.9. Induced hyperaccumulation in *Cannabis sativa*

The rate of uptake of cyanide metal compounds into biomass has been suggested to be higher for nitrogen deficient substrates (Larsen, 2005). As the gossan soil substrate is likely poor in macro nutrients, as it is crushed rock, the uptake of metal cyanide complexes by *Cannabis sativa* should be high. Correspondingly, high metal accumulation in the aerial biomass was observed.

Induced hyperaccumulation using KCN shows that soil B has more available metals than soil A. The results as presented in Table 6.1 showed that hemp has a different metal concentration in its biomass as a function of soil type. Hemp grown on soil B took up a high concentration of copper, nickel, and palladium. In particular, copper uptake was very large, as $6,726 \mu\text{g g}^{-1}$ (0.6 percent) was analysed in the biomass. The concentration of copper as an essential element in plants is recommended between 20 and 30 ppm (Ducic & Polle, 2005). Concentrations higher than this threshold level will cause plant toxicity. Hemp grown on both soil types therefore accumulated copper to a higher concentration than normal plants do. As the threshold level for copper hyperaccumulation is $300 \mu\text{g g}^{-1}$, this study suggested that some individual hemp plants were able to concentrate copper at the hyperaccumulator level, although it must be noted that this hyperaccumulation was induced. Control plants did not hyperaccumulate copper.

The nickel metal uptake ratio showed that nickel in soil B was more available for hemp than in soil A. The range of nickel concentration in hemp was assessed to be from below detection to $184 \mu\text{g g}^{-1}$. According to Rascio and Navari-Izzo (2011), nickel toxicity may occur at concentration between 10 and 15 ppm in the plant tissues, while according to Gagnon et al. (2006) 1 ppm of palladium may cause necrosis as well as death to *Sphagnum* species. Nickel

concentrations in hemp tissues exceeded the toxicity threshold. Hence, this plant species is likely more tolerant to nickel toxicity than normal plants.

Hemp from gossan on soil A extracted a higher concentration of gold than hemp in soil B. Gold is suggested to have no biological effect on plants as no study has associated high gold concentration in plants to phytotoxicity. The current study therefore assumed that no gold toxicity occurred in the plant trials. After induced hyperaccumulation treatment, the gold concentration in *C. sativa* was as high as 9,098 ng g⁻¹ (9 ppm) and 7,635 ng g⁻¹ (7 ppm) for Hemp A and Hemp B respectively.

The current study noted that since palladium has low solubility in the natural environment, this study could assume that an average palladium concentration in natural plants is 10 ng g⁻¹. Therefore any palladium concentration above 1,000 ng g⁻¹ could be suggested as reaching the hyperaccumulation threshold level. Observations for individual palladium uptake concentration showed that *C. sativa* was able to uptake palladium to a concentration as high as 62,420 ng g⁻¹ (62 ppm) from soil B. This concentration could be reported as palladium hyperaccumulation concentration.

In comparison, an induced hyperaccumulation study using artificial substrate as reported by Walton (2002) showed that *Berkheya coddii* could uptake gold at concentration of 75.9 ppm, and 84.2 ppm. Hence in the current study gold uptake in *C. sativa* was low. However, gold uptake at hyperaccumulation level was observed.

Walton (2002) reported that 9.8 ppm palladium was measured in individual *B. coddii* after treated with 1 gram KCN per litre. This work likely was the only previous report for induced hyperaccumulation of palladium. The current study suggests that individual *C. sativa* from this trial was examined to accumulate palladium approximately seven fold more than was reported by Walton. Hence, the current study presents the highest palladium concentration observed under conditions of induced hyperaccumulation.

6.10. Conclusions

In general, the growth trial for *Cannabis sativa* demonstrated that the plant has good growth performance on gossan substrates.

Metal concentrations and aerial parts. *Cannabis sativa* has a different pattern to the distribution of the four studied metals in aboveground tissues. Leaf was identified to deposit copper, nickel, palladium, while stem appeared to be the primary gold storage in this plant species.

Metal contents (mass) and aerial parts. Leaf of Fasamo cultivar was determined to contain the highest concentration and content of copper, nickel, and palladium. Total metal contents from the highest to the lowest from all plant samples in order as: Cu (58.5 mg) > Ni (1.1 mg) > Pd (0.3 mg) > Au (0.07 mg).

Biomass production. Total yield biomass in treatment plant was 15.27 gram. Biomass production in Fasamo and Ferimon cultivars was 8.4 and 6.9 gram respectively.

Metal uptake ratio. Analysis of metal content in biomass versus extractable metal indicated that *C. sativa* growing on soil A has a same pattern to soil B. The metal uptake ratio in both soil types is as follows: Au > Pd > Cu > Ni.

Maximum individual concentration. Significant metal concentration values after KCN treatment were as follows: Copper ($6,726 \mu\text{g g}^{-1}$) > nickel ($184 \mu\text{g g}^{-1}$) > palladium ($62 \mu\text{g g}^{-1}$) > gold ($9 \mu\text{g g}^{-1}$). Copper, palladium, and gold hyperaccumulation was observed.

Chapter 7 - General discussion

7.1. Introduction

Broken Hill can be considered as one of the most rich platinum group metal ore bodies on earth. Previous exploration across this area reported a palladium concentration in surface rocks at a grade of 50 g t^{-1} (Birch, 2007; Impact Minerals Limited, 2015). Hence, Broken Hill is a potential target for palladium phytomining, as indicated in the objective of the Phytocat Project. There are two challenges in order to extract PGMs from the Broken Hill gossan by using plants. First, the Broken Hill substrate was identified to contain high soluble copper which could harm plant species. Secondly, since palladium solubility in soil low, a method is needed to make palladium more available for plant uptake.

The potential of plant species to extract copper, nickel, gold and palladium was reported and discussed in Chapter 4 (native plant species from Broken Hill), Chapter 5 (metal uptake using *Brassica juncea*), and Chapter 6 (induced hyperaccumulation in *Cannabis sativa*). The aim of this study was to achieve high net uptake of palladium to a concentration of $1,000 \mu\text{g g}^{-1}$ in biomass.

This chapter is aimed to discuss significant findings from previous chapters, as well as to synthesise best knowledge which could be used to put palladium phytotextraction into practice.

7.2. Plant growth performance

High metal net uptake in all plant trials was recorded and some difficulties were experienced during this study. *Brassica juncea* was sown in the summer. Recorded temperature reached up to 35°C . As asserted by Angadi et al. (2000), *B. juncea* may experience heat stress and affected to its growth and development. Reported total biomass production from this species was 5.1 gram from 39 pots. Therefore this study concluded that *B. juncea* has poor growth performance on the gossan.

Observations for *Cannabis sativa* noted that this fibre plant has a better growth performance. Germination was observed in 14 days. This species grew very fast, tall, and thin. *Cannabis*

sativa was therefore a good choice to be used in such a trial. However, this plant only can be grown in the summer time and temperature was likely to have been a main factor to support plant growth. *Cannabis sativa* produced more biomass than *B. juncea*. Total harvested biomass was 15.27 gram. It is about 3 fold higher than *B. juncea*. About 3 days after KCN treatment, the plant was harvested. Despite toxicity effects appearance during the plant trials, plants were still alive at final harvest. Presumably the KCN concentration of 8 g L⁻¹ was not toxic to *C. sativa*. Hence it is assumed that the plant trials could have been left longer before be harvest in order to concentrate a higher metal concentration in aerial portions.

7.3. Soil types and metal uptake

Two soil types were used in this study. Soil A (goethite rich) which had a lower concentration of soluble metals, and soil B (hematite rich) which had more soluble metal. Berhe (2015) reported that sample A is rich in goethite (58.4 wt. %), with sample B hematite dominant (49.4 wt.%). Berhe asserted that physically, sample A (goethite rock dominant) was light and porous, and sample B (hematite rock dominant) was heavy and dense rock. The porous and light samples were a product of weathering processes. As the result of weathering, the concentration of platinum group metals in sample A was high compared to sample B. Hence, the goethite mineral (sample A) has higher total (precious) metal concentrations, but lower soluble metal concentrations. Oppositely, the hematite substrate (sample B) has more soluble metal but a lower total metal concentration.

Berhe found that the distribution of palladium in gossan samples from Broken Hill is poorly homogenous. In comparison, the distribution of copper, nickel, ruthenium, and platinum was more equally distributed throughout the rock samples. Further, palladium was found together with copper, nickel and silver, and was associated with barium sulphate crystals.

In order to make palladium more soluble in soil and ready for plant uptake, a chemical lixiviant is needed. This is the underlying principle of induced hyperaccumulation. Sakambari (2015) examined the potential of chemical solvents to increase palladium solubility from the gossan substrates, and also determine its phytoavailability for plant uptake.

In this study Sakambari employed 10 chemical solvents at a concentration of 0.1 M. These solvents were DI Water, KCN, Na₂S₂O₃, HCl, Na-EDTA, NaHS, Humic Acid, KOH, NH₄OH, and NH₄SCN. From this trial, Sakambari concluded that 0.1 M KCN is the best adjuvant to solubilise

palladium from the gossan samples. A summary of extractable copper, nickel, gold, and palladium concentration from the gossan substrate as tabulated at Table 7.1.

Findings from this work have demonstrated that metal solubility as a function of water extraction, was limited. Copper was more soluble than other metals, as much as 11.31 μg soluble copper g^{-1} was detected from hematite rich substrate. However water was not able to solubilise nickel from the goethite rich soil. The solubility of precious metals such as palladium was detected higher in soil B, while gold was found higher in soil A. Subsequently, the application of KCN in metal extraction proved that KCN makes metals more soluble in soil. The greatest increase was for copper in soil B. The soluble copper concentration reached 1,099 μg g^{-1} . Copper solubility in soil B increased up to 9,617%. Moreover, a significant increase in solubility was observed from palladium. Compared to H_2O extraction, palladium solubility in soil B increased by 56,983%, however the most significant palladium solubility was for soil A which increased by 923,233%.

Sakambari's work highlighted that palladium solubility can be optimised by application of 0.1 M KCN. This chemical solvent could then be used for phytoextraction trials. Although after KCN application solubility of palladium increased, KCN also affected an increase in copper solubility. Copper is suggested to cause phytotoxicity if it occurs at high concentration and an increased copper soluble fraction would therefore be a challenge for phytoextraction trials. Accordingly, what is needed is a plant species which can tolerate a high soluble copper concentration.

All greenhouse trials reported high metal uptake by plants grown on soil B. *Brassica juncea* grown on soil B was reported to extract more copper, nickel, gold, and palladium. However, although the reported mean concentration of those metals was high, they were not significantly different to *B. juncea* grown in soil A. Induced hyperaccumulation in *C. sativa* demonstrated that significant uptake occurred for copper, nickel, and palladium from soil B. Gold uptake from this study was suggested to be not significantly different between the two soil types.

Table 7.1.1. Comparison of extractable metal from gossan substrate as factor of H₂O and KCN

Metal	Unit	Soil A (Goethite)			Soil B (Hematite)				
		Total metal	Extractable	Extractable	Total metal	Extractable	Extractable		
			H ₂ O	0.1 M KCN		H ₂ O	0.1 M KCN		
			Percent Increase (%) ^a			Percent Increase (%) ^a			
Cu	µg/g	13,962.2	1.82	445	24,351	10,890	11.31	1,099	9,617
Ni	µg/g	6,354.4	0	5.01	N/A	3,483.5	0.9	35.7	3,867
Au	ng/g	3,804.7	0.16	34.4	21,400	1,565.6	0.09	20.2	22,344
Pd	ng/g	32,650.2	0.03	277	923,233	16,665	0.36	205.5	56,983

^a Percent increase is defined as increasing metal concentration in solution from H₂O extractable to KCN extractable fraction.

In general, more metal was available for plants from the hematite rich substrate than the goethite substrate and mineral differences were examined as key to understanding potential influences on metal uptake in the plant trials. Substitution of metals for iron in natural goethite under weathered conditions has been described in Cornell and Schwertmann (2004). Cornell and Schwertmann asserted that some cations can be substituted into iron oxide minerals. For example Ni^{2+} can be incorporated into the goethite, and Cu^{2+} and Ni^{2+} can be incorporated into the hematite. Metal adsorption into goethite and hematite structure is affected by the surface area of these iron oxide minerals. The surface area of goethite is between 45 and 50 $\text{m}^2 \text{g}^{-1}$, whereas hematite is 14.4 $\text{m}^2 \text{g}^{-1}$. Hence goethite is predicted to adsorb more metal ions than hematite (Giménez, Martínez, de Pablo, Rovira, & Duro, 2007; Liu, Chen, & Frost, 2014).

According to Anderson (personal communication, 2015), goethite mineral was considered, in this work, to adsorb soluble metal from soil solution before plant absorption. It is suspected that goethite become a competitor to plants for soluble metals. Therefore plants grown on the goethite rich substrate had a lower metal concentration, despite the relatively higher total metal concentration in the soil.

7.4. Metal uptake comparison in plant species

Metal uptake as a natural occurrence in the aboveground biomass of five native plants, *Sclerolaena lanicuspis*, *Solanum centrale*, *Tetragonia moorei*, *Ptilotus obovatus*, and *Brassica* sp was examined. Despite a total palladium concentration in the rhizosphere soil of 28.9 ng g^{-1} , the study could not detect any concentration of palladium deposited in the biomass of the five native species.

Presumably palladium is not naturally soluble in the gossan environment. In contrast, some plants were observed to extract gold in their biomass. For instance, an individual *Solanum centrale* specimen was assessed to concentrate 23.2 ng g^{-1} gold in its biomass. This study also assessed native plant species which can survive on soil with a high concentration of copper. The current study noted that *S. centrale* was able to uptake a high copper concentration of 277.4 $\mu\text{g g}^{-1}$.

In comparison, normal plants need copper between 5 and 20 mg kg^{-1} and a copper concentration higher than 20 mg kg^{-1} can be considered as above the toxic level (Reimann et

al., 2001). A threshold level of copper hyperaccumulation was determined at a concentration $> 300 \mu\text{g g}^{-1}$ (van der Ent et al., 2012). *Solanum centrale*, the gossan plant, did not exceed the copper hyperaccumulator threshold, but with the high copper concentration in its tissues, this plant species could be a potential candidate to be used for induced palladium uptake from Broken Hill gossan in the future.

Findings from the field survey analysis highlight that palladium is poorly soluble in the natural environment. In order to study palladium solubility in soil and to promote its uptake in plant species, a greenhouse trial using *Brassica juncea* was conducted. *Brassica juncea* were grown on the gossan substrate and irrigated with water. During the trial, this study reported poor growth performance in *B. juncea* associated with metal stress symptoms. Although total biomass yield from all plant trials was only 5.1 gram, this trial demonstrated that maximum copper concentrations recorded between 440 and 759 $\mu\text{g g}^{-1}$ were observed in individual *B. juncea* plants. These concentrations were classified at the hyperaccumulator level. Observations on the maximum concentrations of nickel recorded a value between 197 and 601 $\mu\text{g g}^{-1}$ and signified that *B. juncea* is a strong nickel accumulator.

Observations on precious metals uptake indicated that *B. juncea* can concentrate palladium from 2,130 to 2,909 ng g^{-1} , and suggested natural palladium hyperaccumulation. Although the gold concentration did not exceed 1,000 ng g^{-1} , reported gold uptake between 609.4 and 633.1 ng g^{-1} demonstrated *B. juncea* were able to bioaccumulate gold in its biomass. As discussed earlier, metal solubility using water as an extractant was low. However, *B. juncea* grown on gossan soil could extract copper, nickel, gold, and palladium at significant concentrations. This result shows that *B. juncea* has ability to solubilise metals in its rhizosphere and can accumulate these in its biomass. These findings highlight the value of *B. juncea* phytomining research.

Metal phytoavailability in a growth medium is the main factor that inhibits plant growth performance as demonstrated from *B. juncea* trial. Induced hyperaccumulation in this research, achieved by adding potassium cyanide (KCN) into the growth substrate, increased metal solubility and became a potential factor to cause metal stress for plant trials (Table 7.1). After KCN extraction, recorded metal availability of copper, nickel, gold and palladium in soil B increased 9,617, 3,867, and 56,983% respectively, whereas the increase in copper, gold, and palladium solubility in soil A was 24,351, 21,400, and 923,233% respectively. Solubility of gold in both soils was slightly different and increased by 1,000%. Palladium in soil A increased

significantly compared to soil B. The solubility of copper was higher in soil A than in soil B, and this was demonstrated to affect plant growth performance in *B. juncea*.

High copper solubility after treatment would be expected to affect plant growth due to the onset of toxic effects. Hence for the induced hyperaccumulation trial, a strong plant species, *Cannabis sativa* was used. Significant findings from this trial demonstrated that copper uptake in *C. sativa* was extremely high with a maximum concentration of $6,726 \mu\text{g g}^{-1}$. The precious metal concentrations were detected at $62 \mu\text{g}$ palladium g^{-1} , and $9 \mu\text{g}$ gold g^{-1} . Thus this study reported hyperaccumulation for copper, palladium, and gold in *C. sativa* after induced metal uptake occurred. In fact, copper toxicity likely affected the ongoing viability of plants and impacted the potential for a higher concentration of palladium uptake. Presumably, exposure to a higher concentration of copper may damage cell plant structures, especially photosynthetic membranes, causing chlorosis and necrosis. Copper toxicity may stop the plant from accumulating more palladium.

Cannabis sativa was suggested as the best plant to be used for the induced hyperaccumulation study. This species is more tolerant to a high copper concentration than *B. juncea* and *C. sativa* was able to concentrate a high concentration of copper, nickel, palladium, and gold. Even at such extreme metal concentrations in its tissues, this species showed limited additional toxicity effects. This finding is in agreement with Arru et al. (2004), who noted that rather than using its vacuoles to localise toxic metals, *C. sativa* uses the upper leaf epidermal cells as the main location for copper accumulation. Typically plants use vacuole to accumulate excess metals in the cells. The current study noted a higher concentration of copper, nickel, and palladium in leaves relative to stems.

7.5. Implication of the study

Metal uptake trials as presented in this study indicated that *Brassica juncea* and *Cannabis sativa* were able to concentrate palladium at hyperaccumulation threshold level ($1,000 \text{ ng g}^{-1}$) in their biomass, but did not achieve target concentration of $1,000 \mu\text{g}$ palladium g^{-1} dry weight even after application of KCN to increase metal solubility in both soil types. Because of different mineral constituents in the two gossan types, greater metal uptake was observed from soil B, which was a hematite rich substrate.

The solubility of copper in the gossan soil is suggested as the main inhibitor for the plant growth trials. *Brassica juncea* was noticeably affected by the high soluble copper concentrations in both soil types. Therefore its growth performance was poor. Despite this fact, some significant concentrations were found in this species. *Cannabis sativa* proved to be a suitable species to grow on Broken Hill substrate. During the trial, less phytotoxicity effects were noted in this plant. It is interesting, that 3 days after treatment of 0.1 M KCN, *C. sativa* was still growing well with only gradual onset of increased toxicity symptoms. Findings from the field survey suggest that *Solanum centrale* could be a possible candidate for further plant growth investigation on the Broken Hill gossan. This native species was assessed to concentrate a naturally high copper concentration in its biomass. This plant may have genetic resistance to copper stress.

This proposed work could be suggested as the second phase of Phytocat research to induce hyperaccumulation of palladium to the target level of $1,000 \mu\text{g g}^{-1}$. This thesis concludes that green biosynthesis of palladium in living plants as described by Parker et al. (2014) is possible by using *C. sativa*, *B. juncea*, and potentially *S. centrale* when palladium is targeted in gossan substrates such as that found at Broken Hill.

7.6. Conclusions

Metal solubility is a function of total metal concentration, but is not necessarily correlated with plant uptake. The parameters pH, Eh and soil composition have a major effect on solubility.

KCN at concentration of 8 g L^{-1} was observed to induce high net metal uptake in *Cannabis sativa*. Copper, gold, and palladium were recorded at the hyperaccumulation level.

Solanum centrale, a native plant species from Broken Hill was identified tolerant to high copper concentration in soil. Further induced hyperaccumulation trials using this species on the gossan are recommended.

The target concentration of $1,000 \mu\text{g g}^{-1}$ palladium in plants set by for the Phytocat project was not reached, but insights gained from the current work can be applied to a second phase of work that can continue research towards this objective.

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