Revegetation and Ecotoxicological Assessment of Abandoned Copper Mine for improved Remediation Utilizing Native Plant Species

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ABBREVATIONS

AML: abandoned mined lands

BCR: Bureau of Community Research

BLM: Bureau of Land Management

EAA: The European Environment Agency

ESEM: Environment Scanning Electron Microscope

ISO: International Organization of Standards

PCA: Principal components analysis

SAA: semi-arid and arid

USEPA: United States Environmental Protection Agency

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Research papers

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property of Australian golden wattle grown on copper mine soils. Ecological Indicators **DOI: 10.1016/j.ecolind.2016.06.062**

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1. **Nirola, R.,** Mallavarapu, M., Saint, C., Aryal, R., Palanisami, T., Venkateswarlu, K., Naidu, R., Beecham, S. (2015). The florescence imaging of above ground parts of stress tolerant golden wattle growing in an abandoned mine site. Australian Mine Rehabilitation (AMR-2015) conference, Adelaide (17-20 August 2015) (Poster presentation). **DOI:10.13140/RG.2.1.2851.4962**

2. **Nirola, R.,** Mallavarapu, M., Palanisami, T., Venkateswarlu, K., Naidu, R. (2015). THE METALLOPHYTES SPEAK LOUDER! A derelict-mine plant-colonist in action to perform phytoremediation. CRC-CARE, Clean-up Communicate Conference 2014 (Poster presentation). **DOI: 10.13140/RG.2.1.4293.2881**

3. **Nirola, R.,** Mallavarapu, M., Saint, C., Aryal, R., Palanisami, T., Venkateswarlu, K., Naidu, R., Beecham, S.2016. *Acacia pycnantha*, a resilient metallophyte in green and gold of Australia. Mine Rehabilitation Conference-2016, Singleton, NSW.

EXECUTIVE SUMMARY

i) Background problem

When mining activities cease, its environmental impact continues as a consequence of which all the adjacent vegetation, soils, groundwater, etc. are exposed to a very high concentration of heavy metals. The toxic heavy metals present in the tailings or 'spoils' of abandoned mines migrate to the surroundings and cause severe and widespread contamination of farmland soils and water bodies leading to the onset of geo-environment disasters. One such example is a recent one in Brazil that released 60 million cubic meters of mine waste due to iron-ore dam burst according to The Guardian daily dated 28 November 2015.

ii) Research gap

To remediate any wide-scale pollution, phytoremediation technology is a cheap and popularly used method. However, less attention is drawn towards the science of metal extraction into plant body that exposes toxic metals to environment with a successive risk of food chain contamination. It is primarily due to the lack of knowledge on specific species of plants and vegetation monitoring including the improper disposal of biomass from a contaminated area. On one hand, growth of plants possessing an ability to tolerate adverse abiotic conditions of mine site increases the soil organic matter, playing important role in immobilizing heavy metals, improving soil structure and fertility, and reducing soil erosion. On the other hand, the accumulated metals on litters pose a danger to getting transferred into the food chain through the detrivores who feed on decaying leaf litter.

Some of the urgent necessity is to amalgamate different remediation technology to tackle the metal pollution problems in the world. The main aspect is to reduce a gap between

bioremediation, phytoremediation technology and emerging remediation technologies that involve the latest digital based analysis system.

Ignorance on the knowledge of plant structure, function and its behaviors are required to be addressed for improved phytoremediation technology. Therefore, screening of metallophyte including the evaluation of metallophytes, toxicity tests and effective ecological monitoring of plants are the major areas of this thesis project.

iii) Research approach

Contextually, the abandoned mines are surrounded by native flora that provides information on the response of native fauna and further into biodiversity information. Besides trees, the ground vegetation is also a highly potential agent of heavy metal transfer into the ecosystem steadily impacting biomagnifications of metals in the flora and fauna. In our study the detrivorous earthworms are used for toxicological experiments to assess the bioavailability of metals in mine leaf litters of *Acacia pycnantha* and *Eucalyptus camaldulensis*. The droppings of animals such as English hare (*Lepus timidus*) are also used in our experiments to get information on whether the herbs they graze are equally responsible for transfer of heavy metals into ecosystem.

An overall research approach adopted is primarily to examine re-vegetation of mine sites, the metal transfer into ecosystem, and the plant stress study using different levels of copper contaminated soils of abandoned mine.

The specific approaches are:

i) To screen ecologically prominent shrubs and trees growing around abandoned copper mine to determine its enrichment factor.

ii) To characterize abandoned mine soils at different sampling sites including the evaluation of interaction of metallophytes with soils.

(iii) To elucidate the potential metal transfer from leaf litter and animal droppings to biota in a microcosm experiment.

(iv) To study plant stress using biochemical production analysis and metal uptake behavior of native Australian legume *Acacia pycnantha*.

iv) Research activities

After determining the research proposal direction, a most appropriate site for experiment was chosen. We wanted to have an abandoned mine examined instead of operational mine to study the behavior of established vegetation. The concerned council was communicated regarding our interest and was agreed upon accordingly. To conduct experiments, various sites were considered for sample collection. The fecal matter of English hare was collected from Adelaide Zoo as a control and the mine site as a contaminated sample. Control leaf litter and soils were collected from Royal Botanical Garden, Adelaide and the University of South Australia, Mawson Lakes premises. The nursery saplings were grown at Realtree Nursery in Adelaide hills SA. Earthworms were sourced from a local farmer at Devoren Park in Playford for exposing to fecal matter of hare. The earthworms used in the plant litter experiments were *Eisenia fetida* cultured in the laboratory.

In the **first experiment** the abandoned copper mine at Kapunda in South Australia was considered as study site and was surveyed accordingly. The plants were sampled for taxonomic as well as laboratory analytical purposes. A sampling strategy was planned accordingly to ensure that all the bushes and trees were represented for screening from diverse range of taxonomic family in and around mine site. The total metal analysis was conducted for each of the plant and soil samples collected from the abandoned copper mine.

The data of plant and soil samples were analyzed to find correlation of metal uptake. Enrichment factors were determined for each plant species. A metal dispersion in soil and pollution in the mine site was also statistically obtained by principal component analysis. All the nine species of plants did exhibit variation in metal uptake factors depending upon species and soil that was exposed to. The mine site soil with predominantly sandy texture had a potential for wind and rain erosion polluting the farmlands and rivers.

The **second experiment** was focused on two plant species among which *Eucalyptus camaldulensis* was a copper accumulator and *Acacia pycnantha* was a stabilizer as per the results of the first experiment. These two plants were sampled from two polluted spots consisting of at least four sampling points around each spot. Soil also was collected from the rhizosphere zone to evaluate the interaction between soil and plant. Roots, stems and leaves were collected for each plant sample. Fresh leaf samples were also collected for microscopic study of cell anatomy and metal localization. The metal enrichment factor, bio-concentration factor and translocation factors were determined for both the metallophytes. The soil samples were thoroughly characterized to find statistical significance and correlation with the selected plant species. The microscopic examinations revealed calcium deposition in the vascular region of *Acacia pycnantha* and in the mesophyll cells of *Eucalyptus camaldulensis*. The two tree species revealed interesting facts in terms of their application for phytoremediation.

The **third experiment** was mostly centered on toxicological experiments using earthworms in microcosm. The detrivorous earthworms were exposed to leaf litter, control soil, contaminated soil, hare fecal matter and OECD soil. The metal uptakes into worms were evaluated among the control and contaminated samples of litter, hare droppings and soil. Microscopic examination of tissue was also carried out. Several statistical models were introduced to study the actual metal bioavailability into the worm's tissue. It was revealed

that litter and faecal matter does transfer heavy metals into the food chain. It was also revealed that worms have a selective nature of metal uptake and rather than copper it was cadmium that was found more likely to transfer into the food chain.

The **fourth experiment** was divided into two sections that included glass house as well as biochemical analysis in the laboratory. *Acacia pycnantha* seeds collected from the mine were germinated in the laboratory. Heat treated and normal seeds were introduced into different types of soil and irrigation types. The saplings were introduced into different soil and substratum types to study metal toxicity and phytoaccumulation level in the pot experiment.

In the **fifth experiment**, plant stress levels were determined by analysis of proline, chlorophyll a, chlorophyll b and carotenoids. A separate microscopic examination was also conducted for elemental uptake study. The experiment revealed that proline stress was due to soil salinity. *Acacia pycnantha* was a copper tolerant plant and was more susceptible to arsenic and lead toxicity and phytoaccumulation.

The **sixth experiment** was dedicated to fluorescence study of different plant extracts using the spectrofluorometer. It revealed that the elemental impacts from soil metal towards plant body was uniform based upon the substratum concentration.

v) Research findings

In conclusion, this research experiment has opened up a new dimension in mine site phytoremediation technology.

a) The *Eucalyptus camaldulensis* emerged out to be a copper phytoaccumulator with its lignin trapping least amount of copper and vascular bundle trapping more copper

b) The Australian golden wattle exhibited least stress related hormone secretion such as proline and this national floral emblem plant *Acacia pycnantha* emerged out to be a resilient plant suitable for revegetation of copper mine.

c) The potential of metal bioaccumulation by detrivores remain high and the metals like cadmium and arsenic are seen to be highly mobile metals in leaf litter of *Acacia pycnantha*.

d) The earthworm reproduction was better in *Eucalyptus camaldulensis* litter compared to *Acacia pycnantha* litter but the overall metal transfer into the earthworms tissue was through soil.

DECLARATION

I declare that this thesis is composed of scientific work carried out by myself and does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university. To the best of my knowledge it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, are clearly acknowledged.

Signed ---------- Date ----------

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Chapter 1

INTRODUCTION

1.1 Global scenario of abandoned mine pollution

A large number of papers on mine tailings and waste, concerning the problems of soil, groundwater and sediment contamination have been published (Donahue et al., 2000; Williams, 2001; Aykol et al., 2003; Megharaj et al., 2011). Reclamation of land is a method used to revive a polluted or disturbed land into its original state, so that the normal process of interaction between biotic and abiotic factors of the ecosystem is restored. To determine land quality of an abandoned mine requires characterization of parameters like mineralogical, physical, and chemical analysis, checking extremes of pH, sodicity and salinity, checking erodibility, analysis of plant nutrients and biological health (Sheoran and Sheoran, 2009).

The biodiversity plays a major role in determining land quality when considering nutrient cycling, soil formation and primary production as indicators to reclamation. In cases of operational mine sites, progressive reclamation can be implemented; whereas, in abandoned mines, the cost burden is high as a result of abrupt attempt to reclaim. Therefore, monitoring and maintenance of soil nitrogen level and containing soil erosion are important areas of priority during the process of reclamation. In a changed physio-chemical nature of the mined habitat, the flora and fauna adapts to deal with pollutants and gradually evolves. As such, it has been revealed that in ancient lead mine at Peak, UK some of the metallophyte species, like *Cochlearia pyrenaica, Minuartia verna, Thalaspi carulescens, Voila lutea,* and invertebrates like *Leptarthrus brevirostris* and *Carabus monilis* including uniquely adapted diverse microorganisms, are found safe and healthy (Batty, 2005).

There has been a global 30 year record high increase in surface mining, which is thought to be a prime cause of ecological losses, downstream impacts and long term pollution problems (Palmer et al., 2010). Ferrous and non-ferrous metals are

generated as a result of tremendous destruction of landscape releasing tons of toxic materials into water bodies and the atmosphere. Even though speciality metals like gold in itself is a least pollutant in nature, the process of producing them is a high energy consuming and polluting affair. As such, a wedding ring is said to produce 20 tons of waste. There are many abandoned sites and metal waste dumps which are constantly polluting the environment. For instance, approximately 550,000 abandoned mine sites in the United States alone have generated 45 billion tons of mine waste, including waste rock and tailing material, with many of the sites found in arid and semiarid regions (USEPA, 2010).

An inventory of abandoned mines from the Bureau of Land Management (BLM) reports 25,281 sites and 65,976 features as of December 31, 2009 (www.geocommunicator.gov). About 20% of the sites have either been remediated, or have reclamation actions planned or underway, or do not require further action. The remaining 80% require further investigation and/or remediation. During surface mining, land is found to be damaged from 2 to 11 times more than underground mining. The direct effects of mining activities can be a disturbed landscape, loss of cultivated land, forest and pasture land, and the overall loss of land productivity (Copeland, 2013). The indirect effects of mining are soil erosion, air and water pollution, toxicity, loss of biodiversity, and ultimately, loss of economic wealth (Wong, 2003). The remediation of abandoned or operational mine closure is therefore of high priority to environmental resource managers, as cleaning up can be a costly task. In 2006, the congressional testimony concluded that it would take nearly $72 billion to clean up abandoned hard rock mines in western United States that produce heavy minerals like silver, gold, uranium and other metals (Clark, 2008).

As per the estimation of USEPA, the surface coal mining practices since 1992 buried almost 1,200 miles of Appalachian streams, including deforestation leading to algal bloom (Stoner, 2011). Over 1,500 mines of metals such as gold, copper, lead and zinc are scattered all over the Republic of Korea, and most of them were abandoned when the ore was depleted (Lee et al., 2005; Kim et al., 2008). Mining waste and acid mine

drainage produced from these abandoned metal mines have released several toxic metalloids or heavy-metals into groundwater, surface water, and geological environments because of their solubility and mobility (Mulligan et al., 2001). In China, currently there are over 6800 mines in operation (Li et al., 2007).

With more than 21% of the world's population living on cultivated land that is less than 10% of the total area available on the Earth, the loss of China's extremely scarce arable lands to industrial expansion, urbanization and mining operation is greatly alarming. For instance, mining activities alone by over 8,000 national and 230,000 private companies resulted in creation of 200,000 km^2 of derelict land, which includes the loss of 370,000 ha of agricultural land. Furthermore, many of the metalliferous mines that have been abandoned have generated about 3.2 M ha of wasteland in China (Li, 2006), and this figure is increasing at a rate of 46,700 ha $year^{-1}$. In Europe, the Iberian Pyrite Belt (IPB) is an extensive mining district that represents the largest concentration of known massive sulfides on Earth. The IPB comprises more than 80 mines in southern Spain and Portugal, including important historical sites such as Rio Tinto, Tharsis, La Zarza, Sotiel and Aznalcóllar in Spain, and Aljustrel, Loussal and Neves-Corvo in Portugal. Most studies have focused on the effects of surface mining and mobilization of heavy metals, and have often revealed a high degree of metal toxicity in mine spoils and soils affected by the oxidation of pyritic material (Pèrez and de Anta, 1992; Taylor et al., 1992, 1993; Monterroso et al., 1999; Clark et al., 2001).

1.2 The scope of study

There appears to be insufficient attention on phytoremediation technology involving sciences of phylogenetics, plant physiology and ecology. Revegetation is a process of planting correct species using the correct method at the correct time to sustain through the odds of ecological factors.

An ecologically harsh condition, such as arid and semi-arid sites, are ideally suited for species of drought tolerant, fast-growing and deep rooting plants, including

grasses that have a more carbon capturing (C4) system of photosynthesis (Sheoran and Sheoran, 2009). Technically, phytoremediation is to rehabilitate a polluted site through revegetation involving different planting methods. Phytoextraction is a metal accumulating or extracting process that takes a long time to accomplish complete remediation to a desirable level. There are also the problems of contaminant exposure and successive risk of improper disposal of biomass from a contaminated area. **Phytostabilization** is another way of sequestration process of pollutants within the soil. It also requires fertilization and amending soils, with a risk of leaching contaminants in long term. Similarly, **phytovolatilization** is another way to pass metabolites into fruit, timber, grains or vegetables and even disperse in air. **Rhizofiltration** is a costly affair as it requires continuous pH monitoring and an elaborate bioreactor establishment (Gratão et al., 2005). These phytoremediation processes can solve problems of continuing impacts of mining like air pollution and waste dump sites besides metal sequestration. However, researchers, stake holders and industrialists are continuously needed to consider environmental side effects such as genetic erosion, allelopathy and species extinction when studying metallophytes.

1.3 Significance of the study

Phytoremdiation being a cheaper 'green' alternative assists in soil enrichment to initiate organic matter cycling (Baker et al., 1994). Phytostabilisation has been used successfully to revegetate mine spoils and areas around smelters (Vangronsveld et al., 1995). The *Green remediation* employs those species of plants native to metalliferous soils with a capacity to accumulate metals such as zinc and nickel to concentrations ≤ 2% in the aboveground parts. Growing such plants under intensive crop conditions and harvesting the dry matter is proposed as a possible method of metal recovery and for 'polishing' contaminated agricultural soils down to metal concentrations below statutory limits.

Plants like *Cynodon dactylon, Juncus usitatus*, *Lomandra longifolia* were identified as being potential species for use in phytostabilisation programs due to their

tolerance to acid soils and tolerance and/or accumulation of significantly higher concentrations of Pb and Cd than other plant species present on the derelict Silver Peak mine near Yerranderie in the Blue Mountains, NSW, Australia (Archer and Caldwell, 2004). Plants like *Alyssum montanum*, *Alyssum bertolonii* were tested hydroponically to show cadmium tolerance and accumulation capacity revealing that metallicolous plants which are least represented and diverse are also suitable for phytoremediation of metals (Barzanti et al., 2011). Dhankhar et al. (2012) reported that *Thalaspi caerulescens* and *Arabidopsis halleri* have phytoextraction ability to uptake Zn up to 40,000 mg kg^{-1} in the above ground parts. An interesting fact is that, some families of plants showing signs of cation diffusion facilitator (CDF) and Zinc-Iron regulated transporter like protein characters (ZIP) have been understood to be zinc phytoextractors.

Since, phytoextraction is an agronomic approach of phytoremediation, it can only be used to remediate contaminated sites with the aim of harvesting the metal-rich plant tissue, followed by treatment to contain the metals off-site (Cunnigham et al., 1995; Huang et al., 1997). A drawback is that the total amount of metal present in the soil may far exceed the capacity of the plants to accumulate that metal, even over an extended time period (Ernst, 1996). Moreover, phytoextraction can only remove metals available in the root zone of the plant, and not in greater depths. Therefore, an alternative to phytoextraction is phytostabilisation, where a constructed ecosystem based on metal tolerant or accumulating plants may be effective in reducing the mobility of heavy metals within the soil (Dahmani-Muller et al., 2000; Gupta et al., 2000). The growth of plants with an ability to tolerate the adverse conditions found in derelict mine sites increases soil organic matter, which plays an important role in immobilising heavy metals, improves soil structure, increases soil fertility and reduces erosion (Gratao et al., 2005). This process may also include the use of nontoxic metal-immobilising or fertilising soil amendments to improve plant growth (Vangronsveld and Cunningham, 1998; Sellers, 1999). The phytostabilizer plants also accumulate metals in their leaves to some extent, and the foliage

accumulation on the ground over a period of time renders a suitable habitat for detrivores like earthworms to multiply.

The present research is focused on studying metallophytes growing naturally over several years in abandoned mines, including the transfer of locked metals in litter into the food chain through the agent of detrivores. Study of plant stress level due to exposure to metalliferous soil is another important aspect of this study to find out whether phytostabilizer also is involved in accumulation under stress. The earthworms and similar organisms dwelling in the mine site draw bio-available metals into their tissue. The bioavailable metals from phytoaccumulators are expected to be higher than the phytostabiliser or non accumulators. However, any metals present in the leaf litter have potential to be transferred into the food chain as a bioavailable metal. Usually faunal adaptation is seen to thrive well with old trees providing shelter, food and assurance; however, the birds are found to adapt equally well in young forests with sufficient understorey (Lindenmayer and Salt, 2008). Moreover, the animals dwelling in mine sites which feed on the plant products from mine areas are thought to be potential agents to transfer heavy metals into the food chain.

1.4 Objectives

The objective of this research is to investigate the role of metallophytes and metalliferous soils in achieving phytoremediation goals, including the study of potential transfer of heavy metals into the food chain. Moreover, the study of stress tolerance in metallophyte at the semi-arid and arid (SAA) environmental condition is incorporated into this research to identify the response of plant species towards polluted soils. The following activities were designed to fulfill thesis objectives:

- Site survey and herbarium collection at an abandoned mine site and screening of vegetation to identify metallophytes

- Determination of metal phytoaccumulation and plant resilience to metals growing in the mine site
- Study of metal uptake factors in roots, stems and leaves of shrubs and trees growing in metalliferous soils
- Study of soil in and around the mine site and its characterization including the BCR metal fraction analysis (Photo plate-I)
- Investigation of leaf litter transfer of heavy metals into the detrivores such as *Eisenia fetida* including the analysis of proline
- Investigation of transfer of heavy metals through the fecal matter of hare grazing the herbs growing in mine site.
- Investigation of metal stress response using chlorophyll and proline analysis of *Acacia pycnantha*, in a glass house pot experiment.
- Investigation of pore water activity of different soils collected from mine sites
- Analysis of metal uptake factors in different plant species including the ESEM study of metal localization in the tissues of earthworms and plants.
- Comparative analysis of metal bioaccumulation using OECD soils, control and contaminated feces and leaf litters.

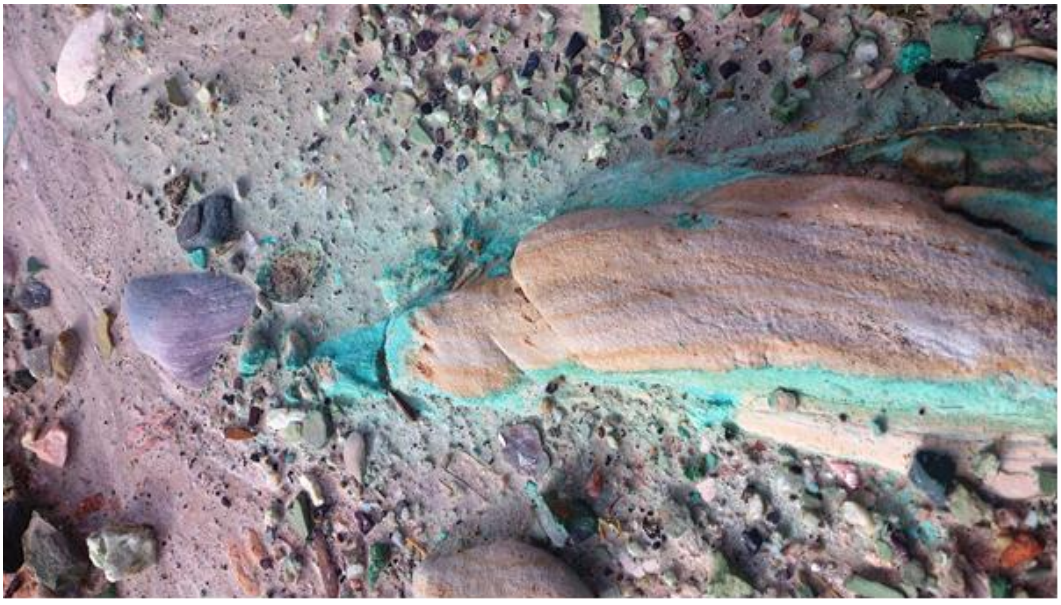


Photo plate I – The erosion of copper rich minerals at Kapunda mine

1.5 Structure of research

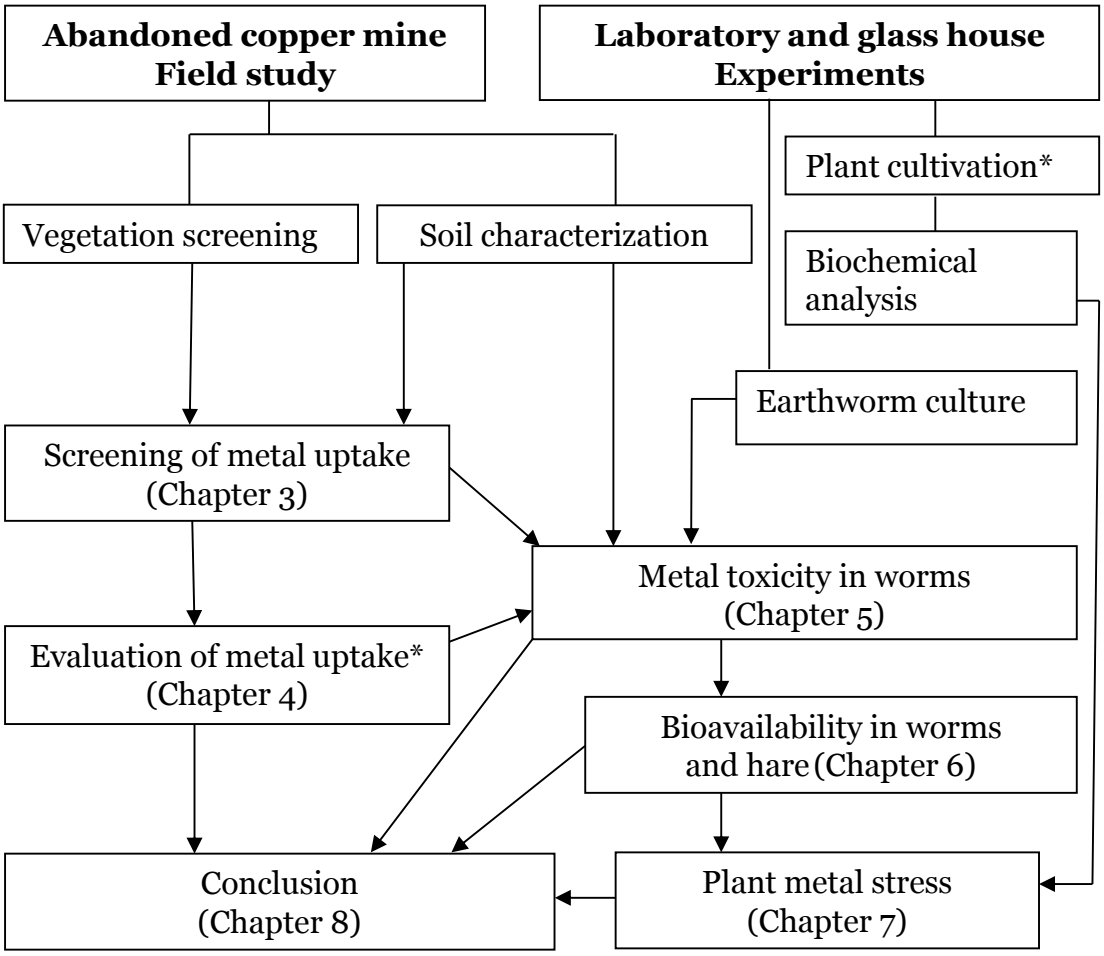


Fig 1.1 Structure of research in a flow diagram

1.6 Layout of Chapters

This thesis consists of eight chapters

Chapter 1 (Introduction) presents an outline of the global problems of open pit mining, heavy metals pollution and challenges of phytoremediation, revegetation and metal transfer into the food chain impacting animals and human health. This chapter also describes the scale of heavy metal pollution and a continuing problem affecting flora and fauna and the efforts put to control the pollution.

Chapter 2 (Literature Review) encompasses the review of all listed metallophytes around the globe with an aim to oversee if the listed plant species are in any way phylogenetically linked to each other. Description of heavy metal pollution through operational and abandoned mine sites has been made. A discussion on phytoremediation in semi-arid and arid environmental conditions with an insight into listed metallophytes and taxonomic discourse up to the rank taxonomic "Order" is made. A new approach towards prospects and challenges of revegetation, phytoremediation and mine site rehabilitation and remediation of contaminated sites has been made.

Chapter 3 presents the screening of metal uptake by plant colonizers growing on abandoned copper mine. Important plant colonists of abandoned copper mine are tentatively classified as accumulators or excluders. The analysis of nine vascular plants growing on abandoned copper mine site including characterization of rhizosphere soil with in-depth analysis of enrichment factor values. The study of metal dispersion at the mine site by principal component analysis is done to identify the major agent of metal dispersion.

Chapter 4 deals with the evaluation of metal uptake factors of native trees colonizing an abandoned copper mine. The metal uptake behaviour of golden wattle and river red-gum including the calculation of uptake factors are conducted. There is a comparative analysis of metal concentration with electron microscopy data with respect to bioconcentration, enrichment and translocation factors. This chapter actually deals with two diverging classes of tree species native to Australia, one as a phytoaccumulator (*Eucalyptus camaldulensis*) and the other as a phytostabilizer (*Acacia pycnantha*).

Chapter 5 examines the metal bioavailability in metallophyte leaf litter and copper mine soil toxicity to earthworms. The leaf litter of hyperaccumulator and non accumulator plant species are tested in the microcosm experiment. The leaf lignin is extracted as well as the metal fractionations in soils are determined to check actual bioavailability. In this experiment the data are subjected to thorough statistical analysis to determine the actual bioavailability source.

Chapter 6 also focuses on the study of metal bioavailability to *Eisenia fetida* through abandoned mine plant and animal litter, a new challenge on contaminated environment remediation. This chapter deals with the use of OECD soils being fed along with English hare faecal matter and leaf litter to determine whether the faecal matter is also responsible to transfer the heavy metals into the food chain. The mine dwelling animals such as rabbit graze over herbs and plant products such as fruits and nuts. It is expected that the detrivore *Eisenia fetida* can bioaccumulate in their tissue and is a potential agent to transfer metals into the higher trophic level.

Chapter 7 investigates the germination, growth, stress tolerance and metal uptake behavior of golden wattle exposed to copper mine soil in ex-situ condition. Right from the germination, growth, enrichment of metal in the plant body and harvest of the biomass, this pot experiment is able to examine the total plant behavior of *Acacia pycnantha* in the glass house. Seeds from mines are germinated into two irrigation system and are planted in the pots to study growth biometry over different soils collected form abandoned copper mine. The assessment of phytochemicals like proline and chlorophylls are done to study their stress. A pore water analysis including the plant metal enrichment has been studied in this experiment.

Chapter 8 focuses on conclusions and recommendations based on findings. It consists of variety of conceptual diagrams and models to explain the overall concept and major features of this research including the major outcomes. Information regarding the comments on best practice application and recommendation for further research has been incorporated.

Chapter 2

LITERATURE REVIEW

2.1 The metal pollution by mines

Humans have always depended on natural resources, while an ever increasing demand for metals and minerals has posed a serious threat to the lives of humans and animals through pollution and health issues (Lottermoser, 2010; Forstner & Wittmann, 2012). One of the most serious forms of pollution is heavy metals discharging from abandoned mine sites, as it has been historically a normal and accepted practice to 'abandon' a mine when mineral extraction is strategically completed (Himley M. 2014; Unger et al., 2015). The most abundant soil contamination areas and mine tailing disposal sites on earth are found in Northern Mexico, the western United States, the Pacific coast of South America (Chile and Peru), south-western Spain, Western India, South Africa and Australia (Mendez & Maier, 2008b).

The European Environment Agency (EEA) estimates that there are around 1.4 million contaminated sites on earth (Haferburg & Kothe, 2010), a figure probably beyond the present capacity of remediation technology. According to van Zyl et al. (2002), approximately 630,309 abandoned mines are spread throughout the world with many remaining undocumented and untraced. A report by Wait (2012) from South Africa put the figure at approximately 5,858 derelict and ownerless mines. Mining Watch Canada in its data reported 5,500 abandoned mines in Japan (Mackasey, 2000).

All these figures however depend on how a mine is defined. In China, small scales mines total 230,000 in number and are found to have a devastating environmental

impact, mostly due to the use of crude technologies to extract metal ores (Li, 2006). Area-wise, the degradation of land in China due to large and small scale ore mining is estimated to be 3.2 million ha (Li, 2006), with a total of 8.5 million ha of land being contaminated by heavy metals due to agriculture and mining (Hongbo et al., 2011).

The USEPA-2011 toxic release inventory data shows 8.66 billion kg of toxic chemicals including heavy metals were released into the environment in the USA in 2010, a 16% increase from that of 2009. In China, one-sixth of the total arable land has been polluted by heavy metals, and more than 40% has been degraded to varying degrees due to erosion and desertification (Lone et al., 2008). Consequently, it is evident that pollution in soil and water bodies are increasing posing chronic health risks and hazards to living organisms including humans (Mc Swane et al., 2015).

The cost burden to contain and remediate polluted land is very high and often lacks sufficient budget to initiate any clean-up work (Pulford & Watson, 2003; Luo & Lerner, 2009). Phytoremediation technologies can be a cost effective tool to target specific toxic metal pollutants (Peters, 1988; Alday et al., 2011). In Australia, remediation of historic mine sites releasing acid-mine drainage was estimated to cost more than $100,000 per hectare of land in 1994 (Archer & Caldwell, 2004).

The global demand for metals is increasing but the efforts to control environmental pollution and human health risks are not keeping pace. For example, global heavy metal consumption per capita during the 1950s was 77 kg, which soared by three times by 2008 to 213 kg (Haferburg & Kothe, 2010). Moreover, the total production of metal related consumables and goods in 2008 was 1.4 billion tons which is 7 times more than that produced in the 1950s (Haferburg & Kothe, 2010).

One of the reasons for this mismanagement of resources is the lack of effective global mechanisms to coordinate resources and restoration processes involving all stakeholders. For instance, in a land restoration research finding in China, the

reclamation studies were found to be separated from restoration practice, as there was no collaboration between the research institution and the entity working to restore the contaminated land (Li, 2006). Moreover, awareness on environmental pollution due to heavy metal mining is poor compared to awareness on green-house gas emissions (Nirola et al., 2013; Dodman, 2009; Haque et al., 2014). As such, an abandoned mine survey in Australia only received a 7% positive response from the participants regarding being fully informed about the issue of heavy metal pollution (van de Graaff et al., 2012). In this context, like with green-house gas emissions, awareness of heavy metal pollution is necessary for resource mobilization and effective implementation of remediation technologies.

The toxic impact and risk evaluation of heavy metals on plants and their responses to heavy metal pollution has long been an area of interest (Nirola et al., 2015a). Even various ancient literatures highlight the importance of plants such as *Ficus religiosa* to purify and protect the environment (Pathak et al., 2011; Singh et al., 2011).

The land and water bodies surrounding mines in semi-arid and arid (SAA) zones are more vulnerable to metal erosion and pollution exposure (Perlatti et al., 2014; Kim et al., 2014; Pascaud et al., 2015). For instance, the abandoned mine in Kapunda (Fig.2.1) has several waste dumps and open cast areas, although even more severe problems are being faced by third world countries in terms of managing abandoned mine sites (Cao, X., 2007). Moreover, rehabilitation attempts on abandoned as well as operational mines have generally involved engineering based technologies rather than biological systems.

The modern science of phytoremediation was conceptualized as early as the 18th century by Carolus Linnaeus (1707-1778), the 'Father' of taxonomy, who discovered a leadwort plant under Plumbaginaceae showing a lead tolerance capacity (Gawronski et al., 2011).

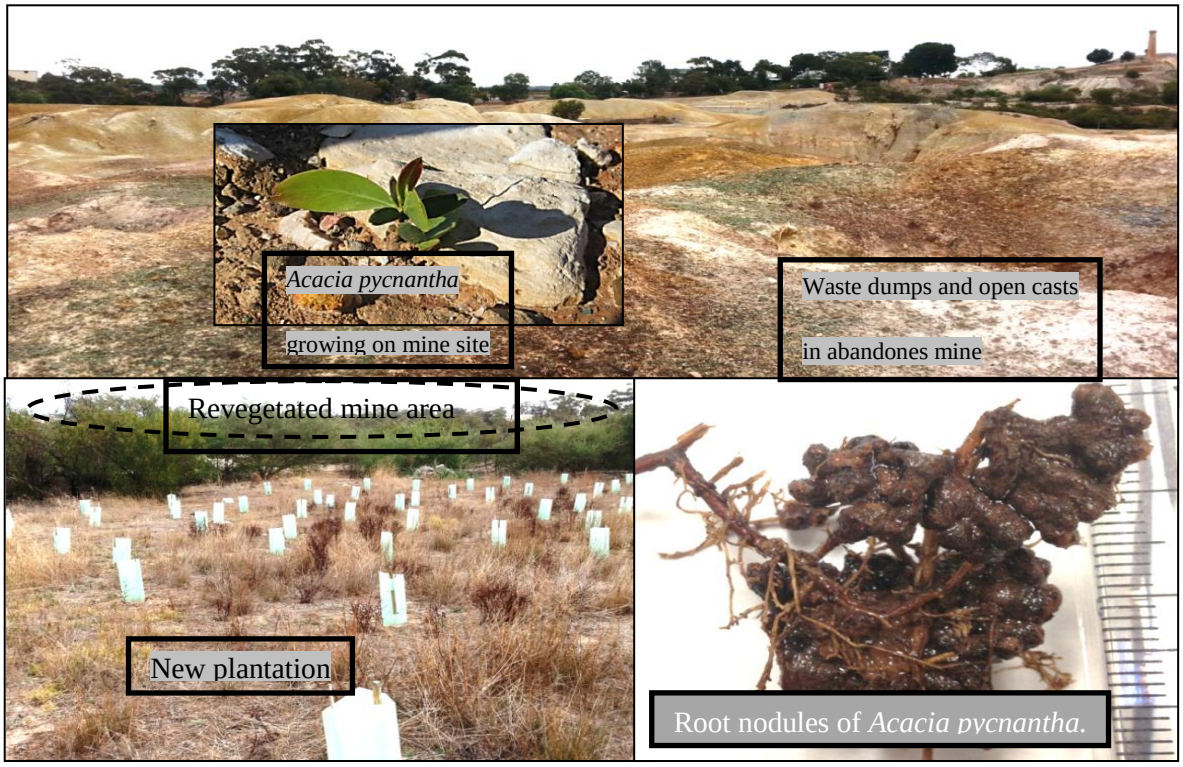


Fig 2.1 Landscape of abandoned copper mine in Kapunda, South Australia showing revegetation using native plants. (Inset, a resilient *Acacia pycnantha* growing on a dry mine site rubble, the dashes-circle shows earlier successful revegetation)

As a result of various trials, sea thrift (*Armeria maritima*), cape leadwort (*Plumbago auriculata*) and some species from the genus *Limonium* are commonly used for remediation of lead polluted sites (Rascio & Navari-lzzo, 2011; Gawronski et al., 2011). Similarly, at a former gold mine in Jales, Portugal, the first attempts to establish a vegetation cover were unsuccessful (Mench et al., 2003) as only a few small patches were colonized by newly planted vegetation of grasses such as *Agrostis castellana*, *Agrostis delicatula* and *Holcus lanatus*.

Various rehabilitation works on mines have attracted scientists, changed public opinion, activated regulators, and concerned administrators. However, modern day budget constraints and an unstable global mining economy are some disturbing factors for effective project implementation. Overall, rehabilitation efforts are likely to be more successful if they consider social and community issues, as well as scientific and legal aspects (Browne et al., 2011).

2.2 Environmental sustainability in mine site rehabilitation

Until recently, not many rehabilitation projects have been able to meet the goals of environmental sustainability (Lamb et al., 2015) often ending with failure to achieving "ecological climax". A process of ecological succession as per Clements (1900-1960) identifies a site transforming 'from bare ground to a climax forest' through an ecological series of revegetation stages (Prach & Hobbs, 2008; Vranjic et al., 2012). In a wider perspective, the Foundation of Ecological Security (2008) states that recovery of degraded, damaged or destroyed ecosystems by anthropogenic and natural agents through ecological restoration or re-greening technologies can be achieved by adopting microbiological, biochemical and bio-engineering methods.

The aim is to revive interactions by involving minerals, water and energy through the patterns of ecological succession (Shrestha & Lal, 2008; Vranjic et al., 2012). In other words, the ultimate aim of eco-restoration is achieving a balanced vegetation cover that stabilizes pollutants within soils and avoids exposure to wind and rain while achieving additional benefits of sequestering carbon, stabilizing the local climate and maintaining the food chain (Tordoff et al., 2000; Moreno-Jiménez et al., 2009; Claveria et al., 2010).

Therefore, phytoremediation is achieved through revegetation leading to rehabilitation of abandoned or polluted land (Shah & Nongkynrih, 2007). Recently, there has been an increasing interest in the use of native and non-invasive plants to

be more specific towards conservation of natural habitat as well as to render phytoremediation (Mendez & Maier, 2008b).

2.2.1 Microorganisms and non-vascular plants

The use of ubiquitous microorganisms by associating with plants at the field scale is a form of bioremediation technology that is gaining momentum today (Dixit et al., 2015). Mandal & Bhattacharyya (2012) define phytoremediation as "an environmental biotechnology using vegetation for *in situ* treatment of contaminated soils, sediments, and water". The phytoremediation process as mentioned earlier uses a synergistic relationship among plants, microbes, water and soil that have evolved for millions of years (Sinha et al., 2010). Microorganisms, including algae and fungi are therefore the most important non-vascular agents to help degradation and detoxification processes by employing biochemical strategies that allow them to digest pollutants (Megharaj et al., 2011; Dixit et al., 2015).

Interaction of bacteria in extreme environments with heavy metals has been studied in relation to adaptation, metabolism, tolerance, and resistance to heavy metals (Prasad & de Oliveira, 2003). A consortium of algae and bacteria comprising of *Chlorella sorokiniana* and *Ralstonia basilensis* has been found capable of removing copper more efficiently at pH 5.0 (Subashchandrabose et al., 2011). In another laboratory test a dried mass of mixed culture of microalgae (such as cyanobacteria, diatoms and bacteria) was used as a biofilter which removed 80% Cu and 100% Cd within the first 5 minutes of contact time (Loutseti et al., 2009).

However, their action under *in-situ* semi-arid and arid (SAA) environmental conditions needs further verification. Others have shown that, rather than the use of bacteria, mycorrhizal fungi as natural biofilters can change the availability of metals in soils and can also work as biofilters for the delivery of metals and nutrients such as nitrogen to plants (Kothe & Haferburg, 2010). In a study of mycorrhizal fungi that form a symbiotic association with roots, Khan et al. (2000) reported that such fungi

protects the roots against toxic substances in soil on the one hand and biodegradation of contaminants on the other. Moreover, arbuscular mycorrhizal fungi (AMF) are reported to supply nutrients such as phosphorous from soil to plants.

Furthermore, symbiotic bacteria such as *Azotobacter*, *Clostridium* and *Frankia* are found to form an important part of the bioremediation process by promoting soil health (Roy et al., 2007; Hayat et al., 2010). However, under field conditions, copper contaminated soil exhibited low microbial biomass which is an indication that microbes find it difficult to multiply and establish in contaminated soils (Guo et al., 2009). A 20-year study of a SAA revegetated mine site showed that plant and microbial diversity was low compared to an adjacent undisturbed site (Mendez & Maier, 2008b). Such evidence demonstrates that field tests are important to establish bioremediation processes in mine sites and to verify their effectiveness. Enhanced microbial establishment in soil could be achieved by application of recombinant DNA technology to produce more aggressive and tolerant strains of metal toxicity to successfully induce microbial enhanced phytoremediation (Evangelou et al, 2013; Gamalero et al., 2009). Also, there are problems associated with genetic engineering, such as decreased levels of fitness and extra energy demands imposed by the presence of foreign genetic material in cells (Ramakrishnan et al., 2011; Megharaj et al., 2011).

Therefore, the inability of microorganisms to compete with indigenous microflora, insufficient microbial activities at the subsurface level, poor support of native as well as pollutant-degrading microflora by limiting nutrients, heterogeneity of bioavailable contaminants, and toxic or inhibitory compounds in pollutant mixture may all be overcome by a plant-microbe symbiosis strategy (Gawronski et al., 2011; Megharaj et al., 2011). Hence, to thrive in SAA climatic zones it is important to look for symbiotic plant species that host microbes and grow fast producing a higher biomass (Gawronski et al., 2011).

2.2.2 Vascular-plants

The processes that phytoremediation covers include phytostabilization, phytofiltration, phytovolatilization, phytodegradation, rhizofiltration, rhizodegredation, and phytoextraction (Banuelos, 2006; Shah & Nongkynrih, 2007; Fulekar, 2010) (Fig. 2.2). These remediation activities dynamically operate either simultaneously or singularly depending upon the nature of the plant species and pollutants (Singh & Prasad, 2015). Vascular plants or higher plants referred to here as metallophytes (dealing with metal pollution) have distinct xylem and phloem bundles to perform advanced photosynthesis and respiration processes. In this context, Chaney et al. (1997) refers to phytoremediation as a "botanical remediation" that uses photosynthesizing plants to decontaminate soil, water and air (Lone et al., 2008).

Plants that have colonized former or abandoned mine sites harbour either tolerant, excluder or accumulator species (Danh et al., 2009; Brunetti et al., 2009; Kim et al., 2014; Nirola et al., 2015b). Based on the degree of tolerance of plants against heavy metals, Ernst et al. (2008) divide metallophytes into hypertolerant, basal tolerant and hypotolerant (Bothe, 2011). Many researchers suggest that it is the capacity of plants to tolerate extreme toxicity in soils that permits their use in revegetation leading to phytostabilization (Song et al., 2004; Whiting et al., 2004; Johansson et al., 2005).

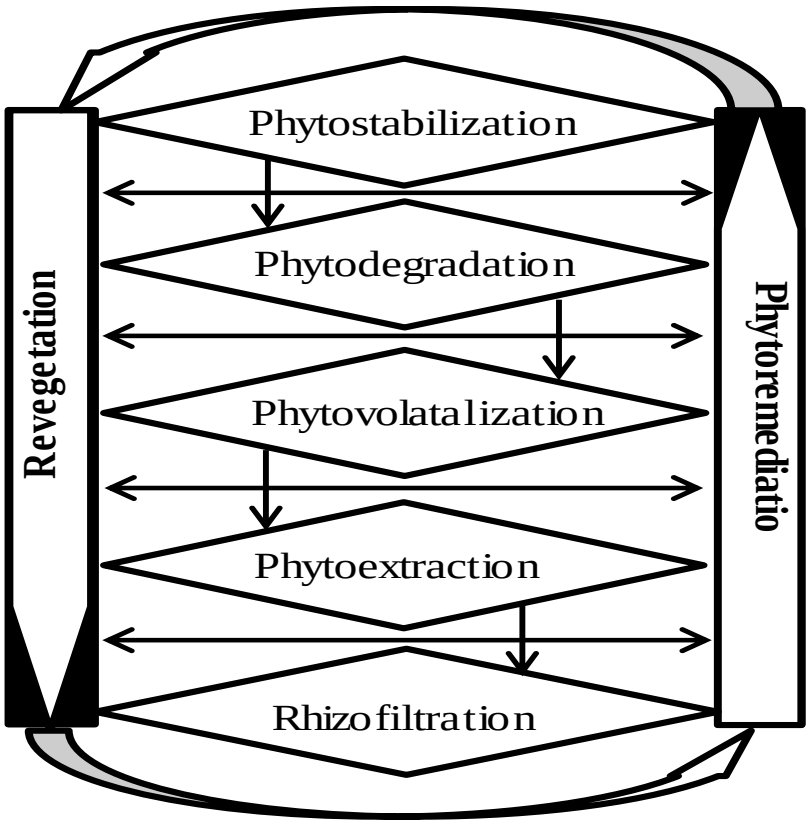


Fig 2.2 A revegetation and phytoremediation dynamics partially (horizontal arrows) or wholly (vertical arrows) involving different remediation processes listed in diamond blocks as decisions.

Evidently, plants cannot prevent metal uptake if they are growing over metalliferous soil but can only restrict the uptake of metals in stems, leaves and roots to varying degrees and are classified as accumulator, tolerant or excluder (Wei et al., 2005; Macnair & Baker, 2008).

Phytostabilization is achieved through sequestration of metals within the rhizosphere zone by restricting metal accumulation in above ground plant tissues (Nouri et al., 2011). Such stabilization by vascular plants render metals to be less bioavailable to livestock, wildlife, and humans thereby reducing exposure and associated health risks (Mendez & Maier, 2008b). Moreover, to arrest the leaching of heavy metals through the adventitious roots of higher plants and associated

rhizosphere microbes, phytostabilization using higher plants is a well acknowledged process of phytoremediation (Rajkumar et al., 2012).

However, such advocacy in favour of phytoremediation is proven successful only through practically demonstrated revegetation examples on heavy metal contaminated sites to sequester metal pollutants within the ground (Krumins et al., 2015). Plantation failure has been reported including the unassisted process of natural colonization of species of plants taking over 100 years (Li, 2006). The length of vegetation establishment in the more adverse climatic conditions of semi-arid and arid (SAA) mine tailings, dams and waste rocks could be much longer (Fig.2.3). Therefore, restoration requires human assistance along with the use of prescribed metallophytes if the restoration goal is expected to be achieved effectively within a reasonable timeframe (Li, 2006).

Gawronski et al., (2011) reported a successful botanical remediation using a group of metallophytes belonging to the taxonomical orders: Asterles, Brassicales, Caryophyllales, Fabales, Malphighiales, Poales, Rosales and Solanales. Detailed physiological and biochemical research was performed on some of these orders by Pollard et al. (2002) to study the mechanism of metal accumulation processes in *Thalaspi*, *Arabidopsis* and *Alyssum* species.

Such studies continue to shed light on the behaviour of different species of plants towards heavy metal pollution and metal accumulation in and around mine sites (Gonzalez & Gonzalez-Chavez, 2006). Hence, the present review is an attempt to evaluate metallophytes reported in the literature with phytoremediation evidence as presented in Table 2.1.

Table 2.1 Metallophytes under different taxonomic classification, target metals and literature source

No	Botanical name	Remediation target Literature source	
	Family: Amaranthaceae		
1	*Amaranthus blitoides*	Toxic chemicals resistant	Gawronski et al. 2011
2	*Amaranthus hybridus*	Pb stabilizer, phytoextractor.	Cetinkaya & Sozen, 2011; Gawronski et al. 2011
	Family: Anacardiaceae		
3	*Pistacia terebinthus*	Cu accumulator, Stabilizer	Johansson et al. 2005; Mendez & Maier, 2008a
4	*Schinus molle*	Cd, Cu, Mn, Pb, Zn Stabilizer	Mendez and Maier, 2008b
	Family: Apocynaceae		
5	*Alstonia macropylla*	Nickeliferous	Claveria, 2009; Sinha et al., 2004
	Family: Asparagaceae		
6	*Lomandra longifolia*	Cd, Pb accumulator, Stabilizer	Archer & Cadwell, 2004
	Family: Asteraceae		
7	*Baccharis neglecta*	As stabilizer	Mendez and Maier, 2008a
8	*Berkheya codii*	Ni accumulator	Singh & Tripathi, 2007; Sinha et al., 2004 Gawronski et al., 2011
9	*Bidens humilis*	Ag, As, Cd, Cu, Pb, Zn stabilizer	Mendez & Maier, 2008a
10	*Carduus pycnocephalus*	Metal tolerant	Brunetti et al. 2009
11	*Cirsium congestum*	Mn,Fe,Zn,Cu stabilizer	Nouri et al. 2011
12	*Helichrysum decumbens*	Pb stabilizer	Cetinkaya & Sozen, 2011
13	*Helianthus annus*	As stabilizer	Gawronski et al. 2011
14	*Isocoma veneta*	Cd, Cu, Mn, Pb, Zn stabilizer	Mendez & Maier, 2008a
15	*Pentacalia* spp.	Ni accumulator	Singh & Tripathi, 2007, Sinha et al., 2004
16	*Silybum marianum*	Metal tolerant	Brunetti et al., 2009
17	*Taraxacum mongolicum*	Zn excluder	Lone et al. 2004; Roy et al. 2007
	Family: Betaceae		
18	*Atriplex hortensis*	Metallophyte	Gawronski et al. 2011

19	*Kochia scoparia*	Metallophyte	Gawronski et al. 2011
	Family: Betulaceae		
20	*Alnus glutinosa*	Cu remediation	Roy et al. 2007; Whitbread & Abrutat, 1997
21	*Betula pendula*	Cd, Zn Stabilizer	Evangelou et al. 2013
	Family: Brassicaceae		
22	*Alyssum bertolonii*	Ni accumulator	Bothe, 2011
23	*Alyssium* spp.	Ni accumulator	Singh & Tripathi, 2007; Sinha et al. 2004
24	*Brassica carinata*	As, Cd, Cu, Pb, Zn stabilizer	Cetinkaya & Sozen, 2011
25	*Brassica juncea*	Metal extractor, Metallophyte	Shah & Nongkynrih, 2007; Gawronski et al. 2011
26	*Dichopetalum gelonioides*	Zn accumulator	Singh & Tripathi, 2007; Sinha et al. 2004
27	*Iberis intermedia*	Th accumulator	Gawronski et al., 2011
28	*Streptanthus polygaloides*	Ni accumulator	Bothe, 2011
29	*Sinapis arvensis*	Metal tolerant	Brunetti et al. 2009
30	*Thlaspi caerulescene*	Zn, Cd, Ni, Pb accumulator	Pulford & Watson, 2003; Singh & Tripathi, 2007, Lone et al. 2008; Sinha et al. 2004; Cetinkaya & Sozen, 2011
31	*Thlaspi rotundifolium*	Pb, Zn accumulator	Shah & Nongkynrih, 2007; Singh & Tripathi, 2007; Sinha et al. 2004
	Family: Caryophyllaceae		
32	*Agrostemma githago*	Salt & heavy metal tolerant	Gawronski et al. 2011
33	*Dianthus carthusianirum*	Salt & heavy metal tolerant	Gawronski et al. 2011
34	*Minuartia verna*	Pb accumulator	Singh & Tripathi, 2007
35	*Silene vulgaris*	Cu tolerant, Salt & heavy metal tolerant	Song et al. 2004; Gawronski et al. 2011
	Family: Casuarinaceae		
36	*Gymnostoma leucodon*	Restoration, revegetation	Whiting et al. 2004
	Family: Celastraceae		
37	*Maystenus bureaviana*	Mn accumulator	Singh & Tripathi, 2007; Sinha et al. 2004
38	*Maystenus sebertiana*	Mn accumulator	Singh & Tripathi, 2007; Sinha et al., 2004
	Family: Chenopodiaceae		
39	*Atriplex lentiformis*	As, Cu, Mn, Pb, Zn stabilizer	Mendez & Maier, 2008 b&c
40	*Atriplex nummularia*	Stabilizer, drought tolerant	Mok et al. 2013
41	*Atriplex canescens*	As, Hg, Mn, Pb Stabilizer	Mendez & Maier, 2008b
42	*Salsola kali*	Cd extractor	Cano- Aguilera et al., 2004
43	*Atriplex semibaccata*	Cu tolerant, drought	Guo et al. 2009;

		tolerant	Bullock, 1936
44	*Teloxys graveolens*	Cd, Cu, Mn, Pb, Zn Stabilizer	Mendez & Maier, 2008a
	Family: Cistaceae		
45	*Cistus creticus*	Cu accumulator	Moreno- Jiminez et al. 2009
	Family: Commelinaceae		
46	*Commelina communis*	Cd excluder	Brewin et al. 2003
	Family: Crassulaceae		
47	*Sedum alfredii*	Zn, Cd accumulator	Yang et al. 2005; Lone et al. 2008; Tian et al. 2011
	Family: Cunoniaceae		
48	*Weinmannia* sp.	Nickeliferous	Claveria et al. 2009
	Family: Cyperaceae		
49	*Ascolepis metallorum*	Cu stabilizer	Saad et al. 2012
50	*Bulbostylis pseudoperennis*	Cu, Co Stabilizer	Saad et al. 2012
51	*Carex hirta*	Metallophyte, salt tolerant	Gawronski et al. 2011
52	*Eriophorum angustifolium*	Acidic soil stabilization	Gawronski et al. 2011
53	*Scripus californicus*	Zn contaminated water	Gawronski et al. 2011
54	*Schoenus juvenis*	Restoratoin, revegetation	Whiting et al. 2004
55	*Uncinia leptostachya*	Uranium accumulator	Khan et al. 2000
	Family: Dennstaedtiaceae		
56	*Pteridium aquilinum*	Cu tolerant	Claveria et al. 2009
	Family: Euphorbiaceae		
57	*Euphorbia sp.*	Cd, Cu, Mn, Pb, Zn stabilizer	Mendez & Maier, 2008a
58	*Jatropha dioica*	Zn accumulator	González & González-Chávez, 2006
59	*Phyllanthus balgooyi*	Ni accumulator	Whiting et al. 2004
	Family: Fabaceae		
60	*Acacia mearnsii*	Metal extractor	Mok et al. 2013
61	*Acacia* spp.	Metal tolerant	Lamb et al. 2010
62	*Amorpha fruticosa*	Metallophyte	Gawronski et al. 2011
63	*Astragalus bisulcatus*	Se stabilizer	Gawronski et al. 2011
64	*Astragalus racemosus*	Se accumulator	Singh & Tripathi, 2007; Sinha et al., 2004
65	*Caragana arborescens*	metallophyte	Gawronski et al., 2011
66	*Crotolaria cobalticola*	Co accumulator, stabilizer	Singh & Tripathi, 2007; Sinha et al. 2004; Saad et al. 2012
67	*Cytisus striatus*	Metallophyte	Gawronski et al. 2011
68	*Dalea bicolor*	Cd, Cu, Mn, Pb, Zn Stabilizer	Mendez and Maier, 2008b

69	*Lupinus albus*	Mn, Pb, Cr(III) & (IV) accumulator	Gawronski et al. 2011
70	*Lupinus angustifolius*	Mn, Pb, Cr(III) & (IV) accumulator	Gawronski et al. 2011
71	*Lupinus hispanicus*	Mn, Pb, Cr(III) & (IV) accumulator	Gawronski et al. 2011
72	*Lupinus* sp.	degrade PAH & PCBs	Gawronski et al. 2011
73	*Medicago Sativa*	Mn, Pb Cr(III) & (IV) accumulator	Gawronski et al. 2011
74	*Meliolotus indica*	Metallophyte	Gawronski et al. 2011
75	*Robinia pseudoaccacia*	Metallophyte	Gawronski et al. 2011
76	*Sesbania drummondii*	Pb accumulator	Shah & Nongkynrih, 2007
77	*Vicia exaltata*	Degrade PAHs & PCBs	Gawronski et al. 2011
	Family: Geraniaceae		
78	*Biebersteinia multifida*	Mn, Fe, Zn, Cu stabilizer	Nouri et al. 2011
	Family: Gleicheniaceae		
79	*Dicranopteris linearis*	Cu tolerant	Claveria et al. 2009
	Family: Hyperiaceae		
80	*Hypericum perforatum*	Cd accumulator	Moreno-Jiminez et al. 2009
	Family: Juncaceae		
81	*Juncus articulatus*	Heavy metal tolerant	Gawronski et al. 2011
82	*Juncus effuses*	Heavy metal tolerant	Gawronski et al. 2011
83	*Juncus lutea*	Ni tolerant	Gawronski et al., 2011
84	*Juncus usitatus*	Cd, Pb accumulator, Stabilizer	Archer & Cadwell, 2004
	Family: Lamiaceae		
85	*Elsholtzia splendens*	Cu tolerant	Song et al. 2004
86	*Haumaniastum robertii*	Co accumulator, metallophyte	Singh & Tripathi, 2007; Sinha et al. 2004; Saad et al. 2012
87	*Stachys inflate*	Mn,Fe,Zn,Cu stabilizer	Nouri et al. 2011
88	*Ziziphora clinopodioides*	Mn,Fe,Zn,Cu stabilizer	Nouri et al. 2011
	Family: Lecithidiaceae		
89	*Lecithis ollaria*	Se accumulator	Singh & Tripathi, 2007; Sinha et al. 2004
	Family: Liliaceae		
90	*Borya nitida*	drought tolerant	Gaff & Churchill, 1976
	Family: Malvaceae		
91	*Triumfetta welwitchii*	Co & Cu accumulator	Saad et al. 2012
	Family: Myrtaceae		
92	*Austromyrtus bidwilli*	As accumulator	Shah & Nongkynrih, 2007

93	*Eucalyptus polybractea*	Metal extractor	Mok et al. 2013
94	*Eucalyptus cladocalyx*	Metal extractor	Mok et al. 2013
95	*Leptospermum scoparium*	Revegetation, Cr accumulator	Rufaut & Craw, 2010; Khan et al. 2000
96	*Melaleuca alternifolia*	Cd accumulator, Stabilizer	Archer & Cadwell, 2004
	Family: Nephrolepidaceae		
97	*Nephrolepis hirsutula*	Cu tolerant	Claveria, 2009
	Family: Onagraceae		
98	*Oenothera biennis*	Cd excluder	Wei et al. 2005
	Family: Orobanchaceae		
99	*Sopubia neptunii*	Co accumulator	Saad et al. 2012
	Family: Phytolaccaceae		
100	*Phytolacca acinosa*	Cu excluder	Ye et al. 2009
	Family: Pinaceae		
101	*Pinus brutia*	Cu accumulator, Stabilizer	Johansson et al. 2005; Cetinkaya & Sozen, 2011
	Family: Plantaginaceae		
102	*Digitalis thapsi*	Cd & Zn accumulator	Moreno-Jiminez et al. 2009
	Family: Plumbaginaceae		
103	*Armeria maritima*	Cu excluder, salt tolerant	Brewin et al. 2003; Bothe, 2011; Gawronski et al. 2011
104	*Limonium* sp.	Pb, Zn stabilizer	Cetinkaya & Sozen, 2011
105	*Lygeum spartum*	Cu, Pb, Zn stabilizer	Mendez & Maier, 2008a
106	*Plumbago auriculata*	Metallophyte	Gawronski et al. 2011
	Family: Poaceae		
107	*Festuca rubra*	Stabilizer	Mendez & Maier, 2008a
108	*Agrostis alba*	Heavy metal tolerant	Gawronski et al. 2011
109	*Agrostis gigantea*	Revegetation	Peters, 1988
110	*Agrostis stolonifera*	Metallophyte	Li, 2006
111	*Agrostis tenius*	Pb accumulator, metallophyte	Singh & Tripathi, 2007; Sinha et al. 2004; Li, 2006; Mendez & Maier, 2008a
112	*Cynodon dactylon*	Metallophyte., Cd	Li, 2006; Archer & Cadwell, 2004
113	*Dasypyrum villosum*	Metal excluder	Brunetti et al. 2009
114	*Deschampia cespitosa*	heavy metal tolerant	Gawronski et al. 2011
115	*Elytrigia repens*	Salinity tolerant	Gawronski et al. 2011
116	*Festuca arundinacea*	Revegetation	Peters, 1988
117	*Festuca rubra*	Revegetation	Peters, 1988; Li, 2006
118	*Glomus intraradices*	Heavy metal tolerant	Gawronski et al. 2011
119	*Leersia hexandra*	Cr accumulator	Gawronski et al. 2011
120	*Phleum pratense*	Revegetation	Peters, 1988
121	*Phragmites australis*	Metallophyte, Cu, Pb, Se stabilizer	Li, 2006; Cetinkaya & Sozen, 2011

122	*Poa compressa*	Revegetation	Peters, 1988
123	*Poa pratensis*	Revegetation	Peters, 1988
124	*Piptatherum miliaceum*	Cu, Pb, Zn stabilizer	Mendez & Maier, 2008a
125	*Stipa austroitalica*	Metal excluder	Brunetti et al. 2009
126	*Vetiveria zizanoides*	Zn, Ni, Pb, Cr accumulator	Singh & Tripathi, 2007; Sinha et al., 2004; Zhang et al. 2014
	Family: Polygonaceae		
127	*Fagoyrum tataricum*	Metallophyte	Gawronski et al. 2011
128	*Polygonum aviculare*	Zn, Hg accumulator	González & G-Chávez, 2006; Bothe, 2011; Gawronski et al. 2011
129	*Rumex acetose*	Copper excluder	Ye et al. 2009
	Family: Proteaceae		
130	*Macadania neurophylla*	Mn accumulator	Peters, 1988; Singh & Tripathi, 2007; Sinha et al. 2004
131	*Grevillea robusta*	Metal extractor	Mok et al. 2013
132	*Pimelea suteri*	Cr accumulator	Khan et al. 2000
	Family: Pteridaceae		
133	*Pteris cretica*	As accumulator	Gawronski et al. 2011
134	*Pteris longifolia*	As accumulator	Gawronski et al. 2011
135	*Pteris* sp.	Cu tolerant	Claveria et al. 2009
136	*Pteris umbrosa*	As accumulator	Gawronski et al. 2011
137	*Pteris vittata*	As accumulator, metallophyte	Shah & Nongkynrih, 2007; Gawronski et al. 2011
	Family: Rubiaceae		
138	*Coprosma arborea*	U accumulator	Khan et al. 2000
139	*Psychotria corinota*	Ni accumulator	Singh & Tripathi, 2007; Sinha et al. 2004
	Family: Rosaceae		
140	*Pyracantha coccinea*	metallophyte	Gawronski et al. 2011
141	*Rosa rugosa*	metallophyte	Gawronski et al. 2011
	Family: Salicaceae		
142	*Populus alba*	Metal extractor	Di Lonardo et al. 2011
143	*Populus monviso*	Cu, Pb Stabilizer	Evangelou et al. 2013
144	*Salix atrocinerea*	Cd & Zn accumulator	Moreno-Jiminez et al. 2009
145	*Salix viminalis*	Cu, Pb Stabilizer	Gawronski et al. 2011; Evangelou et al. 2013
	Family: Scrophulariaceae		
146	*Sopubia neptunii*	Co stabilizer	Saad et al. 2012
	Family: Sicaceae		
147	*Thlaspi goesingense*	Ni accumulator	Bothe, 2011
	Family: Solanaceae		
148	*Datura inoxia*	heavy metal sequestration	Gawronski et al. 2011

149	*Solanum nigrum*	Zn stabilizer	Cetinkaya & Sozen, 2011
	Family: Typhaceae		
150	*Typha angustifolia*	Heavy metal tolerant	Gawronski et al. 2011
151	*Typha latifolia*	Metallophyte, Se stabilizer	Li, 2006; Cetinkaya & Sozen, 2011
	Family: Tamaricaceae		
152	*Tamarix tetrandra*	Salt & metal excluder	Gawronski et al. 2011
153	*Tamarix* sp.	Cu, Zn stabilizer	Cetinkaya & Sozen, 2011
	Family: Violaceae		
154	*Viola baoshanensis*	Heavy metal tolerant	Gawronski et al. 2011
155	*Viola calaminaria*	Heavy metal tolerant	Gawronski et al. 2011
156	*Viola lutea*	Heavy metal tolerant	Gawronski et al. 2011
	Family: Zygophyllaceae		
157	*Zygophyllum fabago*	Zn stabilizer	Cetinkaya & Sozen, 2011

2.2.3 Semi-arid and arid (SAA) plants

Many undocumented plants have developed various xeric characters to tolerate or endure drought conditions with further evolution to adapt to metal toxicity (Cousins & Witkowski, 2012). Drought resistance is a generic term for i) drought escape and ii) drought tolerance (at high and low tissue water potential - ψ_w) achieved through the physiological mechanisms of plants (Paleg & Aspinall, 1981). Metalliferous sites present several environmental constraints for plants to establish including dry and nutrient deficient conditions (Whitbread-Abrutat, 1997; Rufaut & Craw, 2010; Saad et al., 2012). It is more challenging in xeric environmental conditions to revegetate with non-xerophytic plants that might involve nursery sapling plantation or the use of a direct seeding method (Chen et al., 2005). We review potential metallophytes for mine site revegetation based on a set of established characteristics (Pollard et al., 2014; Baker, 2014), namely:

(a) Drought tolerance.

(b) Tolerance to soil acidity and salinity.

(c) Leguminous plants that boost nitrogen assimilation and biomass production.

According to Hudson (1987), semi-arid and arid (SAA) areas are those falling within rainfall zones of 300-600 mm and 0-300 mm, respectively. It is estimated that 50% of the land on earth comes into the SAA category. In this scenario, it is evident that SAA shrub-lands are not likely to have complete plant coverage such as those found in tropical or temperate climates. Many abandoned mine sites fall within SAA zones and this worsens the potential for heavy metal contamination due to acute erosion by extreme rainfall events or seasonal high-speed winds over barren dry-soil (Duque et al., 2015). The plant dynamics in SAA climates are also largely affected by nitrogen deficiency and water use efficiency, in comparison to the temperate plant communities which are influenced by light and nutrition availability (Hudson, 1987; Atwell at al., 1999).

Australia is the driest inhabited continent in the world, with 70% of the land falling in the SAA zone (Hudson, 1987; Duque et al., 2015). There are over 30,000 species of vascular plants recorded in Australia that include angiosperms, seed-bearing non-angiosperms (such as conifers and cycads), and spore-bearing ferns and fern allies (Orchard, 1999). Of these, only around 11% are naturalized species and the remainder are native or endemic species (Orchard, 1999).

Out of the 11% of naturalised species, there are a few **Australian metallophytes** recognised to grow in SAA zones, namely *Cheilanthes lasiophylla* (Adiantaceae), *Rhagodia spinescens* (Chenopodiaceae), and *Nitraria billardierei* (Zygophyllaceae) (Kutsche and Lay, 2003). Research is inadequate on the rehabilitation of mines in SAA zones using exotic or sub-native species of trees, shrubs (Le Houerou, 2000). Among native plants, species of *Atriplex* (salt bush) of the sub-family Chenopodiaceae are also known to be pioneer plants growing on mine tailings in semi-arid Western Australia (Jefferson, 2004).

There is also scope to search for combined metal-drought resilient species by the use of C3 plants which thrive in moderate temperature and sunlight intensity and C4 plants that can thrive in high temperature and sunlight intensity. For instance, there is an unusual adaptation with C3 plants that behave like C4 plants. Normally C3

plants grow in warm temperate and tropical grasslands, savannahs, sand dunes, salt marshes, including semi-deserts and deserts (Kadereit et al., 2003).

Specifically, the most diversified C3 plants like *Gomphrena*, *Amaranthus*, *Atriplex*, *Salsola*, and *Suaeda* prefer C4 habitats including semi-arid and arid (SAA) zones. Moreover, most of the soil enriching leguminous plants of the *Acacia* species are also drought and salt tolerant but careful consideration of such species is needed given the potential weed threat in some regions of the world (Hoffmann et al., 2012).

2.2.4 Revegetation challenges

Chen et al. (2005) state that it is a sustainable exercise to use appropriate plant species or ecotypes or nursery stocks for assisted revegetation of abandoned mine areas contaminated with toxic elements. However, assisted revegetation can be expensive if the area is too large. Therefore, before considering phytoremediation, the adaptive characteristics of plants forms a basis for sustainable rehabilitation of mines in SAA zones (Vranjic et al., 2012). Moreover, various risks and challenges are involved such as polluted water entering and clogging ponds, tailings instability, lack of documentation of faunal diversity for food chain assessment, lack of identification of high risk zones of erosion and human safety hazards (Fig 2.3).

- A taxonomic survey, plant assessment and laboratory research can help avert the negative impacts of ecological processes. One such process is a 'phenomenon of arrested succession' where grasses dominate trees and render them into diseased stumps (Groninger et al., 2007; Kramer et al., 2010; Boyes et al., 2011).
- A further problem of plant establishment is allelopathism where one species competes with the other to limit growth leading to elimination of the latter. An allelopathic effect is also brought about by its own decaying matter such as *Kochia* leaves on its own roots that depress its own growth (Wali, 1999).
- It has also come to light that plants involved in phytoremediation often bear the toxicity stress of Al, Cd, Cu, Mn and Zn that hinder plant establishment and growth (Peters, 1995; Maddocks et al., 2009).

- Moreover, the secondary successional plant communities in SAA climates are more likely to be affected by substantial mortality because of a deficiency of water (Mendez & Maier, 2008b). Therefore, it is necessary to identify the spatial variability and heterogeneity of soil properties for determining the revegetation potential with regards to an availability of soil moisture and nutrients (Guo et al., 2010).
- There have been cases of species-extinction through genetic assimilation or demographic swamping due to the use of alien species of plants in revegetation programmes (Byrne et al., 2011).



Fig 2.3 The ecological and engineering challenges associated with revegetation of SAA abandoned mine sites such as the copper mine in Kapunda. (2014-2015)

2.3 Phylogenetic implications in semi-arid and arid (SAA) lands

There is a very small literature available regarding documented total number of available metallophytes. In one source, 400 species belonging to 45 families are identified as metallophytes based on their heavy metals (Cd, Co, Cu, Pb and Zn) uptake behaviour (Mendez & Maier, 2008b). However, Pollard et al. (2014) report the number to be 500 based on facultative hyperaccumulation. Rascio & Navari-lzzo, 2011 in their review report 450 angiosperms as hyperaccumulators of heavy metals. New listings have appeared in recent times which are either a repeat or are taxonomically related to the earlier identified species or are indeed new additions (Pollard et al., 2014). Fine, 2015 suggests that, as a result of long geographic isolation and climatic influences, there are natural variations in plants leading to environmental evolution. For instance, species behaviour linked through their phylogeny has been proven in the orders Caryophyllales, Apiales and Cucurbitales, because of high ^{36}Cl uptake among these related orders (Fawcett & Willey, 2005). As a result of analyses of molecular data from various sources, the understanding around phylogenetic relationships among angiosperms has greatly increased. For instance, Fior et al. (2006) discuss the repositioning of the family Caryophyllaceae within the order Caryophyllales as the sister to the family Amaranthaceae in a clade, which in turn is sister to the core order Caryophyllales.

The phylogenetic linkage is also seen useful for quantifying and predicting the soil-to-plant transfer of ions as found in some angiospermic plants (Fawcett & Willey, 2005). The molecular level study of phylogenetics of the plant *Limonium* sp. and its related genera under the family Plumbagenaceae shows physiologically similar ion transfer behaviour (Lledo et al, 2005). However, little is known regarding the genetic basis of hyperaccumulation as there are no known cases of major genetic polymorphism, which contrasts the phenomenon of metal tolerance as a result of evolution as discussed earlier (Pollard et al., 2002; Hanikenne & Nouet, 2011).

There is also a lack of understanding of the genetic and regulatory factors influencing the variable gene expression of the hyper-accumulative behaviour of metallophyte

plant species (Pollard et al., 2002). However, it is evident that plant systematics also deals with hybridization and molecular techniques, including comparative genomics and the hyper-accumulative nature of plants (Hanikenne & Nouet, 2011). However, it is expected that any given metallophyte, by virtue of being taxonomically related to another far-related genera, should behave identically with respect to its response to heavy metal remediation. Therefore, all species falling under the genus *Atriplex* as mentioned above should be resilient to SAA mine sites containing saline and sodic soils.

2.3.1 Plant metal interaction in SAA zones

As mentioned earlier, Australia has 70% of its land in SAA zones (Hudson, 1987) and the plants growing in these zones and particularly near abandoned mines such as the Kapunda copper mine in South Australia (Nirola et al., 2015a) could be taxonomically related to metallophytes as reported in various literature (Table 2.1). For instance, the nitrogen fixer *Medicago polymorpha* is native to South Australia (Black, 1909) and has a leguminous relative *Medicago sativa* which is a Mn, Pb and Cr accumulator (Gawronski et al., 2011). Likewise, the legume *Acacia mearnsii* is a SAA metal accumulator (Mok et al., 2013). Several other genera namely *A. victoriae*, A. *tarculensis*, *A. tetragonophylla*, *A. nyssophylla*, *A. rivalis*, *A. careneorum* and *A. ligulata* could be effective in SAA mines for revegetation (Kutsche & Lay, 2003). Similarly, a legume *Crotolaria cobalticola* which is a cobalt accumulator (Table 2.1) is related to SAA plants namely C. *cunninghamii* and *C. eremaea* (Kutsche & Lay, 2003).

Plants belonging to the Brassicaceae family *Brassica tournefortii*, also occur in disturbed soils (Kutsche & Lay, 2003). Plants of the same genera *Brassica carinata* and *Brassica juncea* act as As, Cd, Cu, Pb and Zn stabilizers and accumulators, respectively (Table 2.1). A popular salt bush plant from the Chenopodiaceae family named *Atriplex* sp. is reported to be a pioneer species growing on mine tailings in semi-arid Western Australia (Jefferson, 2004) and the species *A. lentiformis*, *A.*

nummularia and *A. canescens* are listed as proven stabilizers (Table 2.1). Russian thistle *Salsola kali* also from Chenopodiaceae is a Cd extractor along with plants under the same family *Atriplex nummularia*, A. *stipitata*, A. *lindleyi,* A. *holocarpa,* A. *limbata*, A. *quinii* and A. *vilutinella* as a salinity tolerant species (Kutsche & Lay, 2003).

Guo et al. (2009) recommend *Atriplex semibaccata* (Table 2.1) to be a suitable species for mine site soil rehabilitation in SAA environmental conditions. Another Cd accumulator and toxic soil stabilizer namely *Melaleuca alternifolia* (Table 2.1) has a related genus from the family Myrtaceae called *M. glomerata* that tolerates high levels of salinity and is also found to grow in SAA zones (Kutsche & Lay, 2003). The forb *Euphorbia* sp. is a Cd, Cu, Mn, Pb and Zn stabilizer (Mendez & Maier, 2008b) and is also related to a SAA genus *E. drummondi* highly reputed for its soil stabilization capacity (Kutsche & Lay, 2003). Several studies have reported growth of SAA species of the grass family Poaceae; namely *Festuca rubra*, *Agrostis alba*, *Agrostis gigantea*, *Agrostis stolonifera*, *Agrostis tenius*, *Deschampia cespitosa*, *Glomus intraradices*, *Vetiveria zizanioides* and *Phragmites australis* (Li et al., 2006; Kutsche & Lay, 2003). The SAA adapted grass *Phragmites australis* which is a drought tolerant species (Kutsche & Lay, 2003) has been reported to grow on Cu, Pb and Se contaminated sites (Li, 2006).

Kutsche & Lay (2003) report 11 species of Eucalyptus that are found in outback SAA zones of Australia. An additional two species, namely *Eucalyptus polybractea* and *Eucalyptus cladocalyx*, are also reported to be metal extractors growing in SAA soils (Mok et al., 2013). There is also a report of *Grevillea robusta* of the family Proteaceae being a metal extractor (Mok et al., 2013) which is represented by *G. striata*, G. *huegelli*, G. *juncifolia*, *G. nematophylla*, *G. stenobotrya*, *G. treueriana* found in SAA zones (Kutsche & Lay, 2003). A popular weed *Solanum nigrum*, commonly called black night-shade, is a Zn stabilizer (Cetinkaya & Sozen, 2011) and has related species, namely *S. quadriloculatum*, *S. ellipticum*, *S. coactifilerum*, and *S. esuriale* growing in SAA conditions (Kutsche & Lay, 2003).

Another Zn stabilizer belonging to the family Zygophyllaceae is *Zygophyllum fabago* (Cetinkaya & Sozen, 2011) which has a family and genera related to *Z. aurantiacum*, *Z. apiculatum*, *Z. howittii*, *Z. prismatothecum*, and *Z. simile* growing in SAA zones (Kutsche & Lay, 2003). A Pb, Zn stabilizer, *Limonium* sp. (Cetinkaya & Sozen, 2011) is reported to grow on disturbed soils under a SAA type climate as *Limonium lobatum* from the family Plumbaginaceae (Kutsche & Lay, 2003). Other exceptional metallophytes in SAA mine sites are identified as *Atriplex* spp., *Atriplex semibaccata*, *Salsola kali*, *Phragmites australis*, and *Medicago sativa* and these are discussed up to the rank 'Order' in the following topics.

2.3.1.1 Order Caryophyllales

The order Caryophyllales include 33 families, 692 genera and 11,155 species worldwide (Stephens, 2007). Some of the pioneer metallophytes under this order listed in Table 2.1 are from the families Amaranthaceae, Betaceae, Caryophyllaceae, Chenopodiaceae, Plumbaginaceae, Polygonaceae and Tamaricaceae. Most prominently, metallophytes from the family Chenopodiaceae are represented by *Atriplex lentiformis*, *Atriplex nummularia*, *Atriplex canescens*, *Atriplex semibaccata* and *Salsola kali*.

It is reported that forbs such as *Atriplex* sp. are metal tolerant because of their higher bio-concentration factor and are considered to be a candidate species for phytostabilization (Kachout et al., 2012). **Generally, soils that contain high concentrations of Zn >300 mg kg^{-1}, Cu >100 mg kg^{-1}, Ni >50 mg kg^{-1} and Pb >100 mg kg^{-1} are phytotoxic to plants**, but the genus *Atriplex* has not shown phytotoxicity when introduced to higher concentrations than those stated above (Kachout et al., 2012). In another study, *Atriplex* was found to have a stimulating effect with Ni exposure, whereas concentrations of Ni > 1 mg L^{-1} were found to be toxic to other plant species (Amer et al., 2013).

However, in remediation studies of Ni, Pb, and Zn in the same area, it was found that the highest Zn concentration of 4660 mg kg^{-1} was observed in shoots of *Atriplex halimus*, indicating that it is a Zn accumulator. Coincidently, the plant *Atriplex halimus* has been proven to be a good phytostabilizer in SAA conditions exhibiting effective adaptation even without soil amendments (Martinez-Fernandez & Walker, 2012). In a study of accumulation of Cu, Pb, Ni and Zn in a halophyte *Atriplex hortensis*, the metal concentrations in the roots were found to be proportionately higher than in the shoots with increasing concentrations of those metals in soil (Kachout et al. 2012a).

The salinity tolerant plant *Atriplex semibaccata* is found to be a most adaptable metallophyte that modifies its habitat and leaf structure when there is a change in environmental conditions, particularly with respect to the influences of temperature, evaporation, soil moisture and salinity (Bullock, 1936). Genus *A. semibaccata* is a drought tolerant SAA species that was introduced into California and Arizona in the USA in the early 1880s (Bullock, 1936). From 1993 to 2004, field phytoremediation studies were conducted using *A. semibaccata* in central California in an area known to have high selenium concentrations (Banuelos, 2006). Evidently, the concentration of Cu in *A. semibaccata* was at elevated levels in both the shoots (205.8 mg kg^{-1}) and roots (129.5 mg kg^{-1}) (Guo et al., 2009). In their study on the effect of leaching and irrigation on growth of *A. semibaccata,* De Villiers et al. (2006), found this species had the ability to survive both drought and salinity. Another species under order Caryophyllales is *A. rubra* of Chenopodiaceae, which is a suitable plant for phytostabilization of Cu and Ni contaminated sites (Kachout et al., 2012).

Salsola kali commonly called tumble weed or prickly saltwort or prickly Russian thistle also belongs to the family Chenopodiaceae, and was found to survive on 6000 mg L^{-1} of NaCl treatment, exhibiting no symptoms of toxicity (Shekhawat et al., 2006). Although this plant is reported to be a troublesome weed (Hasan et al., 2001),

following hydroponic cultivation experiments and agar exposure, Gardea-Torresdey et al. (2005) reported its positive potential for bioaccumulation of heavy metals.

The ICP/OES and XAS studies conducted by de la Rosa et al. (2004) on this species did not show any phytotoxicity effect when grown in an agar-based medium with 20 mg L^{-1} of Cd (II). The Cd accumulation from dry biomass was 2696 mg kg^{-1} in the roots, 2075 mg kg^{-1} in stems, and 2016 mg kg^{-1} in leaves in this experiment. Yet in another EDTA experiment on Pb phyto-accumulation test of *Salsola kali,* concentrations on dry weight of roots, stems and leaves were 31,000 mg kg^{-1}, 5,500 mg kg^{-1}, and 2,100 mg kg^{-1} respectively (Cano-Aguilera et al., 2007). In a road side study of this species in Iran, Pb which is one of the components of automobile fuel was found significantly absorbed by S. *kali*. Lead concentration in plant organs decreased exponentially with corresponding distance away from the motor vehicle road. Moreover, shoot parts were found to absorb more Pb than below ground parts (Sinegani, 2009). These findings indicate that the family Chenopodiaceae has a good prospect for remediation of heavy metals, especially in semi-arid and arid (SAA) conditions.

2.3.1.2 Order Poales

The order Poales has 20 families (Jacobs & Wilson, 2002) but Bremer (2002) states there to be 18, prominently with Poaceae having 12,070 species and Cyperaceae having 5,500 species. All these grass metallophytes are C4 and resilient SAA plants belonging to Poales and are listed in Table 2.1 belonging to Poaceae, Typhaceae, Juncaceae, and Cyperaceae. Some of the promising plants for phytoremediation technologies as listed under the Poaceae family are *Agrostis alba*, *Agrostis gigantea*, *Agrostis stolonifera*, *Agrostis tenius*, *Cynodon dactylon*, *Dasypyrum villosum*, *Deschampia cespitosa*, *Elytrigia repens*, *Festuca rubra*, *Glomus intraradices*, *Leersia hexandra*, *Phleum pratensis*, *Poa compressa*, *Poa pratensis*, *Piptatherum miliaceum*, *Phragmites australis, Stipa austroitalica*, and *Vetiveria zizaniodes*.

The common reed *Phragmites australis* is a popular plant in environmental science research and is traditionally used for waste-water treatment. In a waste-water remediation experiment, the associated rhizosphere bacterial community of *Phragmites* showed decolourization of distillery effluent from dark brown (high concentration of amino carbonyl polymer, phenolics, heavy metals and sulphate) into light brown colour (Chaturvedi et al., 2006). This grass plant was able to adapt to the harmful effects of cadmium.

For example, it was found that phytochelatin production was increased when *Phragmites australis* was exposed to higher Cd concentrations suggesting that phytochelatin has a role in heavy metal sequestration (Ederli et al., 2004). As per Ali et al. (2002), these common reeds are a recommended species for phytostabilization. This was based on the fact that the bioconcentration factor of Cu was higher in roots than in shoots. Although *Phragmites australis* (Ederli et al., 2004) is reported to be an ideal species for treating industrial effluent and sewage, it may not be ideally suitable for mine site revegetation programs.

2.3.1.2 Order Fabales

According to Stevens (2006), the order Fabales has four families, namely Fabaceae, Polygalaceae, Quillajaceae, and Surianaceae with 95% of the genus appearing only from the family Fabaceae. Also referred to as Leguminosae, this family includes a significant number of SAA metallophytes that are listed in Table 2.1. They are *Acacia mearnsii*, *Amorpha fruticosa*, *Astragalus bisulcatus*, *Astragalus racemosus*, *Caragana arborescens*, *Crotolatia cobalticola*, *Cytisus striatus*, *Dalea bicolor*, *Lupinus albus*, *Lupinus angustifollus*, *Lupinus hispanicus*, *Meliolotus indica*, *Robinia pseudoaccacia*, *Sesbania drummondii*, *Vicia exaltata* and *Medicago sativa*.

The legume *Medicago sativa* also commonly called Alfalfa has been found to have enhanced uptake of metals such as Pb when grown hydroponically in 100 μM of hormone IAA, and 0.2^{-1} mM of EDTA, which increased the Pb accumulation in leaves by approximately 2,800% (Lopez et al., 2005). This is in comparison with Pb content in leaves of *Medicago sativa* when exposed to a Pb/EDTA combination, resulting in only 600% accumulation compared to exposure with hormone Pb/IAA/EDTA. This result suggests that plants could also increase their metal accumulating potential by hormone feeding and chelation even without genetic manipulation (Lopez et al., 2005).

According to Rajendran & Gunasekaran (2007), *Medicago sativa* was found to accumulate 12,360 μg g^{-1} in the roots and 1920 μg g^{-1} in the shoots when exposed to 50 μg mL^{-1} concentration of cadmium. This species was also used to study the uptake of organic pollutants from soil and to establish the role of plant-soil contaminant interactions (Chekol & Vough, 2001). However, the toxic PAH pollutant pyrene had an inhibitory effect on growth of *Medicago*, including its root (Fan et al., 2008). The effects of toxic pollutant uptake of organic matter content in soils were also tested with *Medicago*. As a result, low levels of pyrene and TNT were recovered from soil having a higher (6.3%) concentration of organic matter compared to a lower organic matter concentration (2.3%) (Chekol & Vough, 2001).

Therefore, organic matter and rhizosphere bacteria have a significant influence on the phytoremediation capacity of leguminous plants such as *Medicago sativa*. However, the advantages of using Leguminosae/Favaceae for phytoremediation include its self-sufficiency for nitrogen, its potential for drought tolerance and its capacity to survive even on infertile soil (Gawronski et al., 2011).

2.4 Advances in remediation science

Highly concentrated contaminants on small area of soils or locked water bodies can adopt conventional or engineered methods of remediation along with phytoremediation technologies. Sometimes, phytoremediation alone may be problematic due to higher bioavailability and deeper root penetration leading to phytoextraction by 'free-metal ion solution' mechanism of absorption of metals (Maddocks et al., 2009).

Therefore, from a phytostabilization point of view, it is a bad idea to let deposition of absorbed metal pollutants on the surface unless plants are harvested and disposed of safely (Singare et al., 2013). Therefore, an alternative option is to adopt a non-biological method in extreme contamination cases (Pulford & Watson, 2003; Pilon-Smits, 2005). For instance, along with long term revegetation programs, short term remedial measures that can be adopted are listed below:

- Hydraulic control to prevent leaching of pollutants (Zhang et al., 2015)
- Chemical treatment by chelation and compartmentalization in roots (Komal et al., 2015)
- Bio-augmentation through introduction of microbes and biostimulation (Chekol & Vough, 2001)
- Attenuation and treatment of contaminated soils by vitrification (Wang et al., 2012)
- Vapour extraction, electro kinetics, soil flushing and slurry-phase bioreactors (Kuppusamy et al., 2016), and
- Combined co-metabolic stripping (Russell, 2012).

However, some of the conventional technologies listed here are harmful to soil microbial diversity and are also costly because they often involve sophisticated machinery (Rajkumar et al., 2012).

2.4.1 Plant biotechnology and tissue culture

Bioremediation in general is a clean-up technology that involves an active role of biotic and abiotic components such as soil, plants and microorganisms in an ecosystem over a polluted site (Fester et al., 2014). Although larger trees such as alder in association with fungus and bacteria have been reported to grow in the SAA zones, it is difficult to actually establish them there (Roy et al., 2007). For example, a 20-year study on a SAA revegetated mine site revealed that plant and microbial diversity was low compared to an adjacent undisturbed site (Mendez & Maier, 2008b).

Moreover, even some fast growing timber trees take 30 to 40 years in a favourable condition (Groninger et al., 2007) which indicates prolonged time periods before achieving phytoremediation as discussed earlier. Therefore, as well as assisting with branching and growth of vegetation, microorganisms and fungi have ability to carry out heavy metal detoxification (Singh & Tripathi, 2007; Cetin et al., 2011). Moreover, inoculating plants with beneficial microorganisms including free living and symbiotic associates is likely to improve remediation results by many folds (Kurek & Majewska, 2012).

There are several types of rhizosphere bacteria that could be integrated with metallophytes to develop potential for phytoremediation (Baunthiyal, 2014). A recombinant gene technology can be effective in improving the quality of microbes that are toxicity resistant and pollutant degrading (De-Bashan et al., 2012). Since approximately 80% of vascular plants have an ability to form mutulastic symbiotic associations with arbuscular mycorrhizal fungi, a screening test for metallophyte identification will help predict the response of plants specifically towards pollutants before spending money and time on a field scale plantation (Isayenkov et al., 2004; Doran, 2009). SAA metallophytes that are compatible to mycorrhiza and rhizobia species are potentially the most sought after plants.

For instance, the gene composition of the microbe friendly Brassicaceae plants *Arabidopsis thaliana* and *Thalaspi caerulescence*, both with 5 chromosomes, is similar to another Brassicaceae *Cardaminopsis halleri* (8 chromosomes), all being coincidently metallophytes (Chiang et al., 2006; Rigola et al., 2006; Dahmani-Muller et al., 2000; Bothe, 2011).

Therefore, engineering heavy metal resistant genes that are compatible with rhizobium and mycorrhizal fungi has a good prospect for developing hybrid pools of such species to give rise to transgenic plants (Lal and Srivastava, 2010). As another example, the tissue culture of transgenic plants has been used to produce *in-vitro* clones of *Populus alba* from the Salicaceae family (Table 2.1) (Niosi & Reid, 2007; Rascio & Navari-Izzo, 2011; di Lonardo et al., 2011).

Moreover, plant tissue culture is also useful to decipher a range of enzymes involved in transformation of some xenobiotic compounds and industrial wastes by amplifying their ability to tolerate, detoxify and store high concentrations of heavy metals (Doran, 2009).

2.4.2 Future of remediation technology

Recently, environmental nanotechnology has been gaining popularity as a clean-up technology (Niosi & Reid, 2007; Baruah & Dutta, 2009; Mura et al., 2013). Nanomaterials are prepared by engineering particles with required optical and electronic properties within a controlled shape and size (Sadik, 2011; Fulekar, 2012). Developing nanomaterials for clean-up technologies involves design, characterization and release into polluted sites (Sadik, 2011). However, it is important to qualitatively determine the effectiveness of nanomaterials since the harsh climatic conditions in SAA environments may not permit nanomaterials to be effective at the field scale.

Although, the costs for production and processing of nanomaterials are high, they have been found to be promising in earlier experimental stages to use on contaminated sites to track the fate, transformation and bioavailability of toxic substances (Fulekar, 2012). If nanotechnology is integrated with plant technology it has the capacity to remediate metals, iron oxides and silicates with the help of microorganisms (Sadik, 2011; Fulekar, 2012).

There has also been an attempt recently to encapsulate toxic substances in soil using nanomaterial-based botanical insecticides (De Olivera et al., 2014) although there are few reported studies regarding eco-nanotechnology involving plants. Wang et al. (2015) attempted to develop a nano-agent called sixthio to chelate heavy metal polluted soils but without any attempt to incorporate roles of plants and microbes. A study using Taunit, containing multi-walled carbon nanotubes (MWNTs) showed an improvement in root growth and peroxidase activity (Smirnova et al., 2011) in plants that adds advantage to produce and identify plants with potential for remediation.

2.5 Conclusion

It is important to understand that a significant number of metallophytes are effectively growing on polluted sites such as abandoned mines under exposure to different biotic and abiotic stresses. An innovation that integrates plant and microbial association to execute rehabilitation of SAA operational and abandoned mines is required. Conceptually, the process of phytoremediation is achieved through revegetation leading to rehabilitation. Moreover, afforestation programs using untested species of plants on a contaminated site cannot be considered as a successful remediation process. The question also arises whether the aim is to merely revegetate polluted sites or to revegetate using native species in a sustainable manner by following patterns of ecological succession.

There is a need to identify groups of pioneer and ideal species that can sustainably stabilize heavy metals avoiding exposure to the food chain. At this point, phytostabilization is a potential technology that awaits a better integrated approach with biotechnology and nanotechnology. The integrated remediation technology (IRT) presented in this paper integrates ecosystem function, plant systematics and gene technology, use of transgenic plants and environmental nanotechnology. However, to achieve remediation goals, phytoremediation technology should also be considered using native species that are drought, salinity and heavy metal tolerant. Ideally these should also be non-allelopathic, non-accumulator, nitrogen fixing and should have a potential for high biomass yield.

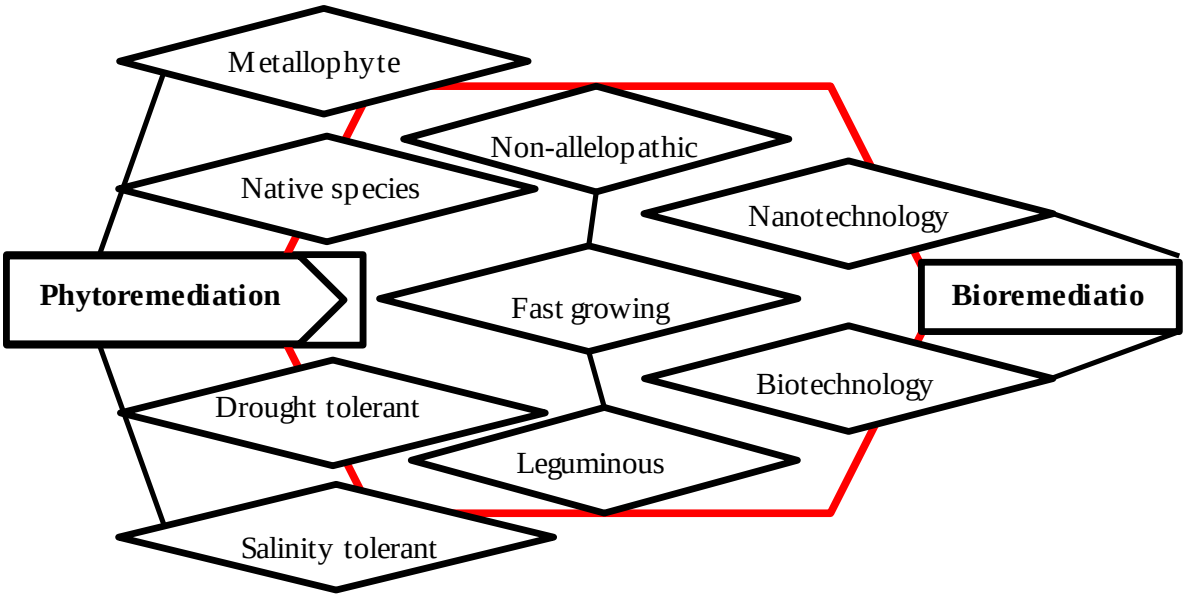


Fig 2.4 Integrated remediation technology (IRT) for revegetation of metal contaminated sites designated to semi-arid and arid (SAA) conditions.

Note: The hexagon in red represents an integrated remediation technology (IRT) concept based on diamond remediation decisions by combination of plants and microbial processes on the opposite sides

Chapter 3

SCREENING OF METAL UPTAKE BY PLANT COLONIZERS GROWING IN KAPUNDA COPPER MINE, SOUTH AUSTRALIA

3.1Introduction

Intensive mining activities worldwide generate major soil contaminants in the form of heavy metals. The soil pollution in Europe is such that 37% of it is contributed by heavy metals produced through industries and related businesses with one of the contaminant metals being copper (Kopittke et al., 2010). Uncontaminated soils are generally expected to have up to 20 mg kg^{-1} of copper (Mackie et al., 2012). However, in the current study area, Guo et al. (2009) found that the copper concentration in soil was far above this threshold value. Avoiding the exposure of such pollutants originating from open pit mines into air and water by degradation has become a global priority (Lande, 1977; Nicholas et al., 2007; Hongbo et al., 2011; Gall et al., 2015). Revegetation or rehabilitation of the disturbed and contaminated land is a long term goal of any industry or government partners. However, everything that is green may not be the process of phytoremediation in a positive perspective. The plants could be excluders, accumulators or stabilizers of the pollutants in the substratum where they are growing (Chambers and Ross, 1998; Mench et al., 2003; Yoon et al., 2006).

The one form of plants called phytoaccumulators is ideal for remediating a small patch of land by harvesting and disposing off the biomass after recovery. However, in vast landscapes like in abandoned open pit mines, the phytostabilizers are better candidates for remediation even though it is very hard for vegetation to grow under unusual edaphic and climatic conditions as in the dry and acidic mine sites (Groninger et al., 2007; Salt & Lindenmayer, 2008). More importantly, such disturbed sites may support a unique vegetation cover of plant colonists among

which there can be metallophytes of special interest to researchers but were never noticed earlier (Batty, 2005; Nirola & Jha, 2011). The present study site is located in semi-arid climate and the plants growing here certainly should have some interesting characters individually to withstand and grow in the harsh edaphic and climatic condition (Mendez and Maier, 2008; Bell, 2001; Mendez & Maier, 2008; Oh et al., 2013; Viehweger 2014). Even those plants growing in abandoned mines do have rhizosphere microbial and micorrhizal activity quite similar to those growing over normal conditions (Guo et al., 2009; Le Houerou, 2000). Particularly, the self-enriching plants popularly known as nitrogen fixers can build plant–microbe (Cetin et al., 2011), microbe–soil (Kurek & Majewska, 2012) and soil–plant (Conesa et al., 2006) functional relationships by establishing well in dry and acidic soils. It therefore requires a further analysis on whether leguminous or non-leguminous plants are better excluders, extractors or stabilizers (McInnes & Brockwell, 2008).

The plants which extract metals to its above ground parts such as stem, branches, leaves, flowers and fruits are rather polluters as they expose metals into the outer environment (Lamb et al., 2010). In the current attempt to characterize some plants, the rate of metal absorption in the plant parts is measured using enrichment factor (EF) which is the quotient of a metal concentration in plant parts and the total soil metal concentration (Sagiroglu et al., 2006; Yoon et al., 2006). Therefore, a plant having EF $\leq$ 1 is effectively an excluder because it restricts metal uptake into the above ground parts (Sagiroglu et al., 2006). These values have also been used earlier for characterizing and identifying metal exclusion and selection of plants for phytostabilization (Sagiroglu et al., 2006; Yoon et al., 2006; Sinha et al., 2007; Nouri et al., 2011; Baker, 1981; Van der Ent et al., 2013). However, the most useful long-term phytostabilizers are thought to be those species which are both excluders and tolerant (Gonzalez & Gonzalez-Chavez, 2006; Hapke, 1996; Nouri et al., 2011).

The present work aims to study the EF of metallophytes that have naturally colonized the area ever since the mine was abandoned. Therefore, the present study is the first attempt to study the status of plant species that are colonizing the abandoned mine site. A vegetation survey, taxonomic identification, analysis of

metal uptake factor and its overall dominance in the area are the parameters that are employed to characterize soil and plants around the mine site.

3.2 Objectives

The overall objective of the experiments reported in this chapter generally examines the abandoned copper mine at Kapunda in South Australia in terms of botanical survey and evaluation of matallophyte plants growing there. The specific objectives of this study include:-

- To classify important plant colonists of abandoned copper mine as accumulators or excluders.
- To determine pioneer plants out of nine vascular plants based upon its response to copper contaminated soil.
- To evaluate the response of rhizosphere soil using enrichment factor values of soils.
- To assess the metal dispersion in the area based upon statistical analysis using principal component analysis.

3.3 Hypothesis

The abandoned mine sites harbor naturally growing metallophytes which among those may be accumulators, stabilizers, exotic or native species.

3.4 Experiments

The major experiments conducted to test the hypothesis and the treatments used in this chapter are listed below.

The major experiments and sequences in chapter 3

Title	Procedure	Analysis
Site survey and visit to light regional council	Survey of landscape, vegetation and safety protocols of mine visits	Sampling of soil and plants. Herbarium preparation for plant identification. Sample processing.
Characterization of Soil and plant samples	Performance of pH, EC, TC, TOC, TC and TN ICP-MS/OES of plant and soil samples	Data recording, entry and statistical analysis
Calculation of metal enrichment factors	Classification of plants based upon metal enrichment analysis	Statistical validation with respect to soil and plant data

3.5 Materials and methods

3.5.1 Site description

The study site Kapunda copper mine (34°20'50.10"S and 138°55'4.78"E) is located 79 km north of Adelaide, South Australia (Figure 1). It was the first and oldest mining town established in Australia in 1842, and was an economic hub of the entire continent until the mine ceased operations in 1879. The mine site area has a semi-arid climate with an annual average rainfall of 493 mm. There is a sparse vegetation cover of endemic, native and exotic species of herbs, shrubs and trees which have structural and floral composition similar to that of Europe and the Mediterranean region (Kloot, 1987).

The dominant species growing all over the mine is the shrub *Acacia pycnantha*. Four site visits were made including during the flowering season to survey and collect plant species. At the abandoned Kapunda Cu mine site, vegetation survey revealed profuse growth in cold and wet August, while in December; after four month lapse of wet season, the site was virtually barren except for perennial bushes and trees as considered in the study. The plants growing around the periphery of the open pit (Mathews Querry) and through 1700 metre downstream consisting of dumps and overburden on both sides of tailing were given consideration.

3.5.2 Sampling and characterization

Sampling locations and the number of plant and soil samples taken are important in making generalizations about abandoned mined lands (AMLs) (Hazelton & Murphy, 2007). In total, 22 locations were chosen for plant and soil sampling using the ecological quadrat method within the distance of 0.1 to 1.7 Km along the slope gradient (Nirola et al., 2011). However, due to the sparse vegetation cover with harsh terrain, only nine sampling plots delivered effective samples with rest of the plots being rejected. Triplicates of each soil and plant samples (roots, stems and leaves) were collected from the periphery of the mine as well as from the tailings and dump areas.

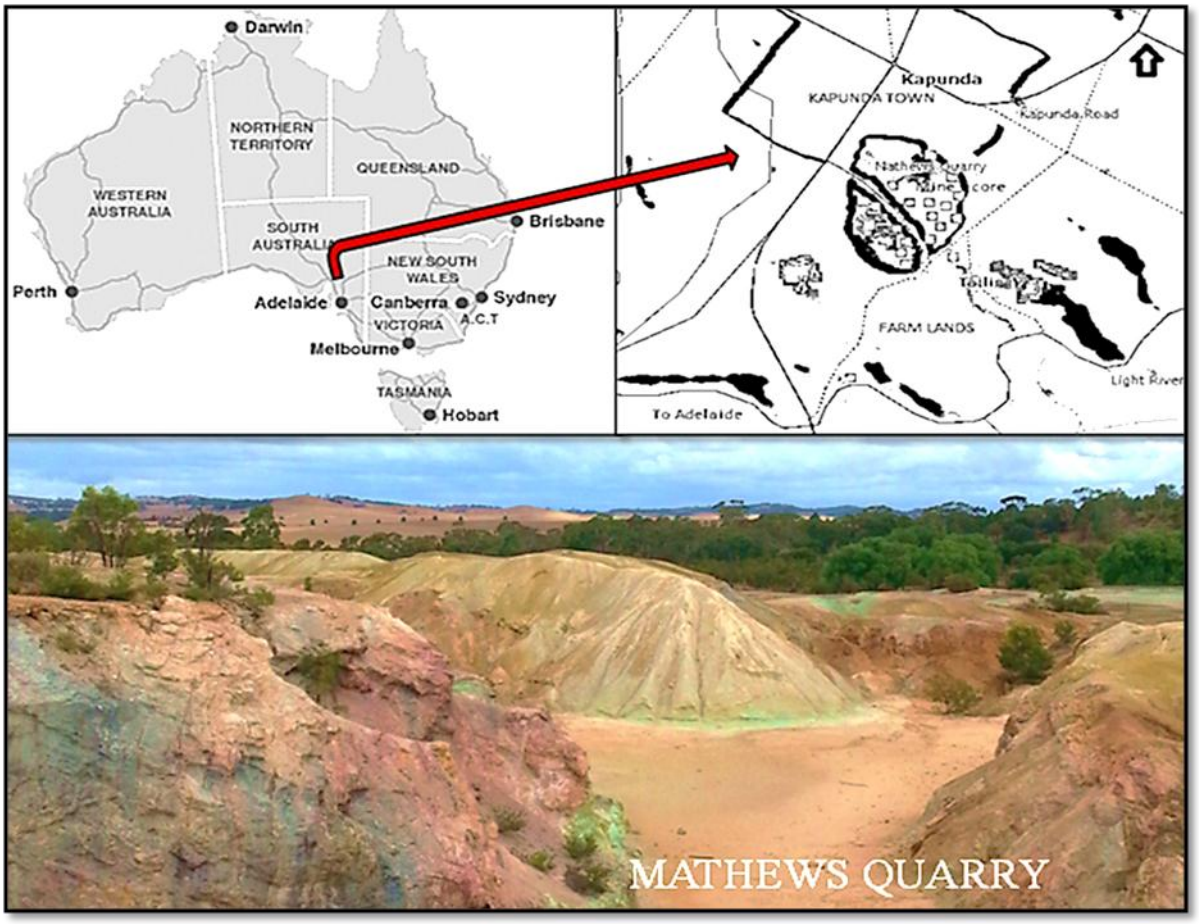


Fig 3.1 Location of Kapunda Copper mine site showing Mathews Quarry

The sampling extended till the origin of tributary of Light River from the core of the main pit called Mathews quarry (Fig. 3.1). Photographs of the plant species were taken and herbarium specimens collected for identification purposes. The soil from the diameter of rhizosphere around each sampled plant were collected and thoroughly homogenized before packing in polypropylene containers. Surface soil samples, collected to a depth of 15 cm were air-dried (25 °C) for 48 h, passed through a 2-mm mesh sieve, and stored in polypropylene containers (Hazelton & Murphy, 2007). Root, stem and leaf plant fractions at specific sampling locations (Table 3.1) were collected, washed three times in deionized water, broken into pieces, oven-dried at 60°C, powdered and then stored in polypropylene containers prior to analysis. The prepared soil and plant samples were characterized following standard

analytical methods. Soil pH and electronic conductivity (EC) were determined in a 1:5 (w/v) soil: water slurry. Total dissolved carbon (TC), total dissolved nitrogen (TN) and total organic carbon (TOC) were estimated by TOC-L Shimadzu (Japan). Total carbon (C %) and total nitrogen (N %) were assessed using a Trumac (Leco® Corporation, Michigan, USA) Carbon-Nitrogen-Sulphur analyzer.

Concentration of metals in *aqua-regia* digested soil and plant samples in microwave digester (Photo plate II) were determined by Inductively-coupled Plasma Optical Emission Spectrophotometry (ICP-OES) using Perkin Elmer Optima 5300V, and by Inductively-coupled Plasma Mass Spectrometry (ICP-MS, Agilent 7500ce, Agilent Technologies, Tokyo, Japan) as outlined in USEPA 3015a and 6020a methods, respectively (http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs). Since the study site was a derelict Cu mine, the sample analysis focused on potentially toxic heavy metals, namely Cu, Cd, Zn, and Pb in relation to an abundance of Acacia in study site considered to be one of the metal resilient species in previous laboratory toxicity study (Lamb et al. 2010).

Table 3.1 Plant colonists at the Kapunda mine site

ID	Distance (Km)	Taxonomic name and authority	Common name
Ap	**0.1**	**Family:Fabaceae** *Acacia pycnantha* Benth. syn. *A. petiolaris* Lehm. *Racosperma pycnanthum* (Benth) Pedley.	Golden wattle, Broad-leaved wattle
Ec	**1.5**	**Family: Myrtaceae** *Eucalyptus camaldulensis* Dehnh.	River red gum, Murray red gum
Rc	**1.7**	**Family: Rosaceae** *Rosa canina* L. Syn. *R. heterostyla* Chrshan.	Dog rose
Lf	**1.5**	**Family: Solanaceae** *Lycium ferocissimum* Miers. Syn. *L. chinense* Mill.	African boxthorn
Oe	**0.3**	**Family:Oleaceae** *Olea europaea* subsp. *africana* L. (Miller)	African olive, Wild olive
Aa	**0.15**	**Family: Fabaceae** *Acacia acinacea* Lindl. Syn. *A. rotundifolia* Hook *Racosperma acinaceum* (Lindl.) Pedley	Gold dust wattle, Round-leaved wattle
Sm	**0.7**	**Family: Anacardiaceae** *Schinus molle* var. *areira* (L.) DC Syn. *S. areira* L.	Peruvian pepper
Ll	**0.8**	**Family:Myrtaceae** *Leptospermum lanigerum* (Sol. ex Aiton) Sm. Syn. *Leptospermum trinerve* Sm.	Woolly tea-tree
Ph	**0.15**	**Family: Pinaceae** *Pinus halepensis* Mill. Syn. *P. ceciliae* Llorens & L.Llorens	Aleppo pine

(Synonyms: www.rbgsyd.nsw.gov.au, www.anbg.gov.au , www.iucnredlist.org)

3.5.3 Data treatment

The enrichment factor (EF), a crude measure of metal accumulation in aerial plant parts, was used to assess the uptake properties of plant colonists (Sagiroglu et al. 2006; Sinha et al. 2007; Nouri et al. 2011).

The EF was calculated as follows:

$$EF = \frac{\text{metal concentration in shoot dry matter (MAGP)}}{\text{total metal concentration in soil (MS)}}$$

Where,

MAGP is the metal in the above ground parts and
MS is the soil metal concentration.

All plant and soil analytical data were subjected to statistical analysis employing principal component analysis (PCA), and Pearson's correlation tests using Microsoft Excel and IBM®SPSS®21 for Windows. Bivariate correlations (r^2) were done between plant species and soil parameters, and metal concentrations in plants and soils against soil properties. The PCA served to assess the relationships between phytoextraction, soil physical properties and soil metal concentration.

3.6 Result

3.6.1 Soil characters and metal uptake

In this study, a total of the nine plant colonists involving a wider range of seven families namely Anacardiaceae, Fabaceae, Myrtaceae, Oleaceae, Pinaceae, Rosaceae and Solanaceae were considered covering most of the mine site. The Kapunda Cu mine site as per hydrometer reading reveals sandy soil (87%) along the open pit.

However, the soil at 1.7 Km distances from the Mathews Quarry reveals 27% clay in the soil. The study site has an average pH ranging from 3.9-8.4 with a mean pH 6.6 (± 0.5) and electrical conductivity of 152-7311 µS with a mean EC 1569 (±802) suggesting that the plant colonists can tolerate a wide range of substrate conditions (Whiting et al., 2004). The total carbon (TC) and total nitrogen (TN) in soil samples ranged from 18.3-371.2 and 4.8-412.4 mg kg^{-1}, respectively (Table 3.2). The range of total organic carbon (TOC) in the entire soil sample was 14.0- 202.6 mg kg^{-1}, which represents a wider range of soil carbon content as evident in the mine soil (Li, 2006).

Table 3.2 Physico-chemical characteristics of the soil samples. (Sample ID corresponds to the name of plants as a spot from where the plants were collected)

Soil Sample ID	**Soil characteristics**				
	pH	**EC (µS)**	**TC (mg kg^{-1})**	**TN (mg kg^{-1})**	**TOC (mg kg^{-1})**
Ap	6.5	436	244	14	164
Ec	7.6	7311	203	36	146
Rc	8.4	356	371	26	203
Lf	8	231	105	5.6	56
Oe	6.9	205	254	22	176
Aa	5.3	1424	67	46	64
Sm	5	506	47	5.6	39
Ll	3.9	3500	48	412	44
Ph	7.3	152	18	4.8	14
Mean ±S.E.	6.6 ± 0.5	1569 ± 802	149 ± 41	64 ± 44	101 ± 24

According to our result, the plant *L. lanigerium* was growing in the most acidic condition whereas *E. camaldulensis* was found to tolerate salt growing in a saline condition of electrical conductivity of 7311 µS. The nitrogen content of soil where *P. halepansis* grew had a poor nitrogen condition of 4.8 mg kg^{1}.

The soil Cu concentration ranged from 65-9941 mg kg^{-1} with an average of 4201 mg kg^{-1} (Table 3.3) which is very close to the average value 3822 mg kg^{-1} reported by Guo et al. (2009) in the same location. The other metals, specifically Zn, Cd and Pb, measured in soil samples were in the range of 7-124, BDL- 19.0 and 2.5-126.0 mg kg^{-1} respectively. The enrichment factor (EF) has been calculated using the data of Table 3.3 as presented in the discussion section of this paper (Fig 3.2).

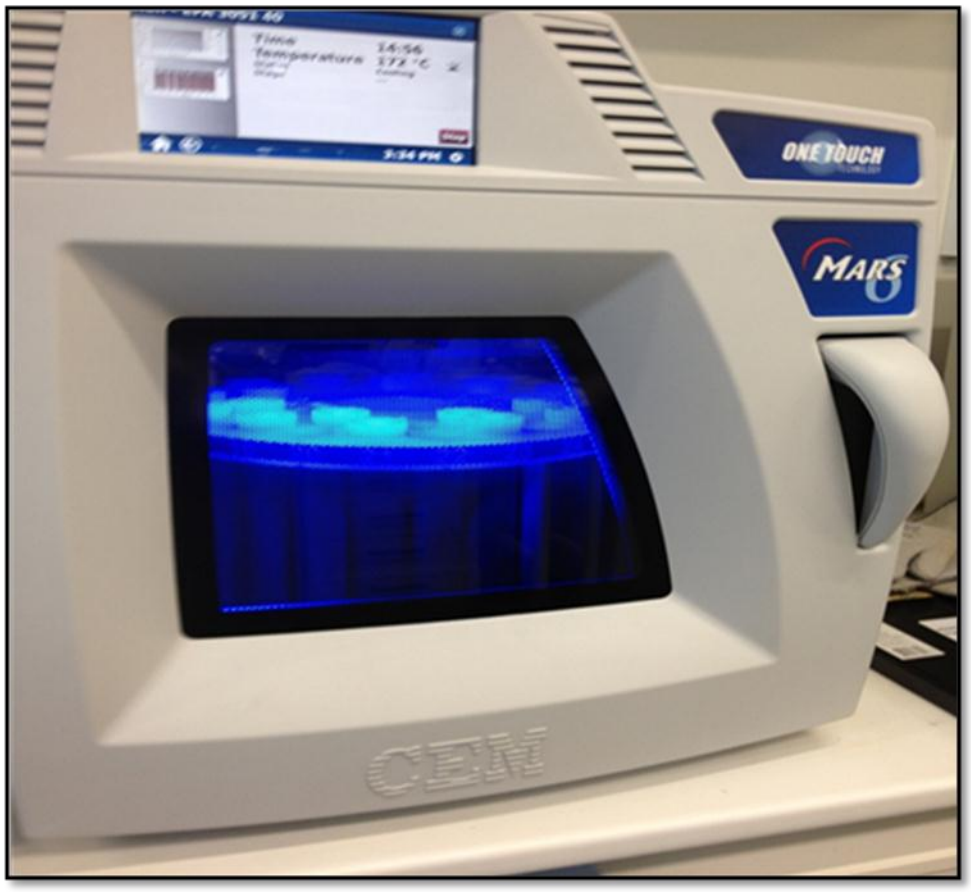


Photo plate II- Microwave digester under operation

Table 3.3 Total metal concentration (mg kg^{-1}) in soil and above ground plant samples. (Sample ID corresponds to the name of plants as a spot from where the plants were collected)

Sample ID	Sample	Metal concentration (mg kg^{-1}) ±S.E.			
		Cu	**Zn**	**Cd**	**Pb**
Ap	Soil	5047 ± 954	77 ± 13	4 ± 0.9	21 ± 4
	Plant	121 ± 24.5	69 ± 15	1.8 ± 0.1	0.6 ± 0.1
Ec	Soil	65 ± 23	59 ± 15	5 ± 2	11 ± 3
	Plant	137 ± 31	99 ± 5	0.9 ± 0.1	14 ± 4
Rc	Soil	1784 ± 695	124 ± 49	3 ± 0.9	37 ± 0.5
	Plant	105 ± 1.5	67 ± 0.6	<0.01	<0.01
Lf	Soil	9941 ± 1039	115 ± 7.1	1 ± 0.04	78 ± 34
	Plant	264 ± 5	81 ± 0.7	0.1 ± 0.2	18 ± 3
Oe	Soil	9125 ± 816	115 ± 19	0.1 ± 0.1	126 ± 13
	Plant	57 ± 3	29 ± 0.9	<0.01	0.2 ± 0.05
Aa	Soil	6773 ± 935	18 ± 0.9	0.5 ± 0.2	9 ± 2
	Plant	36 ± 17	22 ± 3	0.6 ± 0.01	1 ± 0.2
Sm	Soil	1922 ± 108	24 ± 2	BDL	11 ± 0.8
	Plant	15 ± 1	38 ± 2	0.06 ± 0.0	BDL
Ll	Soil	2480 ± 24	27 ± 0.2	0.9 ± 0.2	21 ± 0.2
	Plant	23 ± 2	11 ± 1	1 ± 0.4	0.5 ± 0.4
Ph	Soil	692 ± 19	7 ± 0.4	19 ± 3	2.5 ± 0.06
	Plant	14 ± 5	15 ± 5	13 ± 3	0.5 ± 0.04

3.6.2 Statistical interpretations

Pearson correlation tests between copper in plant species and the soil metal including soil TN and TC indicate a positive correlation for *E. camaldulensis* with r^2 values of 0.81 ($P \leq 0.05$) and 0.74 ($P \leq 0.01$), respectively. However, with the exception of *E. camaldulensis*, soil Pb concentration positively correlated (r^2 0.97; $P \leq 0.01$) for *A. pycnantha*, (r^2 0.96; $P \leq 0.01$) for *R. canina*, (r^2 0.97; $P \leq 0.01$) for *L. ferocissimum*, (r^2 0.97; $P \leq 0.01$) for *O. europaea*, (r^2 0.97; $P \leq 0.01$) for *A. acinacea*, (r^2 0.97; $P \leq 0.01$) for *S. molle*, (r^2 0.97; $P \leq 0.01$) for *L. lanigerum* and (r^2 0.96; $P \leq 0.01$) for *P. halepensis* (Table 3.4).

The Principal component analysis result is presented in Table 3.5 that discusses whether it is plants themselves or the physical agents that are primarily responsible for metal dispersion in the mine site conditions. The metal heterogeneity in component I soil indicates least value of 0.54 factor loadings for Cu.

Table 3.4 Correlation coefficient (r^2) for copper concentrations in above ground plant tissues of samples with soil metal and physical characteristics. (Sample ID corresponds to the name of plants as a spot from where the plants were collected)

Sample ID	Soil Cu	Soil Pb	Soil TN	Soil TC
Ap	0.5	0.97**	0.45	0.49
Ec	0.05	0.2	0.81**	0.74*
Rc	0.51	0.96**	0.48	0.51
Lf	0.5	0.97**	0.46	0.50
Oe	0.5	0.97**	0.44	0.48
Aa	0.5	0.97**	0.45	0.49
Sm	0.5	0.97**	0.45	0.49
Ll	0.5	0.97**	0.45	0.49
Ph	0.5	0.96**	0.46	0.5

**Significant at p< 0.05 level; **Significant at p< 0.01 level*

3.7 Discussions

The pH varied greatly over the sampled area which impacts greatly on metal bioavailability to plant in the substratum (Adriano, 2001; Moreno-Jimenez et al., 2009). This wide range of metal concentration supports the very heterogeneous nature of the surface materials at this site (Smolders and Mertens, 1995; Young, 2013). One of the most outstanding results is evident with Cu concentration in roots of *E. camaldulensis* which ranged from 92.8-185.7 mg kg^{-1}, a value much higher for mine site than that reported by Reichman et al. (2006) in laboratory solution culture. Thus, the relative abundance of Cu in plant including the root and stem compared to soil was higher in *Eucalyptus camaldulensis* due to its wider area of root coverage over the copper mine dumps in tailings area.

The EF data as presented in Fig. 3.2 reveal that *P. halepensis* is a weak accumulator of Zn, whereas *E. camaldulensis* shows better uptake ability of Cu, Zn, Cd and Pb. This is a finding warrants us to considered to be a potential phytoextractor (Meeinkuirt et al. 2012; Mok et al., 2013). The higher values of EF that is observed in *A. acinacea* (Zn, Cd), *S. molle* (Zn), *L. lanigerum* (Cd) and *P. halepensis* (Zn) are comparable to those reported for native flora at one mine site in Spain (Moreno-Jimenez et al., 2009). The EF data further indicates that the non-native tree *O. europaea* is a good phytostabilizer followed by *R. canina, A. pycnantha* and *L. ferocissimum* which could prove to be effective in restoring sites formerly mined for Cu, Zn, Cd and Pb. A similar result has been observed by Ye et al. (2009) at copper enriched sites along the River Yangtse, China where the concentrations of Cu in roots were higher compared to shoots for all species sampled. However, the Cu enrichment in the above ground parts of *E. camaldulensis* was higher compared to soil copper concentration. Therefore, our initial study warrants a more thorough categorization of the species that grow around the abandoned mine to identify those that could serve for phytoremediation in any rehabilitation program (Nawab et al. 2015).

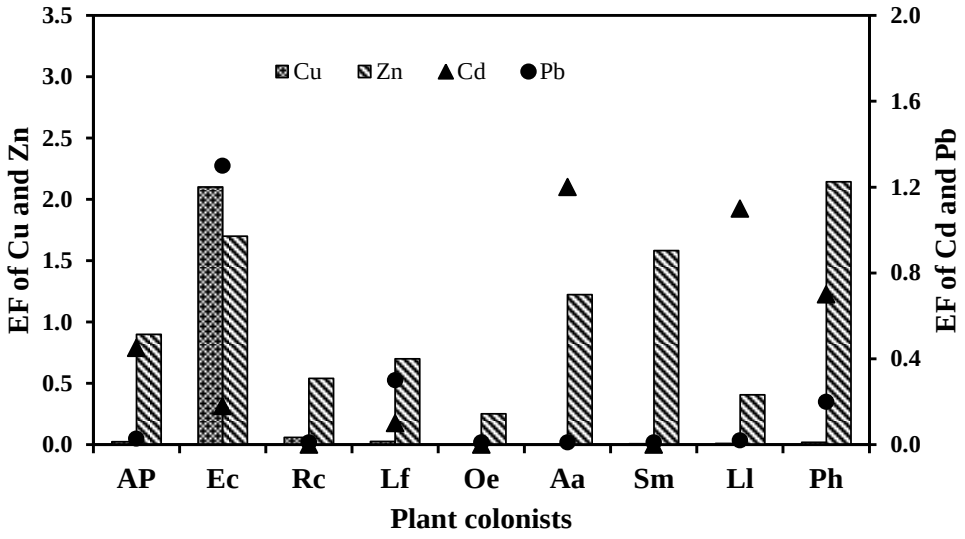


Fig 3.2 Enrichment factor (EF) for Cu, Cd, Zn and Pb of the plant colonists.

Note: The EF of Cu for Ec is high compared to the other species

The outcomes from the study need to be treated with caution as *O. europaea* although show better potential as a phytostabilizer, it is an exotic agronomic plant (Olive) with a risk of metal contamination of the food chain if cropped. The dog rose called *Rosa canina* is an invasive weed, discouraged in many places including South Australia (www.light.sa.gov.au). In the present context, the most successful species surviving the harshness of this arid mine site is *Acacia pycnantha,* the native Australian leguminous plant. This species produces a larger biomass and canopy cover and has deeper root penetration than the other legume studied here, *A. acinacea,* also considered in the present investigation (McInnes and Brockwell, 2008).

Considering the potential of phytostabilization of vast mined lands to sequester toxic metals in the rhizosphere zone of the vegetation standing there, the lack of vegetation often is another reason of erosion. During the dry season, wind potentially

erodes the surface soil layers containing toxic metals to deposit on the surrounding farmlands and in water bodies to alter the normal chemical property of soil and water (Alloway G., 2013). The dispersion of heavy metals from Kapunda is also aggravated by the effects of rain during wet season and the wind during dry season. The principal component analysis (PCA) using metal concentrations in plants and in soils together with soil parameters provide an insight into the degree of heterogeneity of heavy metals in AMLs. This analysis along with EF has further aided to roughly characterize plant being either a hyperaccumulator or a stabilizer (Prasad et al., 2003).

From the analysis of the present dataset - component I, the metal heterogeneity in soil indicate factor loadings of 0.54, 0.95 and 0.98 for Cu, Zn and Pb, respectively, and 0.53, 0.61, 0.61 and 0.66 for TC, TOC, TN and TC, respectively, all at 44.8% total variance (Table 3.5).

Table 3.5 Principal components analysis (PCA)* of metal concentrations in plant and soil samples and soil characteristics with values of factor loadings ≤ 0.5 omitted

Component	**Plant concentration**				**Soil concentration**				**Soil characteristics**			
	Cu	Zn	Cd	Pb	Cu	Zn	Cd	Pb	pH	EC	TC	TOC
I (44.80%)	-	-	-	-	0.54	0.65	-	0.98	-	-	0.53	0.61
II (27.52%)	0.67	0.97	0.90	0.83	-	-	0.98	-	0.68	0.5	0.57	0.54

Component II of the PCA accounts for 27.5% total variance with factor loadings of 0.98 for soil Cd, and 0.67, 0.97, 0.90 and 0.83 for plant Cu, Zn, Cd and Pb, respectively. Factor loadings for soil parameters are 0.68, 0.5, 0.57, and 0.54 for pH, electrical conductivity, TC, TOC, respectively. These values of factor loadings for Zn and Cd in plants and soil pH in Component II of the PCA match the PCA values reported from an abandoned mine in Spain by Moreno-Jimenez et al. (2009).

Relating PCA to EF, the value of Zn (factor loading 0.98) is also high in *E. camaldulensis*, *A. acinacea*, *S. molle*, *P. halipensis* and of Cd (factor loadings 0.97) in *A. acinacea* and *L. lenigerium* also indicating the role of these plants to spread accumulated metals around the surface through its decaying leaf litter. However, the plant *R. canina* is least involved in accumulating Cu, Zn, Cd and Pb for dispersion even though it is a weed that spreads faster (Nestorovic & Konstantinovic; 2011) and can colonize to hold the soil tight but is not an ideal stabilizer for heavy metal like copper (Hodge & Harmer, 1996).

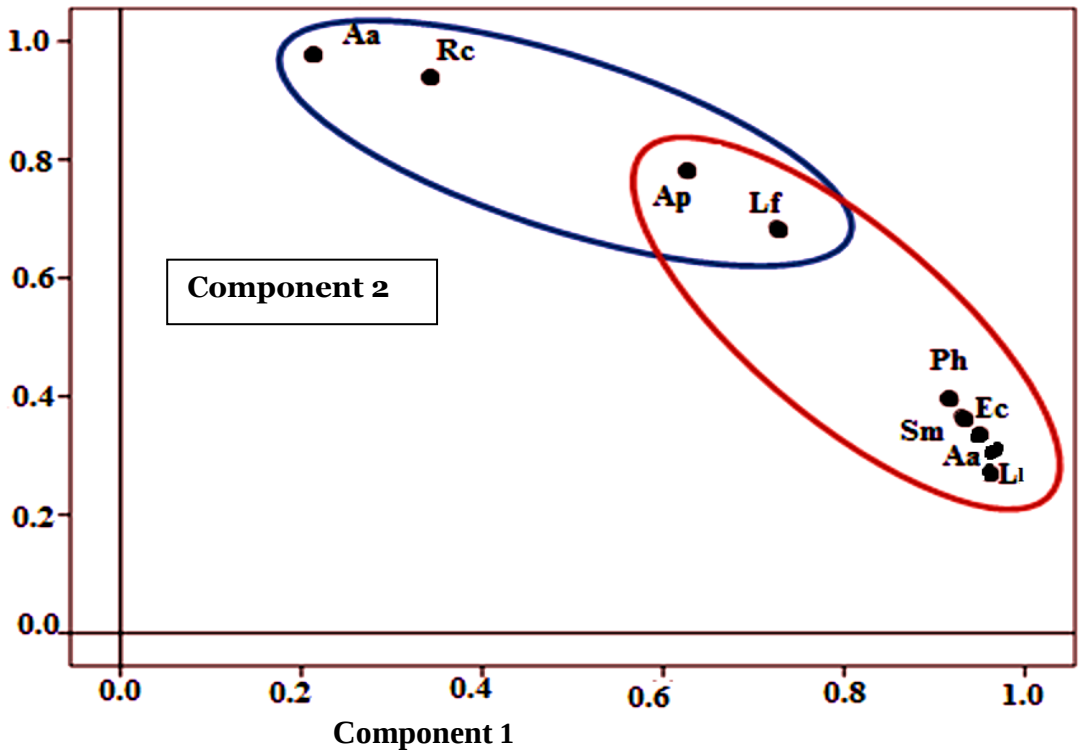


Fig 3.3 The component plots in rotated spaces showing three groups of metallophytes with similar adaptation based on soil character

Further to the PCA analysis, African boxthorn (*Lycium ferocissimum*) and Golden wattle (*Acacia pycnantha*) demonstrate common plots in rotated spaces against the

rest of the species by meeting at the diagonal point between component 1 and component 2 (Fig. 3.3). In essence, the r^2 value of Lf and Ap against copper, lead, TN and TC of soil is similar with Zn- EF response. The Lf is more of a culinary plant used in China and in many other places as a source of food. Therefore, this plant may not be suitable to grow in metal contaminated land like *Acacia pycnantha* (Randall R E., 1992).

3.8 Conclusion

We report an initial attempt to study the existing vegetation and their response to metal enrichment at an abandoned copper mine. Of the nine species studied, five species (*L. lanigerum, A. acinacea*, *S. molle*, *P. halepensis* and *E. camaldulensis*) do show uptake of one or more of these heavy metals. Rest of the species namely *O. europaea, L. ferocissimum, R. canina* and *A. pycnantha* are identified as efficient phytostabilizers for the four heavy metals, in that order. However, *A. pycnantha* (the native Australian legume) is found exceptional as a phytostabilizer for heavy metals like Cu, Zn, Cd and Pb with its better EF value and PCA ratings.

Chapter 4

EVALUATION OF METAL UPTAKE FACTORS OF *ACACIA PYCNANTHA* AND *EUCALYPTUS CAMALDULENSIS* AT A COPPER MINE IN-SITU SOIL

4.1 Introduction

We consider two native tree species growing naturally over many years in mine site as per our earlier study (Nirola et al., 2015). The leguminous plant *Acacia pycnantha* Benth. (golden wattle), and non-leguminous *Eucalyptus camaldulensis* Dehnh. (river red-gum) were included with an aim to find answers to the issues of bioavailability, bio-accumulation and biotransformation of metals. The Australian legume *A. pycnantha* (Ap) has attributes of root nodule formation and an active microbial activity, and give upper hand in growing resilient at the AML. Our earlier survey and screening at the mine site also indicated more Cu accumulation in the root zone for this species (Nirola et al., 2015). Further evidence for such resilience by legume Acacia has been documented for sites in the Sahara Desert (Brockwell et al., 2005).

An estimated annual quantity of nitrogen fixed worldwide by legumes is 70-100 million tonnes with acacias fixing a substantial amount growing in around 5 million hectares of land worldwide (Brockwell et al., 2005). In terms of its drought tolerance mechanism, acacias have a morphological advantage to adapt to arid or xeric conditions including AMLs (Brodribb & Hill, 1993). Their leaves are modified into phyllodes with an abundance of thick sclerenchyma in the outer palisade mesophyll aiding its ability to survive drought conditions (Boughton, 1986; Midgley & Turnbull, 2003). The other native Australian species, *E. camaldulensis* (Ec) is widely distributed and exhibits a substantial drought tolerance capacity which is attributed to deep roots and tough leaf cuticles as seen through the Environment Scanning Electron Microscope (ESEM) image (Boland et al., 2006).

However, most of the species of eucalyptus including *E. camaldulensis* have indicated an allelopathic effect on other vegetation, hampering the germination of seed (Ahmed et al., 2008; Khan et al., 2008; Yang et al., 2009). In the current study site both of these species were found to be dominating the different landscapes of the abandoned Kapunda copper mine. A curious thought to evaluate two taxonomically different plants is expected to add impetus to the issues of mine site rehabilitation with respect to avoiding phytoextraction and encouraging phytostabilization. Moreover, no earlier studies on metal uptake factors are available for widely available *A. pycnantha* in mine site rehabilitation.

The threshold references used for metals in the surface soils are Cd-3 mg kg^{-1}, Cu-60 mg kg^{-1}, Pb-300 mg kg^{-1}, and Zn-200 mg kg^{-1} with a level above 20 mg kg^{-1} for Cd and 300 mg kg^{-1} for Pb considered to be a health hazard (Ash & Truong, 2003). The threshold reference values are important indicators to determine soil pollution level as these values help to measure phytoremediation degree before and after the process. The accumulation of metals in the foliar region could be severe due to the reoccurring of soil pollution by metal accumulation in litter of mine site plants (Nouri et al., 2011). In a study of plant behaviour with soil heavy metals in Turkey, species such as *Euphorbia macroclada*, *Verbascum cheiranthifolium* and *Astragalus gummifer* were found to accumulate 3 to 4 times higher levels of heavy metals than those found in the soil (Sagiroglu et al. 2006).

In another study, Reichman et al., 2006 assert that plant soil interaction explores its capacity to stabilise the heavy metal (HM) using native plants in AMLs. Of all the available rehabilitation technologies, phytostabilisation is a plant's ability to avoid exposure of pollutants to the environment by not translocating metals from soil to the above ground parts (Kurek & Majewska, 2012; Yoon et al. 2006). This is to contain heavy metal pollution by immobilizing the bioavailable components in the earth's biosphere through revegetation of operational, derelict and abandoned mine sites. One analysis of metal toxicity on copper in an abandoned mine in Florida (Yoon et al., 2006) reported the native grass species *Gentiana pennelliana* that

could stabilise heavy metals such as Pb, Cu and Zn based on translocation factor (TF) study.

Therefore, it has been established that species of plants having high metal concentration ratio (≥ 1) of roots to soil is called bioconcentration factor (BCF), a low shoots to roots metal concentration ratio (≤ 1) known as the translocation factor (TF) and ≤ 1 ratio of metal content in above ground plant parts over metal content in soil known as enrichment factor (EF) is considered to be ideal HM stabilizers (Sagiroglu et al., 2006; Sinha et al., 2007). Therefore, in the present context of the use of plant species for mine site rehabilitation, phytoextraction or phytoaccumulation is effectively based on EF since the metal accumulation in above ground parts is exposed to biosphere (Baker, 1981).

4.2 Objectives

The effect of heavy metal toxicity on biota is severe and there is an urgency to control the level of toxicity and impact on public health, particularly from abandoned mine lands (AMLs). Ecological impacts in places such as AMLs can be controlled effectively by evaluating the accumulators against the non-accumulator plants prior to the phytoremediation processes (Ma et al., 2013). A right rehabilitation practice is certainly a tool to control the heavy metal pollution and such practices have been known through the use of phytoaccumulators (Ali et al. 2013).

The phytoremediation technology is employed over polluted sites and have served to be a boon to assist in pollution clean-up efforts (Nirola et al., 2015). As such, copper in particular is one of those toxic metals that have a capacity to bind to albumen and other small molecules in the human body as a 'free copper' to cause nerve damage (Brewer, 2010). An effective phytoremediation would have saved the inhabitants near a mine site in Mexico from elevated levels of lead and arsenic in their blood, and are more severe in children (Gonzalez & Gonzalez -Chavez, 2006). Identification of suitable remediating species of plants could reduce the ecological risks of heavy

metals such as Cu from contaminating water bodies and damage gills causing premature death of fishes Couillard et al., 2008). It is thought to be an important component for identifying the species positively responsible for bioaccumulation of pollutants being transferred from plants into the food chain (Krumins et al., 2015).

A typical practice during industrial revolution was to abandon mines once mineral extraction was conducted. The estimated number of abandoned mines in the developed nations was 6,30,309 by the year 2000 (Van Zyl et al., 2002). Wait (2012) reports 5858 derelict and ownerless mines in South Africa, and Mackasey (2004) records 5500 derelict (abandoned) mines in Japan alone. There are about 1800 final mine voids and 150 operational open cut mines in Western Australia (Doupe & Lymbery, 2005). Moreover, approximately 2000 derelict mine site exists only in New South Wales, Australia that date back to the mid-1800s (Grant et al. 2002). However, the exact figure of abandoned and post operational mine differs depending on the definition of 'what a mine site is'.

The overall objectives of the experiment reported in this chapter were to evaluate the interaction of two native species of wider anatomical and taxonomical deviation. The tree species were stabilizer native leguminous *Acacia pycnantha* and native non leguminous phyto extractor tree *Eucalyptus camaldulensis*.

- To assess heavy metal accumulation in roots, stems and leaves heavy metal for determining the bioconcentration factor (BCF) fnrichment factor (EF) and translocation factor (TF).
- To validate the metal uptake factor using the anatomical study and tissue concentration of elements with the help of environment scanning electron microscope (ESEM)
- To examine the effect of soil factors in the metal concentration in plant parts
- To validate species suitability for phytoabsorption and phytostabilization for the purpose of revegetation of mine sites.

4.3 Hypothesis

Leguminous species are good nitrogen fixers and are self-nourishing to grow on infertile soil conditions. However some native species like *Eucalyptus camaldulensis* are also found very well adapted in abandoned mine soils. *Acacia pycnantha* is more resilient species for phytostabilization of heavy metal contaminated soils in mine sites needs validation.

4.4 Experiments

Fresh samples of leaves of *Acacia pycnantha* and *Eucalyptus camaldulensis* were collected for ESEM analysis. The rest of the experiments to investigate the hypothesis were done as per the procedure listed earlier in section 3.4 (Photo plate-IV)

4.5 Materials and methods

4.5.1 Site description

The abandoned mine at Kapunda is one of the oldest settlements in Australia that mined copper till 1879 AD. The mine site is located 79 km from Adelaide city. The site harbours arid to semi-arid type of vegetation with a calcareous soil. A thorough screening of nine prominent shrub and tree species were undertaken to check their metal enrichment factors namely *A. pycnantha*, *E. camaldulensis*, *Rosa canina*, *Lycium ferocissimum*, *Olea europaea*, *Acacia acinacea*, *Schinus molle*, *Leptospermum lanigerum*, and *Pinus halepensis* (Nirola et al., 2015). Among the nine species, the two species emerged as an outstanding metallophytes growing in the abandoned mine site. The golden wattle (Ap) was found growing around the

periphery of the open cut area whereas the river red gum (Ec) was mostly concentrated towards the clayey soil downstream.

Currently, the local Light Regional Council is responsible for managing the area as a heritage site to control pollution and to promote tourism (http://www.light.sa.gov.au). The master plan of council categorizes the Kapunda mine site into cultural landscape (C), geological landscape (G) and environmental (regenerative) landscape (E) within the area of approximately 2 km (Fig 4.1). Drainage from the site originates mainly from regenerative landscape and flows into the Light River. The currently operated heritage mine track runs about 1.5 km mainly through the geological landscape (G). The geological landscape is a barren ground, fenced as a safety measure to avoid human intrusion. Fresh leaf, stem and roots of Ap and Ec including the corresponding soils were collected from G and E zones, respectively. Some of the sites having the unconfirmed indications of earlier land refilling by off-site soils were avoided.

4.5.2 Sampling and processing

The sampling plots were modelled to extend a maximum coverage (ISO, 2002; Hazelton & Murphy, 2007), starting from the geological landscape (G) towards the environmental landscape (E). Altogether each of 16 *Acacia pycnantha* and *Eucalyptus camaldulensis* plots were sampled including the associated soils as per the ecological quadrat principles (Krebs, 1999). The soil samples were collected from the rhizosphere at 15-20 cm depth using a plastic trowel from two points around the plant diameter of 2 m and homogenized. Plant parts were collected from around the canopy and bulked to form a composite sample to maintain a uniform representation. Fresh leaf samples were collected in an ice filled thermo-col box (5 ^{0}C) for ESEM studies to be analysed within 24 h. Inductively Coupled Plasma Mass Spectrometer/Optical Emission Spectrometer (ICP-MS/OES) to determine the total metal concentration in plant and soil samples were employed (Thomas, 2013).

The plant samples consisting of roots, stems and leaves for ICP-MS analysis were

collected in the polypropylene containers and stored in laboratory at room temperature. The plant samples were washed three times in deionised water and oven dried at 60 ^{o}C. The oven-dried plant samples for ICP-MS analysis were ground separately using an electric stainless steel grinder and were stored in the polypropylene containers. The soil samples were air-dried and sieved using a 2 mm plastic mesh sieve and stored in polypropylene containers for ICP-OES analysis.

4.5.3 Sample characterization

Soil characterization included texture analysis using hydrometer method; standard tests were employed to measure pH, electrical conductivity, total dissolved carbon (TC), total dissolved nitrogen (TN) and total organic carbon (TOC) (Mendez et al. 2012). The potentio-metric measurement of the supernatant suspension of soil: deionised water (w/v) at 1:5 ratio was used to measure the pH and EC (Sposito, 2008). A 5 g soil was separately oven dried to compare and record the moisture content of the soil.

A microwave accelerated reaction system (CEM-MARS X®) served to digest metals in *aqua-regia* for soils and HNO_3 for plants separately following USEPA method 3015a (Agazzi & Pirola, 2000). The digested samples were diluted up to 50 ml using deionised water and were passed through a 0.45 μm filter using a syringe. The final test samples of 10 ml diluted suspension were put into ICP tubes for analysis in the Agilent 7500c (Agilent Technologies, Tokyo, Japan) (Photo plate-III). The TC, TN and TOC were measured using air-dried, 2 mm mesh sieved soil dissolved in 1:5 (w/v) deionised water (You et al., 2015). The soil solutions were agitated in end-over shaker overnight and were centrifuged at 4000 rpm for 10 min. The supernatant was analysed in the Formacs^{series} total organic carbon, total nitrogen analyser using the UV-promoted persulphate digestion methodology.

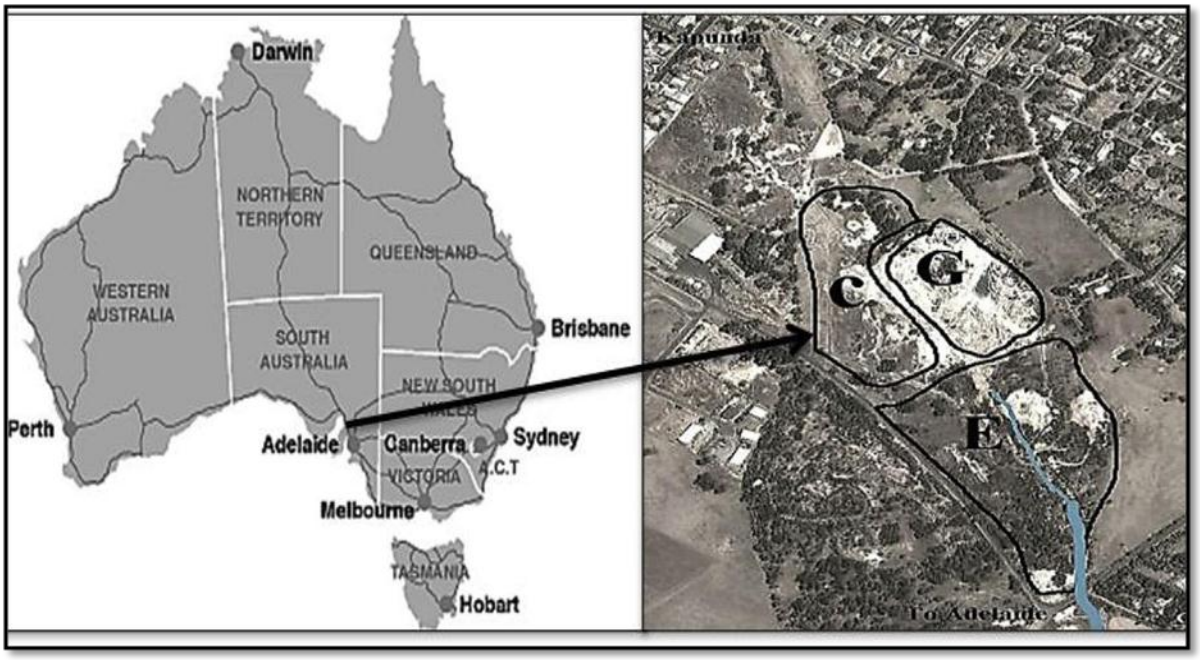


Fig 4.1 Site map of abandoned copper mine at Kapunda in South Australia.

Note: *The letter 'C' represents Conservation zone, 'G' represents the Geological zone and 'E' represents the Environmental zone*

Analyses of total C (%) and N (%) were done by introducing 0.5 g of air-dried 2 mm mesh sieved and ground sample in a Trumac carbon-nitrogen-sulphur (CNS) analyser (Leco® Cor-poration, Michigan, USA). The standard blank, controlled and cross-referencing samples were introduced to maintain quality control after every 10 readings. The pH, EC, moisture content (H_2O %), dissolved TC (mg kg_{-1}), dissolved TN (mg kg^{-1}), dissolved TOC (mg kg^{-1}), total N % and total C % were recorded to average the duplicates of each sample.

4.5.4 Spectroscopic characterization of plant matter

The bioavailability of heavy metals like copper is based on the influence of soil physical factors (Cerqueira et al., 2015). The localisation of the elemental distribution in the leaf sample by ESEM is another appropriate method to track the fate and status of accumulated metals in plant tissues (Stokes et al., 2013). The process for characterization began by selecting and washing ice preserved leaves followed by air drying. Leaf samples were sliced using a surgical blade into the thinnest transverse sections (TS) possible on a watch glass. The specimens were placed on a watch glass for sample sorting. The thinnest sections of both the Ap and Ec were separately chosen using a light micro-scope Digitek QC 3199.

The sample sections were mounted on the aluminum specimen-mounts using double-sided tape and fitted onto the platform. The specimens were loaded into a "FEI Quanta 450 FEG ESEM" with an attached "EDAX" Apollo X SDD Energy Dispersive X-ray (EDX) detector for multiple ex-aminations. High voltage (HV), low voltage (LV) and ESEM modes were utilised to examine the three samples, respectively, namely

1) Coated dry sample with 5 nm chromium by Quorum Q150T coating unit,

2) Uncoated dry sample and

3) Uncoated fresh wet sample.

However, for the uncoated fresh but wet specimen, the temperature of the sample holder was reduced to maintain constant water inside the cells within the optimum pressure as per standard procedure.

Photo images and associated spectral data representing metal concentrations were obtained from leaves of Ap and Ec from each sampling site. As a result, the anticlinal images (TS) of the specimen were produced in the monitor along with corresponding spectral images that were saved for further analysis.

4.5.5 Data analysis

The data were analyzed statistically using Microsoft Excel and IBM®SPSS®21 for Windows. Each sample had separate data arising from triplicate laboratory analysis results that was subjected to ANOVA, including the descriptive statistics. The data and pictures of ESEM anticlinal section of leaves corresponding to their graphical spectrum were carefully examined to compare with the data of ICP-MS. While analyzing the ESEM data, each picture was tagged so that it corresponded to the radiation outcome. The spectral data were then compared with the corresponding ICP-MS data. The ESEM pictures were processed in Microsoft paint program. The mean, range and average were calculated using triplicate result of each sample. Pearson's correlation coefficient determined the correlation between metals and soils, roots, stems and leaves.

4.6 Results

The physico-chemical parameters used in the present investigations include the EC, pH, TC, TN, TOC, N (%) and C (%) as presented in Table 4.1. The soil being tested in our study is from the copper and other metals contaminated dry mine spoil. The *Acacia pycnantha* (Ap) rhizosphere soil was a sandy loam comprising 8% clay whereas the *Eucalyptus camaldulensis* (Ec) rhizosphere soil had 72% clay as per the hydrometer readings (Mendez et al., 2012). The lack of water stagnation and low water holding capacity of soil result into low electrical conductivity and acidic nature of soil (Reuss & Johnson, 2012). In our study, the soil pH at Ap rhizosphere ranged from 4.4 to 6.8 (acidic to slightly acidic) and Ec rhizo-sphere ranged from 6.8 to 8.4 (neutral to basic). The average electrical conductivity of soils in Ap rhizosphere was low 304 mS cm^{-1} compared to the Ec rhizosphere being 7242 mS cm^{-1} (Table 4.1). The decaying leaf litter of Ec might have been responsible for basic soil and high salinity condition as per the result.

Table 4.1 The physico-chemical parameters of soils from the abandoned mine site; ±se (range) n=3

Average	**Plots of** ***Acacia pycnantha* (Ap)**	**Plots of** ***Eucalyptus camaldulensis*(EC)**
pH	5.8 ± 0.41 (4.4-6.8)	7.6 ± 0.75 (6.8-8.4)
EC µS	303.8 ± 121.9 (117-1276)	7242 ± 1929 (1382-13240)
Moisture %	2.8 ± 0.52 (1.6-5.3)	4.8 ±2 (2.8-6.8)
TC mg kg^{-1}	260.5 ± 63.6 (38.7-540.5)	203 ± 101.98 (91-315)
TN mg kg^{-1}	10.6 ± 3.7 (1-34.7)	35.8 ± 9.5 (26-45)
TOC mg kg^{-1}	180 ± 55.4 (27.1-497.8)	146 ± 77.6 (68-223)
N %	0.07 ± 0.02 (0.01-0.18)	0.17 ± 0.13 (0.04-0.29)
C %	1.97 ± 0.4 (0.93-3.6)	2.7 ± 1.67 (1.1-4.4)

The table 4.2 provides information on heavy metals present in soil, root, stem and leaf for plants Ap and Ec. Although the average soil concentration of Cu in *E. camaldulensis* rhizosphere soil was lower with 64.9 mg kg_{-1}, the plant and particularly the stem (139.23 mg kg^{-1}) and leaf (5341.1 mg kg_{-1}) recorded a higher accumulation ability compared to Ap (Baker & Brooks, 1989). The leguminous *Acacia pycnantha* showed a decreasing trend of Cu concentration as it travels from soil to leaf, [1631.7(soil) / 90.4(root) / 152(stem) / 93.6(leaf) mg kg^{-1}] whereas *Eucalyptus camaldulensis* showed a much higher accumulation capacity in root and stem [64.9(soil) / 185.7(root) / 139(stem) / 5341(leaf) mg kg_{-1}] and significantly high in its leaf (Fig.4.2).

Table 4.2 The average concentration of metals in the soil, root, stem and leaf of samples from Kapunda abandoned mine site; ±se (range) n=3

	Soil (mg Kg^{-1})		Root (mg Kg^{-1})		Stem (mg Kg^{-1})		Leaf (mg Kg^{-1})	
	Ap	**Ec**	**Ap**	**Ec**	**Ap**	**Ec**	**Ap**	**Ec**
Na	2425 ±687 (690-6554)	5557 ±3225 (452-14750)	4461 ±8450 (1473-7749)	17075 ±9859 (0.00-34320)	13926 ±4054 (2953-39360)	13670 ±969 (11930-15740)	34823 ±534 (33250-35590)	599 ±174 (203-1652)
Al	3160 ±739 (539-6102)	8155 ±2824 (1801-14750)	293 ±58 (157.5-667.2)	2414 ±732 (1132-3700)	649±7 (367-1002)	946 ±24 (906-1010)	15071 ±2364 (4898-23125)	1913 ±214 (1530-2297)
K	22079 ±4381 (11518-49850)	5034 ±1563 (2057-9017)	39353 ±10611 (12120-101450)	38663 ±4835 (30140-47840)	55277 ±12106 (22835-114450)	57088 ±1560.9 (52870-59750)	102450 ±1680 (100000-107300)	3836 ±654 (1594-6591)
Ca	30489 ±6296 (13730-68340)	5707 ±900 (4164-7823)	95879 ±20433 (54145-220350)	128075 ±13157 (104100 - 153700)	128372 ±19345 (55620-218450)	167325 ±20547 (127900 - 203500)	63528 ±730 (62490-65680)	14334 ±9205 (244-72300)
Fe	5755 ±1195 (835-11010)	28200 ±7240 (12900-47200)	595 ±89 (412-1173)	4059 ±957 (2372-5819)	1288 ±123 (715-1836)	1355 ±50 (1268-1489)	3134 ±408 (2365-3919)	51649 ±7262 (26620 -80170)
Cu	1632 ±241 (458-2544)	65 ±19 (25-117)	90 ±22 (17-199)	186 ±27 (137-233)	152 ±30 (45-282)	139 ±27 (90-195)	94 ±9 (77-110)	5341 ±1226 (946-11710)
Zn	63 ±17 (11-169)	40 ±11 (20-69)	62 ±15 (19-126)	107 ±17 (76-140)	96±16 (35-165)	71 ±10 (52-91)	128 ±4 (119-136)	80 ±13 (31-133)
Cd	0.28 ±0.07 (0.1-0.6)	5 ±1.45 (2.3-9)	0.28 ±0.07 (0.1-0.6)	0.55 ±0.01 (0.5-0.6)	0.19 ±0.05 (0.1-0.4)	0.57 ±0.10 (0.39-0.7)	0.27 ±0.04 (0.2-0.3)	4.1 ±1.0 (1.1-9)
Pb	2.6 ±0.6 (0.0-4.8)	10.9 ±3.1 (4.5-19.3)	2.6 ±0.6 (0.0-4.8)	2.4 ±0.6 (1.4-3.5)	0.8±0.2 (0.1-1.6)	10.3 ±3.9 (3.3-17.6)	2.05 ±0.4 (1.4-2.7)	21 ±3.7 (7.2-37)

The BCF, also referred to as root (below ground) - soil quotient of Ec is ≥ 1 for Cu (3.57) and Zn (2.81), are values regarded as a copper or zinc stabiliser. However, the BCF of Ap is ≤ 1 for Cu (0.43), Zn (0.84), Cd (0.11) and Pb (NA) as the value of BCF ≤ 1 is considered as inferior phytostabilizer (Sagiroglu et al. 2006; Yoon et al. 2006). The heavy metal accumulation in root as in the case of *Acacia pycnantha* is not as harmful as above ground parts accumulation as in *Eucalyptus camaldulensis* in terms of pollution and dispersion (Nouri et al., 2011).

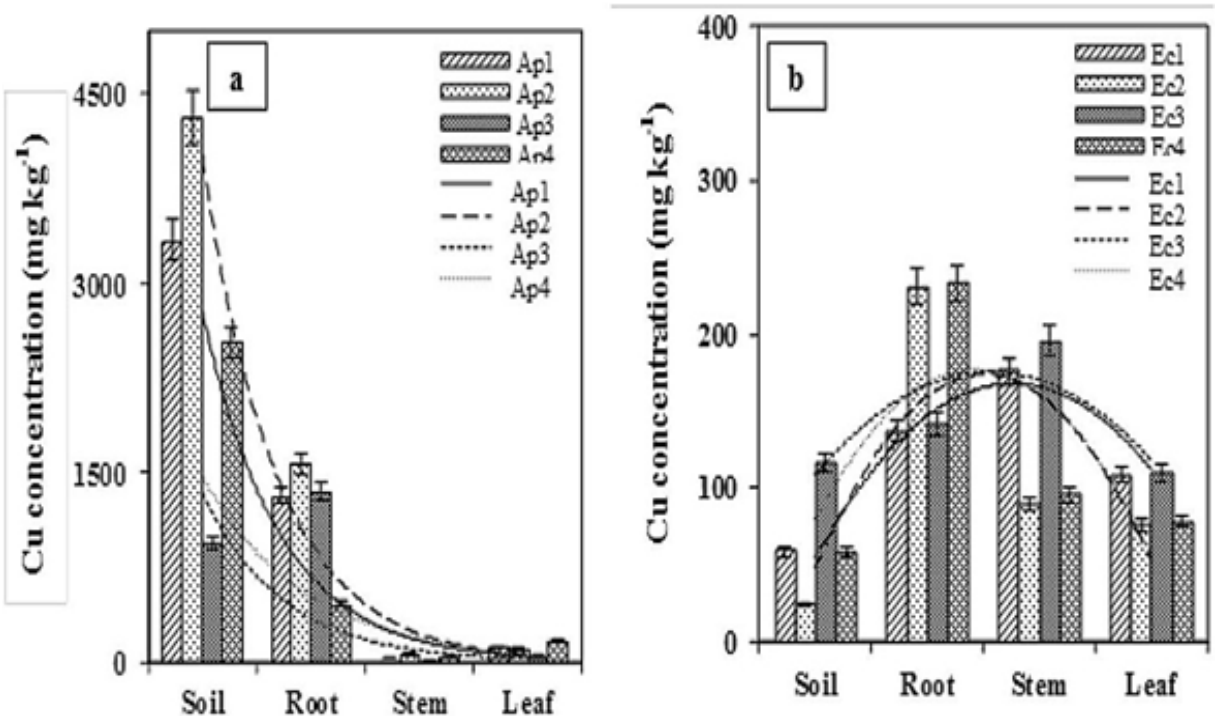


Fig 4.2 The accumulation pattern of underground and above ground parts of metallophyte (SD-%, N=3)

a. Cu concentration in Ap rhizosphere soil (bars) and *A. pycnantha* (Ap1, Ap 2, Ap3, Ap4 = 4x4) root, stem and leaf at 16 sampling locations.

b. Cu concentration in Ec rhizosphere soil (bars) and *E. camaldulensis* (Ec1, Ec2, Ec3, Ec4= 4 x 4) root, stem and leaf at 16 sampling locations.

The TF, also called shoot-root quotient, explains a plant's ability to translocate metal from its roots to shoots and to leaves where a value of ≤ 1 is ideal for phytostabilization. The TF of Ec for Cd (1.04) and Pb (3.86) is ≥ 1, a good candidate for Pb and Cd phytoabsorption but less preferred for phytostabilization (Yoon et al., 2006). The Ap showed a negligible level of copper translocation (TF) along its tissue and such a quality is acknowledged as Cu tolerance (not translocating) ability (Lamb et al., 2010). An EF also called soil-leaf quotient with a value ≤ 1 is considered to be an ideal stabilizer (Sagiroglu et al., 2006; Yoon et al., 2006). Therefore, the EF of copper in Eucalyptus camaldulensis is 2.17, as Cu accumulator and 1.89 as Zn accumulator (Table 4.3).

4.7 Discussion

4.7.1 Soil characteristics and metal accumulation in plants

An evaluation of metal uptake behavior of both the species *Acacia pycnantha* and *Eucalyptus camaldulensis* growing on mine site with respect to its adaptation and soil interaction based on their interaction with heavy metal uptake behavior has been investigated. The extent of root and the physico-chemical properties of soil influenced the uptake or accumulation values analyzed currently using the top soil metal concentrations (Zengin et al. 2008). However, the correlation of soil pH to root copper concentration has R^2 value of 0.58 ($p \leq 0.05$), which is a less significant result which explains the feeble effect of soil pH on metal uptake to root zone of Ap (Fig. 4.2). This indicates plants' ability to adapt to soil ionic impact on metal uptake as reflected with Ap where a highest soil copper yielded less foliar concentration. In contrast, there is relatively higher concentration of metals in Ec leaf and stem compared to soil and root concentration growing on basic pH soil condition (Table 4.2).

Table 4.3 The Translocation factor (TF), Bioconcentration factor (BCF) and Enrichment factor (EF) of *Acacia pycnantha* (Ap) and *Eucalyptus camaldulensis* (Ec) against the metals under investigation; ±se (range), n=3.

Metals	*Acacia pycnantha*			*Eucalyptus camaldulensis*		
	BCF	**TF**	**EF**	**BCF**	**TF**	**EF**
Na	2.4±0.6 (1.2-6)	2.3±0.5 (0.4-4.2)	3.9±0.7 (0.3-6.3)	47±28 (19-75)	0.7±0.0 (0.7-0.7)	29.9±25.6 (4.3-55.6)
Al	0.27±0.1 (0.05-1)	0.15±0.04 (0.03-0.3)	0.02±0.00 (0.01-0.04)	0.3±0.2 (0.08-0.42)	0.6±0.3 (0.3-0.9)	0.09±0.02 (0.07-0.1)
K	1.8±0.4 (0.9-4.5)	0.9±0.2 (0.3-1.7)	1.5±0.3 (0.5-3.4)	14.8±0.13 (14.7-14.9)	2.2±0.5 (1.7-2.6)	31.7±7 (24.7-38.7)
Ca	24±9.5 (0.56-85)	3.5±0.6 (1.4-6.6)	78.9±31.5 (0.8-276)	22.7±5 (17.6-27.8)	1.4±0.5 (0.9-1.9)	29.1±4.8 (24.3-33.8)
Fe	0.24±0.12 (0.03-1)	0.19±0.1 (0.04-0.6)	0.02±0.0 (0.01-0.02)	0.17±0.10 (0.07-0.27)	0.41±0.19 (0.22-0.59)	0.05±0.01 (0.04-0.06)
Cu	0.43±0.14 (0.18-1.42)	*NA	0.02±0.00 (0.01-0.04)	3.6±1.99 (1.58-5.56)	*NA	2.2±0.06 (2.11-2.23)
Zn	0.84±0.22 (0.20-2.21)	1.42±0.56 (0.42-5.23)	0.90±0.22 (0.17-1.77)	2.81±0.33 (2.48-3.14)	0.67±0.03 (0.64-0.70)	1.9±0.31 (1.58-2.2)
Cd	0.11±0.05 (0.02-0.45)	1.20±0.29 (0.33-2.8)	0.21±0.15 (0.02-1.27)	0.13±0.04 (0.09-0.17)	1.04±0.4 (0.67-1.4)	0.15±0.09 (0.06-0.23)
Pb	*NA	*NA	0.02±0.01 (0.00-0.04)	0.27±0.17 (0.10-0.44)	3.9±1.29 (2.6-5.2)	1.3±1.01 (0.25-2.3)

Note: TF of Ec for Pb and of Ap for Cd, BCF of Ec for Cu and Zn and of Ap for Zn and EF of Ec for Cu and Zn and of Ap for Zn are high. BCF > 1, TF and EF < 1 means that it is a good stabilizer, NA- Not available.

Therefore, this result generally indicates that Ap when grown on acidic soils translocate less metals compared to Ec indicating natural adaptation. This result could be also attributed to the metal enrichment in Ec leaves through root uptake

which is called systemic uptake, and through the bark called dermal sorption, or by a combination of both (Chenery et al., 2012; Reichman et al. 2006). Our study shows that the accumulation level of Cu in Ap and Ec is different in root, stem and leaf suggesting the independent behaviour of each species against copper (Fig. 4.2).

4.7.2 Plant-soil interaction

The ESEM spectral analysis shows a higher level of Ca deposition in the vascular bundle of *Acacia pycnantha* (Ap) with EF value 78.9%. Thus, Ap has an affinity to Ca salts as evident from the results shown in Fig. 4.3. According to Maksymiec (1998) there is a physiological effect on cellular processes of higher plants. One such effect is due to Cu^{2+} where enzymatic and leaf senescence is brought about with Ca^{2+} moving into xylem through apoplast pathway. Moreover, the study also points out about the photosynthetic apparatus where Cu^{2+} and Ca^{2+} are involved in the process of escaping metal toxicity.

Accumulation of high Ca in the vascular zone of *Acacia pycnantha* forms calcium flakes clogging the vascular bundles could be a possible explanation for its premature death after almost every 10 years (Brockwell et al. 2005; Gibson et al. 2011; Miller et al. 2011). However, the accumulation of Ca in Ap vascular bundle leading to a mutualistic interaction between plant and microbe can be defined as "a necessary evil". Vadassery & Oelmuller, 2009 assert a similar view regarding the mutual relationship existing between the plant and microbe based on Ca signaling. This relationship has an advantage for Ap with its root nodules being able to survive in contaminated soils due to the ability to draw moisture and nutrition such as nitrogen from the rhizosphere with the help of rhizobia present in root nodules and mycorrhiza present in root tissue (Gibson et al., 2011). Moreover, some species of Acacia (*A. nilotica* and *A. senegal*) were found growing well in calcareous soil supports Ca^{2+} philic and accumulating behavior of this genera (Chaplot, 2013).

One study in Western Australia (Mikli, 2001) reported that *E. camaldulensis* was found to grow well on riverine sites quiet similar to the habitat in the current study site. Mikli, 2001 in his thesis further reports that *Eucalyptus robusta* and *E. camaldulensis* struggled to grow in poor coal mine soils even though they tolerated acidic soils. One of the possible reasons for poor growth of Ec was due to sandy soil as found in arid to semi-arid mine sites that prevented deeper root penetration (Boland et al., 2006). In the stressed soil condition, the root hairs struggle to settle inside the nutrient deficient soil, and preferentially, Ec is found to grow on grey heavy clayey alkaline soils. This result is consistent to Brooker et al., 2005; Costermans, 1989; Haling et al. 2013.

Generally, the acacia species have been widely preferred to eucalyptus for afforestation and greening programs because it can grow in poor soils faster, can fix nitrogen, and revegetate even in nutrient deficient soil (Boyes et al. 2011; Gawronski et al. 2011). Therefore, in our study area containing acidic soil this leguminous Ap species has been evaluated to address the phytotoxicity of metals to plants (Seigler, 2003; Zaets & Kozyrovska, 2012). Ap rhizosphere soil confirmed that this species grows well in acidic soil with low EC, low carbon and nitrogen content as presented in Table 4.1.

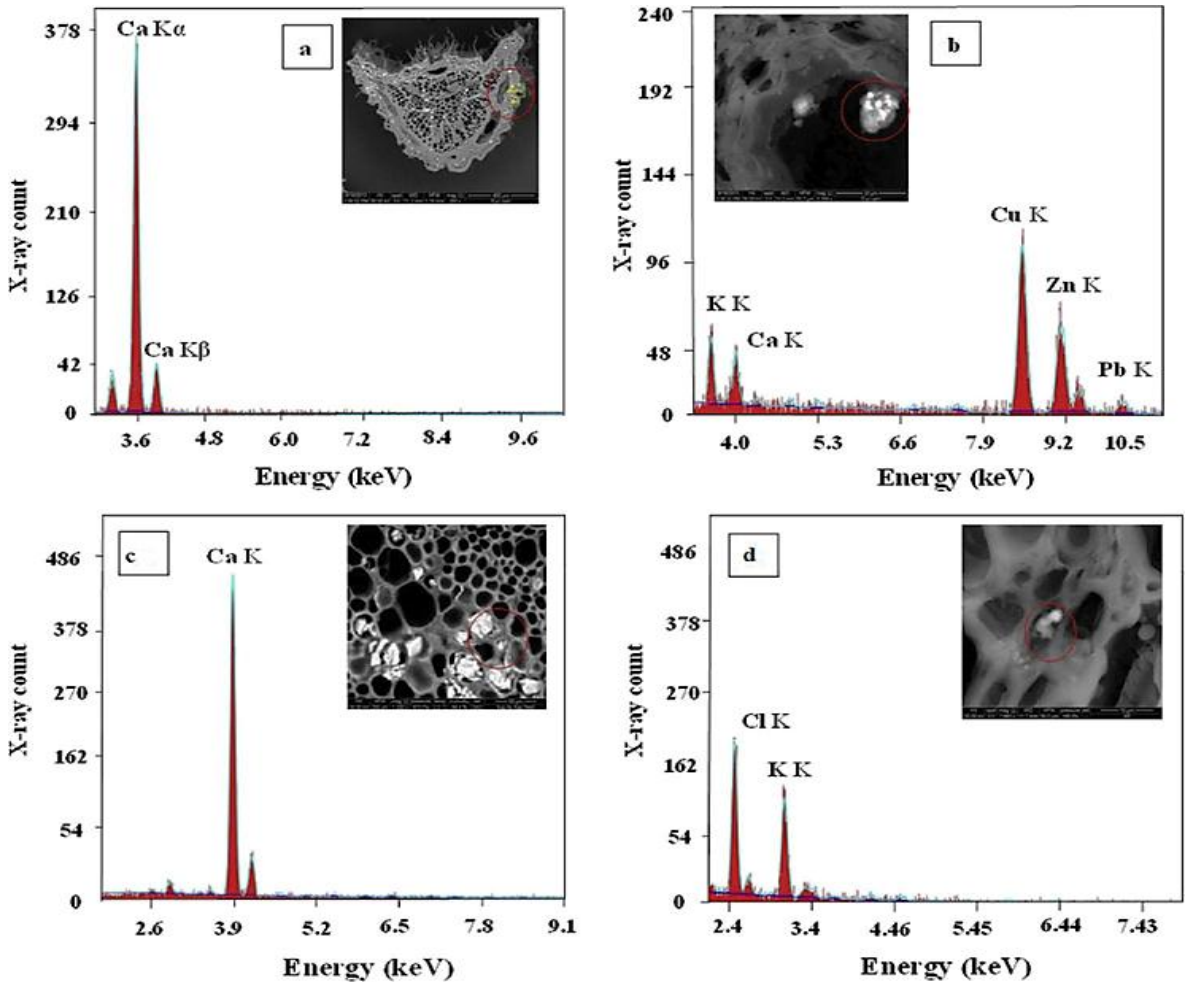


Fig. 4.3 ESEM spectral diagram with images inset

a. The anticlinal section of Ap xylem bundle in the inset with spectral diagram of highlighted area in red circle shows Ca flake deposition

b. The anticlinal section of Ap xylem vessel in inset with spectral diagram of the highlighted area in red circle shows salts of chlorine and potassium respectively, at 2.4 and 3.4 keV

c. The anticlinal section of Ec leaf in inset with spectral diagram of highlighted area in red circle shows Ca deposition in mesophyll zone at 3.6 keV

d. The anticlinal cross-cut of Ec leaf in inset with spectral diagram of highlighted red circle shows deposition of Cu and Zn in xylem vessel.

4.7.3 Accumulation factors of heavy metals on Ap and Ec

The observed metal concentration in leaf, stem and root were used to measure BCF, TF and EF to determine whether a plant is an excluder, extractor or stabiliser (Baker, 1981). As per the current investigation, Ap has a better advantage over Ec because of its high BCF (high root accumulation) and low EF (lower leaf accumulation) ratio (Masvodza et al. 2013). However, there is no convincing earlier data for both species regarding their interaction with heavy metal, particularly in abandoned mine soils. According to Baker & Brooks, 1989; plants that accumulate **more than 1000 mg kg^{-1} of Cu, Co, Cr, Ni or Pb and more than 10,000 mg kg^{-1} of Mn or Zn are called hyperaccumulators,** and the present investigation implies to Ec leaf accumulation.

Therefore, the uptake factor values actually explain the qualitative aspect rather than the quantitative one. The ESEM data of the leaf indicates that Ap is an excluder of Cu because of its low spectroscopic readings (Fig. 4.3). This is in contrast to the findings of Masvodza et al. (2013) who reported hyperaccumulation of Cu in *Acacia saligna* and *Acacia polycantha* grown in mine slime dams in Harare, Zimbabwe. Baker (1981) suggests that it is the foliar concentration that disqualifies a plant to act as a stabilizer. In the present study, the foliar concentration of Cu in Ap was comparatively lower than in Ec, indicating that Ap as a more suitable candidate for phytoremediation.

4.8 Conclusion

The present study of abandoned mine site at a semi-arid climate provides insight into the ability of native trees to interact with metals present in soil. Our data suggest that *E. camaldulensis* is a hyperaccumulator as evident from the EF value. We suggest that the use of a native species like leguminous Ap has a better advantage over Ec to stabilize pollutants such as Cu in a long run. The Ca build-up in the vascular region of Ap, with BCF ≤ 1 for Cu, Zn, Cd and Pb are issues that need further verification in the future. Overall, this study recommends to avoid *E. camaldulensis* growth in copper polluted mine sites.



Photo plate III- ICPMS analysis under progress

Chapter 5

ASSESSMENT FOR METAL TOXICITY AND BIOAVAILABILITY IN METALLOPHYTE LITTERS AND METALLIFEROUS SOILS USING *EISENIA FETIDA* IN A MICROCOSM STUDY

5.1 Introduction

Metals from open cast mines, dumps and tailings constitute a major source of pollutants entering into rivers, lakes and agricultural lands affecting food chain in the ecosystem (Spurgeon & Hopkins, 1996; Perlatti et al., 2014). Abandoned or decommissioned mine soils harbour diverse species of plants called metallophytes that accumulate metals in their above ground parts (Nirola et al., 2015a). There is a possibility that phytoremediation can negatively impact food chain through leaf litter containing hyper-accumulated metals (Chaney et al., 1996; Deportes et al., 1995; Andre et al., 2010; Gall et al., 2015). The phytoaccumulated metals in the above ground parts have a certain fraction of metal in their litter that is further available for organisms feeding on it to bioaccumulate, and this is a bioavailable fraction (Dai et al., 2004; Udovic & Lestan, 2010). Metal accumulator plants are involved in exposing pollutants to organisms such as earthworms (Frouz et al., 2013). In particular, *Eisenia fetida* is one such ecological indicator that is commonly used to assess soil toxicity (Neaman et al., 2012). The earthworms which feed on decaying leaves of metallophytes in vast abandoned mine sites are potential agents in transferring toxic metals into the ecosystem (Posthuma & Van Straalen, 1993).

Contact through soil has been a primary route for transferring metals into worms (Smith et al., 2012) including through skin/dermal contact or by ingestion. Initiation of metal uptake physiology is by “free-ions” available in the gut of worms. During the ingestion of soil or organic matter, metals are absorbed and transported into the inner lining of alimentary canal to deposit into the tissue (Suthar et al.,

2009). Therefore, bioaccumulation of bioavailable metals in earthworms is one of the sources of food chain contamination at higher trophic levels leading to biomagnification (Hsu et al., 2006). The biomagnification of metals occur in such a way that if a bird eats 10 worms that accumulated 0.5 g of a particular bioavailable metal in their tissue, it amounts to eating 5 g metals. The bioavailability of metals in worms is facilitated by protein metallothioneines that plays a vital role in tissue deposition (Edwards et al., 1992; Suthar et al., 2008). Besides such proteins, the abiotic factors such as organic matter (OM) content of soil, temperature, moisture content, particle size distribution also plays a major role in bioavailability of metals to organisms where bound fractions of metals increase their bioavailability to detrivores (Lukkari et al., 2006, Neaman et al., 2012). The metal partitioning and speciation is also influenced by cation-exchange capacity leading to bioavailability and ecotoxicity (Criel et al., 2008).

The extent of bioavailability of metals accumulated in leaf litter from metallophytes or metalliferous soils has not been studied in detail so far. Quantifying the metals bound in lignin is even more essential to establish metal bioavailability through litter consumption by earthworms. Lignin is a polymeric hydrocarbon found in plant vascular walls with many functional groups attracting metals, and it is difficult for organisms to digest lignin (Scheu, S., 1992; Guo et al., 2008). Therefore, to validate the actual source of metal bioavailability in mine-borne leaf litter, we extracted lignin from litter samples to analyze locked up metal concentrations.

We tested whether the metallophyte leaf litter as well as the mined soil makes earthworm responsible for heavy metal transfers into the ecosystem. Thus, we assessed the toxicity and bioavailability of metals by exposing *Eisenia fetida* to metallophyte leaf litters and metalliferous soil originated from an abandoned Cu mine located in Kapunda, South Australia. In a microcosm, we used leaf litters of two ecologically important and predominant native metallophytes, *Acacia pycnantha* and a hyperaccumulator *Eucalyptus camaldulensis* (Nirola et al., 2015b), both collected from the Cu-mined soil, as a source of metal-laden substratum for earthworms to feed. In addition, the survivability and reproduction in earthworms

were monitored since these two factors, if unaffected by the pollutants, are likely to facilitate in the transfer of metals.

5.2 Objectives

The overall objective of this section of study as reported in this chapter is to study metal bioavailability in metallophyte leaf litter and copper mine soil in a microcosm. The specific objective of this experiment includes:

- To see if mine leaf litter and mine soil were toxic to earthworm survival and reproduction
- To study the bioaccumulation of metals in lignin and non-lignin parts of the plants
- To assess if bioavailability of metals from mine soils was dependent on water-soluble fraction of metals
- To study if the metal accumulation in worms were directly proportional to the concentration of metals in samples
- To see if earthworms play a significant role in transfer of heavy metals from mine sites into the food chain.

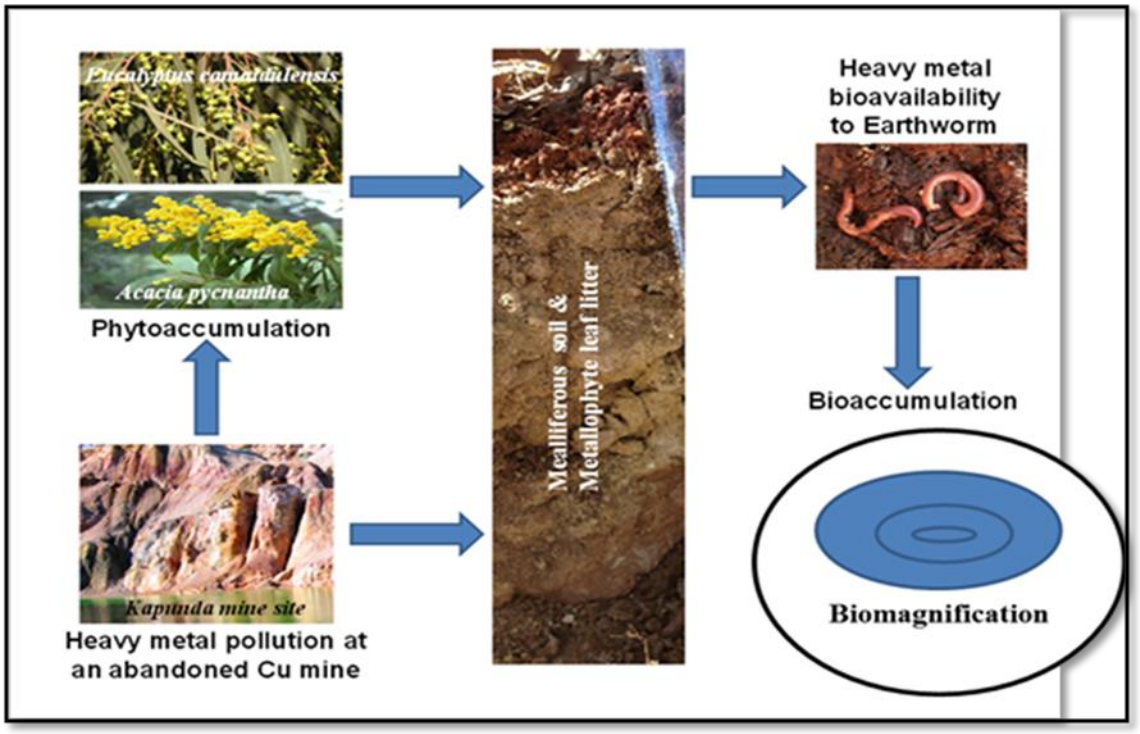


Fig 5.1 The metal cycle from litters to earthworm tissue during the process of bioaccumulation and biomagnifications

5.3 Hypothesis

The leaf litters of tree species, *Acacia pycnantha* (Ap) and *Eucalyptus camaldulensis* (Ec), predominantly growing at an abandoned copper (Cu) mine and mine soils including controls, are toxic and bioavailable to *Eisenia fetida* posing risks to food chain contamination.

5.4 Experiments

An extensive experiment was conducted in a microcosm. With multiple samples of soils from mine site including the mine leaf litter, a well-planned toxicity and

bioavailability study took almost eight months to complete right from the culture of worms. The following table details the overall steps.

The major experimental sequence in chapter 5

Title	Procedure	Analysis
Earthworm culture	Worms were cultured in a laboratory condition	Feeding of worms with oatmeal, vegetable wastes and horse feces were conducted
Sample collection and characterization	Soil and litter samples from mine sites including the control sites	Lignin extraction. Physical characterization of plant and soil samples
Microcosm study	Processing of samples, powdering and treatment preparations	Several experimental pots were prepared to feed the matured worms to study the toxicity and bioavailability
Sample analysis and statistical tools used	ICP-MS/OES, ESEM, Minitab, ANOVA, SPSS tools were used.	Duncans/Tukyes test, test to determine metal uptake using uptake factorial analysis in minitab.

5.5 Materials and methods

5.5.1 Soil and leaf litter samples

Soil and plant leaf litter samples were collected from an abandoned copper mine at Kapunda, South Australia, with map coordinates of 34°20'50.10"S and 138°55'4.78"E. Based on geochemical concepts of Nannoni et al. (2011a), sampling was taken to cover all aspects of environmental homogeneity as far as practicable (Krebs, 1999). Two major sites were chosen. Major site A at 1.7 km away from

Mathews quarry and major site B at 500 m away from of Mathews quarry are presented in Fig.5.2.

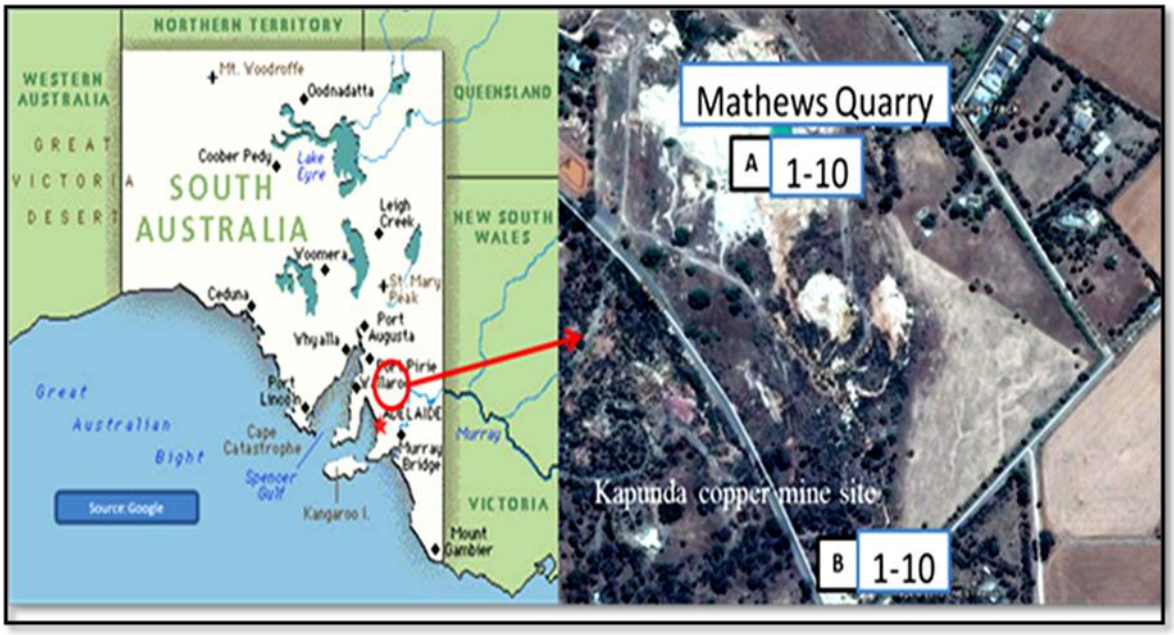


Fig 5.2 Kapunda abandoned mine site in South Australia showing sampling locations

Each major site was subdivided into 10 minor spots to collect mine soil and litter samples of *A. pycnantha* (Ap) or *E. camaldulensis* (Ec). At each sampling site, soil was collected from a depth of 15-20 cm around the plants within a 5 m radius. Samples were placed in separate PVC bags (Krebs, 1999). Control soil and leaf litter samples of Ap and Ec were collected from Mawson Lakes campus, University of South Australia. The soil samples were air-dried, homogenized in concrete mixer, sieved through a 2-mm mesh, and stored in polypropylene containers. The leaf litter samples were manually homogenized, air-dried, triple rinsed with deionized water, and oven-dried at 85 ^{o}C for 24 h. The dried samples were powdered using a steel-blade glass electric grinder and stored in polypropylene containers.

Portions (5 g) of soil were taken in centrifuge tubes and mixed with 5 parts of milli-Q water (1:5) for determining pH and electrical conductivity (Sposito, 2008). Total organic carbon (TOC) and total carbon (TC) and total nitrogen (TN) were estimated in soil-water slurry (1:5) as prepared above. The soil solutions were agitated in a shaker overnight and were centrifuged at 4000 rpm for 10 min. The supernatant was divided into three portions for further analysis in Formacs^{series} TOC analyzer following UV-promoted per-sulphate digestion method. Analyses of TC (%) and TN (%) were done by using 0.5 g soil sample in a Trumac carbon-nitrogen-sulphur (CNS) analyser (Leco® Corporation, Michigan, USA).

Standard blank, controlled and cross-referencing samples were used to check for quality after every 10 readings. The soil texture was determined following hydrometer method (Gee & Bauder, 1979). Soil water-holding capacity (WHC) was determined by oven-drying method of Gardner & Klute, 1986. The cation-exchange capacity (CEC) for each soil sample was measured using Rengasamy & Churchman, 1999 method with special reference to Ross & Ketterings, 1995.

5.5.2 Metal analysis of soil and plant litter samples

Soil samples (0.5 g) were separately taken into Teflon tubes for microwave digestion. A microwave accelerated reaction system (CEM-MARS X ®) was used to digest metals for soils in *aqua-regia* using USEPA-3015a method (Juhasz et al., 2007). After digestion, the metals were recovered by thorough rinsing of the inner walls of each Teflon tube with deionised water until all the digested sample was collected in separately labelled centrifuge tubes. The digested samples were diluted up to 50 ml using deionised water, centrifuged at 4000 rpm for 10 min, and the supernatant was passed through a 0.45 μm filter.

Aliquots (10 ml) of diluted suspension, in triplicates, were transferred into ICP tubes for analysis in the Agilent 7500c (Agilent Technologies, Tokyo, Japan) Inductively

Coupled Plasma-Optical Emission Spectrometer (ICP-OES) USEPA 3015a method. Portions (0.5 g) of dried and powdered leaf litter samples were taken into Teflon tubes and digested using concentrated HNO_3 to determine total metal content following Inductively-coupled Plasma Mass Spectrometry (ICP-MS, Agilent 7500ce, Agilent Technologies, Tokyo, Japan) as outlined in USEPA 6020a method. A blank was introduced after every 10 samples in the instrument for quality control. Dilution was carefully prepared after several initial trials for effective detection of metals by instrument.

Water-soluble and exchangeable metals, reducible metals bound to Fe and Mn, oxidizable metals bound to organic matter and sulphides, and residual metals bound to silicate minerals were investigated (Zemberyova et al., 2006; Oyeyiola et al., 2011) by employing Bureau of Community Research (BCR) method (Rauret et al., 2000). To 1.0 g of soil sample, 40 ml of 0.11 mol L^{-1} acetic acid was added and shaken for 16 h at 25 ^{o}C in a mechanical shaker to extract water-soluble, exchangeable and carbonate-bound metals. The samples were centrifuged at 4000 rpm for 10 min, the supernatant passed through 0.45 μm filter, and loaded into ICP tubes. To the residue, 40 ml of 0.5 mol L^{-1} hydroxylamine hydrochloride was added and shaken for 16 h to extract the reducible metals. To the residue, 10 ml of 8.8 mol L^{-1} hydrogen peroxide was added and digested for an hour at 25 ^{o}C and left for another hour at 85 ^{o}C in a water bath. The solvent was evaporated, and 50 ml of 1 mol L^{-1} ammonium acetate was added to the residue and shaken for 16 h to extract oxidizable metals at pH 2. Finally, *aqua-regia* digestion was carried out in a microwave digester to determine the status of metals in the residue using the USEPA-3015a standard procedure followed by ICP-OES analytical method (Juhasz et al., 2007).

5.5.3 Extraction of leaf lignin

Since the use of harsh chemicals might degrade lignin-bound metals during extraction (Tan et al, 2009), "Kraft" physical and chemical extraction procedure (Gellerstedt and Lindfors, 1984) with modifications was adopted in the present study. Portions (150 g) rinsed and dried leaf litter were placed in 1500 ml glass beaker containing one litre of aqueous Na_2CO_3 (250 g L^{-1}) on a hot plate. Heating at a constant temperature of 80 ^oC was continued for 12 h with occasional topping up with water and stirring. A thick dark decoction was decanted after every 4 h followed by topping up with Na_2CO_3 solution. The lignin portion was separated from the tissue debris physically by twisting the leaves before each fresh topping-up. Finally, the lignin fibers were isolated, washed with milli-Q water, oven-dried and powdered in an electric grinding machine for further digestion in a microwave digester followed by ICP-MS analysis. The bioavailable portion was calculated by subtracting the fraction of a metal contained in leaf lignin from the total metal content present in the leaf litter.

5.5.4 Preparation of microcosm substratum

Portions of crushed and powdered leaf litter, left to decay for three months in an aerated glass bottle in a 40% moist environment, was thoroughly oven-dried at 85 ^oC before exposing it to earthworms in a microcosm study. To each 500 g mined soil, 50 g leaf litter was added to make a total of 550 g (w/w) and was placed in PVC pots. This ratio of soil and litter was followed to make sure of the threshold level of total metals in final volume of the substratum did not cross the Cu-3000 mg kg^{-1} mark, a value of total metal expected in abandoned mine soil (Guo et al., 2009). Separately, only 550 g of mined soil or plant litter was also maintained. Portions (550 g) of soil or leaf litter, collected from the University campus, in pots served as controls. Accordingly, the treatment Ids and their corresponding details are indicated in Table 5.1.

Table 5.1 Different treatments used in the present study

Treatment ID	**Details of treatment**
T1	Control (Ap litter and soil collected from University campus)
T2	Control (Ec litter and soil collected from University campus)
T3	Mine Ap litter and control soil
T4	Mine Ap litter and mine soil A
T5	Mine Ap litter and mine soil B
T6	Mine Ec litter and control soil
T7	Mine Ec litter and mine soil A
T8	Mine Ec litter and mine soil B
T9	Control soil collected from University campus
T10	Mine soil A
T11	Mine soil B
T12	Mine Ap litter
T13	Mine Ec litter

The pots were subjected to an end-over shaker for 24 h to ensure uniform distribution of decayed and dried litter powder and soil samples. The formulated pots were moistened to 30-45%, weights taken and left for two more weeks in the culture room to stabilize before the worms were introduced.

The formula used for calculating Cu, Zn, Cd or Pb in the treatment substratum containing soil and litter is:

Total metal concentration in a treated sample

$= [\{\left(\frac{s}{2}\right) + \left(\frac{50 \times l}{1000}\right)\} \times 1000] \div 550.$ Where,

s = total metal in soils,

l = total metal in litter *(for the total of 550 g sample substratum by weight using 50 g litter)*

(Unit: mg kg^{-1})

5.5.5 Earthworm toxicity and bioavailability test

Earthworm of the strain *E. fetida* Savigny, was acclimatized for three months in a culture room in the laboratory prior to its use in the toxicity experiment. The culture room had a provision for light and dark at a room temperature. In the culture cabinets, mixture of washed vegetable peel, waste fruit and oat meal was used to feed earthworms on a weekly basis at room temperature with sufficient moisture of 30-45 % based on initial weight of pots. After culturing, 390 mature worms possessing clitellum were selected for the experiment. Prior to exposing to substratum, the worms were depurated for 48 h, rinsed using deionised water and patted dry to record their initial weight (in a batch of 5) to calculate change in weight. Overall, each treatment received 30 worms and each treatment consisted of 6 replicates containing 275 g of substratum to facilitate enough room and exposure. The worms were gently introduced one by one into all the 78 pots coming from 13 samples consisting of six replicates for each treatment. They were left at 21 ^{0}C with light and dark provision to facilitate diurnal cycle for eight weeks following a standard protocol (OECD-222, 2004) in a special experiment room. The moisture in each pot was maintained throughout at 30-45%. Aeration was facilitated by drilling 10 tiny holes in the plastic lid of each pot.

After two weeks, mortality and physical deformity in worms were recorded. The physical deformity included tissue decay, fungal infections and injury. The survived worms were reintroduced into the same pots. At the end of first phase, on 28th day, mortality, post-exposure 48-h depuration, and final weights were determined for each treatment. Finally, worms survived in these pots were separately collected and placed in centrifuge tubes and stored at - 20 ^{o}C overnight for analysis of metals in the tissue. The frozen worms in each tube were left in a water bath at 80^{o}C for 25 min, and vortexed with the addition of 10 ml concentrated HNO_3. The process of heating and vortexing was repeated until the tissue was completely dissolved in acid. The tissue samples were then topped up to 50 ml using deionised water, vortexed further and centrifuged at 4000 rpm for 10 min. The supernatant was passed through a 0.45µm filter into 10 ml ICP tubes for measuring total metals in tissue using the ICP-MS method as described earlier. The worms introduced in control soil and litters served as controls.

The substratum (with worms taken out for analysis) in all the pots were left intact for another 28 days for assessing metal toxicity on reproduction that included cocoon hatching followed by juvenile count. The guidelines of OECD (Spurgen et al., 1994) were followed for microcosm study to determine weight change, mortality, juvenile count and metal uptake by the worms (Zemberyova et al., 2006; Perlatti et al., 2014).

The data of juvenile and survival count was converted to percentage value by using the mean juvenile count and survival value of 6 replicates.
Thus, the percentage juvenile was calculated using the formula:

$\frac{\Sigma x}{y} \times 100$ where,

x = mean number of juveniles or survivals,

y = mean number of adults exposed to experiment pots.

The reproductive potential in worms under the influence of metals in the substratum was validated using interactions between juvenile count and nature of the substratum. The per cent weight change was calculated by the formula:

$\frac{\Sigma w2}{\Sigma w1} \times 100$ where,

Σw2 = mean final weight and

$\Sigma w1$ = mean initial weight.

5.5.6 Statistical analysis

Microsoft Excel and IBM®SPSS®22 for Windows were used to process the statistical data. Tests of homogeneity of variances were conducted between treatments T1-T8 (mixture) and T9-T12 (pure), before performing ANOVA and Tukey tests to check if the variances are similar between the groups being compared. The means and standard deviations were plotted using excel bar chart. Bars that are not significantly different were marked with the same letters.

The data of necessity were not balanced in our tests; therefore, the three overlapping subgroups of data analyzed included (a) control soils to enable litter effects only, (b) Ap and Ec samples from mine in a 2 × 4 level factorial, and (c) Ap and Ec samples from mine in a 3 × 3 level factorial. Fisher's (protected) test was used when the overall ANOVA was significant. The statistical assumptions of homoscedasticity was checked using plots of residuals vs fitted values, and the normality of the residuals was checked using Q-Q plots. All the treatments were paired statistically for first post-hoc or least significant difference (LSD) test. They were grouped into (a) only those containing control soil to test the impact of different litter samples, (b) only those with litter from the mine sites to test the effect of soil samples, and (c) those without or with mine litter and mine soil to test the simultaneous effect of litter and soil.

5.6 Results

Soil pH was generally neutral (pH 7.8) in control soil (T9) collected from university campus and the mine soils (T10 and T11) (Table 5.2). Mine soil A (T10) showed a slightly lower level of salinity compared to other two soils (T9 and T11).

Table 5.2 Characteristics of soil samples used in microcosm study (mean ± SE, n =3)

Characteristic	**Control soil (T9)**	**Mine soil A (T10)**	**Mine soil B (T11)**
pH	7.8 ± 0.3	6.7 ± 0.3	6.2 ± 0.4
EC (μS cm^{-1})	125 ± 7.4	98 ± 6.4	169 ± 0.8
WHC (%)	39 ± 2.5	27 ± 1.5	26 ± 2.5
TOC (mg kg^{-1})	37.5 ± 2.2	10.3 ± 0.4	31.7 ± 1.8
Total C (%)	2.1 ± 0.2	1.7 ± 0.1	2.7 ± 0.2
Total N (%)	0.2 ± 0.01	0.1 ± 0.01	0.1 ± 0.01
Sand (%)	68 ± 12	81 ± 11	85 ± 11
Clay (%)	10 ± 4	4 ± 2	3 ± 1
Silt (%)	22 ± 2	15 ± 2	12 ± 1.5
CEC (c mol c kg^{-1})	8.6 ± 3	5.5 ± 3	5.8 ± 2

The WHC of the soil samples ranged from 26-39%. The total organic carbon content in the mine soil A was low (10.3 mg kg^{-1}) compared to the control soil (T9) and mine soil B (T11). The mine soils A and B were sandy with low nitrogen content. The CEC of mine soils A and B was also comparatively lower than the control soil.

5.6.1 Metal bioavailability from soil and litter samples

Analysis of different treatments for total content of Cu, Zn, Cd and Pb indicated that mine soil B alone (T11) or its combination with Ap/Ec leaf litter (T5 and T8) contained significantly higher level of Cu, Cd and Pb compared to any other treatment samples (Table 5.3). In contrast, Zn was more in mine soil A alone (T10) or its combination with mine Ap/Ec leaf litter (T4 and T7). The data on metal fraction in soil obtained by following BCR sequential extraction are presented in Table 5.4. Expectedly, the water-soluble Cu fraction of mine soil B (T11) was significantly higher (2100 mg kg^{-1}) than that of mine soil A (227 mg kg^{-1}), and this value corresponds to ICP-MS data to indicate positive validity of the result.

Table 5.3 Total content of Cu, Zn, Cd and Pb in different (mean ± SE, n =3)

Treatment	Total metal content (mg kg^{-1})			
	Cu	**Zn**	**Cd**	**Pb**
T1	10.7±2	12±2	1.6±1	8±3
T2	10.6±1.23	11±1.4	1.6±0.7	8±1
T3	68±4	22±3.2	1.57±0.4	8.3±1.5
T4	1500±69	230±34	5.4±2	6.5±1.2
T5	13968±756	148±21	6±1	22±0.5
T6	26±7	18±6	1.9±2	10±2
T7	1458±54	226±9	5.8±0.8	8±2
T8	13925±105	144±32	6±1	24±2
T9	11 ± 2	19 ± 2.5	1.7 ± 0.1	15 ± 2.5
T10	1586 ± 182	241 ± 56	6 ± 2	7 ± 2
T11	15300 ± 1650	151 ± 41	7 ± 2	25 ± 2
T12	645 ± 54	128 ± 2	0.27 ± 0	2 ± 0
T13	183 ± 78	80 ± 12	4 ± 0.5	21 ± 1

The residual metals in all the three soil samples were high. However, the Pb in T9 was significant at 21 mg kg^{-1}. The value observed for water-soluble fraction of Cu

between T10 and T11 was similar for reducible, oxidizable and residual forms of Cu. However, the residual forms of Zn and Pb were higher in soil sample collected from the university campus (control, T9).

Table 5.4 BCR result for metal fraction (mg kg^{-1}) in soil samples (mean ± SE, n =3)

BCR	Treat-ment	Cu	Zn	Cd	Pb
Water-soluble	T9	2.1 ± 0.9	9.6 ± 3	0.05 ± 0.01	0.1 ± 0.01
	T10	227 ± 48	20 ± 4	0.02 ± 0.01	0.1 ± 0.01
	T11	2100 ± 588	22 ± 8	0.05 ± 0.01	0.1 ± 0.01
Reducible	T9	0.4 ± 0.1	2.3 ± 1	0.5 ± 0.1	0.05 ± 0.01
	T10	27 ± 5	1.1 ± 0.05	0.5 ± 0.1	0.01 ± 0.01
	T11	327 ± 54	2.4 ± 0.1	0.1 ± 0.01	0.01 ± 0.01
Oxidizable	T9	0.3 ± 0.1	0.7 ± 0.1	0.05 ± 0.01	0.1 ± 0.01
	T10	161 ± 36	ND	1 ± 0.1	0.02 ± 0.01
	T11	460 ± 120	0.14 ± 0.01	1.5 ± 0.1	0.03 ± 0.01
Residual	T9	9 ± 3	28±3	2.03 ± 1	21 ± 2
	T10	1905 ± 200	31 ± 3	3.01 ± 0.5	11 ± 2
	T11	4105 ± 206	18 ± 3	3.01 ± 0.5	8.6 ± 2

Note: ND, not detected. The recovery of Cd and Pb are not according to the values expected in total metal analysis due to dilution and low detection level of instrument.

The total metal content of leaf litter as per ICP-MS data is the total metal present in litter including lignin and rest of the tissue such as epithelial and mesophyll layers. The lignin data presented in Table 5.5 indicate that Cu and Zn were predominant in both (Ap and Ec) leaf litter samples collected from mine sites.

Table 5.5 Metal fraction in lignin from leaf litter samples (mean ± SE, n =3)

Treat-ment	Metal content in lignin (mg kg^{-1})			
	Cu	**Zn**	**Cd**	**Pb**
T1	6.5 ± 3	10 ± 4	0.01 ± 0	0.4 ± 0
T2	5 ± 3.5	8 ± 2.5	0.05 ± 0	1.0 ± 0.1
T12	198 ± 67	17 ± 3	0.04 ± 0	1.2 ± 0.3
T13	115 ± 65	18 ± 3	0.11 ± 0.01	2.8 ± 0.3

However, the leaf litter samples of Ec obtained from mine showed higher amounts of Pb than other three metals considered here. The bioavailable fractions (subtracting lignin) of Cu in the mine leaf litter samples of Ap (T12) and Ec (T13) were 447 and 68 mg kg^{-1}, respectively, and the bioavailable fractions (subtracting lignin) of Zn in the corresponding samples were 111 and 62 mg kg^{-1}, respectively. Although the contents of Cd and Pb in leaf litter samples collected from the mines were low (Table 5.3), significant fractions of these metals are bioavailable is a subject of interest. In particular, the bioavailable fractions of Cd and Pb in mine Ec leaf litter samples (T13) were 3.89 and 18.12 mg kg^{-1}, respectively.

5.6.2 Earthworm weight, survival and reproduction in microcosm

The data in Table 5.6 present change in body weight of worms, survival and juvenile count. The weight loss in worms was maximal in treatments containing mine soil A alone (T10), mine soil B alone (T11), and a combination of mine soil B with mine Ap litter (T5). Moderate loss in body weight was observed in treatments that contained mine Ec litter either with mine soil A (T7) or mine soil B (T8).

Table 5.6 Body weight, survivability and juvenile count of earthworm in microcosm study averaged in each pot out of 5 worms, mean ± SE, n =6.

Treat-ment	Body weight change (g)	Survival (No.)		Juvenile Count
		After 14 d	After 28 d	
T1	-0.4± 0.2	5.0 ± 0	5.0 ± 0	13.0 ± 2.0
T2	-0.3 ± 0.1	5.0 ± 0	5.0 ± 0	11.0 ± 1.8
T3	-0.5 ± 0.2	5.0 ± 0	5.0 ± 0	2.8 ± 1.7
T4	-0.5 ± 0.1	4.8 ± 0.4	4.2 ± 1	0.2 ± 0.4
T5	-1.4 ± 0.2	3.8 ± 1.2	1.7 ± 1.5	0.0
T6	-0.4 ± 0.1	5.0 ± 0	5.0 ± 0	4.0 ± 1.4
T7	-1 ± 0.1	5.0 ± 0	4.8 ± 0.4	0.0
T8	-0.9 ± 0.1	4.7 ± 0.5	3.8 ± 1.6	0.0
T9	-0.2 ± 0.1	5.0 ± 0	5.0 ± 0	15.0 ± 1.9
T10	-1.5 ± 0.3	4.5 ± 0.8	2.5 ± 1.8	0.0
T11	-1.5 ± 0.5	3.0 ± 0.6	1.0 ± 0.6	0.0
T12	-0.6 ± 0.2	5.0 ± 0	5.0 ± 0	3.7 ± 1.2
T13	-0.4 ± 0.1	5.0 ± 0	5.0 ± 0	25.2 ± 11.2

(- value = negative weight change)

There was no weight loss in treatments when the worms were fed with mine Ap litter (T12) or Ec litter (T13). The numbers of juveniles were around 10-25 in treatments that contained only control Ap litter (T1) or Ec litter (T2), control soil (T9), and mine Ec litter (T13). No juveniles were observed in substratum involving mine soil B together with mine Ap litter (T5), with mine Ec litter with mine soil A (T7) or mine soil B (T8), mine soil A alone (T10), and mine soil B alone (T11).

5.6.3 Metal bioavailability to earthworm

Metal bioavailability in survived worms was assessed by determining the amount of a metal accumulated in the tissue after 28 days of exposure to substratum in different treatments. The results presented in Fig.5.3 indicate that accumulation of Cd was maximum (0.8-0.9 mg kg^{-1}) in treatments with control soil (T9), mine Ec litter (T13), and in a mixture of control soil and mine Ec litter as substratum (T6).

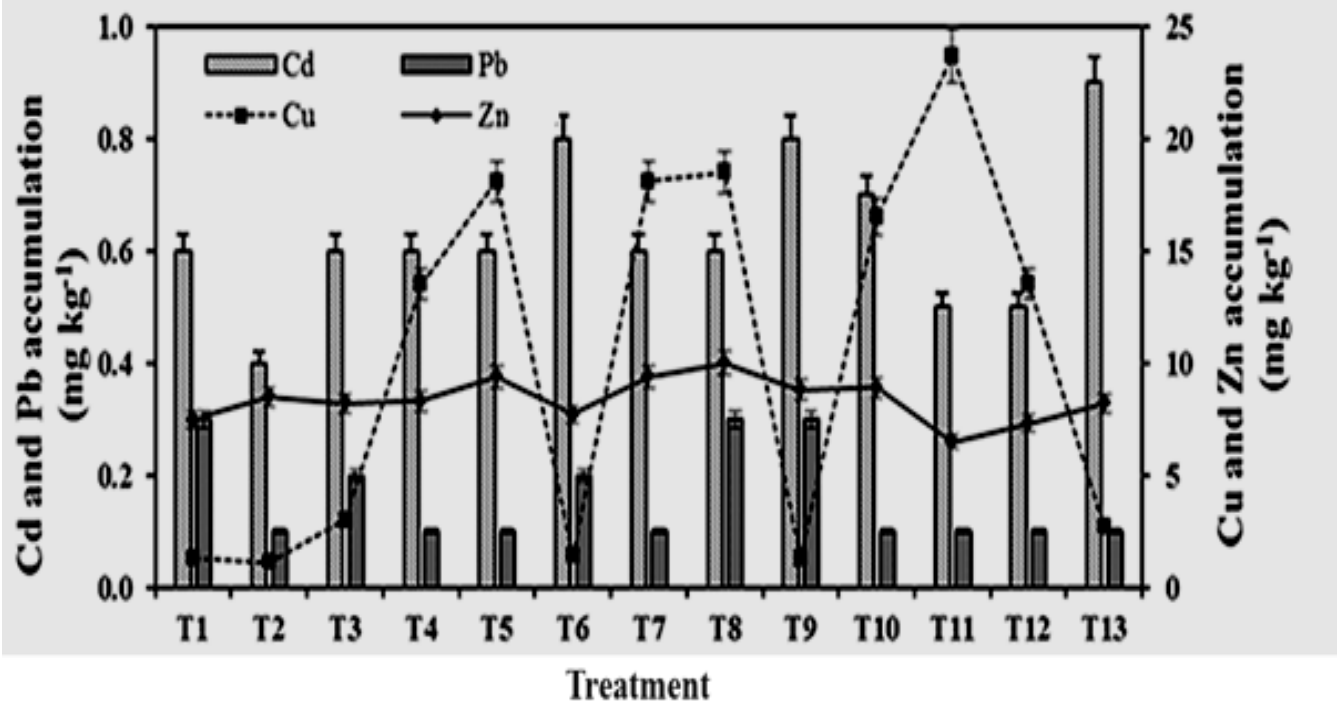


Fig 5.3 Bioaccumulation of Cu, Zn, Cd and Pb in *E. fetida* exposed to different treatments. Values are means ± SE (n=3)

On the other hand, Pb accumualtion was the highest (0.3 mg kg^{-1}) in T1 (control Ap litter), T8 (mine Ec litter + mine soil B), and T9 (control soil only). The Cu bioavailability in tissue was significant when the worms were fed with mine Ap litter and mine soil B (T5), mine Ec litter with mine soil A (T7) or mine soil B (T8), and mine soil B alone (T11). Thus, a maximum of 24 mg kg^{-1} Cu is accumulated in earthworm with treatment T11. As per the standard error value of Cu in leaf litter, the concentration of metal in leaf litter is a defining factor for bioavailability to

worms at normal pH and EC conditions, irrespective of whether it is locked with lignin or not. The tissue accumulation of zinc was almost uniform and around 10 mg kg^{-1} for all the samples.

5.6.4 Statistical test

5.6.4.1 Earthworm body weight

The effect on change in weight of worms was analysed by using a linear model assuming normal errors. The final weight was dominated according to the initial weight of the worms, with a significant negative contribution from soil Cu concentration. Therefore, the final weight of the worms was examined using analysis of variance with the initial weight being a covariate. In analysis of log transformed values for samples with no litter, Ap litter from control site, Ec litter from control site, mine Ap litter and mine Ec litter, the values were -1.12, -1.14, -1.08, -0.54 and -0.68 (LSD, 0.14%), respectively.

In the analysis of group that included the treatments with litter only from the mine sites, there was a significant effect of soils on worm weight. The worm body weights were 1.51, 1.50, 1.23 and 0.80 g in treatments with no soil, control soil, mine soil A and mine soil B, respectively, indicating that the worm weight decreased with exposure to the contaminated soil.

5.6.4.2 Earthworm numbers and juveniles

Initially, a generalized linear model with Poisson errors and a logarithmic link function as described by McCullagh & Nelder (1989) was used to model the number of juveniles present. However, the residuals were found not to follow a Poisson as indicated by very low deviance (about 0.611 in 25 degrees of freedom). Analyses

were therefore performed by logarithmic transformation by the formula, $y = \log_{10}(x+1)$, using a multiple linear regression based on the initial weight, and concentration of Cu in both soil and litter (Fig. 5.4a). A Q-Q plot of the residuals from an analysis of the logarithmically transformed juvenile count data was employed to check the assumption relationship (Fig. 5.4b).

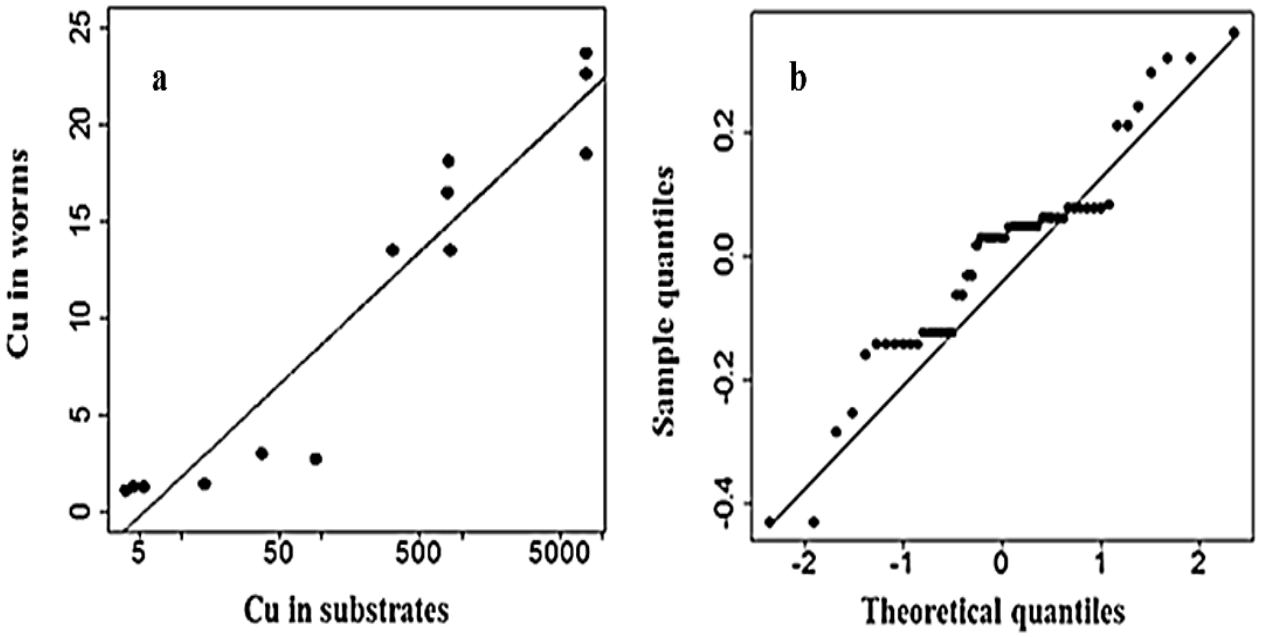


Fig 5.4 with X and Y axis not lebelled as equal.

a. The best fit with worm Cu content being a linear function of the logarithm of the substrate Cu concentration

b. The Q-Q plot of the residuals from an analysis of the logarithmically transformed juvenile count with an effectively s=a straight line relationship indicting that the residuals are normally distributed

5.6.4.3 Copper uptake by earthworms

Since the main component of Cu body burden in worms was soil Cu, the effect of soil Cu was assessed using all the 13 treatment combinations. The results of ANOVA tests with Tukey post hoc option show that there were no significant differences between T1-T8 (mixture of litter and soil) and T9-T13 (pure samples) as all p-values were larger than 0.05. A uniformity of degree of exposure to worms from treatment samples as seen in the analysis is presented in Fig.5.5.

There was a tendency for Cu content in worms to asymptote at about 20 mg kg^{-1}, so the data were fitted in a model by von Bertalanffy-1934 function (Kooijman, 1986) using the formula:

$$Worm\ Cu = max.Worm\ Cu * [1 - expected(k * substrate\ Cu)] + residual.$$

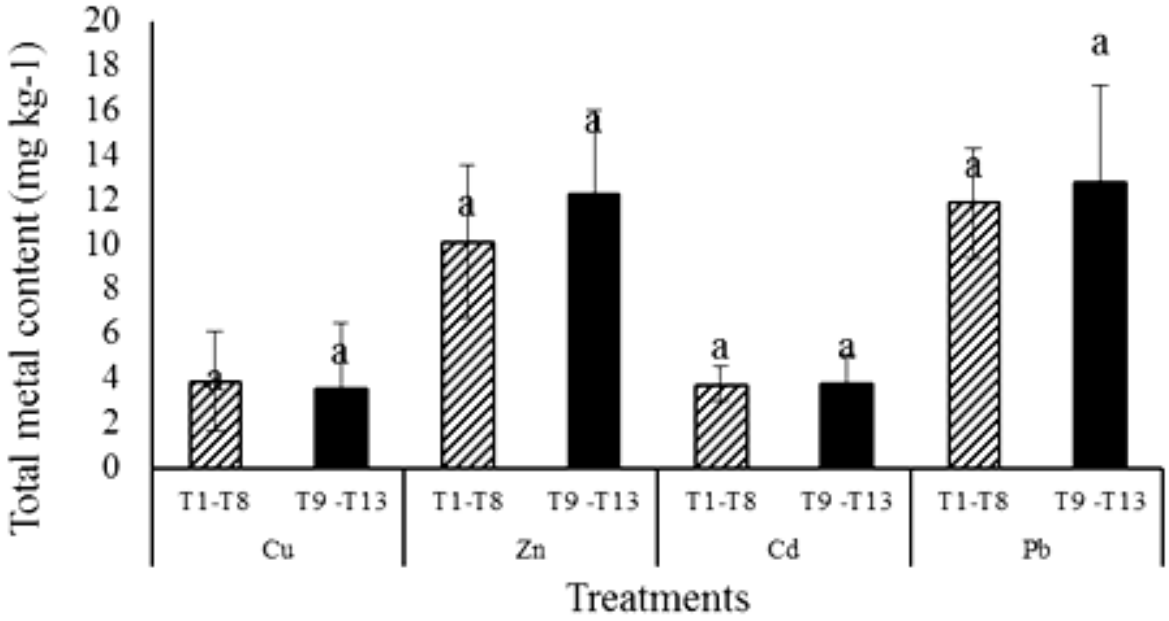


Fig 5.5 Means and standard errors of total metal content of Cu (divided by 1000), Zn (divided by 10), Cd and Pb by different treatments. Columns for each element sharing the same letter are not significantly different at P < .05 (Tukey's test).

5.7 Discussion

The bioaccumulation of metals in earthworm depends on the concentration of contaminants available for uptake (Thakali et al., 2006; Nannoni et al., 2011b). In our findings, T11 treated worms had a higher bioaccumulation value corresponding to its water soluble fraction or even the total metal content in the soil sample. Earthworms are resilient creatures that can dwell on contaminated soil substratum but the information on progression of population is still an anomaly. Since the metal contaminants are sequestrated into organic part of the substratum, when worms ingest, the substratum is digested in gut making the metals bioavailable (Smith et al., 2012). Therefore, the actual bioavailability of metal is prevalent only in the gut after ingestion. Li et al. (2010) suggested that bioaccumulation of metals in *E. fetida* from pig manure was dependent strongly on bioavailability. The bioavailability is however mostly influenced by pH, dissolved organic carbon content and water-soluble portion of metals (Wen et al., 2004). In a secondary poisoning study, it was found that soil-related properties influenced better uptake of metals in worm rather than compound-specific properties (Romijn et al., 1993).

Nonetheless, it is still unclear whether the organic-rich and heavy metal-enriched substratum has less bioavailable metal than the contaminated soil itself due to metal sequestration with organic compounds such as lignin and soil factors affecting the bioavailability (Smith et al., 2012). The influence of water-soluble metals is also seen primarily affecting bioavailability coupled with higher salinity and lower pH that are typical in mine soils (Viventsova et al., 2005; Leveque et al., 2014). In our study the higher Cu bioavailbility in T10 due to higher deposition in worm tissue could be due to a reduced metabolic activity influenced by low salinity, low TOC and low CEC (Criel et al., 2008; McKelvie et al., 2013; Konecny et al., 2014). Therefore, not only does the water-soluble fraction of metal influence the metal uptake, but soil and environmental factors also affect the metal bioavailability (Hobbelen et al., 2006).

The worms exposed to higher metal concentration and copper in particular showed a higher weight loss (Van Gestel et al., 1991; Spurgeon et al., 1994; Helling et al., 2000; Zhou et al., 2013). The average weight of worms exposed to mine Ap litter was 2.3 g while it was 2.5 g with mine Ec litter with LSD of 0.17%, suggesting that mine Ec litter had less detrimental effect than mine Ap litter. In the third group, with or without litter, there was no effect on initial weight, whereas contaminated soil showed significant effect on weight loss. The mean worm weights (g) for the control soil, mine soil A and mine soil B were 1.8, 0.3 and 0.2, respectively, with LSD (5%) of 0.24.

Virtually, no mortality in worms was observed even after 28 days in treatments of control Ap litter (T1) or Ec litter (T2), control with mine Ap litter (T3) or mine Ec litter (T6), control soil (T9), and mine Ap litter (T12) or Ec litter (T13). One of the reasons for better survival and juvenile count in control soil (T9) could be soil CEC that facilitates for organic matter availability to worms (Caravaca et al. 1999). Thus, physical characteristics of soil may have a greater influence on bioavailability, apart from metal concentrations in soil (Saxe et al., 2001; Sanchez-Hernandez, 2006). Only 20% of worms survived in substratum containing only mine soil B (T11) while treatment of mine soil B together with mine Ap litter (T5) resulted in about 40% survival. This tendency of worm's survival in mine soil B is possibly by release of metallothionein that neutralizes the toxic effect of metals (Mustonen et al., 2014). However, the food availability in the form of organic matter and decayed leaf litter is another reason for less mortality besides their exposure to the copper containing substratum. Severe tissue injury together with deformed tissue and exposed digestive organs was observed in mine soil B-treated worms, probably due to oxidative stress imposed by copper (Li et al., 2010; Zhou et al., 2013).

The back transformation of two groups using statistical model indicated that there were approximately four times as many worms in the controls compared to those exposed to metal-contaminated treatments. When the data were confined to treatments with litter from mine site, it was apparent that metal contamination effectively prevented the production of juveniles. There were 25 juveniles per pot

with only mine Ec litter treatment, and 2 to 4 juveniles in other treatments that did not receive metal-contaminated soil. The analysis again showed that there were no juveniles when metal-contaminated soil was present, and the juveniles were more in treatments without or with litter from a mine site. In particular, the worms introduced into Ec mine litter (T13) were able to reproduce more juveniles, clearly indicating the ability of worms to withstand metal toxicity.

In contrast, treatment with mine Ap litter (T12) resulted in significant reduction in juvenile count. This could be due to gradual release of Cu from decaying lignin leading to reproductive failure, a fact similar to the Freundlich model (Qiu et al., 2013). Moreover, reproductive capacity is related to Cu contamination that can lead to higher earthworm mortality and reduced cocoon hatching (Lukkari et al., 2000; Reinecke et al., 2001; Helling et al., 2000). In one of the studies on copper contaminated soil, the reproduction of *Enchytraeus crypticus* was nil in the soils containing 5000 mg kg^{-1} of copper with reproduction effect arising at effect concentration (EC50) of 351 mg kg^{-1} (Konecny et al., 2014). In general, Neaman et al. (2012) also suggests a positive impact of soil organics on juvenile production. Cu accumulation from control soil treatments (T1, T2, T6 and T9) in the present study was low, and this observation is similar to the report of Peijenburg & Jager (2003) on the bioaccessibility and bioavailability of metals.

The highest adjusted R^2 was obtained using the residual fraction of Cu. The high importance of the residual Cu questions its composition. It may have included an organic component responsible for the observed relationship. Pb in residual form was significantly more than water soluble, oxidable and reducible forms. Accordingly, Zn exhibited more water soluble form including the residual amount. Cd was comparatively higher in the residual form outlining their bioavailability. Therefore, the bioavailability of Zn, Cd and Pb also reported by Nannoni et al., 2011b seems to be more influenced by soil fractions concerning reducible and oxidisable amount.

The content of Cu in the worms was found to be dependent on soil Cu content as in expression: - [Cu in worm = -6.91 (± 4.07) + 3.34 (± 1.16) ln (Cu in substrate)]. A stronger relationship was observed when the regression was based on substrate Cu. The resultant relationship was significant ($p < 0.05$) with an adjusted R^2 of 0.85. Therefore, the statistical analysis revealed that the level of Cu in soil had a proportionate impact on bioavailability (Steenbergen et al., 2005; Arnold et al., 2008; Fisker et al., 2011; Smith et al., 2012; Suthar and Singh, 2009; Zhou et al., 2013).

Although Zn concentration ranged from 12 mg kg^{-1} in control soil to 241 mg kg^{-1} in mine soil A (T10) (Table 5.3), its accumulation in the worms' tissue was limited to below 10 mg kg^{-1} in all treatments probably due to the ability of worms to avoid or escape the uptake of Zn (Reichman, 2002). In a study of Bernard et al., 2010 in exposure depuration experiment no significant difference in Zn uptake was found even with different exposure levels.

5.8 Conclusion

Treatments with higher metal concentrations in substratum significantly reduced worm body weight and increased the mortality rate. Feeding on a substratum that contained a mine soil or mine leaf litter resulted in no reproduction of worms. Metal bioaccumulation in worm tissue was dependent on water-soluble fraction of a metal. In treatments that received mine soil without or with mine leaf litter, significant amounts of Cd, Pb and Cu accumulated in worm tissue. Mine leaf litter from Ec was favorable to earthworm survival and juvenile than that of Ap. Validation of the present data on bioaccumulation of metals in earthworm tissue indicated that the mine leaf litter significantly contributed to metal bioavailability. However, the metal concentration in mine soil was also primarily responsible for earthworm toxicity and metal bioavailability. Thus, the present data on toxicity and bioavailability of metals contained in metallophyte leaf litter and metalliferous soils indicate that a detrivore like an earthworm poses danger of biomagnification.



Photo plate IV- The earthworm experiment in snapshot

Chapter 6

METAL BIOAVAILABILITY TO *EISENIA FETIDA* THROUGH *LEPUS TIMIDUS* FECES AND PLANT LITTER.

6.1 Introduction

One of the major sources of heavy metal pollution are former mine sites which were abandoned without following any protocols of decommissioning (Gutierrez-Gines et al., 2015). Albeit, mining activity is responsible for substantial impact on ecosystem and environment due to the exposure of ores, casts and dumps containing metals (Yellishetty et al., 2013). When the living environment is exposed to metals, multiple problems arise due to their direct and indirect effects on reproduction and health (de Vries et al., 2013; Hashmi et al., 2013; Kwok et al., 2014). Since plants have a tendency to grow in habitats that it acclimatizes, most of the species growing in abandoned mines are metallophytes performing either phytoaccumulation or phytostabilization (Nirola et al., 2015).

Phytoaccumulators are plants that uptake metals through xylem vessels based on bioavailability and deposit them into stem or leaf tissue (Chaudhry et al., 1998). Bioavailability is "that fraction of total amount of chemicals stored in a specific environmental compartment which is either available or can be made available for uptake by (micro) organisms or plants" (Peijnenburg & Jager 2003).

There are challenges involved to identify, avoid and control the growth of metal accumulating species. In cases such as abandoned mines, some species are evolved into hyperaccumulators while the others are primarily hyperaccumulators (Pollard et al., 2014). However, any plant that uptakes metal is a concern. The plant debris such as leaf litter deposited on the ground harbour various (micro) organisms including annelid earthworm that follow detritus (decaying matter) food chain to start up as a primary consumer (Odum & Heald, 1975). The ground vegetation over the

contaminated sites is mostly dominated by metallophyte herbs which are grazed upon by animals like rabbits (Gutierrez-Gines et al.,2015). An idea of fecal substratum of mine dwelling animals feeding on herbs contain metals and the worms feeding on it would uptake them for biomagnification (Kohn & Wayne 1997) is also being studied here. The feces of English hare (*Lepus timidus*), which is a most dominant animal in the current study site, was used to test the secondary poisoning and bioavailability in this microcosm study. Little literature is available that deals specifically with faecal matter metal bioavailability to the higher trophic levels including through the mine borne litter (Rawlins et al., 2007; Li et al., 2010; Gong et al., 2012; Lester, 2014; Clair et al., 2015).

Ecologically, earthworms placed at the base of terrestrial food chain pyramid are potential organisms to transfer contaminants to the higher trophic levels composed of birds and mammals (Carpene et al., 2006; Suther & Singh, 2008; Duffus J.H, 2001; Ortiz-Santaliestra et al., 2015). The predatory birds when consume worms exposed to high concentration of metal pollutants, are the secondary consumers in food chain (Romijn et al., 1992; Fry D.M , 1995). For instance, in the Netherlands, breeding birds *Limosa limosa* feeding on earthworms were studied to track the bioaccumulation of heavy metals from soil and it was found that lead, mercury and cadmium were highly bioavailable (Roodbergen et al., 2008). Overcoming these challenges is very rewarding to industrialists, entrepreneurs and government bodies. Avoiding hyperaccumulator plants will have several advantages such as, metal free leaf litter and fodder for animals and reptiles which dwell on contaminated sites. Such clean technology will minimize biomagnification of toxic elements into the environment, avoiding death and elimination of species of plants and animals from the earth (Malik & Zeb 2009; Sfakianakis, et al., 2015).

Native plant species of Australia; *Eucalyptus camaldulensis* as a hyperaccumulator of copper and *Acacia pycnantha* a copper tolerant studied earlier in the same location (Nirola et al., 2015) are used for leaf litter experimentation. Only Cu (the study site being an abandoned copper mine), Zn, Cd and Pb are considered in this study based on the idea of exchange kinetics studied in seven mine sites (Ren et al.,

2015) that play an important role in metal bioavailability. Moreover the control samples of *Eucalyptus camaldulensis* and *Acacia pycnantha* leaf litter are also omitted due to lack of information on threshold level of metal accumulation and low detection by instruments in earlier studies (Nirola et al., 2015a). To test the level of metal bioavailability, ecotoxicity experiment using earthworms as ideal bio-indicators of soil toxicity was conducted (Gish & Christensen, 1973; Udovic et al., 2007; Suthar et al., 2008; Arnold et al., 2008; Andre et al., 2010). *Eisenia fetida* is a popular and appropriate species that is used here for the microcosm study (Suthar et al., 2008; Hsu et al. 2006; Dennis et al. 2010; Kim et al. 2016). Use of OECD (Organisation for Economic Cooperation and Development) soil as a control substratum, control soil and mine borne leaf litter as well as the feces of English hare originating from mine site and control faecal sample has been used. Overall, the experiments are guided by protocols of the Organisation for Economic Cooperation and Development (OECD 207, 1984; OECD 222, 2004).

6.2 Objectives

The objectives of the experiment were to study metal bioavailability to earthworms from fecal matter of mine dwelling animals and fallen litters. Infact, the herbs are equally dangerous agents responsible to transfer the bioavailable heavy metals into the food chain. Therefore, one of our aims was to feed earthworms on dead and decaying matter (detritus) with following specific objectives:

- To determine the metal bioavailability of *Acacia pycnantha* and *Eucalyptus camaldulensis* leaf litter to earthworms
- To find out if animal feces contain bioavailable metals for further bioavailability into earthworms
- To study the tissue concentration and bioaccumulation potential of heavy metals by earthworms and study under ESEM
- To establish if hyperaccumulators do pose environmental challenges in a long term in terms of organism toxicity and metal biomagnification.

- To conduct reproduction test and check if high copper concentration in samples effected the juveniles
- To cross determine bioavailability using OECD soils with control soils
- To check if metals other than copper are involved in bioavailability and biomagnification

6.3 Hypothesis

Heavy metals can enter the food chain through earthworms exposed to the animal fecal matter and leaf litter from abandoned mine site soils.

6.4 Experiments

The use of OECD soils and faecal matter of English hare collected from mine site and zoo are the major focus of the experimental sequence in this chapter as presented in the table below :-

Title	Procedure	Analysis
Earthworm sample and acclimatization	Matured earthworms were sourced from local farmer and acclimatized in laboratory	Feeding of worms with oatmeal, vegetable wastes and horse pooh were conducted
Sample collection and characterization	English hare fecal matter from mine and zoo were collected along with litter samples	ICP-MS analysis after digestion in *aqua-regia* in microwave digester
Microcosm study with OECD soils	Processing of samples, powdering and treatment preparations with OECD as control	Several experimental pots including controls were prepared to feed the matured worms to study toxicity and bioavailability
Sample analysis and statistical tools.	ICP-MS/OES, ESEM, Minitab, ANOVA, SPSS tools were used.	Principal component analysis and dendrobium charts using SPSS.

6.5 Materials and methods

6.5.1 Sampling and processing

The sampling site was a former copper mine abandoned in 1879 at Kapunda, approximately 80 km from Adelaide city centre. The samples were categorised into a) hare faecal matter from mine (MHF), b) control hare faecal matter from the Adelaide Zoo of 5 days collection (CHF), c) *Acacia pycnantha* leaf litter (ApM), d) *Eucalyptus camaldulensis* leaf litter (EcM), e) Control soil from the uncontaminated site from University of South Australia premises (SC) and g) OECD soil from laboratory stock; all sampled and processed as per standard procedures (Putman R.J., 1984, Krebs, C.J., 1999, Nannoni et al., 2011). Each of the mine site samples

including native tree foliage were collected in polypropylene containers in five duplicates covering a radius of 10 m for each category.

In the laboratory, the hare feces were crushed using a glass mortar and pestle and were thoroughly air dried for 48 hours. An aliquot of each of 0.5 g powder was separately loaded into Teflon tubes prewashed in Decon and 2% HNO_3 (*V/V*). The rest of the faecal matter powder was stored for microcosm study. The leaf samples were washed in deionised water, air dried and powdered. An aliquot of 0.5 g leaf litter powder was separately loaded into Teflon tubes for microwave digestion. The rest of the leaf samples were stored in glass jars with air pores bored in the lid for further degradation to feed the worms. The soil samples (including the OECD) were air dried for 48 hours, sieved through a 2 mm mesh and homogenized using an end over shaker overnight.

An aliquot of 1 g each was separated for determining moisture content out of which 0.5 g was separated after oven drying at 80^{o}C. The rest of the soils were kept for microcosm analysis. Using the USEPA-3015a method, microwave digestion (CHEM-MARS X ®-USA) was employed to separately digest soil samples using *aqua-regia* and HNO_3 (v/v) to digest plant litter and hare faecal matter samples. All the digested samples were transferred from Teflon tubes to fresh centrifuge tubes and tubes completely emptied by rinsing into centrifuge tubes. The digested samples were diluted up to 50 ml using deionised water and centrifuged at 4000 rpm for 10 minutes. The supernatants were filtered through 0.45µm filter pads into 10 ml ICP-tubes for ICP-MS/OES analysis. The soil samples were separately analysed using the Agilent 7500c (Agilent Technologies, Tokyo, Japan) Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES). The feces and plant samples were analysed using an Inductively Coupled Plasma-Mass Spectrometer (ICP-MS). In each digestion batch, a blank was introduced after every 10 readings for quality control.

6.5.2 Soil characterization

The pH and electrical conductivity (EC) of soil samples were determined in a 1:5 (w/v) soil: water slurry. Total C (%) and N (%) analysis employed 0.5 g of soil sample in a Trumac carbon-nitrogen-sulphur (CNS) analyser (Leco® Corporation, Michigan, USA). The standard blank, control and cross-referencing samples were introduced to maintain quality control after every 10 readings. Soil texture using hydrometer method, water holding capacity (WHC %), and cation exchange capacity (CEC) were determined for the soil samples using standard procedures (Rengasamy & Churchman; 1999).

Soil samples were separately subjected to metal fraction analysis using the three step Bureau of Community Research (BCR) method as proposed by Rauret et al. 2000 (Zemberyova et al., 2006). An aliquot of 1 g of each soil sample was mixed with 40 g of 0.11mol L^{-1} CH_3COOH. The solution was subjected to mechanical shaking for 16 hours at room temperature. Metals which were water soluble, exchangeable and those bound to carbonates were extracted from the supernatant after centrifugation and filtration as mentioned earlier.

The residue was treated with 40 ml of 0.5 mol L^{-1} of hydroxyl amine hydrochloride ($NH_2OH.HCl$) and subjected to mechanical shaking with a repeat of the same procedure, as mentioned earlier, to extract reducible metals. Further, using the residue it was treated with 10 ml of 8.8 mol L^{-1} of hydrogen peroxide (H_2O_2) for one hour at room temperature and then one hour at 85 ^oC in a water bath. To extract the oxidisable metals in sequence, the resultant solution was evaporated after adding 50 ml of 1 mol L^{-1} of ammonium acetate (CH_3COONH_4) and shaken in a mechanical shaker for 16 hours at acidic condition of pH 2. The left over residue was subjected to a final step of *aqua-regia* digestion in a microwave digester repeating the process as outlined earlier for other soil samples to conduct total metal analysis by ICP-OES method (Nannoni et al., 2011).

6.5.3 Earthworm assay

Each treatment pots were prepared by mixing equal weights of 250 g each of substratum samples predetermined based on ICP result not to surpass high toxicity level to worms beyond 800 mg kg^{-1} copper (Svendsen & Weeks 1997). The treatments include control soil, OECD soil, OECD soil with mine site hare feces, OECD soil with zoo hare faecal matter, OECD soil with mine site *Acacia pycnantha* litter and OECD with mine site *Eucalyptus camaldulensis* litter. The mixture of substratum was homogenized in an end-over shaker for 24 hours and kept for two weeks with 40% moisture content.

In relation to collection and culture of worms; to ensure that earthworms were naturally acclimatized, *Eisenia fetida* feeding on decaying lawn grasses were purchased from a local farmer. The worms were transferred into a laboratory culture room and were acclimatized by maintaining in OECD soil, in a culture vessel for four weeks before starting the experiment. Washed vegetable peels and oat meals were provided as feed to the earthworms. The matured worms possessing well-developed clitellum were sorted to introduce into treatment pots containing formulated substratum. Each treatment pot was inoculated with 5 worms as per the standard protocol of Organisation for Economic Cooperation and Development (OECD-222, 2004).

6.5.4 Microcosm analysis

The worms were picked individually from the culture cabinet, checked for clitellum, and triple rinsed with deionised water in a large Petri dish and carefully transferred into deionised water-wetted cotton pad into a fresh Petri dish to depurate. After 48 hours, the worms were collected one by one and placed over a fresh moist tissue paper platform, triple rinsed, again separately placed in centrifuge tubes (treatment wise) for initial weight reading. A batch of 5 worms from centrifuge tubes were released into respective treatment pots and effectively aerated through tiny holes on pot lid.

After 14 days of experiment, counting and checking was done for each experimental pot to record any mortality or physical damage such as tissue tear, fungal infection etc. A reference earthworm was taken from nursery stock to use in ICP-MS analytical accuracy to yield a baseline value compared to those in the treatment pots. At the end of experiment on 28th day, worms in each pot were carefully transferred onto a moist tissue paper platform, triple rinsed with deionised water, patted dry and left for depuration for 24 hours.

Final weight was taken, labelled and transferred into centrifuge tubes and stored in cold room at 20 ^{o}C overnight. The frozen dry worms were weighed and carefully taken over to fume hood cabinet and added with 10 ml of HNO_3 (v/v) each for digestion. The solution was vortexed before subjecting to heating in water bath inside the fume hood cabinet for metal digestion in tissue. From time to time, centrifuge tubes (containing tissue solution) were topped up with water to avoid drying.

A final 50 ml volume was maintained again vortexed and introduced into centrifuge unit at 4000 rpm for 10 minutes. The supernatant solution was filtered using 0.45 µm filter pad fitted with syringe up to 10 ml and taken in the ICP tubes for total metal analysis. ICP-MS was carried out using the same procedure as explained earlier in 6.5.1. The experimental pots were left in the culture room at suitable environmental conditions for a further four weeks to study reproduction that involved a juvenile count. Oat meal was used to feed juveniles and to ensure that they matured and grew fast to facilitate counting.

6.5.5 ESEM analysis

Each depurated worm from microcosm experiment was separately frozen at -20 ^{o}C in Petri dishes. After 24 hours, the sample was heated in a water bath at 20 ^{o}C for 45 minutes to defreeze. Each sample was separately sliced using separate surgical blades for each sample into the thinnest transverse sections possible. The sectioned specimens were sorted out separately using a light microscope Digitek QC 3199 before proceeding to study the localization of elemental distribution. The aluminum specimen mount was used to load specimens to get fitted onto ESEM platform. The specimens were subjected to analysis with a "FEI Quanta 450 FEG ESEM, with an attached "EDAX" Apollo X SDD Energy Dispersive X-ray (EDX) detector for multiple examinations. High voltage (HV), low voltage (LV) and ESEM modes were utilized to examine each samples. The earthworm anatomical images and associated spectral data and curves representing metal concentrations were obtained in the microscope monitor (Fig.6.3a&b).

6.5.6 Data analysis

Microsoft Excel and IBM®SPSS®21 for Windows were used to process the statistical data. In our analysis, no statistically significant result using ANOVA test was obtained. So, a correlation of samples using dendrogram ward linkage analysis was performed to see the metal distribution pattern in samples. Some mathematical formulae were used to calculate the bioaccumulation factors and bioavailability percentages. The metal bioaccumulation factor was obtained using total metal concentration in worms over total metal concentration in samples used to feed the worms.

The following mathematical expression was used

$BAF(log) = \frac{\text{EfM}}{TmS}$. where

$BAF = bioaccumulation\ factor,$

$EfM = worms\ total\ metal\ content,$

$TmS = treatment\ samples\ total\ metal\ content.$

To calculate the actual bioavailability percent, the BCR fraction of water soluble upon total metals present in BCR treatment pots were used. The following mathematical expression was proposed and used

$aBa(\%) = \frac{WsF}{Tbcr}\ X\ 100.$ where

$aBa(\%) = actual\ bioavailability\ (\%),$

$WsF = water\ soluble\ fraction,$

$Tbcr = Total\ BCR\ metal\ concentration$

The actual bioaccumulation percent was calculated using total metal concentration in worm upon total BCR metal concentration in each treatment pots using the following expression;

$aBc(\%) = \frac{EfM}{(\Delta TP)}$ X 100. where

$aBc(\%) = actual\ Bioaccumulation\ (\%),$

$EfM = worms\ total\ metal\ content,$

$(\Delta TP) = Total\ BCR\ metal\ content\ in\ each\ treatment\ pots$

(Li et al., 2010, Nannoni et al. 2011)

6.6 Results

As presented in Table 6.1, the pH of soils and feces samples was slightly acidic in nature ranging from 6.2-6.8. Both the control and mine site faecal matter samples recorded higher electrical conductivity, Nitrogen (%), Carbon (%) and the CEC in samples compared to control and OECD soils.

Table 6.1 Characterization of soil and fecal samples (±S.E, n=3)

Sample ID	**pH**	**EC** (µS)	**WHC** (%)	**N** (%)	**C** (%)	**CEC** ($cmol^{+}kg^{-1}$)
Control soil (SC)	6.8	125	49	0.2	2.1	8.5
OECD soil	6.5	159	55	0.1	1.5	8.5
Control hare feces (CHF)	6.4	1712	67	14	22	8.9
Mine site hare feces (MHF)	6.2	1621	68	15	22	9.1

The total metal content of all the six samples formulated to use in microcosm experiments is presented in Table 6.2. In the control soil (SC), the iron and lead content in samples was high at 15165 mg kg^{-1} and 19 mg kg^{-1} respectively. However, the concentration of Cu, Zn and Cd was the least among others in SC. The OECD soil was Na and K rich with the presence of 6 mg kg^{-1} Pb. The level of Zn in control hare feces (CHF) was highest with 173 mg kg^{-1}. Similarly, a higher values for Cu (645 mg kg^{-1}) in ApM litter and Ca (15785 mg kg^{-1}) in EcM litter were recorded. Since the control and OECD soils had a lower concentration of toxic metals, the juvenile production was high as expected (Fig. 6.1b). The weight loss was high in control soils and mortality was high in *Acacia pycnantha* leaf litter treated pot.

Table 6.2 Total metal concentrations in control and contaminated samples in mg kg^{-1} (±S.E.n=3)

Index	Na	Al	K	Ca	Fe	Cu	Zn	Cd	Pb
S1 (SC)	161 (±0.8)	1197 (±70)	1860 (±16)	5127 (±7.5)	15165 (±25)	12 (±0.01)	25 (±0.07)	0.07 (±0)	19 (±0.1)
S2 (OECD)	1500 (±10)	2300 (±12.5)	2312 (±11.4)	7324 (±24)	1001 (±12)	22 (±2)	37 (±1.2)	0.04 (±0)	6.0 (±1)
S3 (CHF)	1142 (±8.5)	576 (±4.4)	2001 (±43.5)	10720 (±120)	557 (±8.3)	47 (±5)	173 (±3.35)	0.1 (±0.01)	1.5 (±0)
S4 (MHF)	284 (±27.8)	1938 (±192)	1384 (±91)	8637 (±346)	2099 (±90)	435 (±41)	75 (±3.8)	0.3 (±.04)	2.5 (±0.4)
S5 (EcM)	482 (±0.6)	4765 (±17.5)	1375 (±0.5)	15785 (±5)	5989 (±16)	183 (±0.25)	66 (±.02)	0.23 (±0)	4 (±0.02)
S6 (ApM)	248 (±22.3)	1061 (±102)	349 (±5.7)	7891 (±829)	890 (±6.9)	645 (±59)	13 (±0.05)	0.03 (±0)	0.9 (±0.11)

EcM- *Mine leaf litter of Eucalyptus camaldulensis.*

ApM- *Mine leaf litter of Acacia pycnantha.*

Table.6.3 Metal fraction in control and mine samples by BCR analysis in mg kg^{-1}

FR	Sample	Cu	Zn	Cd	Pb
FR-A	**SC**	2.1	9.6	0.01	0.08
	OECD	8.0	9.5	0.01	0.02
	CHF	13.0	80.0	0.04	0.04
	MHF	29.0	35.0	0.12	0.05
	EcM	69.0	46.0	0.1	2.5
	ApM	292.0	7.0	0.02	0.27
FR-B	**SC**	0.35	2.3	0.02	0.05
	OECD	3.0	1.5	0.01	0.01
	CHF	2.1	11.0	0.03	0.02
	MHF	43.0	12.4	0.05	0.04
	EcM	51.0	4.0	0.01	0.01
	ApM	68.0	3.0	0.01	0.02
FR-C	**SC**	0.27	0.7	0.05	0.14
	OECD	0.75	0.6	0.01	0.01
	CHF	26.0	40.0	0.03	0.57
	MHF	245.0	11.0	0.14	0.07
	EcM	40.0	7.0	0.01	0.01
	ApM	190.0	2.0	0.01	0.01
FR-D	**SC**	9.0	35.6	0.05	18.0
	OECD	11.0	12.5	0.01	4.0
	CHF	7.0	24.6	0	0.68
	MHF	214.0	28	0.09	2.0
	EcM	25.0	9.0	0	0.5
	ApM	70.0	1.0	0	0

**ND:not detected, (FR-Fraction)*

Note:BCR analysis presenting metal concentration fraction available in each samples are indexed into four categories namely;

a) *FR-A: Water soluble, exchangeable, and bound to carbonates*
b) *FR-B: Reducible; bound to Fe and Mn*
c) *FR-C: Oxidisable; bound to organic matter and sulphides*
d) *FR-D: Residual; bound to silicate minerals*

As per the metal fraction data presented in Table 6.3, water soluble (FR-A) copper concentration in ApM is highest with 292 mg kg^{-1} followed by Zn 80 mg kg^{-1} in CHF.

The concentration of residual (FR-D) Pb in SC is higher than other samples (18 mg kg^{-1}). The MHF sample had a highest level of metal copper with 214 mg kg^{-1} in oxidisable part (FR-C). The soil fractionation reveals that more amount of partitioning is present in residual fraction (FR-D). However, for faecal matter and litter, the metal fraction is partitioned more in water soluble (FR-A) and oxidisable (FR-C).

Table 6.4 Total metal concentration in worms exposed to sample index combinations in mg kg^{-1} **(**±S.E. n=3)

Sample	**TP**	**Na**	**Al**	**K**	**Ca**	**Fe**	**Cu**	**Zn**	**Cd**	**Pb**
S1	T1	839 (±33)	23 (±4)	1148 (±198)	556 (±45)	54 (±6)	0.3 (±0.1)	8.8 (±1.1)	0.08 (±0)	0.3 (±0)
S2	T2	709 (±31)	5 (±1)	987 (±54)	956 (±56)	27 (±5)	0.7 (±0.5)	9 (±1)	0.02 (±0)	0.07 (±0)
S2+S3	T3	324 (±31)	10 (±2)	1650 (±98)	1417 (±132)	6 (±1)	3 (±0.1)	13 (±1.2)	0.1 (±0)	0.1 (±0)
S2+S4	T4	886 (±75)	17 (±2)	1821 (±154)	1281 (±127)	24 (±2)	46 (±2.2)	11 (±1)	0.2 (±0)	0.06 (±0)
S2+S5	T5	748 (±26)	10 (±2)	1053 (±145)	791 (±14)	16 (±3)	11.4 (±1)	8.6 (±0.6)	0.2 (±0)	0.04 (±0)
S2+S6	T6	957 (±81)	10 (±2)	1548 (±116)	1391 (±76)	31 (±3)	14 (±2.6)	12 (±1.1)	0.2 (±0)	0.1 (±0)

Note: TP- *Eisenia fetida* introduced treatment pots.

TP-Treatment pots, *S1 as T1, S2 as T2, average of (S2+S3)/2 forming T3, (S2+S4)/2 forming T4, (S2 +S5)/2 forming T5 and (S2+S6)/2 forming T6*.

Table 6.4 presents the data of total metal content in worms after exposure to substratum treated with sample combinations T1 to T6. Essentially S1 and S2 combinations are controls to compare with rest of the results formulated with OECD soil. Control samples of leaf litter were omitted due to the lack of standard metal content information in the leaves of those species. The worms in both T4 containing

OECD soil + MHF and T3 containing OECD + CHF accumulated the highest concentration of copper (46 mg kg^{-1}) and Zn (13 mg kg^{-1}) respectively.

6.7 Discussion

Irrespective of the sample origin, pH, organic matter content and bioavailability factors are critical components for bioaccumulation in worms (Spurgeon & Hopkins, 1996, Oste et al., 2001). According to Hobbelen et al. (2006), earthworm uptake metals through direct dermal contact or by ingestion and absorption through intestine or both, and soil characters play a vital role to facilitate this process. In a moist condition, usually the heavy metal content in soil is absorbed through dermal contact. However, the organic matter containing samples as in the faecal matter of hare and leaf litter is prominently through the intestinal absorption after digestion of sequestered metals possibly by oxidation process (Suthar et al. 2008). Konecny et al., 2014 reported no metal accumulation in *Enchytraeus crypticus*, exposed to Cu levels above 5000 mg kg^{-1} with the reproduction effect concentration (EC50) was 351 mg kg^{-1}, a value within the range of our treatments.

6.7.1 Bioavailable metal and bioaccumulation

Mine soils are generally sandy with low cation exchange capacity (CEC) (Rayment & Higginson 1992). However, with the leaf litter accumulation on the ground, CEC tends to increase leading to metal sequestration into the organic matter. The exchangeable base cations such as calcium (Ca^{2+}), magnesium (Mg^{2+}), sodium (Na^{+}) and potassium (K^{+}) influence the CEC values of soil (Rayment & Higginson 1992) and the earlier study shows a high level of calcium and sodium in mine soil (Nirola et al., 2015; McKenzie et al. 2004). However, our present study does not show much effect of pH and CEC on earthworm metal uptake, a finding similar to Konecny et al. (2014). With regards to higher electrical conductivity (EC) as in treatments 3 and 4 (CHF and MHF respectively), soil salinity seems to have facilitated bioavailability of

metals (Owozori et al., 2008). It is also evident that the water holding capacity (WHC) of substratum is important for earthworms to carry normal physiological functions. In this case the samples CHF and MHF have better moisture retention capacity, therefore aid in bioaccumulation (Table 6.1). Soil with low pH in the mines increase the metal toxicity to plants and organisms. In a study of Peterson et al. (1984), the toxicity of Cd to algal uptake strongly increased with increasing pH. However, no specific literature is available with regards to leaf litter and faecal matter experiment data.

6.7.2 Population assessment in microcosm

The survival of worms is 100% in all the treatments until the second week of exposure to various treatments indicates their ability to initially tolerate mild metal stress (Neuhauser et al. 1985, Clemens S, 2006). Treatment T1 containing control soil shows a good reproduction rate as per result of juvenile count but the weight loss is higher due to the lack of organic substances, a finding similar to Neaman et al. (2012).

There was a low to mild toxicity on the earthworm survival and juvenile rate in T3 and T4 containing OECD and faecal matter formulation. The T5 treated worms had a good reproduction and weight retention rate due to the presence of *Eucalyptus camaldulensis* litter that created a favourable environment for reproduction. The T6 treatment had lowest juvenile production, probably due to a higher level of cadmium toxicity as reflected in Figure 6.1b, a result consistent with the findings of Li et al., 2010.

Besides the earthworms feeding on leaf litter, the herbs grazing by hare and their droppings eaten up in turn by earthworms is contributing bioavailability as presented in Figure 6.1 a. On the contrary, the bioaccumulation of Cd is very high for treatment T6 containing *Acacia pycnantha* litter, even when the total copper concentration is 645 mg kg^{-1} in the source. In T1, the weight loss is due to lack of enough food and in T6 mortality is due to copper toxicity, a result consistent with the

findings of Spurgeon & Hopkins (1995). Overall the survival percent, weight retention and juvenile production were lowest in treatment T6.

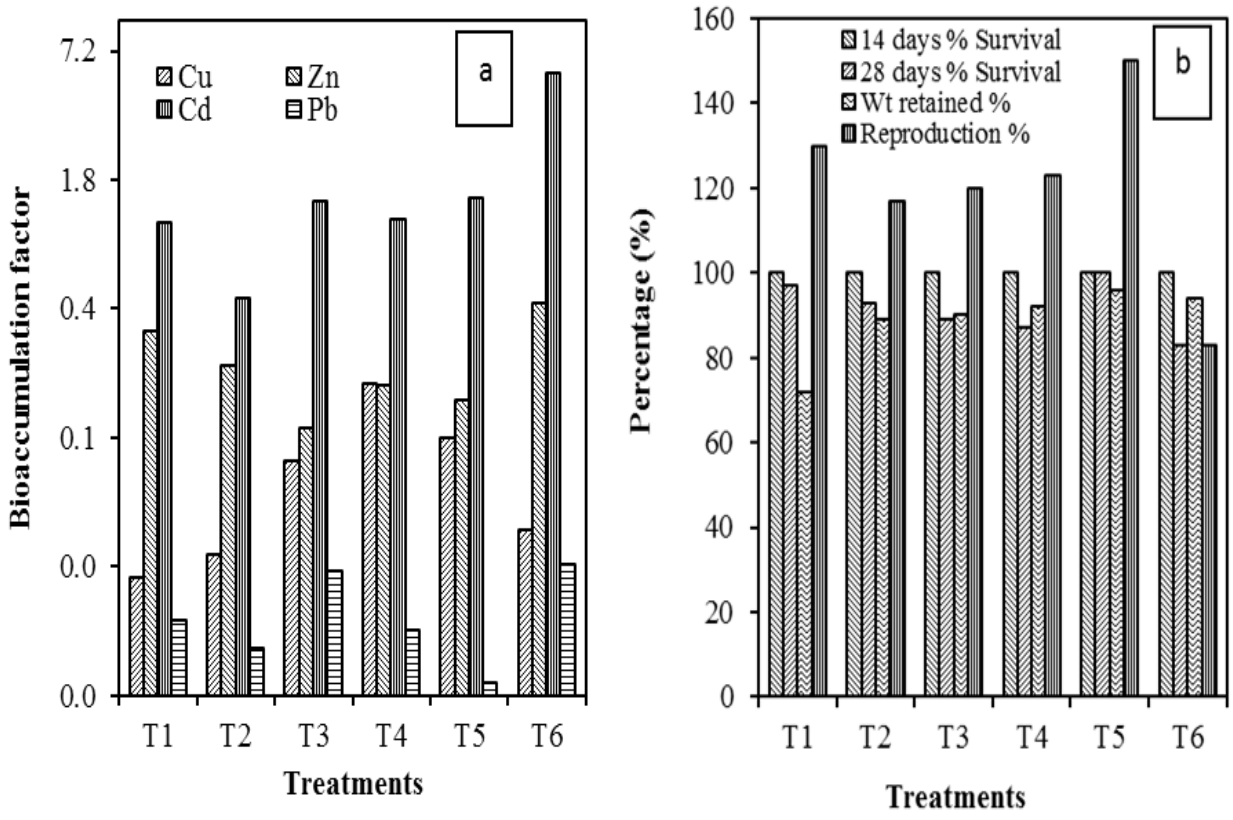


Fig 6.1 with X axis labels different

a. The earthworm microcosm health and population census with different metal concentration and substratum treatments, and

b. Bioaccumulation $_{(log)}$ factor of *Eisenia fetida* against the total metal concentration in treatment samples

6.7.3 Metal fraction and bioavailability

Generally, the water soluble fraction of metals is phytoextracted due to its ionic property to get transported, binding along with water through vascular bundles in plants (Mukhopadhyay & Maiti 2010). Analysing the exposure of heavy metals Cu,

Zn, Cd and Pb to worms through hare faecal matter and leaf letter of trees shows that the water soluble fraction of metals are highly bioavailable, a finding consistent to Li et al (2010) on bioavailable metal concentrations in pig manure.

The eco-toxicological risk is also high for worms feeding on mine site litter treatments as in S5 and S6 where the bioavailability of water soluble fractions of Cd and Pb as shown in Fig. 6.2a is very high (van der Oost et al., 2003). The bioavailability of Zn in artificial OECD soil S2 was higher than control soil S1 is also reflected in bioaccumulation of more Zn in T2 in Figure 6.2b (Spurgeon & Hopkins, 1992).

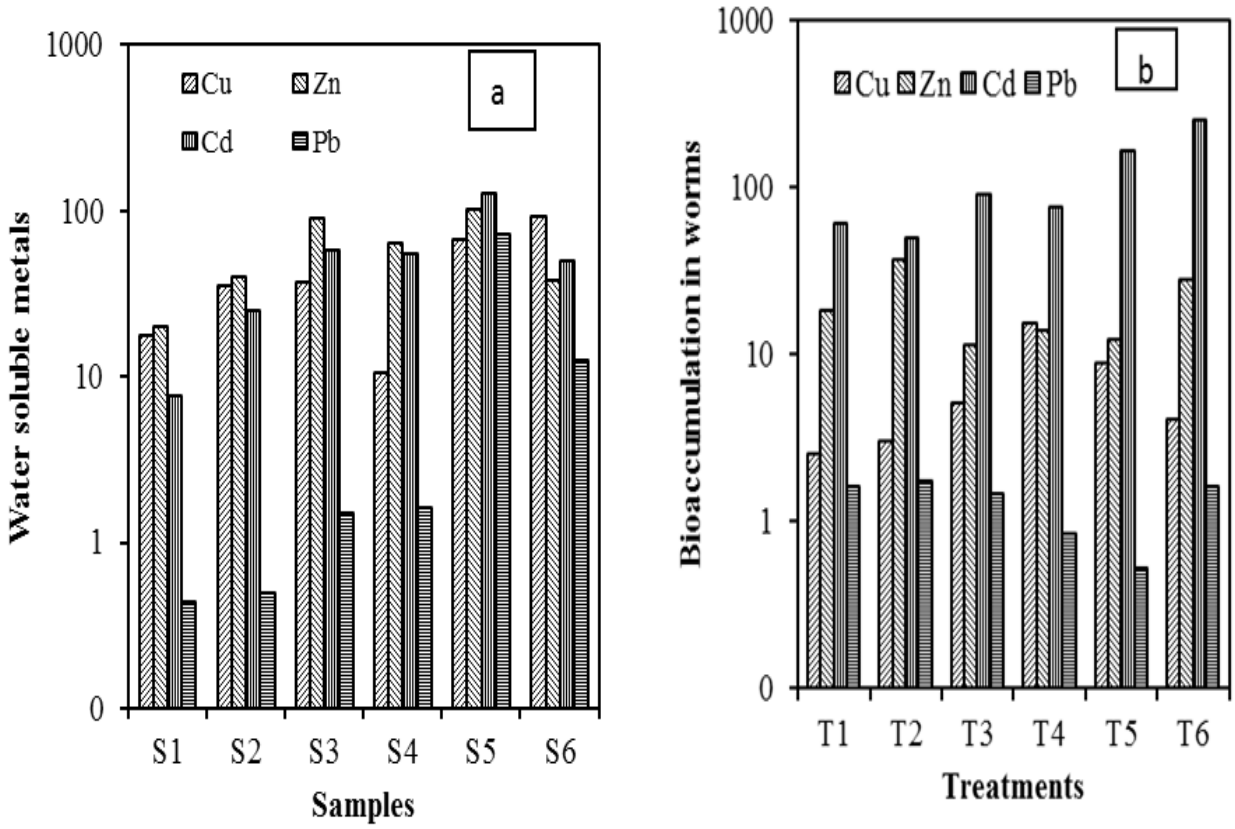


Fig 6.2 Log transformed percent expressions of metal bioavailability in BCR metal analysis with different Y axis labels.

a. Actual bioavailable percent (log) of metals from BCR extracted water soluble metals in substratum

b. Actual bioaccumulation percent $_{(log)}$ in worms from total BCR extracted metal in each treatment pot 6

However, in an earlier study of Spurgeon & Hopkins, 1999; the major source of metal accumulation in earthworm was soil and not leaf as in present case. So, the Zn is possibly coming from OECD soil. The bioavailable metals such as Zn in earthworm tissues exposed to OECD soil could be due to the presence of readily available ions that are water soluble as artificial soil like OECD contains kaolin clay where the metal cations are not sequestrated into such clays and have free charged ions (Spurgeon & Hopkins, 1995).

Figure 6.2(b) presents a high level of Cd bioavailability followed by Zn and Cu, which corroborates with the findings of Chapman *et al.* (1996) on evaluation of bioaccumulation factors. Dai et al. (2004) also reports about bioaccumulation factor of Cd being the highest in the sequence Cd>Zn>Cu>Pb on his work related to heavy metal accumulation by earthworms which is in agreement with our finding (Figure 6.1b & 2b). Ren et al. (2015) reports that the exchange kinetics of isotopic Cd is higher in several mine soils. The highest copper bioaccumulation in T4 may be due to high concentration in sample of hare faecal matter including the water soluble form (Figure 6.2b). In a metal elimination experiment of Spurgeon & Hopkins (1996); it was found that the worms regulated other metal accumulation in their bodies but Cd was the metal that kept increasing in the worm's tissue (Veltman et al., 2007). However, the correct determination of metal transfer cannot be obtained with basic tests and this study is purely focused on mine site litter and hare dropping preliminary study.

6.7.4 Microscopic examination and statistical linkages

A study in the UK on copper tolerant population of worms by Arnold et al. (2008) found that the worms can tolerate a high Cu concentration in soils. The question is to find whether copper gets deposited in the tissue of worms if it can tolerate toxicity.

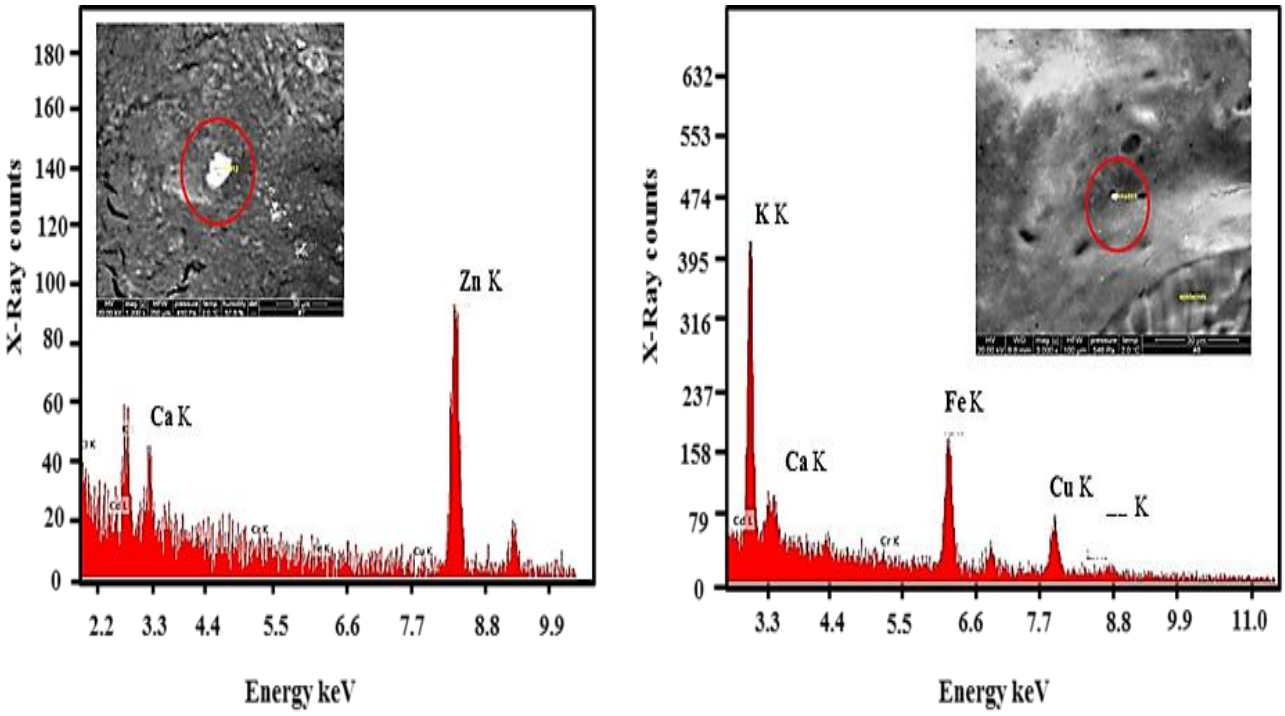


Fig 6.3

a. ESEM image and EDX spectrum of worm exposed to T3

b. ESEM image and EDX spectrum of worm exposed to T4

Although, ESEM examinations cannot be validated, it helps to support the result of total metal analysis by ICP-MS in earthworm tissue. As has been opined earlier, the metal speciation is also an important factor in bioavailability (Li et al., 2010) and is probably dependent on the food source. It is evident from the ESEM analysis that the metals are deposited in the tissue according to the level of concentration of metals in the food source as in T3 and T4 (Figure 6.3a and b).

Generally the values of bioconcentration in worms decline when the soil concentration increases (Romijn et al., 1994), and microscopic examination aids to validate to some extent to explain this issue. According to Romjin et al. (1994), the experimental data of microcosm is suitable for low concentration tests but the microscopic examination of earthworms exposed to highly contrasting treatments can help to compare the effect of extremes of metal exposures. The ESEM spectral data of endodermal layer of *Eisenia fetida* reveals that earthworms introduced in control hare faecal matter show a high level of Zn X-ray counts in T3 whereas the worms introduced in copper mine hare faecal matter (T4) shows high X-Ray counts for Fe and Cu in the tissue (Figure 6.4 a and b) which also corresponds to the results in Table 6.4, and Figure 6.1a.

6.7.5 Soil metal homogeneity and uptake relationship

Bioavailability of heavy metals in living organisms is related not only to its substrate concentration but also to chemical chelation and complexation in the soil and the ecophysiological condition of the organisms (Dai et al., 2004). To draw an idea on chemical complexation, it is necessary to find whether the metals in the samples have some kind of affinity or linkages with the other. In Fig. 6.4a, the alkali and alkali earth metals (Na, K,Ca) fall within one group secluded from transitional metals (Cu,Zn,Cd) projecting a possible selective nature of intake of metals by earthworms. A study of Nannoni et al., 2011 on uptake and bioaccumulation of heavy metals in Northers Kosovo also suggests that the heavy metal bioavailability in tissue for transitional metals showed similar linkages. This indicates a common channel of uptake of alkali metals and transitional metals exists earthworms.

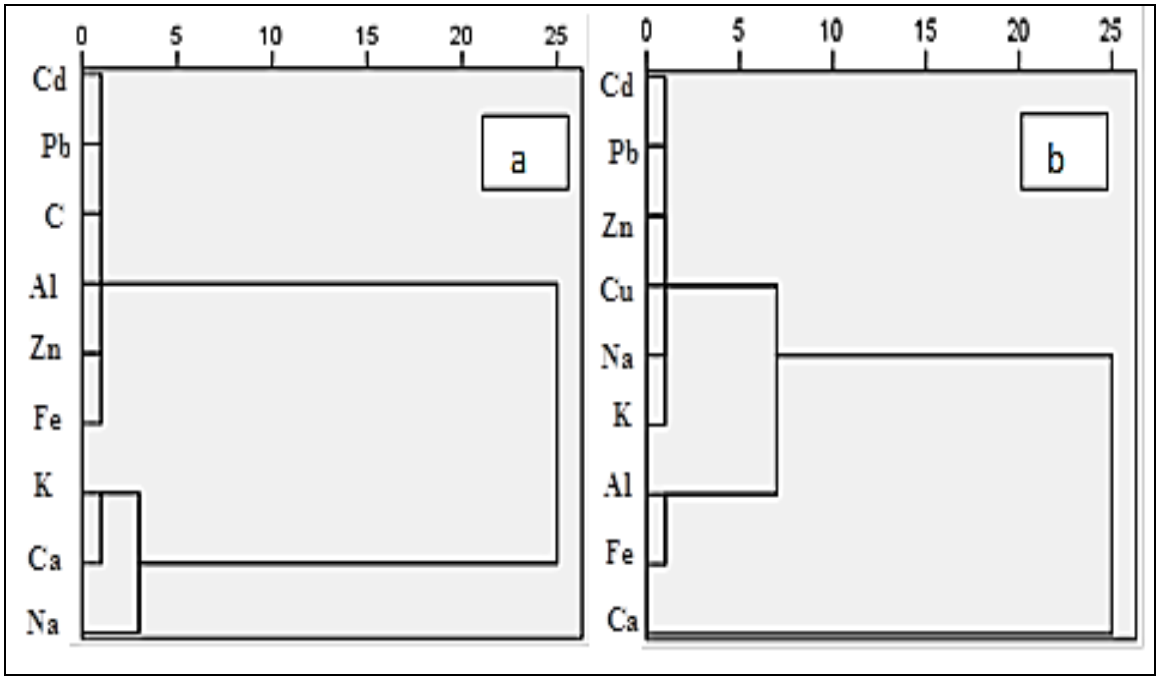


Fig 6. 4

Dendrogram using Ward Linkage with Rescaled Distance Cluster Combine of metal bioavailability and metal homogenisity

a. Metal bioavailability linkage in earthworms' tissue

b. Metal homogenisity linkage in sample treatment soils

Figure 6.4b predicts soil metal homogeity and uptake relationship distribution of all the metals that are homogenous. Though the homogeneity is mostly uniform except for Ca, the uptake of metals by worms are selective. In Fig. 6.4 (a&b), Pb and Cd are linked in tissue as well as in soil supports the findings of Roodbargen et al. (2008), that these two metals including mercury were highly selective and transferable among many other metals. This is being studied in birds the Black-tailed godwit. Hence, it is not only the high soil concentration and homogenisity but it is the bioavailability and uptake potential of metals like Cd that leads to a worst form of food chain contamination (Fig.6.2b) for Cd to be in the range of high bioaccumulation percent (Ren et al., 2015). In another interesting finding in Korea by Kim & Koo (2007), the feather concentrations of Cd and Pb in Black-crowned

Night Heron and Grey Heron chicks were also elevated depending upon amount of Pb and Cd (excepting other metals) concentration in the diet (and possibly worms) to further validate the consequences of metal speciation and homogenisity.

The research prospect for future holds promising with the use of metallophytes of several types of hyperaccumulators to determine the impact on ecosystem and the food chain contamination including human health in the long run. The question of secondary poisoning by metallophyte herbs to worms through feces or through litter to birds via worms is a question that needs to be further investigated. If the metal toxicity impact on organisms gets diluted in the course of time, we need proof so that this chapter gets closed for ever. Some of the technical difficulties during experiment is a length of time involved to determine the entire chain of ecotoxicity in organisms linked to the food chain. The soil sample S1 in Fig.6.2a shows lower water soluble metals, due to low electrical conductivity/salinity as discussed earlier needs further examination. Moreover, the multiple sets of experiment setup is required to carry out such microcosm experiment in different physical conditions.

6.8 Conclusion

The bioaccumulation of heavy metals in worms and specifically copper is not found very significant through leaf litter bioavailability. The total metal concentration in faecal matter is higher with copper being 435 mg kg^{-1} that could bring higher risk to food chain contamination. The leaf litter of *Acacia pychantha* feeding earthworms produced less juvenile (90%) than *Eucalyptus camaldulensis* (152%). With high copper concentrated samples being exposed to worms, the bioavailability factor and bioaccumulation values were Cd directed with as high as 7 in *Acacia pycnantha* litter in our present study. A routine monitoring of post operational mines, contaminated and revegetated sites and operational mines to determine ecological health and metal speciation is highly recommended for industrialists, stake holders and government bodies worldwide.

Chapter 7

STRESS RESPONSES AND SPECIFIC METAL EXCLUSION ON MINE SOILS BASED ON GERMINATION AND GROWTH STUDIES BY AUSTRALIAN GOLDEN WATTLE

7.1 Introduction

An extensive use of heavy metals, pesticides, petroleum oils, hydrocarbons, and chlorinated hydrocarbons are highly responsible for harming the environment (Megharaj et al., 2011). With the trend of rural to urban migration of humans, the scarcity and demand for land has increased pressure to reclaim land for use. As the ancient mining city grew larger, the mined and contaminated land got encroached raising concerns for human health and safety (Balabanova et al., 2010). Copper finds in South Australian cities of Kapunda (our sampling site) and Burra in the early 1840s and gold rush in New South Wales and Victoria in the early 1850's are just a few examples that have created abandoned mines and cities (Lottermoser, 2010).

A scientifically coordinated phytoremediation technology is the need of the modern era because of its low cost green remediation technology (Schwitzguebel, 2004). Metals such as Zn, Ni, Cu, V, Co, W, and Cr bring phytotoxic effects to plants of which Cu, Zn, and Cd are readily absorbed by plants as bivalent cations (Schutzendubel & Polle, 2002; Foy et al., 1978, Clemens, 2006). Globally, the attempts to rehabilitate abandoned and post operational mines have been practised for decades, and the human assisted revegetation program in Broken Hill, Australia in 1936 is probably the first one (Lottermoser, 2010).

Several researchers on this area have added knowledge regarding the metal tolerant plants (Sperlich et al., 2015), but *Acacia pycnantha*, the national flower (green and gold colour) of Australia remains least explored. It is a native, soil enriching (leguminous), drought tolerant and metal resilient plant that thrives well in coastal calcareous soils (McInnes & Brockwell, 2008). Since the semi-arid and arid mines are typically difficult to revegetate (Johnson & Putwain, 1981), golden wattle can

serve as a pioneer species to establish on barren soil (Nirola et al., 2015a). However, some species if Acacia are reported to be a weed in South Africa and other parts of the world (Van Wilgen et al., 2004; Kull et al., 2008). Dixon et al., 1878, p.54 in Australia quotes a farmer *"For every wattle that was growing on my land twenty years ago there are at least a score now"* (Lunt, 1998).

Traditionally, re-greening has been practiced as a low cost alternative to remediate soil pollution (Pilon-Smits, 2005). Sheoran et al. (2010) are of the view that native species are better adapted and are less susceptible to stress for revegetation purposes (Cetinkaya & Sozen, 2011). In Australia, *Acacia acinacea* has been a popular revegetation species for south east region (Broadhurst et al., 2006). Some popular plants used for revegetation program in northwest Africa and Mediterranean regions are *Eucalyptus* spp., *Pinus radiata*, *Acacia cyanophylla*, *Atriplex nummularia*, *A. canescens*, *A. lentiformis*, *A. amnicola*, *A. undulate*, *Agave americana*, and *Prosopis* spp. (Le Houerou, 2000). However, these species are not based on native attribute and belong to different taxonomic positions bearing different phylogenetic backgrounds as well as physiological capacity that adapt to metalliferous soils (Nirola et al., 2015b).

Whether native or not, plants growing over metal contaminated sites are stressed to varying degrees due to pollution, salinity, low pH, lack of soil nutrition and harsh climate posing a serious hindrance to plant restoration (Kramer et al., 2000; Sharma and Dietz, 2009). Very few plant species are adapted to manage physiological stress by avoidance (water conservation in stem by cactuses) (Shepherd & Wynne Griffiths, 2006; Punz & Sieghardt, 1993) or by desiccation tolerance (exhibited by "resurrection plant" *Selaginella lepidophylla* and *Myrothamnus flabellifolia*) (Moore et al., 2009). Stress indications in plants are communicated by hormones and stress related proteins (Dalvi & Bhalerao, 2013; Foy et al., 1978). The earlier studies state that phytochelatins are involved in metal stress through proline production as an expression of defence mechanism (Schützendübel & Polle, 2002;

Bassi & Sharma, 1993). Therefore, measure of stress in plants is evaluated by analysis of proline and chlorophyll concentration (Pätsikkä et al., 2002).

We take germination study, stress and metal uptake behaviour by exposing golden wattle to copper mine soils in glass house. The seeds are exposed to control and contaminated soil environments. Plant biometry is studied over a 16 weeks period and biomass until 14 months after sapling transplantation into the glass house. The analysis of chlorophyll a and b, carotenoids and proline are performed in the laboratory (Maxwell & Johnson, 2000). Response of salt and metal stress and determination of total metal uptake factors including the pore water metal composition are conducted. We aim to investigate physiological traits of golden wattle to check its metal tolerant mechanism.

7.2 Objectives

The objectives of this experiment is to study a metallophyte in detail regarding its germination, growth and stress behaviour in different mine soils. Even though a revegetation program may be accomplished, the real situation needs to be addressed pertaining to the sustainable establishment of the particular plant species. Therefore the aim of this experiment is an eco-physiological study of *Acacia pycnantha* on different level of copper concentrated soils.

- To germinate mine site wattle seeds in laboratory under heat treatment and two irrigation conditions.
- To study the biometry related measurements of golden wattle grown on six types of soil samples from the mining area.
- To determine the uptake factors of golden wattle under controlled growth conditions.
- To elucidate the stress level and impact on plants grown over different soil conditions of the samples from an abandoned copper mine.
- Study of biochemical production from plants such as proline and chlorophyll.

- To try to relate the semi-arid and arid rehabilitation issues based upon the results.

7.3 Hypothesis

The plants are stressed even though they are identified as metallophytes. Does stress make a long term issue to achieve sustainable mine site rehabilitation? Do we need to really consider the plant stress data to consider phytoremediation?

7.4 Experiments

One of the most important part of this thesis is the analysis of proline and chlorophyll production by Acacia pycnantha on different mine site soils as outlined in the following table.

Title	Procedure	Analysis
Soil sample and seeds of *Acacia pycnantha* collection	Strategically sites were selected to collect varied soils. Seeds were collected from the golden wattle bushes	Soil characterization, total metal analysis Seed treatment and germination trials
Plant tube stock preparation, Soil sieving and potting preparations	Nursery planting. Pots were prepared to conduct pore water analysis. Holes on the rim of pots were drilled for rhizon sampler insertion	ICP-MS analysis of soil solution from rhizon samplers. Plants were harvested for root, stem and leaf concentration of different metals
Leaf extracts to determine proline, chlorophyll a,b and carotenoids.	Use of spectrophotometer to determine the biochemicals in three seasons during its growth.	Proline, chlorophyll a, b and carotenoids were analysed based upon the materials and methods outlined in 7.5
Biomass analysis and statistical tools.	Biomass of pre harvest and post-harvest data were used to estimate the stress level. Minitab, ANOVA, SPSS tools are used for statistical analysis.	The biomass chart of above ground, below ground and fresh as well as dry weight in percent as well as the principal component analysis between metals and biochemical were conducted

7.5 Materials and methods

Soil samples were collected from abandoned copper mine site following a 3 x 3 parallel spot quadrat sampling technique to represent forested land and open pit area (Nirola & Jha, 2013). As indicated in Fig.7.1, sample 1 (HC) was collected near the Mathews Quarry, sample 2 (E) from Eucalyptus jungle, sample 3 (ECE) from Acacia-Eucalyptus jungle, sample 4 (RS) is a road side fresh soil, sample 5 (M) from mine tailing and sample 6 (ECR) from Eucalyptus jungle near the road. The seeds of golden wattle *Acacia pycnantha* were collected from 3(ECE) and 5(M) sites.



Fig 7.1 Map of Kapunda mine site with soil and litter sampling spots (marked in numbers) in South Australia

7.5.1 Soil characterization

Fresh soil samples were collected in a polypropylene container at a depth of 15-20 cm from the spot numbers 1-6 indicated in Fig 7.1. The soils were air dried, sieved

through a 2-mm plastic mesh and stored in separate PVC buckets. The electrical conductivity (EC), soils acidity (pH), cation exchange capacity (CEC), water holding capacity (WHC), texture, carbon and nitrogen content of the experimental soils were determined. Soil slurry of 1:5 soil to water ratio (w/v) was prepared to take the EC and pH readings. The values of total dissolved carbon (TC), total dissolved nitrogen (TN) and total organic carbon (TOC) of soil samples were estimated by a CNS analyzer (TOC-L Shimadzu, Japan). A 5 g each of oven dried sample (80 ºC) was loaded into boats for total carbon (C%) and total nitrogen (N%) analyses using Trumac (Leco® Corporation, Michigan, USA) CNS analyzer. To maintain quality control, standard blank, control and cross-referencing samples were introduced after every 10 readings. Soil texture was determined using hydrometer method, water holding capacity (WHC) by oven drying method (Gardner & Klute, 1986) and cation exchange capacity (CEC) by Cu (II)-trien method (Rengasamy & Churchman, 1999; Rayment & Lyons, 2011). As outlined in USEPA 3015a and 6020a methods respectively, digestion of total metals in soils by *aqua-regia* and plant by conc. HNO_3 were conducted and the metal concentrations were determined by either inductively-coupled plasma optical emission spectrophotometry (ICP-OES) (Perkin Elmer Optima 5300V) or inductively-coupled plasma mass spectrometry (ICP-MS) (Agilent 7500ce, Agilent Technologies, Tokyo, Japan) (http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs). Although the study site was an abandoned Cu mine, the sample analysis focussed on all metals and metalloids namely As, Ca, Cd, Cr, Cu, Fe, K, Na, Ni, Pb and Zn in relation to *Acacia pycnantha* in the study site.

7.5.2 Seed germination

Seeds were collected based on an idea from our earlier research about this plant growing successfully over the mined area (Nirola et al., 2015a; Nirola et al., 2015b). The *Acacia pycnantha* seeds were collected from standing and fallen pods. Firm ones were selected and packed in zip-lock bags. In the laboratory, seeds were washed

with deionised water and treated with 1.5 g L^{-1} Mancozeb fungicide (Brown et al., 2003) in fume-hood. Seeds were rinsed, immersed in 2% sodium hypochlorite (NaOCl) solution for 5 sec. The sterilized seeds were divided into separate glass beakers A and B. The beaker A seeds were soaked at room temperature for three days and heat treated for 30 minutes at 40 ^{o}C (Brown et al., 2003). The beaker B seeds were left soaked for 3 days and left without heat treatment. Seeds were incubated/germinated over three category soils namely: pure-sand, mine soil from spot 1(HC) and with a mixture between mine soil and sand in the ratio of 1:1 in a 200 g PVC made commercial germination pots. The three category pots were subjected to two types of irrigation systems. Irrigation type-1 used 2 ml of watering daily in each pot while the irrigation type-2 used 8 ml of watering weekly to roughly mimic daily rainfall and occasional rainfall (Fig 7.2). Each pot was sown with ten seeds and was left for germination at room temperature. The germination numbers were recorded on a weekly basis for next four weeks.

The following scheme was used for germination with ten seeds in each category:

Irrigation	Heat treated (Seeds A)			Non heat treated (Seeds B)		
Daily (Type I)	Sand	Sand & Soil	Soil	Sand	Sand & Soil	Soil
Weekly (Type II)	Sand	Sand & Soil	Soil	Sand	Sand & Soil	Soil
4 weeks →						

7.5.3 Plants cultivation

The entire rhizosphere zone of plantlet was scooped out by a plastic spoon to avoid damage to roots and was carefully transplanted into tube-stock pots containing 230

cm^3 cc of potting mix each. The potting mix comprised of 70% composted pine bark and 30% peat moss. The fertilizer content per cubic metre of potting mix was 1 kg of nutricote slow release fertiliser (NPK = 17.6:2.9:6.9), 500 g of nitroform containing 38% slow release nitrogen, 2.5 kg of calcium carbonate as a powder and 500 g of flowtrace trace elements containing 24% ferrous sulphate, 0.75% copper sulphate, 0.5% manganese sulphate, 0.2% zinc sulphate, 0 .04% molybdenum as sodium molybdite, 0.033% boron as sodium borate. A 500 g m^{-3} of saturaid (an inert soil wetter) for immediate rewetting of the potting mix was also applied. A daily wetting was conducted using automatic irrigation system in a nursery green house for three months before transferring to the glass house.

PVC buckets (10 L) were purchased from local store and a 4 mm diameter hole was drilled at the rim area in laboratory for inserting the rhizon sampler procured from Rhizosphere Research Products, Wageningen, The Netherlands. Each soil sample was wetted with deionised water for one week before transferring into 10 L PVC buckets. Plant saplings in the tubes were acclimatized in glass house for two weeks following which each sapling was carefully transferred into the PVC buckets and filled with soil samples. After two weeks of plantation, biometry recording was continued for 16 weeks by measuring the height and number of leaves every fortnight till the leaves started shedding. Filtered water (300 ml) was irrigated manually every alternate day to each pot using a measuring jar.

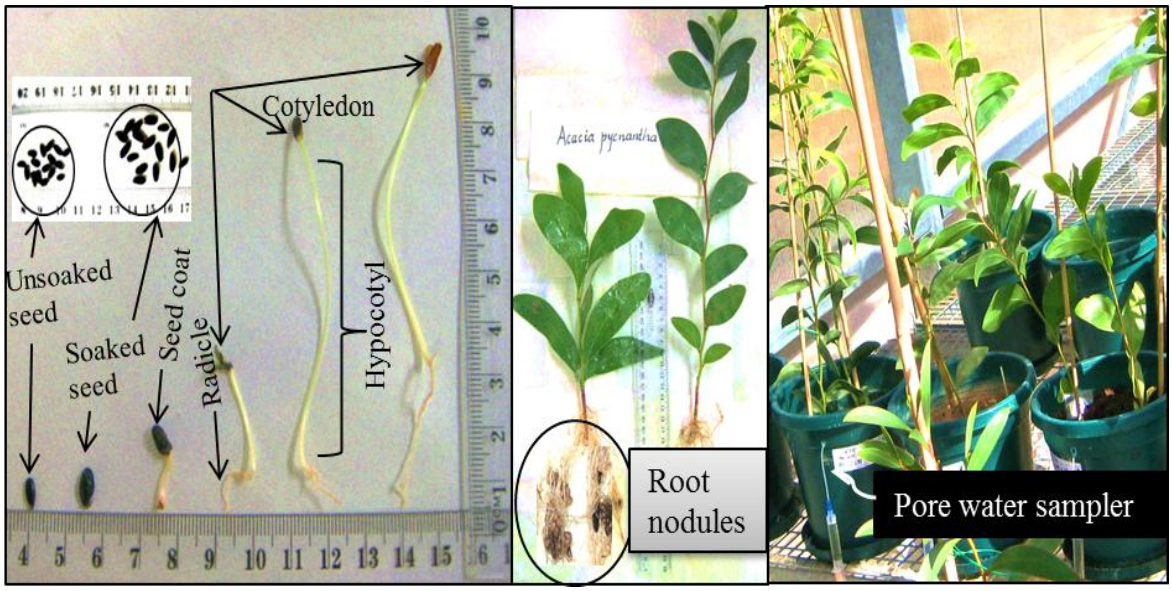


Fig 7.2 Process of *Acacia pycnantha* seed germination, growth and nodulation, and pore-water collection in glass house experiment set-up

7.5.4 Pore-water sampling and analysis

The hydrophilic pore water sampler tips were inserted through the rim hole while loading soil samples at a mark 6 cm above the base and in the centre of the PVC buckets (Fig. 7.3). After every two months, pore water from each experiment pots were drawn out in 10 ml syringes through fitted microfiltration membrane having nominal pore size of 0.12 - 0.18 µm. Once the syringe was plunged out, a wooden plunger holder was fitted at the handle of the syringe to continue sucking pore water into the syringe. The vacuum drew water out from soil pores until the syringe was filled. The wooden holder was detached and sample pore water was transferred into centrifuge tubes for processing in the lab. Pore-water was diluted 1000 times in deionised water and adjusted in 10 ml ICP tubes to perform ICP-OES analysis. Three pre analysis trials were conducted while drawing out pore water and during dilution due to errors in ICP-OES readings (Beesley et al., 2010) and for quality control.

7.5.5 Biochemical analysis of proline

Leaf extracts were prepared by using 1 g of plant material initially homogenizing in 20 mL of 3% aqueous sulphosalicylic acid (w/v) by crushing with pre-chilled mortar and pestle. The extract was purified by centrifugation at 8,000 rpm for 20 min. Supernatant (2 mL) was taken in a test tube and 2 mL glacial acetic acid and 2 mL acid ninhydrin was added. The mixture was heated in water bath for 1 h at 100 ^{0}C. The reaction was terminated by placing the sample laden test tube in ice bath. Another 4 mL toluene was added to the reaction mixture and stirred well for 20-30 sec. Toluene layer was separated and brought to room temperature. The red colour intensity was measured at 520 nm using a UV-3600, Shimadzu Uv-Vis-Nir spectrophotometer. A series of standards with pure proline was prepared by dissolving L-proline (range = 0.1-5.0 µg mL^{-1}) in 3% sulfosalicyclic acid (w/v), and run repeatedly to prepare a standard curve.

The amount of proline in the test sample was finally determined from the standard curve (Tripathi et al., 2013) and expressed fresh weight basis as follows (Equation……. 1)

$$\text{m molg}^{-1}\text{ tissue} = \frac{mg\frac{proline}{mL} X\ mL\ toluene}{115.5} \text{ X } \frac{5}{g\ sample}.$$ where,

115.5 = molecular weight of proline.

7.5.6 Spectrophotometric determination of chlorophyll a, b and carotenoid

A 1 g of leaf sample from each treatment was washed with deionised water and air dried. The sample was ground in 96 % methanol and centrifuged at 1000 rpm for one min. The supernatant was extracted by filtering through two layer cheese cloth (Türlerinde et al., 1998). The process was repeated for residue until all chlorophyll was recovered to finally make 100 ml by adding methanol (Zengin & Kirbag, 2007).

The absorbance was read in BIO-TEK, Synergy HT photometer at lambda 470, 646, 652 and 662 nm. The calculation was done using following expressions (Wellburn, 1994);

(Equation..... 2)

Chlorophyll A ($\mu g\ L^{-1}$) = 15.65 A_{662}-7.340 A_{646}

Chlorophyll B ($\mu g\ L^{-1}$) = 27.05 A_{646}- 11.21 A_{662}

Carotenoid ($\mu g\ L^{-1}$) = 1000 A_{470}-2.860 C_a- 129.2 C_b/245

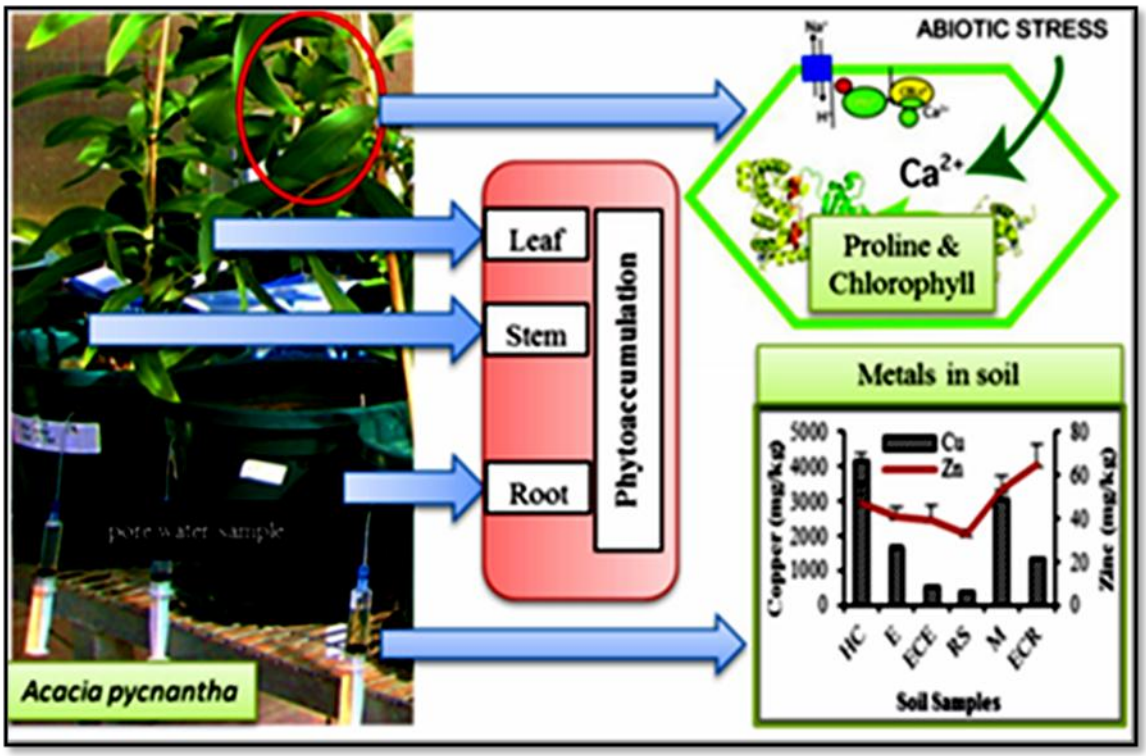


Fig 7.3 Experimental process for analysis of leaf, phytoaccumulation of metals and pore water activity in mine soils.

7.5.7 Total metal analysis of plants

Plants were harvested by separating roots from the soil after 14 months into the glass house. The physical characteristics of roots and their nodule status were recorded. Each plant was washed with deionised water, rinsed thrice; gently dried and fresh

weight was taken, packed in paper bags and oven dried at 85 °C. The dry weight was measured and powdered using a steel grinder. Powdered plant sample (0.5 g) was treated with 10 ml concentrated nitric acid (Ajax Chemical, Australia, 70% w/w), transferred in Teflon vessel and digested in a microwave (CEM-MAR6, USA) digester. The digested extracts were filtered through 0.45 µm nylon filter and diluted with deionised water for analysis. The analysis was carried out using ICP-MS, as outlined in USEPA 6020a methods. The pore water samples were directly determined by ICP-OES using Perkin Elmer Optima 5300V as stated in 7.5.4 above. The metal enrichment factors (Yoon et al., 2006; Sagiroglu et al., 2006; Nouri et al., 2011) were calculated as

(Equation..... 3)

Bio-concentration factor: root soil quotient; ($BCF = \frac{\text{Root}}{\text{Soil}}$) and

Translocation factor: stem root quotient; ($TF = \frac{\text{Shoot}}{\text{Root}}$) and

Enrichment factor: soil leaf quotient; ($EF = \frac{\text{Above ground parts}}{\text{Soil}}$).

7.5.8 Statistical analyses

Microsoft Excel and Microsoft paint were used to prepare the figures. Pearson's correlation tests on data of proline and chlorophyll contents against the metal uptake in plants were employed using IBM®SPSS®21 software package for Windows. Principal Component Analysis (PCA) was performed using Minitab® on metal uptake by plants and biochemical data. One way ANOVA including Tukey's test for root-metal concentration, stem-metal concentration and leaf-metal concentration were also performed.

7.6 Results

The results of soil EC, pH, CEC, water holding capacity, texture, nitrogen and soil organic matter are presented in Table 7.1 below. The average electrical conductivity ranged from 448 to 1364 while the pH ranged from 4 to 7.6. The soil samples had high ranging values of EC and pH while rest of the parameters were almost similar among each other except the total organic carbon in ECE sample was high. The soils were generally sandy with overall low CEC, N% and C% content, a condition generally found in mine soils.

Table 7.1 Characterization of soils from six sampling sites around Kapunda mine site (n=3, ±SD)

Soil	EC	pH	CEC (cmol +kg^{-1})	WHC (%)	Sand (%)	Silt (%)	Clay (%)	TOC mg l^{-1}	N %	C %
HC	1184 (±80)	7.2 (±0.1)	7.9 (±0.1)	23 (±1.0)	77 (±0.6)	21 (±0.5)	2 (±0)	32 (±1.2)	0.1 (±0.0)	2.6 (±0.1)
E	355 (±20)	7.2 (±0.2)	8.2 (±0.0)	21 (±3.8)	82 (±0.6)	15 (±0.2)	3 (±1)	33 (±4.6)	0.1 (±0.1)	2.3 (±0.3)
ECE	213 (±16)	7.3 (±0.1)	8.2 (±0.2)	19 (±1.0)	66 (±1.2)	26 (±1.5)	8 (±1)	68 (±3.6)	0.2 (±0.0)	2.3 (±0.2)
RS	448 (±2)	7.6 (±0.2)	7.5 (±0.1)	22 (±0.6)	89 (±1.5)	14 (±1)	7 (±1)	30 (±1.0)	0.2 (±0.0)	2.8 (±0.1)
M	1364 (±39)	4.0 (±0.0)	7.2 (±0.2)	27 (±1.2)	75 (±1.0)	22 (±1.5)	3 (±0)	10 (±0.6)	0.1 (±0.0)	0.9 (±0.1)
ECR	1256 (±39)	6.5 (±0.0)	8.3 (±0.2)	21 (±1.2)	78 (±1.0)	15 (±1)	7 (±0.5)	12 (±0.6)	0.2 (±0.0)	1.6 (±0.1)

7.6.1 Germination and biometry

Germination percent of heat treated seed was showing up to 85% in contrast to 20 to 30% sprouting per pot for the non-heat treated seeds. The heat treated and daily irrigated seeds germinated faster and vigorously. In daily irrigated treatment, sand was a preferred media for germination while in the weekly irrigation system, mine soil M performed better. Survivals of germinated seeds in heat treated pots were better than the non-heat treated batches (Fig.7.4 a&b). Moreover, the germination was successful even in a saline and acidic soil (M) with poor nitrogen and organic matter content.

The studied plants resulted into varied height gains as growth progressed over time (Fig.7.4 c&d). While HC and ECR plants swiftly gained the height in 8 to 10 weeks, plants in soils E, RS and M were stunted. The number of leaf formation also followed similar trend of growth. The soils HC, RS and ECE had a good proportion of healthy root nodules visible during harvest of biomass (Fig 7.2). The biomass of fresh above ground weight (ag), fresh below ground weight (bg), dry above ground weight (ag) and dry below ground weight (bg) are presented in Fig.7.6 a. The soil in pot 5 containing soil M followed by pot 2 containing soil E had the least biomass. The fresh weight and dry weight above ground value in pot ECE was also low.

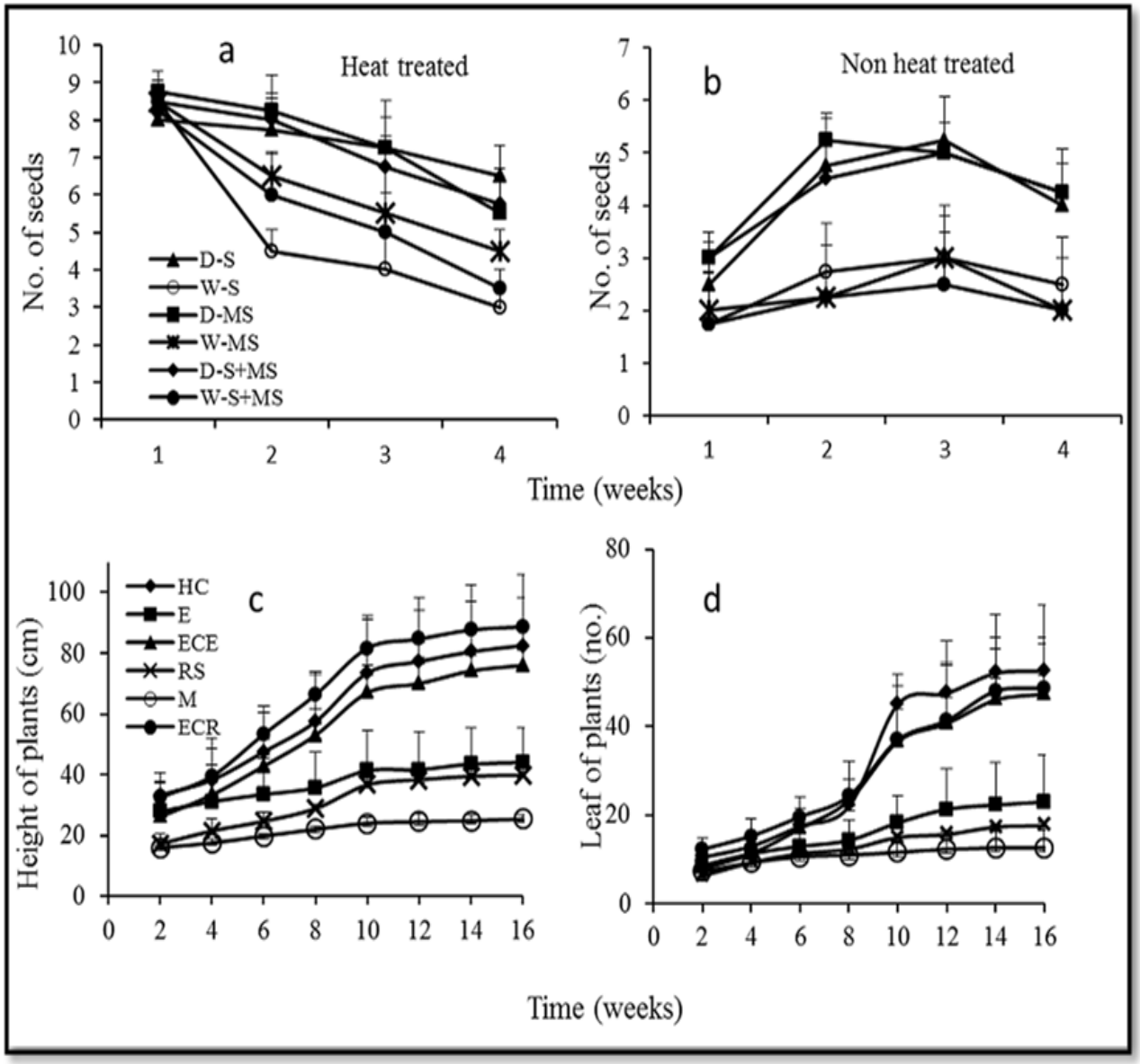


Fig 7.4 Germination and biomass study

a. Germination and survival pattern of heat treated seeds and

b. Non-heat treated seeds under different irrigation system (where *D-S*; daily watering sand, *D-MS*; daily watering mine soil, *D-S+MS*; daily watering sand and mine soil, *W-S*; weekly watering sand, *W-MS*; weekly watering mine soil, *W-S+MS*; weekly watering sand and mine soil);

c. Height gain by *Acacia pycnantha* over 16 weeks in different soils, and

d. Leaf formation of *Acacia pycnantha* in different soils till 16 weeks after transplantation

7.6.2 Pore-water analysis and biochemical attributes

The total Cu availability in pore water collected from sample M was 409 mg kg^{-1} during the first sampling and remained almost constant throughout the growth stages. However, total Cu availability in pore water constantly increased in pot E as experiment progressed. In the pore water of HC, ECE, RS and ECR, the As and Pb availability was low until the fourth month, but got increased till eighth month (Fig.7.5a).

The average proline production in plants grown in soil-M was highest with 2050 µmol g^{-1} tissue followed by 2010 µmol g^{-1} tissue in soil-ECR plant. The RS-soil plant sample showed the lowest 400 µmol g^{-1} tissue of proline among all the analysis. The chlorophyll a level in all the plants showed a decreasing trend as the experiment progressed. Carotenoid activity was almost constant in all the soil samples. The proline activity showed an increased level in HC and RS plants and constant in ECE and ECR plants, but decreased in E and M as the time progressed (Fig 7.5b).



Photo plate V- *Acacia pycnantha* grown in Glass house. Note the different colour of leaf and growth.

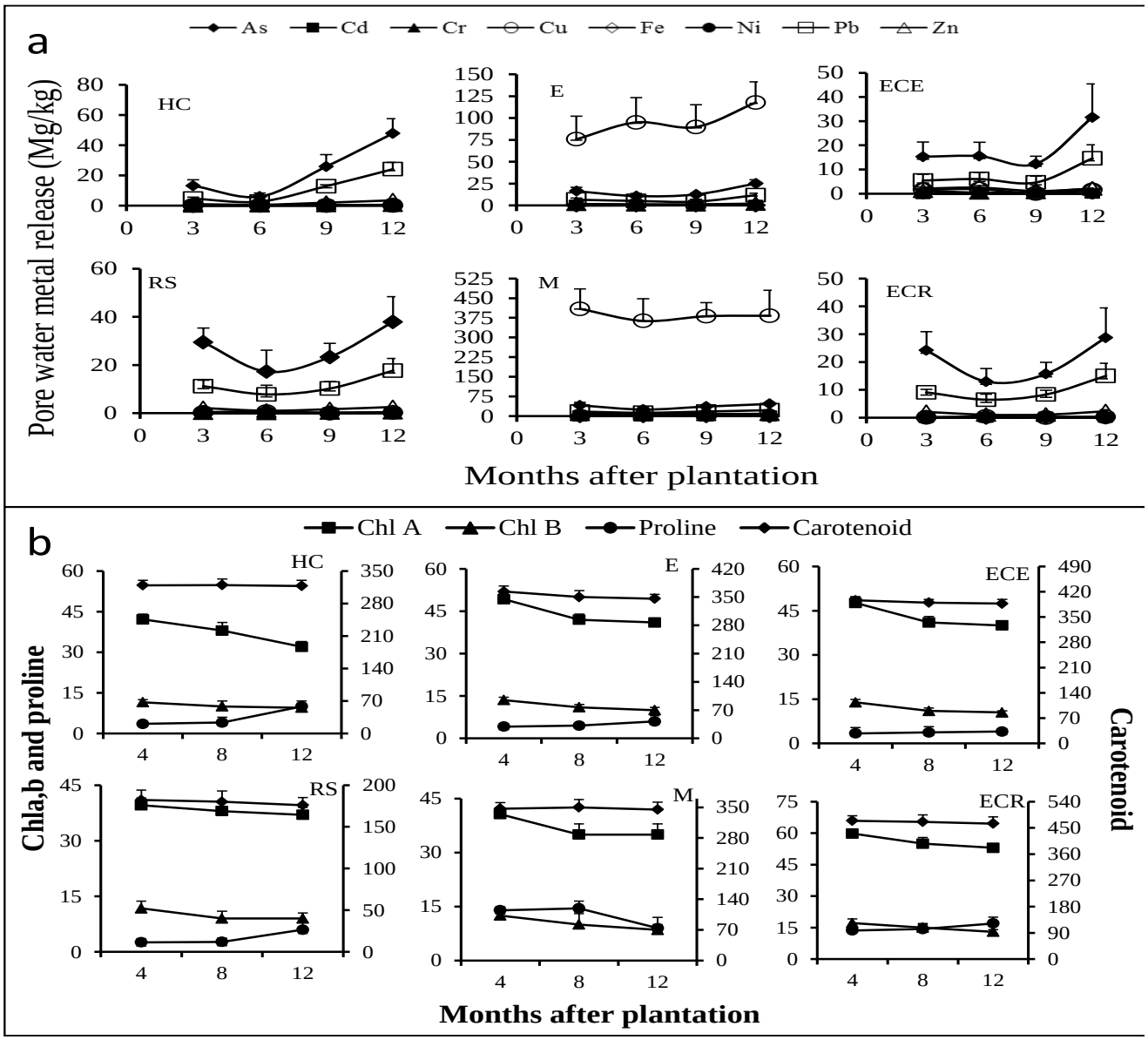


Fig 7.5 Changes in pore water dynamics and biochemical attributes

a. The pore water metal dynamics showing prominent As, Pb and Cu activity

b. Response of proline and chlorophylls over time on metal polluted soil.

7.6.3 Metal accumulation and biochemical correlation.

The metal uptake in plant parts grown on different soils are presented in Table 7.2. Generally *Acacia pycnantha* exhibited a resilient behaviour with below 20 mg kg^{-1} copper accumulation in stem and leaf portion in all pots. With copper concentration

of 4083±103 mg kg^{-1} in soil, the HC leaf accumulated only 18±2 mg kg^{-1}. In case of RS, the copper accumulation in leaf was 7±1 mg kg^{-1}, in line to its low concentration in soil. However, the accumulation of calcium and other salt elements were high in roots (4284±711 mg kg^{-1} in M), stem (6177±389 mg kg^{-1}in M) and leaf (11648±1209 mg kg^{-1} in HC & 10816±389 mg kg^{-1} in M). Cd, Ni and Cr had a very low concentration in mine soils. Zn in soil was below 40 mg kg^{-1} in all samples.

Table 7.2 Total metal in soil and bioaccumulation in different plant parts such as $_S$-Soil, $_R$-Root, $_{St}$-Stem, and $_L$-Leaf. (±SD, n=3)

	HC	**E**	**ECE**	**RS**	**M**	**ECR**
As $_S$	46±1	66±0	69±0	30±0	59±0	31±0
Ca $_S$	14129±1143	19303±70	22282±539	12301±506	21200±200	1001±316
Cu $_S$	4083±103	1667±41	575±22	384±26	3112±16	1382±22
Fe $_S$	1291±42	721±103	1163±151	1111±99	786±46	1000±202
K $_S$	7008±317	7597±635	7833±193	8434±674	7887±289	7829±419
Na $_S$	7657±10	8917±28	10082±110	10946±326	9472±88	8333±133
Zn $_S$	44±4	42±4	34±1	34±5	48±1	62±3
Ca $_R$	4100±11	2486±286	2588±503	2956±982	4284±711	3567±680
Cu $_R$	175±13	343±3	65±2	94±1	156±1	111±2
Fe $_R$	363±10	328±5	391±2	472±2	269±0	445±3
K $_R$	2929±557	1504±860	3195±1641	1695±162	2337±37	2873±708
Na $_R$	510±60	1254±367	688±293	713±53	491±57	485±188
Ca $_{St}$	5628±401	4264±503	2368±528	3442±537	6177±389	3538±917
K $_{St}$	7995±136	7430±503	7271±543	6464±114	5972±762	6149±126
Na $_{St}$	266±147	2499±522	574±76	956±33	240±34	284±57
Ca $_L$	11648±1209	6310±503	6787±528	6387±537	10816±389	7818±91
Fe $_L$	78±9	76±21	101±11	119±12	89±14	62±4
K $_L$	7303±488	4179±503	5166±154	4814±114	6216±762	6692±126
Na $_L$	1243±155	4345±1522	3846±187	4902±33	1117±134	1856±57

Values of metals in soil, roots, stem and leaf below 20 mg kg^{-1} are omitted

A negative correlation between Cr and Cu is found between chlorophylls, carotenoid and proline. The regression values are not significant between metals and chlorophylls or proline either. However, there is a significant negative correlation between Cr and carotenoids likely impacting on plant health as presented in Table 7.3. There is a negative correlation between As, K, Ni, Pb and Zn against chlorophyll a, b and carotenoids including proline. Duncans comparison of sets of mean values in root revealed (2)↑ for Na, Mn, Co, Ni, Cu, and Zn, with (2)↓ for Cr, (3)↑ for Cd and Pb, and (4)↑ for Cu. There was no significant difference in mean values of metal in stem while in leaf the values were as wide as (4) ↑ for K and Ca.

7.7 Discussion

Not much literature is available regarding the plant *A. pycnantha* growth and behaviour over mine soils and similar conditions. The germination, growth and stress tolerance mechanisms against metalliferous soils of abandoned mines are an interesting subject discussed here. Heat treatment of seeds favoured dormancy breaking with 85% germination and quicker growth even under acidic and saline condition. A similar finding was also reported earlier by Martin et al., 1975 and Maslin et al., 1998 on legume seeds and wattle seeds respectively.

As such, the mine soil M containing high Cu concentration with acidic condition did not impact negatively on germination. This may be due to self-sufficiency of seeds with glucose in cotyledons to supplement food. It is evident that sufficient moisture on top of heat excitation strikes germination with activation of respiratory mechanisms leading to growth due to protein synthesis (Kubala et al., 2015). However, the same growth vigour of nursery was not visible after transplantation in the glass house environment, which reflects physical and chemical stress and growth loss prevalent under mine site conditions.

Table 7.3 Correlation table of biochemical parameters and metal uptake in plant parts

	***ChlA*$_4$**	***ChlA*$_8$**	***ChlA*$_{12}$**	***Chl B*$_4$**	***Chl B*$_8$**	***ChlB*$_{12}$**	***Caro*$_4$**	***Caro*$_8$**	***Caro*$_{12}$**	***Pro*$_4$**	***Pro*$_8$**	***Pro*$_{12}$**
As	-0.17	-0.24	-0.23	-0.22	-0.30	-0.33	-0.12	-0.12	-0.13	-0.50	-0.48	-0.54
Ca	0.01	-0.05	0.01	0.01	-0.05	-0.03	0.00	0.01	0.00	-0.36	-0.39	-0.54
Cd	0.30	0.30	0.30	0.36	0.35	0.37	0.31	0.32	0.32	0.71^^	0.65^^	0.61^^
Cr	-0.54	-0.49	-0.47	-0.54	-0.47	-0.44	-0.59**	-0.58**	-0.58**	-0.30	-0.27	-0.32
Cu	-0.31	-0.36	-0.38	-0.33	-0.36	-0.39	-0.22	-0.20	-0.22	-0.09	-0.07	-0.09
Fe	0.64^^	0.64^^	0.66^^	0.70^^	0.70^^	0.74#	0.54	0.55	0.55	0.21	0.13	0.10
K	-0.09	-0.14	-0.10	-0.11	-0.23	-0.24	-0.17	-0.16	-0.18	-0.35	-0.35	-0.41
Na	-0.31	-0.41	-0.35	-0.24	-0.39	-0.40	-0.39	-0.40	-0.41	0.15	0.19	0.16
Ni	-0.22	-0.31	-0.22	-0.17	-0.29	-0.33	-0.15	-0.14	-0.16	-0.20	-0.19	-0.35
Pb	-0.09	-0.16	-0.12	-0.09	-0.20	-0.26	-0.09	-0.08	-0.10	-0.24	-0.24	-0.30
Zn	-0.14	-0.15	-0.15	-0.06	-0.04	-0.04	-0.08	-0.08	-0.07	-0.16	-0.14	-0.06

*The critical value of correlation is 0.576 for p=0.05 and 0.708 for p< 0.01. Symbol ** is negative at p<0.05 levels (no very strong negatives), # positive at p < 0.01, and ^^ significant at p < 0.05*

Biometry data further reveal that in soil sample M, plant was stunted and showed limited leaf formation. This may be due to low pH (4) and high soil copper concentration (3112 mg kg^{-1}) leading plant toxicity. Küpper et al., 2003 reported that metals and particularly Cu, Zn, Ni and Mn in high concentration brought severe phytotoxic effects. The initial sluggish tendency of plant growth and low biomass production in M soil sample indicated that substratum pH had a major role on limiting the plant growth. In HC (Cu- 4083 mg kg^{-1} and pH 7.2), the growth and leaf formation pattern was similar to reference soil sample RS containing low soil copper concentration (384±26 mg kg^{-1} and pH 7.6). Therefore, metal solubility and toxicity was pH dependent, a finding similar to Conesa et al. (2006) in a heavy metal accumulation study in Spain.

The metal uptake in plants also depends upon soil metal bioavailability, influenced by the physical and chemical properties of soil (Punz & Sieghardt, 1993). The accumulation of Fe and Na in leaf was higher than other metals in plants grown on each PVC bucket. However, the regression analysis did not show a significant result with either chlorophyll or proline. The stress tolerance mechanism in *A. pycnantha* is therefore still anomalous even though this species has been identified earlier as copper stabilizer and a metal tolerant plant (Nirola et al., 2015a; Nirola et al., 2015b). The pore water metals have been used earlier to predict bioavailability in plants exposed to different pollutant sources (Kidd et al., 2007). The pore water metal concentration in soil samples E and M exhibited a higher mobility of Cu in soil which coincides with gradual proline production by E and M soil plant. This may be an indication of copper toxicity. Here, free copper ions in the pore water were thought to induce the proline production (up to 15 μg g^{-1} tissue) in metal tolerant *A. pycnantha*. A similar observation was made by Schat et al., (1997), in a metal tolerant plant *Silene vulgaris*. Therefore, bioavailability, phytoaccumulation, and plant stress leading to biochemical production is an interrelated mechanism (Navari-Izzo & Quartacci, 2001).

In the pore water again, As and Pb mobility in HC, ECE, RS and ECR increased from 6 month onwards coinciding with the increase in proline content from 5 μg g^{-1} to 20 μg g^{-1} tissue (Fig.7.5 b). This indicates that metals such as As, other than copper were also perhaps involved in creating the stress to plants (Sharma, 2012) leading to rise in proline, a case of mixed metal contamination. The proline production might not be only due to metal stress but also by other factors like salt, drought, soil organic matter content and pH (Kishor et al., 2005). Clemens (2006), also viewed this behaviour of As and Pb to be a major concerns for plant metal uptake in acidic pH soils. Consequently, there is As and Pb mobility in our samples pore water having basic to neutral pH soils except in M sample. This is unlike the findings of Gambrell et al. (1991) which reports that metals are highly mobile in acidic condition. However, the current As and Pb activity in pore water may be actually due to root and rhizosphere activity (Khan, 2005; Burd et al., 2000) which is yet to be ascertained.

The stress is a point of metabolic state in plant which loses its potential to be fit to grow further in a given environmental condition (Elstner et al., 1988). Metal toxicity over plant leads to stunted growth, disarray in chlorophyll formation and prone to attacks by diseases (Assche & Clijsters, 1990). Several studies have reported about the decrease in chlorophyll content due to metal stress of copper (Singh et al., 2007), including Cu substitution by Mg in the chlorophyll molecule (Küpper et al., 2003).

A negative correlation between Cr and chlorophylls, carotenoid and proline (Table 7.3) suggests that metals such as Cr induce inhibition of chlorophyll synthesis (Backor & Dzubaj, 2004), and this supports our finding that Cr reduced the yield of chlorophyll content in the plants grown on mining soils. Evidently, there is a positive correlation between iron content and chlorophyll concentration (Table 7.3). The metal Cd showed a positive correlation with proline. It indicates that Cd induced *A. pycnantha* to produce proline to overcome stress. In this study, the effect of metal stress was evident with a decreasing value of chlorophyll a, b and carotenoid contents as the growth stages progressed. While reduction of chlorophyll content was due to metal stress, increase in proline content could also be due to salinity and

osmotic stress (Kubala et al., 2015, Nounjan et al., 2012). Checking back the salinity level of the soils and proline content, it was found that the HC (having the highest EC) grown plant showed a sharp increase in proline level (Fig.7.5b) which proves our assumption.

Biomass is related to plant health and chlorophyll content including stress (Filella et al. 1994). Our biomass study of harvest reveals that the initial weight of plant is independent of its gain or loss in biomass after planting into the soil samples. Besides soil HC being highly concentrated with Cu, the plants did gain significant biomass similar to the reports of Lasat (2000). However, the soil M did not yield a better biomass compared to the other soil samples (Fig. 7.6a). As per Figure 7.6 b, metals including Cu analysed in the soil samples are grouped in the negative side of first component revealing their reduced influence on the biochemical response of the plants grown on metal contaminated soils. However, the metals such as As and Ni lied in the positive side of the first component might be contributing to influence the production of chlorophyll, carotenoids and proline. Moreover, the proximity of Ni and As to proline in PCA (Fig.7.6b) also reflected that there is a mixed stress of metals other than Cu.

The As activity in pore water has rendered a higher proline response, the finding reported in Indian mustard by Khan et al. (2009). Iron content in soil has a positive influence on chlorophyll production in *Acacia pycnantha* while chromium negatively influenced the production of carotenoids and cadmium but instead induced proline production (Di Toppi & Gabbrielli, 1999; Sinha & Saxena, 2006). Similarly, in a study of leaf of *Phaseolus vulgaris*, the excess copper did not affect chlorophyll content, but the Fe substitution of copper took place (Patsikka et al., 2002). In wheat seedling, proline production was induced by Zn and Cu toxicity (Bassi & Sharma, 1993). Besides proline, the chlorophyll pigment level in plant could also be influenced by several environmental factors like light intensity, nitrogen deficiency and nutrient imbalances (Turlerinde et al., 1998).

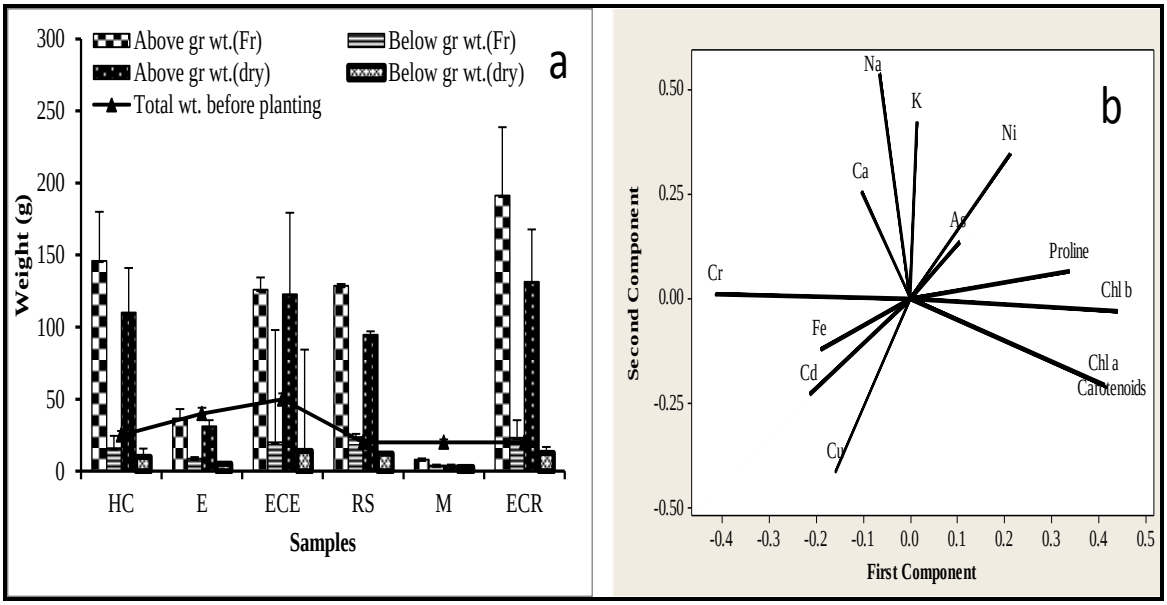


Fig 7.6 Plant growth and metal relationship with bio-chemicals

a. Plant biomass rates of potted plants on different soils harvested after 14 months. (below gr-*above ground, below gr-below ground*).

b. Relationship between soil metal concentration and biochemical parameters of plants.

The uptake factor values of metal in soils revealed that *A. pycnantha* has a mixed ability to exclude copper in soil. Specifically, the BCF values of ≥1 are considered to be characters of stabilizer plants (Lorestani et al., 2011), which did not reflect with *A. pycnantha*. Since BCF is calculated considering the root uptake of metals and not the stem or leaf, it may not be directly hazardous to the environment above the ground. Instead, TF and EF are based on values of metal transfer to aboveground parts such as stem or leaf which holds a significant importance on exposure of metals to environment. Therefore, the value of TF ≤ 1 is considered to be an effective stabilizer (Yoon et al., 2006). As such *A. pycnantha* is a copper excluder due to its low (TF ≤ 1) translocation factor (Table 7.4).

Table 7.4 The plant uptake factor of metals as in BCF (bio-concentration factor) TF (translocation factor) and EF (enrichment factor)

	Soils	HC	E	ECE	RS	M	ECR
As	BCF	0.01	0	0.01	0.01	0.01	0.01
	TF	0.07	0.04	0.11	0.05	0.07	0.04
	EF	0	0	0	0	0	0
Ca	BCF	0.29	0.13	0.12	0.24	0.2	3.56
	TF	**1.37**	**1.71**	0.9	**1.16**	**1.44**	0.99
	EF	**1.22**	0.55	0.41	0.8	0.8	**11.34**
Cd	BCF	0.52	2.05	0.81	0.88	0.98	2.28
	TF	0.74	0.51	0.57	0.16	0.06	0.81
	EF	0.98	**2.48**	**1.93**	0.79	0.54	**4.16**
Cr	BCF	0.06	0.06	0.08	0.07	0.06	0.13
	TF	0.09	0.23	0.11	0.11	0.02	0.05
	EF	0.07	0.07	0.13	0.07	0.08	0.11
Cu	BCF	0.04	0.21	0.11	0.24	0.05	0.08
	TF	0.04	0.03	0.12	0.05	0.04	0.08
	EF	0.01	0.02	0.04	0.03	0.01	0.02
Fe	BCF	0.28	0.45	0.34	0.42	0.34	0.45
	TF	0.03	0.05	0.03	0.02	0.04	0.03
	EF	0.07	0.13	0.1	0.12	0.13	0.07
K	BCF	0.42	0.2	0.41	0.2	0.3	0.37
	TF	**2.73**	**4.94**	**2.28**	**3.81**	**2.56**	**2.14**
	EF	**2.18**	**1.53**	**1.59**	**1.34**	**1.55**	**1.64**
Na	BCF	0.07	0.14	0.07	0.07	0.05	0.06
	TF	0.52	**1.99**	0.83	1.34	0.49	0.59
	EF	0.2	0.77	0.44	0.54	0.14	0.26
Ni	BCF	0.76	0.71	0.08	0.16	0.08	0.29
	TF	0.4	0.25	0.25	0.18	0.3	0.23
	EF	**1.03**	0.73	0.07	0.08	0.12	0.26
Pb	BCF	0.02	0.02	0.02	0.02	0.01	0.02
	TF	0.18	0.23	0.18	0.13	0.13	0.1
	EF	0.04	0.03	0.06	0.02	0.03	0.01
Zn	BCF	0.1	0.39	0.25	0.29	0.07	0.21
	TF	0.96	0.89	**1.3**	0.45	**1.13**	**1.46**
	EF	0.48	0.8	**1.06**	0.57	0.42	0.65

7.8 Conclusion

Our study on the growth of *A. pycnantha* in copper contaminated mine soils revealed this plant as a metal stabilizer. The plants exhibited stress based upon the soil factors. This leguminous species showed resilience to adverse conditions due to low metal enrichment in its above ground parts. Its ability to tolerate salinity, acidity and low nutrition level of soils was evident through its germination and growth responses. This species with adequate watering could germinate and thrive on mine soils and thus could be recommended for revegetation in arid and semi-arid climatic zones. While this wattle species could exclude Cu uptake, the As and Cd seems to be toxic to it.



Photo plate VI- ICP-OES analysis under progress

Chapter 8

GENERAL DISCUSSION AND RECOMMENDATIONS

8.1 Introduction

This chapter concluding the thesis focuses on major research findings including discussion and recommendations derived from the experiments. The major focus of research has been screening of vegetation, ecotoxicological experiments using earthworms and plant stress tests as presented in Fig. 8.1.

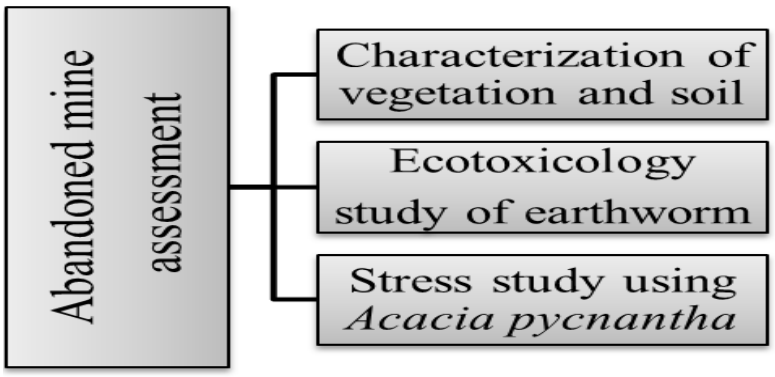


Fig 8.1 Flow chart of major research areas

These experiments and methodologies were also aimed at finding the most revegetation friendly plant species and technology at a given ecological and environmental condition present in arid and semi-arid mines.

As has been mentioned in previous chapters, a rampant revegetation attempt leads to failure of achieving a sustainable rehabilitation goal. This research attempts to establish a basic understanding that the rehabilitation is plant and pollutant specific. In fact, the attempt is to find whether native species alone are sufficient to cater for the revegetation of mines without any ecotoxicological side effects and pollutant exposure as a result of phytoaccumulation. An attempt is also made to understand

the degree of plant stress in achieving a climax community within a vibrant ecosystem.

8.2 Metallophyte screening and ecotoxicity tests

Biodiversity is a key element to sustain life on Earth as its unflinching role of supporting the biogeochemical cycling, provisioning, regulating and beautifying the environment makes this planet habitable. In case of mining industries, effective biodiversity conservation will bring an increased confidence of investors, communities, partners and non-governmental organizations, and reduces risks and liabilities. As time progresses, nature tends to colonize barren land with plant species to convert nude land into a climax community. In the process, plants either adapt to the environment or the most resilient plants survive the harshness of the area. Hence, it is very important to take stock of metallophytes that are successfully colonizing mine affected areas.

Screening of metallophytes help to identify the species of its phytoaccumulative capacity, based on which the plants are either selected or rejected for the purpose of revegetation. Originally, phytoaccumulation was a mode of land reclamation. However, the danger of pollutants being exposed to the environment through above ground accumulation in plant parts is a concern. Therefore, one of the major objectives of this study was to find out how different species of plants have colonized the abandoned mines as metallophytes.

Study of metallophytes by screening using metal uptake factors helped us to evaluate the most successful species and its attributes for growing in the semi-arid and arid conditions. As such, the golden wattle *Acacia pycnantha* stood up to be one of the most resilient and copper tolerating species growing naturally over years in abandoned mine of Kapunda. Some of the agronomic species such as *Olea europea* also appeared to be copper excluder; however, this plant is a non-leguminous and is also discouraged as a commercial crop to grow there.

Our experiments on *Acacia pycnantha* and *Eucalyptus camaldulensis* regarding the leaf litter toxicity to the earthworm *Easina fetida* opens up a novel finding to prove that the phytoaccumulators do pose a risk of food chain and food web contamination. Therefore the need for metal pollutant specific revegetation involving metallophytes is more convincing. Moreover, to test the ecological health, testing the plant stress such as proline is very important. In this process, we have found that *Acacia pycnantha* is an ideal species to revegetate copper mines in semiarid and arid environments. It is due to its ability to uptake less copper in its aboveground parts, not being very hospitable for worms to thrive and also being very resilient to weather conditions and harsh soil.

There is likelihood of many undocumented species already evolving, present or adapted in nature to carry out remediation process naturally but unknown to the scientific world. For instance, the question of *Crotolaria cobalticola* growing over an unknown soil-status area may be phytoaccumulating cobalt. Likewise, sunflower (*Helianthus annus*) growing over uranium contaminated soil could poison food chain and food web. We need scientific basis to answer these given the fact that phytoremediation is a slow process and a juvenile science compared to other disciplines of environmental remediation. The agronomic plants; *Zea mays* and *Vicia faba* are found to remove toxic chemicals like TNT and RDX from soil. It may prove toxic to humans if consumed directly or via food web by the process of biomagnification.

8.3 Measure of metals from litter into organisms

Abandoned mines are open sources of pollutants. There were no proper laws binding the mining operations. Hence, with the passing of time, land leases lapsed, records were lost and the original ownership of the mines became unclear. Though now abandoned, the legacy of the mining era remains in the form of river and floodplain sediments grossly polluted with metals. Accessible adits, shafts and workings at the abandoned mine sites form open hazards to human and animal life. There is a risk of

transformation of metals into more bioavailable forms or more toxic nature as the ecosystem matures in such abandoned areas. Therefore, the study of food chain and food web ecosystem is very important to decipher the level of bioaccumulation in living organisms including humans.

We attempted to take up two major experiment involving OECD soil, animal litter from mines and plant litter from mines to expose it to earthworm in our microcosm study. We found that there is a danger of bioaccumulation and biomagnification as a result of feeding detrivores over the metal laden litter. However, a careful choice of species and regular monitoring of ecosystem is necessary to avoid any long term ecological disaster. The ecotoxicological studies of the revegetated areas are necessary to also determine the post mined-land health. As the leaf litter of phycoaccumulators contains bioavailable metals, there is a need to take up a thorough study to safeguard faunal biodiversity from getting stressed. Besides, a concern regarding the poisoning of fauna by low scale pollutants as a result of biomagnification is an equally worrisome issue. Indeed, *Acacia pycnantha*, the floral emblem of Australia displayed in the coat of arms passed through metal accumulation and vegetation screening tests (not knowing it was emblem plant) before performing the pot experiment.

However, a comparative ecotoxicological test was necessary before determining this plant to be a pioneer for revegetation in arid and semi-arid conditions. As such, the leaf litter experiment of *Acacia pycnantha* revealed that its metals are not hyperaccumulated in the first place. Secondly, its litter is not highly toxic to earthworms and most importantly not preferred much to be habitable by earthworms. This was proven due to less juvenile production in the *Acacia pycnantha* litter. Therefore it is revealed that the chances of metal transfer into higher trophic levels are lower than the hyperaccumulating *Eucalyptus camaldulensis*. Moreover, the metal content in the litter itself is much lower than the other counterpart *Eucalyptus camaldulensis* indicating that leguminous species is a nitrogen fixing species in abandoned mine reclamation and post mine revegetation.

Measures of plant stress levels are important to reflect plant health and revegetation success. It is a noble finding that favours the Australian emblem plant *Acacia pycnantha* behaving exceptionally different than the rest of the nine prominent screened plant species in terms of metal uptake factors being low in above ground parts. As such we performed a glass house experiment by planting same species on different levels of copper contaminated soils in a pot experiment. This stress experiment helped us to compare the effect of metals in different soil samples on plant. Using the seeds collected from mine site helped us to mimic the mine growing conditions as far as possible. Moreover, the study of metal dynamics using the pore water data helped us to study the metal mobility as a result of irrigation. The study revealed that the plants are stressed due to metals present in the soil. However, the plants were tolerating stress as indicated by excessive proline production based on metal concentration in soil. There were no significant reductions in chlorophylls as the time progressed. Moreover, the metal uptake in plants depending upon the amount of metals present in soil was also demonstrated by the pot experiment with *Acacia pycnantha*.

8.3 Scope of this research

Ferrous metals followed by non-ferrous metals were generated as a result of tremendous destruction to the landscape releasing tons of toxic materials into water bodies and atmosphere. Hence, there is still a big financial burden to overcome pollution and sadly there is a lack of effective and augmentative implementation of programmes of phytoremediation. Besides, there is an anomaly over the kind of species of plants to be introduced over polluted land as a result of the issues of genetic erosion, allelopathism and fears of species extinction.

Phytoremediation is a time consuming process with remediation efforts in Chernobyl under action for the last three decades is a question to be answered. Although, growing phytoaccumulators under intensive crop conditions and harvesting the dry matter is proposed as a possible method of metal removal including from

agricultural soils down to metal concentrations below statutory limits, it is not practical to implement it a massive mining area. A drawback is that the total amount of metal present in the soil may far exceed the capacity of the plants to accumulate that metal, even over an extended time period. Moreover, phytoextraction can only remove metals available in the root zone of the plant, and not in the greater depths.

Use of soil amendments such as metal inactivating additives like coal, fly ash or zeolites has been able to make a good progress to remediate toxic contaminated soils. However, the use of synthetic chelators are found to be creating detrimental effect by leaching underground, and are also found to be unfriendly to the microbial action to carry out symbiotic associations with plants. There is no documentation to inform the subsequent increase of land rehabilitation corresponding to the level of mining growth and per-capita metal consumption. Therefore, if population increase is a threat to natural resources, irresponsible mining is a curse to humanity when a huge patch of land is left dry and barren after the extraction is over. As the process of remediation is implemented, there are unforeseen risks and hazards requiring advanced caution.

Therefore, an alternative to phytoextraction is phytostabilisation, where a constructed ecosystem based on metal tolerant or accumulating plants may be effective in reducing the mobility of heavy metals within the soil. The growth of plants with an ability to tolerate the adverse conditions found on abandoned mine sites increases soil organic matter, which plays an important role in immobilising heavy metals, improves soil structure, increases soil fertility and reduces erosion. This process may also include the use of nontoxic metal-immobilising or fertilising soil amendments to improve plant growth.

One of the reasons for Earth's species diversity and richness is its growth, distribution and adaptation based upon the soil properties and climate. With a good approach on molecular and cellular mechanisms of a suitable plant species, genetic engineering can be attempted to further enhance its ability to remediate the contaminants. In this context, transgenic plants are preferred due to the presence of heavy metal resistant genes. However, transgenic plants pose risk of interbreeding

with native and wild population raising the concern about mutation and genetic erosion.

There are examples of using heterologous genes from microorganisms, plants and even the animals to improve the efficiency of transgenic plants to deal with pollutants. By the use of plant tissue culture, a plant genome can be vitalized by incorporating a foreign genome of a better choice, to produce clones with better characters in turn conserved intact within the tissue. There also is a big scope of characterization of root nodule bacteria of *Acacia pycnantha* to further implement the integrated remediation approaches more effectively.

8.5 Recommendations and future work

The presence of biodiversity determines land quality when considering nutrient cycling, soil formation and primary production as indicators to reclamation. In case of operational mine sites, progressive reclamation can be implemented whereas in abandoned mines the cost burden is high as a result of abrupt attempts to reclaim. Therefore, monitoring and maintenance of soil nitrogen level and containing soil erosion, are some of the important areas of priority during the process of abandoned mine reclamation.

The reclamation, rehabilitation, and remediation (RRR) cannot be achieved by merely removing human-made structures but are achieved by restoring the lost natural property called biodiversity. Restoration is still misunderstood as the decommissioning phase of a mine, placing pressure on managers to revegetate or renew within limited time, which actually take at least a decade to start shaping up as a reformed ecosystem. Moreover, an ideal view on restoration is thought to be achieved by inclusion of aesthetic, cultural, moral, historical, political and social aspects besides others. An adaptive management of mined lands gives a scientific approach to remediate and restore with abundant scope for monitoring and research in the future. Some of the recommendations arising out of this research are

- Ecotoxicological studies using mine dwelling organisms such as rodents' birds and detrivores to determine the biomagnification of toxic pollutants into the food web.
- Microbial analysis, DNA extraction, pyrosequencing study of the rhizobium in the *Acacia pycnantha* root nodules.
- Need for environment impact assessment (EIA), documentation of pre-mining local flora and fauna.
- Conserving topsoil to avoid denuded expanses of regolith, bedrock and tailings emplacements, after mining operations.
- Keeping adequate documentation of accidental hazards, unmarked workings, unstable embankments, and unexpected chemical contamination during operation.
- Managing acidic water entry and exit routes of water channels making it easy to assess the impact.
- Identifying mining and processing methods to avoid the impact of activities beyond the precincts of the original site affecting a larger area.
- The sites often have heritage and scientific significance which needs proper considerations. Most sites are isolated from major settlements so that in many cases cost recovery through subsequent sale of remediated land is not generally feasible.
- Sites may have become the habitat for native fauna and relict populations of native flora, sometimes as a direct result of mine disturbance can complicate restoration.
- Documentation and management of presence of exotic noxious plants and weeds to encourage native flora and fauna.

- A field trial to conduct 'Phyto-instauration*', a new term which is coined as a result of this research outcomes.

What is Phyto-instauration*?

This is a new technical term proposed after the outcome of our review and research for ecological phytoremediation. With regards to revegetation of arid and semi-arid areas and specifically the mined lands revegetation, the introduction of *Acacia pycnantha* as a pioneer species is the first phase of remediation. Once the soil is enriched with organic matter and nitrogen the introduction of non hyperaccumulator species based on the climate, location and soil situations are programmed. Therefore, within a ten years' time, Acacias are phased out with programmed native species to take over. However, this hypothesis needs to be validated and tested under field trials before actually releasing as a technology.

The soil fertility must be restored so that the land can be grassed and trees planted by laying topsoil or substitute materials, the application of fertilizers and seeding. Adequate subsoil drainage will also need to be installed. As recreation plays a major role in the life of a community, mined areas have frequently been reclaimed for such a purpose. Parklands, playing fields and other sporting facilities, marinas, etc. often have arisen from areas of former mine sites.

If buildings are to be erected at an abandoned site, the ground requires adequate compaction and/or reinforcement so that the structures are not subjected to risky settlement. Industrial estates are also often located on restored land, as may be new housing estates. In order to rehabilitate remote contaminated sites effectively, on-site analyses should be carried out to ensure that the materials used to rehabilitate the site are not contaminant-bearing. Understanding the geomorphic setting of the rehabilitated areas is also important in understanding where, and for what period, contaminated materials might be stored in fluvial systems downstream of mine workings.

The mine dumps can be managed by building constructed wetlands, grouting, anoxic limestone drains, bactericides, run-off diversion, and the introduction of sulphate-

reducing bacteria. Many mining companies are introducing several of these technologies. Some of the features that complicate rehabilitation projects are due to lack of extensive and detailed planning. In many instances, work on the site may also require complementary off-site works to protect rehabilitation. It may also be necessary to limit the types of activities permitted on sites either for a short or long-term to safeguard the integrity of rehabilitation.



Photo plate VII- Digging deeper into future

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