BIOREMEDIATION POTENTIAL OF METAL RESISTANT BACTERIA ISOLATED FROM ABANDONED GOLD MINE TAILINGS

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DECLARATION

I, the undersigned, declare that this thesis submitted to the North-West University for the degree of Doctor of Philosophy in Biology in the Faculty of Science, Agriculture and Technology, School of Environmental and Health Sciences, and the work contained herein is my original work with exception of the citations and that this work has not been submitted at any other University in part or entirety for the award of any degree.

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DEDICATION

This work is dedicated to Almighty God for his faithfulness, guidance and infinite mercy over my life, to my loving and wonderful husband, Mr Oluwole Peter Fashola, and my lovely kids: Esther Abisola, Isaiah Oluwatomiwa and Ezekiel Oluwaseyi Fashola.

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GENERAL ABSTRACT

Environmental pollution from gold mining activities has resulted in the generation of large quantities of metals laden wastes, a serious environmental concern worldwide. Metal species are non-biodegradable with persistent and hazardous effect in the ecosystem and its living biota. Hence, there is an urgent need to find suitable treatment approach to the menace of metal species in the environments. This research was designed to screen for prospective metals resistant bacteria that can be utilized as a microbial inoculant in the treatment of metals contaminated environments. The physicochemical properties and the concentration of six metal species nickel (Ni), cadmium (Cd), zinc (Zn), cobalt (Co) and arsenic (As) peculiar to gold mine tailings were determined from three abandoned gold mine tailings: mine tailings A (MA), B (MB), Tudor shaft (TS) and their surrounding soil (MAC, MBC and TSC) in Krugersdorp, South Africa. Metals were extracted using aqua regia and their concentrations determined using atomic absorption spectrometry. The pH values of most of the samples were generally acidic ranging from 2.17-6.79 with a mean value of 6.34. The physicochemical properties and metal species contents of the MA, MB and their surrounding soils are similar while TS shows different properties and levels of metals. The physicochemical properties of the three sampling sites shows that they are deficient in nutrient for bacteria growth and activity. The mean concentration of the metals recorded in TS were; As (612.5) Co (490.09), Ni (2,247), Pb (46.11) and Zn (2555.43) in mg/kg which all exceeded the South African recommended values for soil and sediments. The maximum value of 36.25, 260.70 and 48.69 mg/kg recorded for Pb and Zn in MAC and MBC also exceeded the recommended standard values of South Africa soils and sediments, while the metal concentrations recorded in MA and MB still fall within the recommended standard. The elevated levels of metals recorded in these sampling sites, especially the TS, shows that the environment and human health around the tailings are at risk and there is an urgent need to find suitable approach to treat these sites. As a result of high concentration of Ni, Pb and Zn recorded in the sampling sites, bacteria tolerance to higher concentration of these metals and their mechanisms of resistant were carried out using conventional biochemical and molecular techniques. A total of 65 metals resistant bacteria were isolated from the three sampling sites using Luria Berthani agar supplemented with Ni, Pb, Zn, Cd and Co. Twenty eight (28) of these isolates showing distinct morphological characteristics were selected and subjected to higher concentration of Ni, Pb and Zn ranging between 2-9 mM. All the isolates shows tolerance to multiple metals but the most promising isolates, OMF 003 and OMF 532

show resistance to 5, 9 and 7 mM of Ni, Pb and Zn respectively. All the isolates were physiologically and biochemically characterized and the results shows that these isolates belong to the phylum Proteobacteria, Actinobacteria and Firmicutes with the optimum conditions for growth at temperature of 37°C, 5.0 and 7.0 pH and NaCl concentration of 2-4% (w/v). The isolates were further subjected to ternary mixture of metals and 12 isolates showing best growth were selected and their identity confirmed using 16S rRNA. Sequencing of the 16S rRNA gene and phylogenetic analysis of the nucleotide sequences determined from the 16S rRNA gene shows that these isolates belong to the genus *Bacillus, Enterococcus, Enterobacte*r, and *Alcaligenes* sp. with 78-100% sequence similarity. Eight of the isolates did not cluster with other bacterial isolates on the phylogenetic tree which shows that these bacteria strains could be probable novel metal species resistant bacteria based on their distinctness. Screening for the presence of plasmid revealed that most of the isolates possessed a single plasmid and they were screened for the presence of metal species tolerant genes (*NccA, P3 P4, PbrT* and *Czc ABD*) on both plasmid and chromosomal genomes. Multiple metal species tolerance bacteria are abundant in the sampling sites and their identity were confirmed. Among these metal tolerant bacterial isolates, OMF 003 and OMF 532 identified as *B. cereus* KX485323 and *E. asburiae* KY000697 were selected based on their ability to tolerate higher concentrations of multiple metal species. The bioaccumulation capacity of the individual and consortium of these bacterial strains to ternary mixture of Pb, Ni and Zn were carried out using ICP-MS analysis. The environmental conditions affecting uptake of the metals were also determined. The results shows an increasing uptake pattern for the three metals at 48 h at concentration of 1 mM to 3 mM while the uptake declines at 5 mM. The two isolates shows increased percentage removal for the three metals at temperature of 37°C, pH 5 and 7, while reduction in percentage removal was seen at pH 3. The mean percentage removal by *E. asburiae* and *B. cereus* for Zn was (29.36%-38.35%), (34.21-37.31%), Pb (22.17%-28.66%), (23.05%-36.97%) and Ni (16.28%-21.77%), (21.31%-23.68%) respectively at the two incubation temperatures. Consortium displayed higher percentage removal of Zn (60.04%- 65.89%), Pb (57.07%-60.87%) and Ni (35.55% -37.55%) while only 1.86-3.98% reduction was observed in the control for all the metals. High biomass production at increasing concentration of Pb and Zn in the growth medium shows that these isolates are prospective microbial inoculants for bio removal of these two metals in polluted environments. The potential of these isolates in reducing the bioavailable fraction of the three metals in mine tailings were also tested using selective sequential extraction method. The result shows that the bacterial isolates were able to interact better with Pb as compared to Ni

and Zn and the mobile exchangeable fraction of Pb were greatly reduced while higher percentage of the three metals were bound to the reducible, oxidizable and residual fraction. High mobility factor was recorded for Ni and Zn especially in the third sample which indicated high availability and mobility of these metals in the environment. This study shows the potential of two indigenous multiple metal species tolerance bacteria that can be used as microbial inoculant in bioremediation of mine tailings.

LIST OF PUBLICATIONS

Chapter 2: Diversity of Acidophilic Bacteria and Archaea and Their Roles in Bioremediation of Acid Mine Drainage. *Published in British Microbiology Research Journal 2015; 8(3): 443-456.*

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LIST OF ABBREVIATIONS

As----------Arsenic

Co----------Cobalt

Cd----------Cadmium

Pb----------Lead

Ni----------Nickel

Zn----------Zinc

Hg----------Mercury

AMD----------Acid mine drainage

PCR----------Polymerase chain reaction

ORP----------Redox potential

CEC----------Cation exchange capacity

EC----------Electrical conductivity

PRBs----------Permeable Reactive Barriers

ALD----------Anoxic limestone drains

SAPS----------Successive alkalinity-producing systems limestone ponds,

OLC----------Open limestone channels

SSP----------Selective sequential precipitation

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction to this chapter

Metal mining is of great importance to the world economy as it provides diverse mineral products for industries and household consumers. However, the extraction of these minerals has resulted in the generation of large volumes of wastes such as tailings, waste rock and slags (Chattopadhyay and Chattopadhyay, 2013). In a country like South Africa, gold mining has played a tremendous role in the sustenance and development of the economy but it is also a major polluter and degrader of its ecosystem (Edwards et al., 2014). South Africa is the largest producer of gold in the world with the Witwatersrand having the world's largest gold mining basin (Cairncross and Kisting, 2016). Gold mining in the Witwatersrand basin began in 1886 and since this period, over 50 thousand tons of gold has been mined. The Witwatersrand gold mining region of South Africa is a potentially hazardous zone because of the continual release of mining wastes mainly in the form of tailings to the environment which could lead to great irreparable destruction of the ecosystems (Anderson et al., 2014, Heinrich, 2015).

The US Environmental Protection Agency stated that, "problems related to mining waste may be ranked as second only to global warming and stratospheric ozone depletion in terms of ecological risk (Durand, 2012). Mine tailings constitute the main source of environmental pollution in mining activities (Pascaud et al., 2014). These mine wastes are found scattered all over the world in countries with both present and past mining activities. The Department of mineral resources in South Africa reported that, there are over 8000 abandoned and ownerless mines in South Africa which constitute some of the largest in the world. Rehabilitation of these mines will take not less than 800 years at a cost of billions of Rands (Durand, 2012, Naidoo, 2016). Most of the tailing dams are unlined and without

vegetation cover thus providing a source of extensive dust, water and soil (surface and groundwater) pollution (Kossoff et al., 2014). In Johannesburg area, most of the tailings dams have been produced by mines that were closed before 1956 leaving them undisturbed for several years thus giving them enough time to be exposed to oxygenated rainwater.

In abandoned tailings deposits, the groundwater level often lies far below the ground surface, making it easier for oxygen to diffuse deep down into the tailings. This exposure to oxygen, coupled with activities of indigenous bacteria populations catalyzing oxidation reactions cause increase oxidation rates of sulphide minerals such as pyrites (FeS2), arsenopyrite (FeAsS), galena (PbS), chalcopyrite ($CuFeS_2$), and sphalerite (Fe, Zn)S) contained in the tailings. This results in the generation of acid mine drainage (AMD), which may severely affect the surrounding soil, surface and groundwater. Acid mine drainage is characterized by low pH, high sulphate contents and elevated concentrations of metal species (Dold, 2014). Ecosystem threats from mining activities are majorly due to uncontrolled discharge of AMD as a result of poor management of tailings dams. Acid mine drainage poses a serious threat to human health, animals and ecological systems as a result of the release of metal species such as copper (Cu), iron (Fe) manganese (Mn) , Zn, Cd and Pb that have been leached from the tailings (Simate and Ndlovu, 2014).

Metal species are natural chemical elements found in the earth's crust having specific gravity at least five times greater than water (Dubey et al., 2012). They have high electrical conductivity, luster and malleability, and freely lose their electrons to form cations. Some metal species like Zn, Cu, Ni, Co are essential to maintain various physiological and biochemical functions in living organisms when in low concentrations. Conversely they become toxic when they exceed certain threshold concentrations (Khan et al., 2010, van Bussel et al., 2014). Metals become contaminants in soil environments as a result of (a) high rate of generation through anthropogenic sources as compared to natural ones (b)

transferability from mines to random environmental locations where higher potentials of exposure occur (c) higher concentration in discarded products as compared to the ones in the receiving environment (d) the forms or phases (speciation) in which the metal is found in the receiving environmental system may increase its bioavailability (D'amore et al., 2005). Metal species from anthropogenic activities tend to be more mobile and bioavailable when compared to the pedogenic and lithogenic ones (Kabata-Pendias and Mukherjee, 2007).

The concentrations of these metals increases as they pass from lower trophic levels to higher trophic levels (a phenomenon known as biomagnifications) causing various diseases and disorders (Abdel-Baki et al., 2011). Metal species of major environmental concern include Pb, Zn, Cr, Cd, Cu, As and Ni because they are well known to produce a long-term negative impact on soil and water in the surrounding environment (Nagajyoti et al., 2010). Unlike organic contaminants, metal species are indestructible biologically and chemically but can be transformed through methylation and complexation and changes in valence state which affect their mobility and bioavailability in the environment (Violante et al., 2010).

Increased production of AMD in mining environments increases the acidity of the environment making these metal species more mobile and available in the environment. Decant of AMD in West Rand basin of the Witwatersrand since 2002 is a serious concern for the nation (McCarthy, 2011). Metal species released into the environment usually find their way into the soil. The environmental conditions of the soil such as pH, redox potential (OR), cation exchange capacity (CEC), organic matter and clay content have a great influence on metals adsorption, mobility and bioavailability in the ecosystems (Alvarenga et al., 2009). Drainage of acid mine into soils increases the mobility of metals and their potential to impact on the ecosystem. Toxic effects of metal species on human health have been extensively studied (Mudgal et al., 2010; Morais et al., 2012; Alloway, 2013). Various adverse health effects such as growth inhibition, cancer, organ damage, nervous system damage and in

severe cases death have been associated with human exposure to metals. High prevalence of upper and gastrointestinal cancer in human has been correlated with high concentrations of metal species such as Cu, Cd and Pb in fruits and vegetables (Islam et al., 2015). High concentrations of metals have also been reported to affect various physiological and biochemical processes of plants such as growth reduction, lower biomass production and metal accumulation, synthesis of chlorophyll pigments (Feng et al., 2004), and protein synthesis which subsequently leads to severe reduction in crop yields (Nagajyoti et al., 2010).

Metal species also affect soil microbial population, growth, morphology and their activities through functional disturbance, protein denaturation or the destruction of the integrity of cell membranes which may directly influence soil fertility (Colin et al., 2012). Soil bacteria are important in the decomposition of soil organic matter and a decrease in their diversity usually results in the decrease in soil nutrient availability to plants. Several studies based on culture dependent and independent techniques have reported the detrimental effects of metals on soil bacteria diversity (Xie et al., 2013, Xie et al., 2016).

Several studies have been conducted by different bodies and researchers in various parts of South Africa which have revealed the alarming rates at which metal species are released into the environments from mining activities. Mitileni et al. (2011) and Council for Geoscience 1996 reported high concentrations of Zn, Pb and As around Ebenezer dam in Limpopo province (Szczesniak et al. 2001). A similar study conducted in the same province by Matshusa et al. (2012), on another tailing dam (Louis Moore) also shows high concentrations of Ni, As and Mn with maximum values in mg/kg of 937 and 975 (As), 583 and 416 (Ni), and 1161 and 680 (Mn) respectively in tailings and soil. Also, higher concentration of As (13.46–234.6 mg/kg) was recorded in Princess Gold mine tailings in Johannesburg, Gauteng province by Olobatoke and Mathuthu (2016). Elevated concentrations of different metal species have been reported by other researchers in various

tailings dams, soil and sediments from gold mine tailings in other provinces (Aucamp and Van Schalkwyk, 2003, Naicker et al., 2003, McCarthy, 2011).

Environmental concerns over metal species contamination in soils, sediments and aquatic environments have led to the development of remediation strategies targeted at reducing metal solubility and toxicity (Parmar and Thakur, 2013, Akcil et al., 2015). Different methods already in place to remediate metals contaminated environments include: chemical precipitation, oxidation or reduction, filtration, ion-exchange, reverse osmosis, membrane technology, evaporation and electrochemical treatment (Polettini and Pomi, 2012, Sarkar, 2012). Chemical methods generate large volumetric sludge which requires additional costs to be disposed of. The chemical and thermal methods are technically difficult and expensive because they can also degrade the valuable components of the soil (Macingova and Luptakova, 2012). Most of these techniques also become inefficient when the concentrations of metal species are less than 100 mg/L (Colin et al., 2012). Most metals salts are water-soluble and cannot be separated by physical separation methods (Hussein et al., 2003). The conventional method of remediating metals polluted soil involves either onsite treatment or excavation of contaminated soil and subsequent disposal to a landfill site (Tsang et al., 2012). This method results in movement of the contaminant elsewhere along with the hazards associated with transportation of the contaminated soil and migration of contaminants from landfill into neighbouring environment. Another means of removing metals from polluted soil is soil washing, which is known to be costly and produces wastewater rich in metal species that needs further treatment (Rosas et al., 2013). Also, the physico-chemical method used for soil remediation removes all biological activities needed for normal plant growth (Pazos et al., 2012). Considering all the disadvantages of existing methods, there is a need to develop new cost effective techniques for the reduction of metals concentrations in the environment to acceptable levels for better protection of human health and the ecosystem.

The biological methods of remediation of contaminated environments known as bioremediation are natural processes that depend on the action of plants and microorganisms such as bacteria to modify contaminants while these bacteria carry out their usual life functions (Gaur et al., 2014). The metal species is utilized as energy sources through the metabolic activities of the bacteria thereby rendering the contaminants harmless or less toxic in most cases (Kieu et al., 2011). This method possesses high specificity in the removal of metals of interest as well as operational flexibility.

Bioremediation technologies are presently classified into three categories: (i) Bio attenuation which involves monitoring the natural progress of degradation to confirm that contaminant concentration reduces with time (ii) bio stimulation which makes use of nutrients, electron acceptors or substrates to stimulate natural biodegradation or biotransformation of contaminants, and (iii) bio augmentation which involves inoculation of microorganisms with desirable traits to improve the biodegrading activities or bio transforming capacities of contaminated sites (Madueño et al., 2015). These techniques of bioremediation benefit from the vast metabolic diversity of bacteria.

In spite of metals accumulation in the soil, soils activate cleansing mechanisms due to the action of large number of certain bacteria that help in reducing the mobility and bioavailability of the metals (Haferburg and Kothe, 2007). The major mechanisms utilized by bacteria in remediating metals polluted soil are alteration of mobility, intra and extracellular sequestration, active transport facilitated by efflux pumps, enzymatic transformation to other less toxic chemical species by redox reactions, methylation or alkylation, dealkylation and or reduction in the sensitivity of cellular targets to metal ions (Gadd, 2010). These resistance mechanisms are controlled by genes located mostly in the plasmid although chromosomal genes may also be involved in rendering genetic manipulation for strain improvement easy and feasible (Valencia et al., 2013).

The success of these mechanisms depends on many factors among which are: microbiological and mineralogical characteristics of the soil, types and concentrations of the contaminants and the physicochemical characteristics of the soil (Ahmad and Carboo, 2000). The heterogeneity of the soil environments presents a serious challenge for bioremediation because well adapted bacteria are required to bio remediate in specific environments (Becker et al., 2006). Out of the thousands of tailings deposit littered all over South Africa, only few of them have been assessed for their environmental impacts and characterized for indigenous metals resistant bacteria. The distribution of the indigenous metals resistant bacteria and their interaction with the metals, mechanisms governing their growth and activity as well as response to environmental changes and their metabolic capacities in the contaminated sites are essential for effective bioremediation. Knowledge of new isolates and probably new genetic information on metal species resistant bacteria could emanate from this study. Such results will add more knowledge to existing information on diversity of metal species resistant bacteria in different gold mine tailings environments and may provide information concerning potential bioremediators for reclamation of tailings in future.

1.2 Problem statement

Environmental pollution caused by gold mining activities is a serious concern all over the world. This is a result of continual release of mine wastes majorly in the form of tailings to the environment. Tailings are potential source of metal species contamination which could cause AMD, a severe environmental pollution problems to subsurface environment. Metals pollution has increased steadily over the years and has resulted in degradation of soil and water resources, loss of fauna and flora in the environment (Gaur et al., 2014). Water is a scarce resource in South Africa due to its low annual rainfall pattern. However this scarce resources is seriously at the receiving end of metals pollution caused by the extensive gold mining activities of the country. The closure and abandonment of many gold mines several

years back has led to decant of poor quality water characterized by elevated metal species concentrations, low pH and high sulphate contents into subsurface soils and water resources which pose serious danger to human health. It was reported years back that acidic water has begun decanting out of an abandoned mine on Randfontein Estates in the West Rand of the Witwatersrand (Lusilao-Makiese et al., 2013). Concern has heightened lately that acidic water overflowing abandoned mine tunnels under Johannesburg will soon be discharged into the water table of the adjoining Witwatersrand basin, posing serious danger to the health of millions of the occupants (McCarthy, 2011). Metal species generated from mine wastes in this area have been reported to travel several kilometers and impact downstream ecosystems (Tutu, 2012). Most human exposure to metals is linked to contaminated water and soil. High levels of metals have also been reported in soils, plants, sediments, water and vegetables in South Africa (Awofolu et al., 2005, Kootbodien et al., 2012). The recalcitrant nature of metal species enhances its persistence and accumulation in the environments leading to fatal effects on important biological processes in the environments and the high prevalence of neurological, cardiovascular and respiratory diseases in human.

Several physicochemical methods are currently in use to treat metals contaminated sites but most of these methods have been discovered to be expensive, time consuming and generate additional toxic wastes among several others. Hence, it is highly important to search for effective method to remove this metal species from the environments. Thus, this research becomes highly important to find efficient and effective methods for the detoxification of these toxic wastes environmental hazards.

1.3 Justification of the study

Witwatersrand Goldfields in South Africa is known to have produced 40% of gold ever unearthed in the world to greatly support the growth and development of the country for over

100 years (Hart, 2014). Extraction and processing of these precious minerals since 1886 have resulted in the production of enormous quantities of tailings, a hazardous mine wastes that contain elevated load of metal species (Rashed, 2010). High metal concentrations in the environment have great consequences on the environment and human health. Krugersdorp located in the West Rand of Gauteng province harboured quite a number of tailings that have been abandoned for many years, thereby allowing the tailings to release the metals into the environments. Studies assessing levels of metal species, physicochemical properties and effects on microbial population in tailings in this area with the aim to find suitable remediation approach to them are limited, thus necessitating the need for this research. Several physico-chemical methods have been used in the treatment of this mine wastes but several drawbacks have been reported (Macingova and Luptakova, 2012). The use of bacteria in the treatment of metal polluted sites has emerged as alternative approach to the conventional methods with many reports on their effectiveness (Yu et al., 2014, El Baz et al., 2015). This is as a result of the chemical compositon and structure of the bacteria cell walls that enable them to bind metal species effectively (Dadrasnia et al., 2015). Nevertheless, studies on bioremediation of mine tailings are very few (Govarthanan et al., 2013), as most studies on metal species bioremediation are reported on artificial contaminated soil whose features are not the same as mine tailings. This research will give more information on the effects of physicochemical properties of metals polluted soil on metal bioavalability and the resulting effects on bacteria diversities. The study will also determine the effectiveness of bacteria as inoculants in the treatment of gold mine tailings.

1.4 General objective

This study was designed to assess the level of environmental pollution with metal species in some abandoned gold mine tailings in Krugersdorp, South Africa as well as the distribution

of the indigenous metal species resistant bacteria and their phenotypic and genotypic characteristics that could be utilized in bioremediation of the selected sites.

1.4.1 The specific objectives of this study were to:

- Determine the physico-chemical properties and metals content of three abandoned gold mine tailings in Krugersdorp, South Africa.
- Isolate and characterize the indigenous metal species resistant bacteria from the tailings and screen them for the presence of plasmids and metal tolerance genes on chromosomal and plasmid DNA.
- Determine the bioaccumulation of Zn, Ni, and Pb by selected metal species resistant bacteria isolates.
- Determine bioaugmentation of mine tailings by mixed cultures of the selected metal resistant bacteria species.

1.5 Research questions

- Do gold mining activities affect soil properties and build up metal species concentration in the environments?
- What are the various diversities of metal species resistant bacteria encountered in abandoned gold mine tailings?
- Can indigenous metal species resistant bacteria accumulate metals in contaminated sites?
- Can bacteria reduce the bioavailability and mobility of metal species in their environment?

CHAPTER TWO

DIVERSITY OF ACIDOPHILIC BACTERIA AND ARCHAEA AND THEIR ROLES IN BIOREMEDIATION OF ACID MINE DRAINAGE

Abstract

The Mining industry generates wealth, but its long term adverse effects, which include AMD cannot be overlooked. Acid mine drainage occurs as a result of biological and chemical oxidation of sulphide containing minerals with consequent production of acidic metal rich effluents. AMD is a serious environmental pollution problem in both active and abandoned mines worldwide, resulting in continual contamination of surface and groundwater resources with metal species. Acidophilic bacteria and archaea have been known to contribute to the accentuation of this problem by speeding up the reaction time for biological oxidation of sulphide containing mineral waste rock. The dominant metal present in AMD is iron with high sulphate content; the iron may be present in either ferrous or ferric form or both depending on the water pH. Reduction of these two important constituents by generating alkalinity through chemical or biological means has been reported to have a significant effect on AMD impacted water. The metabolic activities of the acidophilic bacteria and archaea through ferric iron and sulphate reduction, a natural attenuation process, also help in remediating this pollution problem by generating alkalinity that immobilizes metals thereby reversing the reactions responsible for the genesis of AMD. This article reviews the various groups of the acidophilic prokaryotic microorganisms and their metabolic activities that help in remediating the problem of AMD in gold mines.

Keywords: Acidophilic microorganisms, acid mine drainage, bioremediation, environmental pollution, metals, mining

2.1 Introduction

Acid mine drainage, also known as acid and metalliferous drainage or acid rock drainage (ARD), is the biochemical oxidation of sulphide bearing minerals, which results in the production of acidic water that contains high concentrations of metal species, sulphate and low pH. Earth disturbances such as construction activities and mining processes in the rocks that contain abundance of sulphide minerals as well as natural rock weathering processes can also contribute to the generation of AMD. This indicates that AMD is the generation of acidic water from sources other than mining. Typically, AMD is characterized by low pH value, high sulphate content and often times, elevated concentrations of ferric iron and other metals such as Cu, Zn Cr, Cd, and Ni. The chemical composition of AMD varies depending on the kind of sulphide mineral associated with coal and metal ores (Ferguson and Morin, 1991, Baker and Banfield, 2003). Acid mine formation is greatly enhanced by the mining process which increases the surface area of the sulphide containing mineral exposed to air and oxygen thereby increasing the rate of acid generation (Yin et al., 2008). Bacterial activity is an important factor in AMD generation because it helps in accelerating the rate of decomposition and oxidation of sulphide minerals.

2.2 Origin of Acid Mine Drainage

Many factors are known to contribute to the development of AMD. Acid mine drainage can be generated as a result of coal and metal mining activity. Some of these sources are; mined materials like spent ore from heap leach operations, spoil, waste dump or tailings, overburdened material, mine structure such as pit walls in surface mining operation and underground workings associated with underground mines and subgrade ore piles. All these are known to contain sulphide minerals like pyrite, which is the most abundant of all sulphide minerals, and others such as galena, covellite, chalcopyrite, realgar and arsenopyrite whose oxidation leads to the formation of AMD (Johnson and Hallberg, 2005). After the extraction

of ores from underground or open pit, 80-90% of the crushed ore is dumped as tailings waste which contains large amounts (between 10 and 30 kg/ton) of sulphide minerals.

2.3 Biodiversity of Acidophilic Microorganisms in AMD

Acid mine drainage usually contains a variety of microorganisms. As a result of the characteristic features of the AMD, prokaryotic microorganisms have been found to be the predominant life forms existing in the environments (Johnson, 1998). These prokaryotes are found in the groups of bacteria and archaea domains with ability to thrive well in the extremely acidic environments. Acidophiles have immense contribution to sulphur and iron biogeochemical cycle (Johnson, 1998, Druschel et al., 2004). Acidophilic microorganisms are a subdivision of the extremophiles which have been gaining a lot of research interest as a result of their ecological and economic importance. The ecology and biodiversity of the acidophilic prokaryotes have been reviewed by Hallberg and Johnson (2001). Acidophiles have been classified using many criteria. On the basis of mineral solubilization, two groups are recognized. The first group are those that accelerate mineral dissolution by an oxidative route (iron and sulphur oxidizers) while the second group uses the reductive route (iron reducers) (Johnson and Hallberg, 2003). Some species (mostly acidophiles) can reduce ferric iron as well as oxidize ferrous iron, depending on the prevailing environmental conditions. The iron and sulphur oxidizers are found in both bacteria and archaea domains and their metabolic activities have been utilized in the extraction of gold from refractory ores (biomining) (Rawlings, 2002). The bacteria are found within the Proteobacteria and comprise (*alpha*, *beta* and *gamma* classes), Nitrospirae, Firmicutes, Actinobacteria and Acidobacteria phyla and in the domain archaea within the Crenarchaeota and Euryarchaeota phyla. The alpha proteobacteria, particularly *Acidithiobacillus ferrooxidans, Acidithiobacillus caldus* and *Acidithiobacillus thiooxidans* are the most extensively studied group (Johnson et al., 2012).

The iron oxidizers are able to thrive in the sulphide mineral rich environments as a result of their ability to use ferrous iron as their electron donor, and they continually regenerate the ferric iron which in turn oxidizes the sulphide minerals present in the acidic liquor. The major iron oxidizers are the *At. ferroxidans* and *Leptospirillum ferroxidans* (Bryan et al., 2006). The genus *Leptospirillum* is the most commonly encountered iron oxidizing organism in mineral leaching environments, due to its high affinity for ferrous iron, tolerance of ferric iron and moderately thermal (>40°C) environments (Hallberg and Johnson, 2001). Others such as *Acidimicrobium ferrooxidans, Sulfolobus metallicus, At. thiooxidans* and *Metallosphaera sedula* also have a central role in the dissolution of sulphide minerals (Johnson, 2003). The number of iron oxidizing cells and their level of activity determines the extent to which these sulphide minerals are oxidized by the microorganisms. Sulphur compounds released during pyrite oxidation can be oxidized to sulphate by ferric iron, and this tends to make the population density of sulphur oxidizers in AMD to be low due to the non-availability of sulphur to support their growth (Druschel et al., 2003). After the initial oxidation of sulphide by the iron oxidizers, the remaining sulphides are oxidized by neutrophilic sulphur oxidizers such as *T. thioparus and T. novellus*. The pH of the AMD is lowered to 4.0 and 4.5 as a result of the oxidative activities of the bacteria and the initial environmental condition. Acidophilic sulphur oxidizers such as *Acidithiobacillus* and *Acidiphilium* species then oxidize the remaining sulphur (Leduc et al., 2002). Ferrous sulphate ($FeSO_4$) is oxidized to ferric sulphate ($Fe_2(SO4)_3$) by iron oxidizing acidophiles (Leduc and Ferroni, 1994, Hallberg and Johnson, 2003). Additional sulphate is produced as a result of further oxidation of FeS_2 and ferric sulphate by iron-oxidizing acidophiles. Elemental sulphur is also oxidized to sulphuric acid (H_2SO_4) by sulphur–oxidizing acidophiles which also lower the pH. Dissimilatory oxidation of iron and /or reduced sulphur compounds are catalyzed by other groups which could be mixotrophic (assimilate organic

and inorganic carbon) or obligate heterotrophs. Heterotrophic microorganisms are known to be abundant in AMD where they rely on carbon originating as waste products from the autotrophs, and it has been suggested that these heterotrophs might supply the autotrophs with additional carbon for their growth (Johnson and Roberto, 1997). Okibe and Johnson (2004), observed in their study that the dissolution of pyrite was accelerated by consortia containing heterotrophic microbes than those of iron-oxidizers and sulphur oxidizers. The authors opined that the heterotrophic contribution must be due to the stimulation of carbon flow between the heterotrophs and autotrophs and not as a result of the generation of ferric iron or removal of sulphur from the mineral surface which aids in the dissolution of the pyrite. Other important groups found in AMD are the neutrophilic iron oxidizers such as *Leptothrix ochracea* (Edwards et al., 2000) and *Gallionella ferruginea* (Hallberg et al., 2006). With the exception of *Leptospirillum* spp and *Ferrovum (Fv.) myxofaciens,* the majority of the iron oxidizing bacteria also reduce ferric iron to support their growth. Based on environmental factors required for growth, acidophiles have been subdivided further into various groups, namely:

Temperature: mesophilic, moderate thermophiles and extreme thermophiles.

pH: acidophiles, alkaliphiles, neutrophiles.

Nutritional type: heterotrophs, autotrophs, mixotrophs as shown in Table 2.1.

Table 2.1: Acidophilic prokaryotic microorganisms

Organisms	Temperature	pH	Nutritional Class	References
Iron oxidizers				
L. ferroxidans	Mesophiles	Acidophiles	Autotrophs	(Bryan et al., 2006)
At. ferrivorans	Mesophiles	Acidophiles	Autotrophs	(Johnson, 1998)
At. ferroxidans	Mesophiles	Acidophiles	Autotrophs	(Bryan et al., 2006)
Ferrimicrobium acidophilus	Mesophiles	Acidophiles	Autotrophs	(Johnson and Roberto, 1997)
Gallionella ferruginea	Psychrophiles/mesophiles	Acidophiles/ Neutrophiles	Autotrophs/ Mixotrophs	(Hallberg et al., 2006)
Ferroplasma acidiphilum	Mesophiles/ thermophiles	Acidophiles	Autotrophs	(Johnson, 1998)
Acidimicrobium ferrooxidans	Moderate thermophiles	Acidophiles	Autotrophs/Mixotrophs/Heterotrophs	(Johnson and Hallberg, 2003)
Sulfobacillus acidophilus	Moderate thermophiles	Acidophiles	Mixotrophs	(Bridge and Johnson, 1998)
S.thermosulfidooxidans	Moderate thermophiles	Acidophiles	Mixotrophs	(Bridge and Johnson, 1998)
At. prosperous	Mesophiles	Alkaliphiles	Autotrophs	(Johnson, 1998)
Metallosphaera sedula	Extreme thermophiles	Acidophiles	Autotrophs	(Johnson and Hallberg, 2003)
Leptothrix ochracea	Mesophiles	Neutrophiles	Autotrophs	(Edwards et al., 2000)
Sulphur oxidizers				
At. thioxidans	Mesophiles	Acidophiles	Autotrophs	(Hallberg et al., 2001)
At. albertis	Mesophiles	Acidophiles	Autotrophs	(Johnson, 1998)
Sulfolobus metallicus	Extreme mesophiles	Acidophiles	Autotrophs	(Johnson and Hallberg, 2003)
Hydrogenobacter acidophilus	Moderate thermophiles	Acidophiles	Autotrophs	(Johnson, 1998)
Thiobacillus denitrificans	Thermophiles	Neutrophiles	Autotroph	(Beller et al., 2006)
Iron-reducers				
Acidiphilum spp.	Mesophiles	Acidophiles	Heterotrophic	(Bridge and Johnson, 2000)
Acidimicrobium ferroxidans	Moderate thermophiles	Acidophiles	Autotrophs/Mixotrophs/Heterotrophs	(Johnson and Hallberg, 2003)
Non-mineral degraders				
Acidocella spp.	Mesophiles	Acidophiles	Heterotrophic	(Johnson, 1998)
Alicyclobacillus spp.	Thermophiles	Acidophiles	Heterotrophic	(Johnson, 1998)
Acidobacterium capsulatum	Mesophiles	Acidophiles	Heterotrophic	(Johnson, 1998)

The archaea-like *Metallosphaera* spp., *Sulfolobus metalicus* and *Acidianus brierleyi* are the most thermophilic growing at a temperature of (65-95°C). They are mostly found inhabiting the most extreme niches on the planet. The mesophiles are the predominant bacterial oxidizers growing at optimum temperature less than 40°C, while the moderate thermophiles grow at optimum temperature of 40-60°C. The mesophilic and moderately thermophilic bacteria are the most extensively studied groups of the acidophilic metal sulphide oxidizing microorganisms. Thermophilic and acidophilic sulphur/iron oxidizers mostly found at a temperature range of 40–60°C are the usual rod shaped, *Sulfobacillus* species (Hallberg and Johnson, 2001, Hallberg and Johnson, 2003). Most life forms which are functional in AMD grow at an optima pH between 2 and 4 or acid-tolerant (pH optimal for growth above that normally encountered in AMD), but can also function in very low pH environments (Johnson, 1995). The concentration of dissolved organic carbon in the majority of extremely acidic environments has been found to be very low (<20 mg L^{-1}). Thus, these environments can be characterized as oligotrophic environments. In abandoned deep mines where light penetration is restricted, the nutrition type that exists will be mainly chemolitho-autotroph, which is the oxidation of ferrous iron and reduced sulphur compounds (Johnson, 1998). The majority of iron and sulphur-oxidizing acidophiles are regarded as autotrophic, but utilization of formic acid as carbon source has also been reported in some of them such as *At. ferroxidans* and they have been found to be responsible for the production of ferric iron and acid (Pronk et al., 1991).The chemolithotrophs are the first prokaryotes isolated from extremely acidic environments and *At. ferroxidans* was the first iron-oxidizing acidophile to be isolated and characterized (Colmer et al., 1950). For this reason, *At. ferroxidans* has been the most well studied isolate in acidophilic microbiology. Reports abound in the literature on its physiology and biochemistry (Leduc and Ferroni, 1994, Barreto et al., 2003, Valdes et al., 2008).

2.4 Pyrite Oxidation

The development of AMD is dependent on six factors, namely, (a) abundance of sulphide minerals, (b) water content (moist environment), (c) oxygen and ferric iron (oxidant) and pH (hydrogen ion concentration), (d) surface area of the exposed sulphide mineral, (e) activation energy and (f) the presence of sulphur and iron oxidizing bacteria (biological activity) (Natarajan, 2008). Sub-surface mining often progresses below the water table, so water must be constantly pumped out of the mine in order to prevent flooding. However, when a mine is abandoned, the pumping ceases, and water floods the mine, which results in the accumulation of contaminated water in the environment (Neal et al., 2005, Younger et al., 2005). The introduction of water is the initial step in most acid mine drainage generation. This results in the production of drainage water that is highly polluting because of low pH which increases mobility and metal content. This low pH occurs as a result of the dissolution of acidic salts that have built up in the pore spaces of the exposed walls and ceilings of underground chambers. Exposing pyrite to oxygen and water leads to an oxidation reaction, where hydrogen, sulphate ions and soluble metal cations are created as shown in the equation below:

$$2FeS_2 + 7O_2 + 2H_2O \rightarrow 2Fe^{2+} + 4SO4^{2-} + 4H+ \quad (1)$$

Further oxidation of ferrous iron Fe^{2+} to ferric iron Fe^{3+} occurs as a result of the availability of dissolved oxygen in water or in the atmosphere

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O \quad (2)$$

Ferric iron (Fe^{3+}) can also precipitate as ochre ($Fe(OH)_3$), the reddish-orange precipitate often observed in AMD waters:

$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H+ \quad (3)$$

Fe^{3+} that did not precipitate from (2) left in solution from (3) will precipitate additional pyrite as indicated in equation (4):

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO4^{2-} + 16H^+ \quad (4)$$

Pyrite oxidation occurs through either direct or indirect pathways and it is very difficult to determine which of the pathways is important in a given situation. The direct pathway involves close proximity of the sulphide bearing mineral with microorganisms such as acidophilic *At. ferroxidans*, *L. ferroxidans* which aids in the oxidation of the sulphide mineral (Myerson and Kline, 1983, Norris et al., 2000, Brierley and Brierley, 2001). In the indirect pathway, sulphide is reduced to ferrous as a result of its chemical oxidation by ferric iron and the ferric iron is further regenerated by iron-oxidizing microorganisms, leading to continuous oxidation of the sulphide mineral. The resultant effect of this reaction is the production of acidic water with characteristic corrosive patterns (Mustin et al., 1992). The acidity of the medium which results from generation of the hydrogen ion makes the metals contained therein highly soluble, thereby preventing their precipitation out of the solution. Indirect production can also occur through the reaction of some metal ions such as Fe^{3+} and Al^{3+} with water (Castro et al., 1999). Further acidity can also be generated by the dissolution of sulphide containing minerals in the anoxic sediment of a constructed wetland or spoil heap as a result of the ferric iron concentrated acidic water infiltrating through them (Vile and Wieder, 1993).

Bacteria play a prominent role in the genesis of acid production because they accelerate the rate of decomposition and oxidation of the sulphide minerals (Johnson and Hallberg, 2003). Evangelou and Zhang (1995), stated that the major reaction which ensures continuous oxidation of the sulphide mineral is continuous regeneration of ferric iron which is reduced to ferrous upon reaction with pyrite. Hence, the primary oxidant is the ferric iron and not molecular oxygen as initially proposed in the classical equation above.

Pyrite oxidation is a two-phase reaction. The first phase involves ferric iron attack on the sulphide mineral, while the second phase is the reoxidation of ferrous iron to ferric, which is an oxygen dependent reaction. The reduced sulphur compounds produced as intermediates

in the reaction are also oxidized to sulphate (Evangelou and Zhang, 1995). Dissolution of the sulphide mineral occurs after its attachment to oxygen and this result in the oxidation of the sulphide moiety which occurs in non-ferrous sulphides such as Cu_2S or of both iron and sulphur in minerals such as pyrite (FeS_2) and pentlandite (FeNiS).

2.5 Disadvantage of Acidophilic Microorganisms

The oxidation of sulphide minerals can be abiotic, but the rate of oxidation is greatly enhanced by several orders of magnitude by sulphur and iron-oxidizing bacteria and archaea (Edwards et al., 2000). Acidophilic bacteria and archaea raise the amount of available ferric iron which increases the rate of pyrite oxidation. *At. ferrooxidans,* for example, uses reduced ferrous iron in AMD areas as an electron donor for energy creation at low pH. Oxidation of sulphur by autotrophic and heterotrophic bacteria and archaea generates sulphuric acid, which if not neutralized by carbonates or other basic minerals present, results in acid generation. Acidophilic bacteria and archaea colonize the concrete surface and its pores, capillaries and micro-cracks and cause biogenic sulphide corrosion of concrete sewer pipes by altering hydrogen sulphide sewage gas into sulphuric acid (Sand and Bock, 1987).

2.6 Different Treatment Technologies for AMD

Acid mine drainage poses a serious threat to human health, animals and ecological systems. Polluted water becomes acidic and contains elevated concentrations of radionuclides and metal species such as Cu^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+}, which are not biodegradable. These metals accumulate in living organisms (bioaccumulation) and the concentrations increase as they pass from lower trophic levels to higher trophic levels (a phenomenon known as bio magnifications) causing various diseases and disorders (Sprynskyy et al., 2006). It is therefore, important to treat acid mine decants effectively before allowing their release into the ecosystem. A wide variety of technologies are available for the treatment of AMD. They

comprise one or more of chemical, physical and biological processes. They involve the following: pH control, adsorption/absorption, complexation, chelation, biological remediation, oxidation/reduction, electrochemistry, sedimentation, flocculation/ filtration/settling, ion exchange and crystallization (Taylor et al., 2005). Two different treatment technologies are utilized for the treatment of acid mine: passive and active treatment.

2.6.1 Passive treatment

Over the years, different types of passive treatment systems have been developed that do not require continuous chemical inputs. Diverse types of passive treatments have gained wide usage as the major modern treatments applied apart from the active treatment. These treatment systems take advantage of naturally occurring chemical and biological processes to cleanse contaminated mine waters or soil and require minimal upkeep (Younger et al., 2002). Treatment involves the use of sulphate-reducing bacteria and/ or lime to neutralize acidity and precipitate metals. Examples of these technologies include wetlands (natural and constructed), anoxic limestone drains (ALD), successive alkalinity-producing systems (SAPS), limestone ponds and open limestone channels (OLC). In treating metals peculiar to hard rock mining such as Zn, Pb, Cd, As, Mo, Au, and Ag, the major principle governing the design of the passive treatment is the ability of bacteria to reduce sulphate thereby generating sulphides as a precipitate from the metal. In case of metal species common to coal mining like Fe, Al, and Mn, the most widely used method is the process that supplies sufficient oxygen with or without addition of an alkaline agent (Costello, 2003). The numerous innovative technologies employed are grounded in similar principles. Permeable Reactive Barriers (PRBs), bio-reactors, and constructed wetland technologies can all make use of alkaline agents and the reducing ability of sulphate reducers to remove metals from AMD. With the exception of PRBs that utilize iron in the treatment of uranium, many of these

technologies are in-situ applications that manipulate natural processes to treat acidic and/or metal contaminated water. They differ only in construction and water source. PRBs have a subsurface reactive section that groundwater flows through to be treated following its natural course (Costello, 2003). The active and passive technologies of treatment also do not allow potentially valuable metals present in mine waters to be recovered and recycled. New innovative developments in mine water remediation technologies now exist. These developments enhance sequential and/or selective removal of dissolved metals and different pollutants from AMD. This results in recycling of additional valuable elements and immobilization of the poisonous pollutants in targeted forms. It involves a combination of the physical-chemical and biological-chemical methods to remove metals and metalloids from AMD. An example of these technologies is the selective sequential precipitation (SSP) which precipitates metals using solutions of sodium hydroxide (NaOH) and hydrogen sulphide produced by sulphate reducing bacteria. This approach produces metals with a high degree of purity and is environmentally friendly (Kaksonen and Puhakka, 2007).

In South Africa, chemical desalination processes, such as CSIR ABC, TUT MBO and Mintek Ettringite processes, can produce drinking water from AMD in a cost-effective way without a resulting sludge disposal problem, as feed chemicals and saleable byproducts are recovered from the produced sludge (Maree et al. 2013). Eutectic freeze crystallization (EFC) is another technology that reduces the temperature of contaminated water like acid mine to eutectic point at which ice and a pure salt will crystallise. This technology does not utilize chemicals and pure water and usable salts can be recovered (Lewis et al. 2010). The use of coal fly ash in the treatment of AMD has also been reported. Fly ash contain high percentage of lime fraction that add alkalinity to acid generated in AMD and thus neutralize the acidity. Fly ash also reduces the hydraulic conductivity of mine spoils. Successful application of fly ash has been reported in the treatment of AMD and amendments of acidic

soils (Gitari et al. 2003, Gitari et al., 2006). Masindi and Gitari (2016), also reported the use of magnesite in the treatment of AMD.

2.6.2 Active treatment

The most widely used approach for remediating mine impacted waters is to aerate (to oxidize ferrous iron to ferric) and add neutralizing chemicals such as calcium carbonate, calcium hydroxide or anhydrous ammonia to raise the pH. This precipitates metals as hydroxides and carbonates, thus reducing the activity of the iron oxidizing bacteria (Kadukova and Stofko, 2007). These methods have a lot of drawbacks such as high energy and chemical requirements, low efficiency and usually the production of large amounts of sludge, from which the separation of precious metals is difficult and high cost and interference by other wastewater constituents occurs (Volesky, 2001).

Metal contamination is one of the most important environmental concerns from mine tailings and various technologies have been developed to treat this environmental hazard. Conventional methods to remediate metals contaminated site include excavation and solidification/stabilization (Alpaslan and Yukselen, 2001). Though these technologies are suitable to control contamination, the metal species cannot be remove permanently. Limitations such as cost effectiveness, generation of hazardous by-products or inefficiency has been reported. Bioremediation methods that involve use of indigenous bacteria that thrive naturally in polluted sites is the alternative and promising approach that can overcome these limitations (Bryan et al. 2006). Ability of bacteria to utilize diverse combination of electron donors and acceptors to drive their metabolism enable them to interact with metals.

2.7 AMD Treatment Mechanism by Acidophilic Microorganisms

The dominant metal present in AMD is iron, with elevated amounts of sulphate due to the breakdown of sulphide containing minerals by microbial activities. The iron may be

present in either ferrous or ferric form, or both, depending on the water pH. Reduction of these two important constituents by generating alkalinity will have a significant effect in AMD impacted water (Johnson and Hallberg, 2003, Johnson and Hallberg, 2005). It is a well-known fact that acidophilic bacteria and archaea help in accelerating the problem of AMD by significantly speeding up the reaction time. Production of acid by ferric iron can take as long as 15 years, but the presence of iron oxidizing bacteria can shorten this reaction time to 8 mins (Macingova and Luptakova, 2012). Acidophilic microorganisms with the ability to generate acid from the reduction of ferric iron have been reported (Bridge and Johnson, 2000, Alexandrino et al., 2011). The acidity generated results in solubilization of the metals present in the rocks. Ferric iron is almost insoluble at neutral pH, whereas in acidic solutions (pH<2.5) its solubility is greatly increased. The principle behind the biotic and abiotic remediation strategies is to induce alkaline condition to reduce metals mobility, thereby alter the reactions accountable for the cause of AMD (Johnson and Hallberg, 2002). Microbiological activities that help in remediating AMD pollution problems are reductive precipitation, chelation, sulphate reduction and metal sulphide precipitation (Gadd, 2004). The metals contained in AMD are non-biodegradable; as a result, their remediation can only be achieved by removing them from the solution. This occurs by precipitation of metals out of solution as insoluble metal sulphides or precipitation of ferric iron as a result of change in their redox state (transformation) (Hallberg, 2010). Since ferric iron is known to be the major oxidant responsible for continuous oxidation of the sulphide minerals and is usually insoluble at neutral pH, removing it from the solution will go a long way in preventing further oxidation of the sulphide minerals.

The acidophilic chemolithotrophic prokaryotes are known for their accelerated oxidative dissolution of pyrite and other sulphide minerals in AMD using inorganic carbon. Other groups known as heterotrophic acidophiles also catalyze the dissimilatory reduction of

iron and sulphur using organic carbon as electron donor and carbon source, thereby reversing the reactions involved in AMD formation (Bridge and Johnson, 1998).

2.8 Ferric Iron Reduction

The rate of dissolution of most sulphide containing minerals is largely dependent on the availability of ferric iron, which has been shown to be the major oxidant of these minerals in the environment (Evangelou and Zhang, 1995, Natarajan, 2008). Acid mine contaminated environments, especially those associated with metal mining, contain high concentration of ferric iron whose solubility is known to be greatly enhanced at acidic pH. Most prokaryotic acidophilic bacteria and archaea use this electron sinks to oxidize organic matters in subsurface environments with high loads of organic matter (Johnson, 1995).

The ability to reduce ferric iron to ferrous iron has been reported in aerobic mesophilic chemoautotrophs (*At. ferroxidans* and *At. thioxidans*) mesophilic heterotrophs (*Acidiphilum* spp. and *Ferrimicrobium acidiphilum*) (Johnson and McGinness 1991, Johnson and Roberto, 1997, Bridge and Johnson, 2000) and moderate thermophiles (iron oxidizing *Sulfobacillus* spp., *Acidimicrobium ferroxidans* and heterotrophic *Alicyclobacillus*-like isolates) (Bridge and Johnson, 1998). With the exception of *At. thioxidans*, ferric iron reduction has been coupled with growth in all these mentioned bacteria (Hallberg et al., 2001). Ferric iron reduction is coupled with the oxidation of many organic compounds in highly acidic heterotrophs. The majority of the *Acidiphilum* species can reduce and solubilize a wide range of ferric iron containing minerals like $Fe(OH)_3$ and jarosite $(XFe_3(SO_4)_2(OH)_6$. Anaerobic dissolution of ferric iron containing minerals: goethite, jarosite and iron hydroxide formed as a result of iron oxidation has been reported in *S. acidophilus* which also oxidizes sulphur compounds using ferric iron. Ferric iron reduction is also common in the Gram-positive mixotrophic iron–oxidizers (Bridge and Johnson, 1998). Although many neutrophilic microorganisms are known to have the ability to reduce ferric iron, the ability to couple

organic matter oxidation exclusively to ferric iron reduction in order to conserve energy to support growth is lacking in the majority of them (Lovley, 1995). To remove soluble iron from AMD, the ferrous iron must first be oxidized to ferric iron. This will enhance the formation of ferric minerals such as schwertmannite and ferrihydrite, which can then be easily precipitated out of solution by the ferric iron reducers. This reduction results in mobilization of iron as well as other metals that may be associated with ferric iron deposit. It is also an alkali generating reaction of high importance in the passive treatment in wetland (Vile and Wieder, 1993). The ability to reduce ferric iron has been reported to be affected by dissolved oxygen concentration in some of these microorganisms. In a study by Johnson and Bridge (2002), to determine the effect of culture condition on the growth of two acidophilic heterotrophic ferric iron reducer's *A. acidiphilum* and *Acidiphilum* SJH in fermenters, it was discovered that growth of the *A. acidiphilum* was affected by dissolved oxygen concentration whereas for *Acidiphilum* SJH the reverse was the case. Also, the expression of the iron reductase system was found to be inducible in the *A. acidiphilum* because it was synthesized in the presence of very low concentration of dissolved oxygen while for *Acidiphilum* SJH it was constitutive because it was able to reduce ferric iron irrespective of dissolved oxygen during growth. Arnold et al. (1990), also reported that the iron reductase system can be both constitutive and inducible in some ferric iron reducers as seen in the case of dissimilatory iron reducer *Shewanella putrifeciens* (strain 200). Dissolved oxygen concentration was shown to have effect on the inducible system because ferric iron reduction was high under limited oxygen supply.

2.9 Sulphate Reducing Bacteria (SRB)

Microbial dissolution of sulphide containing minerals results in high concentration of sulphate in AMD. Sulphate reducers are a morphologically diverse group of bacteria with varying nutritional requirements. They are obligate anaerobes that use sulphate or other

sulphur compounds as an electron acceptor for the dissimilation of organic compounds (Castro et al., 1999). These bacteria are a major part of the total microbial community because they play a vital role in the biogeochemical cycle of carbon and sulphur which help in the regulation of sulphate in the environment (Mudryk et al., 2000, Alexandrino et al., 2011). SRB are usually found in the anaerobic regions of marine, estuarine and mine waste water sediments as well as saline ponds due to the high sulphate content (Castro et al., 1999, Alexandrino et al., 2011).

The sulphate-reducing bacteria include the following genera: *Desulfovibrio, Desulfomicrobium, Desulfobulbus, Desulfosarcina, Desulfobacter* and *Desulfotomaculum* (Garcia et al., 2001, Cabrera et al., 2006). These groups of heterotrophic acidophilic bacteria use sulphate as a terminal electron acceptor, in the process releasing hydrogen sulphide as a result of sulphate reduction. Soluble metals like Cu, Zn, Fe, Cr and Cd present in the solution react with this biologically produced hydrogen sulphide to form insoluble precipitates of the metals as shown in the equations below:

$2H+ + SO4^{2-} + 2C\ (org) \Leftrightarrow H_2S + 2CO_2$ (5)

$M^{2+} + H_2S \Leftrightarrow MS\ (\downarrow) + 2H+$ (6)

M^{2+} represents the dissolved metal

The acidity of the system is reduced as a result of carbon metabolism and inherent ability of the bacteria to reduce the sulphate. These fundamental properties make SRB useful in mitigating AMD (Garcia et al., 2001, Cabrera et al., 2006) and this natural technology has been considered the most promising approach of removing sulphate, acidity and metal species from AMD (Maree et al. 2000, Johnson and Hallberg, 2005, Neculita et al., 2007). The ability of the SRB to achieve the proposed sulphate standard of 500 ppm as well as 250 ppm required for drinking water has been reported (De Vegt, 1998) as compared with the conventional chemical method that can only reduce it to 1500 mg/L. Other advantages over

chemical mitigation methods such as production of more compact sludge which settles faster and is less subject to dissolution, selective precipitation of metal, high efficiency and low cost have also been reported (Martins et al., 2009, Sheoran and Choudhary, 2010).

Efficient sulphide production using SRB can be achieved by the addition of a complementary carbon source because AMD is deficient in carbon donors. Therefore, choosing an appropriate carbon source is very crucial in ensuring long time usage, high efficiency, and economic viability of the system. Three factors are usually considered in selecting this carbon source: availability of the carbon source, its degradability which enhances its capacity to allow complete sulphate reduction by the SRB and its cost per unit of sulphate converted (Van Houten et al., 1994, Liamleam and Annachhatre, 2007). Despite the reported success of SRB in the treatment of AMD, the sensitivities of these bacteria to acidity and metals constitute the major setback in using them; hence there is a need for the addition of a remediation reagent to improve the living conditions so as to enhance their activity (Bai et al., 2012). The optimum pH of growth for SRB is between 7.0 and 7.5 and different metal toxicity levels to SRB have been reported (Utgikar et al., 2001, Martins et al., 2010). Several efforts have been made to address this problem (Wilkin and McNeil, 2003, Karri et al., 2005, Lindsay et al., 2008, Xin et al., 2008, Bai et al., 2012). In the past, it was generally believed that SRB can only thrive between pH 6 and 8 (Widdel, 1988, Hao et al., 1996) but some other reports have punctured such notions, with sulphate reduction at a pH as low as 2.7 and 3.8 being observed in their studies (Gyure et al., 1990, Ulrich et al., 1998). But there are lots of studies in support of the earlier claim (Garcia et al., 2001, Kusel et al., 2001, Lee et al., 2009). It has now been proven that microbial sulphate reduction with efficient metals recovery can proceed in AMD impacted environment (Table 2.2).

Table 2.2: Microbial sulphate reduction and metals recovery of SRB in acidic environments

System	Initial pH	Final pH	% of metals removed			% of Sulphate removed	References
			Cu	Fe	Zn		
Bioreactor	2.75	6.20	99	9	86	61	(Bai et al., 2013)
Column + mining soil	2.3	8.0	>90	>90	>90	90	(Costa and Duarte, 2005)
Fumarole	-	-	97	100	96	91	(Alexandrino et al., 2011)
Reactor	2.7	4.3	99.9	99.9	99.9	90	(Sahinkaya et al., 2011)
Reactor	3.7	5.0	99.9	-	99.9	-	(Macingova and Luptakova, 2012)

Sulphate reduction in acidic environments such as acidic mine tailings, lakes and rivers, wetlands and bioreactors has been documented. Denaturing gradient gel electrophoresis revealed the presence of SRB in Rio Tinto river in Spain with pH as low as 2.3 (Garcia et al., 2001, Gonzalez-Toril et al., 2003, Bai et al., 2013). Acidophilic SRB with the ability to selectively immobilize different transition metals has been reported (Ňancucheo and Johnson, 2012). Successful isolation of acid tolerant SRB shows a promising future for their application in bioremediation purposes. The significant contribution of iron and sulphur oxidizing bacteria in the genesis of AMD has been confirmed by many researchers (Leduc and Ferroni, 1994, Rawlings, 2002, Hallberg and Johnson, 2003) but the microbial diversity in the AMD sites is yet to be well characterized (Auld et al., 2013). The rate of dissolution of sulphide mineral is a function of the population of iron oxidizing cells present and their level of activity in a given environment. Information about the population of iron-oxidizers is important in deducing the microbial impact of AMD (Baker and Banfield, 2003) so that appropriate remediation approach (es) can be taken. Hallberg and Johnson (2003), isolated eight acidophilic moderate iron oxidizers from two abandoned mines in the United States and a pilot-scale constructed wetland at one of the sites with pH 3-6. Analysis of the 16S rRNA gene sequences of these isolates showed that they were previously undescribed residents of the acidic waters. Three of the isolates shows greater than 99% genetic relatedness and have 97% gene identity to a clone deposited in the public data base (Edwards et al., 1999). The closest recognized strain was *Frateuria aurantia,* a neutrophilic acetogenic iron-oxidizer with 93% gene identity, two of the other three sets were found to have 99.6% gene identity and 97.5% to the third isolate. When their gene sequences were compared to the gene sequences in the data bases, *Thiomonas thermosulfata*, a neutrophilic thiosulphate-oxidizer was found to be the closest relative with 96% genetic relatedness. The remaining two isolates had 99.7% gene relatedness to *Propionibacterium acnes,* an unknown anaerobic microbe to inhabit

acidic waters. Auld et al. (2013), in a study using direct sequencing of the 16S rRNA, also isolated three previously unidentified genera in AMD; *Legionella*, a neutrophilic heterotroph, *Alicyclobacillus pohliae*, a Gram positive, aerobic, acidophilic bacterium and *Halomonas ventosae*, a Gram negative, high salt tolerant, halophilic proteobacteria, from AMD tailing ponds.

An enormous diversity of ferrous iron oxidizing prokaryotes exists in the acidic environment (Hallberg and Johnson, 2001) with different affinities to the prevailing environmental conditions. Variation in environmental conditions such as pH, temperature and oxygen contents exist in AMD. This leads to great differences in physiological properties of the acidophilic microorganisms that can be found in these environments. This metabolic variation can be exploited in remediation strategies of various AMD which is the principle behind the emerging strategies of using these acidophiles for oxidation and precipitation of iron from acid mines of different water chemistry (Hallberg, 2010). To speed up iron oxidation rate in the various mine water, it is important to use different consortia due to variations in environmental conditions of the mine waters.

2.10 Conclusion

Though acidophilic microorganisms are known to play a prominent role in the genesis of AMD, various advantages have been derived from their metabolic activities which have been harnessed in bio mining and bioleaching processes in the mining industries (Rawlings, 2002). The diversities of this group of microorganisms are being shaped by geographical conditions prevailing in the various environments which are not uniform. Due to this reason, there is need for more studies on the bacteria diversity, function and the factors affecting the distribution of these microorganisms in the acid mine environments. This will help in designing the appropriate bioremediation strategy for the contaminated sites. Knowledge of these as well as the various metabolic processes and interactions that exist among these

microorganisms will help in identifying the various groups with potential to ameliorate the problem of acid mines. More groups with unknown potentials are being detected every day, and the discovery of acidophilic anaerobic SRB has greatly helped in the recovery of metals from polluted AMD. Further work needs be structured on the metabolic pathways involved in the biomineralization of metal species by the acidophilic bacteria and archaea. To facilitate the processes involved in utilizing these microbes in bioremediation of metals, there is need to study the optimal conditions requires for their growth.

CHAPTER THREE

METAL POLLUTION FROM GOLD MINES: ENVIRONMENTAL EFFECTS AND BACTERIAL STRATEGIES FOR RESISTANCE

Abstract

Mining activities can lead to the generation of large quantities of metal laden wastes which are released in an uncontrolled manner, causing widespread contamination of the ecosystem. Though some metal species classified as essential are important for normal life physiological processes, higher concentrations above stipulated levels have deleterious effects on human health and biota. Bacteria able to withstand high concentrations of these metals are found in the environment as a result of various inherent biochemical, physiological, and/or genetic mechanisms. These mechanisms can serve as potential tools for bioremediation of metal polluted sites. This review focuses on the effects of metals wastes generated from gold mining activities on the environment and the various mechanisms used by bacteria to counteract the effect of these metals in their immediate environment.

Keywords: bioremediation, environmental pollution, metal toxicity, mine wastes

3.1 Introduction

Increased urbanization and industrialization have led to large amounts of toxic contaminants being released into the environment worldwide. Some of these contaminants occur naturally, but anthropogenic sources, especially mining activities, have contributed significantly to their increase. Although mining provides enormous social and economic benefits to nations, the long-term adverse effects on the environment and public health cannot be overlooked (Akabzaa, 2000).

Mining, mineral processing and metallurgical extraction are the three principal activities of gold mining industries which produce wastes. Mineral processing also known as beneficiation aims to physically separate and concentrate the ore mineral(s) using physical, chemical and sometimes microbiological techniques. Metallurgical extraction breaks the crystallographic bonds in the ore mineral in order to recover the desired element or compound (Lottermoser, 2007). Large quantities of waste are produced during this activity particularly in gold mines which release over 99% of extracted ore as waste to the environment (Adler and Rascher, 2007).

The use of bacteria in gold extraction, known as biomining, has received considerable attention due to the potential roles played by these bacteria in the recovery of gold from gold-bearing ores. Acidophilic, chemolithotrophic iron and sulphur oxidizing bacteria such as *Acidithiobacillus* (*At.*) *ferrooxidans, At. thioxidans*, *Leptospirillum (L.) ferriphilum* and *L. ferroxidans*, *Sulfobacillus acidophilus Sulfolobus metallicus* have been identified and utilized in gold extraction. These bacteria help in solubilizing the sulphide matrix of the gold deposits thereby making the gold more reachable to leaching by the chemical lixiviants (Acevedo, 2000, Reith et al., 2007). Biomining is known to be more environmentally friendly than many physicochemical extraction processes. In addition, the wastes generated using bacteria are less biologically reactive compared to those obtained using the physicochemical methods (Rawlings, 2002).

Tailings are the major wastes produced from gold extraction and they contain high amounts of metals. These metals leach out in an uncontrolled manner into surrounding environments on exposure to water or through dispersal by wind. The presence of elevated concentrations of metal species in the environment is a serious health issue worldwide due to their non-degradative nature which makes them persistent and thereby exerts long-term effects on the ecosystem (Singh et al., 2011). Metals affect the natural population of bacteria

in the soils. This leads to loss of bacterial species responsible for nutrient cycling with a consequent negative effect on ecosystem functioning (Piotrowska-Seget et al., 2005). To survive in metal polluted sites, some bacteria have devised various ways to withstand the potentially deleterious conditions. They are known to acquire and utilize diverse cleansing mechanisms such as biotransformation, bioaccumulation and biosorption which can be utilized to clean-up metal contaminant present on the subsurface or the contaminant removed from its original position to be treated on-site or off-site (Gadd, 2010). This review focuses on environmental impacts of increasing metal pollution caused by gold mining activities on human health and the environment and how bacteria interact with these metals.

3.2 Gold Processing and Extraction and the Role Played by Bacteria

Gold mining can be open-pit or deep shaft mixed with other metals such as Cu, silver (Ag) and Pb. Its location determines the type of mining process to be used in the extraction and the amount of wastes that will be generated. In the past, small quantities of waste were generated by mining activities because higher grade ores were being exploited. There was also limited capacity to move large quantities of materials and so the waste generated was discarded within a few meters of the mine opening or pit. Open-pit mining produces eight to 10 times as much waste as underground mines because a greater amount of topsoil, overburden and barren or waste rock has to be removed. Gold mining in South Africa over the centuries has resulted in the accumulation of thousands of voluminous tailings dumps which are scattered all over the country with lots of potentially negative impact on the environments (Dold, 2010).

To separate the gold (Au) from the mineral bearing rock, mercury is mixed with the ores dug from the ground or from stream beds to form an amalgam. The burning of the amalgam leads to vaporization of the elemental mercury into a toxic plume leaving the gold behind. Mercury amalgamation was the initial method used for centuries to process gold and is still in

use today by artisanal and small-scale gold mining (ASGM). Globally, ASGM is the second largest source of atmospheric mercury pollution after coal combustion (Telmer and Stapper, 2012). Another method of Au extraction uses cyanide in a two-stage process; extraction and recovery. Gold is first dissolved using cyanide in the extraction stage and the dissolved gold is then recovered from the cyanide solution by cementing with Zn or adsorption onto activated carbon. The cyanide extraction processes could be heap leach or vat/tank leach depending on the quality of the ores. In ores of higher gold content, the vat/tank leaching is employed, which involves leaching of the crushed and ground ore in large enclosed tanks equipped with agitators to dissolve the gold which then adheres to pieces of the activated carbon. The activated carbon and the gold are then stripped of the solution and the barren solutions together with the leached ore are discarded. The heap leach is used for low-grade ore and involves extraction of crushed oxide gold ore piled onto plastic-lined pads with leaching solvents such as acids or cyanide to dissolve the gold which is collected at the bottom of the pad (Lottermoser, 2007).

The equation below explains how cyanide dissolves gold:

$$4Au(s) + 8NaCN(aq) + O_{2\,(g)} + 2\,H_2O\,(l) \rightarrow 4NaAu(CN)_2(aq) + 4NaOH(aq)$$

The high demand for gold and the fluctuating gold prices have necessitated the need for processing of lower grade ores, waste rock dump materials and scrap residues. Bacteria are now increasingly being used to facilitate the extraction of metals from low grade ores and concentrates (bio mining) that cannot be economically processed by conventional methods. These bacteria help in the enrichment of metals in water from gold ores and mines, in a solubilization process called bioleaching. This process occurs in nature under suitable environmental conditions that favour the growth of the bioleaching bacteria (Rawlings, 2002). The sulphidic nature of many gold deposits hinder accessibility of lixiviants but activity of several acidophilic, chemolithotrophic iron and sulphur oxidizing bacteria has

been reported to assist in the oxidation of the sulphide matrix. The bacteria include; mesophilic iron and sulphur oxidizing *Acidithiobacillus* (*At.*) *ferrooxidans*, sulphur-oxidizing *At. thioxidans*, iron-oxidizing *Leptospirillum* (*L.*) *ferriphilum* and *L. ferrooxidans*, moderately thermophilic bacteria such as sulphur-oxidizing *At. caldus* and sulphur and iron oxidizing *Sulfobacillus* spp. (Golyshina et al., 2009, van Hille et al., 2013). These bacteria obtain energy by oxidizing ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) or elemental sulphur (S^0) or other reduced sulphur compounds to sulphuric acid (H_2SO_4). The released Fe^{3+} and hydrogen ions then break down the sulphide matrix (Belzile et al., 2004). This is summarized in the equations 1 to 4 below using pyrite as a typical example of gold bearing ores:

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O \quad (1)$$

$$2S^0 + 3O_2 + 2H_2O \rightarrow 4H + 2SO_4^{2-} \quad (2)$$

$$FeS_2\,(Au) + 2Fe^{3+} \rightarrow 3Fe^{2+} + 2S^0 + (Au) \quad (3)$$

$$FeS_2\,(Au) + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4 + 16H^+ + Au \quad (4)$$

Bio-oxidation of sulphide contained in refractory gold ores enhances liberation of gold particles from the sulphide matrix thereby rendering the gold amenable to dissolution using lixiviants such as cyanide. Bio-oxidation is a pretreatment method of gold processing that helps to decrease the use of lixiviant for gold solubilization in subsequent parts of the operation and in the long run increasing the gold yields (Reith et al., 2007). This method is usually used in conjunction with other methods since it does not actually solubilize gold. Bacteria also excrete ligands that are capable of stabilizing gold by forming gold-rich complexes and/or colloids. Biologically produced amino acids, cyanide and thiosulphate can also aid gold solubilization (Reith et al., 2007). Gold solubility can also be reduced with the use of bacteria that help in consuming the ligands that bind the gold or by bio-sorption, enzymatic reduction and precipitation and by using gold as micronutrient (Reith et al., 2007).

In addition, bacteria can also indirectly influence gold solubilization by enhancing the permeability of gold-bearing ores bodies (Brehm et al., 2005).

Distinct advantages have been reported for bio-leaching of gold over traditional physicochemical methods. Microbial extraction procedures are more environmentally friendly: (1) they do not produce environmentally noxious gaseous emissions; (2) they do not require high energy consumption used during roasting or smelting; (3) they enhance extraction of low grade gold ores that are too expensive to process using conventional methods; and (4) tailings produced from bio- mining processes are less chemically and biologically active since they are already bio-leached (Reith et al., 2007).

3.2.1 Characteristics of Gold Mine Tailings

Tailings are a mixture of finely ground rock that is left after the retrieval of the precious mineral and water used in processing. Considerable volumes of open-dump tailings are found in many countries where environmental regulations are not strongly adhered to (Baker and Banfield, 2003). The chemical and physical nature of tailings particles can be likened to typical river sand and silt and their properties are determined by the nature of the ore, geochemistry, the processing method used in extracting the ore, the particle size of the crushed material and the type of chemical process used in extracting the ore (Davies and Rice, 2001; Franks et al., 2011). Gold mine tailings are characterized by poor physical properties like poor aggregation, high hydraulic conductivity, fine texture and very limited cohesion ability. These properties make tailings different from soil (Vega et al., 2004, Blight and Fourie, 2005) and the lack of cohesion is responsible for the varied moisture content and temperature seen in this toxic waste. Chemically, tailings contain up to 6% pyrite, high salinity and are nutritionally deficient with low contents of organic matter (Vega et al., 2006). The high sulphides content result in high acidity and high metal concentrations in ground water in the vicinity of the tailings (Vega et al., 2004). Rafiei et al. (2010), reported a pH

value of 7.35 in gold mine tailings in Iran, whereas Mitileni et al. (2011), reported pH values of 3.25–6.28 in South Africa and Harish and David (2015), pH value of 3.48–8.12 in India. Highly acidic pH has also been observed in AMD arising from gold mining activity in other studies (Naicker et al., 2003, Tutu et al., 2008). The characteristic features of gold mine tailings are the elevated concentrations of toxic metal species such as, Cd, Ni, Pb, Cu, Zn, Co, and mercury (Hg) (Da Silva et al., 2004). The largest fraction of the total metal may exist as silicates (Hayes et al., 2009) which are limitedly accessible to microbial life. These characteristics of gold mines result in complex stress for the bacteria inhabiting these environments and lead to selection of different resistant bacterial species. The differences in prevailing environmental conditions, levels of contamination, geographic and geologic origin as well as the site of origin are factors determining the bacterial diversity (Khozhina and Sherriff, 2006).

Aside from the acidophilic mesophilic species known to be involved in bio oxidation of gold, diverse metallophilic Gram positive and negative bacteria belonging to the phylum *Proteobacteria* such as *Pseudomonas*, *Aeromonas, Shewanella, Brevundimonas, Agrobacterium* and *Acinetobacter* and the phylum *Firmicutes* (*Bacillus, Serratia,* and *Exiguobacterium)* and so on have been reported in gold mine tailings using culture-dependent techniques (Akcil et al., 2003, Wei et al., 2009). A number of studies also investigated bacterial diversity in gold mines using culture independent techniques based on bacterial 16S rRNA gene identification. Santini et al. (2002), in the Northern Territory of Australia also discovered the *Agrobacterium/Rhizobium* branch of the *Proteobacteria* while Rastogi et al. (2009), using the same method obtained bacteria diversity mainly composed of phylotypes related to the phylum *Proteobacteria* and other phyla *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Chlorobi*, *Firmicutes*, *Nitrospirae*, *Verrucomicrobia* in deep subsurface homestake gold mine soil in the USA.

3.3 Environmental Pollution from Gold Mine Tailings

Environmental pollution from gold mines is associated mainly with the release of harmful elements from the tailings and other mine wastes. The infiltration of water through sulphide- containing tailings piles and ponds, surface and underground workings, waste and development rock leads to leaching of large volumes of metals like Zn^{2+}, Ni^{2+}, Pb^{2+}, As^{2+}, Cu^{2+} and sulphate ions into stream and river ecosystems (Durkin and Herrmann, 1994, Edwards et al., 2000). This results in AMD with severe detrimental effect on the receiving water bodies. Metals pollution and AMD is a very important environmental concern where waste materials containing metal-rich sulphides from mining activity have been stored or abandoned (Concas et al., 2006). Tailings and rock dumps are associated with the surface impacts which greatly affect surface and ground water quality. The underground impacts are caused by the influx of water into the underground workings and the subsequent dewatering of the aquifer (Banister et al., 2002). Another source of environmental pollution from gold mines is the chemicals used in processing the gold. An estimated 1400 metric tons of mercury was used in 2011 by ASGM and an annual average of 1000 metric tons of inorganic Hg was discharged. One-third of this estimated value goes into the air and the rest is mixed up in heaps of tailings, soil and waterways (Telmer and Veiga, 2009). Mercury can also be released into the environment as a result of present reprocessing of some old gold tailings dumps. Pacyna et al. (2010), reported that Hg emissions in South Africa are second only to China. The cyanidation method of extraction also gives rise to the emission of hydrogen cyanide, global warming and production of huge amounts of tailings a potential source of metals due to the extraction of low-grade ores (Bambas-Nolen et al., 2013).

3.3.1 Metal Toxicity in Gold Mine Environment

Metals play a vital role in metabolic and physiological processes of plants, humans and microorganisms. Some metal species such as Cr, Zn, Co, Ni and Cu are micronutrients that

are essential in redox-processes. They are important in the maintenance of molecules through electrostatic interactions, control of osmotic pressure and cofactors for numerous enzymes and electron transport chains. Hence, metal ions play an essential role in complex biochemical reactions (Bruins et al., 2000). The non-essential metals like Ag, As, Cd, Pb and Hg are of no biological importance to living organisms and are very toxic when found in the ecosystem.

The disruption and acceleration of the natural process of the geochemical cycle through anthropogenic activities like gold mining has led to most soils of rural and urban settings accumulating metals above the recommended levels (D'amore et al., 2005). Studies of the effect of metals in soil, plants and water have been reported by Concas et al. (2006), Bitala et al. (2009), as well as Ndeddy Aka and Babalola (2016).

Elevated levels of metal species in gold mine tailings greatly affect the diversity, total number and general activity of bacteria. Metals affect the metabolism, growth and morphology of soil bacteria as a result of functional disturbance, destruction of cell membrane integrity or protein denaturation (Smejkalova et al., 2003, Chakravarty and Banerjee, 2008). Bacteria are essential in the decomposition of soil organic matter and any decline in bacterial diversity or biomass may have a profound effect in nutrient absorption from the soil to plants (Ndeddy Aka and Babalola, 2016). Many studies using culture dependent and independent techniques have shown that metals contamination gives rise to shifts in microbial populations (Bajkić et al., 2013, Xie et al., 2016).

Diverse toxicological and biological effects of metals in the environment occur as a result of the different forms (oxidation) in which the metal species exists which also relates to compounds with great variation in toxicity. The oxidation state is a function of the type and quantity of the metal's redox potential, pH and microbial activity (Yong and Mulligan, 2003). The noxious effect of metals is as a result of alteration in the structural arrangement of the

nucleic acids, proteins or by interference with osmotic balance and oxidative phosphorylation (Yaoa et al., 2008). Some metal species like Cd^{2+}, Ag^{2+}, Hg^{2+} can attach to the sulfhydryl (SH) groups of important enzymes used in microbial metabolism, thereby hindering the activity of sensitive enzymes (Turpeinen, 2002). This metal species may enter the food chain as a result of their uptake by edible plants (Alirzayeva et al., 2006).

3.3.1.1 Cadmium

Cadmium is one of the most toxic metal to most organisms. Its concentration in unpolluted soil is usually 1 mg/kg (USEPA, 2001), but in gold mine tailings, concentrations ranging between 6.4 and 11.7 mg/kg have been reported in Tanzania (Bitala et al., 2009). It occurs in gold bearing orebodies as an isometric trace element in sphalerite and its concentration depends on the concentration of the sphalerite in the ore body. Cadmium is of serious concern as a result of its accumulation in the food chain, drinking water and soil. It has an exceptionally long biological half-life (>20 years), highly mobile in soil-plant systems and can also exert a great effect on the proper functioning of the ecosystems (USEPA, 2004). The bioavailability of Cd and associated toxicity to soil bacteria depends on the bacterial species, concentration, environmental factors, time, speciation, soil properties and ageing (Vig et al., 2003). Cadmium affects many metabolic activities of soil bacteria such as nitrogen mineralization, carbon mineralization, CO_2 production and enzyme activities. Negative effect of Cd at concentrations of 50 and 500 mg Cd/kg were observed on dehydrogenase activities in soil bacteria by Landi et al. (2000), while Smolders et al. (2001), also noted 14% decrease in nitrification activity of soil bacteria in a soil having pH 6.6 at Cd concentration of 2 mg/Kg.

3.3.1.2 Zinc

Zinc also occurs in gold ore bodies in the form of sphalerite (ZnS) which is often associated with galena. The average natural level of Zn in the Earth's crust is 70 mg/kg (dry weight), ranging between 10 and 300 mg/kg (Malle, 1992). In gold mine tailings, concentrations ranging between 8.9 and 65.7 mg/kg have been reported in South Africa by Mitileni et al. (2011), while a higher concentration of 177.56 mg/kg was reported by Bempah et al. (2013) in Ghana. Though a micronutrient needed by plants, bacteria and human beings for vital cell functions, its presence beyond the normal physiological value is toxic due to its interaction with sulfhydryl groups or replacement of other essential metals in a wide range of proteins (Kox et al., 2000). Zinc speciation is very important in determining its toxicity to bacteria because it varies considerably with pH. High concentrations of Zn shows varied inhibitory or toxic effect on cellular activities and growth of bacterial cells. Mertens et al. (2007), noted that the nitrification process by *Nitrosospira* sp. was reduced by 20% in soil contaminated with Zn at pH 4.8–7.5.

3.3.1.3 Lead

Lead is toxic at the lowest concentration and naturally non-degradable unless it is removed from the medium where it is found. Standard mean concentration for Pb in surface soils worldwide averages 32 mg/kg with a range of 10–67 mg/kg (Kabata-Pendias and Pendias, 2001) but concentration ranging between 80 mg/kg (Abdul-Wahab and Marikar, 2012) and 510 mg/kg (Ogola et al., 2002) have been reported in gold mine tailings. It occurs in the form of galena (PbS) in gold ore and this form is found when sulphide concentration of the ore is high (Matocha et al., 2001). Lead exists in various oxidation states (0, I, II, IV) and the most stable forms are Pb(II) and lead-hydroxy complexes. The ionic form, Pb(II) is the most reactive and most common form which forms mononuclear and polynuclear oxides and hydroxides. This ionic form together with lead oxides and hydroxides are the forms that are

released into surface water, ground water and soil. Lead gains access to bacterial cells through the uptake pathways for essential divalent metals like Mn^{2+} and Zn^{2+} and exerts its toxic effects on bacterial species by changing the conformation of nucleic acids, proteins, inhibition of enzyme activity, disruption of membrane functions and oxidative phosphorylation as well as alterations of the osmotic balance of the bacterial cells (Bruins et al., 2000).

3.3.1.4 Chromium

Chromium is widely distributed in soils and rocks where it occurs in minerals such as chromite [(Fe, Mg, Al) Cr_2O_4]. Chromium concentration ranges between 2 and 60 mg/kg in unpolluted soil (Dhal et al., 2013) but a higher concentration of 486 mg/kg was reported in gold mine tailings in Oman (Abdul-Wahab and Marikar, 2012). Chromium is mainly found in chromate $FeCr_2O_4$ having 70% of pure Cr_2O_3. It can be found in the environment in several forms (with oxidation states from −2 to +6) depending on pH and redox conditions but Cr(III) and VI are the most stable forms with differing chemical and physical features as well as biological effects (Oliveira, 2012). Chromium (III) species are less soluble and relatively immobile as a result of their ability to adhere to clays and oxide minerals at acidic pH while low solubility at pH above 5 is as a result of $Cr(OH)_3$. Hexavalent chromium (Cr(VI)) is the most oxidized form, a potentially dangerous substance due to its high solubility and mobility which allow it to infiltrate biological membranes and pollute soil and water (Sultan and Hasnain, 2003). This is the form usually found at contaminated sites, its major species being chromate CrO_4^{2-} and dichromate ($Cr_2O_7^{2-}$). Studies have shown that Cr(VI) is 100 times more toxic and 1000 times more mutagenic and carcinogenic compared to Cr(III) (Costa, 2003). It damages bacterial DNA and this genotoxic ability has been attributed to its intracellular reduction to Cr (III) through reactive intermediates. The two types of resulting

DNA damage produced are (1) oxidative DNA damage; and (2) Cr(III)-DNA interactions (Sobol and Schiestl, 2012).

3.3.1.5 Nickel

Nickel levels in soils greatly depend on the concentration of the parent rocks and this concentration was between 3 to 100 mg/kg for world soils (Abdullahi, 2015). In gold mine tailings, a higher concentration of 583 mg/kg was found by Matshusa et al. (2012) in South Africa. Bitala et al. (2009), also reported concentrations as high as 11,200 mg/kg in Tanzania. Nickel exists in gold bearing ore as pyrrotite ($Fe_{(1-X)}S$), which can contain up to 5% Ni and pentlandite FeNi)S_8. Other mineral sources are chalcopyrite (CuFeSz) and gersdorffite (NiAsS). It exists in the 0 and +2 oxidation states and less often in the −1, +1, +3 and +4 oxidation states. Among its species, the +4 oxidation state is known to be more toxic and carcinogenic compared to +2 (Higgins, 1995). Nickel toxicity arises due to its tendency to substitute other metal ions in proteins, enzymes or attach to cellular compounds (Cempel and Nikel, 2006). It also intermingles with not less than 13 essential elements in living organisms. The major toxicity of Ni to bacterial cells include: (1) replacement of essential metal of metalloproteins; (2) attachment to catalytic residues of non-metalloenzymes; (3) allosteric inhibition of enzyme; (4) oxidative stress that enchance DNA damage, protein impairment, lipid peroxidation along with increased titers of oxidative stress defense systems (Macomber and Hausinger, 2011).

3.3.1.6 Arsenic

Arsenic is one of the most dangerous metals of worldwide environmental concern (Choudhury et al., 2011) due to its potential toxicity. It occurs as arsenopyrite [FeSAs], realgar [As_2S_2] and orpiment [As_2S_3] in gold bearing rock (Nriagu et al., 2007). Elevated levels of As have been reported in gold mine tailings at Obuasi, Ghana. Ahmad and Carboo

(2000), reported 8305 mg/kg while Bempah et al. (2013) found a concentration of 1752 mg/kg. The Obuasi region has been reported to be one of the regions in the world with elevated levels of As which has been attributed to the richness of arsenopyrite (FeAsS) mineralization in the gold-bearing ore (Ahmad and Carboo, 2000, Bernard et al., 2007). The highest toxicity level of As is seen in the inorganic forms As(III) and arsenate As(V) which are the predominant forms in mine tailings. The arsenate acts like phosphate and can therefore gain access to microbial cells via the transport system meant for the uptake of this essential salt. Once inside the cell, it inhibits oxidative phosphorylation due to its interference with the phosphate based energy generating processes. Arsenite, on the other hand, enters through a different path (aqua-glycerolporins) and targets a wider range of cellular processes, binding to the thiol groups in essential cellular proteins such as pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase (Lloyd and Oremland, 2006).

3.3.1.7 Copper

Copper is widely distributed in sulphides, arsenites, chlorides and carbonates in gold ores. The mean concentration of 5 to 70 mg/kg exists in unpolluted soil while higher concentrations are found in contaminated environments like mining sites. Bempah et al. (2013), found a concentration of 92.17 mg/kg in gold mine tailings in Ghana. Gold mining has greatly increased Cu concentration in the environment which upon release binds to particles of organic matter, clay minerals and sesquioxides leading to great accumulation in the soil (Ranjard et al., 2006). Copper exists in two states, oxidized state Cu(II), and reduced state, Cu(I). The ability to exist in these two states makes this metal potentially toxic because the conversion between Cu(II) to Cu(I) could lead to a generation of superoxide and hydroxyl radicals (Stern, 2010). Excessive Cu concentration has deleterious effects on soil microbes (Dell'Amico et al., 2008). Copper toxicity is as a result of its harmful effects on the bacterial

cell membranes and nucleic acid structure as well as its ability to alter enzyme specificity and disrupt cellular functions (Bruins et al., 2000).

3.3.1.8 Mercury

Large amounts of Hg are discharged into the environment due to its usage in gold extraction. About 1.32 kg of Hg is lost for every 1 kg of gold produced which goes directly into water, soil and streams as inorganic Hg and later converted into organic forms (Matshusa et al., 2012). Several researchers have reported on its high concentration in gold mine tailings. Rafiei et al. (2010), reported 100 mg/kg concentrations of Hg in Iran whereas Matshusa et al. (2012) reported concentrations as high as 1920 mg/kg in Limpopo, South Africa. Some of the inorganic Hg that reaches aquatic ecosystems is converted by microbes into organic methylmercury (MeHg), which accumulates in fish. Mercury is also inhaled during the mining and roasting processes and dangerous levels remain suspended in air due to its volatile nature. When inhaled by humans, this could lead to a series of health conditions outlined in Table 2.1. Mercury compounds cause oxidative stress to bacterial cells due to imbalance between pro-oxidant and anti-oxidant homeostasis. They have high affinity for thiol group containing enzymes and proteins that serve as a line of cellular defense against Hg compounds. On gaining access to the cell, both Hg II (Hg^{2+}) and MeHg form covalent bonds with cysteine residues of proteins and deplete cellular antioxidants (Valko et al., 2006). The various toxicological effects of metal species in human and microbes are summarized in Tables 3.1 and 3.2.

Table 3.1: Effects of metals on human health

Metals	Effects	References
As	Peripheral vascular disease, lung, skin, kidney and bladder cancer, severe disturbances of the cardiovascular and central nervous systems which may lead to death, bone marrow depression, haemolysis, hepatomegaly, melanosis, polyneuropathy, and encephalopathy may also be observed.	WHO (2001)
Cd	Bronchial and pulmonary irritation, kidney stone, liver damage, various system disorders such as nervous and immune system, blood, bone and Itai disease.	(Satarug et al., 2011)
Cr	Skin rashes, stomach and ulcers upset respiratory problems, weakened immune systems, kidney and liver damage, alteration of genetic material, lung cancer and death chromium hinder enzyme activity, DNA damage, altered gene expression and causes mutations.	Bagchi et al. (2002)
Cu	Accumulation in liver, kidney, brain and cornea leading to cellular damage and Wilson's disease, upper respiratory tract and nasal mucous membrane irritation, hemolytic anaemia, epigastric pain, nausea, dizziness, headache and death may occur.	Martınez and Motto (2000)
Pb	Blood related disorders such as colic, constipation and anemia, high blood pressure, decrease of hemoglobin production, kidney, joints, reproductive and cardiovascular systems disorder, long-lasting injury to the central and peripheral nervous systems, loss of IQ, low sperm count, loss of hearing.	Lam et al. (2007), Shahid et al. (2012)
Hg	Affect gene expression, kidney damage, tremor, restlessness, anxiety, depression and sleep disturbance, paresthesia and numbness in the hands and feet while high doses may lead to death. Total brain damage can occur in early exposure while late exposure results in localized damage to the cerebellum, motor cortex and the visual cortex.	Weiss et al. (2002), Curtis and Klaassen (2010)
Ni	Hypoglycemia, asthma, nausea, headache, cancer of nasal cavity and lungs.	Rattan et al. (2005)
Zn	Tachycardia, vascular shock, dyspeptic nausea, headache, cancer of nasal cavity and lungs, asthma, vomiting, diarrhea, hypoglycemia, pancreatitis and damage of hepatic parenchyma, impairment of growth and reproduction.	Salgueiro et al. (2000), Rattan et al. (2005)

Table 3.2: Toxic effects of metals on bacteria

Metals	Mechanisms of Action	References
Hg, Pb, Cd	Denaturation of protein	(Bánfalvi, 2011)
Hg, Pb, Cd and Zn	Inhibition of cell division	(Khan et al., 2009)
Hg, Pb, Ni, Cu and Cd	Disruption of cell membrane	(Khan et al., 2009, Yuan et al., 2015)
Hg, Pb, Cd, Cu, Ni and Zn	Inhibition of enzyme activities	(Khan et al., 2009,Wyszkowska, 2013)
Hg, Pb, As and Cd	Damage of nucleic acid	(Bánfalvi, 2011, Yuan et al., 2015)
Hg, Pb, Cd	Inhibition of transcription	(Gundacker et al., 2010)

3.4 Bacterial Interaction with metals

Bacteria are the most abundant microorganisms in the soil, with 10^6–10^9 viable cells cm^{-3} of soil. Due to their small size they have a high surface to volume ratio which affords them a large contact area for interaction with their immediate environment. At a higher concentration, metal ions are known to form toxic compounds in bacteria cells (Nies, 1999), and their increasing concentrations in microbial habitats caused by environmental and natural processes, have led to bacteria developing various mechanisms to withstand their presence. Bacteria are known to possess the ability to convert toxic metals into insoluble substances which enhance easy mobility and dissolution in dump-sites (Gadd, 2001). They accumulate metals from the environment as a result of the negative net charge of their cell envelope through a metabolism-independent passive or a metabolism-dependent active process that is determined by absorptivity of the cell envelope and ability to take up metals into the cytosol (Hrynkiewicz and Baum, 2014). This ability to accumulate metal can be utilized to concentrate, recover and remove metals from mine tailings and industrial effluents (Malekzadeh et al., 2002).

In mine tailings, the redox potential, physicochemical conditions, metal speciation and co-contaminants limit bacteria-metal interactions and bacterial activity. Despite this limitation, SRB such as *Syntrophobacter sulfatireducens, Syntrophus gentianae Desulfobacca acetoxidans, Desulfosporosinus* sp. and *Desulfotomaculum* sp., have been reported in both acid base-metal tailings and pH neutral gold mine tailings where they assist in natural bioremediation of mine tailings by precipitating toxic metal species and increasing pH (King et al., 2001, Liu et al., 2004). The level of tolerance shown by bacteria found in various gold mine tailings contaminated environments is determined by the concentration of the metals present in such environments.

Several researchers have reported bacterial interactions with metals in various metals contaminated mining sites. Anderson and Cook (2004), isolated 6 members of the genera *Exiguobacterium, Aeromonas, Bacillus, Pseudomonas, Escherichia,* and *Acinetobacter* resistant to arsenate from two sites contaminated with gold mine tailings in New Zealand and observed that two of the bacteria, *Exiguobacterium* strain WK6 and *Pseudomonas* strain CA1 are well adapted and gained metabolic energy from the utilization of 50 mM and 30 mM of the arsenate which increased their total cell yield two fold. Similarly, Chang et al. (2008), evaluated bacterial interaction with arsenic from arsenic-contaminated gold-silver mines in the Republic of Korea and discovered 15 isolates that were able to oxidize and reduce two different species of arsenic [As(V) and As(III)]. Two of the isolates, *Pseudomonas putida* strains OS-3 and OS-18 completely oxidize 1 mM of arsenite III to V within 35–40 h of growth, while two of the four arsenate reducers obtained (*P. putida* strains RS-4 and RS-5) were able to grow and efficiently utilize 66.7 mM of arsenate V. Bacterial interaction with Hg was also investigated by Ball et al. (2007), in tailing ponds located in gold mining area of El Callao, Venezuela. High rates of resistance to both inorganic Hg and organomercurials were detected among the bacterial isolates. The minimal inhibitory concentrations (MIC) determined shows a broad range of resistance levels. As much as 73.58% of the isolated bacteria strains were able to grow in the presence of 0.1 mM of Hg and when grown in the presence of 0.01, 0.02, 0.04 and 0.07 mM of Me-Hg, the percentage resistances were 71.5%, 59.6%, 48.08% and 30.77% respectively. El Baz et al. (2015), isolated 59 metals resistant bacteria from various abandoned mining sites in Morocco that belong to *Amycolaptosis* and *Streptomyces* genera. Their results shows different levels of metal resistance, the MIC recorded in mM was 1.66 for Pb, 0.51 for Cr and 0.53 for both Zn and Cu. Bacterial interactions with metals have several impacts on the environment as they play crucial roles in

the biogeochemical cycling of toxic metals as well as in cleaning or remediating metal contaminated sites (Gadd, 2010).

3.4.1 Effects of metals on Bacteria

Microbes are usually the first biota to be affected by metal pollution (Gutierrez-Gines et al., 2014). Bacterial communities have been reported to be the most affected by high metal concentration as compared to fungal communities (Rajapaksha et al., 2004). The beneficial or detrimental effect of metal species to microbial cells is a function of its concentration and the form in which it exists in the environment. The essential metals help in building the structure of an organism or assist in metabolic functions as a component of enzymes (Haferburg and Kothe, 2007). The Presence of metal species like Zn, Cu, Ni, Co and Fe at low concentrations is fundamental for numerous microbial activities as they aid in the metabolism and redox processes (Haferburg and Kothe, 2007). Exposure to high metal concentration results in selective pressure on the microbial community leading to the establishment of metal resistant microbial populations with reduced diversity when compared to unpolluted environment. The community profile is affected by reducing the number, biomass, alteration of morphological structure and loss of activity in microbially assisted soil processes such as nitrification, denitrification and decomposition of organic matter. The decrease in diversity can also result in soil erosion due to reduced soil aggregation and poor soil structure. Metals also interfere with the life cycle of microbes and causes decrease in pigmentation of microbial cells (Piotrowska-Seget et al., 2005). Smejkalova et al. (2003), studied the effect of three metals (Zn, Cd and Pb) on colony forming unit (CFU), enzymatic activities and microbial biomass carbon: oxidizable carbon content (C-biomass: Cox ratio) of a soil's microorganisms. They discovered that all the measured parameters were significantly affected by the metals concentrations. Considerable reduction was observed on CFU which was most significant in

the spore-forming and oligotrophic bacteria. Major inhibition of C-biomass was observed in these soils and the C-biomass: ox ratio decreased with increasing soil pollution.

3.4.2 Mechanisms of Bacterial Resistance to Some Selected Metal Species

Many bacteria are able to resist and survive metals-induced stress. When the acceptable limits of metal a bacterial cell can withstand are exceeded, mechanisms of resistance are triggered in order to survive in the adverse environment (Kim et al., 2010, Dupont et al., 2011). Metal tolerant bacteria have been isolated from metal laden environments with some able to survive while others are endemic to their environment and the prevailing environmental conditions may have favoured their selection (Ahemad and Malik, 2012). The ability to survive in these extreme conditions depends on acquired biochemical and structural attributes, physiological, and/or genetic adaptation such as changes of cell, morphological and environmental alterations of metal speciation (Abou-Shanab et al., 2007). Bacteria have developed several types of resistance mechanisms which aid in the maintenance of intracellular homeostasis of the vital metals and normalize resistance against toxic metals which is the principle governing bioremediation processes.

3.4.2.1 Bioaccumulation

This is an energy-dependent metal transport system that involves the retention and concentration of metals by living cells. Metals present outside bacterial cell are transported into the cytoplasm through the cell membrane and the metal is later sequestered (Pandey et al., 2001) intracellularly by metal binding metallothioneins which form complexes with the metal. Metallothioneins are small cysteine rich metal binding proteins that are induced by metals stress conditions in bacteria. They are important in protecting bacterial metabolic processes catalyzed by enzymes which immobilize toxic metal species. Studies have shown the presence of metallothioneins in many cyanobacterial and bacterial strains.

Metallothioneins from *Synechococcus* sp. strain PCC 6301, *Synechococcus* sp. strain PCC 7942 (SmtA) and *Pseudomonas putida* (BmtA), *Oscillatoria brevis* (BmtA), *Anabaena* PCC 7120 (SmtA), *Pseudomonas aeruginosa* (BmtA) have been described by Blindauer et al. (2002). The *smt* locus consists of two divergently transcribed genes, SmtA and SmtB which confers resistance to Zn and Cd in *Synechococcus* spp. (Botello-Morte et al., 2013). This mechanism is subject to environmental modification, availability and toxicity of the metal, intrinsic biochemical and structural properties as well as genetic and physiological adaptation. It includes ion pumps, ion channels, carrier mediated transport, endocytosis, complex permeation, and lipid permeation. Typical examples of this active mechanism are seen in the transport of Zn, Pb, Cu, Cr and Ni (Rani and Goel, 2009). Bioremediation of metals using growing bacteria cells allow both biosorption and bioaccumulation to occur simultaneously. Several authors have reported metal bioaccumulation by bacterial cells as a promising approach for clean-up of metal contaminated sites (El Baz et al., 2015). Wei et al. (2009), reported intracellular accumulation of four metal species by bacteria strain CCNWRS33-2 isolated from root nodule of *Lespedeza cuneate* in gold mine tailings in China. This bacterium was found to have 98.9% similarity to *Agrobacterium tumefaciens* LMG 196 by 16S rRNA. The result obtained shows that 0.101 mM of Cu was accumulated after 4 h, while Cd accumulation increased from 0.225 mM at 4 h to 0.353 mM at 12 h and Pb accumulation reached 0.2 mM at 12 h.

Despite the promising results observed from the use of growing bacterial cells for bioremediation in many studies, there are still some significant limitations to the use of this approach in treatment of metal polluted sites. Uptake of metals by bacterial cells encounters significant practical limitations such as sensitivity of the systems to extremes of pH, high salt concentration, the availability of the contaminant to the bacteria, interactions with co-ions and requirement of external metabolic energy (Kaduková and Horváthová, 2012,

Zabochnicka-Świątek and Krzywonos, 2014). Metal interaction is an important factor that needs to be considered as a result of antagonistic and synergistic interactions of metals due to their competition for the same binding sites which determine their uptake in contaminated environments like mine tailings.

3.4.2.2 Biosorption

This is a non-enzymatic immobilization of metals by dead or living microbial biomass. Dead biomass is better when compared to living biomass, because it is cheaper to obtain as waste, it is not affected by nutritional supply as well as metal toxicity or unfavourable operating conditions. Biosorption denotes the totality of all passive interactions of metal ions with the cell wall, which include adsorption reactions, surface complexation reactions and ion exchange reactions with the functional groups at the cell surface (Sahmoune and Louhab, 2010). In the light of reliance on metabolism, biosorption processes can be divided into metabolism dependent and metabolism independent processes. Depending upon the area where the metal removal takes place, biosorption can be categorized as extracellular accumulation/precipitation, cell surface adsorption/precipitation, and intracellular accumulation. In viable cells, biosorption is dependent on cell metabolism because it is associated with an active defense system of microorganisms, metal is transported across the cell membrane resulting in intracellular accumulation of the metal. Metabolism-independent biosorption using dead biomass occurs due to the physicochemical interaction between the metal and the functional groups (carboxyl, imidazole, sulfhydryl, amino, phosphate, sulfate, thioether, phenol, carbonyl, amide, and hydroxyl moieties) present on the cell surface of the microbial cell. This passive uptake of metal is rapid and reversible and the examples are; physical adsorption, ion exchange, and chemical sorption. Microbial cell walls comprised polysaccharides, proteins, glucans, chitin, mannans, and phosphomannans and have abundant metal-binding groups such as carboxyl, sulphate, phosphate, and amino groups. These ligands

are known to be involved in metal chelation (Ahalya et al., 2003, Ahluwalia and Goyal, 2007). In adsorption, metal ions bind non-specifically to extracellular cell surface associated polysaccharides and proteins (Rani and Goel, 2009).

Metal uptake capability by some bacteria has been reported as successful in many studies, Dorian et al. (2012), evaluated biosorption capacity of *Delftia tsuruhatensis* isolated from mine tailings in Mexico. This bacterium showed resistance to 6 mM Pb and 25 mM Zn and maximal absorption for Pb and Zn was observed to be 0.216 mM/g and 0.207 mM/g respectively. Isotherm curves generated from equilibrium batch sorption experiments and effect of process parameters have been extensively researched (Sag et al., 2000). In addition, desorption of adsorbed metals using dilute eluents and cyclic use of regenerated biomass has also been studied (Dixit et al., 2015). However, research that takes into consideration the physicochemical conditions seen in mine tailings such as cocktail of metals, low nutrient contents of the tailings, salinity and other important factors that dictate the effectiveness of this process for efficient metals removal in mine wastes such as tailings is limited. Also, there are still limitations with respect to most studies carried out on biosorption because information on absorbent characterization which is an important prerequisite for repeatability of the results is still lacking. Surface characterization of the bio sorbent in terms of surface area, surface morphology, functional group and particle size has now recently been included (Ramrakhiani et al., 2011, Kirova et al., 2012). There is also a need for more research on characterizations as well as final disposal of the bio sorbent used in order to develop a reliable biosorption process.

3.4.2.3 Biotransformation

Bacteria are able to interact with metal species and alter the metal structure through mechanical and biochemical mechanisms which affect the speciation and mobility of the metal (Uroz et al., 2009). Chemical transformations of metals are brought about through

many processes such as oxidation, reduction, methylation, and demethylation which are sometimes by-products of normal metabolism of the bacteria (Surjit et al., 2014). Biological transformation of metals is a significant detoxification mechanism that is carried out by different bacterial species. The biological action of bacteria on metals results in changes in valency and/or conversion of metal species into organometallic compounds that are volatile or less toxic (Gadd, 2010). In an oxidation-reduction reaction, bacteria mobilize or immobilize metal ions, metalloid and organometallic compounds, thus promoting redox processes. Metals reduction by bacteria leads to metal solubility which enhances efficient mobilization of the metal. Mobilization reduces the metals to a lower oxidation state which gives rise to metallic elements (load zero) thereby reducing the metal toxicity. For example, the oxidation of arsenite As(III) to arsenate(V) and the reduction of mercury ions to metallic mercury (Hg^{2+} to Hg^{0}) greatly increases the volatility of Hg and may contribute to its transport away from the microorganism's immediate environment. In bio methylation, the transformation of metals such as Hg, As, Cd and Pb leads to their increased mobility and suitability for involvement in processes that lead to the reduction in their toxicities. It is an enzymatic mechanism that involves the transfer of methyl group (CH_3) to metals and metalloids. The resulting methylated compounds formed differ in solubility, volatility and toxicity compared to the original metal (Gadd, 2004). For example, the inorganic forms of Hg are more toxic when compared to methyl and dimethyl mercury and also the inorganic forms of As are more toxic than methylated species (acids and methyl-As dimethyl-As) (Tabak et al., 2005). Numerous studies have reported the conversion of metal species by bacterial cells. Govarthanan et al. (2013), reported the conversion of lead nitrate $Pb(NO_3)_2$ to lead sulphide (PbS) and lead silicon oxide ($PbSiO_3$) by *Bacillus* species isolated from mine tailings. In addition to this is the extracellular conversion of Pb ions to PbS by phototrophic *Rhodobacter sphaeroides* reported by Bai and Zhang (2009).

3.5 Genetic Determinant of Metal Resistance

Genetic determinants responsible for resistance to metals are found in several bacterial strains. These resistance determinants are mediated by the chromosomal genome, plasmids or transposons and involve many operons like czcD, nccA, pco, cop, mer, ars, etc. (Nies, 2003). The resistance-encoding genes seem to be plasmid mediated mainly and these findings have led to suggestions that these plasmids are most likely to be spread by horizontal transfer (Rensing and Grass, 2003).

Zinc resistance is mediated by two efflux mechanisms which are P-type ATPase efflux and resistance nodulation cell division (RND) driven transporter system (Spain and Alm, 2003). Efflux system is the most studied of all mechanisms of metal resistance in bacteria and involves an active system of transport that actively pumps back toxic ions that entered the cell via an ATPase pump or diffusion (a chemiosmotic ion or proton pump). This mechanism is mediated by plasmids and involves the P-type ATPase which catalyzes the reactions of ATP hydrolysis forming a phosphorylated intermediate (Nies, 1995). Metal is transported from the cytoplasm to the periplasmic space by the energy released from ATP hydrolysis. This mechanism is one of the pathways responsible for metal resistance in Gram-negative bacteria. Xiong et al. (2011), isolated a novel and multiple metalloid resistant strain, *Comamonas testosteroni* S44 having up to 10 mM resistance to zinc. Whole genome sequencing of this bacterium, revealed 9 putative Zn^{2+} transporters (4 znt operons encoding putative 4znt operons which encode Zn^{2+} translocating P-type ATPases and 5 czc operons encoding putative RND family protein). The RND is a family of proteins that take part in transport of metals. It pumps metal from the cytoplasm directly to the extracellular space and is powered by the proton gradient across the cell wall in Gram-negative bacteria (Nies, 1999, Spain and Alm, 2003).

Cupriavidus metallidurans strain CH34 that was isolated from various metal laden environments is a good example of a bacterium to describe plasmid-borne determinants. This type strain possesses two large plasmids pMOL28 and pMOL30 that contain the different types of metal resistant genes. Plasmid-borne czc confers resistance to Cd, Zn, Co, ncc to Ni, Co and Cd and cnr to Co and Ni cation efflux metal resistance operons (Mergeay et al., 2003). The czc locus is located on pMOL30 which is approximately 250 kb in size while the ncc and cnr were reported to be located on pMOL28 (180 kb) (Monchy et al., 2007). The *cnr*YXHCBA operon of *R. eutropha* CH34 plasmid is the most well studied of the determinants that facilitate medium levels of (up to 10 mM) of Ni and Co resistance (Mergeay et al., 2003). The mechanism of resistance mediated by cnr is inducible which as a result of an energy-dependent efflux system driven by a chemo-osmotic proton-antiporter system (Taghavi et al., 2001). Another Pb resistance operon found in *Cupriavidus metallidurans* strain CH34 is the pbr, which functions in uptake, efflux and accumulation of Pb. These resistance loci are made of five structural resistance genes which are: (i) pbrT, coding for Pb uptake protein; pbrA, coding for a P-type Pb efflux ATPase; (iii) pbrB, coding for a predicted integral membrane protein whose function is unknown; (iv) pbrC, codes for a predicted prolipoprotein signal peptidase; and (v) pbrD gene, that codes for a Pb binding protein, was identified in a region of DNA, which was essential for functional Pb sequestration (Borremans et al., 2001).

The pco and cop operon comprises four structural genes ABCD and an additional one pcoE with two regulatory trans-acting genes pcoRS and copRS (Brown et al., 1995). The arsenic resistance system also comprises three genes Ars ABC. Arsenite is transported by the arsenic resistance efflux using either a two-component (ArsA and ArsB) ATPase or a single polypeptide (ArsB) which functions as a chemiosmotic transporter. The *arsC*, encodes an

enzyme that converts intracellular arsenate [As(V)] to arsenite [As(III)], the substrate of the efflux system (Nies and Silver, 1995).

The mer operon on the other hand allows bacteria to detoxify Hg^{2+} into volatile metallic mercury through enzymatic reduction. This operon varies in structure and is made up of genes that encode the functional proteins for regulation and transport (merC, merE, merF, merG, merT) of Hg^{2+} to the cytoplasm where it is reduced by merA. The merB is also found downstream of merA, a periplasmic scavenging protein (merP) and additional one or two regulatory proteins (MerR, MerD) (Lin et al., 2012). The genetic determinant responsible for multiple metal (As, Pb, Cd, Hg, Ni, Co and Cu) resistance patterns observed in 45 Gram positive and Gram negative bacteria isolated from the rhizosphere of *Alyssum murale* and Ni rich soil was examined by Abou-Shanab et al. (2007), using polymerase chain reaction in combination with DNA sequencing. The genes responsible for this resistance *(nccA, czcD, mer, and chr*) were discovered to be present in these bacteria.

3.6 Alteration of Cell Morphology

Another mechanism that bacteria adopt to withstand metal stress is the alteration of cell morphology. This was observed in phototropic bacteria *Pseudomonas putida* and *Enterobacter* sp. on exposure to metalloid oxyanions (Nepple et al., 1999) in the presence of noxious organic compounds (Neumann et al., 2005). It was also reported that high temperature brought about morphological changes in *E. coli* (Bennett et al., 1992) and *Pseudomonas pseudoacaligenes* (Shi and Xia, 2003). Exposure of bacteria to unfavourable environmental conditions encountered in polluted sites such as mine tailings with toxic metals/metalloids, highly acidic or alkaline pH and the high and low temperature observed typically induced a stress response which gives rise to characteristic changes in cell shape and arrangement. These responses assist in protection of vital processes, restoration of cellular homeostasis and increase in cellular resistance against subsequent stress challenges (Foster et

al., 2000). Chakravarty et al. (2007), reported the effect of four metal species (Cd, Cu, Ni and Zn) on acidophilic heterotrophic *Acidocella* strain GS19h that was isolated from a Cu mine. This bacterium by passes the noxious effect of the metals by reducing its surface area in relation to volume ratio. This change was due to alteration of cell morphology as a result of the penicillin binding proteins present on the bacterial cell envelope which give shape to the bacteria cell. The divalent metals structurally resemble the calcium cation and it was proposed that the metals bind in place of calcium to the binding sites as a result of their similar ligand specificities.

3.7 Future Prospects

Considering the extreme conditions that are found in gold mine tailings, future work may look at how the resistant bacteria interact with metal species in this environment. To develop an efficient bioremediation approach for gold mine tailings, better understanding of bacterial interactions with metals in this environment is required.

3.8 Conclusion

Gold mining has played a tremendous role in the growth and sustenance of the economies of many countries with a huge price to pay in the form of generation and release of toxic waste products which have profound impacts on the ecosystem. Although some metals are required for normal functioning of life processes, elevated concentrations of these metals like those found in mining environments today can be toxic to bacteria that are responsible for biogeochemical cycling of nutrients which are therefore beneficial to human health. Bacterial interactions with metals in contaminated environments have important environmental and health implications and these interactions could result in clean-up of metal-contaminated sites. Most studies on bioaccumulation have focused on accumulation of individual metal ions when exposed to test organisms. Only a limited number of studies

utilized growing bacterial cells with multiple mechanisms of metal sequestration and thus may hold greater metal uptake capacities. Nevertheless, such challenges can be overcome by strain selection and supply of nutrients to support the bacteria growth. The screening and selection of metal resistant strains peculiar to contaminated environments is paramount to overcome the limitation of utilizing living cell systems. Resistant cells are anticipated to bind substantial amounts of metals which will greatly enhance bio precipitation/intracellular accumulation and development of an efficient bioremediation process. Understanding the various ways bacteria interact with these metals can explain the ability of the bacteria to remove noxious ions from the environment.

CHAPTER FOUR

PHYSICO-CHEMICAL ASSESSMENTS AND METAL PROFILES OF SOME ABANDONED GOLD MINE TAILINGS IN KRUGERSDORP, SOUTH AFRICA

Abstract

Mining of gold has resulted in the production of enormous quantities of mine wastes in the form of tailings which have great impacts on the environment and human health. This study aimed to assess the physicochemical properties and concentrations of selected metal species and metalloids Cd, Zn, Ni, As, Cr, and Pb characteristic of three abandoned gold mine tailings: mine tailings A (MA), mine tailings B (MB), Tudor shaft (TS) and their surrounding soil (MAC, MBC and TSC) in Krugersdorp, South Africa. Metals were extracted from samples using aqua regia and their concentrations determined using atomic absorption spectrometry. The physicochemical parameters were determined using standard laboratory methods. The pH values of the soil and tailings samples ranged from 2.17-6.79 with a mean value of 6.34. Low values were recorded for cation exchange capacity (CEC), nitrogen, organic matter and nitrate contents in the samples. Mean concentrations of metals in samples from Tudor shaft (TS) were 612.25 mk/kg, 6.6 mg/kg, 490.1mg/kg, 2,247.0 mg/kg, 46.1mg/kg and 2555.4 mg/kg for As, Cd, Co, Ni, Pb, Zn respectively. High concentrations were also recorded in the surrounding soil (As; 737.4 mg/kg, Cd; 5.5 mg/kg, Co; 856.6 mg/kg, Ni; 2786.8 mg/kg, Pb; 136.8 mg/kg), and Zn; 4269.3 mg/kg). Concentration of all the metals in tailings and soil samples from Tudor shaft and Pb and Zn in samples MAC and MBC exceeded the South Africa recommended values for soil and sediments. The elevated levels of metals recorded showed that the environment and human health around the tailings are at risk and there is an urgent need to find a suitable approach to treat the polluted sites.

Keywords: Bacteria, Metals, Soil properties, Mine tailings, Tudor shaft

4.1 Introduction

Witwatersrand in South Africa houses some of the largest gold reserves in the world, making the country one of the world leaders in gold mining (Hart, 2014). Gold mining has resulted in the generation of thousands of tailings dumps along the gold mining corridor from Nigel to Randfontein (Bobbins, 2015). Mine tailings is one of the major concerns of gold mining (Mileusnić et al., 2014). Gold mine tailings are known to present great risk of contamination to soils, plants, surface and groundwater because of the dissemination of particles containing potentially toxic metals and metalloids through wind action and/or by runoff from the tailings to streams that drain these tailings (Naicker et al., 2003). In many of the residential areas around abandoned mines like Krugersdorp located in West Rand, dust from tailings has been a long-term environmental hazard because mining operations release huge amounts of metal species containing dust into the environment (Bobbins, 2015).

Metal contamination associated with gold mining is one of the foremost environmental concerns in South Africa. Elevated levels of aluminum (Al), uranium (U), manganese (Mn), Ni, Cd, As, Zn, Hg, Co and Cu, have been reported in soil around Tudor shaft, an old radioactive mine dump (Bambas-Nolen et al., 2013) and other gold mine tailing dumps and water resources in their vicinity in south Africa (McCarthy, 2011, Mitileni et al., 2011, Matshusa et al., 2012, Kamunda et al., 2016, Olobatoke and Mathuthu, 2016) and all over the world (Rafiei et al., 2010, Bempah et al., 2013). The adverse effects of metals are linked to their transfer in the trophic chain from soil through plants to animal and human (Chary et al., 2008). High levels of metal species in soils may lead to their uptake by plants which reduce crop yields as a result of their inhibitory effect on the physiological metabolism of the plants (Singh and Kalamdhad, 2011). Contamination of aquatic environment with metals may affect aquatic ecological balance and biodiversity (Forstner and Wittman, 2012).

Mining activities also disrupt nutrient dynamics of the soil as a result of alteration in the physical, chemical and microbiological properties of soil (Adewole and Adesina, 2011). Numerous studies have emphasized the significance of soil properties; organic matter, particle sizes, redox potential (ORP), electrical conductivity (EC), moisture content, CEC, clay content and pH on the behaviour of metals in soils (Šourková et al., 2005, Keskin and Makineci, 2009). High contents of organic matter enhances metals adsorption thereby reducing their availability in the environment. Elevated concentrations of metals in soil could decreases soil organic matter content because only plants and bacteria with the ability to thrive in the stressful conditions imposed by the metals can survive (Ogar et al., 2015). During acidic condition, metals in soils are more soluble enabling them to go into soil solution easily (Sheoran and Choudhary, 2010). These soil properties also have an effect on the abundance of microbial groups and their functional diversity in the soil environment (Fijałkowski et al., 2012). Changes in pH can upset microbial metabolism due to its effect on the activities of certain enzymes and nutrient availability which could be toxic to soil bacteria (Kapoor et al., 2015). Medium textured loam and clay soils favour microbial activity compared to sandy soils with lower water retention potentials (Turbé et al., 2010) highlighting the signiifcnce of soil texture in soil microbial activities. Fu et al. (2010), reported that severe destruction in soil structure caused by mining activities could lead to serious soil erosion. These effects cause dramatic changes in bacterial diversities and activities, disrupt enzymatic activities and change the community structure thereby leading to loss of soil fertility (Ahmad et al., 2012) and decline in ecosystem functioning. Mining activities also cause land degradation as a result of (1) removal of topsoil and cover plants that add litter and protect the soil, (2) disturbance of surface and subsurface hydrologic regimes, (3) digging and dumping of rock and soil that cover the minerals to be extracted (overburden). The present study investigated selected physicochemical parameters and

concentrations of six metal species (Pb, Ni, Zn, Cr, Cd, Co) in some abandoned gold mine tailings dams in Krugersdorp, South Africa, to determine how mining has effected these soil properties and the implications on metal behavior and microbial activities in soil.

4.2 Materials and Methods

4.2.1 Geographical description of sampling sites

The sampling sites are located in Gauteng province, which is the wealthiest province in South Africa. This province houses the Witwatersrand basin that has the largest gold deposit in the world. The basin is made up of the Kosh basin, the Free state gold fields, far West Rand , West Rand, Central Rand and East Rand which have been mined for over a century with 40 percent of gold ever unearthed from the earth coming from these areas (EEB, 2000). The basin covers an area of 1600 km^2 and has led to a legacy of some 400 km^2 of mine tailings dams and 6 billion tons of pyrite tailings containing 430,000 tons low-grade uranium (CSIR., 2009).

Gauteng province is in the Highveld of South Africa, positioned at an altitude of 1700 m (5580 ft) above sea level. The sampling site, Krugersdorp is located in the West Rand district in Gauteng province with coordinates $26^0 6^1$S and $27^0 46^1$E and covers a total area of 247.22 km^2 (95.45 sq. mi) with a population of 378, 821 and 570/km^2 (1,500/sq. mi) making it the 8th biggest city in Gauteng province. This province has a sub-tropical climate with total annual rainfall of about 759 mm. A temperature range of between 15°C and 26°C is experienced in summer while winter periods are usually between 4°C and 16°C occasionally dipping below the freezing point. Brief summer thunderstorms accompanied by thunder and lightning with hail are usually experienced in the late afternoons while winters are crisp and dry with frost occurring in Southern parts. Light wind speed (4 ms^{-1}) is generally observed except during thunderstorms with evaporation rate ranging between 109 and 246 mm/mol

(Otto, 1996). Some parts of the area are conserved and classified as endangered as a result of habitats loss, higher degree of fragmentation and developments of threats.

The oldest rock formation in Gauteng province is the granite dome situated between Pretoria and Johannesburg, the younger sedimentary and volcanic rocks of the Transvaal and Witwatersrand Supergroups are deposited on this granite dome. Large expanse of Gauteng contains the Proterozoic era formations of the Transvaal supergroup, notably containing the gold-bearing "Black Reef" quartz-pebble conglomerate, which has been mined on the East and West Rands. The ridges of conglomerate of the Witwatersrand basin give rise to ease west ridges on resistant quartzite and the Ventersdorp volcanic lavas ridge in the Klipriviersberg hills south of Johannesburg and to the east and west of Heidelberg. The Transvaal Supergroup is made up of malmani dolomites which house the world famous fossil while sinkholes and subsidence of the dolomites occur in the West and East Rand (Viljoen, 1999).

The vegetation around the study area is made up of grassland and savanna biomes comprising 71% and 29% respectively of Gauteng area. The Gauteng savanna comprise nine different vegetation types in which the most common are the Central sandy Bushveld and Marikana Thornveld comprising 6.3% and 5.8% respectively. Savanna are the richest of all the biome with respect to animal biodiversity. Seventeen percent (17%) of Gauteng area are classified as urban land uses while the remaining area showed complex and land capability patterns. About 23.1% of the deep, well drained, apedal soils of the Hutton type give soil with arable potential and another 25.3% is considered "marginally" arable, with the remaining 51.6% suitable for grazing and wildlife. The soils are dominated by plinthic, duplex and hydromorphic soil, which make the land and soils unsuitable for agricultural crop production (Bredenkamp, 2002).

4.2.2 Sample collection and preparation

The sampling points were identified based on the reports of high metal pollution from gold mines in Krugersdorp area. Soil samples were collected at depths of 0-15 cm with a Dormer steel soil auger from three abandoned gold mine tailings in Krugersdorp between the months of February to September, 2014. The three sites were site MA (27.80764 E, -26.14265 S), site MB (27.81576, -26.12771) and site TS (27.80362, -26.13191) commonly known as Tudor Shaft (Photo 4.1 and Figure 4.1). Adjacent native soils were also collected from the surroundings of the tailing dumps designated as MAC, MBC and TSC (Figure 4.1). Three subsamples were collected a few meters apart randomly from each site and combined to obtain a composite sample representative of the sites. The samples were packaged in labelled plastic bags and transported to the laboratory for analysis. According to ISO (2006 ISO 11464) standard, the samples were air dried, crushed, and sieved through a 2 mm sieve and the sieved soils were used for analyses. Selected physical and chemical parameters of the samples were analyzed using standard laboratory procedures.

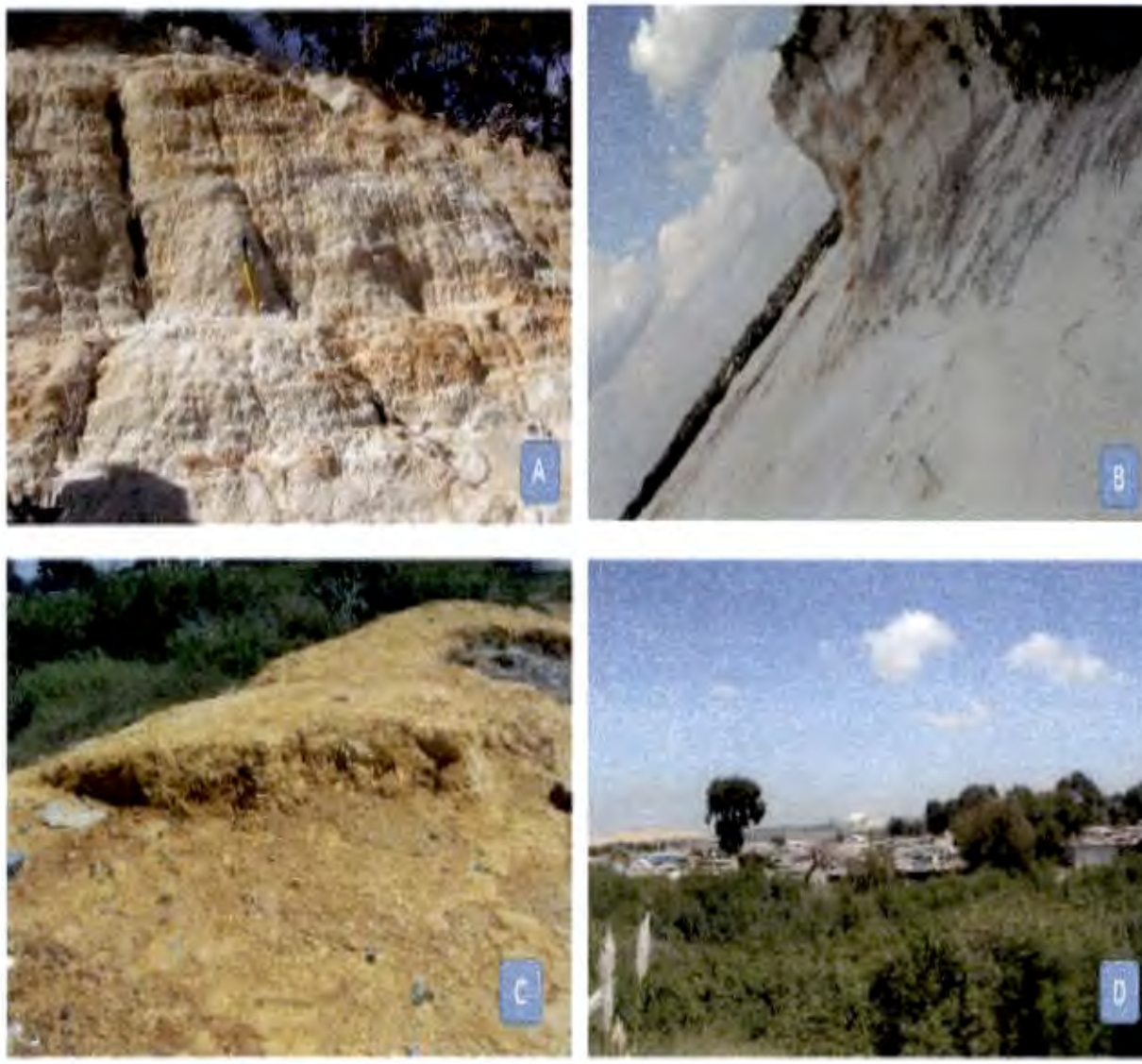


Photo 4.1: Showing the sampling sites. A = Sampling site (MA), B= Sampling site (MB), C= Sampling site (TS), D= Residential sites close to TS

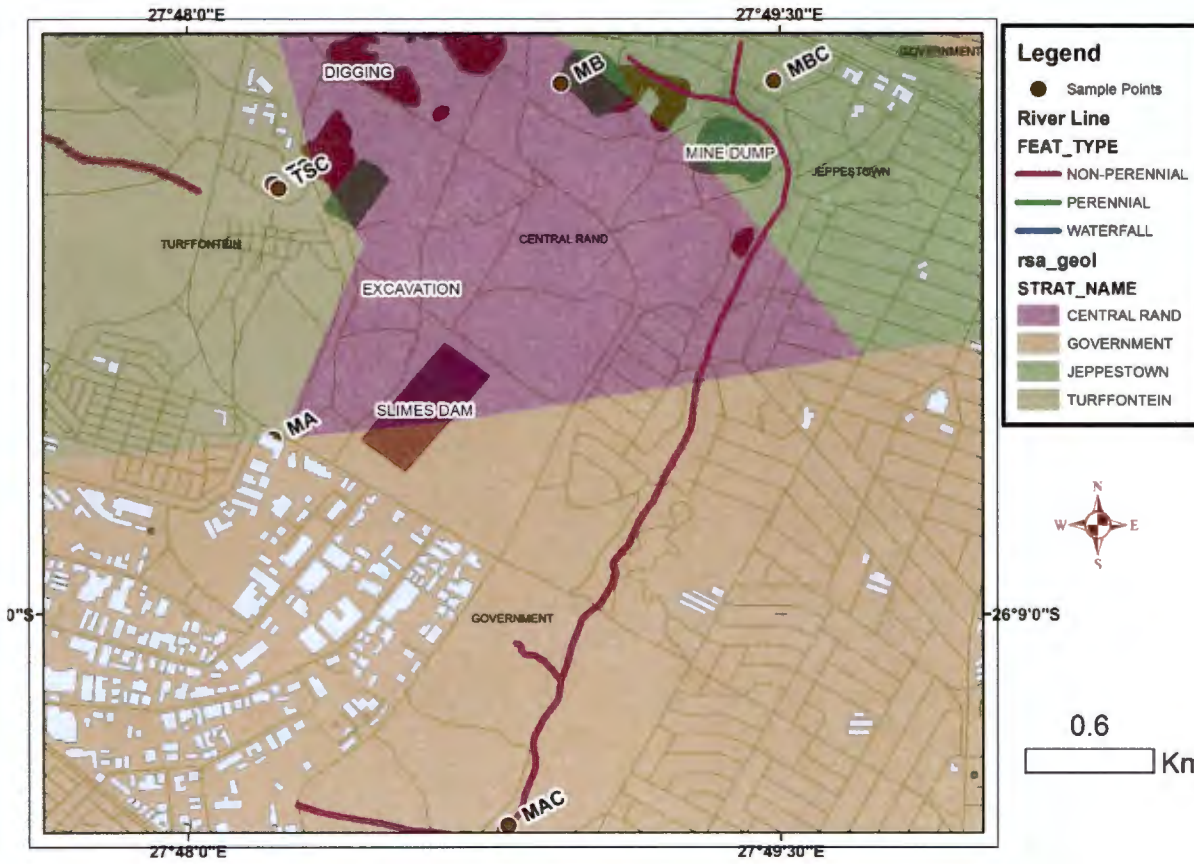


Figure 4.1: Map showing the detailed sampling points

4.2.3 Determination of physicochemical parameters of the samples

4.2.3.1 Determination of soil moisture

The moisture content of the tailings and surrounding soils was determined according to the method described by Odu et al. (1986). Hundred grams (100 g) of each tailings sample (W_1) was weighed into a clean pre- weighed 150 mL beaker and kept in an oven at 105°C ± 3°C for 24 h. The difference in the weight of beaker and soil before (W_2) and after drying in the oven (W_3) was recorded and used to calculate moisture content of the tailings and soil samples as indicated in the formula below.

$$\text{Percentage moisture} = \frac{(W3-W1)-(W3-W1)}{W3-W1}$$

Where W1= Weight of empty can

W2 = Weight of soil and beaker before oven drying

W3 = Weight of soil and beaker after oven drying

4.2.3.2 Determination of particle size

Particle size distribution of samples was determined using the < 2 mm fraction using the Bouyoucos hydrometer method (Gee et al., 1986 , Filgueira et al., 2006). Fifty gram (50 g) of 2 mm sieved air-dried soil sample was weighed into 500 ml beaker and 100 ml of the dispersing solution (hexametaphosphate, $Na_6(PO_3)_6$) and 200 ml of distilled water added. The mixture was homogenized with electric mixer for 5 mins after which the dispersed soil was transferred into 1000 ml measuring cylinder and made to volume with distilled water. A plunger was used to homogenize the soil water suspension, after which the cylinder was shaken by turning end on end and then placed on a stable surface. A hydrometer and thermometer were then inserted into the suspension and hydrometer and temperature readings

taken exactly 40 sec after the cylinder was placed on the table. According to Bouyoucos (1962), sand particles would have settled at the bottom of the cylinder after 40 seconds leaving silt and clay particles in suspension. The hydrometer reading after 40 seconds therefore measures the density of the suspension of silt and clay of the sample. The cylinder was again shaken to homogenize the suspension and again placed on the table. The hydrometer and temperature readings were again taken 6 hours 52 min after placing the cylinder on the table. After this time, only clay particles are expected to be in solution. The 6 hrs reading therefore represents the density of clay suspension of the sample.

The following equations are used to calculate the percentage of sand, silt and clay in the sample:

$$\% \text{ clay} = \% \text{ clay} = \frac{corrected\ hydrometer\ reading\ after\ 6\ hours}{mass\ of\ sample\ (50g)} \text{ x } 100$$

$$\% \text{ silt + clay} = \frac{corrected\ hydrometer\ reading\ after\ 40\ seconds}{mass\ of\ sample\ (50g)} \text{ X } 100 \text{ .. \% clay}$$

% sand = 100 % - (% silt + % clay)

The weight % sand, silt and clay obtained was then used to determine the textural class of each sample using the soil textural triangle (USDA, 1999).

4.2.3.3 Determination of sample pH, electrical conductivity (EC) and redox potential (ORP)

Ten grams of each soil sample was weighed into a beaker and mixed with 25 ml of distilled water to obtain 1:2.5 (m: v) soil-water suspension. The mixture was allowed to shake for 1 h on a rotary shaker for pH measurement with a pH meter Jenway 3520 (Van Reewijk, 1992). The combined electrode was inserted into supernatant; the solution just above the sand layer. Hydrogen ion concentration (pH) values and ORP of the samples was recorded simultaneously, electrode was washed with distilled water after each reading. The EC of soil

samples were determined by immersing equiptronics digital electrical conductivity bridge in the supernatant solution used for pH determination and the EC values recorded. Each measurement was replicated three times. The means of the three readings were therefore recorded as the sample pH and EC.

4.2.3.4 Determination of CEC and extractable cations

The ammonium acetate extraction technique was used to determine the CEC of samples. Twenty five grams (25.0 g) of each soil sample was weighed into a 500 ml Erlenmeyer flask and 125 ml of the 1 M NH_4OAc added. The suspension was shaken thoroughly and allowed to stand for 5-7 h after which the solution was allowed to leach through a leaching tube. The clear filtrate obtained from each sample was used for determination of the extractable sodium, potassium, calcium and magnesium (Na, K, Ca, and Mg) in the samples. The NH_4^+ saturated soil in the leaching tube was then washed with 150 ml 50% ethyl alcohol followed by 30 ml 90% ethyl alcohol to remove the excess NH_4^+. The adsorbed NH_4^+ in the soil was leached with 25 ml of the replacing solution (1M KCl) four to five times. The leachate was transferred to a 250 ml volumetric flask and brings to volume with additional 1 M KCl. The concentration of NH_4^+-N in the KCl extract of each sample as well as in the blank was determined by distillation. The CEC of each sample was then calculated as follows:

CEC (cmolc/kg) = (NH_4-N in extract - NH4-N in blank) / 18

Where NH_4-N is reported in mg NH4/L:

If mg/L of NH_4 is quantified in the leachate, 18 mg NH_4 is used

4.2.3.5 Determination of Organic Matter

Organic carbon was determined by the modified Walkley-Black method (Abollino et al., 2002). One gram (1 g) of air-dried samples was weighed into 500 ml Erlenmeyer flask

and 10 ml of 1 N potassium dichromate solution added, followed by 20 ml of concentrated sulphuric acid. The flask was swirled and left in a fume hood for 30 mins during which time it was periodically swirled. After 30 mins, none of the sample developed a green colour and so 200 ml of deionised water and 10 ml concentrated orthophosphoric acid were then added to the solution followed by 12 drops of diphenylamine indicator (1 g diphenylamine in 100 ml concentrated sulfuric acid). The mixture was then titrated with 0.5 M ferrous ammonium sulphate until a colour change from violet-blue to green was observed. Reagent blanks were also prepared in which no samples was added.

The normality of the $FeSO_4.7H_20$ solution used was obtained using the reagent blank as follows

$$\text{Normality of } FeSO_4.7H_2O = \frac{\text{Total volume of } K_2Cr_2O_7 \text{ used x Normality of dichromate}}{\text{Total volume of } FeSO_4.7H_2O}$$

Volume of $K_2Cr_2O_7$ reduced =

(Volume of 1N $K_2Cr_2O_7$ used — (Volume of $FeSO_4.7H_20$ X Normality of $FeSO_4.7H_20$)

Organic carbon was then calculated as follows

$$\%\ O.C = \frac{0.395 \text{ X volume of } K_2Cr_2O_7 \text{ reduced}}{\text{Mass of soil}}$$

% organic matter = 1.72 x % organic carbon

4.2.3.6 Determination of sulphate

Ten grams (10 g) of air-dried, sieved soil samples were each weighed into 50 ml Erlenmeyer flasks and 25 ml of the extracting solution (39 g NH_4OAC and 1 liter of 0.25 M acetic acid) added. The mixture was shaken at 200 oscillations per minute for 30 mins after which 0.25 g of activated charcoal was added. The mixture was again shaken for an additional 3 mins. The resulting solution was then filtered through a sulphate free filter paper (Whatman no 42) which had been washed with the extracting solution. Ten milliliter of the filtrate was then pipetted into a 50 ml Erlenmeyer flask and 1 ml of acid seed (6 M HCl + 20

mg of K_2SO_4 and 50 ml of 40 mg standard solution plus 50 ml of concentrated HCl) solution was added. The solution was swirled and 0.5 g of $Bacl_2.H_20$ crystals was added and mixture was allowed to stand for a min and swirled frequently to dissolve the crystals. Sulphate content in the resulting solution was determined by taking the optical density or transmittance within 3 to 8 mins intervals using a UV spectrophotometer at a wavelength of 420 nm.

4.2.3.7 Determination of Carbon, Nitrogen and Sulphur (C, N and S)

The content of C, N, and S in each sample was determined using a LECO CNS Trumac Analyzer. One gram of each tailing samples was weighed into a large ceramic boat and loaded into the purge chamber located in the front of the horizontal ceramic high temperature furnace. The system utilizes combustion techniques to determine carbon, sulphur and nitrogen content of a sample. Entrained atmospheric gas was purged from the samples and the ceramic boat was introduced into the furnace regulated at a temperature of 1100°C to 1450°C. Complete oxidation of the sample was ensured by a pure oxygen environment within the furnace, with additional oxygen being directed onto the sample via a ceramic lance. A reference soil standard (CNS LECO part no 502-309 having carbon percentage (%) (11.98 ±0.44), nitrogen % (0.93 ± 0.04) and sulphur % (0.136 ± 0.009) was used to calibrate the equipment. The instruments settings and operations conditions were done in accordance with the manufacturers' specifications (Table 4.1). The C N S in the samples were calculated as follow

$$\%\ Analyte\ = \frac{grams\ Analyte\ x\ 100}{sample\ mass} - Atmospheric\ blank$$

Where atmospheric blank is calculated by analyzing the same sample mass encapsulated and in an open container, dissolving in water or palletizing the sample. The difference in the results is the atmospheric blank.

Table 4.1: LECO operating conditions

Elements parameters	Nitrogen	Carbon	Sulphur
Baseline delay time	6 sec	0	0
Minimum delay time	35	15	15
End line time	2	2	2
IR base line	1 secs		
Auto detect data missed time	3 secs		
TC baseline time	10 secs		
Burn cycle	Lance flow	Purge flow	Time (secs)
1	Off	On	5 sec
2	On	On	End
Equilibrate time	30 secs		
Not filled out time	300 secs		
Equilibrate pressure time	4 secs		

4.2.3.8 Determination of nitrate

Nitrate content of the samples was determined using the equilibrium extraction method described by Bremmer and Keeney (1965). A 10.0 ± 0.05 g of ≤ 2.0 mm of air-dry soil was placed into a 250 cm^3 wide mouth extraction bottle and 25 ml of 2.0 M KCl extracting solution (150 g of KCl in 500 ml deionized water diluted to 1000 ml) was added, stoppered and shaken for 30 mins on a shaker. The solution obtained was filtered to get a clear extract and the nitrate content was determined in the clear extract using a spectrophotometer automated flow analyzer.

4.2.3.9 Determination of metal contents

Metal contents of the samples were determined by aqua regia extraction put forth by Nieuwenhuize (1991). Conventional aqua regia (3:1 HCl: HNO_3) digestion was performed according to the method of Chen and Ma (2001). A well-mixed dried and powdered sample of 1g was weighed into 100 ml conical flask, 15 ml of aqua regia was added and the flask was covered with a watch glass. The mixture was placed on a hot plate and digested for 3 h at 110^0C. After evaporation to near dryness, the sample was removed from the hot plate, allow to cool and the extracts were diluted with ultra-pure water to a final volume of 100 ml in a volumetric flask. The contents were mixed together and left standing overnight to settle. The supernatant was analyzed for the concentrations of Pb, Ni, Zn, Cr, Co and Cd using a contraAA 300. The operating conditions of the equipment and the concentrations of the standard used are shown in Table 4.2.

Table 4.2: Operating conditions for metals determination using Contra AA varian 300

			Standards					
Elements	Wavelength	Burner height (mm)	Cal-zero	Cal-std 1	Cal-std 2	Cal-std 3	Cal-std 4	Cal-std 5
As	193.6960	7	0	7.5	7.5	30	60	120
Cd	228.8018	4	0	2.5	5	10	20	40
Co	240.7254	5	0	2.5	5	10	20	40
Ni	232.0030	4	0	2.5	5	10	20	40
Pb	217.0005	7	0	2.5	5	10	20	40
Zn	213.8570	6	0	7.5	15	30	60	120

4.2.4 Statistical analysis

Descriptive statistics such as mean, maximum, minimum, range and standard deviation of the physicochemical parameters were done. Also inferential statistics such as correlation coefficient and ANOVA were used to compare the relationship between the metal species and the physicochemical properties in order to establish the relationship between the tailings properties and metal contents using the SPSS statistical package programme (version 21) at the significance level of 5%.

4.3 Results and Discussion

4.3.1 Physicochemical properties of soil and tailings sample

The fate and activities of metals in soils and sediments are known to be controlled by the physicochemical properties of the soils which dictate their mobility and bioavailability (Osakwe, 2010). Hydrogen ion concentration (pH), CEC, EC, ORP, sulphate and organic matter are known to be the major soil properties affecting metal behaviour in the mining environment. Other physicochemical properties of the tailings such as the particle size, moisture content, also have a significant role to play in metals availability in the soil.

Detailed results of the physicochemical properties of the samples are presented in Appendix 1. The mean percentage moisture content of the samples are presented in Figure 4.2. The highest moisture content was recorded in samples from TS followed by those from MA, whereas samples from MB recorded the least values. The tailings samples contained more moisture 7.23%-12.07% with mean value of 6.61% than the surround soils 3.39%-6.56% and mean value of 4.22%. These differences were however insignificant (p >5). Moisture content in mine soil is not a stable parameter; it is affected by sampling period, organic carbon content, height of dump, stone content, the texture and thickness of litter layers on the dump surface (Maiti, 2006). Maiti et al. (2002), reported percentages as low as 2-3% in summer while average content of 5% was found in winter. The finding of this study conforms to that reported in the study of Adewole and Adesina, (2011), Sadhu et al. (2012), Mapindusi et al., (2016). High percentages of moisture were also observed in autumn.

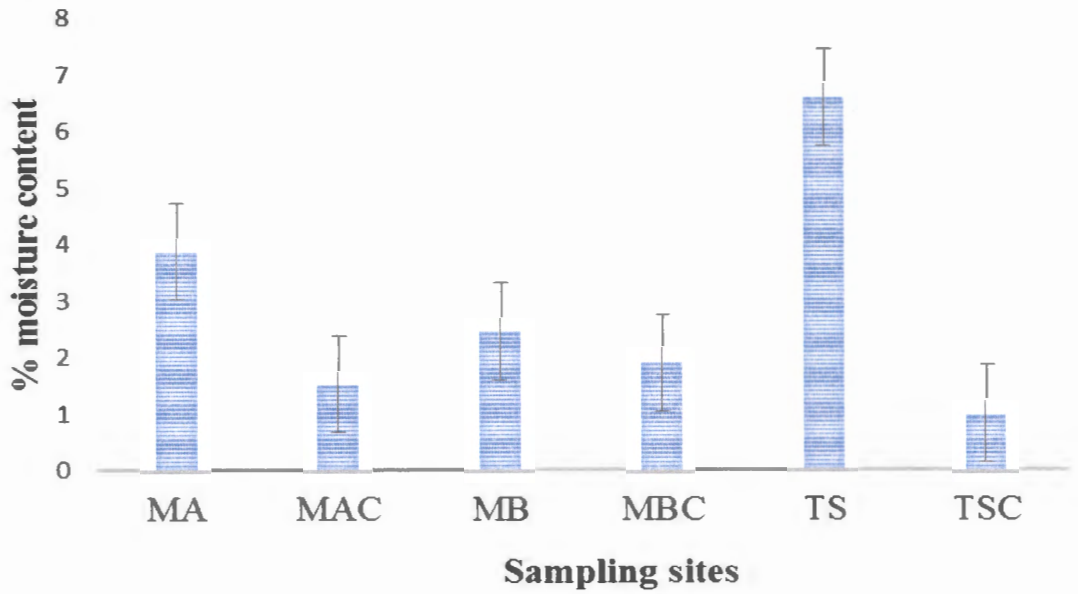


Figure 4.2: Mean % of moisture content in the sampling sites

The percentage of clay, silt, and sand in the samples range between 16-28%, 12-44% and 28-64% respectively (Table 4.3).

Table 4.3: Particle size distribution (mean of three samples) and texture of the tailings

Samples			Proportion of soil separates			Textural class
	Codes	Location	Clay (%)	Silt (%)	Sand (%)	Textural class
Mine tailing A	MA	Kagiso	16	28	56	Sandy loam
Surrounding soil	MAC	Kagiso	24	12	64	Sandy clay loam
Mine tailing B	MB	Chamdor	24	12	64	Sandy loam
Surrounding soil	MBC	Chamdor	28	44	28	Silt loam
Tudor shaft dump	TS	Tudor shaft	26	18	56	Sandy clay loam
Surrounding soil	TSC	Tudor shaft	28	34	38	Sandy clay loam

The highest percentage of sand was recorded in samples from MB and MAC followed by those from MA and TS while samples from MBC recorded the least value. Samples TSC and MBC recorded the highest percentage of clay followed by tailings from TS, MB and MAC with the lowest value in MA. Highest percentage of silt was recorded in MBC followed by TSC, MA and TS while same value was recorded for MAC and MB as shown in Table 4.3. According to the soil textural triangle (USDA, 1999), the tailings samples were classified as either sandy, loam-sandy, or clay loam which was markedly different from the surrounding soils classified as silty loam (Figure 4.3). Soil texture is an important properties of the soil that exert a great influence on soil behaviour, such as water holding capacity, nutrient leaching, nutrient retention and supply as well as drainage. The generally sandy texture of the soils implies a low surface area, poor ability to retain and supply enough nutrient to plants and soil fauna. The diversity and function of bacteria and other organisms present in such soil may be compromised (Reeve et al., 2010). The sandy texture will also result in increased bioavailability of metals in this environment as a result of low binding capacity of sandy soil for metals. Hence, metal mobility in these environments may be high.

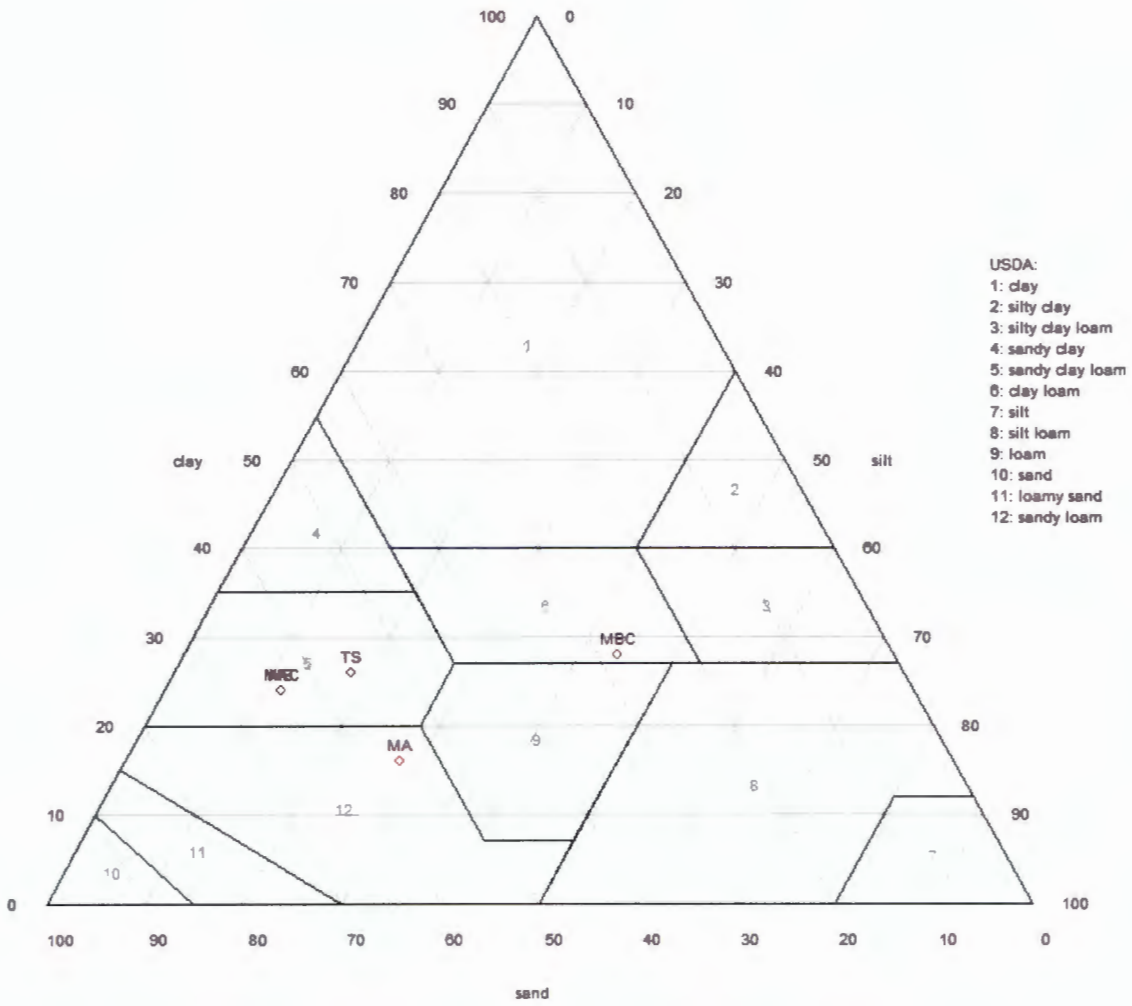


Figure 4.3: Textural triangle indicating textural classification of samples

There were significant differences in the pH of the samples from the three sites (P = 0.000). Almost all the samples analyzed were acidic (Figure 4.4) with the pH values ranging from 2.17-6.79 with mean value of 6.34. Samples from sites MA and MB were the most acidic with their surrounding soils having similar pH values. The pH values of samples from TS were however different (Figure 4.4).

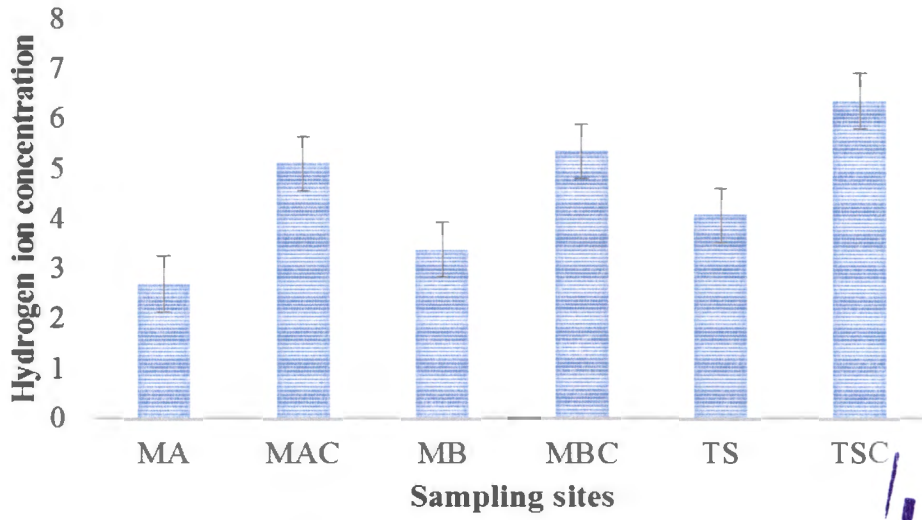


Figure 4.4: Mean values of the hydrogen ion concentration (pH) in the sampling sites

The pH of the soil system is a very significant parameter that has a direct influence on sorption/desorption, precipitation /dissolution, complex formation, and oxidation-reduction reactions. The result of this study corroborate with the previous result of García-Lorenzo et al. (2012).

The acidic values recorded in the tailing soils may be due to AMD as indicated by Dold (2014). Acid mine drainage is the biochemical oxidation of sulphide bearing minerals, which result in low pH and the variation in pH recorded among sites could be due to different mineral composition of the tailings. Generally, redox level varied between 194 - 380 mV, showing highest level in samples from MA followed by MB and TS and lowest level (147-195 mV) in surrounding soils with mean value of 126.50- 301.00 mV (Figure 4.5). These results are substantiated with the findings of Stoltz and Greger (2006), Ňancucheo and Johnson (2011) that also reported similar values in mine tailings.

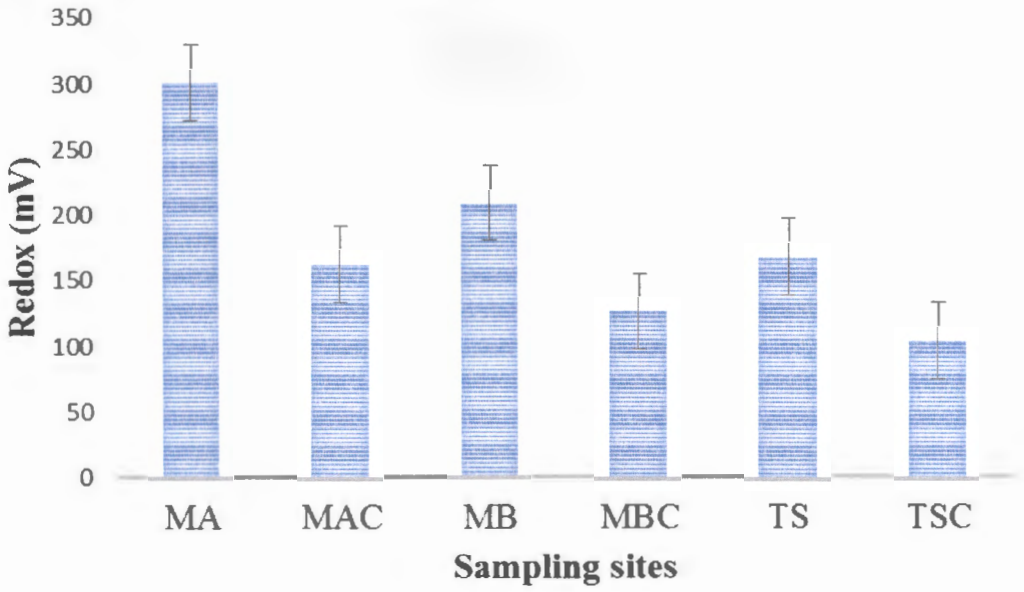


Figure 4.5: Mean values of the redox potential in the sampling sites

Redox potential indicates the state (oxidizing or reducing) of metal species in the environment. When in reducing conditions which are common in contaminated sites, the acidophiles and metallophiles present utilize the metals for their growth and metabolic activities and this increases their solubility which in turn increase their availability in the environments (Chuan et al., 1996). Redox potential and pH jointly affect metals speciation and its release, since metals are bound to particles (inorganic or organic) or precipitate at high pH and become more available or mobile under acidic conditions. Solubility and mobility of metals in soil are major drivers of soil/plant/microorganism systems (Ogar et al., 2015). Randall et al. (2004), reported that concentration of mercury in mercury containing mine wastes maintained at pH 3.2 increased as the oxidation potential increased until the redox value reached 0.2 mV. The acidic nature of the samples in the study site, coupled with the sandy nature of the soils would imply high mobility of metal in the three different sites.

The mean values of EC, CEC and % organic matter is presented in Figure 4.6-4.8 below

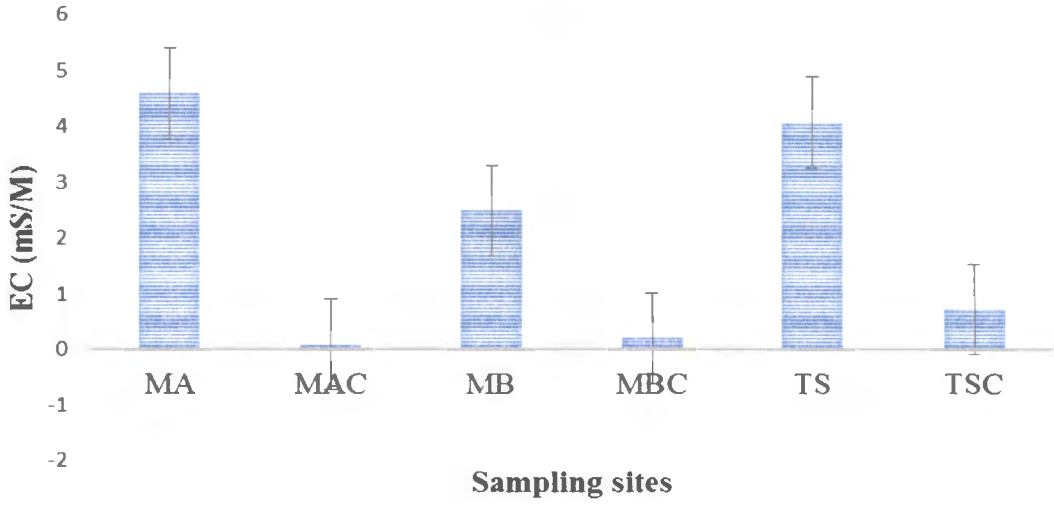


Figure 4.6: Mean values of the electrical conductivity (EC) in the sampling sites

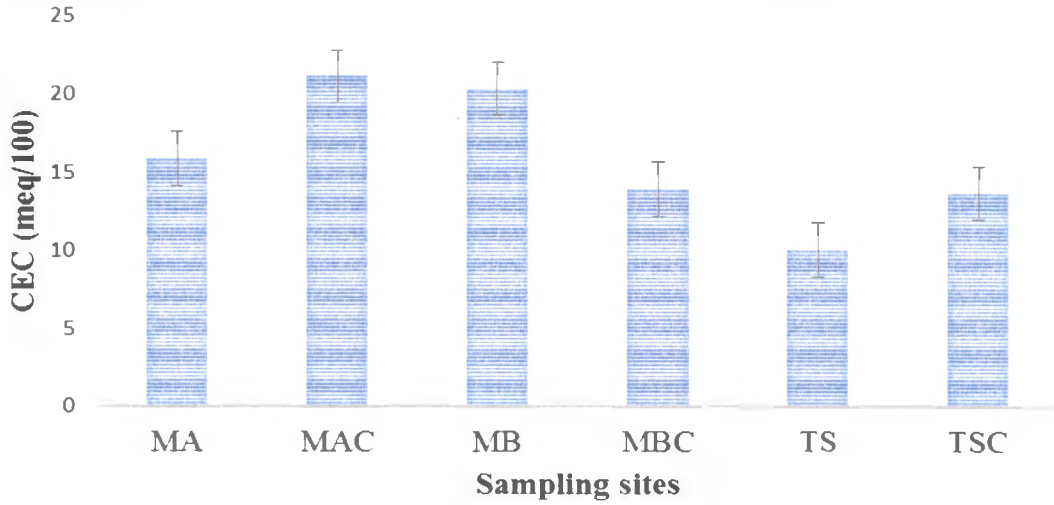


Figure 4.7: Mean values of the CEC in the sampling sites

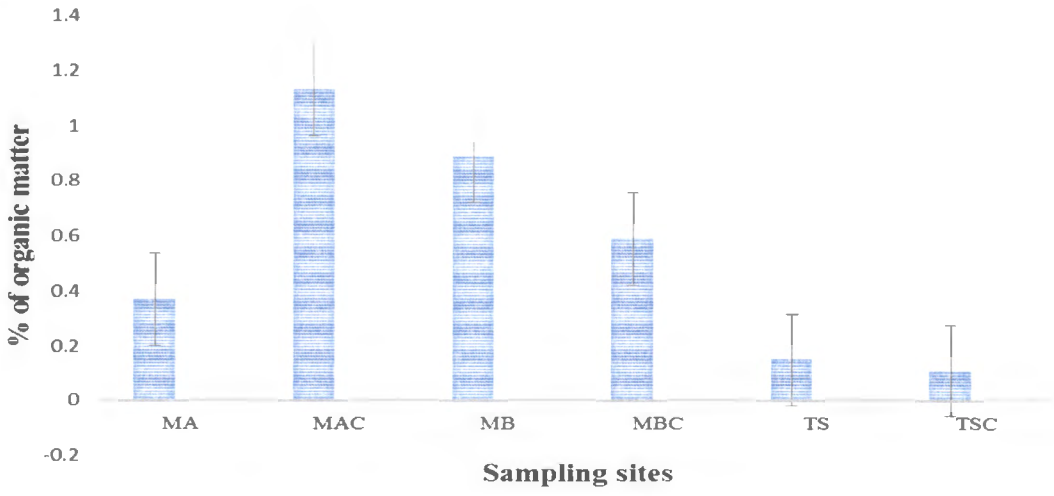


Figure 4.8: Mean % of organic matter in samples in the sampling sites

The EC recorded in the three sites varied with values ranging between 5.33 mS/m to 11.52 mS/m in the tailings whereas the surrounding soils had an EC range of 0.49 mS/m to 3.39 mS/m with mean values of 0.09-4.59 mS/m as shown in Figure 4.6. These differences observed in the EC of the mine tailings and the surrounding soils were significant ($p < 0.05$) and indicate different levels of salinity in the samples. High levels of salinity has a negative impact on microbial activities as a result of decrease in microbial biomass and basal soil respiration (Liao and Xie, 2007). This site may therefore have low microbial diversity and activity as well as low soils productivity because of reduction in the rate of soil organic matter decomposition and the mineralization of carbon, nitrogen and phosphorous which are the responsibilities of soil microbes.

The CEC varied from site to site, with values ranging between 20.83-22.43 Meq/100 and an average value of 10.07-20.31Meq/100 in the tailings while the surrounding soils shows values of 23.44-23.90 Meq/100 with average value of 13.65-21.10 Meq/100 (Figure 4.7).

The differences in CEC values recorded in the sampled tailings and soil were statistically significant (P= 0.021). The values obtained are reflective of the amount of clay present in the samples as presented in Table 4.3. The result obtained in this study is in accordance with the findings of Taberima et al. (2010), who also reported low to medium CEC values ($\leq$ 20 me/100g). The CEC values are generally low and fall within the range of CEC values for 2:1 clays. These values indicate poor ability of the soils to retain cations. This further indicates a high potential for metals availability in these environment.

The organic matter recorded in the three sampling sites differered significantly with the lowest mean concentration recorded in samples from TS whereas the highest mean values was recorded in the control sample MAC. The values for OM content in samples recorded range from 0.19-1.88% and mean value of 0.89% in the tailings whereas the surrounding soils recorded values ranging from 1.24% -2.81% and average value of 1.13% as presented in Figure 4.8.

The result obtained in this study is consistent with the findings of Rösner and Van Schalkwyk (2000), Yang et al. (2003) and Ashraf et al. (2012) which shows that mine soils generally lack organic matter. Soil organic matter in mine tailings has been reported to be low (Sadhu et al., 2012) due to reduction in the number and type of plants and bacteria diversities responsible for nutrients turn over in the soil. Low content of organic matter observed in the sampling sites will also increase metals bioavailability in the tailings (Vamerali et al., 2010).

Low nitrate content was also recorded in all the samples as compared to arable soils. Sampling sites MA and MB shows similar level while the highest level was recorded in the third sampling site, TS as shown in Figure 4.9

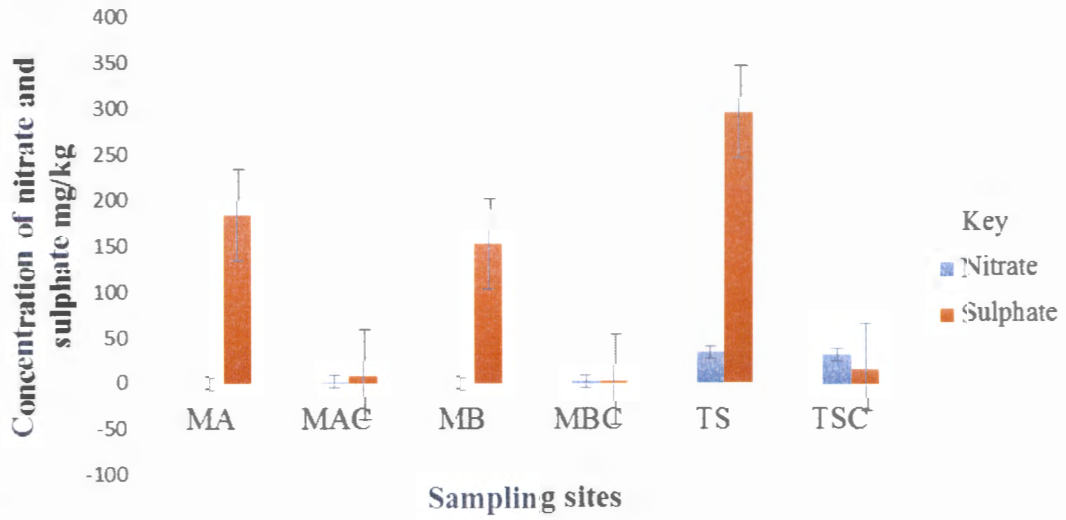


Figure 4.9: Mean concentration of nitrate and sulphate in the sampling sites

Sulphate content recorded in the three sampling sites followed the pattern TS > MA > MB as shown in Figure 4.9. The highest concentration recorded in TS is above the permissible limit of 200 mg/ml stipulated by (WHO, 2004). High sulphate content is an indication of AMD. Sulphate originates from exposure of sulphur-containing minerals such as pyrite, chalcopyrite present in the Au host rock to water and atmosphere during mining excavations leading to the formation of sulphuric acid (Bosman, 2009).

The mean percentage of the tailings nutrients (nitrogen, carbon and sulphur) is presented in Figure 4.10 below.

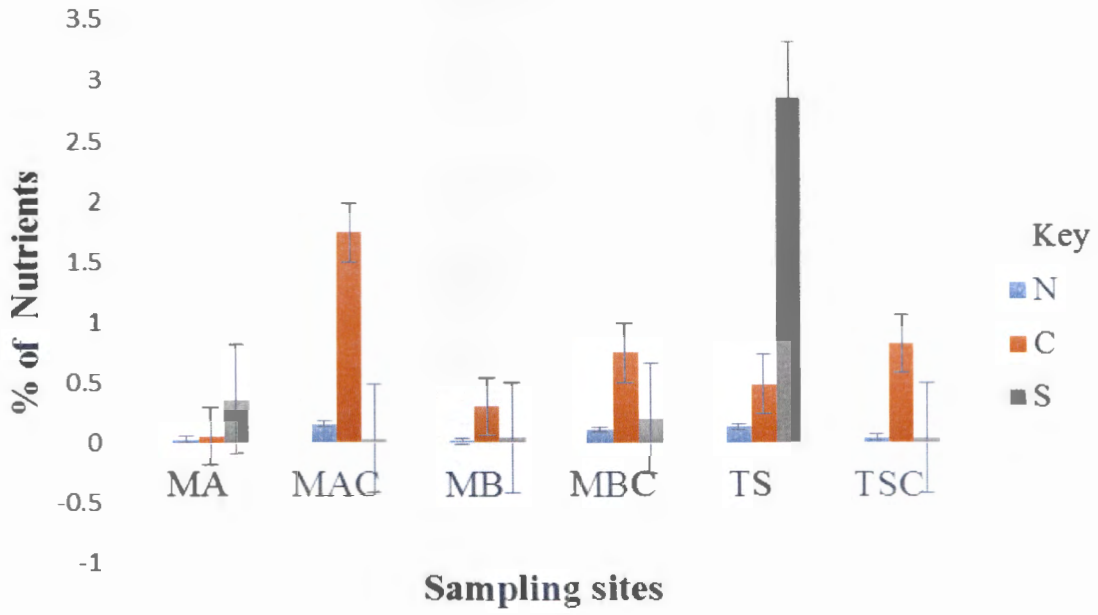


Figure 4.10: Mean % of N, C and S in the sampling sites. *NB: N is nitrogen, C is carbon, and S is sulphur*

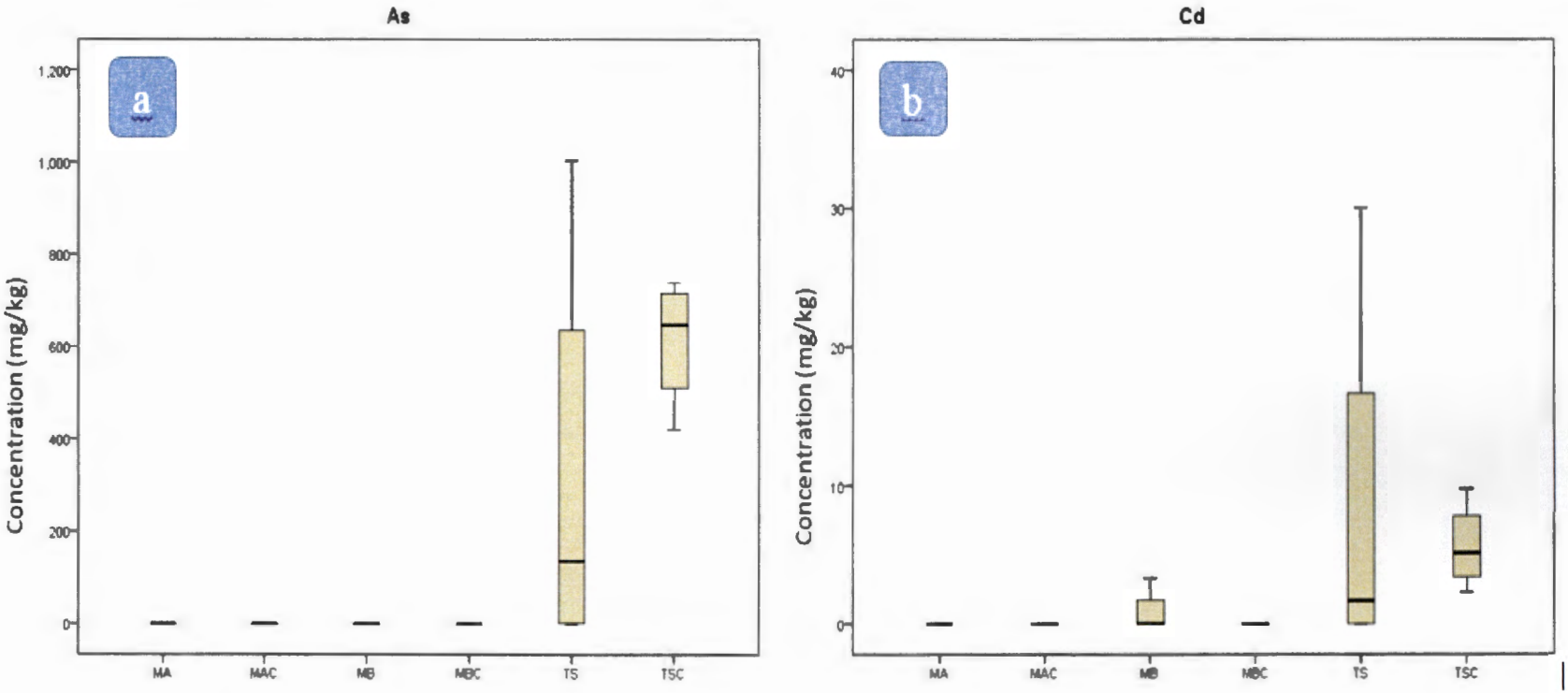
Highest percentage of sulphur was recorded in samples from site TS as compared to the two other sites. Differences in sulphur content of the tailings was highly significant (P= 0.000) with a maximum value of 3.22% implying that the tailings could still be undergoing oxidation. According to Rosner (2007), a total sulphur content of approximately 0.1% within the first metre below the surface of tailings indicates that the tailings have almost fully oxidized. The carbon content in the samples ranged between 0.40-1.12% with mean value of 0.48% in the tailings while samples from the surrounding soil recorded values of between 0.29-3.27% and average value of 1.61%. Low nitrogen content of 0.04-0.22% with mean value of 0.01-0.13 % as compared to arable land was recorded in both the tailings and the surrounding soils as shown in Figure 4.10.

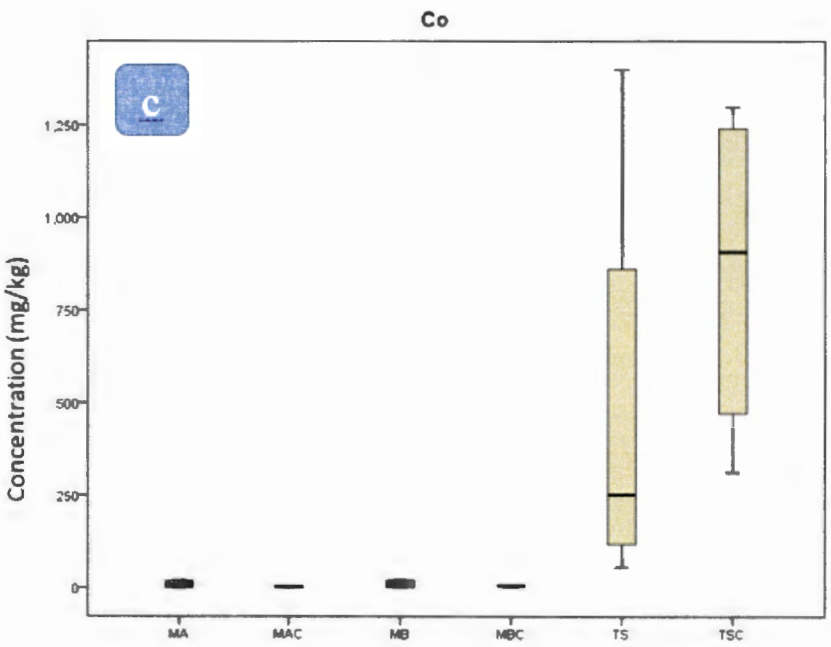
The nitrogen content recorded in this study also corroborates with the values reported by Saviour and Stalin (2012), who also noted that the inadequate mineralization of organic nitrogen and reduce mineralization rates of mine dumps lower the nitrogen contents. Nitrogen is generally known to be deficient in mine dumps (Sheoran and Choudhary, 2010) because of a decreased microbial population and unfavorable conditions for maintenance of soil vegetation cover and formation of soil humus. Large amount of nitrogen could also have been

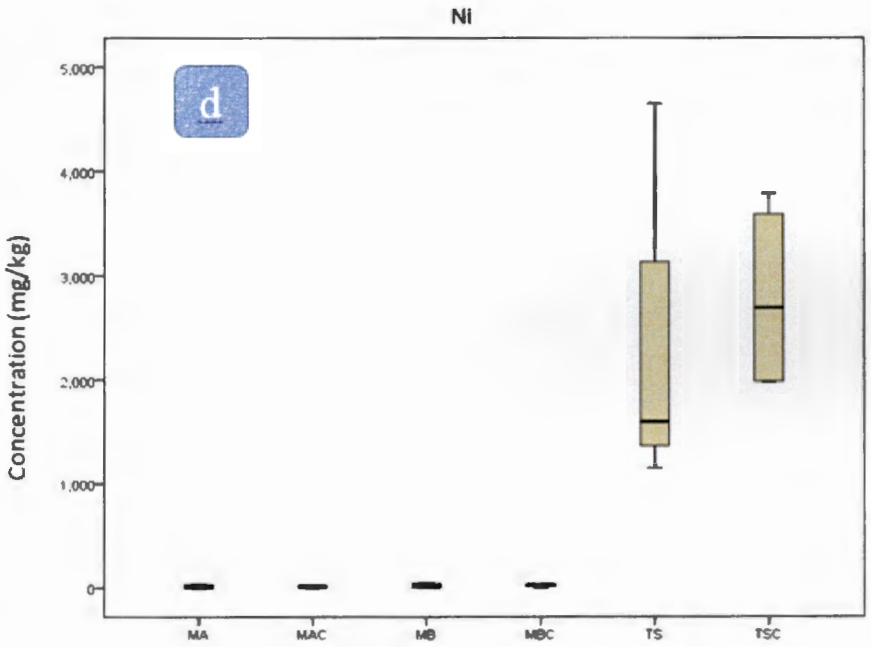
lost from soil surface as a result of removal of natural vegetation through mining activities (Aghasi et al., 2011).

4.3.2 Metals concentration in the samples

The concentrations of the six metal species: As, Cd, Co, Ni, Pb and Zn recorded in the samples after acid digestion are shown in Figure 11 (a-f).


As
a
Concentration (mg/kg)
1,200
1,000
800
600
400
200
0
MA
MAC
MB
MBC
TS
TSC
Cd
b
Concentration (mg/kg)
40
30
20
10
0
MA
MAC
MB
MBC
TS
TSC


Co
c
Concentration (mg/kg)
1,250
1,000
750
500
250
0
MA
MAC
MB
MBC
TS
TSC


Ni
d
Concentration (mg/kg)
5,000
4,000
3,000
2,000
1,000
0
MA
MAC
MB
MBC
TS
TSC

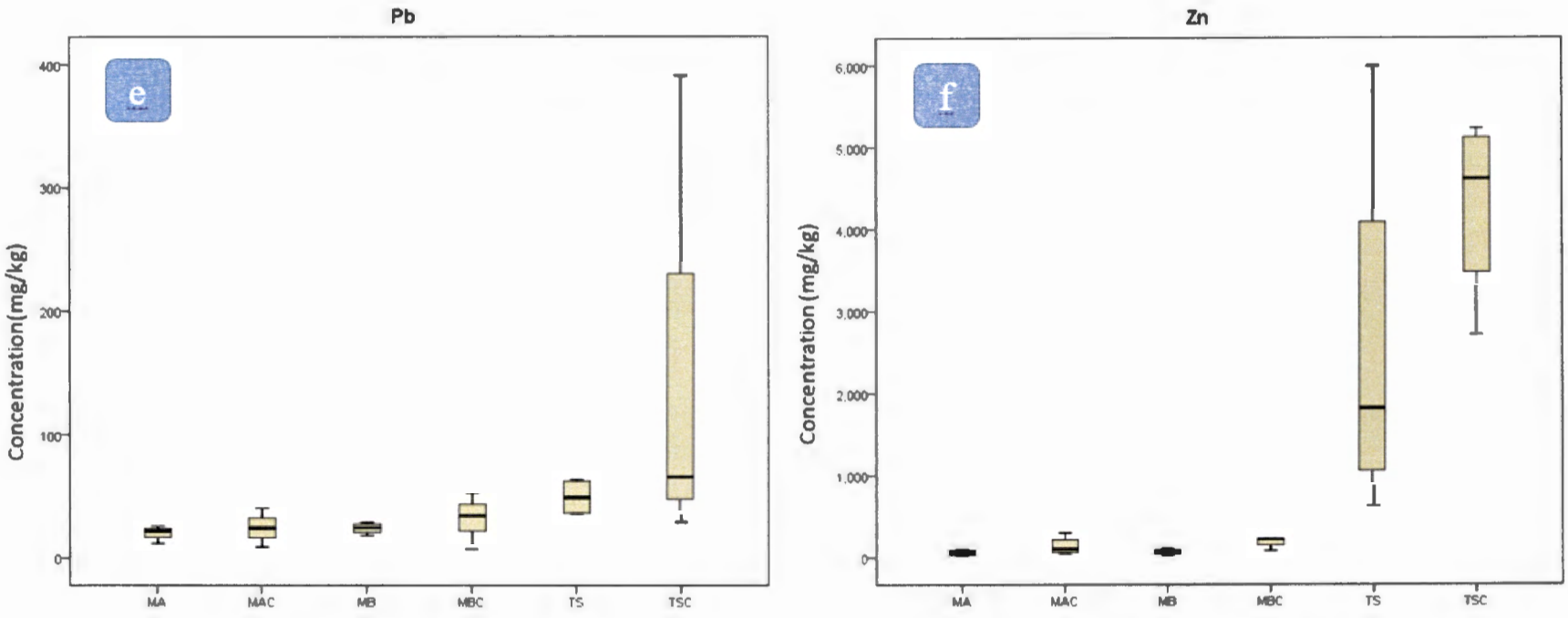


Figure 4.11 (a-f): Total concentrations of the (a) As (b) Cd (c) Co (d) (Ni) (e) Pb (f) Zn recorded during the different sampling periods

The total concentration of As detected in samples MA and MB and their surrounding soils (MB and MBC) were below the detection limit of the ContraAA 300 used for the analyses. The maximum values of Pb and Zn (Figure 4.11 e and f) and the mean values recorded in MAC and MBC as shown in Table 4.4 exceeded the recommended values stipulated by South Africa standards for soils and sediment quality guidelines, Dutch pollutant standard and Canadian soil quality guidelines for the protection of human and environmental health as outlined in Table 4.4. However, the concentration of Cd, Co and Ni found in these two sites falls within the acceptable limit recommended by the three standard bodies. Highest concentration of all the metals in both tailings and soils were recorded in sample from TS (Figure 4.11 (a-f). The mean values recorded (Table 4.4) shows that all the metals exceeded the recommended values of the standard bodies as shown in Table 4.4. Cadmium in samples from TS however had values which still fall within the permissible levels recommended by South Africa standards for soils and sediment quality guidelines but above the Canadian and Dutch standards.

Table 4.4: Recommended standards of metals in soils and sediments

Element	MA		MB		TS		South Africa[a]	Canada [b]	Netherlands[c]
	MA	MAC	MB	MBC	TS	TSC			
As	< 1	< 4	<1	< 4	261.65	612.50	5.8	30	12
Cd	< 1	< 1	<1	< 1	6.61	5.54	7.5	1	1.4
Co	9.17	2.57	10.87	5.09	490.09	856.60	300	20	NA
Ni	17.37	13.44	24.33	26.68	2247	2786.75	91	35	50
Pb	16.46	20.82	20.47	29.47	46.11	136.79	20	85	50
Zn	61.11	102.16	69.01	155.61	2555.43	4269.25	240	140	200

Ref a: National Norms and Standards for Remediation of Contaminated Land and Soil Quality in South Africa.

Ref b: Canadian Soil Quality Guidelines for the Protection of Human and Environmental Health

Ref c: Dutch pollutant standard.

With the exception of Pb and Cd, significant differences were observed in the concentration of the six metals in the samples with P values of 0.037, 0.004, 0.000 and 0.000 for As, Co, Ni and Zn respectively. Gold mining activity in South Africa has resulted in deterioration of the surrounding environment through contamination of soil by metals as observed in this study. This observation agrees with several studies conducted on gold mine tailings dams as potential source of metals contamination in adjoining soils and sediments in South Africa (Bempah et al., 2013, Olobatoke and Mathuthu, 2016).

4.3.3 Implications of the physicochemical properties of soils and tailings on metal behaviour in surrounding environment

The acidic pH recorded in most of the samples in this study implied that greater amounts of the metals will be dissolved in the soil solution thereby increasing their bioavailability in the environment. Zinc solubility in soil has been reported to increase by 100 at every unit decrease in soil pH (Mortvedt et al., 1991). Chuan et al. (1996), also reported increasing solubility of Pb, Cd and Zn in contaminated soils as the pH decreases from 5.0 to 3.3.

The Lower content of organic matter recorded in the samples is an indication that metals will be mobilized in the environment and thus increase bioavailability of the metals to plants and microbial populations present in the soil. Organic matter provides sites for cation exchange as a result of the carboxyl and phenolic groups that provide strong affinity to metals that form metal complexes or chelates. These complex formations occur in great part with the fulvic and humic acids, present in the organic matter in large quantities (Mellis, 2004). Hernandez-Soriano and Jimenez-Lopez (2012), reported decreasing mobility and bioavailability of Cd, Cu, Pb and Zn in three soils amended with different levels of organic materials.

The CEC values recorded (Appendix 1) shows that metals will be retained in the tailings and consequently released into the environments as the values recorded are not significantly high. Likewise, the different values recorded for the redox potential can cause dissolution and precipitation of some minerals ubiquitous in mining contaminated environments which can increase metal load in the environment. This may result in leaching of metals bound in the tailings to overlying surface water. Antić-Mladenović et al. (2016), reported increased rate of mineral weathering as a result of high redox values recorded in their study.

Bioavailability and mobility of metal species will be high at the sampling sites as a result of lower contents of clay minerals recorded in this study. This will result in decrease rate of metals adsorption because clay minerals possess edges that are important sites for metals retention. Maskall et al. (1995), reported that migration of Pb and Zn down the soil profile was greatly reduced due to the attenuative properties of clays.

A very strong negative and positive correlation relationship was observed in most of the analyzed parameters. Of all the metals, only Co and Zn showed strong positive correlation with pH ($P \leq 0.05$ and r value of 0.408 and 0.033) (Appendix 4) respectively. This shows that pH determines the bioavailability of this metal species. All the metals except Pb were highly positively correlated ($P \leq 0.01$ and $P \leq 0.05$) with r values of 0.432 for Cd and As, 0.534 for Co and As, 0.592 for Ni and As, 0.582 for As and Zn, 0.861 for Co and Cd, 0.863 for Ni and Cd, 0.848 for Zn and Cd, 0.964 for Co and Ni, 0.974 for Co and Zn and 0.976 for Ni and Zn. This shows that with the exception of Pb, the remaining five metals are associated with each other and their presence could be majorly due to gold mining activities. The presence of Pb could be from other anthropogenic inputs aside from gold mining activities. Among the physicochemical parameters, pH and EC was negatively correlated ($P = 0.006$, $r = -0.548$) (Appendix 4) which shows that acid generation will result in accelerated dissolution of the

solid matrix leading to increased cations and anions in solutions (Shu et al., 2001). Hydrogen ion concentration (pH) and ORP was also correlated (P = 0.000, r = -0.696) (Appendix 4), this confirmed that these two parameters function jointly together in controlling mobility and solubility of metals. Moisture content and CEC also showed negative correlation (P = 0.006, r = -0.548).

4.3.3.1 Implications of physicochemical properties of the tailings, soils and metals contents on bacterial activities and diversities

Mining activities alter soil physicochemical properties which have a direct influence on soil bacteria. Dumping of mine wastes such as tailings on the soil alters soil pH which can reduce the availability of soil nutrients to bacteria. This will lead to the reduction in diversities of soil bacteria as they require adequate nutrients for their metabolic activities. Low pH value have also been reported to reduce rate of respiration and substrate utilization in bacteria as well as affects the activity of the bacteria cell surface, the functional groups which consist of carboxylate, phosphate and amino acid groups of their cell wall (Lemire et al., 2013).

Bacteria are usually the first to be affected by the direct and indirect effects of metal species release into the environment by mining activities. Metals affect the metabolism, growth and morphology of soil bacteria as a result of functional disturbance, destruction of cell membrane integrity or protein denaturation. This results in the alteration of the rates of key biological processes that underlie ecosystem functioning (Singh et al., 2014).

Bacteria lives in soil by adsorbing to soil particles, plant roots or animal surfaces, pH has been reported to play a prominent role in bacteria attachment to surfaces. Any decrease in pH will bring about decrease in the exchange of bacteria surface (Borrok et al. 2004). Exchange of genetic material between bacteria in soil can also be influenced by pH. Uptake of naked DNA from bacteria external environment via transformation process has been

known to require competent of the bacteria cell, which is a state requiring formation of competence inducing proteins. Low pH has been reported to affect this induction. As a result of the low pH and high metals concentrations recorded in the tailings and soil samples, the metallophilic and acidophilic sulphur and iron oxidizing bacteria such as the *Acidithiobacillus* spp, *Acidiphillum* spp are the predominant bacteria that can survive (Natarajan, 2008). Several other bacteria such as *Bacillus* spp, *Arthrobacter* spp, *Pseudomonas* spp, *Achromobacter* sp, *Streptomyces* spp and many others with ability to thrive in the acidic and high metals conditions have also been identified (Jamaluddin et al., 2012 , El Baz et al., 2015).

The low content of organic matter observed in this study will result in reduction of bacterial biomass and extractable carbon as well as bacterial community structure and biodiversity (Šourková et al., 2005 , Laudicina et al., 2015). Insufficient moisture content in the soil would further lower microbial activity as microbes require adequate moisture for their growth and activities. High CEC value will reduce the rate of organic matter decomposition because cations necessary for bacterial activity will be bound to organic matter thereby reducing availability of nutrients to soil bacteria (Gogo and Pearce, 2009). High redox state of the sampled tailings and soil will increase the rate at which metal species are being released into the environments as a result of increased oxidation rates of the sulphide minerals present in the tailings. This will affect transport of protons and other substances through the bacteria membranes and the activity of the enzymes found in the membrane.

4.4 Conclusion

This study looked at the physicochemical parameters and distribution of six metal species (As, Ni, Pb, Co, Cr and Zn) in some abandoned gold mine tailings dumps and surrounding soils in Krugersdorp mine areas. The results obtained shows that the first two

sites have similar soil properties and metals concentrations, while a significant difference was observed in the third site. Comparison of the various concentrations of the metals in the soil and tailings with the South Africa standard recommended for soils and sediments shows elevated concentrations of the metals in almost all the studied sites which was also observed to be spreading to adjacent environment as a result of wind and water erosion. The physicochemical properties of the tailings and soil recorded in the three different sites will enhance availability of metals in the environments. This will greatly reduce the number and functional diversities of the bacteria communities present with negative impacts on ecosystem functioning. This implied that the predominant bacterial populations that will be present are the metallophilic and acidophilic bacteria with potentials to adapt to the adverse environmental conditions created by the metals. The levels of the metal species recorded will constitute major health risk to the local populace which calls for urgent attention. However, because the total concentrations of metals is not a good predictor of metals availability and mobility in soils, there is need for assessment of readily available species to determine the likelihood of these metals transferring from the soil to other components of the ecosystems. Understanding the extent and sources of metals contamination is important for environmental management. It is also essential in reducing risks to human health, ensuring food safety, and managing contaminated soil.

CHAPTER FIVE

SCREENING AND CHARACTERIZATION OF METAL RESISTANT BACTERIA FROM AN ABANDONED GOLD MINE TAILINGS IN KRUGERSDORP, SOUTH AFRICA

Abstract

Bacteria are known to possess several detoxifying mechanisms to withstand the toxic effects of metal species which make them serve as key agents in biotransformation and removal of these metals. A total of 65 metal species resistant bacterial were isolated from 3 abandoned gold mine tailings in Krugersdorp, South Africa using I mM of Ni, Pb, Zn, Cd, Cr and Co. Twenty eight (28) of these isolates showing distinct morphological characteristics were selected and subjected to higher concentration of Ni, Pb and Zn ranging between 2-9 mM. All the isolates show tolerance to multiple metals but the most promising are OMF 003 and OMF 532 which show resistance to 5, 9 and 7 mM of Ni, Pb and Zn respectively. All the isolates were physiologically and biochemically characterized and the biochemical characterization shows that they belong to the phylum Proteobacteria, Actinobacteria and Firmicutes. The optimum conditions for their growth were temperature of 37°C, pH of 5.0 and 7.0 and NaCl concentration of 2-4% (w/v). The selected isolates were further subjected to multiple metal tolerance test, 12 isolates with ability to survive in the multiple metal mixtures were selected and their identity confirmed based on the partial 16S rRNA gene sequences. The bacterial isolates were correlated with other species of the genera in the database library using BLAST search and phylogenetic analysis. Computational analysis of the amplified 16S rRNA confirmed that the bacteria belong to the genus *Bacillus, Enterococcus, Enterobacte*r, and *Alcaligenes* sp with 78-100% sequence similarity. Eight of the isolates: OM6 142, OM9 107, OM4 274, OMF 532, OM7 132, OMF 008, OMF 003 and OM8 321 did not cluster with other bacterial isolates on the phylogenetic tree drawn which shows that these bacterial strains

could be probable novel metal species resistant bacteria based on their distinctness. The 16S rRNA sequences of the bacterial isolates were submitted to the GenBank under the accession numbers: KX485322-KX485325, KY000694-KY000700 and KY125908. The identified isolates shows optimum growth at 37°C, 5.0 and 7.0 pH and at NaCl concentration of 2-4% (w/v). Most of the isolates possessed plasmid and polymerase chain reaction was used to determine the genetic mechanism responsible for the metal species resistant on the DNA and plasmid of the twelve bacterial isolates. The *ncc* genes responsible for resistance to Ni was found to be present in two of the bacterial isolates while resistance to Pb and Zn was not detected with the primers used. *B. cereus* strain OMF 003 and *Enterobacter asburiae* strain OMF 532 shows potential as microbial inoculant in metal species polluted sites and were chosen for bioremediation studies.

Keywords: Bacteria, gold mine tailings, metals, multiple metal tolerance, metal resistant genes

5.1 Introduction

Metal pollution emanating from mining activities is one of the serious environmental threat worldwide (Qing et al., 2015). Metal species such as Ni, Zn, Cu and Co are essential micronutrient for living beings at lower concentration (Osredkar, 2012, Alloway, 2013) while some like As, Cd, Pb and Cr are of no biological importance. An anthropogenic activity such as mining has led to increased concentrations of these metal species in the environments. The physicochemical properties of the soil are known to enhance the mobility and bioavailability of metal species in the environments which make them available to living organisms. Excessive concentration of metal species in the environments is highly toxic to bacterial community showing great effect on their community structure by reducing their diversity, biomass and functionality. This leads to great impact on ecosystem functioning because bacteria play a major role in maintaining soil fertility and structure (Singh et al., 2014).

Bacteria are very sensitive and respond quickly to changes in environmental condition; they are therefore considered to be efficient bio-indicators of soil quality (Valverde et al., 2011).

Bacteria in metals contaminated sites are able to overcome the stress imposed by metal species and thus make them to have a functional role in remediation of the polluted sites. The stress imposed has led to the establishment of various defense mechanisms by the native bacteria community to tolerate the metals (Dixit et al., 2015). Numerous studies have shown that indigenous bacteria isolated from metal species contaminated sites effectively interact with toxic metals via direct and indirect mechanisms. Bacteria interaction with metals can cause mobilization and or immobilization of the metals (Bolan et al., 2014). These mechanisms include: biotransformation reactions such as oxidation-reduction of metals (Akhtar et al., 2013), uptake of metals into the bacterial cells, biosorption by cell surface polymers (Dadrasnia et al., 2015) and induction of metal precipitation (Achal et al., 2012) or modification of metal speciation due to microbial induced redox changes in the environment (Fonti et al., 2015). The various resistance ability is a direct response to the particular metal species and consequently a given bacteria may directly and/or indirectly rely on many survival mechanisms (Ruiz-Díez et al., 2012). Due to this reason, bacteria are seen as tools for the treatment of metal species polluted sites in biological processes known as bioremediation. They serve as alternative method to physico-chemical processes (Kamika and Momba, 2013). To effectively use these bacteria in bioremediation, it is compulsory to isolate, cultivate and select the desired strains because bacteria are known to be metabolically heterogeneous. Having a better knowledge of the mechanism controlling growth and activity of the bacteria in contaminated sites, their metabolic capabilities and response to environmental changes will also enhance their successful application.

Despite large numbers of abandoned gold mine tailings in South Africa, there is limited information on metal species resistant bacteria from the various hazardous wastes.

Most research have focused on metal species resistant bacteria associated with AMD (Kim et al., 2014, Sánchez-Andrea et al., 2014). Therefore, further research on indigenous metal species resistant bacteria associated with mine tailings is important to know the diversity and physiology of metal species resistant bacteria that are thriving under unfavourable metal pollution. Hence, the aim of this research was to isolate metals resistant bacteria associated with gold mine tailings and soils as well as identify and characterize them for their use as a prospective inoculant in bioremediation of metals polluted sites. Nickel, Zn and Pb were selected for further study as a result of their elevated concentration reported in the previous study and because of the differences in their toxicity level to microbial cells.

5.2 Materials and Method

5.2.1 Sample collection

The description, collection and physicochemical properties of the tailings samples used for the isolation of the metal species resistant bacteria have been described in chapter 2.

5.2.2 Preparation of stock solution of metals

All glassware was autoclaved prior to use. The metal salts, $ZnSO_4$, $NiCl_2 \cdot H_2O$, $CoCl_2 \cdot 6H2O$, $Crcl_2$, $CdCl_2 \cdot 2H_2O$ and $Pb(NO_3)_2$ used were of analytical grade purchased from Sigma Aldrich, South Africa. One molar stock solutions of Pb, Ni, Zn, Cd and Co were prepared by dissolving $Pb(NO_3)_2$, $NiCl_2 \cdot H_2O$, $ZnSO_4$, $CdCl_2 \cdot 2H_2O$, $Crcl_2$ and $CoCl_2 \cdot 6H_2O$ respectively in milli-Q grade deionized water and later filter sterilized using 0.20 µm pore size membrane filter. The glass wares used were treated in 2 N HNO_3 rinsed several times with distilled water before use to avoid metal contamination (Hassen et al., 1998).

5.2.3 Isolation of metal resistant bacterial strains

Metal tolerant bacteria were isolated from the samples using the spread plate method. One gram of duplicate composite samples was suspended in 9 mL of saline solution (8.5 g/ L

of NaCl) in distilled water and vortexed for 1-2 mins at room temperature. These were serially diluted (10^{-1} to 10^{-7}) and aliquots 0.1 ml dilution from 10^{-4}, 10^{-5} and 10^{-6} were spread with a glass rod over triplicate Luria–Bertani (LB) medium supplemented with 1 mM of $ZnSO_4$, $NiCl_2 \cdot H_2O$, Pb $(NO_3)_2$, $CrCl_2$ $CdCl_2 \cdot 2H_2O$ and 0.5 mM $CoCl_2 \cdot 6H_2O$ (Van Nostrand et al., 2007). To minimize metal ion complexation, the medium was adjusted to pH 7 using 1.O N NaOH or 1.O N HCl. Plates were incubated at 37°C and 25°C for 24 - 48 h. Growth was observed and morphologically distinct colonies were selected and purified in the same medium and growth conditions (Raja and Selvam, 2009). All cultures were stored at -80°C in LB broth with 20% glycerol for further studies.

5.2.4 Determination of maximum tolerable metal concentration of the isolates

Maximum tolerable concentration (MTC) for Pb, Cr, Zn, Ni, Cd and Co was determined by agar dilution method (Kannan and Krishnamoorthy, 2006). A log-phase culture of the isolates was spot inoculated onto LB agar plates supplemented with increasing concentration of metals. The plates were incubated at 37°C and 25°C for 24 - 48 h and observed for bacterial growth. The highest concentration of the metal at which no bacteria growth was seen was designated as the MTC. All metal salts were added to the LB agar after autoclaving and cooling to 50°C from filter-sterilized stock solutions. The concentrations in mM of the metal species used are stated below:

Pb^{2+} : 1, 2, 3, 4, 5, 6, 7, 8, 9

Ni^{2+} : 1, 2, 3, 4, 5

Zn^{2+} : 1, 2, 3, 4, 5, 6, 7, 8, 9

Co^{2+} : 0.5, 1, 2

Cd^{2+} : 1, 2, 3

Cr^{2+} : 1, 2, 3, 4, 5, 6, 7, 8, 9, 10

5.2.5 Determination of multiple metal mixtures tolerance

Multi elemental studies were done using combinations of two metals: Ni+Co, Ni+Pb and Zn+Pb. A log-phase culture of the isolates was inoculated into 10 µl of LB broth supplemented with 1mM each of Ni, Pb, Zn and 0.5 mM of Co. The plates were incubated at 37°C and 25°C for 24 h - 48 h and observed for bacterial growth by checking the bacterial OD at 600 nm using the UV spectrophotometer. All metal salts were added to the LB broth after autoclaving and cooling to 50°C from filter-sterilized stock solutions.

5.2.6 Characterization and identification of the isolates

5.2.6.1 Biochemical characterization

To identify the isolates, the following biochemical tests were performed: starch hydrolysis, Voges–Proskauer test, methyl red, citrate utilization, catalase, oxidase, nitrate reduction, hydrogen sulphide production and gas production of sugars like glucose, lactose, sucrose and maltose. Pure cultures of the bacterial isolates were identified according to the Bergeys manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

5.2.6.2 Physiological characterization

In selecting metal tolerant strains, it is crucial to determine the physiological conditions necessary for their growth. The physiological conditions that are of great importance to bacteria growth are temperature, pH and salt (NaCl) concentration.

5.2.6.3 Salt tolerance

To determine salt tolerance of the isolate, 24 h pure cultures of each bacteria strains were streaked inoculated on LB agar supplemented with 1 mM Pb and 1- 10% NaCl which acts as a selective medium and Petri plates were incubated at 25°C and 37°C for 24 - 48 h.

Bacteria were marked positive or negative for their ability to grow at different salt concentrations (Damodaran et al., 2013).

5.2.6.4 pH profile

Hydrogen ion concentration (pH) has a profound effect on bacteria growth. To determine optimal pH necessary for growth, LB agar supplemented with 1 mM of Pb was used to grow the isolates. It was adjusted to different pH ranging from 3.0 – 9.0 using 1.0 N HCl and NaOH and then inoculated with the pure cultures of the isolates and the plates were incubated at 25°C and 37°C for 24 - 48 h. Growth was measured by the presence or absence of growth on the solidified agar medium.

5.2.6.5 Temperature profile

To determine the optimum temperature, the pure isolates were inoculated into LB agar plates supplemented with 1 mM of Pb. Overnight incubation was done at different temperatures ; 25, 37, 40 and 50°C. The growth was measured by checking for the presence or absence of growth on the solidified agar plates.

5.2.7 Molecular characterization of bacterial isolates

5.2.7.1 Genomic DNA extraction

Genomic DNA was extracted from the selected bacteria by growing in 10 ml of metal supplemented LB broth. The bacterial cultures were grown for 24 h in a shaking incubator (150 rpm) maintained at 37°C and 1.5 ml of each culture was transferred to sterile Eppendorf tubes which was centrifuged at 1000 rpm for 5 min. Supernatant were discarded and cells were re-suspended in 650 µl of TE buffer. Total genomic DNA was extracted from each bacterial suspension using a ZR soil microbe DNA mini prep ™ DNA extraction kit (Zymo Research, USA) according to the manufacturer's protocol.

5.2.7.2 Plasmid extraction

Pure cultures of the bacterial isolates were grown overnight in 10 ml of sterile LB broth and cell pellet were harvested by centrifuging 1.5 ml of each culture in microfuge tubes for 5 min at 6,000 rpm (revolutions per min) and screened for the presence of plasmid DNA using high pure Zyppy™ plasmid miniprep kit (Zymo Research, USA) according to the manufacturer's instruction. The extracted plasmid DNA was stored at -80°C for further use.

5.2.7.3 Agarose gel electrophoresis

The presence of the genomic DNA and plasmid DNA was confirmed in a 1.0% (w/v) agarose gel electrophoresis prepared by dissolving 1.0 g of agarose (Bio-Rad, SA) in 100 ml of 1X Tris-acetate-ethylenediaminetetraacetate (TAE, pH 8). The mixture was heated in a microwave oven for 3 mins and allowed to cool after which 10 µl of ethidium bromide (Bio-Rad, SA) was added to the molten agar which was poured in a gel casting tray and allowed to solidify. After solidification, the combs were removed and the gel was carefully placed in the electrophoresis tank containing 1X TAE buffer (40 mM Tris, 20 mM Acetic acid, and 100 mM EDTA pH 8.0). DNA samples were prepared by mixing 5µl of the genomic DNA with 5µl of 6X DNA loading dye (Thermo Scientific™) and this was carefully loaded in the pre-formed wells in the gel. A GeneRuler™ DNA Ladder (1 kb) was used to estimate the sizes of the genomic DNA. The electrophoresis was carried out at 100 V, 450 mA for 1 h. Gels were visualized and photographed using a gel documentation system (Gel Doc 2000, Bio-Rad).

5.2.7.4 Polymerase chain reaction (PCR) amplification

The 16S rRNA gene was amplified from genomic DNA obtained from bacterial cultures by PCR using universal bacterial 16S rRNA primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGTTAC CTT GTT ACG ACT T-3′) previously described by Liu et al. (2007). Polymerase chain reaction (PCR) was performed in a total volume of 50 µl

containing 25 µl of 2x PCR master mix (0.05 U/µl *Taq* DNA polymerase, 4 mM $MgCl_2$ and 0.4 mM dNTPs) (Thermo Scientific™), 2 µl of genomic DNA template, 1.0 µl of each forward and reverse primer and 22 µl of nuclease free water. The amplification reaction mixture was subjected to 30 cycles in a C1000 thermal cycler (BioRad, USA). The thermal cycling condition used was an initial denaturation, 95°C for 5 min; followed by denaturation at 95°C for 1 min; annealing, 58°C for 30s; extension, 72°C for 1 min and final extension, 72°C for 7 min. The PCR amplicons were analyzed by electrophoresis in 1% (w/v) agarose gel and the sizes of the bands were determined using 1 kb molecular marker. The gel containing ethidium bromide (10 µg/ml) were visualized and photographed using a gel documentation system (Gel Doc 2000, Bio-Rad) to confirm the expected size of the PCR products. PCR products were gel extracted (Zymo Research, Zymoclean™ Gel DNA Recovery Kit).

5.2.7.5 PCR analysis targeting genes encoding metal tolerance in bacteria

Genes associated with nickel (*ncc*), zinc (*Czc*) Pb (*P3 P4* and *PbrT*) in each bacteria isolate were screened by PCR amplification. The oligonucleotide sequences used as primers for the partial amplification of these genes are expressed in Table 5.1.

Table 5.1: Oligonucleotide primers for PCR amplification of genes involved in metal tolerance

Gene	Forward sequence	Reverse sequence	Reference
NccA	ACGCCGGACATCACG AACAAG	CCAGCGCACCGAGACTCATCA	(Abou-Shanab et al., 2007)
CzcA	GTTTGAACGTATCATT AGTTTC	GTAGCCATCCGAAATATTCG	(Nies et al., 1989)
CzcD	CAGGTCACTGACACG ACCAT	CATGCTGATGAGATTGATGATC	(Nies et al., 1989)
CzcB	CTATTTCGAACAAAC AAAAGG	CTTCAGAACAAAACTGTTGG	(Abou-Shanab et al., 2007)
P3 P4	GGTGGATCCCCATGA ACAGCGAAACCT	GGTGAATTCTCAGGGCGAGAT CGGGTCGC	(Blindauer et al., 2002)
PbrT	ATGGTGATTGCTTTA GTT	TTAGGCTTGCTTCTTTTT	(Shin et al., 2012)

Metal resistant genes of the bacterial isolates were amplified in 50 µl reactions volumes containing 22 µl of nuclease-free water, 23 µl of Failsafe™ PCR 2x premix buffers containing a buffered salt solution with all 4 dNTPs and various amounts of $MgCl_2$ and FailSafe PCR Enhancer (with betaine) (Epicentre Technologies, Madison, WI, USA), 0.5 µl of each primer (100 mM), 0.5 µl of 1.25 U Master Amp *Taq* DNA polymerase. Templates for PCR amplification included 1 µl of the total genomic and plasmid DNA from the selected highly metal resistant gram-positive and gram-negative bacteria. The thermal cycler (C1000 BioRad) was programmed with the following five steps: initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturation (94°C for 30 s), annealing (52°C for 30 s), elongation (72°C for 2 min); and a final elongation step at 72°C for 5 min for *nccA* and *Czc* ABD genes while the Pb resistant genes were amplified using the following PCR conditions initial denaturation at 95°C for 4 min, 35 cycles at 95°C for 30 s, 50°C for 1.5 min, 72°C for 2 min and a final extension step of 72°C for 7 min. Aliquots of PCR reactions were electrophoresed on a 1% agarose gel stained with ethidium bromide to check for amplification.

5.2.7.6 Sequencing reaction

Both the forward and reverse primers were used in the sequencing of the purified PCR products. The sequencing was done at Inqaba Biotechnical industrial (Pty) Ltd, Pretoria, South Africa with PRISM TM Ready Reaction Dye Terminator Cycle Sequencing Kit using dideoxy chain termination method and electrophoresed with a model ABI PRISM® 3500XL DNA Sequencer (Applied Bio systems, USA) by following the manufacturer's instructions. Purified sequencing products (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™) were analyzed using CLC Main Workbench 7.

5.2.7.7 Phylogenetic analysis

5.2.7.7.1 Sequence similarities and phylogenetic analysis

The chromatograms resulting from sequencing reaction were edited using Chromas Lite version 2.4 software (Technelysium, 2004). The resulting nucleotide sequences were then analyzed and edited using Bio Edit Sequence Alignment Editor (Hall and CA, 2004). The consensus 16S rRNA sequences generated were compared with other reference sequences available in the NCBI (www.ncbi.nlm.nih.gov) database using the Basic Alignment search tool (BLASTn) (Altschul et al., 1997) and the sequences were deposited in the GenBank. Multiple alignment of the nucleotide sequences were done using Mafft version 7.0 (Katoh and Standley, 2013). Phylogenetic and molecular evolutionary analyzes were conducted using softwares in MEGA version 6.0 (Tamura et al., 2013). Evolutionary distance matrix was generated and a phylogenetic tree was drawn by neighbour joining method (Saitou and Nei, 1987). The significance levels of interior branch points were evaluated by bootstrap method (Felsenstein, 1985) based on 1000 resampling of the neighbour-joining data set.

5.2.7.7.2 Nucleotide sequences accession numbers

The 16S rRNA gene sequences of the twelve selected metal resistant bacterial isolates determined in this study were deposited in NCBI/ EMBL nucleotide sequence GenBank database, under the accession numbers: KX485322-KX485325, KY000694-KY000700 and KY125908.

5.3 Results and Discussion

5.3.1 Isolation and selection of metal resistant bacterial strains

Metal tolerant bacteria are usually found in sites polluted by high concentrations of metal species as a result of anthropogenic activities such as mining (Wei et al., 2009, Xie et

al., 2016). The presence of elevated metal concentration has been reported to result in the emergence of bacteria population with potential to tolerate the metal species, but usually with reduced diversity, metabolic activity and population size (Zampieri et al., 2016). This study also highlights the presence of metal species in the tailings sample studied and confirms wide spread occurrence of metal species resistant bacterial population in the gold mine tailings. A total of 65 culturable metal species resistant bacteria were isolated from the three sites under study using 1 mM concentrations each of Pb, Zn, Ni, Co, Cr and Cd. Twenty eight (28) of these isolates shows distinct colony characteristics like size, pigmentation, shape, elevation and margin surface (Table 5.2 and Appendix 5). These isolates were chosen and characterized by biochemical tests performed according to the Bergeys manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

Table 5.2: Total number of metal species resistant bacteria isolated from the three sites

Sampling Site	Total number of isolates	Number of selected isolates	Designates
MA	19	10	OM6 142,OMF 002, OMF 811, OMF 812, OMF 813, OMF 814, OMF 815, OMF 816, OMF 810,OMF 809
MB	22	8	OMF 001, OM4 274, OMF 808, OMF 807, 0M9 107, OMF 806, OMF 805, OMF 804,
TS	24	10	OMF 532, OMF 008, OM7 132, OMF 003, OMF 803, OMF 802, OMF 801, OMF 800, OMF 005, OM8 321
Total number of isolates	65	28	28

Key: TS (Tudor shaft), MA (Mine tailings A), MB (Mine tailings B).

The bacteria isolated in this study showed the ability to withstand varying concentration of the tested metals like those observed in studies reported by Abou-Shanab et al. (2007), Bajkic et al. (2013), Hookoom and Puchooa (2013) from different contaminated sites.

5.3.2 Colonial and cellular morphology of the bacterial isolates

The bacterial isolates shows various colonial and cellular morphology expressed in Figure 5.1 and appendix 5. Forty six percent (46%) have raised elevation while the remaining 54% are flat, 64% are creamy in colour and 82% shows entire margin while 75% are circular in shape. Most of the isolates (85%) have rod shape and the remaining 15% are cocci. Out of the 28 isolates obtained, only 6 (21.43%) are Gram negative while the remaining 22 (70.57%) are Gram positive.

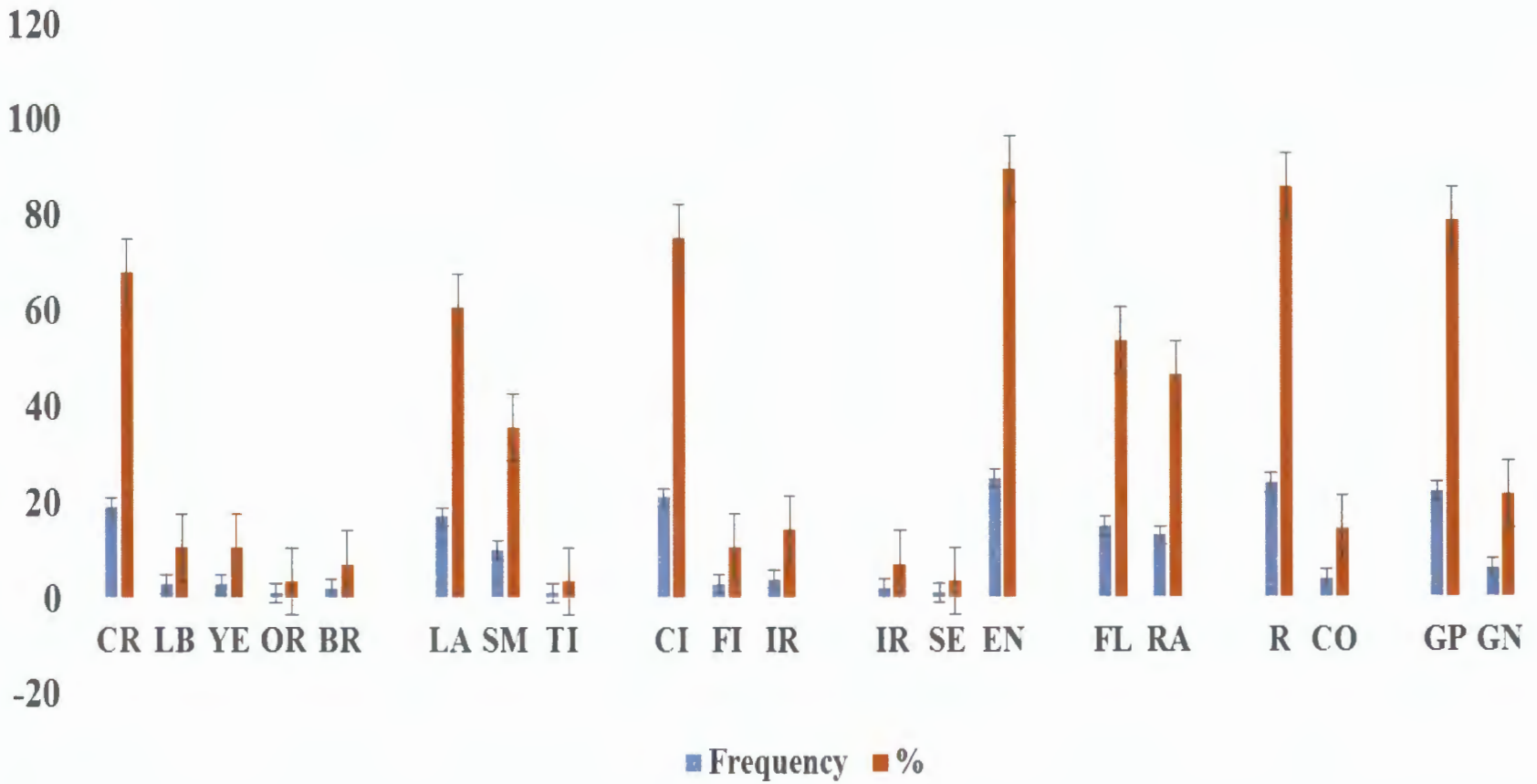


Figure 5.1: Colonial and cellular morphology of the bacterial isolates

Keys: CR, LB, YE, OR and BR = cream, light brown, yellow, orange and brown coloration, LA, SM and TI = large, small and tiny sizes, CI, FI and IR = circular, filamentous and irregular shape, IR, SE and EN = irregular, serrated and entire margin, FL, RA = flat and raised elevation, R and C = rod and cocci cell shape, GP and GN = Gram positive and Gram negative Gram reaction

5.3.3 Bacteria tolerance to metals

The MTCs growth pattern of the bacterial isolates to the metal species are shown in Table 5.3

Table 5.3: Maximum tolerable concentration of the tested metals against the bacterial isolates

Isolate codes	MTC (mM)					
	Co^{2+}	Ni^{2+}	Pb^{2+}	Cd^{2+}	Zn^{2+}	Cr^{2+}
OMF-012	1	2	4	1	3	8
OMF-811	1	2	5	1	3	9
OMF-812	1	2	4	1	4	9
OMF-813	1	3	5	2	5	7
OMF-814	1	3	6	2	5	9
OMF-815	1	2	6	2	6	8
OMF- 816	1	3	4	1	4	8
OM6-142	1	5	6	2	7	9
OMF-810	1	5	6	2	7	9
OM8-321	1	4	6	2	7	9
OMF-001	1	4	5	2	7	9
OMF-809	1	4	6	3	7	9
OM4-274	1	4	6	3	6	7
OMF-808	1	2	3	1	3	9
OMF-807	1	2	4	2	4	10
OMF-107	1	4	7	2	9	9
OMF-806	1	4	6	2	5	9
OMF-805	1	4	6	2	4	8
OMF-804	1	2	5	2	4	8
OMF-532	1	5	7	3	9	10
OMF-008	1	4	6	3	6	9
OM7-132	1	4	6	2	7	9
OMF-003	1	5	7	3	9	10
OMF-803	1	4	5	2	5	9
OMF-802	1	4	5	2	5	9
OMF-801	1	4	5	1	5	9
OMF-800	1	3	6	2	5	10
OMF-005	1	5	7	2	7	9

All the bacterial isolates tested could not grow beyond 1 mM of Co but they were able to tolerate varying concentrations of Ni, Pb, Zn, Cd and Cr as shown in Table 5.4. Cadmium tolerance range between 1-3 mM and five isolates; OMF 809, OM4 274, OMF 532, OMF 008 and OMF 003 could tolerate up to 3 mM concentration of this metal. Bacterial tolerance to Ni^{2+} range between 1- 5 mM, isolates OMF 532, OMF 003, OM6 142 and OMF 810 shows highest tolerance of 5 mM to this metal. Tolerance to Pb also range between 1-7 mM for the isolates; OM9 107, OMF 532, OMF 003 and OMF 005 shows the highest tolerance of 7 mM. Similarly, tolerance to Zn was also found to range between 1-9 mM and three isolates OMF 532, OM9 107 and OMF 003 were able to tolerate up to 7 mM concentration of this metal. Chromium was also well tolerated by the isolates, high tolerance of 7-10 mM was recorded. Isolates OMF 807, OMF 532, OMF 800 and OMF 003 shows highest tolerance of 10 mM. Bacterial isolates OMF 532 and OMF 003 were observed to tolerate the highest concentration of 5 mM Ni, 7 mM Pb, 9 mM Zn and 10 mM Cr^{2+} of the metal species tested. The high level of resistance and the widespread tolerance shown by the bacterial isolates against multiple metals could be attributed to the elevated concentrations of the metal species recorded in the different samples where the bacteria were isolated from (see chapter 4). Mean values of the metal species recorded in Tudor shaft where the two isolates were obtained from are 2,247 mg/kg of Ni, 59.96 mg/kg of Pb, 4,269.25 mg/kg of Zn and 856.50 mg/kg (see Table 4.4 chapter 4) which were well above the South Africa standards for soils and sediments. The other two sites equally shows higher levels of the three metals above the stipulated values. The pattern of toxicity of the metals to the bacterial isolates are Co > Cd > Ni > Pb > Zn > Cr, which shows that Co is the most toxic out of the six metal species to all the bacterial isolates.

Bacteria have devised various physiological and genetic mechanisms needed for their adaptation to metal contaminated environments. Efflux system has been reported in Gram-negative bacteria, which enables bacteria to pump out metals from the cytoplasm to the

periplasmic space with the help of ATPase's found in the internal membrane of the bacteria (Blair et al., 2014). Other mechanisms such as complexation, precipitation, biotransformation, bioaccumulation, oxidation- reduction reactions and biosorption have also been reported to be responsible for bacterial tolerance to metals (Wei et al., 2009, Sahmoune and Louhab, 2010, Govarthanan et al., 2013). The MTC values recorded for Ni, Pb and Zn by the bacterial isolates obtained in this study are much higher than those reported by Choudhary and Sar (2009) and Govarthanan et al. (2013), in mine polluted tailings and soil. However, the Ni tolerance level recorded is lower than the values reported by Bajkic et al. (2013), where Ni tolerance as high as 11 mM was attained by the bacteria strain MS108 identified as *Staphylococcus* sp. from the copper mining and Smelting complex Bor in Serbia. The differences in metal tolerance by the bacterial isolates could be attributed to many factors such as the medium strength, presence of negatively charged ions like chloride, organic constituents, and nature of the medium which determines availability of the metals to the bacteria (Kannan and Krishnamoorthy, 2006). The multiple metals resistant patterns observed by the bacterial isolates could be attributed to the fact that, mine tailings are usually contaminated with cocktails of metals, and hence, bacteria surviving in mine tailings could possess the ability to withstand the presence of different metals. For example, *Staphylococcus* sp isolated from copper mining soil was able to tolerate Cr, Ni and Cd (Bajkic et al., 2013). Similarly, the bacterial strain CCNWRS33-2 isolated from *Lespedeza cuneate* in gold mine tailings also shows high resistant to Cu, Cd, Pb and Zn (Wei et al., 2009). The present study also shows that the bacterial isolates have multiple metal resistance (Table 5.3).

5.3.4 Multiple mixtures tolerance test

Metals are usually present as mixtures in contaminated sites and it is important to determine their interaction. All the isolates were grown on LB broth supplemented with combination of two metals. The isolates shows different tolerance to the multiple mixtures of

metals tested. It was observed that Ni+Co combination was generally toxic to the bacterial isolates as observed in their reduced growth rate (Table 5.4). The bacterial isolates were also observed to show the least tolerance to Co during the initial screening for metal resistant and Ni was also seen to be the most toxic metal to the bacterial isolates out of Pb and Zn in the MTC test. This could explain the inability of the two metals to interact together. The bacterial isolates were able to tolerate the combination of Ni+Pb and Zn+Pb better than Ni+Co. Out of the 28 isolates, twelve isolates shows better growth in the presence of the multiple metal mixtures but the isolate OMF 003 and OMF 532 shows the best growth. All the isolates were further characterized for their physiological and biochemical characteristics to determine the probable identity of bacteria that could tolerate multiple metals in the mine tailings.

Table 5.4: Optical density of the bacterial isolates in the multi metal mixtures tolerance test

Isolate codes	Ni+Co			Ni+Pb			Zn+Pb		
	1	2	3	1	2	3	1	2	3
OMF-012	0.148	0.229	0.163	0.235	0.248	0.263	0.138	0.226	0.119
OMF-811	0.005	0.027	0.025	0.996	1.055	1.164	0.547	0.783	1.115
OMF-812	0.017	0.018	0.017	0.267	0.226	0.156	0.281	1.115	0.713
OMF-813	0.017	0.064	0.046	0.813	1.319	0.693	0.204	1.170	0.830
OMF-814	0.079	0.161	0.173	0.287	0.234	0.103	0.369	0.885	0.588
OMF-815	0.021	0.045	0.050	0.155	0.120	0.014	0.645	1.054	1.194
OMF-816	0.160	0.216	0.199	0.227	0.170	0.130	0.207	0.377	0.225
OM6-142	0.037	0.065	0.075	1.223	1.051	1.509	0.350	0.968	1.398
OMF-810	0.014	0.027	0.031	0.633	1.362	1.636	0.626	1.380	1.460
OM8-321	0.117	0.998	0.183	0.316	0.412	0.813	0.371	0.761	1.084
OMF-001	0.068	0.165	0.112	0.243	0.612	0.912	0.171	0.461	0.953
OMF-809	0.015	0.031	0.033	0.751	1.290	1.530	0.750	1.236	1.298
OMF-274	0.026	0.115	0.120	0.158	0.411	0.723	0.011	0.419	0.891
OMF-808	0.010	0.386	0.211	0.424	0.383	0.404	0.776	0.808	0.946
OMF-807	0.023	0.027	0.033	0.204	0.482	0.861	0.216	0.599	0.791
OMF-107	0.006	0.019	0.024	1.156	1.966	1.450	0.018	0.322	0.822
OMF-806	0.216	0.269	0.280	0.241	0.349	0.118	0.114	0.763	0.683
OMF-805	0.033	0.030	0.367	0.230	0.215	0.603	0.251	0.750	0.687
OMF-804	0.174	0.241	0.247	0.208	0.171	0.241	0.578	0.997	1.085
OMF-532	0.050	0.113	0.480	1.221	1.991	2.117	0.856	0.897	1.621
OMF-008	0.358	0.385	0.151	0.618	0.845	0.661	0.817	1.109	1.211
OM7-132	0.010	0.016	0.029	0.049	0.174	0.986	0.220	0.690	1.042
OMF-003	0.168	0.201	0.335	1.107	0.760	1.050	0.116	1.018	1.561
OMF-803	0.222	0.029	0.021	0.592	0.882	0.926	0.285	0.508	0.871
OMF-802	0.035	0.037	0.054	0.653	1.101	1.727	0.125	0.885	0.727
OMF-801	0.048	0.058	0.045	1.229	1.600	0.804	0.327	0.914	0.547
OMF-800	0.021	0.033	0.047	0.594	0.812	1.208	0.309	0.466	0.626
OMF-005	0.060	0.128	0.133	0.366	0.437	0.766	0.927	1.250	1.196

Key: 1, 2, 3 = incubation period of bacteria growth at 24 h, 48 h and 72 h respectively

5.3.5 Characterization and Identification of the isolates

5.3.5.1 Biochemical characterization

The biochemical studies of the bacterial isolates were determined according to Bergey's manual of determinative bacteriology and the results presented in Table 5.5 below.

Table 5.5: Biochemical characteristics of the bacterial isolates

ISOLATE CODES	TESTS																					
	MOT	CAT	OXI	NIT	IN	VP	MR	CIT	URE	SH	GL	H_2S	MAN	XYL	GLU	ARA	SUC	GAL	FRU	MAL	LAC
OMF 012	+	+	+	+	+	−	−	+	−	+	+	−	−	+	+	+	+	+	+	+	+
OMF 811	−	+	−	+	−	+	−	+	+	+	−	−	−	+	+	+	+	+	+	+	+
OMF 812	−	+	−	+	−	+	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+
OMF 813	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	+	−	−	+
OMF 814	+	+	+	+	+	−	−	+	−	+	+	−	−	+	+	+	+	+	+	+	+
OMF 815	−	+	+	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
OMF 816	+	+	+	−	−	+	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+
OM6 142	−	+	+	+	−	+	+	−	−	+	−	−	+	+	+	+	+	+	+	+	+
OMF 810	+	+	−	+	−	+	+	+	−	+	+	−	+	+	+	−	+	+	+	+	+
OM8 321	+	+	+	−	−	+	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
OMF 001	−	+	+	+	−	+	+	+	−	+	+	−	+	−	+	−	+	+	−	+	−
OMF 809	+	+	−	+	−	+	−	+	−	+	−	−	+	+	+	+	+	+	+	+	+
OMF 274	+	+	+	−	−	+	−	+	−	+	−	+	−	+	−	−	−	+	−	+	+
OMF 808	−	+	−	+	+	−	−	−	−	+	−	−	+	+	+	+	+	+	+	+	+
OMF 807	+	+	−	+	+	+	+	−	−	+	+	−	+	−	−	+	−	−	+	+	−
OMF 107	+	+	+	−	−	+	+	−	−	−	−	−	−	−	+	−	+	+	+	+	+
OMF 806	−	+	−	+	+	−	−	−	−	+	−	−	+	−	+	+	+	+	+	+	+
OMF 805	+	+	−	+	−	+	+	+	−	+	−	+	+	+	+	+	+	−	+	+	+
OMF 804	−	−	+	−	−	+	−	+	+	−	+	−	+	−	−	−	−	−	−	+	−
OMF 532	−	+	−	+	−	−	+	+	−	+	−	−	+	+	+	+	+	−	+	+	+
OMF 008	−	+	+	+	−	+	−	−	−	+	−	+	+	+	+	−	−	+	+	+	+
OM7 132	−	+	−	+	−	+	−	+	+	+	−	−	−	+	+	+	+	+	+	+	+
OMF 003	+	+	+	−	−	−	−	+	+	−	+	−	−	−	+	+	+	−	+	+	+
OMF 803	−	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
OMF 802	−	+	+	−	−	−	−	+	−	+	+	−	+	−	+	+	+	+	−	+	+
OMF 801	−	+	−	+	+	−	−	+	+	+	+	−	+	−	+	+	+	−	+	+	+
OMF 800	+	+	−	+	−	+	−	+	+	+	−	−	+	+	+	+	+	+	+	+	+
OMF 005	−	+	+	+	+	+	+	−	−	+	−	−	+	+	+	+	+	+	+	+	+

Key : MOT (Motility), CAT (Catalase), OXI (Oxidase), NIT(Nitrate), IN (Indole), VP (Voges Proskaeur), MR (Methyl red), CIT (Citrate), URE (Urease), SH (Starch hydrolysis), GL (Gelatin liquefaction), MAN (Mannose), XYL (Xylose), GLU (Glucose), ARA (Arabinose), SUC (Sucrose), GAL (Galactose), FRU (Fructose), MAL (Maltose), LAC (Lactose).

The biochemical results shows that 46% of the bacterial isolates were motile, 71% were able to reduce nitrate to nitrite while only 20% of the isolates were positive for indole test, 75% were able to hydrolyze starch and 39% hydrolyzed gelatin. Majority (75%) of the isolates were urease negative while 57% produced the oxidase and catalase enzyme. Most of the sugars were utilized by the isolates: 89% utilized glucose, 75% arabinose, 82% sucrose, 67% mannose, 64% xylose, 21% galactose, 22% fructose, 26% maltose and 85% lactose. Metal pollution usually gives rise to great decrease in the activities of various soil enzymes such as urease (Oste et al., 2001) as observed in this study. The ability of the bacterial strains to utilize the sugars signifies aerobic metabolic pathways because these sugars could be easily incorporated and directly enters the Tri Carboxylic Acid (TCA) Cycle (Samanta et al., 2012). The results of the biochemical tests (Table 5.5) revealed that the bacterial isolates belong to three phyla: Actinobacteria, Proteobacteria and firmicutes (Table 5.6). Previous researches has shown the abundance of these phyla in metals impacted environments (Jamaluddin et al., 2012, Bajkic et al., 2013, Hookom and Puchooa 2013). Their ability to survive in these environments has been attributed to the composition of their cell walls that are able to interact and bind effectively with the metals (Shin et al., 2012) as well as various genetic mechanisms that enable them to overcome the effects of the toxic metals (Rensing and Grass, 2003, Abou-Shanab et al., 2007, Dupont et al., 2011).

Table 5.6: Probable identity of the bacterial isolates

Isolates code	Putative identity
OMF 012	*B. amyloliquefaciens*
OMF 811	*Bacillus* sp.
OMF 812	*Enterobacter* sp
OMF 813	*B. methylotrophicus*
OMF 814	*Bacillus* sp
OMF 815	*Micrococcus yunnanensis*
OMF 816	*B. pumilus*
OM6 142	*E. faecalis*
OMF 810	*B. subtilis*
OM8 321	*Bacillus sp*
OMF 001	*B. pumilus*
OMF 809	*B. anthracis*
OMF 274	*E. aerogenes*
OMF 808	*B. cereus*
OMF 807	*Arthrobacter oxydans*
OMF 107	*B. thuringiensis*
OMF 806	*Arthrobacter* sp
OMF 805	*Enterobacter* sp
OMF 804	*Rhodococcus* sp
OMF 532	*E. asburiae*
OMF 008	*Alcaligenes* sp
OM7 132	*Enterobacter* sp
OMF 003	*B. cereus*
OMF 803	*Arthrobacter* sp
OMF 802	*A. nigatensis*
OMF 801	*B. cereus*
OMF 800	*B. psychodurans*
OMF 005	*Enterococcus* sp

5.3.6 Physiological characterization

5.3.6.1 Effect of temperature, pH and salt tolerance on bacterial growth

Physiological state of bacteria is an important factor to be considered in selecting metal resistant bacteria as it determines their uptake capacity. Temperature is an important factor that determines the rate of growth of a bacterial cell. The effect of temperature, pH and salt on bacteria growth in the presence of metal is shown in Table 5.7.

Table 5.7: Physiological characterization of the bacterial isolates

Isolate codes	Temperature (°C)					pH				Salt Tolerance (%)				
	25-30	30-35	35-40	40-45	45-50	3	5	7	9	2	4	6	8	10
OMF 012	++	++	++	++	++	−	++	++	++	++	++	++	++	++
OMF 811	++	++	++	++	−	−	++	++	−	++	++	++	++	−
OMF 812	++	++	++	++	−	−	++	++	++	++	++	++	++	−
OMF 813	++	++	++	++	++	++	+	+	+	++	++	++	++	++
OMF 814	++	++	++	++	++	−	++	++	+	++	++	+	+	−
OMF 815	++	++	++	++	−	−	−	++	++	++	++	++	−	−
OMF 816	++	++	++	++	−	−	+	++	−	++	++	++	++	++
OM6 142	++	++	++	++	−	++	++	++	+	++	+	+	+	−
OMF 810	++	++	++	++	−	+	++	+	+	++	++	++	++	−
OM8 321	++	++	++	++	−	++	++	+	+	++	++	−	−	−
OMF 001	++	++	++	−	−	−	++	++	+	++	++	+	−	−
OMF 809	++	++	++	−	−	++	++	++	+	++	++	++	+	−
OMF 274	++	++	++	+	−	−	+	+	+	+	+	+	−	−
OMF 808	++	++	++	+	−	++	++	++	++	++	++	+	+	−
OMF 807	++	++	++	++	−	−	++	++	+	++	++	+	−	−
OMF 107	++	++	++	++	++	++	++	++	++	++	++	++	++	++
OMF 806	++	++	+	+	−	+	++	++	+	++	++	+	+	−
OMF 805	++	++	+	+	−	+	++	+	+	++	++	++	+	−
OMF 804	++	++	+	+	−	+	++	++	+	+	−	−	−	−
OMF 532	++	++	++	++	++	++	++	++	++	++	++	++	++	++
OMF 008	++	++	++	+	−	+	++	++	+	++	++	+	−	−
OM7 132	++	++	++	++	++	++	++	++	+	++	++	++	++	++
OMF 003	++	++	++	++	+	++	++	++	++	++	++	++	+	−
OMF 803	++	++	++	+	+	++	++	++	+	+	+	+	+	−
OMF 802	++	++	+	−	−	−	++	++	+	++	++	+	−	−
OMF 801	++	++	+	+	−	−	+	++	−	−	−	−	−	−
OMF 800	++	++	+	+	−	−	++	++	−	++	+	+	−	−
OMF 005	++	++	++	−	−	+	++	+	+	+	+	−	−	−

Key: ++ Good growth + Moderate growth − No growth

The results shows that the optimum temperature for maximum growth by all the bacterial isolates was between 30°C-35°C, although majority of the isolates were also able to grow between 35°C-40°C. Decline in growth was observed when the temperature was raised to 40°C which could be due to the decrease in metabolic activity as a result of increase in temperature above the optimum value the bacteria can withstand. High temperature of the growth medium affects the arrangement and stability of bacterial cell wall, ionized chemical moieties and also leads to disruption of the enzymatic activities which consequently slow down the metabolic activities of the bacteria (Adamo et al., 2005). Similarly, low temperature also reduced bacterial growth because enzymes are inactivated at this temperature which decreases the rate of metabolism (Feller, 2013).

Hydrogen ion concentration (pH) is another crucial factor affecting metal uptake by bacteria. It has been reported that the solubility and ionization state of the functional groups of bacteria cell wall such as carboxylate, phosphate and amino acids are affected by the medium pH. (Varghese, 2012). The result of the effect of pH on bacterial growth indicated that optimum pH range for the bacterial growth is 5 and 7 while a decline in growth was observed at pH 3 and 9. The ability of the isolates to grow at the observed pH could be due to the pH of the samples where they were isolated from which was recorded to range between 2.17- 6.79 (see chapter 4). The effect of pH on bacteria growth was not carried out above pH 9 because most metal ions usually form hydroxyl-complexes that result in metals precipitation (Izatt, 2016). Ability to tolerate salt is another important factor to consider in selecting metal resistant bacteria because it affects the rate at which they respire.

The effect of salt on bacterial growth shows that increasing concentration of salt in the growth medium reduces the bacteria growth rate, maximum growth was observed at 2% followed by 4%, which implies that the bacteria are able to produce osmolytes like sugar and amino acids to protect themselves against the hypertonic environment created by the salt

(Bacilio et al., 2004). At higher concentration of 6% -8%, growth was greatly reduced, some of the isolates could not withstand the high osmotic gradient created by the salt again while at 10% only isolates OM6 142, OM8 321, OMF 003, and OMF 532 could tolerate the high salt concentration in the growth medium. Bacterial tolerance to salt concentration observed in this study indicated that bioavailable salt present in the tailings could be high.

Based on the result of metal tolerance, multi metal mixtures tolerance test as well as the physiological characterization, twelve isolates: OMF 001, OMF 003, OMF 005, OMF 008, OMF 532, OMF 810, OM4 274, OM6 142, OM7 132, OM8 321, OM9 107, OMF 811 that shows high metal tolerance and ability to withstand varied environmental conditions were chosen for further molecular characterization.

5.3.7 Molecular characterization of bacterial isolates

The amplification of the16S rRNA genes from the genomic DNA of the bacterial isolates using the universal primers (27F, 1492R) yielded a 1.5 kb fragment as shown in photo 5.1.

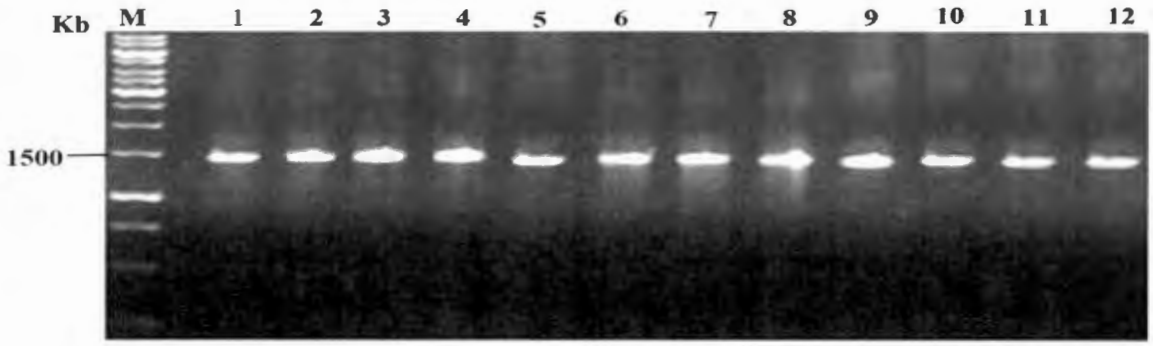


Photo 5.1: Agarose gel photograph showing amplicons of approximately 1500 bp for 16S rRNA gene amplification of the metal resistant bacteria. M= 1Kb molecular weight marker. Line 1: OMF 001, Line 2: OMF 003, Line 3: OMF 532, Line 4: OM6 142, Line 5: OMF 810 Line 6: OMF 0MF 003, Line 7: OMF 811, Line 8: OMF 274, Line 9: OMF 321, Line 10: OMF 132, Line 11: OMF 008 Line 12: OMF 005

Identification of the bacterial isolates was confirmed by computational analysis. BLAST search was conducted in the NCBI database, based on the analysis of partial sequences of the 16S rRNA gene to identify the isolates up to genus and species level (Table 5.8).

Table 5.8: Partial 16S rRNA sequence alignment results from NCBI blast searches for the bacterial isolates

Isolate	Accession no	Blast ID (closest cultured representative)	Accession no	Similarity	E- value
OMF 001	KX485322	*B. pumilus*	KP772344	100	0.0
OMF 003	KX485323	*B. cereus*	KU983642	99	0.0
OMF 005	KX485324	*Enterococcus* sp	AJ271847	78	0.0
OMF 008	KX485325	*Alcaligenes faecalis*	EF195168	86	0.0
OMF 532	KY000697	*E. asburiae*	AM184247	99	0.0
OMF 810	KY000695	*B. subtilis*	KX454058	99	0.0
OMF 811	KY125908	*Enterobacter sp*	KM253094	99	0.0
OM4 274	KY000694	*E. aerogenes*	AM184247	99	0.0
OM6 142	KY000696	*E. faecalis*	KX674048	99	0.0
OM7 132	KY000698	*Enterobacter sp*	KF010358	99	0.0
OM8 321	KY000699	*Bacillus sp*	DQ416781	99	0.0
OM9 107	KY000700	*B. thuringiensis*	CP016588	99	0.0

The BLAST query grouped the bacterial isolates into *Bacillus* (5 isolates), *Enterobacter* (4 isolates), *Enterococcus* (2 isolates) and *Alcaligenes* (1 isolate) which also correlate with the result obtained from the biochemical characterization. Gram positive and Gram negative bacteria have been reported to thrive in metal polluted soils and tailings, but in this study, Gram positive were observed to predominate in the tailings samples which is in accordance with the results of Hookoom and Puchooa (2013), Jaafar et al. (2015) who also reported the prevalence of Gram positive bacteria in their studies. Both the Gram positive and negative bacteria isolated in this study shows high tolerance to the metal species. Exposure to metals has been reported to usually lead to tolerance in population of Gram positive and Gram negative bacteria present in contaminated sites (Abou-Shanab et al., 2007) as observed in this study. Bacterial surfaces are known to be negatively charged as a result of the ionization of their functional group which contribute to metal binding (Tiraferri and Elimelech, 2012) and these metal binding properties make them excellent agent in detoxification and biotransformation of metal species.

5.3.7.1 Phylogenetic analysis

The twelve selected metal resistant isolates were subjected to sequencing and phylogenetic analysis. The 16S rRNA sequences of the twelve isolates were aligned with 26 reference sequences of the 16S rRNA of closely related taxa retrieved from the GenBank data library; and *Pantoea agglomerans* as the out-group (Figure 5.2). The relationships were based on evolutionary distances using the Maximum Composite Likelihood method (Tamura et al., 2004). The distance based method inferred the evolutionary relationship using NJ (Neigbour Joining) clustered-based algorithm. The concatenated NJ revealed the optimal tree of 65.92582460 branch length with 364 positions in the final dataset. Based on the cluster algorithm, NJ tree revealed the percentage of evolutionary relationship with the metal resistant bacteria based on the degree of differences among the sequences.

The concatenated NJ revealed that OMF 001 possessed the highest similarity index of 99% with *B. invictae, B. altitudinis, B. safensis and Endophytic* bacterium. Equally, OMF 005 shared very high similarity of 99% with *Enterococcus* sp. In addition to this OMF 811 also exhibited high level of similarity of 98% with *E. asburiae*. The high similarity value expressed by the metal resistant bacteria is above 70% borderline of degree of relatedness suggested by Wayne et al. (1987). In addition to this, similarities expressed by these metal resistant bacteria with the reference taxa belonging to different species, is as a result of high similarity value exhibited in DNA reassociation values which fall below the 70% threshold values (Stackebrandt et al., 2002). This shows high genetic relatedness that is increasingly reliable because they cannot be wiped out overnight, according to Konstantinidis and Stackebrandt 2013. In OMF 810 very low similarity index of 51% was also observed with *E. ludwigii*. This relatedness is not strong it may be wipe out over time. Moreover, eight of the strains including: OM6 142, OM9 107, OM4 274, OMF 532, OM7 132, OMF 008, OMF 003 and OM8 321 exhibited very low bootstrap value having distinct clades that is less than 50% with *Enterococcus* and *Bacillus* spp as their closest relative. These metal resistant bacteria did not cluster with any strains, which implied that OM6 142, OM9 107, OM4 274, OMF 532, OM7 132, OMF 008, OMF 003 and OM8 321 could be probable novel metal resistant bacteria based on their distinctness (Konstantinidis and Stackebrandt, 2013; Aremu and Babalola, 2015).

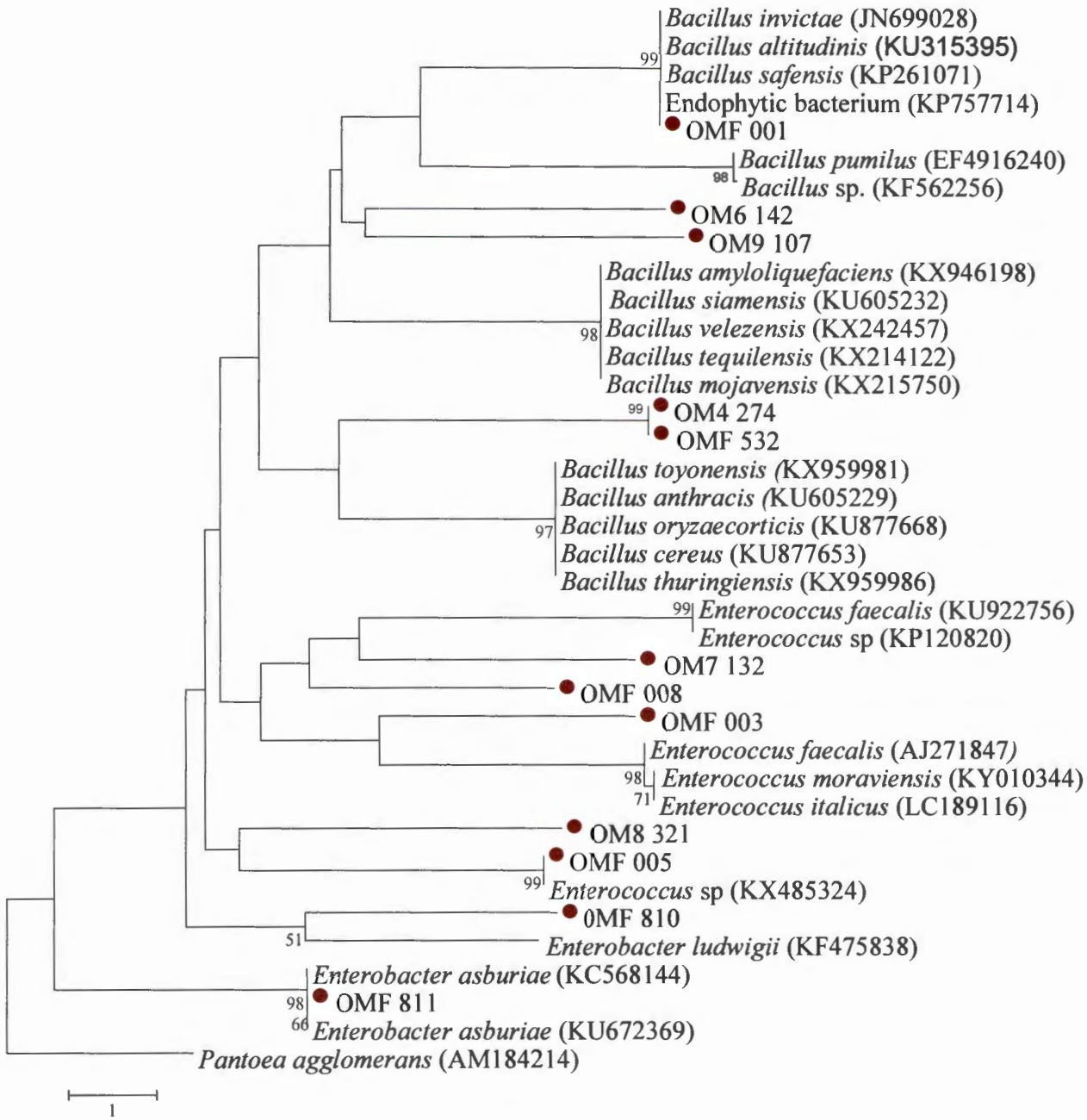


Figure 5.2: Evolutionary relationships of taxa using Neighbour-Joining method of phylogenetic tree based on partial 16S rRNA gene sequence, showing the phylogenetic relationships between metal resistant bacteria and the most closely related strains from the GenBank. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values > 50% are shown

5.3.7.2 Detection of plasmids

In this study, isolate OMF 005 identified as *Enterococcus* sp was dropped for OM6 146 which was also identified as *Enterococcus faecalis* and plasmid was extracted from the remaining eleven isolates. A single band of plasmid DNA was observed to be present in nine out of the eleven isolates as shown in Photo 5.2. Gel electrophoretic separation shows that the plasmid size is 9.4 kb using the Lambda DNA/Hind III marker. Out of the eleven isolates, isolate OMF 532 and OMF 003 shows higher concentration of plasmid (Photo 5.2). Genetic resistant to metals are found in both Gram positive and Gram negative bacteria. These bacteria are known to apply various types of resistance mechanisms which could be plasmid or chromosomally encoded but these resistance has been mostly reported to be plasmid mediated (Nies, 2003, Rensing and Grass, 2003). In a study conducted by Raja and Selvam (2009), resistance to Pb and Ni by *Pseudomonas aeruginosa* isolated from metal polluted waste water was found to be plasmid mediated. Similarly, Piotrowska-Seget et al. (2005) also reported bacterial resistance to Zn in their study to be plasmid mediated. Although plasmid was detected in most of the bacterial isolates, it was checked further to confirm if it is actually responsible for their tolerance to the metal species.

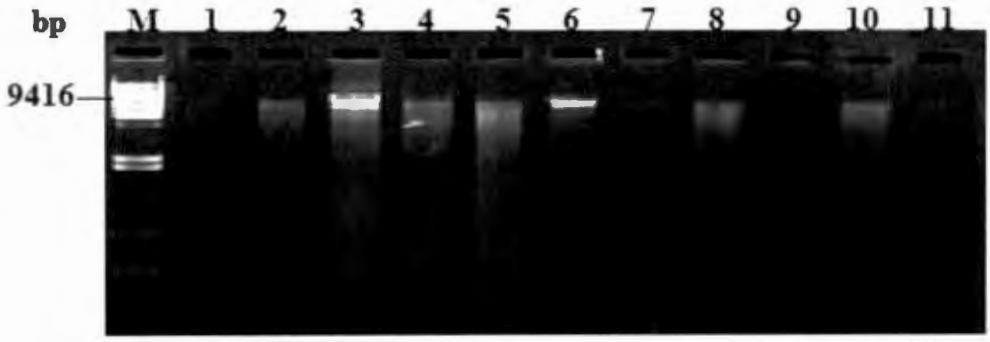


Photo 5.2: Electrophoretic separation profile of plasmid DNA isolated from bacterial isolates. Lane M= Lambda DNA/Hind III, Line 1: OMF 001, Line 2: OMF 003, Line 3: OMF 532, Line 4: OM6 142, Line 5: OMF 810 Line 6: OMF 0MF 003, Line 7: OMF 811, Line 8: OMF 274, Line 9: OMF 321, Line 10: OMF 132, Line 11: OMF 008

5.3.7.3 Amplification of metal tolerance genes

Bacteria tolerance to metal could be natural or genetically acquired which are known to involve many operons like *czcD, nccA, pco, cop, mer, ars*. Metal tolerance genes in the bacterial isolates were performed using primer-specific PCR amplifications on total genomic and plasmid DNA fractions to detect the involvement of genetic systems responsible for Ni, Pb and Zn tolerance in the bacteria. No amplicons were detected on the chromosomal DNA of all the 11 bacteria isolates using all the primers (Appendix 10). This evidently demonstrated that the metal resistance mechanisms of the bacterial isolates are not chromosomally encoded. Using the primers *nccA*, only two isolates Gram negative *E. asburiae* KY000697 and Gram positive *B. cereus* KX485323 reproducibly shows the presence of 1141 base pair fragment (Photo 5.3). Abou-Shanab et al. (2007), also reported that Ni resistance genes are widely distributed in Gram positive and Gram negative bacteria obtained from *A. murale* rhizosphere and Ni- rich soils in their study.

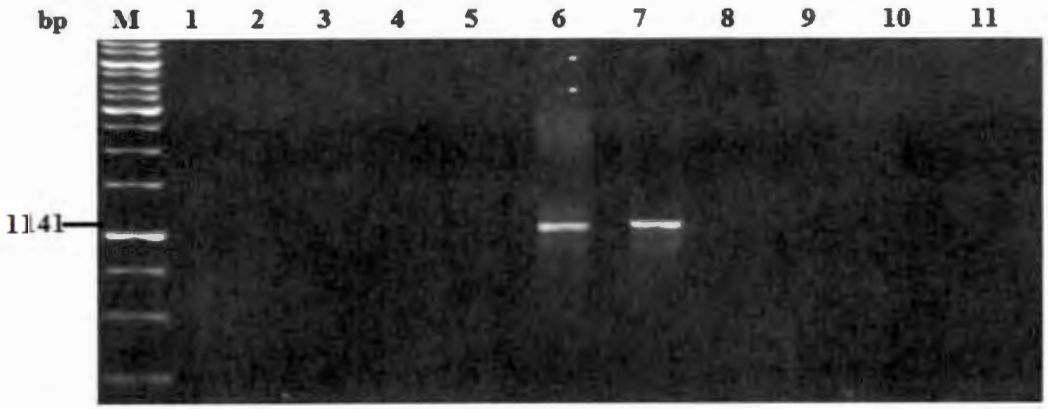


Photo 5.3: Agarose gel electrophoresis of nccA PCR product Lane M= 1500 DNA ladder, Line 1: OMF 001, Line 2: OMF 003, Line 3: OMF 811 532, Line 4: OM6 142, Line 5: OMF 810 Line 6: 0MF 003, Line 7: OMF 532, Line 8: OMF 274, Line 9: OMF 321, Line 10: OMF 132, Line 11: OMF 008

Most of the isolates yielded no amplification products with the primer sets *Czc ABD, P3 P4* and *PbrT. E. asburiae* KY000697 and *B. subtilis* KY000695 yielded smaller multiple bands size other than the expected size with the primer *PbrT* while the isolates OMF 003 also yielded a product size not up to the expected band size with primer *Czc B* and this could not be reproduced. Similar reports were observed by Trajanovska et al. (1997), using the primer pairs *CzcB* for the multiple metal resistance to Cd, Zn and Co. This shows that these genetic systems have limited or no contribution to the mechanism responsible for metal ion resistance of the bacterial isolates. It is also possible that the bacteria utilize other mechanisms different from the efflux system. The result obtained for Pb resistant mechanism using primer *PbrT* (membrane transport protein) and *P3 P4* (metallothioeins encoding gene, *BmtA*) revealed that the bacteria may harbour a different type of uptake protein or that the primer is not suitable for the amplification of the membrane transport protein gene and the metallothioeins encoding gene. The result obtained using the primer *PbrT* is in accordance with the report of (Govarthanan et al., 2013; Govarthanan et al., 2015) using the same primer *PbrT* which showed no amplification of the membrane transport protein gene in the *Bacillus* sp studied. Efforts to obtain positive control strains to ascertain the results obtained in this study proved abortive.

Most isolates yielded no amplification products with primers *P3 P4, PbrT* and *CzcABD*. Due to the poor amplification and reproducibility of results of most bacterial isolates with the, *nccA, PbrT, P3 P4* and *Czc ABD* primers, sequencing and nucleotide translation for these genes were not pursued further. However, the absence of amplicons in the bacterial isolates does not infer that they did not possess the genes for the metal resistant as bacterial tolerance to Pb and Zn could be due to other metal resistance mechanisms. The presence of metal resistance determinants on plasmids has suggested that these genes may be transfered to divergent bacteria through horizontal gene transfer (Polz et al., 2013). The

presence of the *nccA* gene in *E. asburiae* KY000697 and *B. cereus* KX485323 in this study is an indication that these genes are shared within as well as among the Gram positive and Gram negative bacterial communities. Thus bacteria possessing metal resistant genes are ideal candidate for bioremediation of metal polluted environments. The presence of these genes in *E. asburiae* KY000697 and *B. cereus* KX485323 shows their suitability as microbial inoculants for bioremediation of metal polluted sites and they were selected for bioremediation studies.

5.4 Conclusion

This study shows the presence of Gram positive and Gram negative metal resistant bacteria from three abandoned gold mine tailings in Krugersdorp. Twelve of the bacterial isolates showing multiple tolerances to varying concentration of Ni, Pb and Zn were screened for the presence of genes conferring resistance to them on their plasmid and chromosomal DNA. None of the isolates shows resistance to the metal species on their chromosomal DNA confirming the earlier claim that resistance to metal are majorly located on plasmid. Mechanisms to Ni resistance genes (*nccA*) was detected on only two of the isolates while others shows no amplification or fragment lower than the expected sizes with the primer. Zinc and Pb resistance mechanisms could not be detected with the primer sets *Pbr, P3 P4* and *CzcABD* implying that the mechanisms conferring resistance to this metals in the bacterial isolates are not the mechanisms the primers sets are designed for and this could be pursued in future studies. The two bacterial strains OMF 532 and OMF 003 characterized with remarkable tolerance to multiple metals were selected for bioremediation studies, as they could be suitable agents for the developments as microbial inoculants in bioaugmentation of mine tailings.

CHAPTER SIX

BIOACCUMULATION OF LEAD, ZINC AND NICKEL BY METAL TOLERANT BACTERIAL SPECIES ISOLATED FROM GOLD MINE TAILINGS

Abstract

The present study investigated the bioaccumulation capacity of the growing culture of individual and consortium bacterial species to ternary mixture of metals (Ni, Pb and Zn). The effect of initial metal concentration, pH, temperature and contact time (exposure period) on metals uptake by pure cultures of *E. asburiae* and *B. cereus* isolated from abandoned gold mine tailings and soil in Krugersdorp, South Africa, were investigated using ICP-MS analysis. These bacteria isolates were chosen due to their multiple metal resistant patterns (7 mM Pb, 9 mM Zn, and 5 mM Ni) observed in the previous study. The results established increasing uptake pattern for the three metals at 48 h at concentration of 1 mM to 3 mM while uptake declined at 5 mM. On the average, the two isolates shows significant ($P < 0.05, 0.10$) increased percentage removal for the three metals at temperature of 37°C. Maximum growth and metal uptake was also observed at pH 5 and 7, while reduction in percentage removal was seen at pH 3. The mean percentage removal by *B. cereus* for Pb, Ni and Zn was 22.17-27.00, 16.28-20.57 and 19.36-31.73 while *E. asburiae* also recorded 23.04-34.37, 12.05-21.72 and 34.20-37.03 respectively for the three metal species at the two incubation temperatures. Consortium displayed higher percentage removal of Zn (60.04%- 65.89%), Pb (57.07%-60.87%) and Ni (35.55% -37.55%) while only 1.86-3.98% reduction was observed in the control for all the metals. The pattern of metals bio-removal by the isolates was found to be Zn > Pb > Ni. High biomass production at increasing concentration of Pb and Zn in the growth medium shows that these isolates are prospective microbial inoculants for bio removal of these two metals in polluted environments. The two bacteria strains tested

presented distinct uptake capacities which showed their potential as suitable agents that can be utilized in efficient metal removal in metal polluted soil and water and better results can be obtained using the consortium.

Keywords: Metal species, *B. cereus*, *E. asburiae*, consortium, ICP-MS.

6.1 Introduction

Anthropogenic activities, primarily associated with mining operations are known to be among the principal causes of soil contamination in South Africa since the country has the largest mining industry in the world (Durand, 2012). Mining activities increase metal loads in the soil and this contaminate ground water, surface waters, sediments and soils with great impacts on biological and ecological systems (Lusilao-Makiese et al., 2013, Masto et al., 2015). Many metal ions are essential as trace elements but at higher concentrations they become toxic. Environmental pollution with metals like Zn, Ni and Pb pose potential danger to the ecosystem and human health (Macomber and Hausinger, 2011, Shahid et al., 2012, Zhang et al., 2012). Lead has been reported as the second most hazardous substance after arsenic based on the frequency of occurrence, potential for human exposure and toxicity (Flora et al., 2012). It persists in the environment with half-life of approximately 5000 years and biomagnifies through the trophic levels, causing series of health hazards in plants (Shahid et al., 2012) humans (Flora et al., 2012) and microorganisms (Yuan et al., 2015). Studies have shown that free ionc Zn (Zn^2+) is a potent killer of neurons, glia and other cell types in human (Shuttleworth and Weiss, 2011). Nickel on the other hand has become a serious concern during the last decade as its concentration has reached up to 26,000 ppm in polluted soils (Kabata-Pendias and Mukherjee, 2007). This metal is a potential immunomodulatory and immunotoxic agent in humans (Das et al., 2010). It is a more recalcitrant pollutant compared to other metal ions and many metal tolerant bacteria have a relatively low binding capacity for it.

Contaminated water bodies and land need to be rectified to free them from metals thereby ensuring healthier environment for the living biota and human health. Many physicochemical technologies such as vitrification, soil incineration, soil washing/ flushing, electrochemical treatment, oxidation/reduction, excavation and landfill are being used to remove metal ions from contaminated sites. However, most of these methods are too expensive, labor-intensive, prone to secondary pollution, harmful to soil characteristics and often less efficient at low concentration of metals (Al Aji et al., 2012). Thus, the use of metal-resistant bacteria for the remediation of metal contaminated sites has received a great deal of attention as an attractive alternative owing to their low cost and high efficiency. Studies have shown that bacteria inhabiting metal polluted environments; such as mining sites, are endowed with many resistant mechanisms to withstand high metal concentrations usually encountered in these environments (Colin et al., 2012, Dadrasnia et al., 2015). It has also been revealed that such metal tolerant bacteria possess the ability to adsorb or accumulate metal ions from their surroundings either actively (bioaccumulation) or passively (biosorption) by a combination of both processes (Sahmoune and Louhab, 2010, El Baz et al., 2015). Bioaccumulation is one of the mechanisms that serve as a bioremediation tool for the removal of metal species from contaminated sites. This process occurs only in living cells and involves binding and transport of toxic metals or contaminants to the cell which accumulate intracellularly in the bacteria cell (Chojnacka, 2010, Zabochnicka-Świątek and Krzywonos, 2014).

Thus, the aim of this study was to characterize the metal (Pb^{2+}, Ni^{2+} and Zn^{2+}) bioaccumulation behaviour of *B. cereus* and *E. asburiae*. For this purpose, various factors affecting the bioaccumulation, such as, pH, temperature, contact time and initial metal concentrations of the solution, were investigated under laboratory conditions. The

effectiveness of a consortium of bacteria working together compared to single isolate in the removal of the three metals as well as interactions among the metals was also determined.

6.2 Materials and Methods

6.2.1 Selection of bacterial strains

In the present study, two bacterial species isolated in the previous studies *B. cereus* strain OMF 003 (KX485323) and *E. asburiae* strain OMF 532 (KY000697) were used. These strains were selected based on their tolerance to high concentrations of multiple metal species (Zn, Pb, Ni, Cd, and Cr).

6.2.2 Preparation of metal solutions

Stock solutions (1M) of the metals were prepared by dissolving analytical grade (Sigma Aldrich) chloride salts of Zn, Ni and Pb in deionized water. Each stock solution was sterilized by filtration and stored at 4°C. Three concentrations (1, 3 and 5 mM) of the metals were then prepared from the stock solutions. The pH of each test solution was adjusted to the required value (3, 5 and 7) by using 0.1 M NaOH or HCl. All plastic and glassware used were acid-washed in 2M HNO_3 and thoroughly rinsed several times with deionized water before use to avoid metal contamination.

6.2.3 Preparation of bacterial inoculum

Bacterial inocula were prepared by growing the two bacterial strains, *B. cereus* and *E. asburiae* in separate 500 ml Erlenmeyer flasks containing 250 ml of Luria Berthani broth. The flasks were kept under agitation in a rotary shaker maintained at 150 rpm at 27 ± 2°C and the bacteria cells were harvested at their mid- log phase. The cells were separated by centrifugation at 10,000 rpm for 15 min using a Hi Cen SR super speed (Germany) and washed three times with sterile saline solution (0.85%). Cell pellets were re-suspended in

saline solution (0.85%) and then adjusted to an absorbance of 1.0 at 600 nm using spectroquant pharo 300 UV Visible Spectrophotometer (Merck, South Africa).

6.2.4 Bioaccumulation of metal species

Bioaccumulation of the metal species by the two bacterial species was carried out in Erlenmeyer flasks containing 100 mL of LB broth spiked with three (1, 3 or 5 mM) ternary concentrations of Ni, Pb and Zn and $8x10^8$ cells/ml of bacteria cells were inoculated singly as well as consortium in the culture medium. To ensure equilibrium, cells and metal solution were maintained in contact for 72 h under constant agitation at 150 rpm to check the growth and metal removal ability of the bacteria at 25°C and 37°C. Controls consisted of metal containing medium without the bacteria cells.

6.2.5 Optimization of growth parameters

To determine the effect of different factors such as pH, temperature, contact time, metal ions, pure and mixed cultures of the bacteria strains on bioaccumulation of metals, flasks were inoculated and maintained under the different conditions. The culture broth was adjusted to three different pH (3, 5 and 7) using 0.1N HCl and 0.1N NaOH solutions and incubated at 25°C and 37°C in an incubating shaker at 150 rpm for 3 days. At an interval of 24 h, growth was monitored as a function of biomass by measuring the optical density at 600 nm (spectroquant pharo 300 UV visible spectrophotometer) and the content of each flask was centrifuged at 10,000 rpm for 10 mins with Hi Cen SR super speed (Germany) to remove the bacterial biomass.

6.2.6 Digestion of the supernatant and metal analysis

The supernatant was digested according to the method described by Mohammed et al. (2012). One milliliter of concentrated HNO_3 was added to 5 mL of the supernatant and heated

to about 70°C for 3 min in a water bath. The resulting solution was transferred to 100 ml volumetric flask, diluted to the mark and then stored in a refrigerator until further analysis. The amount of residual metals (Ni, Pb or Zn) left in the supernatant was determined using a Perkin Elmer Nexion 300 Q ICP- MS. The instruments settings and operations conditions were done in accordance with the manufacturers' specifications and these were stated in Table 6.1.

6.2.7 Determination of metal bioaccumulated by the bacteria biomass

The amount of metal ion removed by the bacterial strain was calculated as follows

$$R = \frac{C1 - C2}{C1} X\ 100$$

Where R is the bioaccumulation %

C1- initial concentration of metals used (mg/L)

C2 – concentration of metals in treated solution (mg/L)

Bacterial isolates grown without metals were used as blank for spectrophotometric analysis.

Table 6.1: ICP-MS operating conditions

Plasma forward power	1.3 KW
Coolant flow rate	13.5 L/min
Auxillary flow rate	0.6 L/min
Nebulizer flow rate	0.78 L/min
Solution uptake rate	0.6 mL/min
Spray chamber temperature	15°C
Data Acquisition	
Detector mode	Pulse counting
Replicate integrations	3
Mass range	8-240 amu
Dwell time	320 μs
Number of MCA channel	2048
Number of scan sweep	85
Total acquisition time	3 mins per sample

6.2.8 Statistics

Data were subjected to mixed analysis of variance procedures using the SPSS statistical programme (version 21) and significance was reported at P = 0.01, 0.05, and 0.10.

6.3 Results and Discussion

Bioaccumulation of multiple metals (Ni, Pb and Zn) using two actively growing bacterial species was evaluated in this study. These bacteria were isolated from mine tailings with elevated level of metal species above the permissible limits in previous study (chapter 5). Hence, the isolates show remarkably multiple high metals resistance patterns to Pb, Ni, Zn, Cd and Cr. Metal tolerance of *E. asburiae* (OMF 532) was in the order of Cr (10 mM) > Zn (9 mM), Pb (9 mM) >Ni (5 mM) > Cd (3 mM) while that of *B. cereus* was in the order of Cr (10 mM) > Zn (9 mM) > Pb (7 mM) > Ni (5 mM).(See chapter 4) The removal efficiency of individual and mixed cultures (consortium) to mixtures of metals (Pb, Ni and Zn) in LB medium at different pH, temperature and time was evaluated and the results are presented in Figure 6.1-6.6

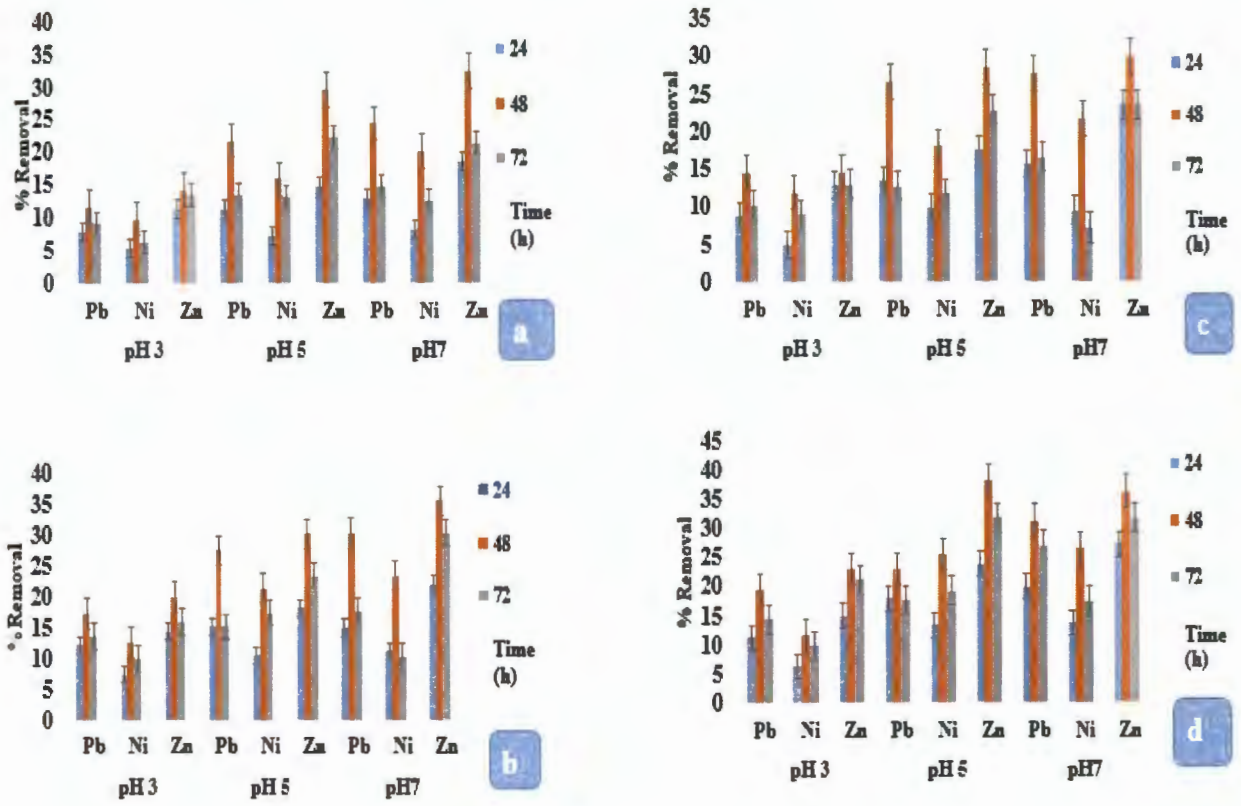


Figure 6.1: Effects of pH (3, 5 and 7) and temperatures 25°C and 37°C on bioaccumulation of Ni, Pb and Zn by *B. cereus* (a, b) and *E. asburiae* (c, d) at 1 mM conc of each metal

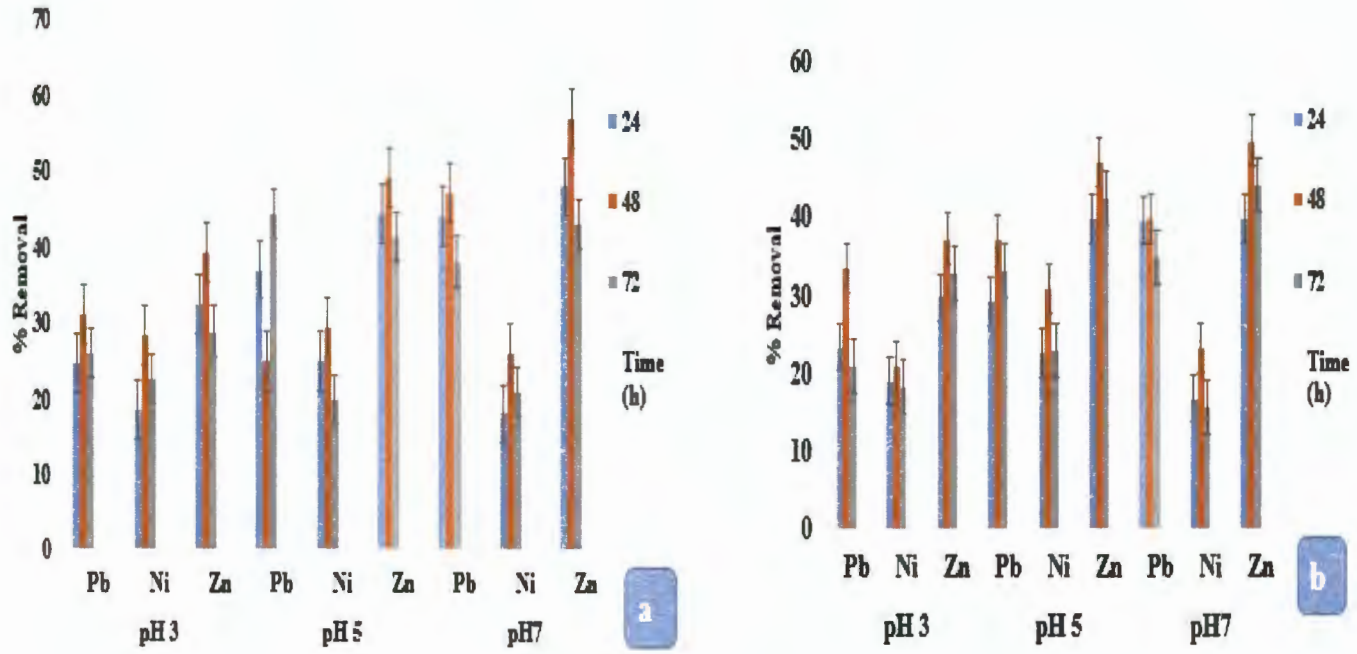


Figure 6.2: Effect of pH (3, 5 and 7) and temperatures (a) 25°C (b) 37°C on bioaccumulation of Ni, Pb and Zn by *B, cereus* and *E. asburiae* (consortium) at 1 mM conc of each metal

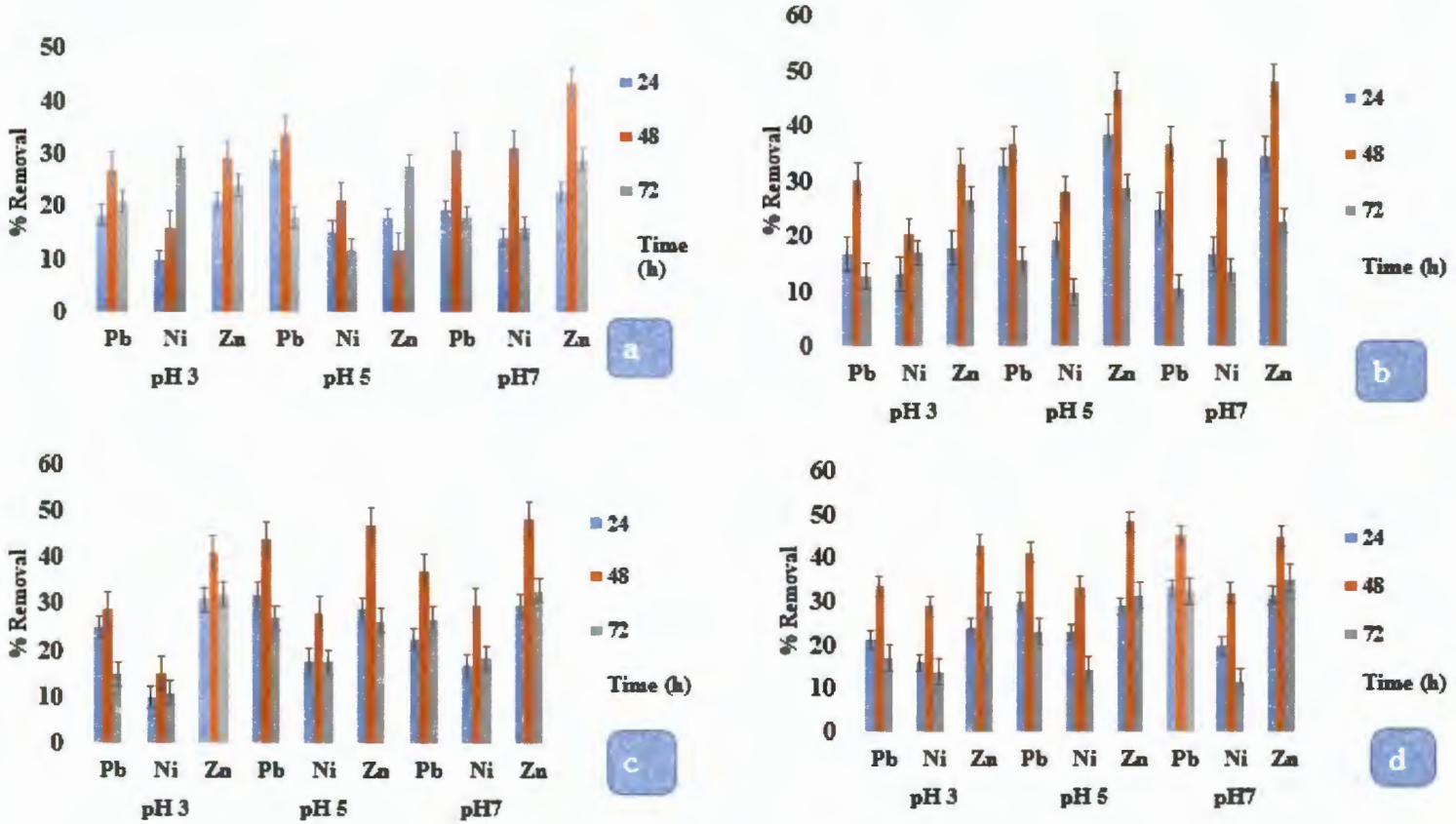


Figure 6.3: Effects of pH (3, 5 and 7) and temperatures 25°C and 37°C on bioaccumulation of Ni, Pb and Zn by *B. cereus* (a, b) and *E. asburiae* (c, d) at 3 mM conc of each metal

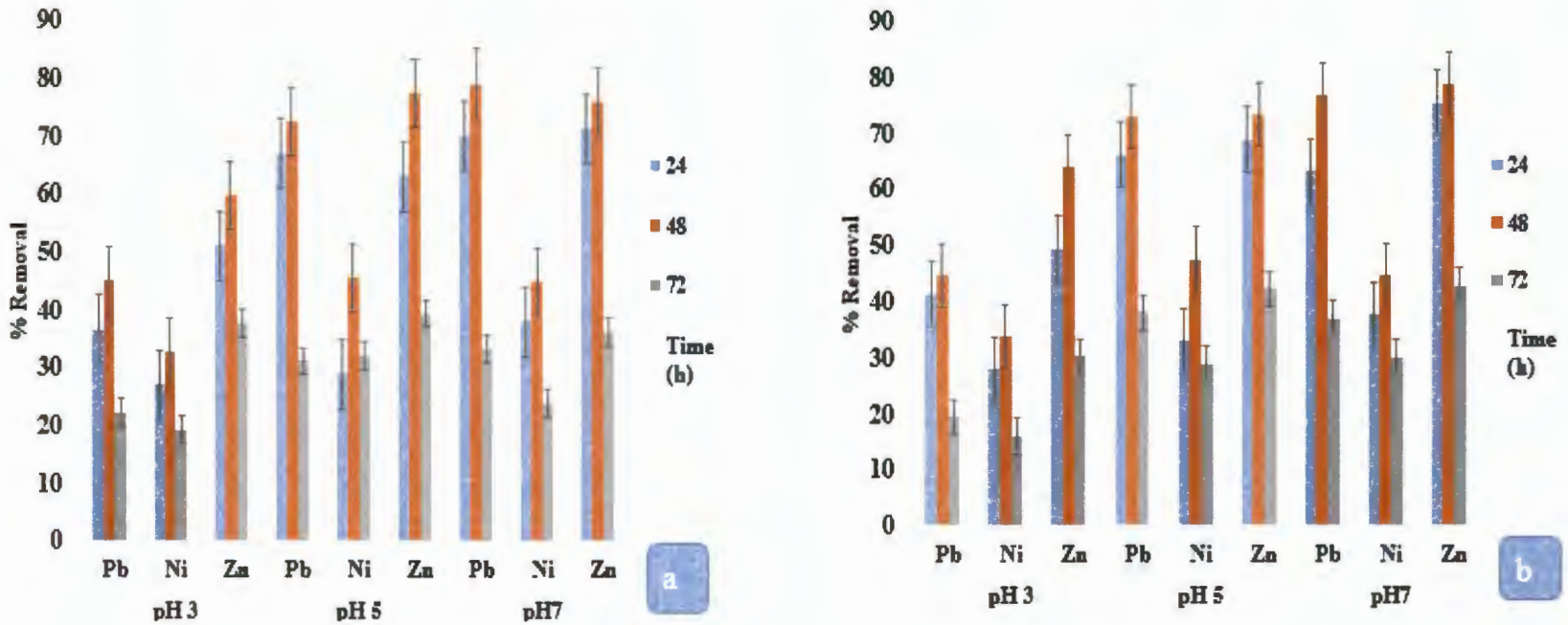


Figure 6.4: Effects of pH (3, 5 and 7) and temperatures (a) 25°C and (b) 37°C on bioaccumulation of Ni, Pb and Zn by *B. cereus* and *E. asburiae* at 3mM conc of each metal

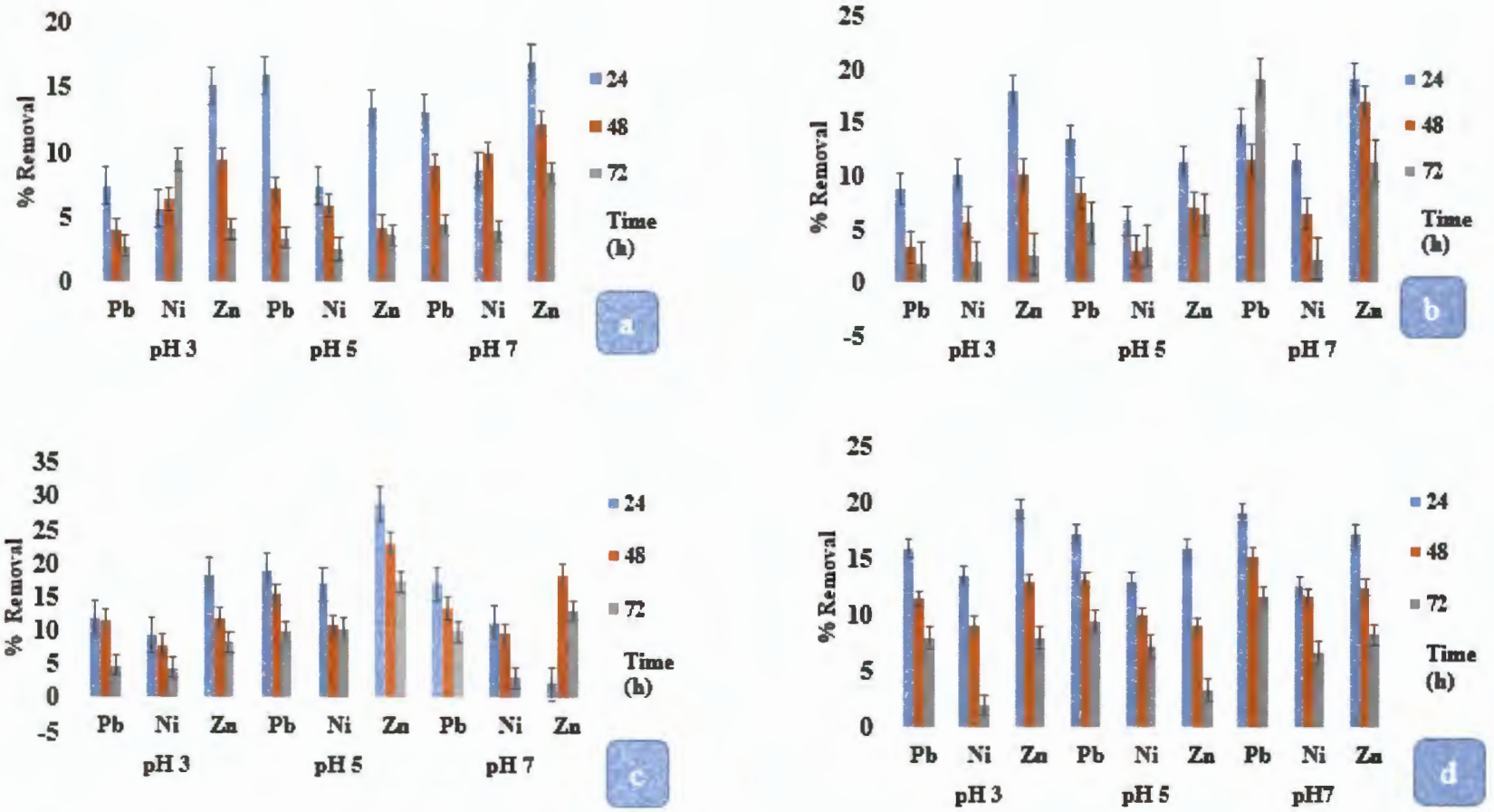


Figure 6.5: Effect of pH (3, 5 and 7) and temperatures 25°C and 37°C on bioaccumulation of Ni, Pb and Zn by *B. cereus* (a,b) and *E. asburiae* (c,d) at 5 mM conc of each metal

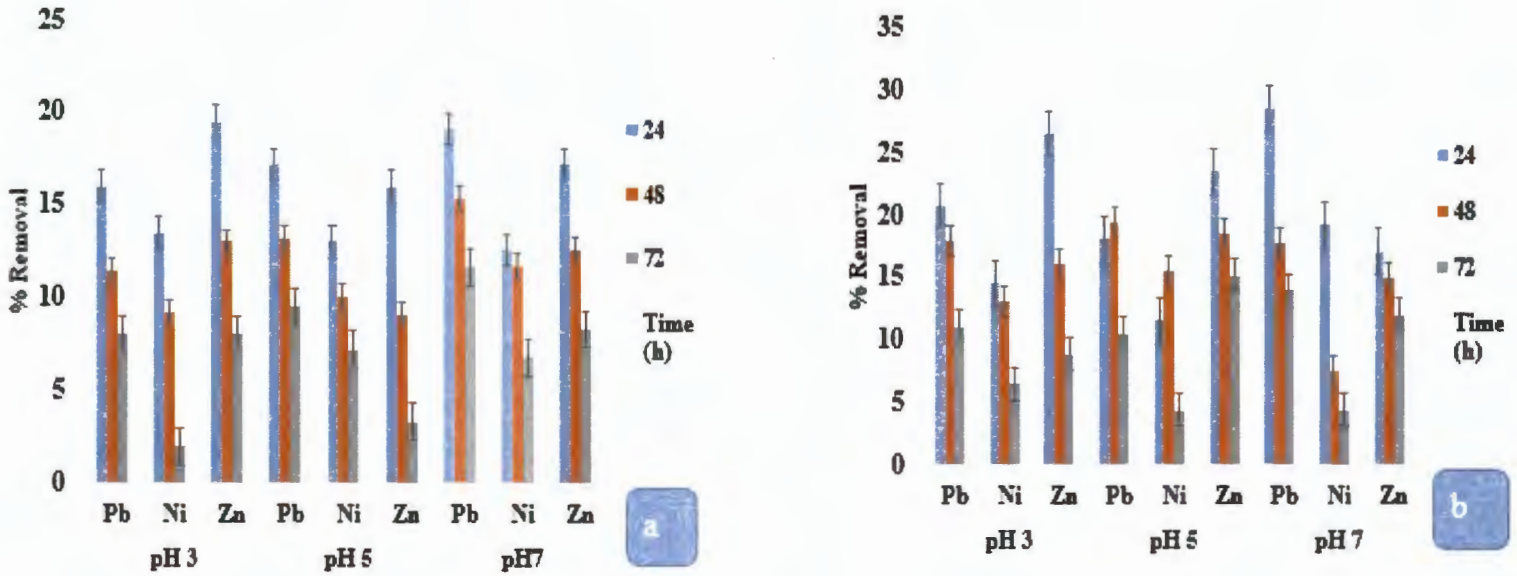


Figure 6.6: Effect of pH (3, 5 and 7) and temperatures (a) 25°C (b) 37°C on bioaccumulation of Ni, Pb & Zn by *B, cereus* and *E. asburiae* (consortium) at 5 mM conc of each metal.

6.3.1 Effects of pH on metal removal

One of the important rate limiting factors observed is the hydrogen ion concentration (pH) of the culture medium. Reduction in percentage of metal removal was observed at pH 3 while increased accumulation was observed mostly at pH 7 for the two species. Substantial uptake was also noticed at pH 5, implying that these bacteria isolates also have the ability to accumulate the metals at pH 5 (Fig 6.1-6.4). The two isolates shows maximum percentage removal for the three metals at pH 7. A significant increasing interaction between metal accumulation and pH at temperature of 37°C, pH of 7 and 5 mM concentration of the metals was observed for *E. asburiae* (Table 6.2) which shows that accumulation was more enhanced for all the metals at those conditions.

Hydrogen ion concentration affects the activity of the cell surface metal binding sites, functional groups, property and solution chemistry of the metal ions (Lemire et al., 2013). The functional groups consist of carboxylate, phosphate and amino acid groups of the cell wall. The phosphate and carboxylate groups are known to carry negative charges that enable the cell wall components to be effective in scavenging cations (Varghese et al., 2012). The lower uptake capacity observed at pH 3, may be attributed to the competition of hydrogen ion and metal cations for non- specific removal sites on the biomass. Thus, at reduced pH due to high proton concentration at the binding site, negative charge intensity on the site is reduced which leads to inhibition or reduction for the binding of metal ion. This generates repulsive ionic environments that result in decrease in binding of cations which consequently reduce the metals uptake (Yan and Viraraghavan, 2003). The observed increased uptake of the metals by the bacteria at higher pH may be due to the ionization of functional group and an increase in the negative charge density on the cell surface which is as a result of deprotonation of the metals binding sites that enabled uptake of the metals (Dudev and Lim, 2013). Similar findings were also reported by Varghese et al. (2012), Oves et al. (2013) and

Sati et al. (2014). In contrast to this observation was the findings of Alam and Ahmad (2011), who reported increased uptake of Cr^{6+} by *Exiguobacterium* sp. ZM-2 and *S. maltophilia* ZA-6 at pH 2.5 and decrease uptake at pH 5.5 while for Cr^{3+} maxima uptake was observed at pH 4.5. This difference suggests that the effects of pH on metal uptake are due to chemicals interaction of the bacteria cells with the metals.

Table 6.2: Increasing interaction in metal accumulation at 37°C

Treatment	DF	F	Level of significance
BC @37@1	1	94.323	***0.000
BC 03@37@3	2	0.532	0.613
BC 03 @37@5	4	2.542	**0.094
EA @37@1	2	4.028	*0.027
EA @37@3	4	2.854	**0.071
EA @37@5	4	4.695	*0.016
EA+BC@37@ 1	2	5.769	*0.040
EA+BC@37@ 3	2	1.378	0.322
EA+BC @37@ 5	2	0.001	0.999

Key: BC: *B. cereus,* EA: *E. asburiae.* * P = 0.05, ** P= 0.10***, P= 0.01

1, 3, 5 = pH at 1, 3 and 5

37 = Temperature at 37°C

6.3.2 Effects of temperature on metal removal

The temperature of adsorption medium is an important factor in energy dependent mechanisms involved in metal removal by bacterial cells. Temperature can bring about reduction in metal removal by bacterial cells as a result of its effect on the binding sites. This is due to the destruction of cell wall configuration and stability of the bacteria cell as well as ionization of chemical moieties (Chakravarty and Banerjee, 2012). The effect of temperature on metal uptake by the two isolates was studied at two different temperatures (25°C and 37°C) as shown in Fig 6.1-6.6. It was observed that the rate of accumulation of the three metal species by these isolates was only slightly affected by the two temperatures. The two isolates shows significant increased percentage removal for the three metals at temperature of 37°C (Table 6.2 and Figure 6.1, 6.3 and 6.5). Zinc and Pb was efficiently removed by two isolates at the two incubation temperatures while Ni bio-removal was the lowest of the three metal species. The pattern of metal bio-removal by the isolates was found to be Zn > Pb > Ni. The ability of the *B. cereus* and *E. asburiae* to efficiently remove the three metals at the two temperature values (25°C and 37°C) were comparable to the temperature range that was used in isolating them. This implied that the selection of these isolates is not only due to the presence of the metals but also as a result of the temperature at which they were isolated. This trend was also observed by Congeevaram et al. (2007), Öztürk, (2007), Ganguly et al. (2011).

6.3.3 Effects of contact time on bioaccumulation

Contact time between the bacterial cells and metal solutions is a crucial factor affecting metal uptake. The effect of contact time on bioaccumulation was studied at 24 h, 48 h and 72 h. The results (Figure 6.1-6.6) show the bio removal efficiency of *B. cereus* and *E. asburiae* (BC and EA) for the metals at all environmental conditions tested as a function of contact time. The Zn >Pb > Ni uptake increase with an increase in contact time up to 48 h and after this period, decline in removal rate was observed. Increase in time led to increase in

biomass of the two selected bacterial species in this study. Correspondingly with an increase in biomass, the percentage of metals accumulated also increase. The initial metal absorption rate has been attributed to increase in surface area which improves the adsorptive nature or increase the number of active binding sites on cell surface while the following slower sorption was ascribed to the interior penetration (Bai and Abraham, 2002, García et al., 2016). These results agree with that of Ganguly et al. (2011), which reported gradual increase in cadmium consumption by *B. cereus* with highest removal rate seen at 48 h and similar findings were reported by Elsilk et al. (2014) and Sati et al. (2014).

6.3.4 Effect of bacteria consortium on metal uptake

Effective bioaccumulation of the metal species by Gram negative and Gram positive bacteria has been reported (Choi et al., 2009; Sulaimon et al., 2014). This is as a result of the presence of specific anionic polymers in their cell wall structure and other surface structures such as para-crystalline, phospholipids, proteins, and exopolysaccharide that confer metal binding properties on them (Dadrasnia et al., 2015). Higher percentage removal was observed in the consortium compared to individual species (Figure 6.2 and 6.4) but growth was greatly inhibited at higher concentration of 5 mM as seen in Figure 6.5 and 6.6. A significant interaction existed between metal accumulation and pH at temperature of 37°C and 1 mM concentration of the metals as shown in Table 6.2. For all the metals, increased accumulation was noticed at 3 mM across all the treatments (Table 6. 3, 6.4 and Figure 6.4, 6.6). Several authors have reported on efficient bioaccumulation of metals by *Bacillus* species (Sulaimon et al., 2014; Banerjee et al., 2015) and *Enterobacter* species (El-Shanshoury et al., 2013) but most of these studies reported bioaccumulation using pure culture for individual metal. Only limited studies have considered multiple metal removals by bacteria cells as represented in this study. The finding is in accordance with the work of Sannasi et al. (2006), which shows efficient removal of Cr, Cd, Cu, Pb and Ni using bacteria consortium in industrial effluents.

Table 6.3: Mean percentage (%) removal of the metals at 25°C and 3 mM concentration

Treatment	Pb			Ni			Zn		
	3	5	7	3	5	7	3	5	7
BC	22.17	27.00	22.80	18.57	16.28	20.57	31.73	19.36	31.73
EA	23.05	34.37	28.76	12.05	21.34	21.72	34.69	34.20	37.03
Consortium	34.90	57.07	28.76	26.52	35.55	21.72	49.70	60.04	61.24
Control	1.86	2.32	2.20	2.01	3.01	3.23	2.70	3.51	3.98

Key: BC: *B. cereus,* EA: *E. asburiae*

Table 6.4: Mean percentage (%) removal of the metals at 37°C and 3 mM concentration

Treatment	Pb			Ni			Zn		
	3	5	7	3	5	7	3	5	7
BC	20.17	28.66	24.33	17.07	19.29	21.77	35.47	38.35	35.47
EA	24.06	31.46	36.97	19.71	23.68	21.31	32.12	36.34	37.31
Consortium	35.25	59.12	59.20	26.02	36.59	37.55	48.01	61.60	65.89
Control	2.20	2.41	3.02	2.73	2.90	3.20	2.50	2.98	3.01

Key: BC: *B. cereus,* EA: *E. asburi*

6.3.5 Effect of co-ions on metal uptake

The interaction of metallic elements with living systems is governed by the properties of metal ions as Lewis acids (electron pair acceptors) or complex anions as Lewis bases (electron pair donor). One of the theories sometimes used in discussing metal interactions is the Lewis acids and bases theory (Jaworska et al., 2000). Zinc and Ni are borderline metal Lewis acid. Hence, it is expected that the interactions between ions of particular atoms can be caused by competition for the same binding site if these ions belong to the same group of Lewis acid or bases. Higher percentage removal was observed for Zn and Pb as compare to Ni (Figure 6.1-6.6). Isolates BC accumulated mean % of 35.47 and EA 37.3 while the consortium accumulated 65.89 % of Zn at the same environmental conditions. The maximum percentage of Ni accumulated by the two isolates (BC and EA) were found to be 21.77% and 23.68% and the consortium was 37.55% while Pb was 28.66% and 36.97% and the consortium 60.87% respectively (Table 6.3 and 6.4).

The high accumulation rate recorded for Zn can also be attributed to the fact that, it is one of the essential metals for bacteria metabolic activities which only become toxic to the bacteria when the concentration was increased to 5 mM and the percentage removal decreased to 3.33%, 8.5% by BC and EA and 8.85% for the consortium. The simultaneous presence of Pb and Zn also greatly inhibited Ni uptake. Lead is known to be toxic to all living forms even at low concentration but it was surprising to see similar uptake pattern for Pb like what was obtained for Zn. This could be due to the fact that increased exposures to Pb have led to development of resistant mechanisms by the bacteria to adapt to its toxic effect. Also the antagonistic effect of Pb over Ni could also be due to the fact that it has a polarizing efficiency over Ni. A similar finding was reported by Sannasi et al. (2006), the bacteria consortium used was found to remove higher percentage of Pb (76%) as compared to Ni (71.5%).

6.4 Conclusion

Bioaccumulation of the three metal species (Pb, Ni and Zn) was conducted using *B. cereus* and *E. asburiae* species. The two isolates showed maximum accumulation of the metals at 3 mM concentration and became saturated above this concentration. The pattern of metals uptake in the mixed metal solutions was Zn > Pb > Ni. The bioaccumulation process was greatly affected by the pH of the culture medium and was favored at pH value of 5 to 7, 48 h pre-culture times and temperature of 37°C. Thus, the presence of metal in the growth medium allowed the maintenance of tolerance at a level comparable with that observed in isolation. This study indicated that the living biomass of *B. cereus* and *E. asburiae* could be used as efficient bio sorbent material and bio removal would be enhanced using the consortium.

CHAPTER SEVEN

BIOAUGUMENTATION OF MINE TAILINGS IN THE SOIL MICROCOSM BY MIXED CULTURES OF BACTERIAL SPECIES

Abstract

Bacillus cereus strain OMF 003 and *Enterobacter asburiae* strain OMF 532 showing high tolerance to Ni, Pb and Zn were isolated from abandoned gold mine tailings and tested for their bioremediation potential in mine tailings. The ability of these bacteria isolates to reduce the bioavailable fraction of the three metals was tested in field moist-microcosms. Sequential extraction of the bioaugmented tailings shows that these bacteria were able to remediate Pb by reducing the mobile fraction of Pb in the tailings as reflected by the decrease in Pb mobility factor in microcosms. There was no significant difference in the efficiency of the isolates as bioremediators under both sterile and unsterile condition. Higher mobility factor was recorded for Ni and Zn after bioremediation especially in the Tudor shaft sample which indicated higher availability and mobility of these metals in the environment compared to those from the other sites. This study shows the potential of the two bacterial strains in bioremediation of metal species in mine tailings. Their efficiency could be improved when supplied with additional nutrients.

Key words: Bioaugmentation, *B. cereus, E. asburiae,* microcosm, mine tailings, mobility factor, sequential extraction

7.1 Introduction

Dispersion of metals from abandoned mines and the resulting contamination of nearby agriculture soils and streams are among the current environmental concerns in South Africa (Mitileni et al., 2011, Matshusa et al., 2012). The various conventional methods used to control the dispersion and bio magnification of these metals have been reported to be ineffective with lots of shortcomings (Ok et al., 2011, Yao et al., 2012). Bioremediation,

involving bioaugmentation has emerged as an economical and eco-friendly approach that is most advantageous soil and water clean-up technique for sites contaminated with metals (Tyagi et al., 2011). Bioaugmentation is the addition of pre grown highly specialized microbial cultures (single strains or consortia) to degrade or transform pollutant present in contaminated soil and water (Tyagi et al., 2011). This technique may be necessary for contaminated sites that do not have sufficient pollutant-degrading microbial cells or where the native population does not possess the metabolic routes necessary to metabolize the compounds under concern (Colin et al., 2012).

Unlike the conventional methods that determine the efficiency of the process during the treatment period by measuring the total concentrations of the metals, bioremediation methods involving bacteria usually determines the bioavailable fractions (Hurdebise et al., 2015). It has been known that total concentration of metals do not provide detailed information needed to understand the different forms, bioavailability, mobility or potential threat of metals in the environment. Determination of the various forms in which metals exists in the environment provide a good indicator of the ecosystem functioning (Wali et al., 2015). Metals accumulate in soils in various geochemical forms, i.e. water-soluble, exchangeable, carbonate associated, Fe–Mn oxide-associated, organic-associated and residual forms. Water-soluble and exchangeable fractions are considered to be bioavailable; oxide, carbonate and organic matter-bound fractions may be potentially bioavailable; while the mineral fraction is mainly not available to either plants or microorganisms (He et al., 2005). The various fractions can be determined in the soil matrices using sequential extraction methods such as Community Bureau of Reference (BCR) (Rauret et al., 1999). Bioremediation of contaminated sites using bioaugmentation approach has recorded great success recently. Numerous pollutants such as poly aromatics hydrocarbons, heterocyclic hydrocarbons, nicotine, cyanides, quinolones and pyridine, chlorinated and fluorinated

compounds, metals and many other toxic organic compounds have been successfully remediated using bioaugmentation (Alisi et al., 2009, Fashola et al., 2013, Salam et al., 2015). Achal et al. (2012), reported increase in carbonate-bound fraction of As as well as significant reduction in exchangeable fraction in contaminated soil by the bacterium *Sporosarcinia ginsengisoli*. Similarly, Govarthanan et al. (2013) and Govarthanan et al. (2015), using sequential extraction also shows the ability of *Bacillus* sp to reduce the exchangeable fraction as well as increase the carbonate fraction of Pb in their studies.

In the previous study, three highly contaminated mine tailings sites in Krugersdorp were identified. These sampling sites were found to be contaminated with various metals like Co, Pb, As, Ni and Zn above the stipulated South African standard for soils and sediments. The impact and long-term ecological effects of metal pollution on the biosphere have resulted in an increased interest to evaluate the interactions between metals, the environment and the biota. Metals appear in groups, one element by itself is rarely the source of contamination. Hence, synergistic and antagonistic interactions of metals should be taking into consideration in assessing metal bioavailability. A good understanding of bacteria metal interactions in mine tailings is a critical requirement for the use of efficient bioaugmentation. Only limited studies have reported bioaugmentation of mine tailings as a result of the redox potential, physicochemical conditions, metal speciation and co-contaminants that hinder bacteria metal-interactions and bacterial activity (Govarthanan et al., 2013). In addition, most available studies have reported bioremediation in artificially contaminated soils. Hence, the objectives of this study were (a) to determine the efficiency of bioaugmentation of the mine tailings with indigenous metal resistant bacteria as a method of bioremediation in mine tailings contaminated soils (b) to understand the interaction between the indigenous metal resistant bacteria and the tailings (c) to determine the effect of varying concentrations of Pb, Ni and Zn on efficiency of indigenous metal resistant bacteria as bioremediators.

7.2 Materials and Methods

7.2.1 Tailings sample collection and preparation

Tailings samples used were collected from three highly contaminated mine tailings sites in Krugersdorp (MA, MB and TS). In the previous chapter, the mean concentrations of Pb, Ni and Zn in these samples were found to be Ni (2,786.75 mg/kg), Pb (136.79 mg/kg), and Zn (4269.25 mg/kg) above the stipulated South African standard for soils and sediments. The physicochemical properties of the samples have been presented in Chapter 4 of this report. Four hundred grams of samples from each sampling sites were weighed into 1000 ml beakers and the various concentrations and combinations of the metals: $ZnCl_2$, $NiCl_2$ and $Pb(N0_3)$, were dissolved in 50 ml of ultra-pure water to give final concentrations of 1000 mg/kg, 500 mg/kg and 250 mg/kg as shown in Table 7.1. The samples were spiked with these metal concentrations to determine the effects of different metal concentrations on bacteria interaction and uptake of the metals and also to see the extent these bacteria could tolerate higher concentration of the metal species. All the samples were subjected to two experimental conditions: sterilized samples (S) with three metal combinations for each sample as shown in Table 7.1. Tailings MA consist of three samples (MAAS, MACS, MAES), MB (MBAS, MBCS, MBES) TS (TAS, TCS, TES); unsterilized tailings (U) + three metal combinations for each sample: MA (MAAU, MACU, MAEU), MB (MBAU, MBCU, MBES), TS (TAU, TCU, TES).

In the first condition (sterilized) which served as control, the spiked samples were autoclaved at 121°C for 15 mins to remove the microbial loads whereas the sets of spiked samples were not autoclaved. The samples were then homogenized and packed in pots for a month to for the spiked metals to mix properly with the tailings (Bahafid et al., 2013, Salam et al., 2015).

Table 7.1: Concentration and combinations of the metals used in spiking of the samples

Metal combination	combination	Concentration (mg/kg)
Pb+Ni+Zn	A	1000+500+250
Pb+Ni+Zn	C	500+250+1000
Pb+Ni+Zn	E	250+1000+500

7.2.2 Source of bacteria inoculant and preparation of bacterial suspension

The tested isolate *B. cereus* strain OMF 003 and *E. asburiae* strain OMF 532 used in this study were isolated from abandoned gold mine tailings in Krugersdorp South Africa in the previous study (chapter 5). Pure cultures of the isolates were maintained in glycerol- LB broth (50:50) at -20°C. The bacterial cells were cultured in LB broth at 25°C for 24 h and the cells colonies growing on LB agar supplemented with 1 mM HM were harvested by centrifugation at 5000 rpm for 5 min and resuspended in 0.05 M phosphate buffer (pH 7). The cells concentration was adjusted to 1.5 optical density (OD) at 600 nm using UV spectrophotometer and the bacteria suspension was used for the bioremediation studies.

7.2.3 Microcosm studies

Microcosm studies were carried out in 500 ml Erlenmeyer flasks containing 20 g of the HM spiked tailing samples mixed with 10 ml suspension of *B. cereus and E. asburiae.* The flasks were incubated at 37°C for 8 weeks. The inoculated bacteria in the spiked samples were tested both in sterilized microcosms (control) and non-sterilized microcosms. Un-inoculated samples in both sterile and unsterile conditions were also analyzed to determine natural antenuation of the metals and this also served as negative control. Replicate flasks with samples were sacrificed at week 2, 5 and 8, air dried and the contents analyzed for concentrations of Pb, Ni and Zn in the different chemical fractions of the bioaugumented tailings using the modified Community Bureau of Reference (BCR) sequential extraction procedure described by Nemati et al. (2011) and Davutluoglu et al. (2011). The sequential extraction procedure involved three main steps and a fourth step as added by Rauret et al. (1999). Details of the BCR procedure are provided below.

7.2.3.1 Step 1: Extraction of Exchangeable and Soluble fraction of metal (Fraction 1)

One gram of air dried tailing samples was weighed into 50 mL capacity bottles, 40 ml of 0.11 mol L^{-1} acetic acid solution was added and the bottles placed in a rotary shaker at 30°C at revolution of 150 rpm for 16 h. The extract was then separated from the solid residue by filtration and the residue collected in polyethylene bottles. The residue obtained was washed by shaking for 15 min with 20 ml ultra-pure water and the supernatant carefully decanted.

7.2.3.2 Step II: Extraction of the Reducible fraction of metal (Fraction 2)

In step II, 40 mL of 0.1 mol/L hydroxyl ammonium chloride solution adjusted to pH 2.0 with HNO_3 was added to the residue from the first step and the mixture was re-suspended by mechanical shaking for 16 h at room temperature. The extract was separated through filtration and the residue also washed as described in the step 1.

7.2.3.3 Step III: Extraction of the Oxidizable fraction of metal (Fraction 3)

Ten milliliters (10 mL) of 8.8 mol L^{-1} of H_2O_2 solution was carefully added to the residue from the second step and covered with a watch glass. The contents were digested for 1 h at room temperature and an additional 1 h with occasional manual shaking at 85 ± 2°C until the volume reduced to less than 3 ml by evaporation. A second aliquot of 10 mL of H_2O_2 was added to the mixture and the tube covered with a watch glass and again digested for 1 h at 85 ± 2°C until the volume of the residue reduced to about 2 to 3 ml. The residue was allowed to cool and was further mixed with 50 ml of 1 mol L^{-1} ammonium acetate solution, adjusted to pH 2 with HNO_3 and shaken for 16 h at room temperature. The extract was separated through filtration, and the residue was washed in the same manner as described in previous steps.

7.2.3.4 Step IV: Extraction of the Residual fraction of metal (Fraction 4)

The residual fraction was extracted as described by Rauret et al., (1999). The residue from the third step was digested using aqua regia (3:1 HCl: HNO_3). In each extraction step, blank samples were also analyzed.

The concentrations of the metals in the extracts from the various steps were determined using a ContraAA300 atomic absorption spectrometer. The operating conditions for the equipment and the concentrations of the standard used are described in chapter 4 (see section 4.2.3.9, Table 4.2).

The metal mobility was calculated as mobility factor; MF (Badawy and El-Motaium, 2003) using the equation below :

$$\mathrm{MF} = \frac{F1}{F2 + F3 + F4}$$

Where F1: metal concentration in the exchangeable and carbonate fraction using BCR method, F2: reducible, F3: oxidizable and F4: residual fractions of the tailings respectively.

7.2.4 Quality assurance

Analytical grade reagents supplied by Merck (South Africa) were used to digest the samples.and ultra-pure water was used to prepare the standards for the calibration. Standards were prepared from the certified stock solutions as described in (chapter 4). All glass wares used for the preparation of the reagents and sample digestion were washed thoroughly, soaked in 20% nitric acid and rinsed three times with deionized water to prevent cross contaminations from glass wares. WhatMan number 42 filter paper was used in the filtration process.

7.2.5 Statistical analysis

The experiment was repeated twice and each sample was analyzed in duplicate. The mobility of the metal species was calculated as described by Badawy and El-Motaium (2003). The concentration of the metals obtained was verified using quality control standards outlined in Table 4.2. in Chapter 4.

7.3 Results and Discussion

7.3.1 Metal speciation

Lead concentration in fraction 1 in samples from sampling site MA and MB was greatly reduced in all metal combinations (A, C and E) in both sterile and unsterile conditions while in the samples from sampling site TS, Pb concentration in fraction 1 of treatment A was higher in the sterile condition compared to its concentration in the unsterile condition as shown in Figure 7.1.

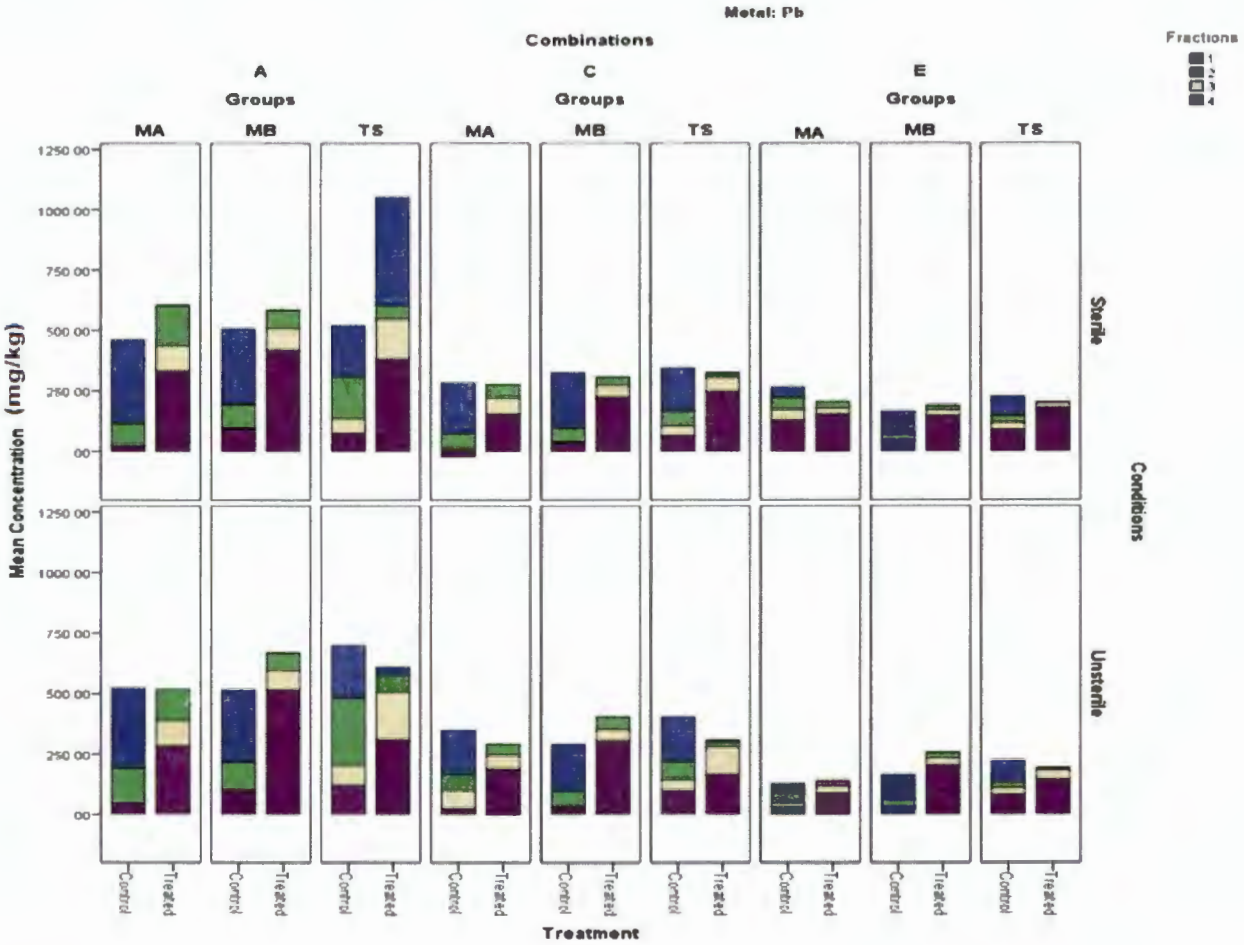


Figure 7.1: Pb concentration in the different fractions of the treated and control samples

Group A: Metal combination A, Group B: Metal combination C, Group E: Metal combination E

Multiple analysis of variance (M-ANOVA) shows that Pb concentration in the control was significantly higher than in the treatment ($P = 0.000$ and lambda 0.055). Pairwise comparison also shows that Pb concentration in the control sample and the inoculated samples were higher at the end of the incubation period in the F1 fraction for all the samples treatment ($P < 0.05$). On the other hand, higher percentage of Ni and Zn were found in the fraction 1 in all the sample treatments and conditions as shown in Figure 7.2-7.3. Similar finding was reported by Esshaimi et al. (2013), who also found high percentage of Zn in the F1 fraction in their study on speciation of metal in mine tailings using the BCR method.

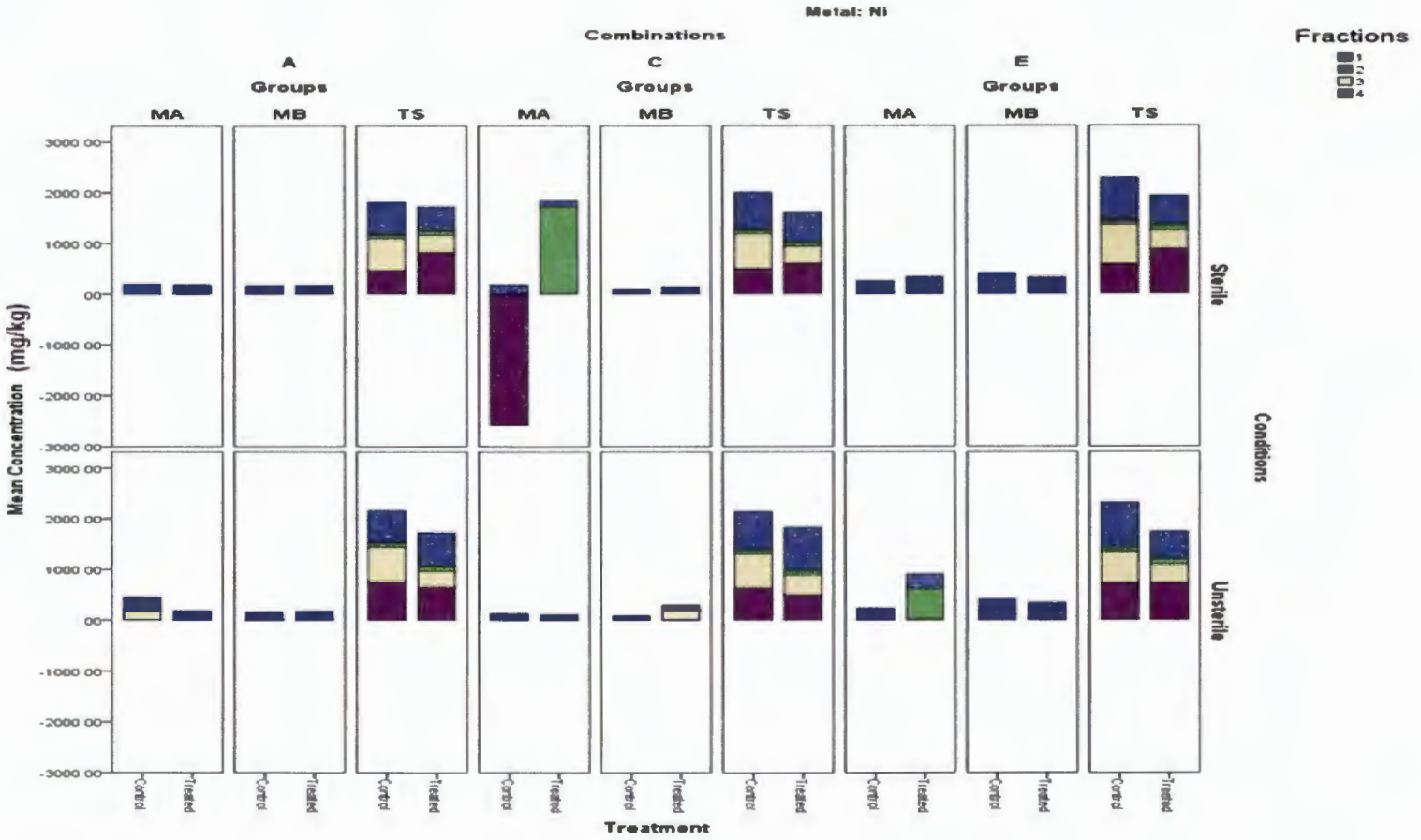


Figure 7.2: Ni concentration in the different fractions of the treated and control samples

Group A: Metal combination A, Group B: Metal combination C, Group E: Metal combination E

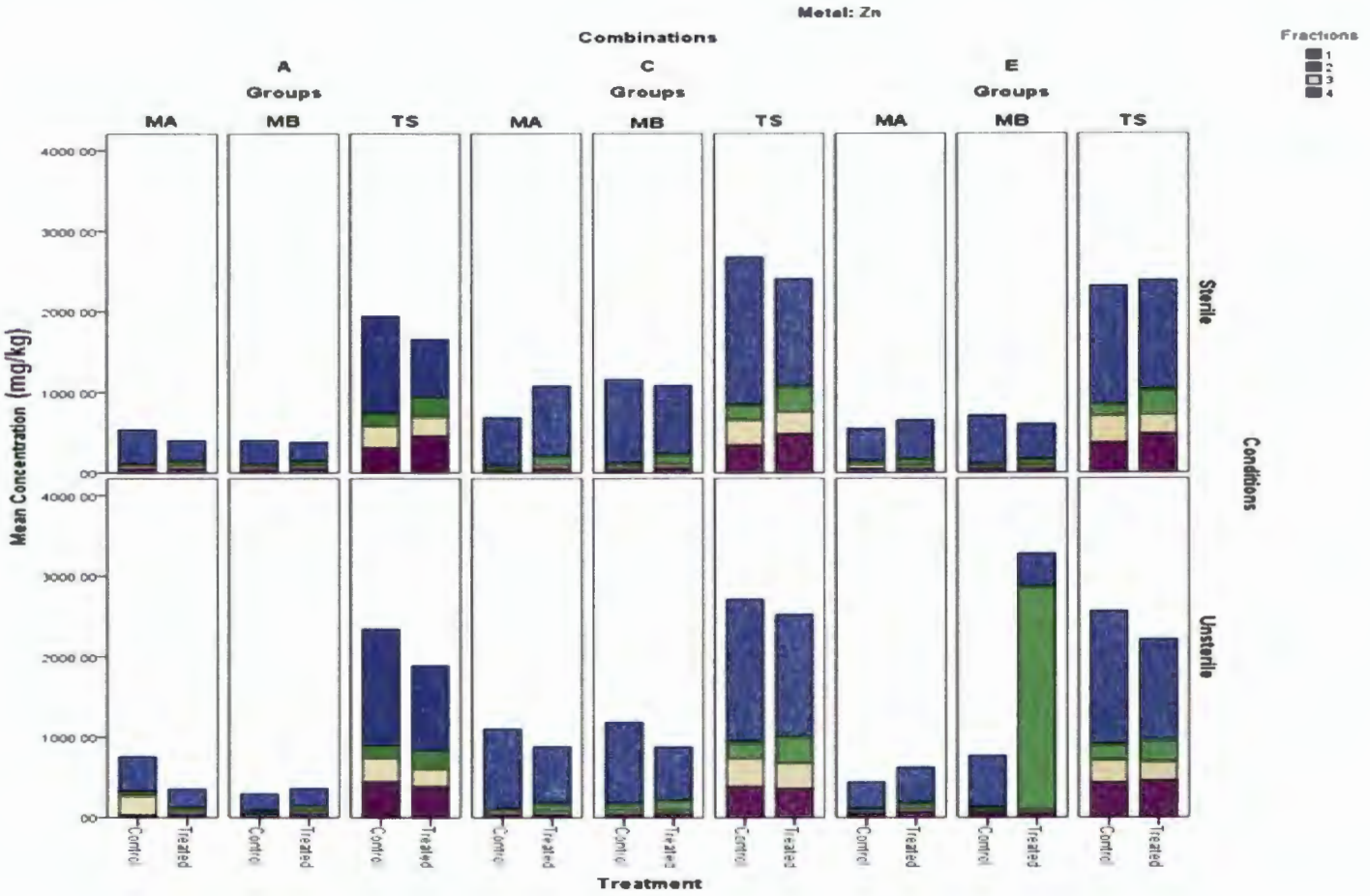


Figure 7.3: Zn concentration in the different fractions of the treated and control samples

Group A: Metal combination A, Group B: Metal combination C, Group E: Metal combination E

Multivariate test shows that Ni concentration in both the control and the inoculated samples was significantly lower in all the metal combinations (P = 0.000 and λ 0.051) (Appendix 4). Significant lower differences in Zn concentration was also observed in the F1 fraction across all the analyzed samples (F = 24.349, P = 0.000) (Appendix 6). Analyses of sample two, five and eight weeks of inoculation, shows that there were no differences in the Zn concentration across all the samples.

The first fraction of the BCR sequential extraction method also known as acid soluble fractions (F1) are fraction of metal absorbed in clay and soil humus which are susceptible to environmental changes like pH that enhance their transformation and migration under acidic condition. These fractions comprise the water soluble, ion exchange and carbonate binding states (Cao et al., 2015).

Metal concentration in the F1 fraction is very important because metals in this fraction are highly available and mobile in the environment. Therefore, it is important for metal in the F1 fraction to be reduced to prevent their toxicity in the environment and their bioaccumulation and biomagnification in the food chain. The potentials of the two bacterial strains as bioremediators of Pb can be deduced from the reduction in the concentration of Pb in the F1 fraction. This result is in accordance with previous studies by Achal et al. (2012), Govarthanan et al. (2013), Govarthanan et al. (2015), who reported a notable decrease in the bioavailable fraction of metals after bioaugumentation using *Bacillus* sp. The higher percentage of Ni and Zn left in the F1 fraction after bioremediation shows that these bacteria could interact better with Pb than with Ni and Zn in the tailings. Zinc has been reported to be highly mobile in the soil environment compared to Pb. Considering the high mobility and potential bioavailability of metal in the F1 fraction and the total concentration recorded for Ni and Zn in the previous study (chapter 4), the mine tailings can be considered as potentially hazardous in the environment. The high concentrations of Ni and Zn in the F1 fraction

highlight the risk of these metals being been taken up by plants growing in the soils. In addition, these metals have been reported to have adverse effects on microbial activities in the soils which may have affected the ability of the bacteria to bioremediate them. Ni is known to replace essential metals of metalloproteins needed for bacteria metabolic activities and causes oxidative stress that destroy DNA, as well as damage bacteria protein (Macomber and Hausinger, 2011). Zinc on the other hand exerts its toxic effects on cellular activities and growth of the bacterial cell. These affects bacteria diversity and functionality in the environment (Majzlik et al., 2011) and may have affected their efficiency as bioremediators.

A higher percentage of the three metal species were associated with the F2 fraction which is the reducible fraction. The concentration of Pb in this fraction varies across the different samples, treatments and conditions. Differences in concentrations of Pb in the F2 fraction at week 2 and 8 were significant ($P = 0.028$ and 0.051) (Appendix 6) whereas differences in Pb concentration in the same fraction between week 2 and 8, and week 2 and 5 across all the samples were insignificant. Nickel and Zn were observed to be exceptionally high in the reducible fraction although statistically the differences were insignificant. The reducible fraction is the fraction of metal linked with Fe and Mn oxides which is difficult to release as a result of strong ionic interaction (Cao et al., 2015). Binding of metals to the reducible fractions indicates the affinities of the metals for the acid-reducible fractions of the tailings are very high. Although this fraction was not considered as a major binding fraction for metals in the tailings, it can be released when exposed to reducing conditions by microorganisms as a result of organic matter decomposition.

The association of the three metal species with the F3 fraction is very high despite the fact that the organic matter contents recorded in the samples in the previous study was low. This may be due to the fact that bacteria biomass is organic and during sequential extraction this will be confined in the F3 fraction thus increasing the interaction of the metal with the F3

fraction. Bacteria are known to play a major role in speciation, fate and transport of metals in soil and associated environments. They are highly efficient in accumulation of both soluble and particulate metals. This is as a result of their negatively charged cell surfaces which comprise diverse type of negatively charged functional groups such as phosphoryl, hydroxyl and carboxyl that can adsorb and retain metal by mineral nucleation (Violante et al., 2010). This result is in contrast to what was reported by Cao et al. (2015), which recorded low binding of Ni, Pb and Zn to the oxidizable fraction due to low content of organic matter recorded in their research. But this cannot be compared with this study because it does not involve bacteria interaction with metals. The F3 fraction is the fraction of the metal bound to the organic matter which is not bioavailable due to its association with stable humic substances, but this fraction may be remobilized back into the environment during degradation of organic matter. Statistical analysis shows that differences observed between the concentration of the three metal species in the control and the bioremediated tailings at day 2, 5 and 8 were significant ($P < 0.05$) in all the samples treatment.

In the last fraction F4, the concentration of the three metals was also high, highest concentration of Ni and Zn was observed in all the samples treatment in TS. This could be due to the fact that fraction F4 is the residual fraction and upon total digestion high concentration of Pb and Ni will be recorded. This is because this fraction of metal species are not bioavailable to the bacteria for interaction, they are locked in the aluminosilicate matrix through strong chemical bonds and can only be broken through strong chemical agents. Another factor that could contribute to metals adsorption is the mineralogy, and particle size of the sample and the surface area available for adsorption. Metals bound to the fourth fraction F4 are the most stable and consists of the fraction of metals bound in the crystal lattice of the soil. This can only be mobilized during weathering process which usually take longer time thus not bioavailable (Cao et al., 2015).

Adsorption and availability of metals in soil has been known to be controlled majorly by pH (Casagrande et al., 2008). According to Houben et al. (2013), increasing adsorption of metals to soil occur at pH above 7. The relationship between metal adsorption on exchangeable surfaces of soil colloids and pH is as a result of the competition of H^+ for adsorption sites at low pH which leads to reduction in metal adsorption (Xiaoqiang et al., 2016). Soares et al. (2011), reported increased adsorption of Ni as pH increase from 4- 6. The acidic nature of the tailings (2.17-6.79) reported in chapter 4 shows that the high percentage of Ni associated with the mobile fraction F1 could be due to the acidic nature observed in all the sampling sites. Also, the activity of the inoculated bacteria during the bioremediation process did not significantly raise the pH of the tailings. (Appendix 9) Generally, the effect of pH on the binding of Pb, Ni and Zn to soils has been reported to follow the pattern Pb > Zn > Ni (Harter, 1983). Zinc is also known to have adsorption edge at higher pH which enhances its mobility (Casagrande et al., 2008). Similarly, Usman (2008), reported that soil in solution having Pb, Ni, Cu and Cd, Pb has higher adsorption affinity as compared to Zn and Ni and shows that the adsorption affinity for the metals in soil solution follow the order Pb > Cu > Zn > Ni > Cd.

Concentration is another factor known to have effect on the metal sorption. At higher metal concentration, metals compete strongly for binding sites (Covelo et al., 2004). Saha et al. (2002), reported that metals usually adsorb onto specific adsorption sites at low metal concentration but at higher metal concentration, soils lose some of their ability to bind metals due to the similarity in adsorption site which in turn leads to reduction in metal sorption.

The decreased interaction of the bacterial strains for metals could also be as a result of the physicochemical properties of the tailings recorded in previous study. The organic matter contents recorded in the tailings was very low which might have resulted in decreased adsorption of the metals.

Another factor that could contribute to metals adsorption is the particle size and the surface area available for adsorption which can affect bioavailability of the metals. Clay particles allow more adsorption as a result of its small particles size with large surface-area to mass ratios as compared to sand with large particles and smaller surface-area to mass ratios. Bioavailability of the metals in the environment increases as a result of reduced adsorption which increases the concentration of dissolved metals in the soil. The high percentage of sand recorded in the tailings in the previous study possibly led to the decrease adsorption of the metals. Furthermore, coarse sand fractions are known to have high concentrations of labile carbon and nitrogen majorly derived from plant residues while clay and silt fractions usually contain high concentrations of reasonably stable organic carbon and nitrogen (Six et al., 2000). Also high contents of soil organic matter are usually found in clay and silt fractions, but in sandy soil low contents of soil organic carbon are present (Kandeler et al., 2000).

7.3.2 Effect of bacteria suspension on the Mobility of Pb, Ni and Zn in tailings samples

The mobility factor recorded for Pb in the control tailing MA (without bacteria) in treatments A and C were higher in both the sterile and unsterile condition compared to the inoculated samples. At the end of the 8 week bioremediation period of the tailings in sterile and unsterile condition, the mobility factor recorded was greatly reduced in the treatments as shown in Figure 7.4 (a-d).

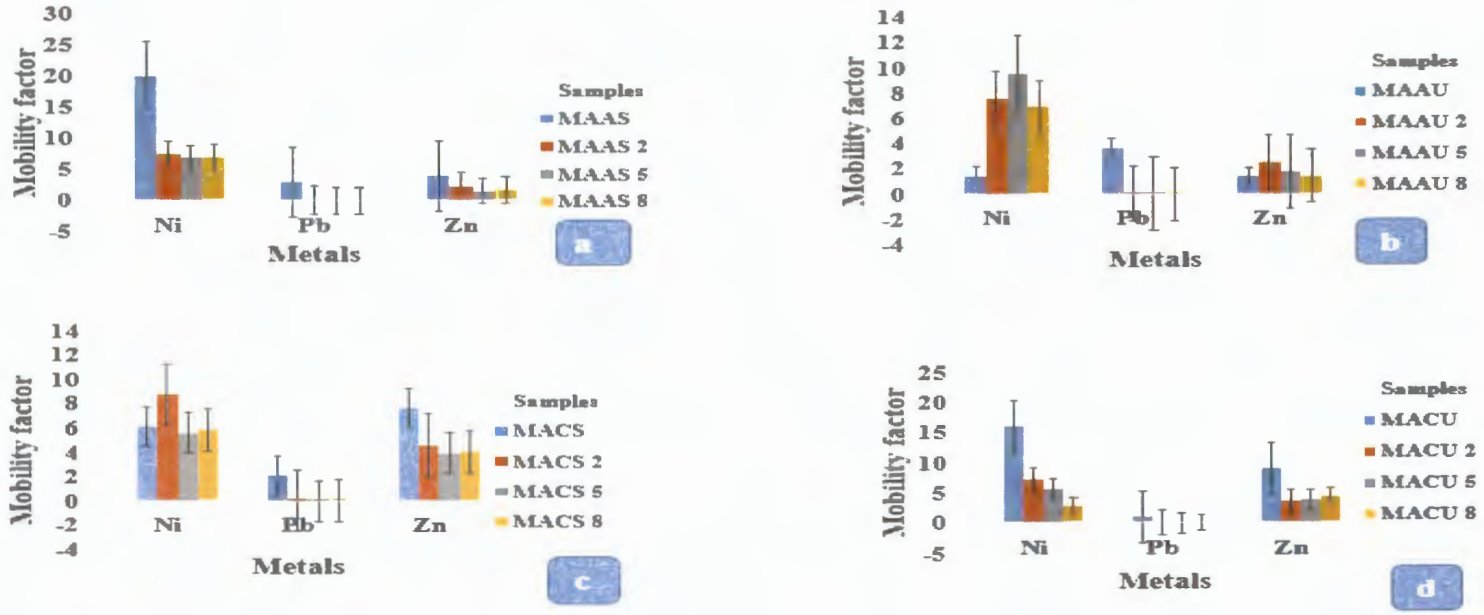


Figure 7.4: Mobility factor of Ni, Pb and Zn in sample MA (a-d)

MAAS: Sterile tailings MA with metal A combination, MAAS 2, 5 and 8: Sterile tailings MA with metal A combination after 2, 5 and 8 weeks of bioremediation, MAAU : Unsterile tailing MA with metal A combination, MAAU 2, 5 and 8 : Unsterile tailings MA with metal A combination after 2, 5 and 8 weeks of bioremediation, MACS: Sterile tailings MA with metal C combination, MACS 2,5, 8 : Sterile tailings MA with metal C combination after 2, 5 and 8 weeks of bioremediation, MACU : Unsterile tailings MA with metal C combination, Unsterile tailing MA with metal C combination after 2,5 and 8 weeks of bioremediation

In the second sample MB, the mobility factor recorded was also similar to what was recorded in sample MA. Higher mobility factor was recorded in the control compared to the inoculated tailings. At the end of the 8 week treatment period, reduction in the mobility factor was observed ranging from 0.002-0.012 in all the treatment as shown in Figure 7.5 (g and h) and Figure 7.6 (i- l).

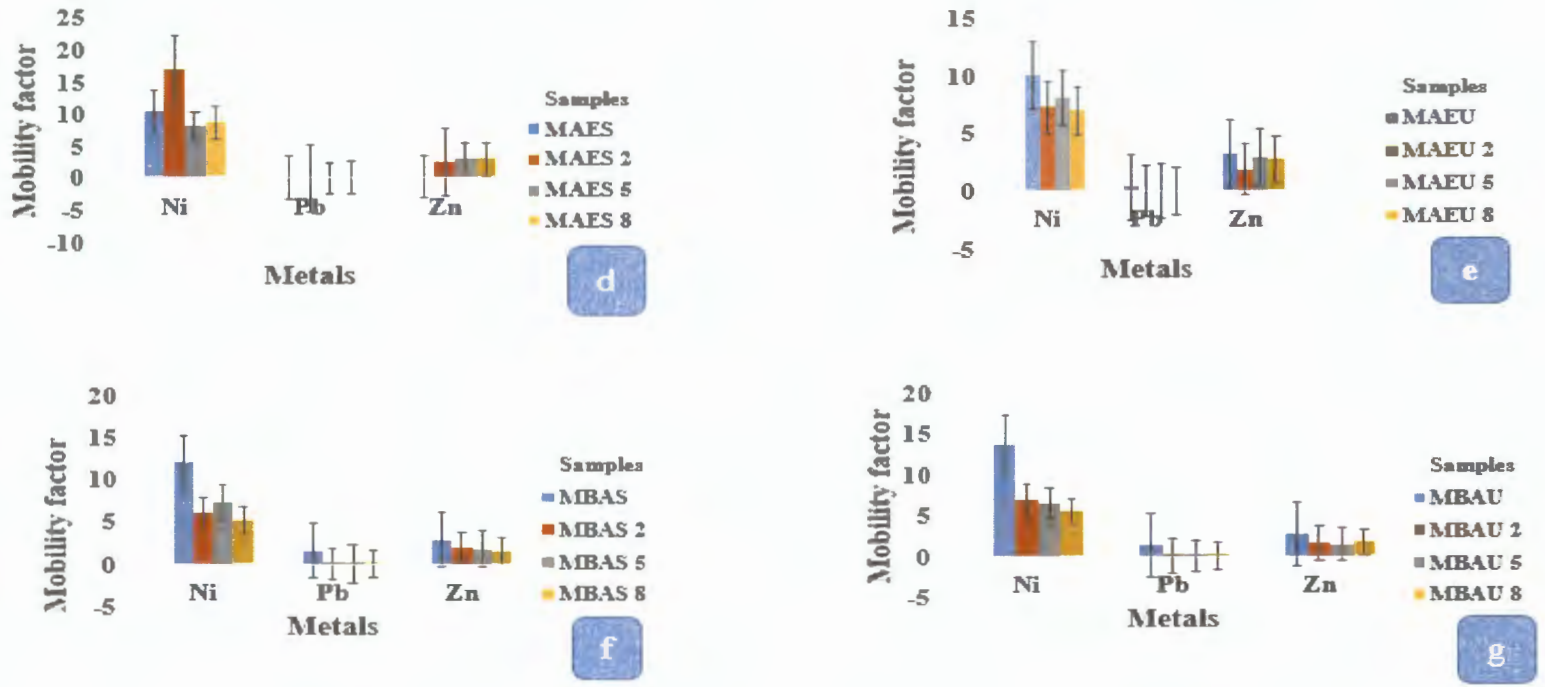


Figure 7.5: Mobility factor of Pb, Ni and Zn in sample MA (e and f) MB (g and h)

MAES: Sterile tailings MA with metal E combination, MAES 2, 5 and 8: Sterile tailings MA with metal E combination after 2, 5 and 8 weeks of bioremediation, MAEU : Unsterile tailing MA with metal E combination, MAEU 2, 5 and 8 : Unsterile tailing MA with metal E combination after 2, 5 and 8 weeks of bioremediation, MBAS : sterile tailing MB with metal A combination, MBAS 2, 5 and 8 : sterile tailing MB with metal A combination after 2,5 and 8 weeks of bioremediation, MBAU : Unsterile tailings MB with metal A combination, MBAU : Unsterile tailings MB with metal A combination after 2, 5 and 8 weeks of bioremediation

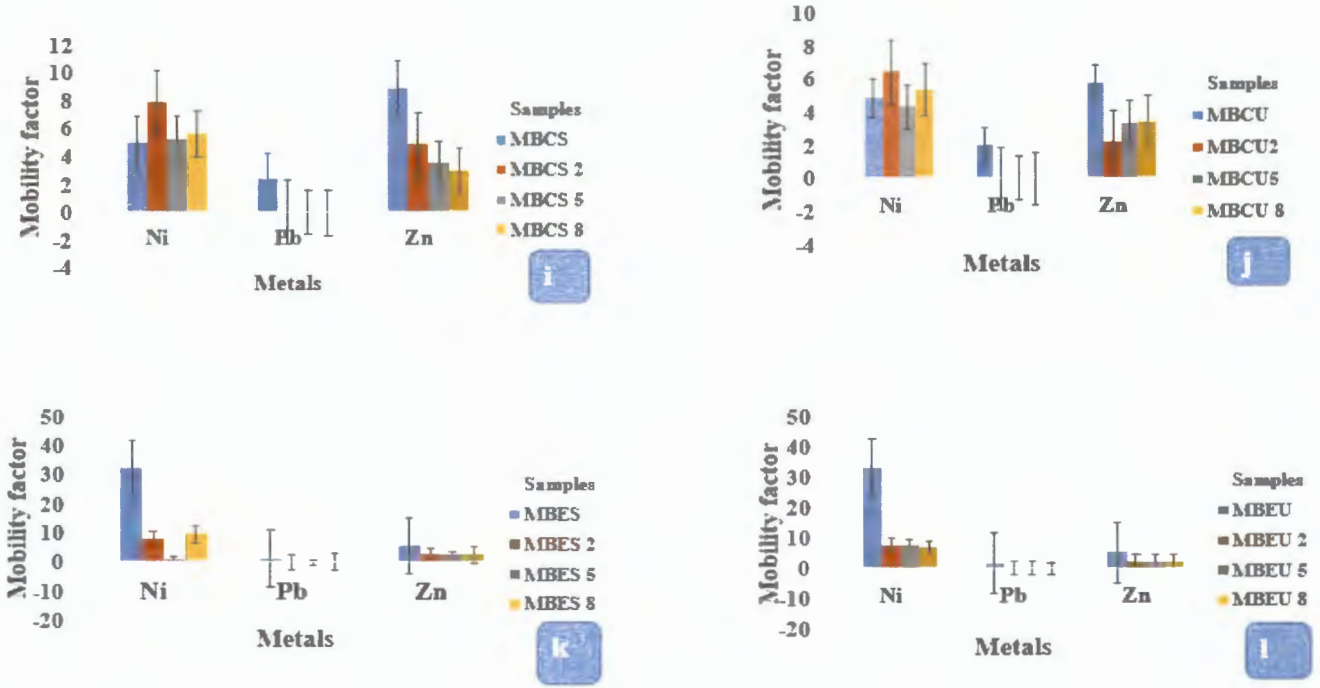


Figure 7.6: Mobility factor of Pb, Ni and Zn in sample MB (i –l)

MBCS: sterile tailings MB with metal C combination, MBCS 2, 5, 8: sterile tailings MB with metal C combination after 2, 5 and 8 weeks of bioremediation, MBCU : Unsterile tailing MB with metal C combination, MBCU 2, 5 and 8 : Unsterile tailing MB with metal C combination after 2, 5 and 8 weeks of bioremediation, MBES : sterile tailing MB with metal E combination, MBES 2, 5, 8 : Sterile tailing MB with metal E combination after 2, 5, and 8 weeks of bioremediation, MBEU : Unsterile tailings MB with metal E combination, MBEU 2,5 and 8 : Unsterile tailings MB with metal E combination after 2, 5 and 8 weeks of bioremediation

The mobility factor recorded for Pb in the third sampling site TS (Figure 7.7-7.8) was different from what was recorded in the first two sites (MA and MB) as shown in Figure 7.4-7.6.

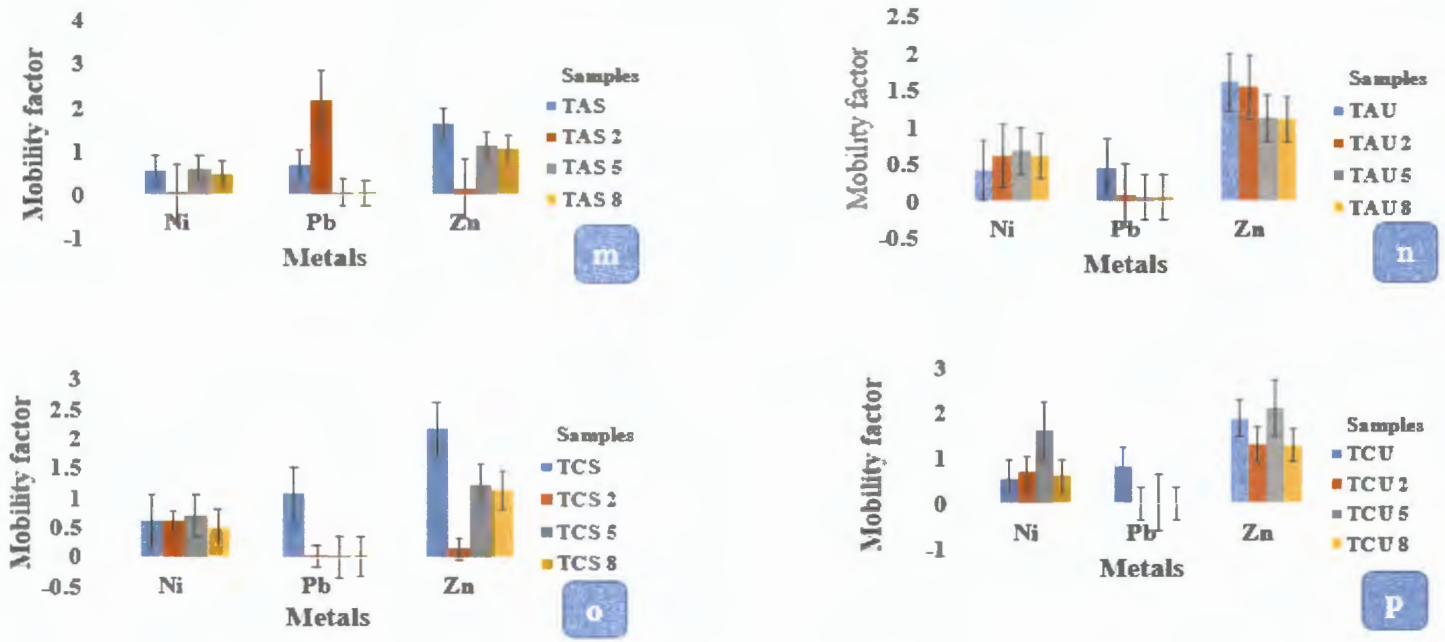


Figure 7.7: Mobility factor of Pb, Ni and Zn in sample TS (m-p) TAS: Sterile tailings TS with metal A combination, TAS 2, 5 and 8: Sterile tailings TS with metal A combination after 2, 5 and 8 weeks of bioremediation, TAU : Unsterile tailing TS with metal A combination, TAU 2, 5 and 8 : Unsterile tailing TS with metal A combination after 2, 5 and 8 weeks of bioremediation, TCS : sterile tailing TS with metal C combination, TCS 2, 5, 8 : sterile tailing TS with metal C combination after 2,5 and 8 weeks of bioremediation, TCU : Unsterile tailings TS with metal C combination, TCU 2, 5 and 8 : Unsterile tailings TS with metal C combination after 2, 5 and 8 weeks of bioremediation

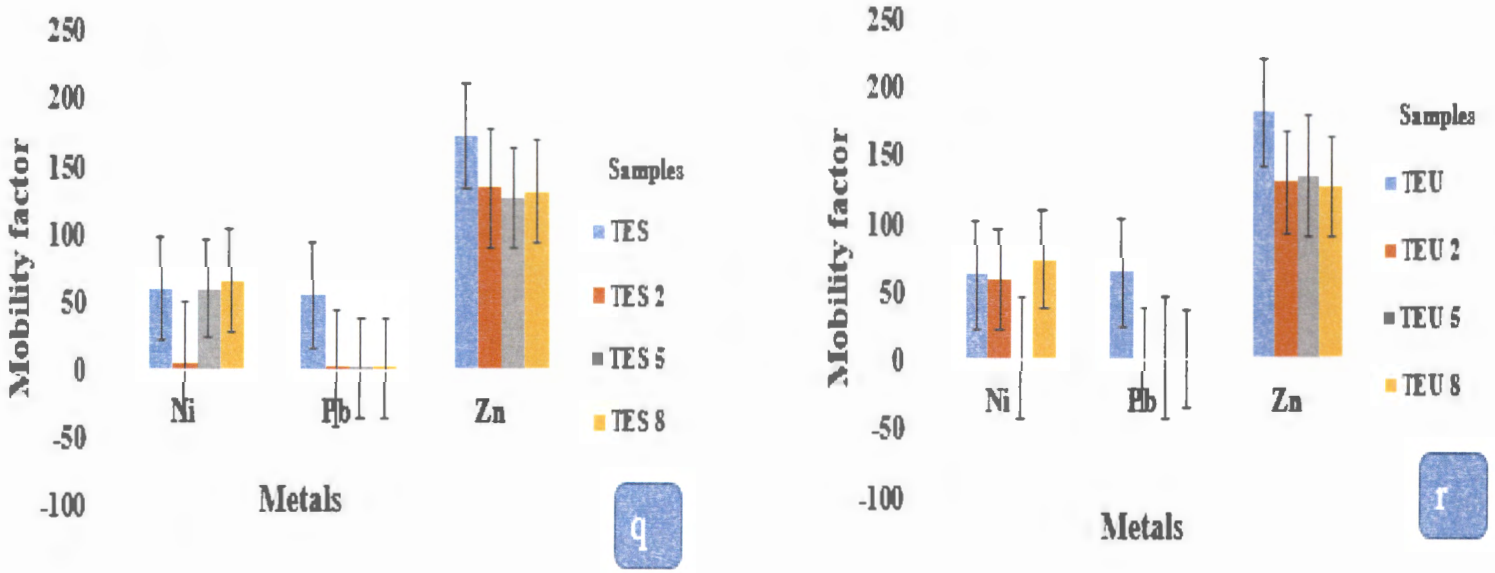


Figure 7.8: Mobility factor of Pb, Ni and Zn in sample TS (q-r)

TES: Sterile tailings TS with metal E combination, TES 2, 5 and 8: Sterile tailings TS with metal E combination after 2, 5 and 8 weeks of bioremediation, TEU : Unsterile tailing TS with metal E combination, TEU 2, 5 and 8 : Unsterile tailing TS with metal E combination after 2, 5 and 8 weeks of bioremediation

At the end of the treatment period, the bacteria were able to reduce the mobility factor to value ranging from 0.042- 0.68 (Figure 7.6). The mobility factor recorded in the un-inoculated control treatment E was very high (Figure 7.6 (i-l) but at the end of the treatment period, mobility factor of 0.5 and 0.68 was achieved in the unsterile and sterile sample respectively. The inoculated bacteria were able to reduce the Pb in both sterile and unsterile condition showing that they are capable of competing effectively in association with other indigenous bacteria present in the tailings and can also function alone. The higher the value of the mobility factor recorded, the greater the bioavailability of the metal and the potential toxicity of the metal in the tailings to the environment.

The mobility factor recorded for Ni in the control tailing MA and the different treatments were very high in the sterile and unsterile samples respectively. As the treatment period progressed in the bacteria inoculated tailings in sterile and unsterile condition, the mobility factor recorded slightly reduced as shown in Figure 7.4 (a-d) but the values recorded were still high indicating high bioavailability of Ni in the tailings which could be leached into the environment. The second sample MB also follows similar trend as sample MA as shown in Figure 7.5 (e-h) and the mobility factor recorded also shows high availability of Ni in the sample. In the third sampling site TS, the mobility factors recorded in the control were low in the treatment A and C and very high in E as shown in Figure 7.7-7.8. In the inoculated tailings, the mobility factor gradually reduced as the treatment progresses in the treatment A and C but for treatment E, the mobility factor recorded was very high as observed in the control in sterile and unsterile respectively (Figure 7.4 (a-d). The mobility factor of Zn was also observed to range from low to very high values. In sample MA, the mobility factor in the control were only low in treatment E in the sterile condition and A in the unsterile condition. At the end of the 8 week treatment period in the bacteria inoculated tailings, the mobility factor recorded was reduced as shown in Figure 7.4 (a-d) above. In sample MB, higher

mobility factor were recorded in treatment C and E in the sterile and unsterile respectively (Figure 7.5). At the end of the treatment period, the mobility factor was lower. The highest mobility factor for Zn was found in the samples from site TS, the values recorded in the control were high in treatment C in both sterile and unsterile conditions, as well as E in the unsterile condition. At the end of the treatment period, the mobility factor recorded was still very high in treatment E as shown in Figure 7.8 above.

According to Subida et al. (2013), mobility factor less than 1 indicates no pollution, 1 and 2 show light pollution and 2 and 3 indicate moderate anthropogenic pollution. The mobility factor recorded for Pb in all the sampling sites shows that Pb availability in the tailings pose no threat to the environment as a result of its reduction in fraction 1 which is the highly mobile fraction. Mobility factor for Ni was high in all the three sites showing similar values in the sample from site MA and MB while TS recorded the highest value 73.55 in the sterile and unsterile samples of treatment E. Zinc mobility factor was also found to follow the same pattern to what was recorded for Ni with the highest value of 131 recorded in the same treatment as Zn. This result shows that Zn and Ni are highly bioavailable in the environment which may pose a danger to the environment.

This study shows that the bioaugumentation of mine tailings with indigenous metal resistant bacteria hold promising future for bioremediation of toxic mine wastes. Bioaugumentation process is highly economical compared to conventional methods of treatment. Bacteria cultures are cheaper to cultivate and environmental factors affecting their growth can easily be monitored to enhance their performance. In addition, this technology can be apply directly at the site of contamination, thus preventing exposure of the clean-up personnel and spread of contaminants to other areas.

7.4 Conclusion

Gold mine tailing is an important source of environmental pollution with metal species.

This study shows the potential of indigenous *B. cereus* OMF 003 and *E. asburiae* OMF 532 isolated from abandoned gold mine tailings in immobilization of metals. The results shows that the two bacterial strains interact better with Pb than Ni and Zn in the tailings which could be due to many factors. There is no significant difference in the bioremediation efficiency of the bacterial strains in both sterile and unsterile conditions showing their potential to compete with other indigenous bacteria as well as their ability to function alone in the bioremediation process. High percentage reduction in mobility was observed for Pb in the exchangeable fraction whereas the concentration of Ni and Zn bound to the exchangeable fraction was still relatively higher. Greater concentration of the three metals was found in the reducible, oxidizable and residual fraction. The mobility factor recorded for the three metals shows that Pb bioavailability was low as compared to Ni and Zn after bioremediation. Further work should look at how to bio stimulate the activity of the inoculated bacterial strains, increase the incubation period and study the various environmental conditions that can affect the bacteria activity to improve their bioremediation efficiency.

CHAPTER EIGHT

8.0 CONCLUSIONS AND RECOMMENDATIONS

The present study provides evidence that abandoned mines are threat to agricultural soils, streams and residents living around mining environments. The physicochemical properties and metals contents of three abandoned gold mine tailings (MA MB and TS) and their surrounding soils (MAC, MBC and TSC) collected from Krugersdorp in Gauteng Province, South Africa were assessed. All the samples tested were acidic with reduced moisture contents and low nutrient status for survival of microbial species. The particle size are predominantly sand with high redox potential, low CEC values and high concentration of sulphate. Excessive levels of metal species such as As, Pb, Ni, Zn, and Co in the samples were well above recommended South African standards for soil and sediments.

A total of 65 metal resistant bacteria species were isolated from the sampled mine tailings. High resistance to Pb, Ni and Zn were observed in the isolated bacteria strains. Biochemical and molecular characterization shows that these bacteria belong to the phylum Proteobacteria, Firmicutes and Actinobacteria consisting of the genus *Bacillus, Enterococcus, Enterobacter*, *Arthrobacter* and *Alcaligenes* sp. Eight of the identified isolates: *E. faecalis* OM6 142, *B. thuringiensis* OMF 107, *E.aerogenes* OM4 274, *E.asburiae* OMF 532, *B.cereus* OM7 132, *Alcaligenes* sp OMF 008, *B. cereus* OMF 003 did not cluster with other bacterial strains on the phylogenetic tree drawn, which shows that these bacterial strains could be probable novel metal resistant bacteria based on their distinctness. Physiological characterization of the bacteria shows that the bacterial isolates have the ability to survive at optimum temperature of 37°C, pH of 5.0 and 7.0 and NaCl concentration of 2-4% (w/v). Metal resistant genes of the bacterial isolates were located on the plasmid. Only the nccA genes conferring resistance to nickel was detected in two of the bacterial isolates while

resistance to Pb and Zn could not be detected with the primers *P3 P4, PbrT* and *Czc ABD* used. This implies that these bacterial isolates used other mechanisms of resistance to Pb and Zn aside from the one the primer sets are designed for, and this will be pursued further in future studies.

Bioaccumulation of Pb, Ni and Zn in ternary mixtures by the two highly metal resistant isolates *E. asburiae* OMF 532 and *B. cereus* OMF 003 shows increasing uptake of the three metals at concentration of 3 mM, 48 h preculture time, temperature of 37°C and pH of 5 and 7. Bacteria consortium was observed to bioaccumulate more metal species as compared to individual specie. Highest bioaccumulation rate was observed for Zn followed by Pb while Ni recorded the lowest bioaccumulation rate.

The two bacterial strains *B. cereus* OMF 003 and *E. asburiae* OMF 532 tested in field moist-microcosms show their suitability in bioremediation of metal species. The two isolates were able to reduce bioavailable fraction of Pb. Selective sequential extraction of the bioaugmented tailings shows that the bacteria have a mutualistic relationship with each other as well as with other indigenous bacteria present in the mine tailings. Better interaction was observed for Pb compared to Ni and Zn and the isolates were able to reduce the mobile fraction of Pb.

Higher percentage of the three metals were bound to the reducible, oxidizable and residual fraction. High mobility factor were recorded for Ni and Zn in the TS which shows high availability and mobility of these metals in the environment.

This study to the best of my knowledge provide the first report in South Africa on the potential of two indigenous bacterial strains isolated from abandoned gold mine tailings in Krugersdorp that can be used as microbial inoculants in bioremediation of mine tailings.

Recommendations

The sampled sites need to be remediated urgently and measures should be taking to prevent water and wind erosion of the tailings as this will lead to further dispersal of metal species in the environment.

Soil amendments should be added to the tailings to improve the organic content of the mine tailings which will improve the microbial activities present in the tailings and also reduce the mobility of metals in the soil since most of the metals segregated into the organic matter rich oxidizable fraction of the soil.

More funding and support should be provided by South Africa government to encourage implementation of bioremediation technology on a large scale as this hold promising future for the treatment of the enormous mine wastes generated by the country.

There is need for collaboration between environmental engineers, geochemists and microbiologists to bring this promising technology to fruitfulness

Recommendations for future work

More studies are needed to develop and improve future applications of metal resistant bacteria for cleaning up polluted environments. There is need to further determine how the physicochemical properties of the tailings influence bioavailability and mobility of metal species in the tailings and how these physicochemical properties influence microbial activities present in the mine tailings. Interactions between various metals need to be studied as well as this affect their uptake.

Further study to evaluate the abandoned mine tailings of Krugersdorp as a reservoir of metal resistant bacteria or metal resistance/tolerance genes is needed. Genetic determinants conferring resistance to several other metals must be screened. Culture independent method should be combined with the conventional culture techniques to screen for the diversities and

functionalities of the metal resistant species. Novel culturing techniques and media need to be used to obtain a greater diversity of culturable bacteria and their genetic make-up. The link between diversities and metabolic activities and pathways of metabolic activities involved in biomineralization of the metals need to be established. Appropriate primers need to be designed to target the metal resistant genes and also further characterization of plasmid to ascertain their role in metal resistant. Further characterization may also provide new genetic tools for future analysis of metal tolerance mechanisms in bacteria.

The presence of plant growth promoting traits by metal resistant bacteria need to be studied as this play an important role in soil remediation strategies. Factors affecting proliferation of bacteria used for bioaugmentation such as the chemical structure and concentration of pollutants, the availability of the contaminant to the bacteria, the size and nature of the microbial population and the physical environment need to be further study for effective application of the technology. Moreso, a suitable organic amendment needs to be sourced to increase the organic matter content of the mine tailings due to their deficiency in nutrients which limits bacteria activities. Interaction of this organic amendment with the bacteria activities is also equally important.

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APPENDICES

Appendix 1: Descriptive statistics of the physicochemical parameters of the samples

Sampling Sites	Descriptive statistics	pH	EC (Ms/m)	Redox (mv)	Moisture content (%)	Organic matter (%)	CEC meq/100	NO_32- (mg/kg)	S04 (mg/Kg)	Nitrogen (%)	Carbon (%)	Sulphur (%)
MA	Mean	2.69	4.59	301.00	3.88	0.37	15.84	0.91	184.25	0.03	0.05	0.36
	STD	0.68	4.81	53.50	5.51	0.16	8.95	0.28	232.14	0.01	0.23	0.34
	Min	2.10	0.46	268.00	0.60	0.16	2.42	0.59	7.12	<0.01	<0.05	0.06
	Max	3.50	11.52	380.00	12.07	0.50	20.83	1.27	502.57	0.10	0.40	0.72
	Range	1.40	11.06	112.00	11.47	0.34	18.41	0.68	495.45	0.12	0.46	0.66
MAC	Mean	5.09	0.09	162.50	1.53	1.13	21.10	2.95	9.19	0.15	1.74	0.03
	STD	0.73	0.11	24.34	1.32	1.16	2.39	2.97	6.41	0.10	1.64	0.02
	Min	4.40	0.02	136.00	0.60	0.17	18.07	0.60	0.23	0.05	0.83	0.02
	Max	5.80	0.26	195.00	3.39	2.81	23.90	7.16	15.43	0.29	4.19	0.06
	Range	1.30	0.24	59.00	2.79	2.64	5.83	6.56	15.20	0.24	3.37	0.05
MB	Mean	3.37	2.49	209.75	2.46	0.89	20.31	0.82	152.64	0.01	0.29	0.04
	STD	1.42	2.21	92.69	3.21	0.76	0.56	0.85	206.03	0.04	0.55	0.02
	Min	2.20	0.06	71.00	0.40	0.06	19.84	0.29	11.66	0.03	<0.05	0.02
	Max	5.20	5.33	264.00	7.23	1.88	21.09	2.08	450.46	0.04	1.12	0.07
	Range	3.10	5.27	193.00	6.83	1.82	1.24	1.79	438.80	0.07	1.15	0.05
MBC	Mean	5.34	0.20	126.50	1.91	0.59	13.93	3.58	3.72	0.11	0.74	0.20
	STD	0.99	0.21	15.02	2.87	0.62	9.17	4.63	3.31	0.13	0.54	0.36
	Min	4.70	0.04	113.00	0.20	0.15	5.98	0.75	1.08	<0.01	<0.05	0.02
	Max	6.80	0.49	147.00	6.20	1.51	23.52	10.46	8.54	0.29	1.16	0.75
	Range	2.10	0.45	34.00	6.00	1.36	17.54	9.71	7.46	0.31	1.19	0.73
TS	Mean	4.05	4.07	167.75	6.61	0.15	10.07	34.87	296.62	0.13	0.48	2.85
	STD	0.29	3.28	19.36	3.47	0.05	8.25	49.44	239.72	0.08	0.18	0.41
	Min	3.70	1.97	148.00	3.33	0.08	5.90	2.41	15.16	0.04	0.23	2.29
	Max	4.40	8.88	194.00	10.38	0.19	22.43	108.50	523.35	0.22	0.65	3.22
	Range	0.70	6.91	46.00	7.05	0.11	16.53	106.09	508.19	0.18	0.42	0.93
TSC	Mean	6.34	0.73	104.25	4.22	0.45	13.65	33.16	17.77	0.14	1.61	0.26
	STD	0.90	0.45	112.81	2.57	0.53	9.94	51.84	11.88	0.13	1.14	0.16
	Min	5.30	0.29	15.00	1.01	0.11	5.09	5.91	6.40	0.04	0.82	0.04
	Max	7.50	1.15	269.00	6.56	1.24	23.44	110.90	32.44	0.33	3.27	0.39
	Range	2.20	0.86	254.00	5.55	1.13	18.36	104.99	26.04	0.29	2.45	0.35

Appendix 2: Descriptive statistics of metals content

Sampling sites	Descriptive statistics	Arsenic	Cadmium	Cobalt	Nickel	Lead	Zinc
MA	Mean	< 4	< 1	9.17	17.37	16.46	61.11
	STD	322.20	1.06	10.87	20.16	5.70	36.84
	Min	< 4	< 1	-0.44	0.03	8.34	22.17
	Max	< 4	< 1	20.97	40.88	21.66	103.60
	Range	759.80	2.48	21.41	40.85	13.32	81.43
MAC	Mean	< 4	< 1	2.57	13.44	20.82	102.16
	STD	296.55	1.32	1.71	10.71	12.51	111.30
	Min	< 4	< 1	0.28	0.01	5.62	14.65
	Max	< 4	< 1	4.16	22.14	36.25	260.70
	Range	706.20	2.78	3.88	22.13	30.63	246.05
MB	Mean	< 4	< 1	10.87	24.33	20.47	69.01
	STD	2790.60	3.95	11.52	22.54	4.45	36.67
	Min	< 4	< 1	0.83	3.52	14.78	21.82
	Max	< 4	3.30	22.07	50.06	25.06	110.04
	Range	5642.80	9.10	21.24	46.54	10.28	88.22
MBC	Mean	< 4	< 1	5.09	26.68	29.47	155.61
	STD	395.75	0.87	2.35	15.77	17.03	68.30
	Min	< 4	< 1	1.63	3.17	7.21	53.45
	Max	< 4	< 1	6.57	36.90	48.69	195.70
	Range	928.30	2.05	4.94	33.73	41.48	142.25
TS	Mean	261.65	6.61	490.09	2247.00	46.11	2555.43
	STD	534.19	16.01	616.50	1616.32	14.59	2372.40
	Min	< 4	< 1	55.55	1150.00	32.97	628.70
	Max	1003.00	30.06	1400.00	4651.00	59.96	5989.00
	Range	1231.30	35.09	1344.45	3501.00	26.99	5360.30
TSC	Mean	612.50	5.54	856.60	2786.75	136.79	4269.25
	STD	139.56	3.13	465.04	944.92	170.26	1123.41
	Min	421.20	2.27	312.50	1975.00	29.08	2711.00
	Max	737.40	9.73	1299.00	3791.00	391.00	5193.00
	Range	316.20	7.46	986.50	1816.00	361.92	2482.00

Appendix 3 : Analysis of variance table of the sampling sites showing physicochemical parameter and metal contents

			Sum of Squares	df	Mean Square	F	Sig.
pH * SS	Between Groups	(Combined)	36.779	5	7.356	9.058	.000
	Within Groups		14.618	18	.812		
	Total		51.397	23			
EC * SS	Between Groups	(Combined)	78.760	5	15.752	2.418	.076
	Within Groups		117.250	18	6.514		
	Total		196.010	23			
Redox * SS	Between Groups	(Combined)	98285.375	5	19657.075	4.648	.007
	Within Groups		76118.250	18	4228.792		
	Total		174403.625	23			
Moisture content * SS	Between Groups	(Combined)	71.185	5	14.237	1.233	.334
	Within Groups		207.772	18	11.543		
	Total		278.957	23			
Organic matter * SS	Between Groups	(Combined)	2.578	5	.516	1.177	.359
	Within Groups		7.886	18	.438		
	Total		10.464	23			
Cation exchange capacity * SS	Between Groups	(Combined)	357.639	5	71.528	1.273	.318
	Within Groups		1010.998	18	56.167		
	Total		1368.637	23			
Nitrate * SS	Between Groups	(Combined)	5473.675	5	1094.735	1.272	.318
	Within Groups		15489.463	18	860.526		
	Total		20963.138	23			
Sulphate * SS	Between Groups	(Combined)	288484.524	5	57696.905	2.248	.094
	Within Groups		461978.435	18	25665.469		
	Total		750462.959	23			
Nitrogen * SS	Between Groups	(Combined)	.078	5	.016	1.670	.193
	Within Groups		.167	18	.009		
	Total		.245	23			
Carbon * SS	Between Groups	(Combined)	9.865	5	1.973	2.536	.066
	Within Groups		14.004	18	.778		
	Total		23.869	23			
Sulphur * SS	Between Groups	(Combined)	24.122	5	4.824	64.635	.000
	Within Groups		1.344	18	.075		
	Total		25.466	23			
Arsenic * SS	Between Groups	(Combined)	21394024.026	5	4278804.805	3.042	.037
	Within Groups		25321925.682	18	1406773.649		
	Total		46715949.708	23			
Cadmium * SS	Between Groups	(Combined)	569.926	5	113.985	2.396	.078
	Within Groups		856.418	18	47.579		
	Total		1426.344	23			
Cobalt * SS	Between Groups	(Combined)	2637451.118	5	527490.224	5.305	.004
	Within Groups		1789775.567	18	99431.976		
	Total		4427226.685	23			
Nickel * SS	Between Groups	(Combined)	33821111.925	5	6764222.385	11.574	.000

	Within Groups		10519967.133	18	584442.618		
	Total		44341079.058	23			
Lead * SS	Between Groups	(Combined)	42676.490	5	8535.298	1.724	.180
	Within Groups		89097.444	18	4949.858		
	Total		131773.935	23			
Zinc * SS	Between Groups	(Combined)	64518701.731	5	12903740.346	11.204	.000
	Within Groups		20730245.630	18	1151680.313		
	Total		85248947.362	23			

Appendix 4 : Correlation analysis of physicochemical parameters and metal species

	pH	EC	Redox	MC	OM	CEC	NO3	SO4	N	C	S	As	Cd	Co	Ni	Pb	Zn
pH	1																
EC	-.548**	1															
	.006																
Redox	-.696**	.291	1														
	.000	.168															
MC	-.014	.082	.099	1													
	.948	.704	.644														
OM	.211	-.176	-.185	-.368	1												
	.322	.412	.386	.077													
CEC	-.264	-.010	.003	-.548**	.180	1											
	.213	.962	.990	.006	.399												
NO3	.177	-.064	.091	.196	-.216	-.432*	1										
	.408	.766	.671	.358	.312	.035											
SO4	-.516**	.589**	.278	.084	-.218	.094	.024	1									
	.010	.002	.188	.695	.306	.663	.911										
N	.471*	-.351	-.325	-.087	.148	.037	-.006	-.080	1								
	.020	.093	.122	.686	.491	.864	.979	.712									
C	.366	-.227	-.443*	-.147	-.145	.161	-.012	-.182	.283	1							
	.078	.285	.030	.494	.500	.452	.955	.395	.180								
S	-.161	.426*	-.067	.436*	-.357	-.276	.254	.532**	.061	-.171	1						
	.451	.038	.756	.033	.087	.192	.231	.007	.778	.424							
As	.373	.074	-.319	.246	-.293	-.269	.398	.104	.250	.229	.349	1					
	.072	.730	.129	.247	.165	.204	.054	.630	.239	.282	.094						
Cd	.175	.021	-.200	.120	-.111	-.269	.816**	.069	.057	.105	.336	.432*	1				
	.414	.921	.348	.576	.604	.203	.000	.750	.790	.627	.108	.035					
Co	.408*	-.068	-.234	.260	-.281	-.444*	.818**	-.037	.048	.183	.241	.534**	.861**	1			
	.048	.753	.272	.221	.183	.030	.000	.864	.823	.393	.257	.007	.000				
Ni	.371	-.003	-.273	.311	-.298	-.458*	.794**	.039	.148	.144	.423*	.592**	.863**	.964**	1		
	.075	.990	.197	.138	.157	.024	.000	.856	.489	.501	.040	.002	.000	.000			
Pb	.165	-.138	-.298	-.052	.072	.161	.104	-.088	.488*	.098	-.014	.276	.208	.188	.326	1	
	.442	.520	.158	.810	.738	.453	.628	.684	.016	.649	.947	.192	.329	.379	.120		
Zn	.435*	-.070	-.329	.274	-.289	-.376	.747**	-.027	.164	.244	.299	.582**	.848**	.974**	.976**	.325	1
	.033	.747	.117	.196	.171	.070	.000	.899	.444	.251	.155	.003	.000	.000	.000	.121	

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Appendix 5: Colonial and cellular morphology of the bacterial isolates

Isolates Code	Color	Size	Shape	Margin	Elevation	Cell shape	Gram reaction
OMF-012	Cream	Large	Circular	Entire	Flat	Rod	+ve
OMF-811	LB	Large	Circular	Entire	Raised	Rod	-ve
OMF-812	Cream	Small	Circular	Entire	Flat	Rod	-ve
OMF-813	Cream	Small	Circular	Entire	Flat	Rod	+ve
OMF-814	Yellow	Small	Circular	Entire	Raised	Cocci	+ve
OMF-815	Cream	Large	Filamentous	Irregular	Flat	Rod	+ve
OMF- 816	Cream	Large	Circular	Entire	Raised	Rod	+ve
OM6-142	Cream	Large	Circular	Entire	Flat	Cocci	+ve
OMF-810	Cream	Large	Filamentous	Irregular	Raised	Rod	+ve
OM8-321	Cream	Large	Filamentous	Entire	Flat	Rod	+ve
OMF-001	Cream	Small	Circular	Entire	Flat	Rod	+ve
OMF-809	Cream	Large	Circular	Entire	Flat	Rod	-ve
OM4-274	Cream	Small	Circular	Entire	Flat	Rod	+ve
OMF-808	Cream	Large	Irregular	Entire	Raised	Rod	+ve
OMF-807	Cream	Large	Circular	Entire	Flat	Rod	+ve
OMF-107	Orange	Small	Irregular	Entire	Flat	Rod	+ve
OMF-806	Cream	Large	Irregular	Entire	Raised	Rod	+ve
OMF-805	Brown	Large	Circular	Entire	Flat	Rod	-ve
OMF-804	Brown	Tiny	Circular	Entire	Raised	Cocci	+ve
OMF-532	LB	Large	Circular	Entire	Raised	Rod	-ve
OMF-008	Cream	Small	Circular	Entire	Flat	Rod	+ve
OM7-132	LB	Large	Circular	Serrated	Flat	Rod	-ve
OMF-003	Yellow	Large	Circular	Entire	Flat	Rod	+ve
OMF-803	Cream	Large	Irregular	Entire	Raised	Rod	+ve
OMF-802	Cream	Small	Circular	Entire	Raised	Rod	+ve
OMF-801	Cream	Small	Circular	Entire	Raised	Rod	+ve
OMF-800	Cream	Large	Circular	Entire	Raised	Rod	+ve
OMF-005	Yellow	Small	Circular	Entire	Raised	Cocci	+ve

LB: Light Brown

Appendix 6 : Multivariate Tests for Nickel

Multivariate Tests

	Value	F	Hypothesis df	Error df	Sig.
Pillai's trace	1.579	1.956	68.000	204.000	0.000
Wilks' lambda	0.051	3.190	68.000	190.684	0.000
Hotelling's trace	8.530	5.833	68.000	186.000	0.000
Roy's largest root	7.496	22.487a	17.000	51.000	0.000

Each F tests the multivariate effect of Sample. These tests are based on the linearly independent pairwise comparisons among the estimated marginal means.

Appendix 6 : Univariate tests for Zn showing significant lower differences in Zn concentration in the F1 fraction

Dependent Variable		Sum of Squares	df	Mean Square	F	Sig.
Zn1	Contrast	13497876.80	17	793992.753	24.349	0.000
	Error	1728289.297	53	32609.232		
Zn2	Contrast	14955620.46	17	879742.380	0.950	0.524
	Error	49083204.77	53	926098.203		
Zn3	Contrast	848339.021	17	49902.295	49.714	0.000
	Error	53200.388	53	1003.781		
Zn4	Contrast	2234018.914	17	131412.877	47.614	0.000
	Error	146277.556	53	2759.954		

The F tests the effect of sample. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Zn 1: fraction 1, Zn 2 : fraction 2, Zn 3: fraction 3, Zn 4 : fraction 4

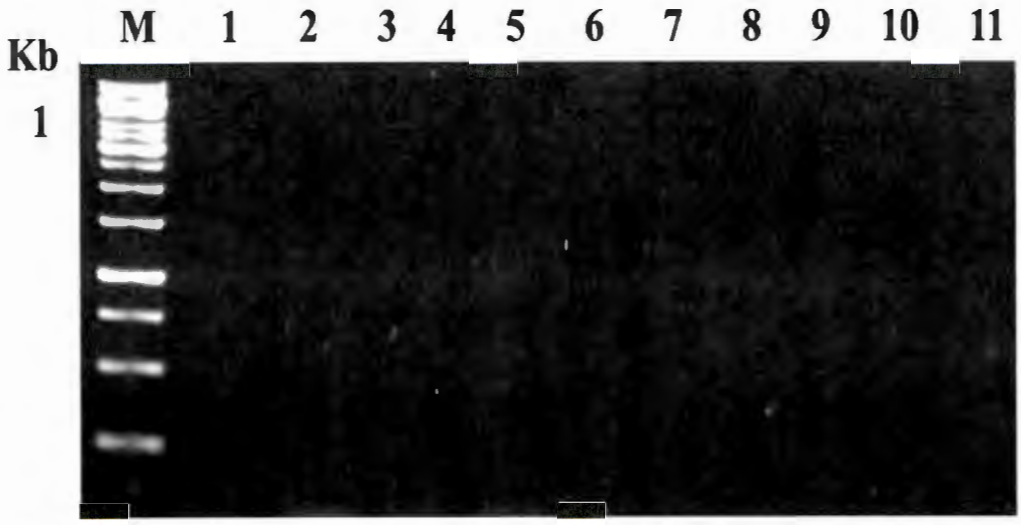
Dependent Variable	(I) week	(J) week	Mean Difference (I-J)	Std. Error	Sig.b	95% Confidence Lower Bound
	2	0	-36.904*	16.473	0.028	-69.785
		5	-7.343	16.473	0.657	-40.224
		8	-3.668	16.714	0.827	-37.029
	5	0	-29.561	16.473	0.077	-62.441
		2	7.343	16.473	0.657	-25.537
		8	3.675	16.714	0.827	-29.685
	8	0	-33.236	16.714	0.051	-66.597
		2	3.668	16.714	0.827	-29.693
		5	-3.675	16.714	0.827	-37.036
Pb3	0	2	-40.636*	16.560	0.017	-73.690
		5	-62.139*	16.560	0.000	-95.193
		8	-50.847*	16.802	0.004	-84.384
	2	0	40.636*	16.560	0.017	7.581
		5	-21.503	16.560	0.199	-54.557
		8	-10.211	16.802	0.545	-43.748
	5	0	62.139*	16.560	0.000	29.084
		2	21.503	16.560	0.199	-11.552
		8	11.292	16.802	0.504	-22.245
	8	0	50.847*	16.802	0.004	17.310
		2	10.211	16.802	0.545	-23.326
		5	-11.292	16.802	0.504	-44.829
Pb4	0	2	-169.686*	39.251	0.000	-248.031
		5	-206.991*	39.251	0.000	-285.336
		8	-188.826*	39.824	0.000	-268.315
	2	0	169.686*	39.251	0.000	91.341
		5	-37.304	39.251	0.345	-115.649
		8	-19.140	39.824	0.632	-98.629
	5	0	206.991*	39.251	0.000	128.646
		2	37.304	39.251	0.345	-41.041
		8	18.164	39.824	0.650	-61.325
	8	0	188.826*	39.824	0.000	109.337
		2	19.140	39.824	0.632	-60.349
		5	-18.164	39.824	0.650	-97.653

Appendix 7: pH of the bioremediated tailings

Sample	EC	pH	(Week)						
		1	2	3	4	5	6	7	8
MAA	Unsterile	7.10	7.21	7.27	7.34	7.35	7.41	7.46	7.88
MAC		7.00	6.87	7.10	7.15	6.92	7.11	6.98	7.33
MAE		6.98	6.86	7.26	7.33	7.02	7.21	7.04	7.48
MAA	Sterile	7.34	7.17	7.45	7.62	7.26	7.25	7.39	7.83
MAC		6.98	6.85	7.32	7.44	7.14	7.13	7.24	7.67
MAE		7.04	6.96	7.37	7.29	7.17	7.32	7.27	7.65
MBA	Unsterile	6.94	7.15	7.32	7.33	6.98	7.09	7.10	7.53
MBC		6.82	7.21	7.24	7.27	6.98	6.81	7.13	7.40
MBE		6.96	6.94	7.05	7.27	6.82	7.03	6.89	7.32
MBA	Sterile	7.05	6.94	7.26	7.33	7.01	7.09	7.10	7.53
MBC		6.98	6.85	7.25	7.35	6.99	7.13	7.06	7.38
MBE		7.05	6.94	7.26	7.33	7.01	7.12	7.16	7.52
TSA	Unsterile	5.31	5.04	4.91	5.25	5.04	5.24	5.25	5.36
TSC		5.35	5.13	5.05	5.23	5.06	5.25	5.29	5.42
TSE		5.29	5.07	5.13	5.27	5.05	5.20	5.18	5.32
TSA	Sterile	5.21	4.94	4.91	5.24	4.97	5.18	5.23	5.29
TSC		5.26	4.93	4.85	5.25	5.02	5.18	5.36	5.29
TSE		5.30	4.97	5.01	5.20	5.23	5.19	5.30	5.29

EC: Experimental condition

Appendix 8:



Agarose gel photograph showing non-amplification for chromosomal metal resistant genes. M= 1Kb molecular weight marker. Line 1: OMF 001, Line 2: OMF 003, Line 3: OMF 532, Line 4: OM6 142, Line 5: OMF 810 Line 6: OMF 0MF 003, Line 7: OMF 811, Line 8: OMF 274, Line 9: OMF 321, Line 10: OMF 132, Line 11: OMF 008.