Microbial diversity and metal pollution from a platinum mine tailings dam in the North-West Province (RSA)

by

Molemi Evelyn Rauwane

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Faculty of Natural Science

North-West University (Potchefstroom Campus)

Supervisor

Prof. M.S. Maboeta

Co-Supervisor

Prof. C.C. Bezuidenhout

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DECLARATION

I declare that, this dissertation for the Degree of Master of Science (Microbiology) at the North-West University (Potchefstroom Campus) hereby submitted, has not been submitted by me for a degree at this or another University, that it is my own work in design and execution, and that all material contained herein has been duly acknowledged.

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Molemi Evelyn Rauwane Date

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But thanks be to God, who gives us the

victory (making us conquerors) through

our Lord Jesus Christ, I Corinthians 15:57

ABSTRACT

The aim of this study was to determine the effects of the heavy metal pollution on microbial diversity along the gradient from a platinum mine tailings dam using culture-dependent (plating methods) and molecular methods. Tailings and soil samples were collected from seven sites (6 samples per site) at increasing distances from the tailings dam. Samples were collected over a two year period and included two rainy and two dry periods. Concentrations of various heavy metals were determined using an inductively coupled plasma mass spectrometer (ICP-MS). The results demonstrated that seasonal variations in metal concentrations occurred and also that concentrations were significantly different ($P < 0.05$) between the experimental sites for each metal. The relative relationship between metals was in the following order: Al > Ni > Cu > Cr. Since soil metal concentration benchmarks for South Africa are lacking, the concentrations were compared to the Canadian microbial benchmarks (MB) and Netherlands maximum permissible concentrations (MPC). Concentrations of most of the heavy metals exceeded the MB and MPC. Levels and diversity of culturable fungi and bacteria at each site were determined using plate count methods. Results indicated that levels of bacteria and fungi were not suppressed by high concentrations of heavy metals. Significantly higher levels ($P < 0.05$) of fungi were found at the sites on the tailings dam (higher concentrations of heavy metals), compared to sites more than 300 m away. A commonly used soil health index (Shannon-Weaver diversity index) was used to compare microbial community diversity at each site and to evaluate whether or not the heavy metal contamination impacted negatively on these soil bacterial and fungal communities. Shannon-Weaver diversity indices were higher at sites on and close to the tailings dam than sites more than 300 m away. However, ratio of fungal to bacterial levels as determined by plate counts was inconsistent. Representatives of bacterial species that were grouped using colony morphology and whole cell protein profiles were identified by 16S rDNA sequences as *Bacillus barbaricus* (*B. barbaricus*) and *Paenibacillus lautus* (*P. Lautus*). Restriction enzyme digest, SDS-PAGE and random amplified polymorphic DNA (RAPD) analyses provided supporting evidence that representatives were correctly grouped. Cluster analysis results demonstrated that the RAPD profiles of the metal

tolerant *P. lautus* representatives were sufficiently dissimilar to discriminate between individuals from the spatially separated sites. The spatially separated sites also represented sites with high and low heavy metal concentrations. Observed genetic variability was thus also associated with varying levels of heavy metals. In conclusion, this study demonstrated the potential of using RAPD analysis as biomarkers for genotoxic effects of heavy metals on bacterial genomes.

Keywords: Heavy metals, microbial diversity, *B. barbaricus*, *P. lautus*, Shannon-Weaver diversity indices, 16S rDNA sequences, RFLP, SDS-PAGE, RAPDs

OPSOMMING

Die doel van hierdie studie was om die effekte van swaarmetaalbesoedeling op mikrobiese diversiteit te bepaal langs 'n gradiënt vanaf 'n platinummyn slikdam deur gebruik te maak van kultuurafhanklike (plateringsmetodes) en molekulêre metodes. Slik- en grondmonsters is vanaf sewe persele (6 monsters per perseel) versamel teen toenemende afstande vanaf die slikdam. Monsters is oor 'n periode van twee jaar versamel, wat twee reën- en twee droë seisoene ingesluit het. Die konsentrasies van metale is bepaal deur van 'n induktief gekoppelde plasmamassaspektrometer ("ICP-MS") gebruik te maak. Resultate het getoon dat daar seisoenale variasies in metaalkonsentrasies was en dat konsentrasies betekenisvol verskil ($P < 0.05$) het tussen die eksperimentele persele vir elke metaal. Die relatiewe verhouding tussen metale was in die volgende volgorde: Al > Ni > Cu > Cr. Aangesien daar geen standaarde bestaan ten opsigte van konsentrasies vir Suid Afrikaanse grond nie, is gemete konsentrasies vergelyk met die Kanadese mikrobiese standaardwaardes (MS) en Nederlandse maksimum toelaatbare konsentrasies (MTK). Meeste van die swaarmetaalkonsentrasies het die MS en MTK oorskry. Die vlakke en diversiteit van kweekbare fungi en bakterië by elke perseel is bepaal deur gebruik te maak van plaattellingsmetodes. Die vlakke van die bakterieë en fungi nie negatief beïnvloed is deur hoë swaarmetaalkonsentrasies nie en betekenisvolle hoër vlakke ($P < 0.05$) fungi is op die slikdam gevind, in vergelyking met die persele 300 m en verder weg. 'n Algemene grondgesondheidsindeks (Shannon-Weaver diversiteit indeks) is gebruik om die mikrobiese gemeenskapsdiversiteit tussen persele te vergelyk en te evalueer of swaarmetaalkontaminasie 'n negatiewe impak op bakterie- en fungi gemeenskappe gehad het. Die Shannon-Weaver diversiteit indekse was hoër op en naby aan die slikdam in vergelyking met persele meer as 300 m weg. Die vehouding van fungi tot bakterieë bepaal met behulp van plaattellings metode was egter inkonsekwent. Verteenwoordigers van bakteriële spesies wat gegroepeer is deur gebruik te maak van kolonie morfologie en heelsel proteïen profiele is geïdentifiseer as *Bacillus barbaricus* (*B. barbaricus*) en *Paenibacillus lautus* (*P. Lautus*) met behulp van 16S rDNA volgordes. Restriksie ensiem vertering -, SDS-PAGE - en "random amplified polymorphic" DNA (RAPD) analises het ondersteunende bewyse gelewer dat

die verteenwoordigers korrek gegroepeer is. Groepsanalitiese resultate het duidelik gedemonstreer dat die RAPD profiele van die metaaltolerante *P. Lautus* verteenwoordigers voldoende verskillend was om te onderskei tussen individue van die ruimtelik geskeide gebiede. Hierdie gebiede verteenwoordig ook gebiede met hoë en lae swaarmetaalkonsentrasies. Waargenome genetiese variasie was dus ook geassosieer met wisselende vlakke van swaarmetale. Ten slotte het hierdie studie het die potensiaal om RAPD analises as biomerkers vir genotoksiese effekte van swaarmetale op bakteriële genome gedemonstreer.

Sleutelwoorde: Swaarmetale, mikrobiese diversiteit, *B. barbaricus*, *P. lautus*, Shannon-Weaver diversiteitsindeks, 16S rDNA volgordes, RFLP, SDS-PAGE, RAPDs

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CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION AND PROBLEM STATEMENT

Soils are heterogeneous and complex habitats consisting of inorganic minerals, organic matter and living biota, supporting a tremendous microbial diversity (Ranjard *et al.*, 2000a). Microorganisms mediate soil processes important to soil quality, such as regulating organic matter decomposition and nutrient availability, initiating and maintaining soil structure (Johnson *et al.*, 2003; Crecchio *et al.*, 2004). Although soils are regarded as the ultimate sink for heavy metals discharged into the environment, relatively little is known about the way that heavy metals are bound to soils and the ease with which they may be released (Banat *et al.*, 2005). Field studies of metal contaminated soils have demonstrated that elevated metal loadings can result in decreased microbial community size and decreases in activities such as organic matter mineralization and leaf litter decomposition (Konoopka *et al.*, 1999; Kelly *et al.*, 2003).

Bacteria and fungi are the main constituents of soil microbial biomass and both play a role in the decomposition of organic material except specific members, such as mycorrhizal fungi and nitrifying bacteria (Bååth and Anderson, 2003). Since fungi and bacteria have different carbon (C) and nitrogen (N) requirements, variations in their relative biomass will affect the C:N ratio of the whole microbial biomass. This is considered important in explaining different nitrogen mineralization processes (Bååth and Anderson, 2003). Information with reference to microbial diversity in soil is incomplete, since both traditional plating and microscopic techniques developed have important limitations (Kozdroj and van Elsas, 2001a). It has been suggested that at least 99% of bacteria observed under the microscope cannot be cultured by common laboratory techniques (Torsvik *et al.*, 1998). This may be because the unculturable bacterial species are simply in a physiological state that eludes the ability to culture them (Torsvik *et al.*, 1998; Robe *et al.*, 2003). However, if the aim is to investigate impacts of heavy metals on the genome of microbes, then traditional plating methods may be more suitable than culture-independent methods.

Tailings and wastewater are large-volume wastes produced in the mining industry, and may contain a variety of contaminants posing possible environmental impacts. They may cause pollution on the surrounding environment and physiological impacts on animals and plants and DNA damage (Nadig *et al.*, 1998; Liu *et al.*, 2005). Methods to assess DNA damage include non-specific techniques such as the comet assay (Angelis *et al.*, 2000) as well as DNA profiling methods such as randomly amplified polymorphic DNA fingerprinting (RAPD). The latter technique has been used in studies of plants (Ronimus *et al.*, 2003; Liu *et al.*, 2005) and animals (Nadig *et al.*, 1998) but little information is available regarding this aspect (DNA damage) on bacterial species. There is a need for studies dealing with impacts of pollution on organisms in general, but microoganisms in particular. Impacts of pollution on microorganisms had for a long time been neglected and recent studies (Ronimus *et al.*, 2003; Liu *et al.*, 2005) have demonstrated the importance thereof.

1.2 AIM

The aim of this study was to determine microbial diversity and metal pollution from a platinum mine tailing dam in the North-West Province (RSA), at different distances away from the aforementioned tailings dam.

1.3 OBJECTIVES

1. To determine the physical characteristics of soil and tailings in terms of particle size distribution and its chemical characteristics with reference to pH, percentage organic carbon and heavy metal concentrations.
2. To determine the diversity of bacterial and fungal isolates in the tailings soil using plating methods.
3. To use standard biochemical tests viz, SDS-PAGE, DNA sequencing and PCR-RFLP to identify selected bacterial isolates.

4. To evaluate the potential of RAPD fingerprinting in the assessment of DNA damage occurring in bacteria isolated from sites on and around the platinum mine tailings dam.

Culture-dependent methods were used to isolate microorganisms whilst the molecular methods were used to identify the microorganisms as well as demonstrate the impacts of heavy metals on their genotype.

CHAPTER 2

LITERATURE REVIEW

2.1 SOIL MICROBES AND METAL CONTAMINATED SITES

Soils are highly complex environments that act as a reservoir for microorganisms. Their activity vary over space and time, which results from multiple interacting parameters e.g. soil texture and structure, water content, pH, climate variations and biotic activity (Torsvik and Øvreas, 2002; Robe *et al.*, 2003; Wellington *et al.*, 2003). In addition, soils also perform essential functions such as nutrient cycling to support plant growth, attenuation and transformation of potentially toxic compounds and the maintenance of biodiversity, making it central to the sustainability of ecosystems (Bååth *et al.,* 1998). Soil microbes (bacteria, fungi, etc.) (Ranjard *et al.*, 2000a) play significant roles in the maintenance of soil structure, detoxification of noxious chemicals, the control of pests and plant growth (Giller *et al.*, 1998; Chen *et al.*, 2006). Although soil microbes perform many critical processes, functional capabilities vary and in many cases their exact roles are unknown (Bååth *et al.,* 1998) because of their abundance, diversity and multiplicity of metabolic activities (Ranjard *et al.*, 2000a). They have the potential to reflect the history of the environment making it essential to understand this interrelationship. This is done by studying the structural and functional diversity of soil microbial communities and their responses to anthropogenic disturbances (Ranjard *et al.*, 2000a). Pollution of soil by metals is critical because soil pollutants (metals) accumulate in it (Adamo *et al.*, 2003) and one such disturbance is mining, which is a major contribution to solid waste in South Africa.

Contamination of soils by metals originating from agricultural (e.g. fertilizers and sewage sludge) or industrial activities (e.g. metal mining and smelting) is one of the major environmental problems in many parts of the world (Mulligan *et al.*, 2001; Gremion *et al.*, 2004; Corami *et al.*, 2008). Soils contaminated with metals have increased markedly in the last 75 years owing not only to the increased consumer use of materials containing metals, but also to technological developments (Garcia *et al.*, 2004; Ferreira *et al.*, 2007). Significant increases in these metal contents are found in

areas of high industrial activity where accumulation may be of several times higher than the average content in non-contaminated areas (Loska *et al.*, 2004). Mining concentrating ores and tailings disposal provide possible sources of contamination in the soil environment (Jung, 2001), with soil microorganisms subjected to stress rendering them unable to maintain the same overall biomass as in uncontaminated soils (Giller *et al.*, 1998).

The impact of metal pollution on ecosystems due to natural processes (Hernandez *et al.*, 2003) and anthropogenic activities (Maboeta *et al.*, 2005) has been frequently investigated. These investigations aimed to understand the behaviour of metals such as chromium (Cr), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), cadmium (Cd) and lead (Pb) in soils. One such an example is platinum mines (North-West Province, South Africa), which produce large amounts of inorganic tailings containing elevated levels of metals. Pollutants produced by platinum mining viz. Al, Cr, Cu, and Ni (Maboeta *et al.*, 2005), might inhibit enzymatic activity in soil even if they are present in relatively low concentrations (Ashman and Puri, 2002; Maboeta *et al.*, 2005).

Metal contaminants cause soil substrate and groundwater pollution, soil structure deterioration, increase in nutritional deficiencies, destruction of the ecological landscape, and tremendous decreases in biological diversity (Hao *et al.* 2004). Because of excessive phytotoxicity in soils containing high levels of metals, the natural vegetation cover could disappear leaving bare soil without vegetation (Yun-Guo *et al.*, 2006). Essential trace elements, metals above certain concentrations and exposure times are toxic to soil animals and affect the abundance, diversity and distribution of the animals (Šmejkalová *et al.*, 2003; Lukkari *et al.*, 2004; Wang *et al.*, 2007a). This might also be true for soil microbial diversity. Ranjard *et al.* (2000b) also reported impacts of metal pollution at the community level using phenotypic or genetic fingerprinting techniques.

Recently metal contamination in tailings received attention since there is a growing need to reclaim these sites after mining operations have ended (Liao and Xie, 2006). Most studies however, focus

on the process of vegetation, restoration and engineering technology rather than underground soil microbe rehabilitation and effects on ecological systems (Tordoff *et al.*, 2000). Furthermore, metals in tailings could be transported, dispersed and accumulated in plants and animals, and then passed through the food chain to humans (Yun-Guo *et al.*, 2006). The main problem associated with metal pollution is that, in contrast to organic pollutants, metals cannot be degraded, which increases their relevance as a serious group of contaminants (Pérez-de-Mora *et al.*, 2006). It has been suggested that by affecting the structure of microbial communities, metals might have significant effects on processes, which are important for the maintenance of soil fertility such as mineralization of organic matter, nitrogen transformations, enzyme activities and degradation of organic pollutants (Giller *et al.*, 1998).

Traditionally, determination of the environmental risk of metals towards soils and sediments are based on quantification of total metal concentrations after digestion with strong acids followed by chemical analysis (Ivask *et al.*, 2004). This however, does not portray ecologically relevant risk since metals may be leached, absorbed by vegetation or retained by the soil and their toxicity is determined by factors such as concentration, speciation and bioavailability (Alvarez *et al.*, 2003). It is generally accepted that accumulated metals may reduce soil microbial biomass and enzyme activities, resulting in a decrease in the functional diversity of the soil ecosystem and changes in microbial community structure (Pérez-de-Mora *et al.*, 2006). However, metal exposure may also lead to the development of metal tolerant microbial populations (Ellis *et al.*, 2003). This makes it possible to utilize soil microbes in ecotoxicological studies when assessing the risks of metal contaminants.

Microorganisms and microbial communities can provide an integrated measure of soil quality, an aspect that cannot always be obtained with physical and chemical measures or analysis of higher organisms (Winding *et al.*, 2005). Frey *et al.* (2006) reported reduced soil microbial activities and biomass as well as changes in microbial community structure following the application of metals to

soil, therefore knowledge of the microbial community function and structure represents a first step towards understanding soil function in response to metal pollution. As a result, the total concentrations of metals in soils are not good indicators of metal bioavailability, or the only tool to assess potential risk assessment (Wang *et al.*, 2003). This is due to the different and complex distribution patterns of metals among various chemical species or solid phases.

Soil microorganisms are the first biotas that are directly and indirectly impacted on by metals in soil (Piotrowska-Seget *et al.*, 2005). Metals affect these microorganisms by reducing their number, biochemical activity, diversity and community structure (Ellis *et al.*, 2003). Short-term and long-term exposure of toxic metals to soil have been frequently investigated and proven to result in reduction of microbial diversity and activities in soil (Sandaa *et al.*, 2001; Ranjard *et al.*, 2000b; Gremion *et al.*, 2004; Rajapaksha *et al.*, 2004; Wang *et al.*, 2007b). The introduction of metals to the environment can produce considerable modifications to microbial communities and their activities (Hassen *et al.*, 1998).

Environmental pollution with metals has led to the appearance of metal resistant microorganisms in soil and water of industrial regions (Giller *et al.*, 1998). The genes controlling metal tolerance/resistance could be found on the chromosome or could be plasmids borne (Piotrowska-Seget *et al.*, 2005; Li *et al.*, 2006). The basic mechanisms by which the heavy metal resistance are obtained include enzymatic detoxification of the metals, binding of metals into the cell wall, intracellular binding by specific components, blocking the cellular uptake of the metals and pumping the metals rapidly out of the cytoplasm (Li *et al.*, 2006). In many cases, resistance to heavy metals is determined by plasmids, which can be used for the creation of novel microbial strains with a high detoxifying activity against metals (Aleem *et al.*, 2003). A study by Piotrowska-Seget *et al.* (2005) demonstrated this by investigating the association of Zn and Cd tolerant bacterial species, in relation to occurrence of plasmids and high levels of the metals in soils. Long-term exposure may thus lead to selection of metal tolerant bacterial populations.

2.2 INDICES OF SOIL HEALTH

Soil microbial diversity is an important index of soil ecosystem health (Johnson *et al.*, 2003; Chen *et al.*, 2006). The notion is that the higher the diversity the greater the stability and resilience of the ecosystem should be (Entry *et al.*, 2008). Since microorganisms have relatively short life cycles, they respond quicker to anthropogenic activities than do plants and animals. They may thus be sensitive indicators to changes in land management practices (Yang *et al.*, 2000; Johnson *et al.*, 2003; Chen *et al.*, 2006; Entry *et al.*, 2008). Yet, relatively little is understood about the diversity and ecology of microbial communities in soil (Nakatsu *et al.*, 2000; Chen *et al.*, 2006). Fungi and bacteria are the drivers of major soil processes such as carbon and nutrient cycling (Hafeel *et al.*, 2004). High functional species diversity of soil fungi and bacteria make quantifying their relative contribution to soil biomass challenging (Feng *et al.*, 2004). For this reason ecological indices such as fungal:bacterial ratios, Shannon-Weaver index, and others were proposed (Atlas and Bartha, 1998; Ingahm, 2007).

The Shannon-Weaver diversity index is used to measure variation or diversity and has been used to reflect the structural diversity of microbial community contributions in agricultural and polluted soils (Yang *et al.*, 2000; Camargo *et al.*, 2005; Entry *et al.*, 2008). Entry *et al.* (2000) used Shannon-Weaver diversity index to demonstrate the impacts of irrigation on microbial diversity in agricultural soils and Camargo *et al.* (2005) used it to demonstrate the biodiversity of chromium resistant bacteria in different soil types.

Fungal:bacterial ratio is commonly measured by a number of methods including substrate induced respiration (SIR), selective inhibition (SI) techniques (Hafeel *et al.*, 2004), phospholipid fatty acids (PLFA) (Schwieger and Tebbe, 1998; Kozdroj and van Elsas, 2001a; Bailey *et al.*, 2002; Tscherko *et al.*, 2004; Stemmer *et al.*, 2007) as well as DNA-based fingerprinting (van Elsas *et al.*, 1998; Feris *et al.*, 2004a; Hong *et al.*, 2007; Hu *et al.*, 2007; Wang *et al.*, 2007b). The latter two are culture-independent methods, each with their own advantages and shortcomings. Although culture-

dependent methods also have limitations, they may be useful in certain studies particularly when a secondary goal of the study is to characterize individual members of the microbial population (Atlas and Bartha, 1998).

Highly productive agricultural soils tend to have fungal to bacterial ratios near 1:1 or somewhat less (Ingahm, 2007). This ratio could be different in polluted environments. In such environments microbial communities are under stress and for this reason and diversity is normally lower (Camargo *et al.*, 2005; Gopal *et al.*, 2007). According to Atlas and Bartha (1998), such stressed communities are less adapted to deal with further environmental fluctuation, thus lower levels and diversity of representative groups occur. Fungi are regarded as being more tolerant to heavy metals than bacteria (Gremion *et al.*, 2004). This may change the composition of the soil microflora and select for metal-resistant microorganisms that may alter the fungal:bacterial ratio (Frey *et al.*, 1999; de Vries *et al.*, 2006; Mench *et al.*, 2006). In studies where the impacts of soil pollution is being investigated., fungal:bacterial ratios may therefore be a useful index (Frey *et al.*, 1999).

2.3 METHODS TO DETERMINE MICROBIAL DIVERISTY

Culture-dependent and culture-independent methods may be utilized for determining diversity in soils (Schwieger and Tebbe, 1998; Tiedje *et al.*, 1999; Yao *et al.*, 2000; Roose-Amsaleg *et al.*, 2001). Even though culture-dependent methods are greatly criticised and have their own limitations, these methods are still being used for determining microbial diversity in soils (Atlas and Bartha, 1998; Piotrowska-Seget *et al.*, 2005; Gopal *et al.*, 2007). Piotrowska-Seget *et al.* (2005) used culture-dependent methods to investigate metal tolerant bacteria occurring in heavily polluted soils of a mine spoil and successfully used these methods to isolate and identify metal tolerant bacteria. The usefulness of these methods was also demonstrated by Gopal *et al.* (2007), who determined the impacts of azdirachitin (an insecticidal allelochemical) on soil microflora enzyme and respiratory activities. They used this approach to obtain levels of tolerant and sensitive bacteria and analysed the data using the Shannon-Weaver diversity index method.

In recent years, studies were performed to describe bacterial diversity and community changes in various pollutant degrading bacterial communities (Kanaly *et al.*, 2000; Kaplan and Kitts 2004). A number of molecular methods, in particular polymerase chain reaction (PCR), have been developed for describing and comparing the dynamics of such complex microbial communities (Schneergut and Kulpa, 1998; Hong *et al.*, 2007). PC R involves the amplification DNA using particular conditions that simulate natural DNA replication (Hong *et al.*, 2007). The development of this technique resulted in an explosion of new DNA profiling techniques as more applications were published (Kubista *et al.* 2006; Hong *et al.*, 2007). Data from DNA profiling methods based on PCR amplification could either be a rough overview of taxonomically distant groups within communities or provide a deeper insight into selected eco-physiological groups (Crecchio *et al.*, 2004; Hong *et al.*, 2007). Furthermore, a large number of studies also used methods based on phospholipid fatty acid (PLFA) profiles to investigate microbial diversity and function in various environments (Schwieger and Tebbe, 1998; Kozdroj and van Elsas, 2001a,b; Bailey *et al.*, 2002; Tscherko *et al.*, 2004; Stemmer *et al.*, 2007). This culture-independent technique also has intrinsic advantages and limitations.

A disadvantage of direct extraction and analysis of DNA (or phosholipids) is that, after analysis, there are no viable examples of specific microbes that were impacted on by the pollutants. Studies that further investigate the impacts of the specific pollutants on the affected microbes are thus not possible. An approach that combine isolation of bacterial or fungal species on culture media and preliminary grouping them based on phenotypic and genotypic means is useful (Schwieger and Tebbe, 1998; Hong *et al.*, 2007). However, combining culture-dependent and culture-independent methodologies would thus be a very powerful approach to study impacts on microbial diversity.

2.4 MOLECULAR METHODS FOR IDENTIFICATION OF BACTERIA AND GENOTOXICITY STUDIES

The PCR based restriction fragment length polymorphism (PCR-RFLP) technique involves amplifying DNA by PCR and size fractionation with restriction endonucleases followed by resolving the resulting DNA fragments by electrophoresis (Babalola, 2003). The presence and absence of fragments result from changes in enzyme recognition sites (Dowling *et al.*, 1990). This technique is regarded as sensitive for strain identification and several bacterial strains have been widely studied using this technique (Kabadjova *et al.*, 2002). This method is most suited to studies at the intra-specific level or among closely related taxa. Two examples (Waleron *et al.*, 2002; Yang *et al.*, 2007) of how this approach was used are provided in Table 2.1. In one example (Waleron *et al.*, 2002), the potential of using this method (PCR-RFLP) for identification of bacterial plant pathogen is illustrated. Another example listed here (Table 2.1) refers to how the methods was used for identification of a fungal species (Yang *et al.*, 2007).

Table 2.1: Some applications of 16S rDNA sequencing, PCR-RFLP, RAPD and SDS-PAGE to genetic diversity of bacteria. Selected applications of the RAPD assay to gentoxicity studies are also listed

	Taxon	**Application**	**Taxonomic level**	**Methods used**	**Reference**
Bacteria	*Bacillus barbaricus*	Indentification of new species	Genus	16S rDNA sequencing, Fatty acid profiles, biochemical data, SDS-PAGE	Täubel *et al.*, 2003
Bacteria	*Bacillus subtilis*	Geographic diversity	Genus	16S rDNA sequencing, RAPD	Istock *et al.*, 2001
Bacteria	*Bacillus thuringiensis*	Epizoonic epidemiology	Genus	RAPD, SDS-PAGE	Konecka *et al.*, 2007
Bacteria	*Bacillus* spp.	Geographic diversity	Genus	16S rDNA sequencing, rep-PCR	Fajardo-Cavazos and Nicholson, 2006
Bacteria	Thermophillic bacteria	Food Microbiology	Genus	16S rDNA sequencing, RAPD	Ronimus *et al.*, 2003
Bacteria	*Vibrio* spp.	Aquaculture	Genus	RAPD	Sudheesh *et al.*, 2002
Bacteria	*Salmonella* spp.	Poultry farming	Species, population	RAPD	Seo *et al.*, 2006
Bacteria	*Pseudomonas ozyzihabitans*	Epidemiology	Species, population	RAPD, 16S rDNA sequencing, antibiogram, electron microscopy	Dussart-Baptista *et al.*, 2007
Bacteria	*Erwinia* spp.	Plant pathology	Genus	PCR-RFLP	Waleron *et al.*, 2002
Fungi	*Pleurotus* spp.	Auto screening	Genus	PCR-RFLP, Computer program	Yang *et al.*, 2007
Plant	*Oryza sativa* L	Genotoxic effects of cadmium	Population	RAPD	Liu *et al.*, 2007
Plant	*Hordeum vulgare*	Genotoxic effects of cadmium	Population	RAPD, protein yield	Liu *et al.*, 2005
Animal	*Lepomis auritus*	Genotoxic effects of chemicals	Population	RAPD	Nadig *et al.*, 1998

Sequencing of specific DNA fragments is regarded as the ultimate method for providing data that could be analysed for DNA variation. This method is also commonly used for identification of bacterial and fungal species. Table 2.1 provide some examples where sequencing of 16S rDNA fragments were used for answering various phylogenetic questions and identification of novel bacterial species (Istock *et al.*, 2001; Ronimus *et al.*, 2003; Täubel *et al.*, 2003; Fajardo-Cavazos and Nicholson, 2006; Dussart-Baptista *et al.*, 2007)

Random amplified polymorphic DNA (RAPD) analysis is also a PCR-based technique. It amplifies random DNA fragments using genomic DNA as template and single short primers of arbitrary nucleotide sequence under low annealing conditions (Liu *et al.*, 2005). This technique is used extensively for species classification, genetic mapping and phylogeny (Table 2.1). The appeal of the RAPD technique is the simplicity of the procedure and its requirements of only small quantities of DNA. No prior knowledge of the genome being analyzed is necessary and many genetic loci can be potentially assessed (Nadig *et al.*, 1998). This technique is particularly useful in genetic studies of natural populations (Sudheesh *et al.*, 2002; Liu *et al.*, 2007). Many laboratories have found that this method can produce consistent and highly reproducible banding patterns provided that the PCR reaction conditions are rigidly standardized and kept constant (Nadig *et al.*, 1998; Istock *et al.*, 2001; Sudheesh *et al.*, 2002; Ronimus *et al.*, 2003; Seo *et al.*, 2006; Dussart-Baptista *et al.*, 2007; Konecka *et al.*, 2007). Furthermore, RAPD assays were successfully used to demonstrate genotoxic effect of heavy metal pollution and other mutagens in plants and animals (Table 2.1) (Nadig *et al.*, 1998; Mengoni *et al.*, 2000; Liu *et al.*, 2005; 2007).

Analysis of soluble whole cell proteins by SDS based polyacrylamide gel electrophoresis (SDS-PAGE) is a common technique in various fields of traditional biochemical studies (Hames and Rickwood, 1990; Täubel *et al.*, 2003; Konecka *et al.*, 2007). In this method extracted proteins are strictly separated according to their size and profiles of inter- and intra-specific specificity can be generated in this manner (Täubel *et al.*, 2003). In Table 2.1, two examples are provided where this

method was used in conjunction with other methods for two separate applications (Täubel *et al.*, 2003; Konecka *et al.*, 2007). Täubel *et al.* (2003) used this method in conjunction with 16S rDNA sequencing, biochemical and fatty acid profile data to, for the first time describe a novel *Bacillus* spp. (*Bacillus barbaricus*).

2.5 SUMMARY OF THE LITERATURE REVIEW

The literature review presented an overview and insight into the aim and main objectives as stipulated in Section 1.3. It was divided into 4 sections, each dealing with a specific aspect of the study. In the first section, insights into soil microbial diversity as well as the negative impact of high concentrations of heavy metal on such environments were discussed. This section also dealt with risks of high concentrations of heavy metals on the environment and briefly discussed potential impacts on humans. In the second section, it was demonstrated that various indices could be used to evaluate soil health. Here the usefulness of the Shannon-Weaver index and fungal:bacterial ratios were discussed. The third section, dealt with methods that could be used for determining microbial diversity in soils. It provided some advantages and disadvantages of culture-dependent and culture-independent methods and how these independently or in combination could provide useful data. Lastly, the fourth section, dealt with the principles and applications of selected molecular methods. Some examples were also provided and briefly discussed. It was particularly indicated how powerful a combination of these methods could be to group and identify organisms. This section also briefly mentioned how RAPD data were used in identification as well as genotoxicity studies.

CHAPTER 3

MATERIALS AND METHODS

3.1 SITE DESCRIPTION AND SAMPLE COLLECTION

The study was conducted in Rustenburg (North-West Province, South Africa) at a platinum mine, which is the second largest platinum mine in the world. The mine has the largest tailings footprint in the southern hemisphere, covering an area of 964 ha and has been "moderately" rehabilitated. Fertilizers which were applied for rehabilitation was Super phosphate; $(NH_4)_2SO_4$ and potassium chloride (KCL) (Wahl, 2007). Figure 3.1 represents a satellite picture on and away from the tailings dam. Sampling was done during August and December 2005, as well as March and May 2006. The sampling regime included wet (December 2005 and March 2006) and dry periods (August 2005 and May 2006). Sampling was carried out on and away from the tailings dam at the following distances and coordinates, 0 m (Site 1, S25 30.394 E27 13.598), 70 m (Site 2, S25 30.358 E27 13.583), 150 m (Site 3, S25 30.323 E27 13.565), 300 m (Site 4, S25 30.245 E27 13.542), 500 m (Site 5, S25 30.127 E27 13.516), 850 m (Site 6, S25 29.945 E27 13.459) and 1350 m (Site 7 and S25 29.681 E27 13.401).

Annual ambient temperature for this area generally range between 2°C and 35°C. Spring and summer temperatures (September to April) range between 22°C to 35°C. Autumn and winter temperatures (May to August) range between 2°C to 20°C.

The mine is in a summer rainfall area and this is demonstrated by the graph in Figure 3.2. The values provided (S.A Weather, 2007) are totals for each month. During the first sampling period (August 2005), 5 mm of rain was recorded for this area. The second sampling was in December 2005 and was preceded by a total of 50 mm of rain in November 2005 and more or less the same amount in December 2005. During January and February (2006) totals of 240 mm and 150 mm of rain, respectively, fell in this area. The third sampling period was in March 2006 when the area had

25 mm rain. There was thus considerable rain during the second and third sampling periods. During April 2006 an average of 10 mm and in May 2006 less than 10 mm of rain were recorded, indicating that the fourth sampling was taken during a dryer period.

Figure 3.1: Aerial photo of the investigated tailings dam in Rustenburg. Sites 1-7 (S1-S7), are the seven sampling points on, closer and further away from the tailings dam.

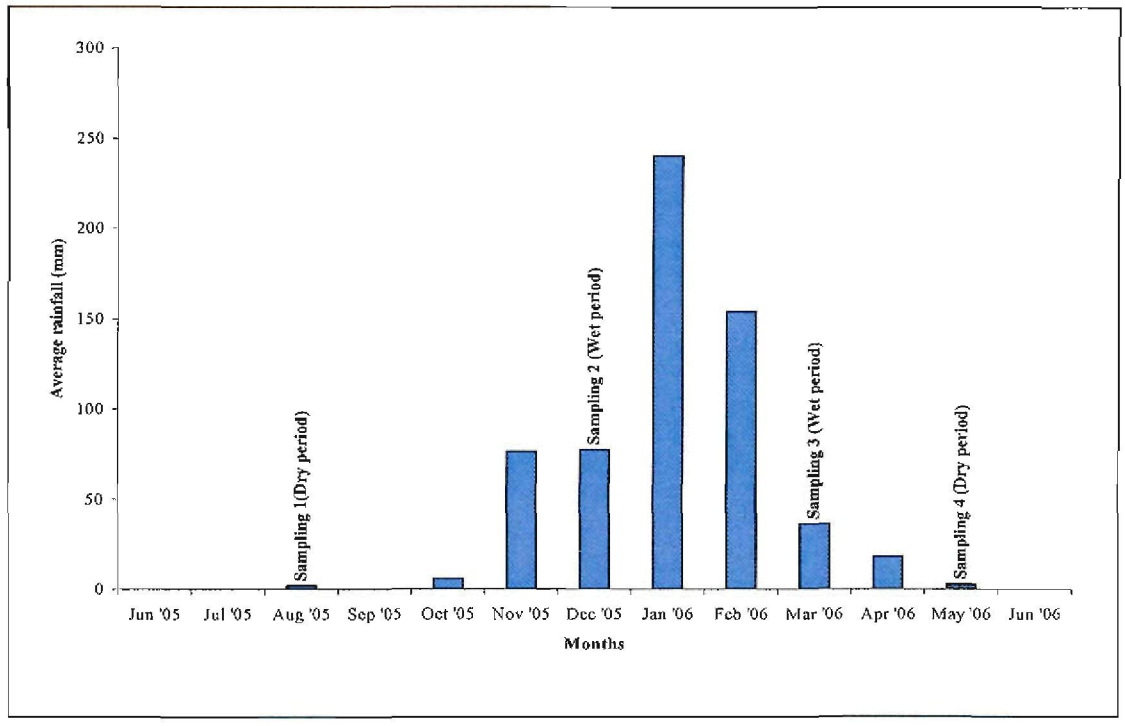


Figure 3.2: Monthly rainfall (mm) for the Rustenburg area from June 2005 - June 2006 (S.A Weather Service, 2007).

3.2 SOIL CHEMICAL AND PHYSICAL CHARACTERISTICS

Six random soil samples (10 cm deep) were collected per sampling site, then transported to the lab in a cooler box and analysed for physical and chemical characteristics. For the determination of pH, 5 g of the sieved soil was mixed with 10 ml of double distilled water and shaken for 30 minutes. Thereafter, the pH of the soil was measured using a calibrated pH meter (WTW multi 350i, Germany).

Sand, silt and clay contents for each sampling sites were determined by means of the hydro-method. The method as described here was reproduced from Wahl (2007). The study of Wahl (2007) was parallel to this study and identical methods were thus used to determine physical and chemical characteristics of soils and tailings material. One hundred grams of each soil/tailings sample was weighed and sifted through a 2 mm sieve. Fifty grams of the sifted soil/tailings material was placed into a 500 ml container, soaked with distilled water and 10 ml hydrogen peroxide was carefully

10 minutes, it was stirred well and heated for 4 hours. The suspension was then cooled and 125 ml Calgon, which contains sodium hexametaphosphate was added and stirred well. A 53 μm sieve was then placed into the funnel, which was set up to drain into a 1000 ml sedimentation cylinder. Suspensions were transferred into the funnel and washed with running water and a small brush until water runs clear. The fraction of soil in the funnel was then placed into a glass beaker, dried in an oven, sifted through a 53 μm sieve and then weighed. The 1000ml suspension in the cylinder was shaken and the first reading was taken after exactly 40 seconds and the second reading was taken 7 hours later.

For the determination of carbon content, soil/tailings samples were dried through a 0.35 mm sieve. One gram of soil (0.5 g if the soil had a dark colour, which would indicate a high carbon content) was then weighed off and placed in an Erlenmeyer flask. A blanco mixture without soil or tailings was also made. Twenty millilitres potassium dichromate as well as 20 ml concentrated sulphuric acid were added to the soil sample in the flask. The flask was gently stirred until the reagents and the soil sample have mixed completely. The flask was cooled for 30 minutes and 150 ml deionized water was added and mixed. Ten millilitres concentrated ortho-phosphiric acid and 1 ml bariumdiphenileaminesulphate indicator were also added to the flask and mixed. The mixture was the titrated with iron (II) ammonium sulphate solution. The percentage of carbon was determined as follows:

$$\text{Concentration iron (II) ammonium sulphate (M) mol/l} = \frac{20\text{ml } K_2Cr_2O_2 \text{ X } 0.167 \text{ X } 6}{\text{ml } Fe(NH_4)_2(SO4)_2 \text{ (ml blanco mixture)}}$$

$$\text{Oranic C\%} = \frac{[\text{ml } Fe(NH_4)_2(SO_4)_2 \text{ blanco} - \text{ml } Fe(NH_4)_2(SO_4)_2 \text{ sample}] \text{ X M X } 0.3 \text{ X f}}{\text{weight of soil per sample (g)}}$$

where M =concerntration $Fe(NH_4)_2(SO_4)_2$ in mol/l and f = 13

Complete dissolution of soil samples for metal determination was performed using an acid digestion method. This entails, transferring 5 g of the sieved soil sample into a beaker, digesting the sample

in a mixture of HNO_3 (60%) suprapure and HCL (40%) suprapure (Merck, Germany). An ICP-MS (Agilent 7500c), utilising a Cetac ASX-510 auto sampler and peristaltic pump was used to determine total metal concentrations in the tailings and soil collected.

3.3 MICROBIOLOGICAL ANALYSIS

Enumeration of bacteria and fungi were performed according to the method by Piotrowska-Seget *et al.* (2005). Once the samples were in the laboratory, each sample was sieved (<2 mm) to remove organic matter and larger inorganic matter. Five grams of each sample was placed in separate Erlenmeyer flasks containing 45 ml of sterile phosphate buffer (0.1 M K_2HPO_4, 0.1 M KH_2PO_4, 0.85% (w/v) NaCl) pH 7.0 and shaken on a rotor shaker at 100 rpm for 30 minutes. A series of tenfold dilutions (up to 10^6) were prepared for enumeration of culturable bacteria and fungi. One hundred microlitres of the diluted samples were used to prepare spread plates on 0.1 strength nutrient agar (bacteria) and potato dextrose agar (fungi). All plates were incubated at 27°C for 4 days.

The number of different viable colonies that developed after 4 days of incubation were counted and based on morphology and colour; been classified, recorded and expressed as cfu/g of soil (Appendix C). Bacterial types were purified by successive streaking of selected single colonies onto appropriate media. Gram stain according to standard procedures (Prescott and Klein, 2002), was used to confirm cell morphology and whether the organisms were Gram-positive or Gram-negative. No further biochemical tests were conducted but the identities of selected isolates were determined by molecular methods (Sections 3.4-3.9). Ratios of fungal to bacterial levels were calculated using the plate count (cfu/g) data (Appendix B).

3.4 DNA EXTRACTION

A commercial DNA extraction kit was used for isolation of genomic DNA (peqGold Bacterial DNA Mini Kit, peqLab, Germany). This method included using 2 ml from the pure culture (overnight

version 6.08.04 software (SynGene, UK). A 100 bp DNA ladder (O`GeneRuler, Fermentas, US) was used as molecular size marker.

PCR amplified fragments were sequenced (Inqaba Biotech, RSA). The sequences were used to confirm the identity of the amplicons using the Chromas Pro Version 2.13 software (www.technellyslum.com.au) and GeneBank BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Five microlitres of the PCR products were digested with *EcoRI*, *AvaI*, *Taq*I and *Kpn*I (fast digest) in buffers and instructions provided by the manufacturer (Fermentas, US). Products were incubated for 30 minutes at room temperature. Digested products were separated by electrophoresis through 1% (w/v) agarose gels in 1XTAE running buffer. Ethidium bromide stained images were captured using a GeneGenius Bioimaging system (SynGene, UK) and GeneSnap version 6.08.04 software (SynGene, UK). A 100 bp DNA ladder (O`GeneRuler, Fermentas, US) was used as molecular size marker.

3.7 RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) FINGERPRINTING

DNA samples of the isolates were further analyzed using the RAPD fingerprinting method. Primers were supplied by Operon technologies (Cologne, Germany) and the PCR reagents by Fermentas (US). Three primers, two from the OPA kit and one from the OPB were tested. Amplifications were carried out in a 25 µl reaction volume consisting of 2X PCR Master mix (0.05 U/µl *Taq* polymerase, 4 mm $MgCl_2$, and 0.4 mm dNTPs), 2.5 U additional *Taq* polymerase (Super-therm *Taq*, J.M Holdings, UK), 50 pmol primer, 100 ng DNA, and PCR free water in an iCycler (BioRad, UK) using the PCR conditions 95°C for 5 minutes (initial denaturing), 95°C for 1 minute (denaturing), 37°C for 1minute (annealing), 72°C for 2 minutes (extension) for 40 cycles, and 72°C for 5 minutes (final extension). Amplified products were size separated on a 2% (w/v) agarose gel. Ethidium bromide stained images were captured using a GeneGenius Bioimaging system (SynGene,

UK) and GeneSnap version 6.08.04 software (SynGene, UK). A 100 bp DNA ladder (O'GeneRuler Fermentas, US) was used as molecular size marker. Fingerprints were compared using GeneTools Version 3.07.03 software and the bands that appeared consistently were evaluated. Presence, absence and intensity of bands were further analyzed using Statistica version 7.0 (StatSoft, US) software. Ward's method and Euclidean distance algorithms were used for cluster analysis.

3.8 PROTEIN EXTRACTION AND SDS-PAGE

Protein extraction was carried out using a sodium azide (NaN_3) extraction method. Briefly: 1.5 ml of an overnight culture (each) was pipetted into a 1.5 ml microfuge tube and centrifuged at 13400 rpm for 5 minutes and the supernatant was discarded. To increase the biomass this step was repeated at least twice. The pellet was resuspended in 1 ml phosphate buffered saline and centrifuged at 13400 rpm for 5 minutes. The supernatant was discarded and pellet was resuspended in 500 µl of 10 mM NaN_3, then centrifuged again at 13400 rpm for 5 minutes and supernatant was discarded. Finally, the pellet was resuspended in 200 µl of extraction buffer (0.125 mM Tris pH 6.8, 4% (w/v) sodium dodecyl sulphate (SDS), 20% (v/v) glycerol, and 10% (v/v) 2-mercaptoethanol) and incubated at 100°C in a dry bath for 10 minutes. Glass beads (50 µl) were added to the samples and vortexed for 2 minutes. The resultant supernatant was transferred to a fresh microfuge tube by making a hole in a microfuge tube containing the sample using a gauge needle. This tube was placed into a second microfuge tube and centrifuged at 1500 rpm briefly to transfer the protein sample that is free from the glass beads into the new microfuge tube. Protein concentration was then determined using the RC Protein Assay kit (BioRad, UK). Bovine serum albumin was the standard ranging of 0 - 3.5 mg/ml.

Extracted proteins were resolved using the SDS-PAGE. Each protein sample (20 µg) was prepared in a protein loading buffer (0.125 mM Tris-HCL pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, and 0.002% (w/v) bromophenol blue). Protein samples were then loaded on SDS-PAGE composed of 12% resolving gel and 4% stacking gel. Unstained protein molecular

weight marker (Fermentas, US) was loaded on each gel. Proteins were visualized by staining the gel with 0.13% (w/v) Coomassie brilliant blue R-250 (Saarchem, S.A) in 50% (v/v) methanol:10% (v/v) glacial acetic acid:40% (v/v) double distilled water for 1 hour, and destained overnight in 10% (v/v) methanol:10% (v/v) glacial acetic acid:80% (v/v) double distilled water. Images were captured using a GeneGenius Bioimaging system (SynGene, UK) and GeneSnap version 6.08.04 software (SynGene, UK).

3.9 NUMERICAL AND STATISTICAL ANALYSIS

Averages and standard deviation of bacterial levels were determined along the gradient of the tailing dam. Shannon-Weaver index values (H) for each site was determined using data from culture-dependent methods.

$$H = -\sum P_i \log P_i$$

Where P_i is the relative probability of finding species at a specific site. H is calculated on the basis of the number of species at specific sites. The relative probability, P_i was calculated as:

$$Pi = n_i / N$$

Where n_i is the relative quantity of a specific species and N is the sum of all the relative quantities of species at a specific site.

Correlations between the diversity of different distances and metal concentrations from tailings dam were calculated using SigmaStat (SYSTAT Software Incorporated, US). For the analyses parametric or non-parametric tests with $P < 0.05$ as level of probability was used.

CHAPTER 4

RESULTS

4.1 SOIL CHEMICAL AND PHYSICAL CHARACTERISTICS

Soil organic matter, particle size and pH for each of the sampling sites are represented in Table 1. The percentage C was lower on the tailings dam (Sites 1 and 2) and increased in distance further away from the dam (Sites 3 to 7). Particle size distribution of sand %, silt % and clay % were different between the sites with sites 1–3 having low fractions of clay and particles > 2mm in contrast to sites 4–7. The pHs of soils from the different sampling sites were higer ($p<0.05$) in sites 1–4 when compared with those from sites 5–7.

Heavy metal contents of collected soil samples are listed in Tables 4.2 – 4.5. The metals listed were those generally associated with platinum mining namely: aluminium (Al), chromium (Cr), copper (Cu) and Nickel (Ni). A table that includes concentrations of all the metals measured is presented in Appendix A. Since there was no significant differences between any of the metals from the different sites during December 2005, only those of August 2005, March and May 2006 will be discussed.

During August 2005 Al concentrations in Sites 1 and 2 were lower ($P < 0.05$) than in Sites 4–7, however, Sites 1 and 3–7 were significantly different ($P < 0.05$) from Site 2. Site 1 was higher than Sites 4–7, in May 2006. In addition, concentrations at Sites 1 and 2 were significantly different ($P>0.05$) from Site 3 and then different from Site 4 which were significantly different from Sites 5 to 7. December 2005 and March 2006 concentrations were not significantly different ($P < 0.05$) at all sites.

In August 2005, Cr concentrations at Sites 1-4 were significantly higher ($P < 0.05$) than those from Sites 5 -7. Similar patterns were observed for March 2006 (Site 1 > Sites 2–3 > Site 4 > Site 5 > Site 6 > Site 7) and May 2006 (Sites 1–3 > Sites 4–7).

Cu concentrations during August 2005 and March 2006 at Sites 1–3 were significantly higher ($P < 0.05$) than those in Sites 4–7. Similar patterns emerged for May 2006 (Site 1 > Site 2 > Site 3 > Sites 4–7).

Concentrations of Ni showed that those from Sites 1 and 2 were higher ($P < 0.05$) than Sites 3 and 4, which were higher ($P < 0.05$) than Sites 5–7 during August 2005. Nickel concentrations during March and May 2006 were similar *viz.* Site 1 > Site 2 > Site 3 > Sites 4–7.

Concentrations of heavy metals were compared to the soil Netherlands maximum permissible concentrations (MPCs, Crommentuijn *et al.*, 1997) and Candian microbial benchmarks (MBs, Efroymson *et al.*, 1997). Most of the heavy metal levels that were measured were very high compared to those of the Netherlands and Canadian benchmarks. The general pattern that emerged for all of the presented metals was that they decreased in concentration further away from the studied tailings dam. When looking "worst case scenarios" (based on the presented data and microbial benchmarks) for the different metals, irrespective of sampling date, the following trends can be observed: Aluminium exceeded the microbial benchmark up to 1350 m away from the tailings dam, Cr up to 300 m, Cu up to 70 m and Ni up to 70 m. With regards to the MPC values, both Cu and Cr exceeded these values up to 1350m, whilst Cr was lower and no MPC exists for Al.

The benchmarks used in this study indicate the percentages of available heavy metal concentrations from the field and laboratory studies. Laboratory bioassays with several organisms (bacteria, plants, arthropods, oligochaets) have been performed in metal contaminated soils originating from the neighbourhood of a zinc smelter works at Budel. The results from these bioassays were compared with the results from experiments performed with the same species in standardized soils spiked with metals, and the benchmarks were then derived from both results. To add to the quality of these benchmarks, methods used from the previous reports of the project "Setting Intergrated Environmental Quality Objectives", results from the literature survey on the background

concentrations in water, soil and sediments in the Netherlands (Crommentuijn *et al.*, 1997), updates of ecological data perfomed in the context of Setting Intergrated Environmental Quality Objectives and other projects, and evaluating procedures to test the coherence of independently derived MPCs for water, soil and air were included when derived. It is important to know about these concentration levels because high concentrations may be harmful to the environment as well as human and animal life.

Table 4.1: Mean (±SD) of soil organic matter (% carbon), pH and the particle size distribution (sand, silt and clay content < 2mm) for each sampling site.

	Organic matter	**pH**	**Particle size distribution**			
Site	**% C**		**>2mm**	**Sand %**	**Silt %**	**Clay %**
1	0.14 ± 0.03^{a}	7.08 ± 0.12^{a}	0.0	68.3	19.0	12.7
2	0.13 ± 0.02^{a}	7.04 ± 0.08^{a}	0.0	76.3	13.8	9.9
3	1.01 ± 0.06^{b}	7.05 ± 0.21^{a}	2.3	45.9	28.0	26.1
4	1.05 ± 0.11^{b}	7.03 ± 0.08^{a}	11.5	28.1	27.1	44.8
5	1.19 ± 0.10^{b}	6.88 ± 0.01^{b}	6.1	26.4	25.4	48.2
6	1.11 ± 0.06^{b}	6.83 ± 0.07^{b}	5.6	32.9	16.0	51.1
7	1.13 ± 0.07^{b}	6.96 ± 0.18^{b}	4.9	24.8	22.8	52.5

$^{a/b}$ similar letters indicate no significant differences ($P > 0.05$) between values and different letters significant differences ($P < 0.05$) between values.

Table 4.2: Summary of heavy metal concentrations (mg/kg^{-1}) at different distances on and away from a platinum mine tailings dam during August 2005 compared to the maximum permissible concentrations (α) of metals from the Netherlands (Crommentuijn *et al.*, 1997) and microbial benchmarks (θ) from Canada (Efroymson *et al.*, 1997).

Sites	Al MB=600$^{\theta}$	Cr MPC= 100$^{\alpha}$ MB=10$^{\theta}$	Cu MPC=40$^{\alpha}$ MB=100$^{\theta}$	Ni MPC=38$^{\alpha}$ MB =90$^{\theta}$
1	4210.5±1.22$^{\theta ab}$	36.08±07.62$^{\theta a}$	81.88±11.90$^{\alpha a}$	95.21±16.49$^{\theta \alpha a}$
2	3306.1±1.01$^{\theta a}$	31.07±6.24$^{\theta a}$	109.67±44.51$^{\alpha \theta a}$	90.21±17.19$^{\theta \alpha a}$
3	6263±1924.70$^{\theta b}$	33.65±16.20$^{\theta a}$	90.49±84.10$^{\alpha a}$	69.33±42.48$^{\theta \alpha b}$
4	5533±1546.50$^{\theta b}$	34.73±29.4$^{\theta a}$	52.63±17.63$^{\alpha b}$	54.61±23.24$^{\theta \alpha b}$
5	5929.8±773.70$^{\theta b}$	10.86±1.44$^{\theta b}$	32.3±16.49$^{\alpha b}$	28.85±3.78$^{\theta c}$
6	5168.3±108.90$^{\theta b}$	8.00±1.36^{c}	57.17±78.94$^{\alpha b}$	24.91±2.45$^{\theta c}$
7	6240.8±646.10$^{\theta b}$	10.74±1.29^{b}	48.35±45.38$^{\alpha b}$	34.06±2.90$^{\theta c}$

$^{a-c}$ similar letters indicate no significant differences (P > 0.05) between values and different letters significant differences (P < 0.05) between values.
MB: microbial benchmarks
MPC: maximum permissible concentrations
α: > MP
θ: > MB

Table 4.3: Summary of heavy metal concentrations (mg/kg^{-1}) at different distances on and away from a platinum mine tailings dam during December 2005 compared to the maximum permissible concentrations (α) of metals from the Netherlands (Crommentuijn *et al.*, 1997) and microbial benchmarks (θ) from Canada (Efroymson *et al.*, 1997).

Sites	Al MB=600$^{\theta}$	Cr MPC= 100$^{\alpha}$ MB=10$^{\theta}$	Cu MPC=40$^{\alpha}$ MB=100$^{\theta}$	Ni MPC=38$^{\alpha}$ MB =90$^{\theta}$
1	3777.6±844.7$^{\theta a}$	28.04±25.1$^{\theta a}$	353.76 ±727.9$^{\theta \alpha a}$	69.13±44.0$^{\alpha a}$
2	3052.3±1212.8$^{\theta a}$	20.98±12.5$^{\theta a}$	45.18±21.1$^{\alpha a}$	56.86±26.2$^{\alpha a}$
3	3636.6±1682.7$^{\theta a}$	22.34±12.2$^{\theta a}$	38.52±20.1^{a}	53.42±16.9$^{\alpha a}$
4	3743.5±2411.7$^{\theta a}$	12.99±5.9$^{\theta a}$	31.71±17.4^{a}	38.24±17.0$^{\alpha a}$
5	3967.3±1588.4$^{\theta a}$	13.77±8.8$^{\theta a}$	32.85±28.3^{a}	41.25±32.2$^{\alpha a}$
6	4278.8±224.9$^{\theta a}$	14.03±12.0$^{\theta a}$	23.73±14.7^{a}	36.71±22.6^{a}
7	3913.3±1447.2$^{\theta a}$	12.72±5.5$^{\theta a}$	29.48±17.2^{a}	39.81±14.1$^{\alpha a}$

$^{a-f}$ similar letters indicate no significant differences (P > 0.05) between values and different letters significant differences (P < 0.05) between values.
MB: microbial benchmarks
MPC: maximum permissible concentrations
α: > MP
θ: > MB

Table 4.4: Summary of heavy metal concentrations (mg/kg^{-1}) at different distances on and away from a platinum mine tailings dam during March 2006 compared to the maximum permissible concentrations (α) of metals from the Netherlands (Crommentuijn *et al.*, 1997) and microbial benchmarks (θ) from Canada (Efroymson *et al.*, 1997).

Sites	Al MB=600$^{\theta}$	Cr MPC= 100$^{\alpha}$ MB=10$^{\theta}$	Cu MPC=40$^{\alpha}$ MB=100$^{\theta}$	Ni MPC=38$^{\alpha}$ MB =90$^{\theta}$
1	1261.0±304.2$^{\theta a}$	10.35±2.08$^{\theta a}$	14.15±2.808^{a}	24.33±5.72^{a}
2	943.6±110.9$^{\theta a}$	7.38±0.826^{b}	11.96±1.197^{a}	18.75±2.79^{b}
3	911.5±89.85$^{\theta a}$	5.77±2.15^{b}	7.42±6.177^{a}	10.24±4.06^{c}
4	836.4±73.23$^{\theta a}$	2.61±0.10^{c}	2.28±0.583^{b}	6.62±0.90^{d}
5	947.5±100.6$^{\theta a}$	2.19±0.24^{d}	3.64±4.001^{b}	6.08±1.53^{d}
6	816.10±44.08$^{\theta a}$	1.56±0.10^{e}	1.44±0.384^{b}	4.16±0.64^{d}
7	790.9±50.55$^{\theta a}$	1.92±0.17^{f}	2.24±1.627^{b}	5.31±0.69^{d}

$^{a-d}$ similar letters indicate no significant differences (P > 0.05) between values and different letters *significant differences (P < 0.05) between values.*
MB: microbial benchmarks
MPC: maximum permissible concentrations
α: > MP
θ: > MB

Table 4.5: Summary of heavy metal concentrations (mg/kg^{-1}) at different distances on and away from the mine tailings dam during May 2006 compared to the maximum permissible concentrations (symbol-α) and microbial benchmarks (symbol-θ) of metals from the Netherlands (Crommentuijn *et al.*, 1997) and Canada (Efroymson *et al.*, 1997).

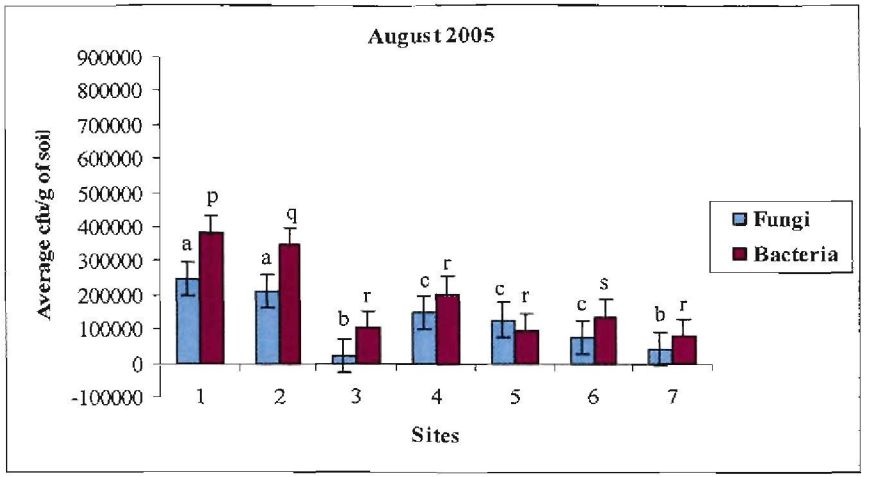
Sites	Al MB=600$^{\theta}$	Cr MPC= 100$^{\alpha}$ MB=10$^{\theta}$	Cu MPC=40$^{\alpha}$ MB=100$^{\theta}$	Ni MPC=38$^{\alpha}$ MB =90$^{\theta}$
1	4103.3±782.5$^{\theta a}$	33.29±4.28$^{\theta a}$	60.71±1.66$^{\alpha a}$	101.86±8.89$^{\alpha a}$
2	3504.4±1316.2$^{\theta a}$	24.61±3.69$^{\theta a}$	56.39±2.23$^{\alpha b}$	84.66±10.32$^{\alpha b}$
3	3420.8±873.4$^{\theta b}$	24.39±13.3$^{\theta a}$	21.95±10.80^{c}	46.72±16.58$^{\theta \alpha c}$
4	3049.1±1610.5$^{\theta c}$	7.92±4.31^{b}	5.9±2.97^{d}	23.91±12.64^{d}
5	3495.9±499.6$^{\theta d}$	6.52 ±1.19^{b}	6.36±1.63^{d}	19.81±4.45^{d}
6	3844.3±572.8$^{\theta d}$	6.08 ±0.86^{b}	5.71±1.04^{d}	21.91±3.48^{d}
7	3474.4±617.6$^{\theta d}$	6.17 ±1.09^{b}	4.82±1.41^{d}	23.88±3.98^{d}

$^{a-f}$ similar letters indicate no sig nificant differences (P > 0.05) between values and different letters significant differences (P < 0.05) between values.
MB: microbial benchmarks
MPC: maximum permissible concentrations
α: > MP
θ: > MB

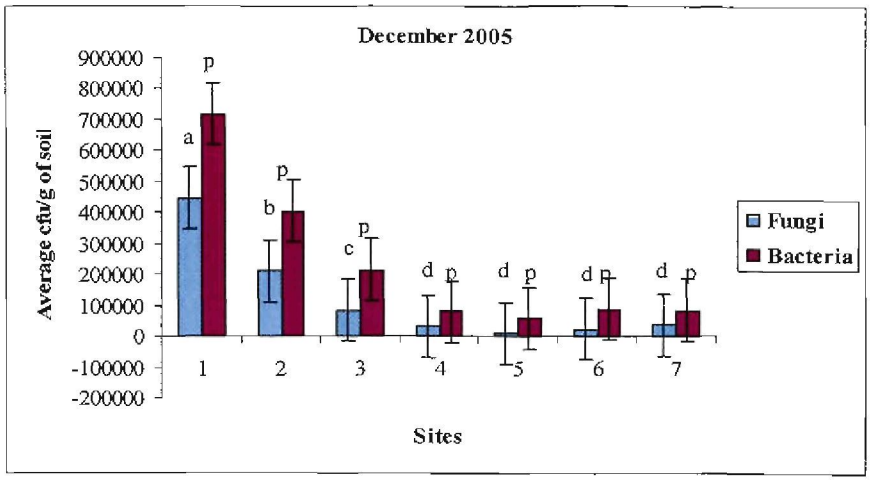
4.2 CULTURABLE BACTERIA AND FUNGI

The levels of bacteria and fungi obtained during different sampling periods are shown in Figure 4.1 (a-d). A table that includes calculated numbers of fungi and bacteria from different sites is shown in Appendix B. When comparing the levels (cfu/g of soil) of bacteria and fungi detected at each of the sites for the different sampling periods, generally no average log differences were observed.

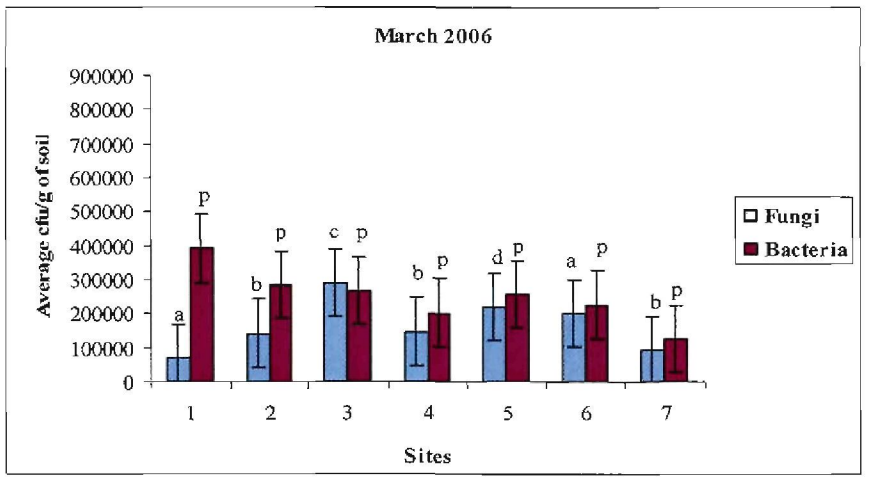
There were significant differences ($P < 0.05$) in fungal levels between sites on and close to the tailings dam for sampling periods during August 2005, December 2005 and March 2006. However, during the May 2006, no significant differences ($P > 0.05$) were observed. On the other hand, no significant differences ($P > 0.05$) in bacterial levels were observed for sampling periods during December 2005, March 2006 and May 2006. The only significant differences in bacterial levels were observed in August 2005. In this case, the sites on the tailings dam (Sites 1 and 2) were significantly different from those close to and further away (Sites 3-7) from the tailings dam.



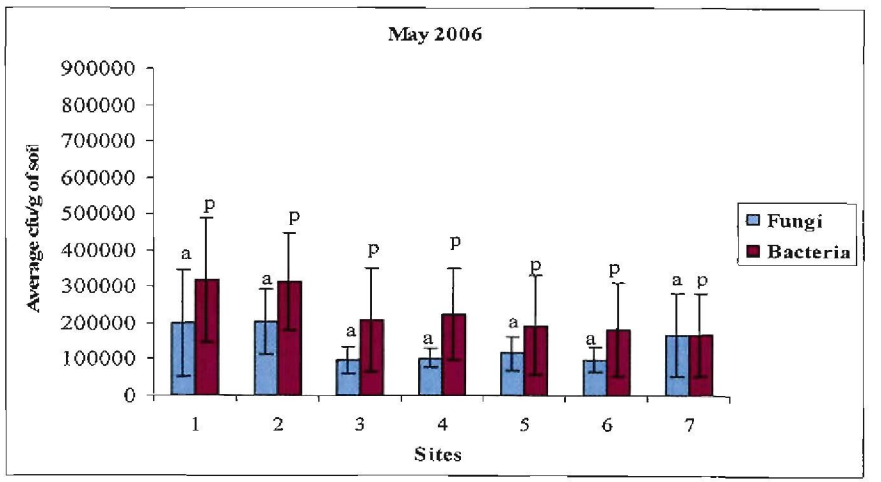
(a)



(b)



(c)



(d)

Figure 4.1: Fungal and bacterial colony forming units per gram of soil collected during (a) August 2005, (b) December 2005, (c) March 2006 and (d) May 2006 from the platinum mine tailings dam. Error bars indicate the standard deviation. a-d indicate that there are no significant differences (P>0.05) between the values with same letters and there is a significant difference (P<0.05) between the values with different letters for fungi. p-s indicate that there are no significant differences (P>0.05) between the values with same letters and there is a significant difference (P<0.05) between the values with different letters for bacteria.

Shannon-Weaver diversity indices based on plating methods were calculated for bacterial as well as fungal diversity at each site for one dry and one wet sampling period. The data used to calculate these were based on the levels of various bacterial and fungal morphotypes observed, at each site, during the study (Section 4.3). Figures 4.2 and 4.3 depict the resultant Shannon-Weaver diversity indices.

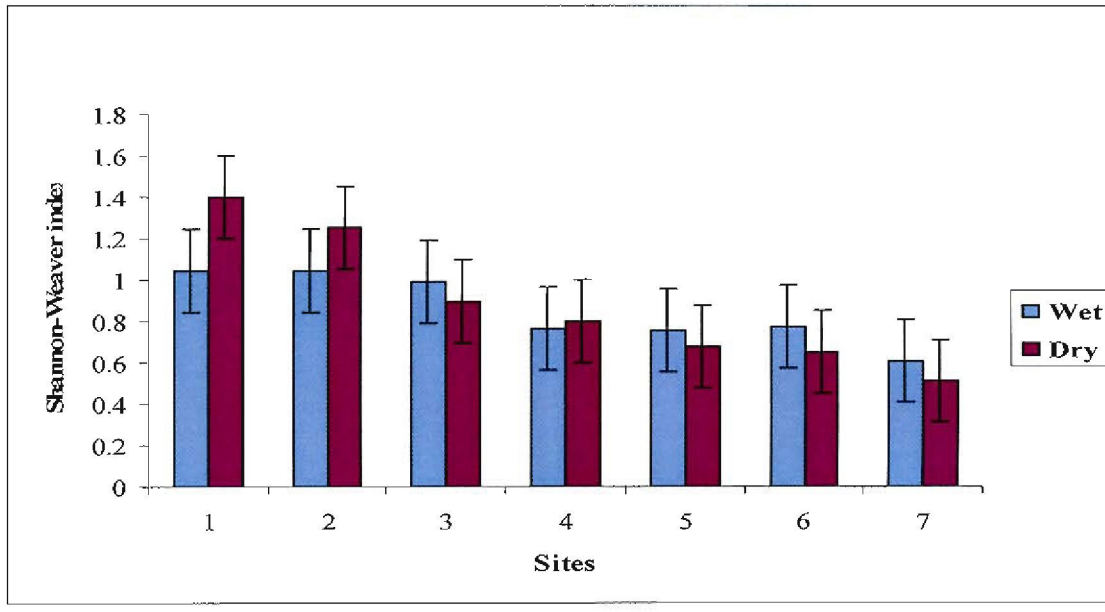


Figure 4.2: Shannon-Weaver diversity indices of wet and dry seasons of bacteria on and away from the mine tailings dam.

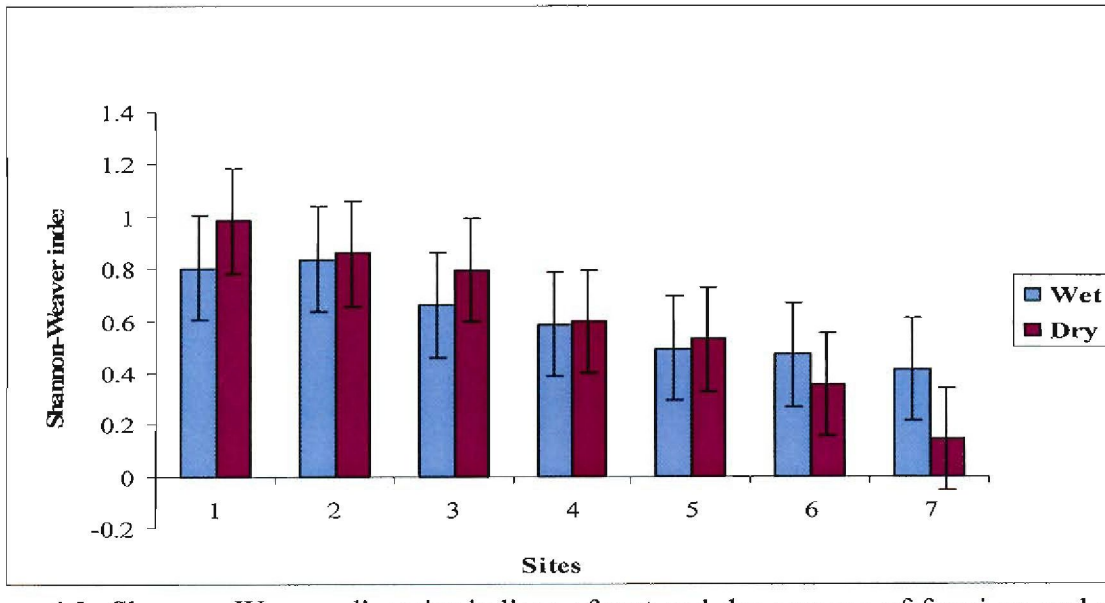


Figure 4.3: Shannon-Weaver diversity indices of wet and dry seasons of fungi on and away from the mine tailings dam.

From Figures 4.2 and 4.3 it is evident that the Shannon-Weaver indices for both bacteria and fungi are greater on and closer to the tailings dam compared to the sites more than 300 m away. This could be the result of the rehabilitation regimes of the tailings dam (Section 3.1). It may thus be that the Shannon-Weaver diversity indices as observed for heterotrophic organisms are artificially high.

Ratios of fungal to bacterial numbers of different sampling periods from different sites on and away from the tailings dam were also calculated and are shown in Figure 4.4. The ratios varied considerably and may thus not be useful for further consideration possibly due to plating methods.

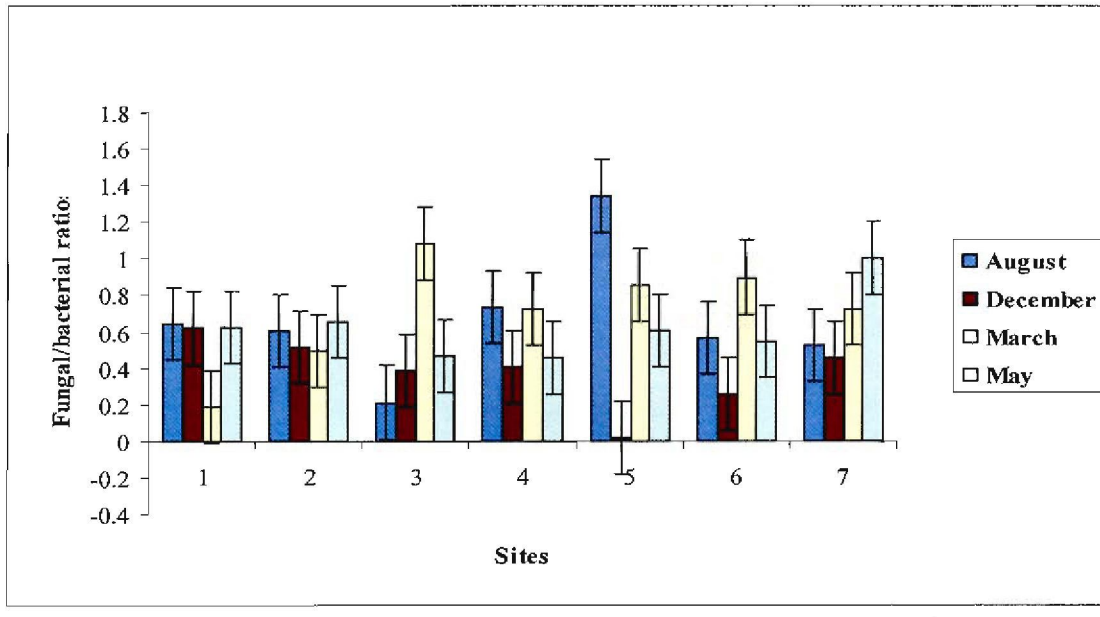


Figure 4.4: Ratios of fungal to bacterial numbers of soil in different seasons from different sites on and away from the mine tailings dam. Error bars indicate the standard deviation.

4.3 SELECTION AND MOLECULAR IDENTIFICATION OF BACTERIA

From the eight bacterial colony types (morphotypes) observed on the 0.1 strength nutrient agar plates (Appendix C), one type that occurred at all the sites was selected and purified by successive streak plating of single colonies onto the same medium. Once purified and the morphology visually checked for consistency, all representatives were Gram stained to ensure that only a single bacterial type was present. These representatives were all Gram-positive bacilli and were used for further analysis.

Furthermore, heavy-metal tolerant species, also isolated from all the sites were obtained from a parallel study done by Daniels (2008 M.Env.Sc dissertation in progress) and were also used for further analysis. These isolates were also Gram-positive bacilli and were identified by Daniels (2008) as *P. lautus*.

The DNA from all the representatives of both species types were extracted using the peqGOLD kit and procedure according to the manufacturer (Section 3.4; Appendix D). The DNA was analysed using spectrophotometric as well as electrophoretic methods (Section 3.5). Electrophoresis results are indicated in Figure 4.5. This is an image of an ethidium bromide stained 1 % (w/v) agarose gel indicating the quality and quantity of the isolated DNA for representatives of *P. lautus* and the other bacterial type.

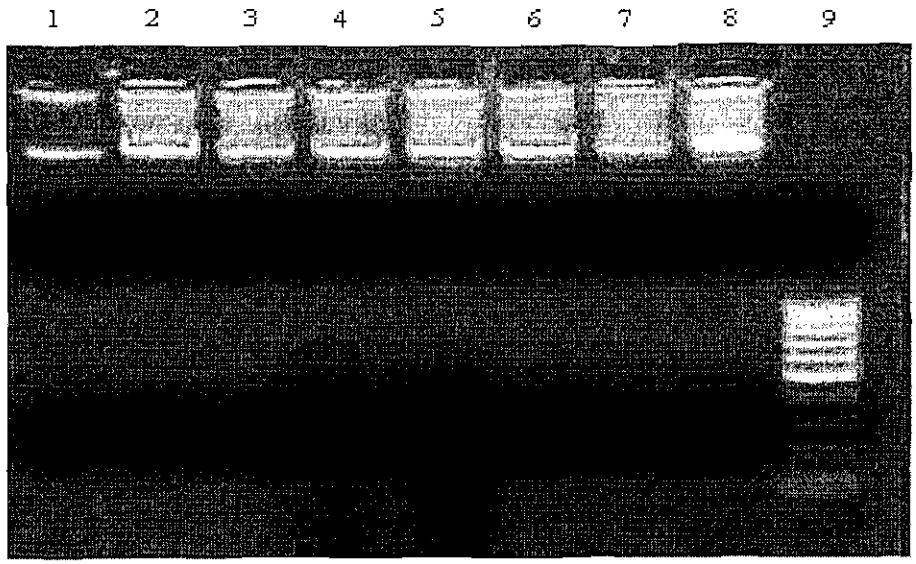


Figure 4.5: An ethidium bromide stained agarose gel (1% agarose) of DNA extracted from pure bacterial cultures (lanes 1 to 8) using peqGOLD bacterial DNA kit. Lane 9 represents DNA markers (100 bp DNA MW marker, Fermentas, US).

From Figure 4.5, it is evident that the DNA was of a consistent good quality and that the yield was considerably high. There were no streaks in any of the lanes indicating that there was no fragmentation during the DNA isolation procedure. Spectrophotometric analysis indicated that the DNA isolated had a A_{260nm}/A_{280nm} ratio between 1.5 and 2.0. This is regarded as DNA of a good quality since pure DNA had a A_{260nm}/A_{280nm} ratio of 1.8 (Csaikl *et al.*, 1998). DNA concentration varied between 49.4 ng/µl and 199.2 ng/µl. The DNA integrity, quality and quantity of the DNA was thus classified as excellent and suitable for PCR based analyses (sequencing, PCR-RFLP and RAPDs).

One hundred nanograms (ng) of DNA was used for each PCR reaction. Figure 4.6 depicts PCR results when the primers GM5F and 907R (16S rDNA) were used. The DNA templates used were from representatives of *B. barbaricus* and *P. lautus* isolated from soil from various sites of the tailings dam.

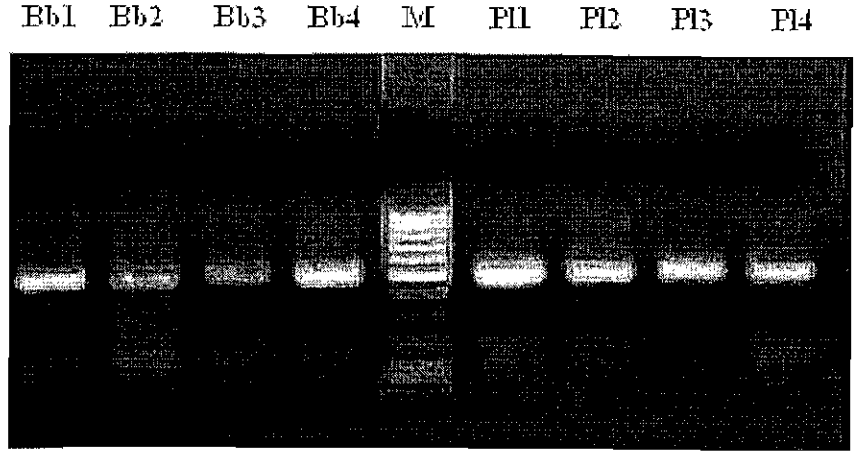


Figure 4.6: Examples of PCR amplification products of 16S rDNA of *B. barbaricus* (Lanes Bb 1 to Bb 4) and *P. lautus* (Pl 1 to Pl 4). In Lanes M is the 100 bp molecular size marker (100 bp DNA MW marker, Fermentas, US).

From the result, it was evident that the PCR amplification worked well and that bands of 550 bp were amplified, as expected. Some of these fragments were sequenced and the data used for BLAST searches in GeneBank, employing Chromas Pro version 2.13 software. Chromatograms of the sequences are shown in Appendix F. From this it is evident that good quality sequences, containing very little or no background noise were obtained. The morphotype isolated in this study was identified as an uncultured *Bacillus* species (94% identity; GeneBank accession number EU196519.1), and the closest cultured one was *B. barbaricus* (94% identity; GeneBank accession number DQ870771.1). It was decided to use the name of the cultured bacterium (*Bacillus barbaricus*; *B. barbaricus*). Thus in the subsequent analysis two species names are referred to namely *B. barbaricus* (from this study) and *P. lautus* (Daniels, 2008). The latter species was the metal tolerant one. Metal tolerance analysis for *B. barbaricus* was not conducted and none assumed.

4.4 PCR-RFLP ANALYSIS AND SDS-PAGE

Using sequencing data for identification is accurate and considered the ultimate method (Barken *et al.*, 2007, Sudhakaran *et al.*, 2008). However, this is relatively expensive. Two arbitrary selected samples were thus used to confirm identification of morphotypes identified as *B. barbaricus*. The

16S rDNA sequence data of these two as well as those for representative of *P. lautus* were firstly subjected to theoretical restriction fragment length polymorphism (RFLP) analysis using the Chromas Pro version 2.13 software. From this it was evident that certain of the enzymes could provide RFLP profiles that were distinctive of the two species, respectively (Table 4.5; Figure 4.7).

Table 4.6: Theoretical and experimental restriction fragment length data indicating the various enzymes that could be used to demonstrate DNA differences and similarities between *B. barbaricus* and *P. lautus*.

Species	**Enzyme**	**Restriction site sequence**	**Expected band sizes**	**Experimental band sizes**
B. barbaricus	*Eco*RI	G↓AATTC	300 bp, 250 bp	350 bp
	*Kpn*I	GGTAC↓C	490 bp	490 bp
P. lautus	*Eco*RI	G↓AATTC	300 bp	250 bp and 350 bp
	*Kpn*I	GGTAC↓C	No digestion	No digestion
	*Ava*I	C↓PyCGPuG	240 bp	No digestion

From Table 4.5 restriction maps could be determined and these are provided in Figure 4.7.

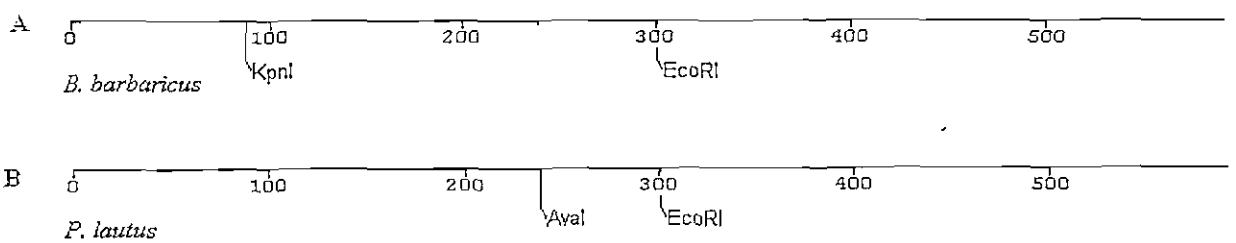


Figure 4.7: Restriction maps of the 16S rDNA sequence indicating the positions where the enzymes, *Eco*RI, *Kpn*I and *Ava*I should restrict the intact PCR amplified DNA. The top map (A) represents *B. barbaricus* and the bottom one (B) *P. lautus*.

To test this, DNAs that were PCR amplified as indicated in Figure 4.6 were then subjected to restriction digests using the various enzymes as indicated in Table 4.5. Images of the fragments resolved on agarose gels (2% w/v) are provided in Figures 4.8 and 4.9. In Figure 4.8 the fragments of the *Eco*RI digests for both species are represented.

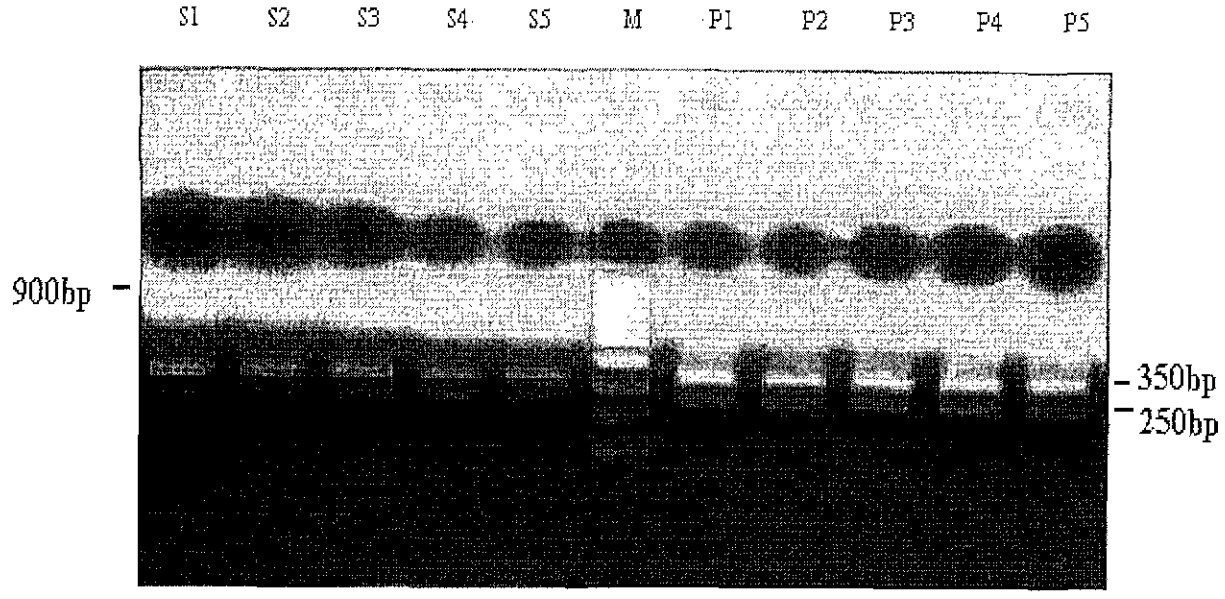


Figure 4.8: RFLP profiles of *B. barbaricus* and *P. lautus* digested with *Eco*R1. The lanes number S1 to S5 indicate representative of *B. barbaricus* isolates. Lane M is molecular size marker (100 bp, Fermentas, US), and lanes numbered P1 to P5 are the profiles of representatives of *P. lautus* isolates.

Considering Lanes S1 to S5 (*B. barbaricus*, Figure 4.8) it appears as if incomplete digestion of the 16S rDNA fragment occurred. However, on closer inspection it is evident that this fragment is 900 bp and may be a non-specific artefact (Figure 4.8). This was absent from all the *P. lautus* isolates. What was clearly evident for all the representatives from these two species is that the digestion of the 550 bp fragment occurred and that 2 fragments of 350 bp and 250 bp were observed (Figure 4.8). This confirmed that the fragment was 16S rDNA but also that the patterns as indicated in Figure 4.8 could be used to distinguish the two bacterial species. These profiles also, to some extent, confirmed the similarity of the isolates that are grouped based on morphological characteristics.

The enzymes *Ava*I and *Kpn*I that were selected for confirming species identification gave inconsistent results. *Ava*I did not digest the *P. lautus* PCR amplified 16S rDNA fragments as expected (Table 4.5). On the other hand, *Kpn*I digested some of the *B. barbaricus* PCR amplified 16S rDNA fragments completely and others partially (Figure 4.9; Lanes S1 to S7). This enzyme also digested *P. lautus* DNA partially (Figure 4.9; Lanes P1 to P7 and P9). Partial digestion of the

P. lautus DNA could be explained by the observation that in the region 90 to 120 bp of the *P. lautus* sequence (Appendix E) base conformations occurred that resembles the restriction site of *Kpn*I. However, from the results it was evident that the restriction patterns observed for the two restriction enzymes were sufficiently different for the two selected species to be used for further analysis.

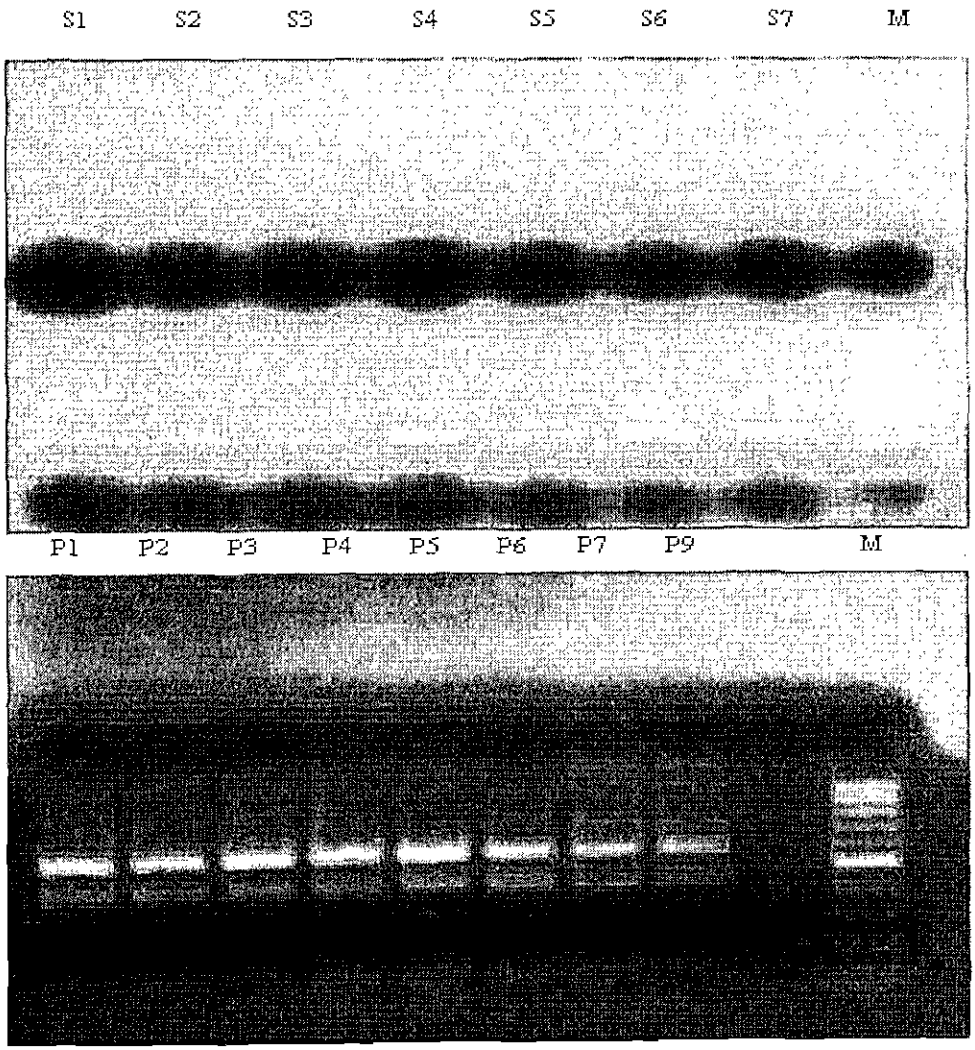


Figure 4.9: Restriction enzymes of *B. barbaricus* and *P. lautus* digested with *Kpn*I. Lanes S1 to S7 indicate *B. barbaricus* isolates, and Lanes P1 to P7 indicate *P. lautus* isolates, P9 is a duplicate of Site 7 and M is a molecular size marker (100 bp, Fermentas, US) for both gels.

To further review the differences between *P. lautus* and *B. barbaricus* soluble proteins of these species representing different sites were extracted and characterized by SDS-PAGE. Before proteins were loaded on the gel, concentrations were determined using the RC protein Assay kit as described in Section 3.8. Concentration ranged from 30 μg/ml to 50 μg/ml. The standard curve is shown in Appendix G. Samples were all diluted and 20 μg was loaded onto SDS-PAGE gels.

Images of Coomassie stained gels are depicted in Figures 4.10 and 4.11. The profiles of the *B. barbaricus* (Figures 4.10) were clearly different from *P. lautus* (Figures 4.11) and confirmed that these were different species but also the grouping of these species was probably correct.

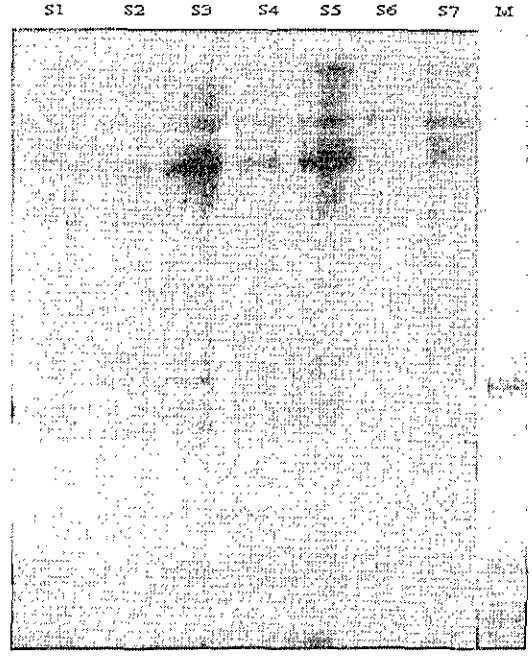


Figure 4.10: Protein profiles of *B. barbaricus* extracted using a sodium azide method. S1: Site 1-Sample 1; S2: Site 2-Sample 1; S3: Site 3-Sample 1, S4: Site 4-Sample 1, S5: Site 5-Sample 1, S6: Site 6-Sample 1, S7: Site 7-Sample 1, and M is the molecular weight marker.

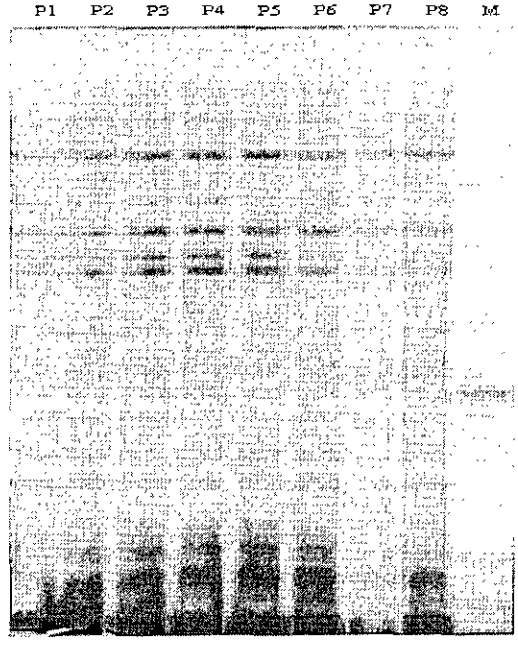


Figure 4.11: Protein profiles of *P. lautus* extracted using a sodium azide method. P1: Site1-Sample1, P2: Site 1-Sample 3, P3: Site 1-Sample 6, P4: Site 3-Sample 1, P5: Site 3-Sample 3, P6: Site 3-Sample 6, P7: Site 7-Sample 1, P8: Site 7-Sample3 and M is the molecular weight marker.

The protein profiles of the representatives from different sites, those on and close to the mine tailings dam on the one hand, and those further way were also scrutinised for differences that could potentially be linked to impact of the soils. In both cases (Figures 4.10 and 4.11) no such clear differences were evident.

Both PCR-RFLP and SDS-PAGE methods are less costly than DNA sequencing. These methods are also relatively quick to perform. The results in this section demonstrated the potential of using data from these two methods for classifying (or grouping) of bacterial isolates that have similar morphological characteristics.

4.5 RAPD FINGERPRINTING

The goal with this part of the study was to evaluate the potential of RAPD fingerprinting in the assessment of DNA damage occurring in bacteria isolated from sites on and around the platinum mine tailings dam. The two species selected for this purpose were *P. lautus* and *B. barbaricus*. Similarity and differences were confirmed using DNA sequencing and molecular profiling (PCR-RFLP and SDS-PAGE) techniques. For each of the two species, three representatives from five of the seven sites and three primers (OPA-01; OPA-02; OPB-01, Operon Technologies) were used to produce RAPD fingerprints (Figures 4.12 to 4.17). The profiles were numerically analysed using GeneTools, MicroSoft Excel and Statistica software (Section 3.8).

From the images of the three primers (OPA-01, OPA-O2 and OPB-01; Figures 4.11 to 4.16) marked differences were observed in the banding patterns. When the profiles of the two species for the same primers were compared, these appeared to be generally quite different. The number of the amplified fragments varied from 5-18 for *B. barbaricus* and 5-16 for *P. lautus*, per primer per sample (Figures 4.12 to 4.17; Appendix H). RAPD profiles generated by the selected primers were highly reproducible with consistent fragment patterns. There were greater variations in the RAPD profiles of *P. lautus* compared to *B. barbaricus*. The profiles of these two species were distinctly

different and this lead to the conclusion that RAPDs may be useful for typing of *B. barbaricus* and *P. lautus* isolated from platinum tailings dams.

A total of 630 bands were analysed. For the analysis sites on and close to the tailings dam (Sites 1, 2 and 3) were grouped separately from those further away (Sites 5 and 7). Table 4.6 is a summary of the presence/absence data for the two species (*P. lautus* and *B. barbaricus*) and the various primers. Scoreable bands were between 2955.6 bp and 63.3 bp. When the total and average number of bands occurring at the sites on and around the tailings dam (Sites 1, 2 and 3) are compared to those further away (Sites 4 and 5) no significant differences ($P>0.05$) were observed in the profiles of both species. How ever, when the present/absent data is combined with band intensity data (Appendix I) then significant differences ($P<0.05$) are observed. The usefulness of such differences warrants further investigation and should be considered in future studies.

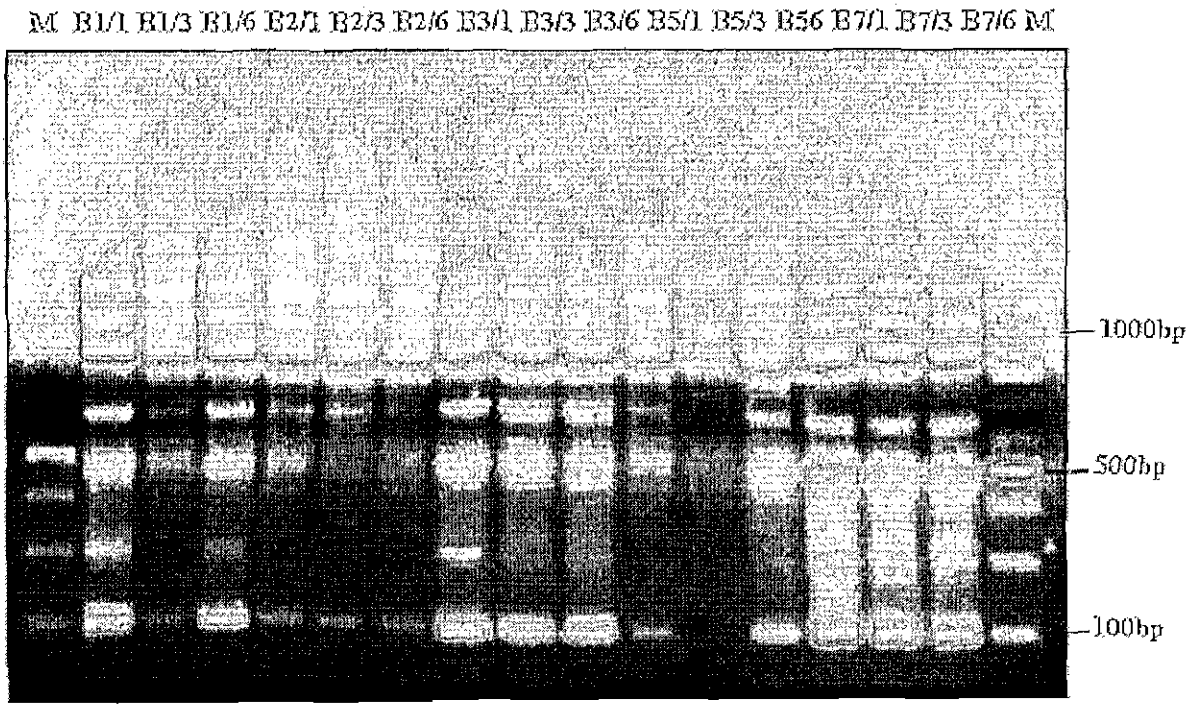


Figure 4.12: RAPD fingerprinting patterns of *B. barbaricus* isolates using primer OPA-01. M is the molecular size marker. The origin of the various isolates are indicated by the following (B = *B. barbaricus*; 1/1 = Site 1, sample site 1. Thus B2/3 = *B. barbaricus* isolated from Site 2, sample site 3 etc.

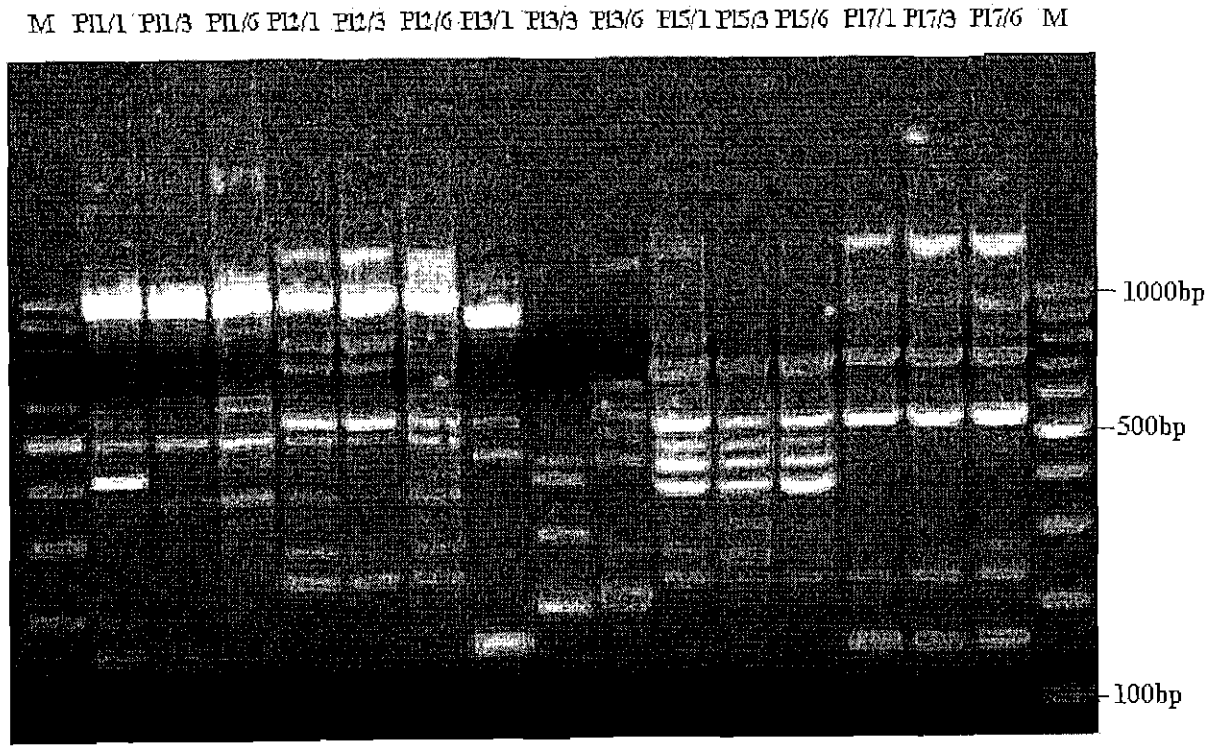


Figure 4.13: RAPD fingerprinting patterns of *P. lautus* isolates using primer OPA-01. M is the molecular size marker. The origin of the various isolates are indicated by the following (Pl = *P. lautus*; 1/1 = Site 1, sample site 1. Thus Pl 2/3 = *P. lautus* isolated from Site 2, sample site 3 etc.

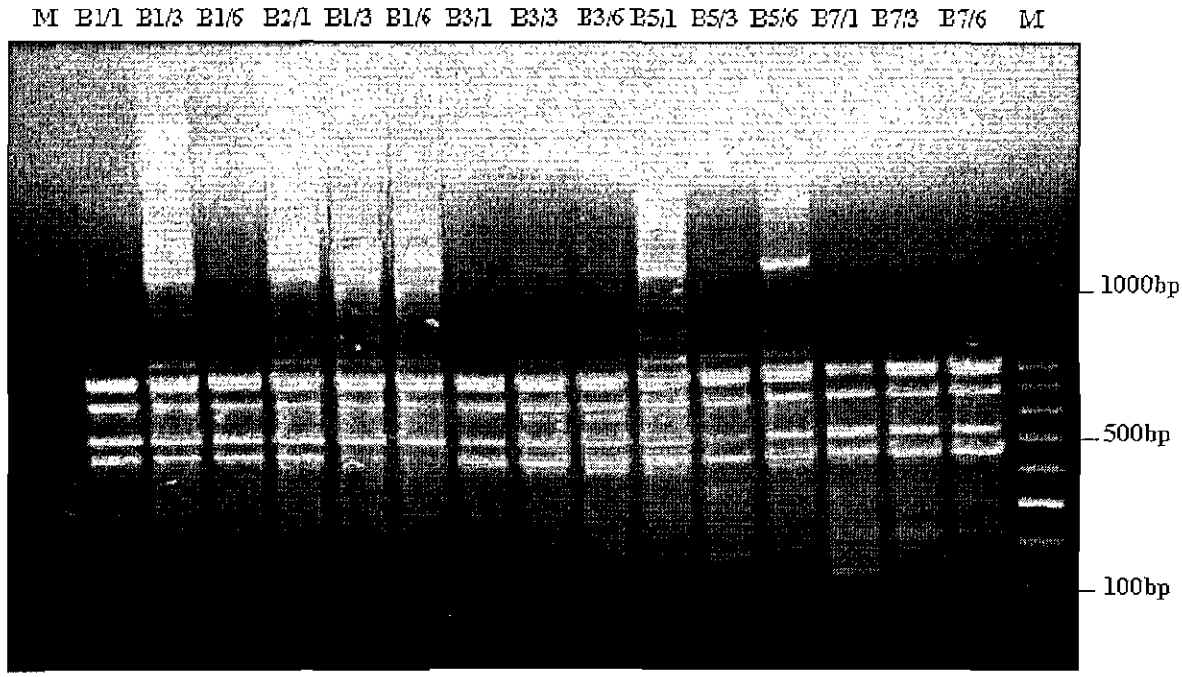


Figure 4.14: RAPD fingerprinting patterns of *B. barbaricus* isolates using primer OPA-02. M is the molecular size marker. The origin of the various isolates are indicated by the following (B = *B. barbaricus*; 1/1 = Site 1, sample site 1. Thus B2/3 = *B. barbaricus* isolated from Site 2, sample site 3 etc.

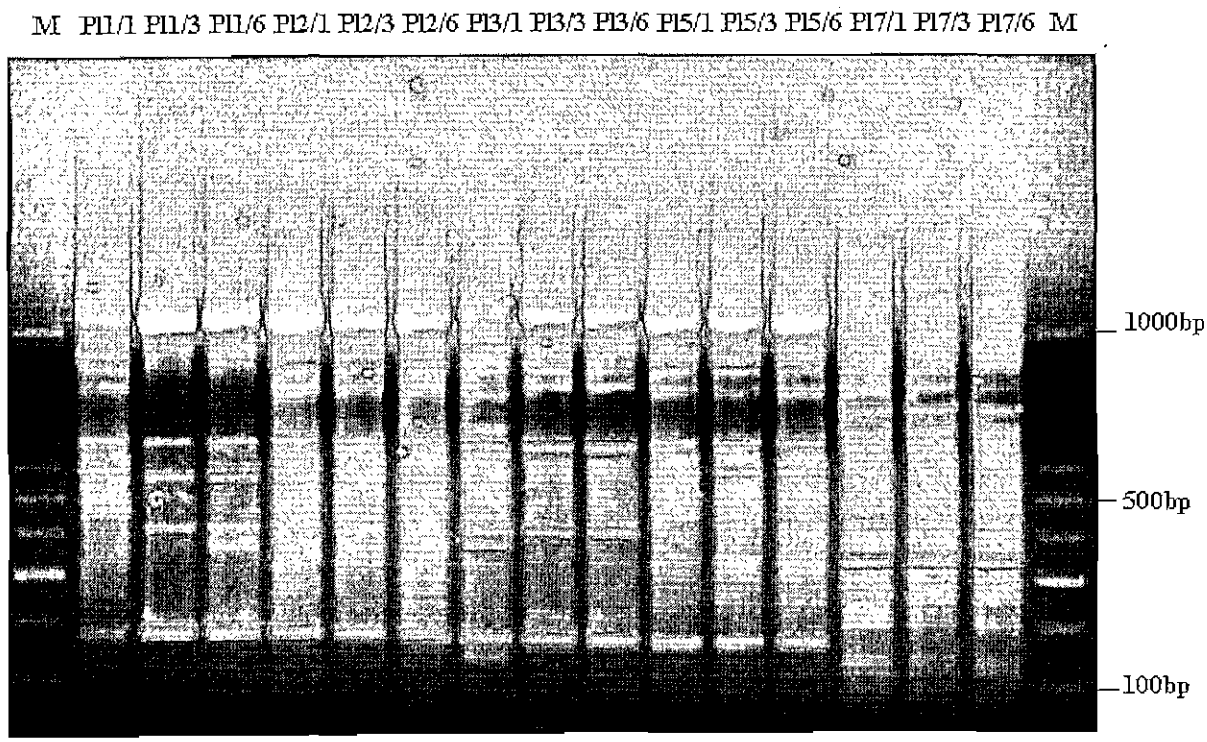


Figure 4.15: RAPD fingerprinting patterns of *P. lautus* isolates using primer OPA-02. M is the molecular size marker. The origin of the various isolates are indicated by the following (Pl = *P. lautus*; 1/1 = Site 1, sample site 1. Thus Pl 2/3 = *P. lautus* isolated from Site 2, sample site 3 etc.

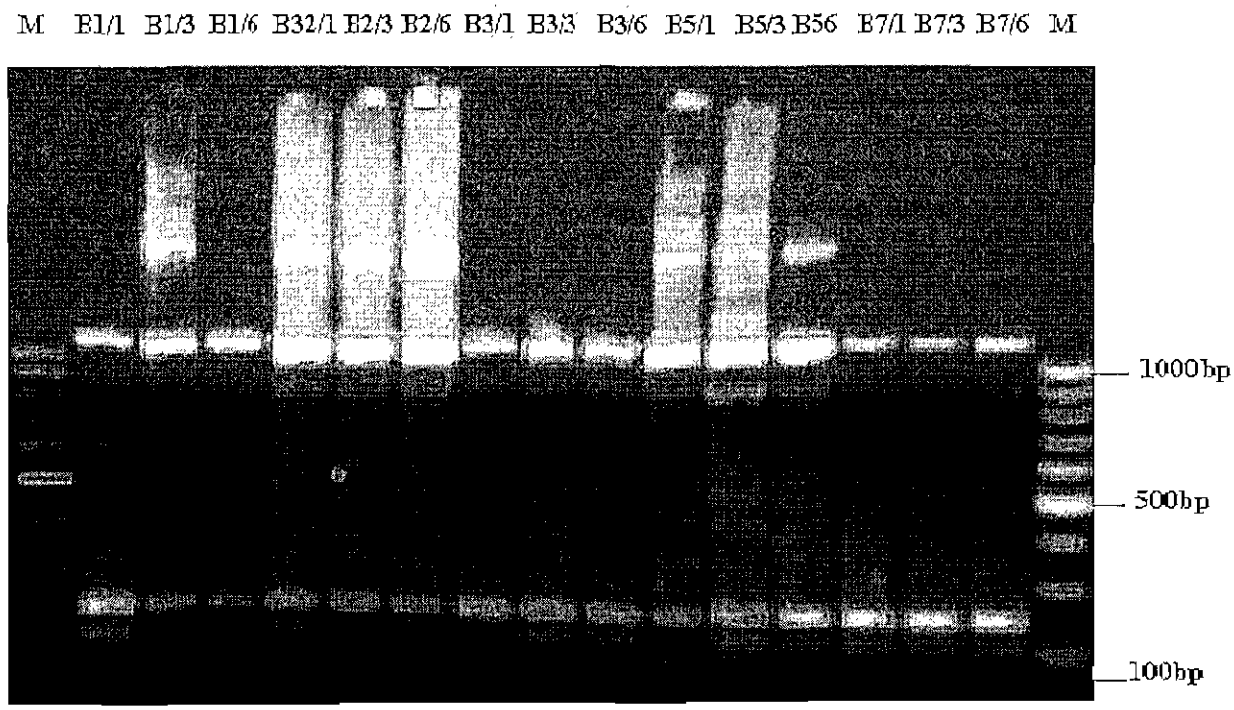


Figure 4.16: RAPD fingerprinting patterns of *B. barbaricus* isolates using primer OPB-01. M is the molecular size marker. The origin of the various isolates are indicated by the following (B = *B. barbaricus*; 1/1 = Site 1, sample site 1. Thus B2/3 = *B. barbaricus* isolated from Site 2, sample site 3 etc.

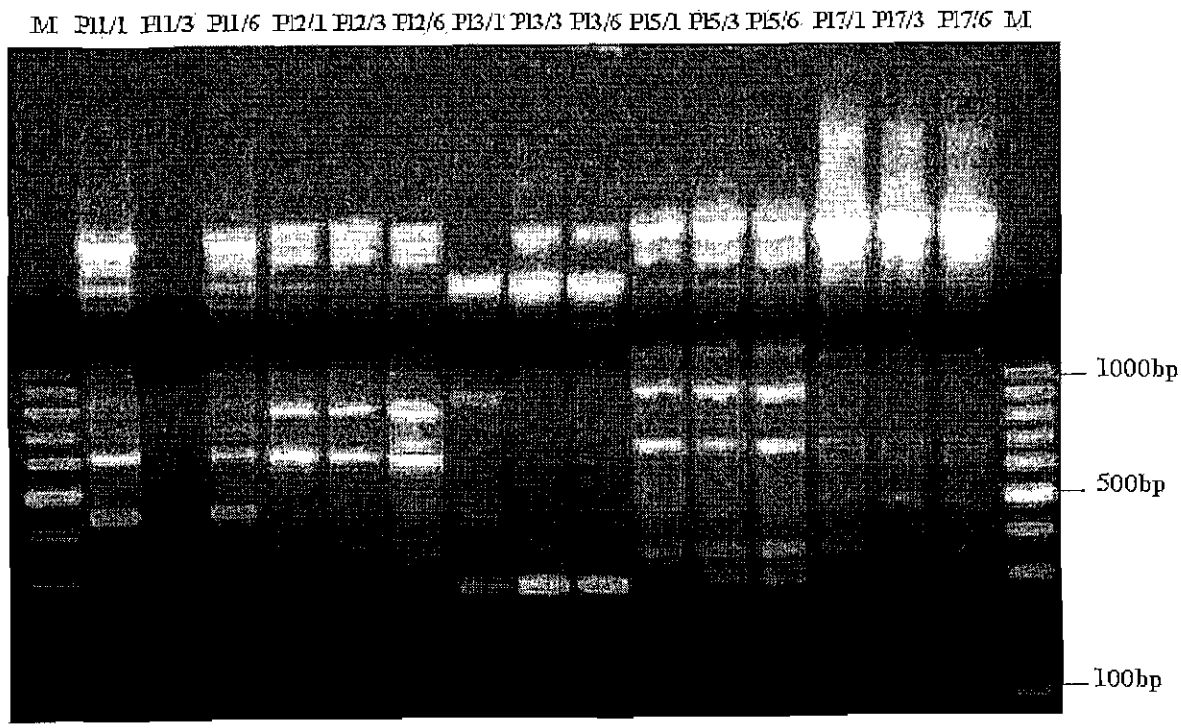


Figure 4.17: RAPD fingerprinting patterns of *P. lautus* isolates using primer OPB-01. M is the molecular size marker. The origin of the various isolates are indicated by the following (Pl = *P. lautus*; 1/1 = Site 1, sample site 1. Thus Pl 2/3 = *P. lautus* isolated from Site 2, sample site 3 etc.

Table 4.7: A summary of the present/absence RAPDS profile data presented in Appendix H. The total numbers of bands as well as the number of polymorphic bands were calculated from this data. However, the size range was determined using Gene Tools software.

Primers	Total No. of bands at Site 1,2 and 3 (Average per site)	No. of polymorphic bands (Average per site)	Size range of bands (bp)	Primers	Total No. of bands at Sites 5 & 7 (Average per site)	No. of polymorphic bands (Average per site)	Size range of bands (bp)
B. barbaricus							
OPA-01	76 (25.3)	4 (1.3)	1561.6 – 205.6	**OPA-01**	40 (20.0)	4 (2.0)	1499.6 – 200.0
OPA-02	69 (23.0)	23 (7.7)	2803.9 – 617.0	**OPA-02**	27 (13.5)	18 (9.0)	2955.6 – 203.8
OPB-01	37 (12.3)	12 (4.0)	2844.8 – 225.4	**OPB-01**	19 (9.5)	7 (3.5)	2212.7 – 211.3
Total	182 (60.7)	39 (13.0)		**Total**	86 (43.0)	29 (14.5)	
P. lautus							
OPA-01	69 (23.0)	12 (4.0)	1561.6 – 197.7	**OPA-01**	47 (23.5)	20 (10.0)	1735.1 – 151.2
OPA-02	88 (29.3)	9 (3.0)	1284.3 – 63.3	**OPA-02**	71 (35.5)	12 (6.0)	1242.7 – 133.8
OPB-01	42 (14.0)	21 (7.0)	2109.1 – 277.8	**OPB-01**	45 (22.50	10 (5.0)	2323.0 – 296.7
Total	199(66.3)	42(14.0)		**Total**	163 (81.5)	42 (21)	

4.6 CLUSTER ANALYSIS OF *P. lautus* AND *B. barbaricus* RAPD PROFILES

The presence/absence of bands and the band intensity data (Appendix H) generated by all the primers for *P. lautus* and 15 *B. barbaricus* were subjected to cluster analysis using Statistica Software. RAPD data were subjected to Ward's algorithm and Euclidean distance analysis. The resultant dendrograms are depicted in Figure 4.18.

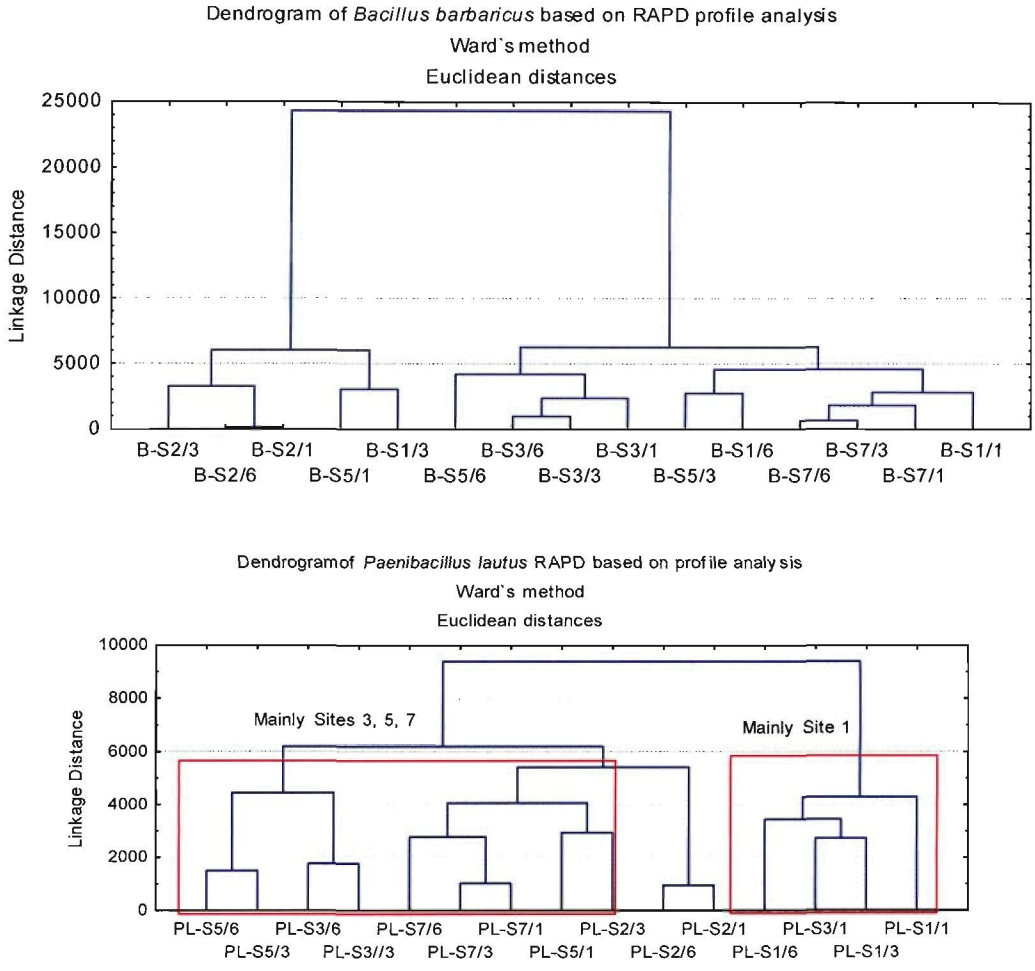


Figure 4.18: Dendrograms of the relationships between the isolates from the two test species obtained from various sites on, close to and a distance away from a platinum tailings dam.

From Figure 4.17 it is evident that the RAPD profiles of *B. barbaricus* did not display any characteristics that could separate these individuals into site specific groups particularly based on distance from platinum mine tailings dam, indicating potential effects of heavy metal pollution. However, when the dendrogram generated for the RAPD profiles of *P. lautus* was analysed for relationships, it was evident that two main clusters were observed (Figure 4.18). On the one hand a

cluster consisted mainly of individuals from on the tailings dam (from Site 1), one consisted of individuals further away (Sites 3, 5, 7). Inclusion of more individuals from the various sampling sites as well as more primers may enhance the resolution of this analysis procedure. The clustering of the individuals from Site 1 with those from the sites more than 300 m away could not be explained. However, the result demonstrates the potential of using RAPD profiling in studying impacts of pollution on certain habitats.

4.7 SUMMARY OF RESULTS

Total heavy metal concentrations from all the soils were measured and observed to be inconsistent between the sites during dry periods than wet periods. Concentrations of certain metals were significantly higher ($P < 0.05$) at sites on and closer to the tailings dam compared to sites further away. Metal concentrations were also compared to the Canadian microbial benchmarks and the Netherlands maximum permissible concentrations, and some of the metals (Cu, Cr and Ni) were higher than the screening benchmarks as well as MPC. Plate count analysis revealed that fungi and bacteria were not suppressed by high concentrations of heavy metals. Fungal levels were generally significantly higher ($P < 0.05$) on the tailings dam (Sites 1 and 2) compared to the sites closer and further away (Sites 3-7). Shannon-Weaver diversity indices of fungal and bacterial populations were significantly higher ($P < 0.05$) at sites on and closer to the tailings dam than the sites further away. Ratios of fungal to bacterial levels from all the sampling periods were inconsistent and not used for any further analyses. Bacterial isolates from different sites on and away from the tailings dam were purified and clustered using colony morphology data. These were subjected to sequencing and PCR-RFLP of 16S rDNA fragments as well as SDS-PAGE analysis. Some of the isolates were identified as *Paenibacillus lautus* and *Bacillis barbaricus* using BLAST searches of 16S rDNA sequences. *P. lautus* and *B. barbaricus* representatives from all the sites were then subjected to RAPDs analysis. The results supported the classification/clustering of the isolates based on colony morphology, PCR-RFLP of 16S rDNA fragments as well as SDS-PAGE.

Furthermore, RAPDs analysis provided evidence of potential genotoxic effects of heavy metal in soil on a metal tolerant bacterial species (*P. lautus*).

CHAPTER 5

GENERAL DISCUSSION

5.1 SOIL CHEMICAL CHARACTERISTICS

The first objective of the study was to determine some chemical characteristics with reference to pH and heavy metal concentrations of soil and tailings on selected distances away from a platinum mine tailings dam. Data of rainfall figures was obtained from the S.A. Weather, (2007). Futhermore, data provided in Section 4.1 showed that metal concentrations varied between the dry and wet periods and between sites. A study by Garcia-Delgado *et al.* (2007) on seasonal and time variability of heavy metals in sludges showed significant ($P < 0.05$) seasonal differences existed for Cd, Cr, Cu, Ni, Pb and Zn concentrations. The concentrations were significantly higher ($P < 0.05$) during the summer period (wet) when compared to the concentrations of the winter period (dry). These authors, however, did not provide any rainfall data. Even though this example is from a study on sludges it demonstrates that seasonal variations of metal concentrations may occur and may possibly be affected by environmental factors such as rainfall.

Certain metal concentrations (Cr, Cu and Ni) were higher at the sites on and closer to the tailings dam (Sites 1, 2 and 3) compared to those sites more than 300 m away (Sites 4 to 7). These results show that pollution on the tailings dam was greater than the sites further away. Statistically, metal concentrations measured from different sampling periods were significantly different ($P < 0.05$) between the sites for each metal. Maboeta *et al.* (2005) also did a study at the platinum mine tailings dam and found similar results to the present study. In a study by Boularbah *et al.* (2006), sites on and close to the mine tailings dams from 5 different mines (a Cu and Mo mine, 2 polymetallic mines, Cu mine and a Mn mine) were investigated in Southern Morocco. From all these mines, heavy metal concentrations were higher on and close to a mine tailings compared to the sites further away.

Metal concentrations from the mine tailings dam were compared to the Netherlands maximum permissible concentrations (Crommentuijn *et al.,* 1997) and the Canadian microbial benchmarks (Efroymson *et al.,* 1997) as discussed in Section 4.1. This was done because South Africa does not have soil screening benchmarks in contrast to existing water quality benchmark proposed by the Departtment of Water Affairs and Forrestry of South Africa (1996). The results from this study demonstrated that most of the metals (Al, Cu and Mn) present in soils were higher than the Canadian microbial benchmarks. A similar trend was also observed for the Netherlands maximum permissible concentrations. If these benchmarks were to be used "as is" for South African conditions (disregarding aspects like climate, soil chemistry, physical attributes of soil, etc.) it could be concluded that the tailings dam would have an effect on soil microbes up to 1350m and further. These metals may end up in ground water, crops and vegetation causing ecosystem problems. According to Giller *et al.* (1998), as well as Liao and Xie, (2006) this might impact human as well as animal health. The tailings dam in this study was near a residential area and heavy metal concentrations (Al, Cr, Cu and Ni) were very high compared to the Canadian microbial benchmarks. Recent studies by Drago *et al.* (2007) and Lima *et al.* (2007) demonstrated the impacts of metals such as Al, Cu, Zn and Fe on neurological processes, particularly development of disease states such as Alzheimer's disease. The study of Lima *et al.* (2007) demonstrated the effects that Al could have on cellular components (DNA) and processes (mitosis). The high levels of metals reported in this study, if leached from the soil, could thus be hazardous to surrounding communities if they land in the groundwater that are used for drinking purposes.

According to the Canadian microbial benchmarks, levels from our study are very high and are seriously harmful to the environment and something needs to be done with those levels. However, we don't know how serious because these are old studies and there are not many studies done for heavy metal concentrations and the determination of soil benchmarks, especially in South Afica. These values might however make a valuable contribution to critical baseline data needed to determine reliable benchmarks.

5.2 DIVERSITY OF BACTERIAL AND FUNGAL ISOLATES: PLATING METHODS

Plate count analysis revealed that the numbers of culturable bacteria and fungi were not suppressed by high concentrations of metals found in soils along the gradient from the tailings dam. The highest levels of bacteria and fungi were observed at the sites that mostly had highest metal concentrations. This could mean that most of the bacteria and fungi isolated from these sites may be tolerant to high concentrations of heavy metals. Statistical analysis (one-way ANOVA) indicated that the levels of bacteria and fungi at the sites on and closer to the tailings dam were in some cases significantly different ($P < 0.05$) from those sites further away.

Several studies (Nahmani and Lavelle, 2002; Feris *et al.*, 2004a;b; Concas *et al.*, 2006; Liao and Xie, 2006; Wang *et al.*, 2007b) demonstrated the negative impact of high concentrations of metals on soil microbial community structure and function. Most of these studies were based on culture-independent methods, which included phospholipids fatty acid (PLFA), denaturing gradient gel electrophoresis (DGGE) and single strand conformation polymorphism (SSCP). The impacts of high metal concentrations were either reduced biomass or fungal:bacterial ratios or species diversity or a combination of these parameters. Since culture-dependent methods only select for those culturable microorganisms, the levels and diversity of bacteria detected is lower than the culture-independent methods. It is thus logical to expect that a very low number of bacterial types that are tolerant to metals are detected in culture-dependent approaches as described by Piotrowska-Seget *et al.* (2005). In a present study conducted by Daniels (2008), it was demonstrated that the mine tailings dam investigated was dominated by metal-tolerant species (*P. lautus*). Similar habitats dominated by one species (e.g. metal resistant *Bacillus cereus* or *Enterobacter cloacae*) were reported from Pb-Zn tailings in Beijing, China (Hu *et al.*, 2007).

Shannon-Weaver diversity index (Figure 4.2 and 4.3) for fungi and bacteria were high at the sites on and close to the tailings dam compared to the sites further away (+300 m). On the other hand, ratios of fungal to bacterial levels (Figure 4.4) varied between the sites and sampling periods. The

method used to determine ratios of fungal to bacterial numbers from the present study was evaluated for usefulness, but did not work. However, the Shannon-Weaver diversity index approach evaluated in this study was useful and could potentially be used in other studies when microbial community dynamics in polluted environments are determined.

5.3 SDS-PAGE, DNA SEQUENCING AND PCR-RFLP TO IDENTIFY SELECTED BACTERIAL ISOLATES.

When the 16S rDNA of each of the isolates was amplified by PCR, an amplification fragment of 550 bp was obtained. Representative fragments were sequenced by Inqaba Biotech and the details of the results were provided in Section 4.6. Using Chromas Pro. Version 2.13 software and BLAST searches for two representatives, both were identified as *B. barbaricus* (94%) homology. Täubel *et al.* (2003) also indicated that a relatively low 16S rDNA sequence homology of *B. barbaricus* (94.2 to 94.6%) in relation to other *Bacillus* species existed. This was similar to what was observed in the present study. *P. lautus* was identified in the study of Daniels, (2008) and sequences showed 98% identity. In a study by Ogawa *et al.* (2007), the closest strain of *P. lautus* was 81.5 to 87.3% similar to sequences of the isolated strains. In the present study, representatives of these two species, isolated form Sites 1, 2, 3, 5 and 7 (three from each site) were used for subsequent analysis. Partial sequencing of the 16S rRNA gene is normally good enough to identify bacteria. It has been shown that sequencing of the 5` end of the 16S rRNA may be sufficient for this purpose (Barken *et al.*, 2007). However, no consensus definition exists when defining genus or species, using the 16S rRNA sequence (Barken *et al.*, 2007). When identifying *B. barbaricus* in this study, there was some background in the sequences. Several examples were provided in Section 2.3 to indicate successful application of 16S rDNA sequence data for species identification.

All *B. barbaricus* and *P. lautus* representative s were subjected to restriction enzyme analysis (RFLP) to confirm the identity of the representatives of these two species. Restriction analysis was carried out on the PCR amplified 16S rDNA of each representative using enzymes *Eco*RI, *Kpn*I and

*Ava*I. Firstly, RFLP analysis successfully provided support for the deduction that the PCR amplicons were 16S rDNA fragments. The RFLP patterns obtained were compared and were found to be diagnostic for the two species. However, in the case of *Ava*I (Appendix F), the PCR fragment for both *P. lautus* and *B. barbaricus* were not digested. This was contrary to the theoretical data that indicated *Ava*I should digest *P. lautus* and not *B. barbaricus* 16S rDNA PCR fragments. In a study by Coelho *et al.* (2003), PCR-RFLP of the 16S rDNA and 23S rDNA was used in the characterization of nitrogen-fixing *Paenibacillus* species. It was observed that the 16S rDNA produced more genotypes than those of the 23S rDNA. This showed that the 16S rDNA is useful for discriminating between *Paenibacillus* species. Lopez and Alippi, (2007) also used 16S rDNA fragments digested with *Alu*I and *Taq*I to determine diversity of *Bacillus cereus* species isolated from honey. The DNA patterns were useful in discriminating the *Bacillus cereus* from other *Bacillus* species. Furthermore, with the support from another genotyping method, it was also demonstrated that a high degree of diversity existed amongst the *B. cereus* isolates. This suggested that the contamination in the honey was from various sources such as pollen, equipment and dust. These examples thus demonstrated, provide the usefulness of 16S rDNA RFLP data and support for the approach in the present study.

In the characterization of *Bacillus* species from medieval wall paintings, Täubel *et al.* (2003) firstly used colony morphology to group isolates. The SDS-PAGE protein patterns were then compared and it was established that some of the *Bacillus* spp. isolates had similar patterns. Furthermore, phenotypic and biochemical data, rDNA sequencing and fingerprints of genomic DNA demonstrated that some of the isolates represented a novel species for which the name *Bacillus barbaricus* spp. *nov.* was proposed. In the present study, 16S rDNA sequences, PCR-RFLP and SDS-PAGE data could discriminate between the two genera used. The observation that the closest relative of the *Bacillus* spp. isolated in this study is *B. barbaricus* is probably valid. Further analysis in this regard should be conducted in which ATCC representatives of other *Bacillus* spp. are included. For the purpose of this dissertation, the name *B. barbaricus* was maintained.

The results of this work report the possibility of using protein profiles in grouping different bacterial species. A c omparison of *P. lautus* and *B. barbaricus* SD S-PAGE profiles showed differences for the two species, but there were similarities between representatives of the same species isolated from different sites. This result was supported by the PCR-RFLP data. Konecka *et al.* (2007), successfully applied SDS-PAGE and RAPD fingerprinting to answer questions about the diversity of *Bacillus thuringiensis* during an epizootic episodes in *Cydia pomonella* L. In this case, the SDS-PAGE results also demonstrated the potential of using protein profile analysis in epidemiological investigations of infections caused by *B. thuringiensis.*

5.4 POTENTIAL OF RAPD FINGERPRINTING TO ASSESS DNA DAMAGE IN BACTERIA ISOLATED FROM THE TAILINGS DAM

To study the degree of genetic variability due to heavy metal pollution, *P. lautus* and *B. barbaricus* isolates were subjected to random amplified polymorphic DNA (RAPD) fingerprinting. Representatives of these species were selected to represent sites on and close to the tailings dam and sites 850 m and 1350 m away. Soils at sites on and close to the tailings dam had higher levels of several heavy metals. DNA of these isolates was amplified with primers OPA-01, OPA-02 and OPB-01 as described in Section 3.7. OPA-02 an OPB-01 patterns were the least informative for *B. barbaricus* but the same was not true for *P. lautus*. The RAPD profiles of both *P. lautus* and *B. barbaricus* isolates showed banding patterns that were species specific. A degree of site specificity was also observed in the profiles of the individual primers. Numerical analysis of individual primers was not sufficiently discriminatory. However, a combination of profile data of all primers was more informative. This data were useful for discriminating isolates from the various sites, particularly for *P. lautus.* The approach of combining RAPD profile data for numerical analysis is not uncommon. Konecka *et al.* (2007) used combination of profiles from different primers to distinguish *Bacillus thuringiensis* strains, and the results were satisfactorily discriminating individual strains of this species.

The objective of this study was to use RAPD fingerprinting method to determine the possibility of DNA damage caused by heavy metal pollution by assessing the similarities and differences of the banding patterns of the representatives of the same species isolated from sites on a mine tailings dam and the representatives from sites more than 850 m away. Cluster analysis results demonstrated that the profiles of the metal tolerant *P. lautus* representatives were sufficiently dissimilar to discriminate between individuals from the spatially separated sites.

The RAPD technique is particularly useful for population genetics studies of natural populations, and had been widely applied to various phylogenetic questions of microorganisms and others. Since the RAPD bands are identified by molecular weights and not by nucleotide sequence, it is possible that two DNA fragments with similar molecular weights but different sequences may be identified and scored as a single band (Nadig *et al.*, 1998; Liu *et al.*, 2007). There have also been questions about the reproducibility of the method (Konecka *et al.*, 2007; Liu *et al.*, 2007), yet the method is still a popular one and has recently been used in application ranging from typing of bacteria (Konecka *et al.*, 2007; Ronimus *et al.*, 2003) to demonstrating the genotoxic effects of heavy metals (Nadig *et al.*, 1998; Mengoni *et al.*, 2000; Liu *et al.*, 2005, 2007). In the latter studies it was clearly demonstrated that RAPD analysis could be applied as a suitable biomarker assay of genotoxic effects of heavy metals. Results from the present study indicated the existence of genetic variability based on RAPDS data that are associated with variable heavy metal concentrations. Statistical evaluation of these results suggested that the potential of the RAPD assay as a biomarker assay for the genotoxic effects of heavy metals when a metal tolerant bacterial species (*P. lautus*) was used. This aspect, however, needs to be further investigated using a set of carefully designed experiments and sufficient controls. A further outcome of this part of the study was that the RAPD profiles of the various representatives of the respective species had sufficient intra species similarities to confirm the results of the SDS-PAGE and PCR-RFLP.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

The aim of this study was to determine microbial diversity and metal pollution from platinum mine tailing dams using culture-dependent (plating methods) and molecular methods. Results and the discussion thereof indicated that this aim was achieved and objectives as set out in Section 1.3 were reached.

6.1 CHEMICAL CHARACTERISTICS OF SOIL

From the present study, it was demonstrated that metal concentrations were generally inconsistent at sites on and close to the tailings dam and the sites further away. However, some of the metals had high concentrations at the sites on and closer to the tailings dam (Sites 1, 2 and 3) compared to the sites further away from the tailings dam (Sites 4 to 7). These results are not unique and were also documented by previous studies as discussed in Section 5.1. Metal concentrations were compared to the Canadian microbial benchmarks (MB) and maximum permissible concentrations (MPC) from the Netherlands, and most of the metals were found to exceed both the MB and the MPC. Concentrations above the screening benchmarks are known to be harmful to the environment and to organisms occurring there. This is of concern for the environment, animals and human health. Rehabilitation methods and environmental management plans should be employed to avoid potential harmful effects.

6.2 DIVERSITY OF BACTERIAL AND FUNGAL ISOLATES

The highest number of culturable bacteria and fungi were observed at the sites on and closer to the tailings dam compared to the sites further away. This could also mean that bacteria and fungi isolated from the mine tailings dam may be tolerant to high concentrations of heavy metals as discussed in Section 5.2. Shannon-Weaver diversity indices were higher at sites on and close to the tailings dam than sites more than 300 m away.

Two bacterial types isolated from all the sites were selected for subsequent analysis. These species were *B. barbaricus* and *P. lautus* identified by 16S rDNA sequences. Restriction fragment length polymorphism of PCR fragments (PCR-RFLP) and SDS-PAGE indicated intra-species similarities for the species representatives isolated from the various sites. Furthermore, these methods also showed sufficient inter-species differences. Both PCR-RFLP and SDS-PAGE were thus useful in the preliminary clustering of *B. barbaricus* on the one hand, and *P. lautus* on the other.

The combination of DNA patterns obtained by RAPD method using 3 different primers was also useful in typing *B. barbaricus* and *P. lautus*. Species specific RAPD fingerprints obtained from 3 primers, protein patterns and RFLP data were sufficient to demonstrate that individuals could be grouped. Additionally, further analysis of the combined RAPD data also indicated that individuals of *P. lautus* obtained from sites on and close to the tailings dam clustered separately from those from further away. Considering that concentrations of certain metals (Al, Ni, Cu, Cr) were higher in samples from on and close to the tailings dam than those more than 300 m away, potential physiological and genotoxic effects were anticipated. The anticipated and observed results may thus provide support for the hypothesis of this dissertation i.e. that RAPDS may be useful in providing biomarkers of the genotoxic effect of metals.

6.3 RECOMMENDATIONS AND PROSPECTS FOR FUTURE RESEARCH

This study provided preliminary data of the potential genotoxic effect of heavy metals on the genomic DNA of bacteria, using RAPD analysis. For this to be successfully further investigated it is critical that future studies include controls such as ATCC cultures of the species selected; experiments where the bacteria are grown on media with and without heavy metals; genotoxic and proteomic effects of various concentration of heavy metals on the bacterial species should also be investigated. Future studies should also investigate the cause of microbial communities' resistance to heavy metals. Data from studies such as this one should be conveyed to the responsible parties to

ensure that rehabilitation processes are put in place to prevent metals from leaching into water sources of human and animals.

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APPENDIX A

Table A1: Total heavy metals (mg/kg^{-1}) from different sites along the gradient from a platinum mine tailings dam in August 2005.

Sample:	Al	As	Ba	Be	B	Cd	Cr	Co	Cu	Fe	Pb	Mn	Ni	Se	Tl	V	Zn
S1 1	5,290	0.43	13.14	0.02	3.11	0.03	43.07	5.47	93.09	4,023	3.93	61.73	100.1	1.96	0.08	2.299	21.42
S1 2	4,243	0.41	11.88	0.02	2.96	0.05	31.62	5.51	105	3,977	3.31	53.25	95.1	2.11	0.09	1.978	29.28
S1 3	9,790	0.42	29.14	0.04	2.92	0.04	58.13	9.39	99.53	5,864	6.19	150.3	152	1.55	0.10	4.685	23.52
S1 4	5,707	0.51	91.67	0.19	2.98	0.08	14.52	23.72	62.44	2,097	4.93	997.6	47.84	1.85	0.14	13.33	31.85
S1 5	6,538	0.44	95.90	0.23	2.61	0.04	11.14	18.46	62.61	1,885	5.82	885	26.93	1.81	0.10	13.75	38.34
S1 6	4,478	0.40	58.49	0.16	3.04	0.02	7.10	14.65	34.77	1,295	4.16	692.4	22.26	2.55	0.08	9.142	21.25
S1 7	6,576	0.56	94.34	0.27	2.56	0.17	11.16	20.67	41.27	1,987	5.73	952.7	30.76	1.77	0.12	16.55	26.58
S2 1	5,245	0.43	13.51	0.01	2.75	0.05	42.31	6.13	95.91	4,256	3.94	59.03	111.1	1.59	0.10	2.378	24.52
S2 2	4,593	0.38	12.10	0.01	2.90	0.07	35.60	5.67	108.6	4,077	3.64	58.34	99.22	1.62	0.11	2.18	26.48
S2 3	4,936	0.31	43.77	0.09	2.52	0.05	28.09	11.66	77.62	3,006	5.11	427.7	57.95	2.55	0.12	7.766	24.8
S2 4	6,012	0.46	75.21	0.19	2.50	0.06	18.87	14.08	55.41	1,950	5.51	568.3	37.35	1.77	0.12	10.44	27.98
S2 5	4,722	0.47	61.51	0.19	3.93	0.04	8.68	16.88	39.46	1,425	4.63	742.9	24.73	2.07	0.12	9.762	76.72
S2 6	5,064	0.47	71.94	0.19	2.81	0.05	7.85	16.54	35.15	1,511	4.71	795.7	27.1	2.07	0.11	11.22	20.81
S2 7	6,687	0.47	110.6	0.26	2.12	0.06	11.99	24.27	24.31	2,182	6.46	1,120	34.7	2.03	0.12	16.8	15.99
S3 1	2,247	0.21	7.33	0.01	2.83	0.05	23.35	3.47	67.11	2,369	2.79	33.23	64.48	0.88	0.10	1.248	12.88
S3 2	2,080	0.27	7.20	0.01	2.88	0.03	21.41	3.72	74.78	2,430	2.63	34.88	66.65	1.33	0.08	1.298	12.31
S3 3	4,736	0.30	53.66	0.12	2.56	0.04	20.42	13.15	32.32	2,260	4.42	543.5	40.28	1.62	0.10	8.683	16.27
S3 4	4,383	0.38	58.11	0.16	2.44	0.06	11.57	16.41	34.98	1,646	4.94	768.4	29.77	1.48	0.11	8.791	17.05
S3 5	6,152	0.39	96.26	0.20	2.57	0.03	11.03	18.68	28.43	1,841	5.33	862.1	26.57	2.11	0.11	13.71	15.04
S3 6	5,271	0.32	67.96	0.20	2.45	0.04	7.66	17.34	21.85	1,507	4.44	822.3	25.04	1.77	0.11	10.54	13.41
S3 7	4,944	0.35	73.35	0.21	2.50	0.04	8.29	22.53	23.48	1,558	5.49	1,054	30.37	1.40	0.11	9.98	15.05
S4 1	4,932	0.22	12.05	0.01	2.09	0.04	34.09	5.12	79.99	3,846	3.69	51.94	100.7	0.96	0.10	1.82	16.71
S4 2	3,540	0.29	10.51	0.01	2.11	0.06	31.05	5.79	110.9	3,526	3.57	50.55	112.3	1.96	0.10	1.77	18.95
S4 3	5,663	0.36	66.62	0.15	2.10	0.06	20.94	15.85	43.1	2,504	5.07	668.6	44.17	2.25	0.12	12.44	27.33
S4 4	6,165	0.36	77.09	0.12	2.02	0.04	66.64	16.26	67.07	4,074	5.23	689.6	85.74	2.369	0.12	10.28	13.99
S4 5	5,978	0.36	99.00	0.21	2.56	0.05	9.77	22.57	21.08	1,835	5.28	1,159	32.99	1.62	0.12	17.52	13.01
S4 6	5,840	0.31	83.55	0.20	2.57	0.04	9.52	17.28	19.61	1,636	5.20	804.1	26.57	1.74	0.10	12.88	16.89
S4 7	6,474	0.45	131.3	0.26	2.04	0.05	10.70	25.35	138.4	2,018	6.65	1,191	34.68	2.33	0.10	17.03	14.99
S5 1	4,220	0.34	12.31	0.01	4.25	0.04	41.06	6.06	85.59	3,162	4.12	54.91	104.5	1.44	0.10	2.17	15.2
S5 2	3,016	0.32	10.39	0.01	3.43	0.10	39.30	5.34	192.2	3,208	4.54	147.3	95.25	1.55	0.12	1.54	190.80
S5 3	5,249	0.30	55.35	0.13	3.09	0.06	24.52	13.69	36.78	2,782	5.29	515.5	46.03	2.99	0.07	9.82	19.02

S5 4	3,233	0.26	11.69	0.01	3.27	0.02	77.83	9.87	69.01	4,854	3.53	55.73	81.02	1.44	0.07	2.43	13.58
S5 5	5,367	0.44	90.73	0.18	3.02	0.05	12.64	17.39	21.12	1,758	6.39	853.5	34.09	1.40	0.08	13.8	13.86
S5 6	3,599	0.30	46.96	0.13	3.00	0.03	6.22	14.51	217.4	1,130	4.65	1,052	21.55	1.40	0.08	8.03	14.22
S5 7	6,386	0.48	129.7	0.27	3.33	0.10	11.57	27.08	44.87	2,121	8.48	1,251	37.36	2.25	0.10	16.23	45.81
S6 1	3,329	0.27	8.93	0.01	2.41	0.09	32.62	5.19	69.61	3,907	3.66	50.89	90.43	1.59	0.06	1.59	8.53
S6 2	2,365	0.24	9.27	0.02	2.36	0.04	27.45	4.69	66.59	2,958	3.49	47.04	72.74	0.85	0.06	1.37	18.31
S6 3	7,042	0.24	32.92	0.05	2.68	0.05	49.81	11.18	253.6	4,864	3.82	267.4	75.6	1.51	0.06	4.61	19.15
S6 4	7,698	0.35	122.50	0.25	2.47	0.05	18.95	26.91	26.88	3,007	6.19	1,261	45.98	2.14	0.07	19.84	46.49
S6 5	6,822	0.44	102.00	0.21	2.22	0.05	11.93	16.72	21.14	2,014	6.15	814.5	27.79	1.59	0.06	15.24	12.74
S6 6	6,758	0.31	97.04	0.25	2.22	0.05	9.70	17.87	14.26	2,023	5.33	809.4	26.95	1.88	0.07	17.95	12.22
S6 7	6,378	0.46	122.10	0.26	2.27	0.05	10.73	26.27	17.82	2,083	7.26	1,216	36.52	1.77	0.07	14.4	12.98

Table A2: Total heavy metals (mg/kg^{-1}) from different sites along the gradient from a platinum mine tailings dam in December 2005.

Sample:	Al	As	Ba	Be	B	Cd	Cr	Co	Cu	Fe	Pb	Mn	Ni	Se	Tl	V	Zn
S1 1	2,914	0.21	11.69	0.01	3.80	0.05	29.27	4.90	80.13	3,188	2.68	41.74	97.97	1.77	0.05	1.487	34.17
S1 2	1,728	0.16	5.59	0.01	3.10	0.04	19.50	2.99	52.72	2,038	2.08	26.4	57.07	1.40	0.04	0.905	25.04
S1 3	2,164	0.21	8.05	0.01	3.20	0.12	23.56	3.54	45.69	2,285	3.84	39.21	66.08	1.40	0.04	1.286	14.95
S1 4	2,652	0.28	7.33	0.01	2.75	0.08	22.44	3.53	58.11	2,481	2.73	33.45	69.22	1.62	0.04	1.153	10.74
S1 5	2,929	0.23	9.59	0.01	2.95	0.09	30.89	5.16	79.89	3,290	2.96	42.89	101.1	1.62	0.05	1.5	9.88
S1 6	1,167	0.10	3.58	0.01	2.57	0.03	13.04	2.17	35.29	1,380	2.05	19.94	42.71	1.07	0.03	0.641	13.77
S1 7	1,705	0.19	6.66	0.01	2.68	0.04	16.60	3.46	56.72	2,100	2.65	37.6	59.26	1.48	0.04	1.047	14.31
S2 1	2,824	0.23	8.68	0.01	2.57	0.05	26.30	4.84	87.59	3,399	2.99	44.56	90.8	1.62	0.04	1.399	16.04
S2 2	2,993	0.20	9.35	0.01	2.60	0.03	26.83	4.70	67.69	3,368	3.38	44.62	90.36	1.33	0.04	1.434	20.12
S2 3	2,025	0.21	6.70	0.01	2.43	0.04	20.27	3.32	60.62	2,165	2.41	32.66	65.04	1.22	0.04	1.126	13.38
S2 4	1,280	0.19	5.66	0.01	2.51	0.02	12.97	2.55	46.18	1,432	2.38	26.67	43.42	1.51	0.04	0.778	16.72
S2 5	1,547	0.24	5.47	0.01	2.49	0.03	15.39	2.88	55.66	1,679	2.70	26.38	53.6	1.55	0.04	0.946	13.12
S2 6	7,413	0.34	82.70	0.16	2.93	0.08	38.05	20.84	48.6	4,062	8.37	793.8	79.73	1.96	0.05	13	35.26
S2 7	4,498	0.32	51.41	0.12	2.24	0.03	19.78	14.19	28.86	2,392	4.51	500	41.53	1.888	0.04	9.112	22.21
S3 1	3,792	0.29	42.52	0.08	1.84	0.08	19.87	11.44	83.81	2,268	5.08	449.6	49.86	1.296	0.04	5.328	21.48
S3 2	2,082	0.23	10.11	0.02	2.53	0.05	19.32	4.65	51.94	2,549	3.04	68.07	65.44	1.592	0.04	1.825	13.32
S3 3	4,388	0.21	37.60	0.08	2.04	0.05	35.40	10.65	41.17	2,674	5.24	358.2	53.3	1.629	0.05	5.048	26.47
S3 4	2,515	0.21	27.47	0.07	1.98	0.09	16.03	7.66	31.61	1,746	3.18	273.2	34.54	1.629	0.03	4.877	23.08
S3 5	5,798	0.19	64.05	0.20	1.87	0.03	10.65	12.35	21.27	1,921	4.28	426.8	16.05	1.629	0.05	9.947	22.78
S3 6	2,663	0.20	35.05	0.09	2.16	0.02	6.75	9.87	13.61	1,013	2.92	409.1	19.53	1.962	0.03	4.891	17.96
S3 7	5,013	0.42	74.93	0.16	2.23	0.06	16.03	23.57	42.93	1,762	5.53	1,176	51.61	1.37	0.10	9.414	45.27
S4 1	4,903	0.33	21.68	0.03	2.28	0.03	76.12	8.78	89.16	5,064	6.36	81.88	130.3	1.814	0.06	3.295	19.08
S4 2	2,622	0.20	9.11	0.01	2.32	0.03	42.36	5.41	60.24	3,155	4.08	41.92	75.24	2.666	0.04	1.543	16.55
S4 3	2,254	0.24	10.73	0.01	2.20	0.05	36.92	5.79	55.4	2,823	3.39	43.26	68.67	1.037	0.05	1.613	15.03
S4 4	8,243	0.47	119.30	0.26	2.71	0.06	13.15	24.09	24.08	2,355	7.87	1,171	33.6	2.851	0.06	16.64	18.18
S4 5	3,585	0.25	56.46	0.12	1.72	0.05	8.42	12.14	14.61	1,224	4.58	582.8	21.29	1.74	0.03	7.352	17.00
S4 6	5,804	0.35	97.76	0.20	2.07	0.05	10.97	17.94	15.47	1,792	6.08	882.2	27.88	1.37	0.04	12.32	20.66
S4 7	2,791	0.21	48.89	0.11	2.19	0.06	7.02	9.84	19.72	985	5.36	461.3	20.8	1.925	0.04	5.78	18.18
S5 1	3,660	0.23	58.28	0.14	1.80	0.06	9.20	12.87	15.74	1,264	4.80	773.2	22.24	1.74	0.04	7.006	16.54
S5 2	3,899	0.29	63.53	0.14	1.71	0.10	8.14	14.64	23.64	1,308	4.81	655.5	20.92	1.111	0.04	8.269	24.50
S5 3	5,572	0.29	91.59	0.20	2.03	0.05	8.08	16.84	15.51	1,662	5.80	808.8	25.34	2.332	0.05	12.55	19.73
S5 4	4,003	0.28	47.86	0.15	2.11	0.05	6.33	14.27	15.91	1,320	3.81	605.8	19.24	2.221	0.05	9.442	22.64
S5 5	4,688	0.29	62.03	0.17	1.92	0.04	7.39	14.65	11.47	1,448	4.83	697.2	21.97	1.518	0.04	9.151	14.18
S5 6	3,922	0.24	51.58	0.14	1.63	0.03	6.50	13.28	13.71	1,267	4.29	648.9	21.21	1.444	0.04	7.915	15.55

S5 7	3,891	0.27	51.89	0.13	1.53	0.03	6.65	16.31	12.52	1,308	4.31	844.7	29.17	1.222	0.05	9.06	13.55
S6 1	4,573	0.24	66.25	0.16	1.94	0.04	7.52	15.79	11.97	1,426	5.02	732	23.64	2.073	0.04	9.586	12.79
S6 2	4,990	0.33	85.63	0.23	1.73	0.08	9.73	24.73	14.9	1,652	7.34	1,125	32.13	2.11	0.05	9.717	11.77
S6 3	5,417	0.29	109.50	0.24	1.89	0.04	9.82	26.78	12.78	2,111	6.41	1,335	42.09	1.37	0.07	14.86	15.18
S6 4	3,768	0.29	54.17	0.18	1.94	0.04	7.08	20.86	14.4	1,319	5.22	1,018	29.47	1.222	0.04	8.185	12.45
S6 5	5,257	0.40	114.40	0.22	1.88	0.03	9.92	23.43	14.2	1,793	7.23	1,125	33.51	1.666	0.05	13.59	12.10
S6 6	4,704	0.36	89.39	0.23	1.76	0.05	8.90	21.60	15.74	1,525	6.52	983	29.25	1.259	0.04	9.409	20.87
S6 7	5,582	0.34	116.30	0.23	1.94	0.05	10.24	25.87	16.15	1,997	6.45	1,177	36.49	1.444	0.07	14.6	20.31

Table A3: Total heavy metals (mg/kg^{-1}) from different sites along the gradient from a platinum mine tailings dam in March 2006

Sample:	Al	As	Ba	Be	B	Cd	Cr	Co	Cu	Fe	Pb	Mn	Ni	Se	Tl	V	Zn
S1/S1	1,717	0.12	3.73	0.014	1.21	0.013	13.44	1.46	17.63	1,127	0.82	14.66	32.36	0.38	0.08	0.78	3.00
S1/S2	1,368	0.09	3.26	0.009	0.95	0.01	10.95	1.09	11.23	874	0.60	11.25	22.62	0.30	0.06	0.65	1.47
S1/S3	1,297	0.11	3.39	0.006	0.79	0.012	11.43	1.40	17.48	1,022	0.59	13.56	29.35	0.28	0.05	0.67	1.89
S1/S4	888	0.07	2.26	0.007	0.72	0.004	7.87	1.01	12.44	705	0.42	9.00	21.86	0.32	0.04	0.44	1.53
S1/S5	951	0.06	2.44	0.007	0.61	0.006	8.20	0.79	11.96	636	0.46	8.18	16.33	0.38	0.03	0.43	1.11
S1/S6	1,345	0.08	3.35	0.009	0.56	0.009	10.24	1.13	14.19	845	0.69	11.12	23.51	0.30	0.03	0.61	2.68
S2/S1	1,123	0.08	2.65	0.006	0.50	0.011	8.56	1.03	12.39	783	0.48	10.29	21.96	0.41	0.03	0.53	1.95
S2/S2	999	0.05	2.43	0.005	0.47	0.004	8.06	1.02	13.66	808	0.63	10.33	20.87	0.36	0.02	0.50	2.29
S2/S3	904	0.06	2.30	0.006	0.45	0.004	7.42	0.94	12.01	700	0.49	9.35	19.19	0.33	0.02	0.43	2.25
S2/S4	964	0.06	2.42	0.003	0.40	0.006	7.27	1.01	11.96	725	0.79	9.99	19.78	0.27	0.02	0.45	9.29
S2/S5	813	0.07	2.42	0.005	0.35	0.006	6.61	0.83	11.84	598	0.55	9.27	15.46	0.30	0.01	0.41	2.87
S2/S6	859	0.05	2.35	0.003	0.32	0.003	6.40	0.78	9.94	568	0.49	8.39	15.26	0.28	0.01	0.41	2.08
S3/S1	876	0.04	3.43	0.009	0.31	0.002	7.16	1.16	9.25	664	0.52	19.20	16.45	0.25	0.01	0.69	2.41
S3/S2	839	0.05	9.97	0.029	0.29	0.005	3.25	2.02	2.52	433	0.60	74.67	6.29	0.31	0.01	1.83	2.39
S3/S3	892	0.05	13.19	0.039	0.30	0.007	3.07	2.46	3.78	396	0.65	96.65	6.18	0.22	0.01	2.37	1.97
S3/S4	1,085	0.05	11.79	0.028	0.24	0.007	6.11	2.38	3.87	587	0.66	84.66	8.74	0.25	0.01	2.12	2.00
S3/S5	923	0.04	4.48	0.008	0.23	0.006	8.33	1.73	19.07	768	0.46	33.22	13.31	0.18	0.01	0.85	26.72
S3/S6	855	0.04	5.44	0.016	0.23	0.005	6.76	1.54	6.09	585	0.51	32.30	10.52	0.23	0.01	1.14	1.74
S4/S1	882	0.04	14.82	0.039	0.23	0.005	2.69	3.64	2.36	357	0.96	154.20	7.46	0.27	0.01	2.13	1.90
S4/S2	807	0.05	14.30	0.042	0.21	0.006	2.63	2.35	2.35	347	0.70	98.01	5.51	0.22	0.01	2.03	2.15
S4/S3	800	0.05	14.34	0.036	0.23	0.011	2.41	2.33	3.35	343	0.87	99.83	5.59	0.30	0.01	2.01	2.86
S4/S4	959	0.03	17.58	0.05	0.20	0.009	2.70	2.73	1.85	319	0.75	119.20	6.93	0.22	0.01	1.97	3.64
S4/S5	818	0.05	13.62	0.041	0.18	0.005	2.61	2.24	1.71	419	0.62	78.82	6.63	0.29	0.01	1.87	1.43
S4/S6	753	0.05	36.12	0.033	0.20	0.011	2.67	2.52	2.11	324	0.66	166.40	7.62	0.31	0.01	2.58	3.49
S5/S1	1,040	0.06	20.47	0.048	0.17	0.08	2.17	4.33	1.66	385	0.99	222.10	8.26	0.19	0.01	2.77	2.51
S5/S2	1,035	0.05	19.51	0.041	0.15	0.009	2.28	3.40	2.11	371	1.00	134.30	5.56	0.27	0.01	2.50	1.33
S5/S3	1,000	0.05	19.13	0.046	0.19	0.008	2.62	4.00	2.80	424	0.92	166.50	7.54	0.30	0.01	2.70	2.64
S5/S4	927	0.05	17.24	0.039	0.14	0.009	1.94	2.70	1.89	322	0.79	122.40	4.87	0.30	0.01	2.37	1.38
S5/S5	775	0.04	14.27	0.038	0.15	0.005	2.17	2.21	11.76	297	0.76	93.77	5.94	0.21	0.01	1.94	16.30
S5/S6	908	0.04	16.80	0.051	0.14	0.015	1.98	2.43	1.62	322	0.72	105.20	4.33	0.21	0.01	2.18	1.29
S6/S1	792	0.04	14.69	0.046	0.15	0.004	1.71	2.18	1.19	277	0.66	93.57	3.81	0.18	0.01	2.07	1.57
S6/S2	791	0.04	13.78	0.045	0.15	0.005	1.46	1.94	1.27	271	0.69	82.91	3.59	0.23	0.01	2.07	1.38
S6/S3	758	0.03	12.78	0.035	0.13	0.007	1.46	1.88	1.18	266	0.65	78.64	3.43	0.23	0.01	1.90	1.57
S6/S4	880	0.04	15.86	0.049	0.15	0.007	1.55	2.60	1.43	313	0.69	111.80	5.01	0.18	0.01	2.43	1.86

S6/S5	840	0.04	15.96	0.043	0.15	0.134	1.56	2.50	2.20	297	0.89	108.00	4.59	0.17	0.01	2.12	3.11
S6/S6	835	0.03	15.69	0.046	0.14	0.01	1.67	2.35	1.45	290	0.74	101.40	4.54	0.24	0.01	2.17	1.18
S7/S1	713	0.05	17.95	0.047	0.18	0.002	1.78	3.31	1.62	279	0.86	140.10	5.13	0.17	0.01	1.87	1.63
S7/S2	802	0.04	17.11	0.051	0.17	0.006	1.90	3.00	1.57	331	0.81	127.00	4.65	0.30	0.01	2.50	5.21
S7/S3	863	0.05	21.15	0.054	0.14	0.061	1.96	3.78	1.36	347	1.11	160.70	5.80	0.25	0.01	2.40	1.22
S7/S4	759	0.04	17.29	0.053	0.15	0.006	1.74	2.75	2.26	299	0.96	119.60	4.82	0.28	0.01	2.03	1.96
S7/S5	801	0.04	16.18	0.052	0.17	0.149	2.23	2.92	5.48	478	0.95	120.60	6.46	0.18	0.01	2.33	11.40
S7/S6	808	0.04	20.66	0.048	0.18	0.004	1.93	3.14	1.16	313	0.84	134.10	5.03	0.29	0.01	2.25	1.49

Table A4: Total heavy metals (mg/kg^{-1}) from different sites along the gradient from a platinum mine tailings dam in May 2006

Sample:	Al	As	Ba	Be	B	Cd	Cr	Co	Cu	Fe	Pb	Mn	Ni	Se	Tl	V	Zn
S1/S1	2,689	0.14	10.39	0.012	0.19	0.016	25.31	3.95	60.74	2,363	2.72	37.44	86.40	0.20	0.023	1.40	3.34
S1/S2	3,771	0.17	14.58	0.012	0.18	0.028	31.66	4.54	62.14	3,076	3.34	51.04	99.98	0.26	0.031	1.70	3.36
S1/S3	4,775	0.17	16.78	0.014	0.24	0.019	36.74	5.03	58.49	3,511	3.75	58.10	110.40	0.31	0.035	2.05	4.51
S1/S4	4,215	0.18	15.51	0.011	0.20	0.021	36.16	5.02	62.67	3,348	3.49	56.54	110.30	0.26	0.029	1.95	3.58
S1/S5	4,477	0.17	16.12	0.013	0.18	0.023	34.78	4.44	61.19	3,240	3.98	53.97	100.00	0.21	0.034	1.85	3.83
S1/S6	4,693	0.17	16.06	0.013	0.28	0.019	35.10	4.88	59.03	3,262	3.32	56.21	104.10	0.25	0.034	2.07	3.55
S2/S1	4,020	0.15	14.80	0.016	0.20	0.023	26.10	4.42	57.47	2,803	2.81	51.24	90.38	0.22	0.031	1.79	3.30
S2/S2	2,816	0.13	11.30	0.01	0.17	0.023	25.36	4.24	58.79	2,690	2.64	40.30	89.73	0.26	0.024	1.53	2.90
S2/S3	2,560	0.16	10.33	0.005	0.17	0.02	22.07	3.70	54.76	2,468	2.46	35.16	79.19	0.24	0.022	1.31	2.50
S2/S4	3,481	0.16	14.04	0.013	0.20	0.023	25.35	4.21	58.81	2,481	2.66	50.54	85.60	0.23	0,028	1.69	4.32
S2/S5	5,861	0.25	73.60	0.207	0.18	0.035	29.82	16.00	53.72	3,075	5.75	584.60	96.11	0.37	0.049	10.16	5.16
S2/S6	2,289	0.13	9.66	0.007	0.21	0.018	19.00	3.27	54.83	1,874	2.26	31.43	66.97	0.28	0.025	1.23	3.96
S3/S1	2,404	0.11	12.88	0.02	0.15	0.018	19.50	4.09	34.75	2,118	2.50	68.53	62.71	0.18	0.02	1.81	3.52
S3/S2	4,214	0.16	63.60	0.182	0.14	0.026	17.43	15.72	13.68	2,002	3.84	546.60	39.19	0.25	0.022	11.62	5.72
S3/S3	3,535	0.11	72.54	0.179	0.14	0.024	9.81	11.90	8.32	1,383	3.13	460.10	24.88	0.30	0.019	12.62	3.88
S3/S4	3,380	0.13	54.99	0.131	0.14	0.029	16.98	9.93	15.40	1,585	3.48	345.00	35.75	0.30	0.016	8.18	4.82
S3/S5	4,527	0.13	43.55	0.067	0.13	0.021	41.28	9.94	29.87	2,716	5.19	263.10	68.10	0.28	0.022	4.89	6.07
S3/S6	2,465	0.11	21.34	0.05	0.17	0.016	40.90	9.00	29.70	2,498	2.44	135.40	49.72	0.28	0.025	4.17	4.76
S4/S1	3,035	0.12	58.89	0.169	0.15	0.023	7.51	10.62	5.69	910	3.10	442.30	25.07	0.28	0.023	6.29	3.51
S4/S2	4,800	0.17	90.53	0.274	0.15	0.024	12.54	15.49	9.10	1,589	4.55	630.00	32.14	0.42	0.027	12.52	4.36
S4/S3	3,286	0.12	68.95	0.174	0.13	0.022	7.35	12.37	5.88	1,175	2.97	525.30	20.82	0.32	0.018	10.98	3.21
S4/S4	3,527	0.18	67.66	0.176	0.13	0.03	9.32	12.96	5.96	1,157	3.52	545.10	32.04	0.32	0.026	8.80	3.97
S4/S5	500	0.02	0.09	0.001	0.12	0.005	0.10	0.02	0.56	14	0.32	0.55	0.11	0.12	0.004	0.03	0.49
S4/S6	3,642	0.17	70.15	0.192	0.12	0.032	10.75	13.59	8.21	1,177	3.82	591.00	33.33	0.29	0.026	9.40	4.47
S5/S1	3,379	0.14	72.34	0.199	0.12	0.028	6.34	12.46	6.21	915	3.83	548.80	19.39	0.31	0.017	9.53	3.21
S5/S2	3,389	0.13	75.14	0.191	0.12	0.024	6.65	12.35	6.58	924	4.11	544.10	20.19	0.34	0.019	8.77	3.41
S5/S3	4,045	0.17	80.43	0.243	0.14	0.041	8.58	14.29	9.33	1,065	5.60	624.50	27.93	0.40	0.019	10.22	4.40
S5/S4	3,806	0.14	75.84	0.238	0.12	0.02	6.11	11.65	5.46	989	3.73	511.30	17.73	0.32	0.023	10.37	3.05
S5/S5	3,737	0.13	79.20	0.193	0.12	0.023	6.59	12.28	6.16	1,004	4.00	547.40	19.13	0.31	0.02	9.90	3.49
S5/S6	2,620	0.10	53.76	0.149	0.12	0.021	4.89	9.37	4.44	745	2.94	412.30	14.52	0.25	0.018	7.63	2.48
S6/S1	4,381	0.17	87.27	0.247	0.12	0.035	7.18	15.48	6.62	1,212	4.91	672.30	25.69	0.31	0.025	12.36	4.08
S6/S2	4,458	0.17	85.21	0.249	0.12	0.032	6.53	15.31	4.99	1,229	4.19	650.40	24.46	0.34	0.03	12.05	3.18
S6/S3	3,606	0.13	73.12	0.207	0.12	0.024	5.52	12.46	4.76	1,000	3.88	525.90	19.17	0.40	0.024	9.87	2.91
S6/S4	3,871	0.15	85.08	0.228	0.11	0.025	6.11	13.91	5.70	1,054	4.60	585.50	21.60	0.32	0.022	11.27	3.62

S6/S5	2,886	0.11	63.32	0.175	0.10	0.019	4.72	9.82	4.92	794	3.15	430.60	16.60	0.31	0.017	9.03	3.07
S6/S6	3,864	0.18	86.69	0.215	0.13	0.03	6.42	13.68	7.32	1,018	4.52	606.60	23.97	0.25	0.022	11.63	3.72
S7/S1	4,138	0.20	98.73	0.263	0.11	0.029	7.61	18.75	5.25	1,357	5.10	801.60	28.56	0.44	0.031	13.82	3.59
S7/S2	2,587	0.12	61.53	0.184	0.12	0.02	4.51	11.88	3.14	825	3.30	512.00	18.57	0.36	0.026	7.90	2.43
S7/S3	4,152	0.18	91.19	0.275	0.10	0.029	7.12	19.80	7.34	1,236	5.34	850.70	28.57	0.43	0.038	13.58	3.84
S7/S4	3,630	0.14	69.31	0.245	0.12	0.018	6.28	15.33	4.09	1,106	4.47	641.10	21.80	0.31	0.031	8.74	3.10
S7/S5	3,243	0.15	77.98	0.233	0.11	0.024	5.83	16.07	4.54	953	4.37	711.60	23.64	0.30	0.022	10.09	3.20
S7/S6	3,097	0.14	83.60	0.226	0.11	0.028	5.72	14.97	4.61	926	4.56	628.90	22.19	0.34	0.023	9.59	3.17

APPENDIX B

Fungal and bacterial levels calculated from different sampling sites in August 2005 (cfu/g of soil)

Fungi	sample1	sample2	sample3	sample4	sample5	sample6	Average/site	stdev/site
Site 1	415000	265000	235000	140000	270000	170000	249166.7	9.65E+04
Site 2	215000	45000	270000	245000	185000	320000	213333.3	9.46E+04
Site 3	60000	0	10000	25000	35000	5000	22500	2.25E+04
Site 4	195000	115000	235000	335000	10000	15000	150833.3	1.28E+05
Site 5	45000	160000	165000	135000	110000	150000	127500	4.50E+04
Site 6	25000	180000	45000	130000	50000	35000	77500	6.27E+04
Site 7	80000	35000	30000	65000	20000	30000	43333.33	2.36E+04

Bacteria	sample1	sample2	sample3	sample4	sample5	sample6	Average/site	stdev/site
Site1	445000	410000	350000	390000	390000	320000	384166.7	4.41E+04
Site2	525000	30000	430000	460000	370000	285000	350000	1.77E+05
Site3	55000	25000	15000	20000	310000	210000	105833.3	1.24E+05
Site4	295000	150000	90000	410000	150000	140000	205833.3	1.21E+05
Site5	55000	50000	90000	115000	200000	60000	95000	5.71E+04
Site6	120000	145000	90000	130000	105000	235000	137500	5.15E+04
Site7	100000	70000	70000	125000	70000	60000	82500	2.48E+04

Fungal and bacterial levels calculated from different sampling sites in December 2005 (cfu/g of soil)

Fungi	sample1	sample2	sample3	sample4	sample5	sample6	Average/site	stdev/site
Site 1	450000	325000	360000	465000	550000	525000	445833.3	8.88E+04
Site 2	620000	390000	30000	115000	65000	35000	209166.7	2.42E+05
Site 3	120000	75000	30000	150000	10000	110000	82500	5.44E+04
Site 4	30000	5000	15000	30000	95000	20000	32500	3.21E+04
Site 5	10000	5000	5000	15000	10000	15000	10000	4.47E+03
Site 6	15000	0	0	10000	5000	105000	22500	4.08E+04
Site 7	20000	60000	15000	15000	10000	105000	37500	3.78E+04

Bacteria	sample1	sample2	sample3	sample4	sample5	sample6	Average/site	stdev/site
Site1	590000	540000	1500000	575000	630000	470000	717500	3.87E+05
Site2	470000	1500000	125000	160000	80000	90000	404166.7	5.56E+05
Site3	225000	255000	225000	130000	140000	300000	212500	6.61E+04
Site4	20000	205000	5000	170000	30000	45000	79166.67	8.56E+04
Site5	60000	30000	50000	100000	85000	25000	58333.33	2.98E+04
Site6	105000	130000	35000	205000	40000	15000	88333.33	7.24E+04
Site7	60000	35000	275000	60000	55000	10000	82500	9.63E+04

Fungal and bacterial levels calculated from different sampling sites in March 2006 (cfu/g of soil)

Fungi	sample1	sample2	sample3	sample4	sample5	sample6	Average/site	stdev/site
Site 1	70000	120000	25000	65000	70000	70000	70000	30166.21
Site 2	260000	310000	70000	95000	75000	45000	142500	112638.8
Site 3	360000	350000	350000	310000	200000	170000	290000	83666
Site 4	300000	125000	150000	75000	135000	95000	146666.7	79916.62
Site 5	295000	255000	200000	260000	180000	135000	220833.3	59448.86
Site 6	335000	295000	265000	155000	115000	45000	201666.7	113783.4
Site 7	200000	160000	80000	50000	30000	30000	91666.67	71949.06

Bacteria	sample1	sample2	sample3	sample4	sample5	sample6	Average/site	stdev/site
Site 1	765000	410000	570000	85000	170000	350000	391666.7	251568.4
Site 2	495000	70000	410000	360000	170000	210000	285833.3	161505.9
Site 3	405000	380000	205000	190000	220000	200000	266666.7	98268.34
Site 4	330000	260000	155000	215000	155000	105000	203333.3	82138.1
Site 5	320000	265000	205000	295000	270000	195000	258333.3	49362.6
Site 6	390000	310000	270000	155000	150000	85000	226666.7	115397.9
Site 7	265000	160000	150000	105000	45000	35000	126666.7	85244.75

Fungal and bacterial levels calculated from different sampling sites in May 2006 (cfu/g of soil)

Fungi	sample1	sample2	sample3	sample4	sample5	sample6	Average/site	stdev/site
Site 1	445000	215000	50000	70000	260000	155000	199166.7	145100.5
Site 2	375000	120000	220000	165000	190000	160000	205000	89666.05
Site 3	160000	110000	70000	85000	60000	95000	96666.67	35730.47
Site 4	135000	105000	90000	60000	125000	100000	102500	26598.87
Site 5	160000	120000	185000	80000	95000	60000	116666.7	48131.76
Site 6	145000	135000	55000	95000	75000	90000	99166.67	34701.1
Site 7	360000	250000	95000	95000	140000	70000	168333.3	113695.5

Bacteria	sample1	sample2	sample3	sample4	sample5	sample6	Average/site	stdev/site
Site 1	610000	410000	175000	210000	320000	180000	317500	170227.8
Site 2	540000	395000	260000	175000	300000	210000	313333.3	134820.9
Site 3	490000	165000	90000	155000	205000	140000	207500	143344
Site 4	470000	205000	175000	160000	220000	115000	224166.7	125913.3
Site 5	455000	215000	150000	140000	95000	95000	191666.7	136369.6
Site 6	405000	260000	140000	100000	100000	85000	181666.7	126833.2

APPENDIX C

Morphological types of bacteria selected from different sites on and away from the tailings dam during the wet periods.

Morphological type	G+/G-	Site1 Ave/ stdev	Site2 Ave/ stdev	Site3 Ave/ stdev	Site4 Ave/ stdev	Site5 Ave/ stdev	Site6 Ave/ stdev	Site7 Ave/ stdev
White round colony	G+	195000± 131263.1	173333.3± 165005.1	753333.3± 187047.2	63500± 32488.4	26833.3± 10186.59	23666.6± 26348.94	22500± 22722.2
Cream white colony	G+	11383.3± 13957.14	7500± 5468.089	20333.3± 21620.98	2500± 3987.48	833.33± 1169.045	7216.6± 3960.008	1950± 3022.41
Yolk yellow colony	G+	916.66± 2010.39	1388.3± 2153.327	555± 1359.467	0	0	555± 1359.46	278.33± 681.77
Orange colony	G+	0	1666.6± 2658.32	0	0	0	0	1666.66± 2581.989
Pink round colony	G+	250± 446.0942	0	0	278.33± 681.77	0	0	1111.66± 2723.016
White oval-shaped colony	G+	0	0	278.33± 681.7746	0	278.33± 681.7746	0	0
Peach colony	G+	0	0	0	278.33± 681.77	0	0	0
White and yellow colony	G+	0	0	173460.6± 194979.1	92551.3± 13578.51	53773.3± 33559.09	0	0

Morphological types of bacteria selected from different sites on and away from the tailings dam during the dry periods.

Morphological type	G+/G-	Site1 Ave/ stdev	Site2 Ave/ stdev	Site3 Ave/ stdev	Site4 Ave/ stdev	Site5 Ave/ stdev	Site6 Ave/ stdev	Site7 Ave/ stdev
White round colony	G+	7500± 12985.0	38055.5± 93216.6	1666.6± 2581.9	23666.6± 56182.1	23361.1± 56329.4	38888.8± 92829.9	24194.4± 55950.4
Cream white colony	G+	201111.1± 85046.84	31388.88± 76886.75	24194.43± 55950.41	157500± 172532.6	76416.65± 117676.3	129166.7± 141495	43361.1± 105317
Yolk yellow colony	G+	61416.65± 87253.4	38611.12± 92149.75	11694.43± 27756.95	0	50000± 90633.58	98333.3± 103997.9	0
Orange colony	G+	68944.4± 53940.47	0	35861.12± 54850.36	1138.88± 2028.727	23611.1± 34695.75	0	0
Pink round colony	G+	17055.57± 29721.25	0	0	6055.56± 7692.48	0	0	6055.5± 7692.48
White oval-shaped colony	G+	8055.55± 12266.35	3611.1± 4138.78	4833.33± 6320.169	3444.45± 6312.84	0	2777.767± 6312.84	0
Peach colony	G+	0	0	0	3444.45± 6312.845	0	0	2777.7± 4303.2
White and yellow colony	G+	2777.76± 4303.298	1666.66± 2357.017	8933.34± 6824.80	1944.43± 3402.054	5777.783± 7767.949	0	0

Morphological types of fungi selected from different sites on and away from the tailings dam during the wet periods.

Morphological type	G+/G-	Site1 Ave/ stdev	Site2 Ave/ stdev	Site3 Ave/ stdev	Site4 Ave/ stdev	Site5 Ave/ stdev	Site6 Ave/ stdev	Site7 Ave/ stdev
Black spongy colony	G+	833.33± 2041.241	35861.12± 54850.36	0	76388.9± 116420.8	0	0	86944.45± 111417.2
Cream smooth colony	G+	34695.75± 34695.75	52285.7± 35287.16	2777.7± 4303.298	1666.66± 2357.017	8933.3± 6824.809	1944.4± 3402.054	5777.7± 7767.949
Yellow colony	G+	32238.09± 34165.54	14833.33± 26906.2	32476.19± 33908.27	12476.19± 25286.39	0	0	0
Black rough colony	G+	0	0	0	0	57166.66± 117269.8	0	141690.5± 137256.9

Morphological types of fungi selected from different sites on and away from the tailings dam during the dry periods.

Morphological type	G+/G-	Site1 Ave/ stdev	Site2 Ave/ stdev	Site3 Ave/ stdev	Site4 Ave/ stdev	Site5 Ave/ stdev	Site6 Ave/ stdev	Site7 Ave/ stdev
Black spongy colony	G+	96690.46± 121724.9	134285.7± 136017.6	0	0	0	0	64761.9± 121793.8
Cream smooth colony	G+	5000± 4647.58	23833.3± 32338.32	6121.66± 6658.59	278.33± 681.77	833.33± 1602.082	278.33± 681.77	555± 1359.467
Yellow colony	G+	73809.51± 126053.7	76388.9± 87253.4	0	86944.45± 111417.2	0	0	0
Black rough colony	G+	0	0	0	0	0	0	23666.65± 56182.14

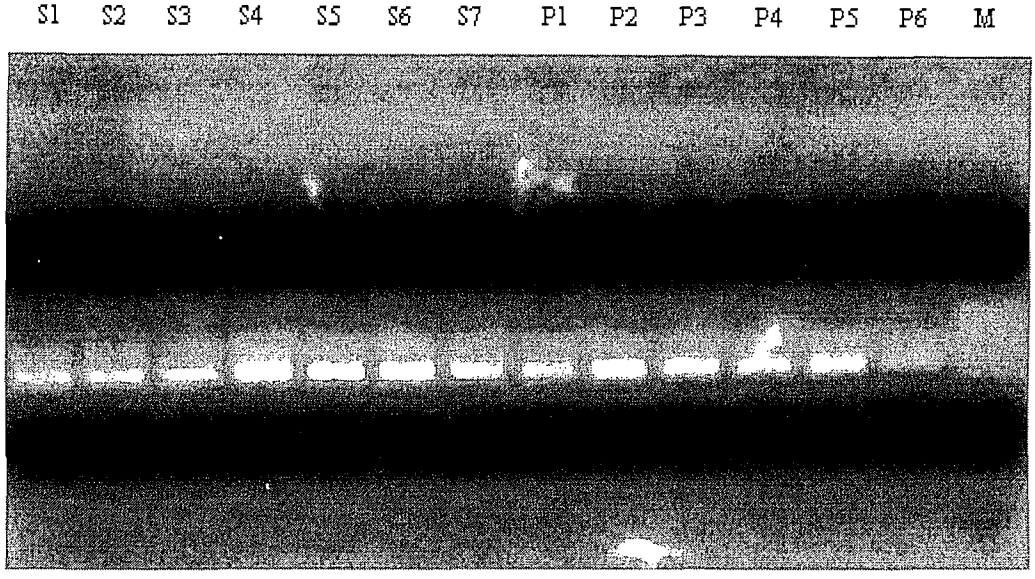
APPENDIX D

peqGOLD Bacterial DNA isolation kit method

Each sample (2 ml) from the pure culture (overnight culture) was added to a 2 ml microfuge tube and centrifuged at 4000 rpm for 10 minutes at room temperature to pellet the cells. The pellet was resuspended in 100 µl of TE Buffer. Then 100 µl of 10 mg/ml lysozyme was added, followed by 10 minutes incubation at 30^0C. A digested sample was centrifuged at 5000 rpm for 5 minutes, and the supernatant was discarded. A pellet was resuspended in 200 µl of buffer BTL and 25 µl of OB-Protease solution, vortexed to mix well and incubated at 55^0C for 1 hour. RNase A (10 µl) was added to the microfuge tube (containing the sample) and incubated at room temperature for 2 minutes. Thereafter 220 µl of buffer BDL was added to the tube, vortexed and incubated at 70^0C for 10 minutes. Absolute ethanol (220 µl) was then added to the tube and mixed thoroughly by vortexing. HiBind DNA spin-column was assembled to the 2ml collection tube (provided). The entire sample was transferred to the HiBind DNA spin-column assembled to the 2 ml collection tube and centrifuged at 8000 rpm for 1 minute to bind DNA. A collection tube was discarded and the HiBind DNA spin-column was placed into the new 2ml collection tube. DNA was washed by pipetting 650 µl of DNA wash buffer diluted with ethanol and centrifuged at 8000 rpm for 1 minute. The flow-through and the collection tube were discarded, the column was placed on a new 2 ml collection tube and the wash step was repeated. The flow-through was discarded and the HiBind DNA spin-column was put back to the same 2 ml collection tube, centrifuged at maximum speed for 2 minutes to dry the column matrix. A HiBind DNA spin-column was then placed into a new sterile 1.5 ml microfuge tube and 100 µl of preheated (70^0C) elution buffer was added, centrifuged at 8000 rpm for 1 minute to elute the DNA. Eluted DNA was stored at 4^0C for further molecular analysis.

APPENDIX E

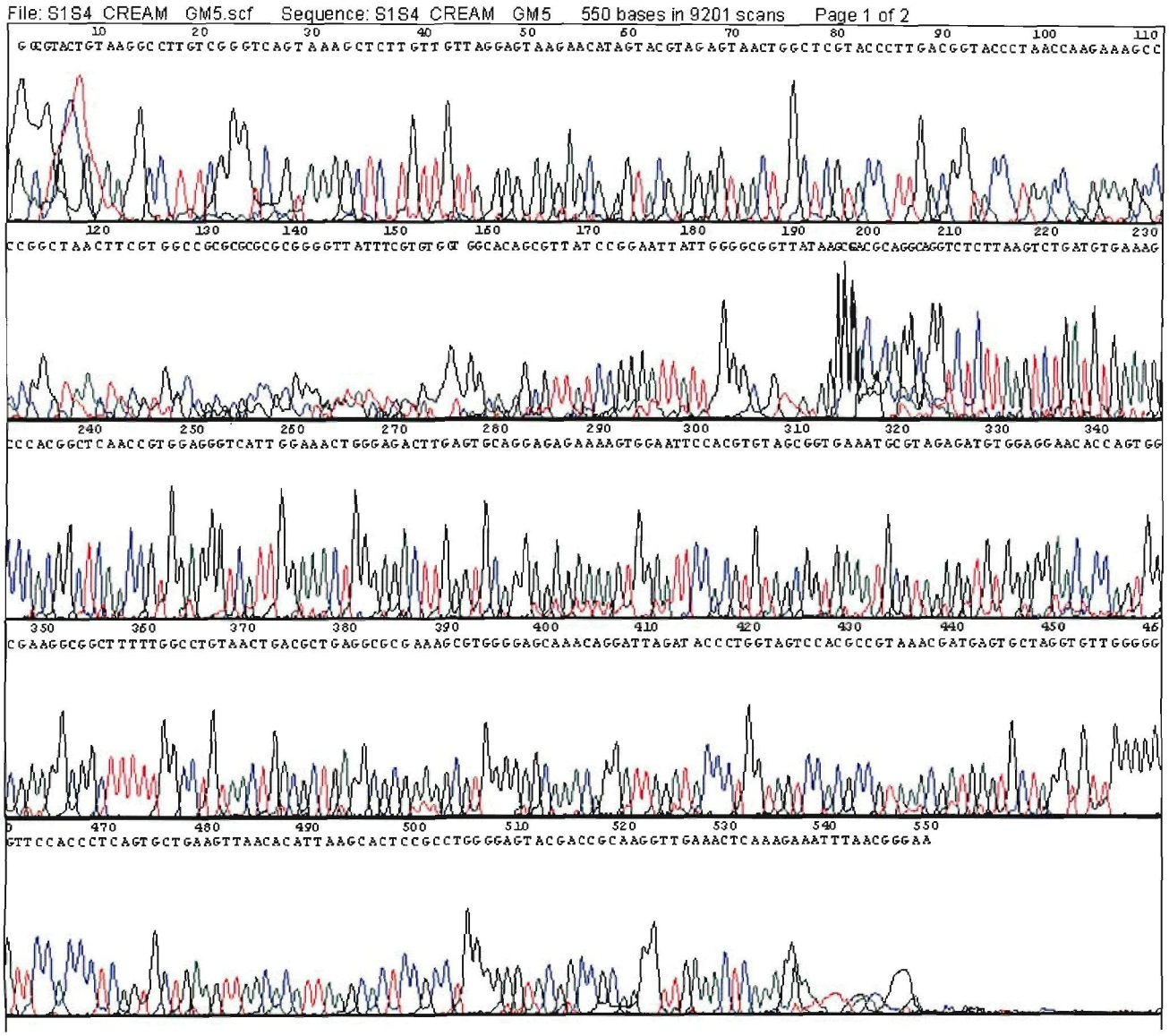
Restriction enzymes digest of *Paenibacillus lautus* and *Bacillus barbaricus* digested with *AvaI.*



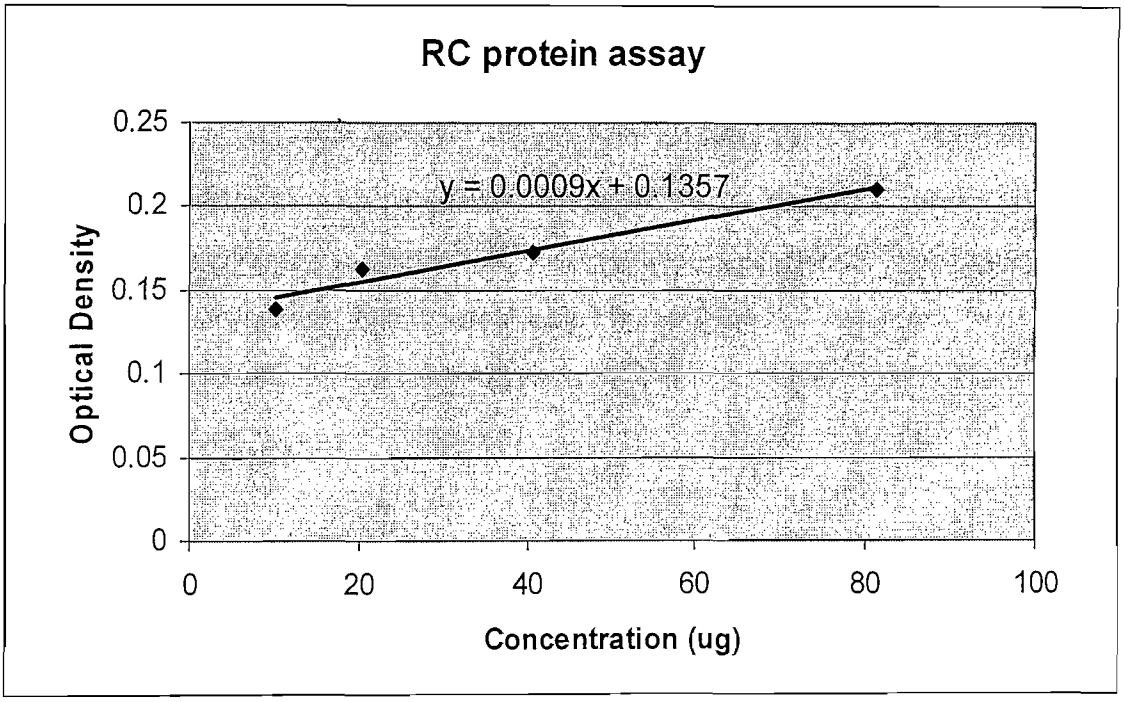
S1-S7, are seven *Bacillus barbaricus* samples digested with *AvaI,* P1-P6 are *Paenibacillus lautus* samples digested with *AvaI* and M is the molecular size marker.

APPENDIX F

Example of DNA Sequence data (16S rDNA fragment) of the isolate that was identified as *Bacillus barbaricus*



APPENDIX G



Standard curve of the proteins extracted from different sites along the gradient from the platinum mine tailings dam.

APPENDIX H

RAPD band sizes of *Barbaricus bacillus* (B) using primers OPA-01, OPA-02 and OPB-01

	B-S1/1	B-S1/3	B-S1/6	B-S2/1	B-S2/3	B-S2/6	B-S3/1	B-S3/3	B-S3/6	B-S5/1	B-S5/3	B-S5/6	B-S7/1	B-S7/3	B-S7/6
OPA-011	0.0	1561.7	1463.6	1536.6	0.0	1511.9	0.0	0.0	0.0	1499.7	1524.2	0.0	0.0	0.0	0.0
OPA-012	1275.2	1254.7	1275.2	1224.6	1214.7	1195.2	1234.6	1224.6	1224.6	1224.6	1296.1	1214.7	1224.6	1234.6	1224.6
OPA-013	1084.4	1075.7	1075.7	1049.8	1049.8	1049.8	1049.8	1058.4	1058.4	1049.8	1049.8	1041.4	1049.8	1058.4	1049.8
OPA-014	0.0	929.7	929.7	914.7	914.7	914.7	907.3	907.3	907.3	914.7	907.3	900.0	907.3	900.0	900.0
OPA-015	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
OPA-016	685.4	690.3	690.3	690.3	685.4	685.4	690.3	685.4	680.7	685.4	685.4	680.7	643.5	643.5	639.1
OPA-017	542.8	542.8	537.8	542.8	542.8	542.8	537.8	528.1	523.3	523.3	523.3	523.3	500.0	500.0	500.0
OPA-018	328.2	308.2	331.2	302.7	313.8	302.7	316.6	343.3	340.2		0.0	0.0	0.0	0.0	331.2
OPA-019	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	270.8	0.0	0.0	0.0	0.0	0.0
OPA-	221.3	223.4	223.4	225.5	221.3	221.3	211.4	207.5	205.6	205.6	201.9	201.9	201.9	201.9	200.0
OPB-011	0.0	2844.8	0.0	2776.5	2799.1	2687.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
OPB-012	0.0	2361.0	0.0	2230.8	2230.8	2177.2	0.0	0.0	0.0	2212.8	2212.8	0.0	0.0	0.0	0.0
OPB-013	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
OPB-014	0.0	1975.4	0.0	1897.0	1866.5	1836.5	1866.5	1881.7	1912.4	1866.5	0.0	1912.4	0.0	0.0	0.0
OPB-015	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
OPB-016	1204.9	1185.5	1185.5	1166.5	1138.5	1138.5	1157.1	1138.5	1129.3	1120.2	1338.8	1138.5	1166.5	1176.0	1166.5
OPB-017	710.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
OPB-018	281.3	289.1	289.1	291.8	286.5	281.3	273.6	271.1	266.1	261.3	261.3	256.5	251.8	249.5	249.5
OPB-019	238.3	0.0	0.0	0.0	0.0	0.0	0.0	229.6	225.5	0.0	0.0	0.0	217.3	215.3	211.4
OPA-021	0.0	2803.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2955.6	0.0	0.0	0.0	0.0	0.0
OPA-022	0.0	2523.6	0.0	2561.8	2523.6	2523.6	0.0	0.0	0.0	2660.1	0.0	2430.4	0.0	0.0	0.0
OPA-023	0.0	2220.5	0.0	2271.2	2237.3	2237.3	0.0	0.0	0.0	2340.6	0.0	0.0	0.0	0.0	0.0
OPA-024	0.0	2028.8	0.0	2075.1	2044.1	2044.1	0.0	0.0	0.0	2138.5	0.0	0.0	0.0	0.0	0.0
OPA-025	0.0	1606.6	1668.2	1631.0	1618.7	1631.0	0.0	1680.8	1680.8	1668.2	0.0	1745.3	0.0	0.0	0.0
OPA-026	0.0	1559.0	0.0	1341.1	0.0	1341.1	0.0	0.0	0.0	1361.5	0.0	1207.0	0.0	0.0	0.0
OPA-027	0.0	1136.5	0.0	1162.4	0.0	1153.7	0.0	0.0	0.0	1171.2	0.0	0.0	0.0	0.0	0.0
OPA-028	0.0	0.0	1022.8	1022.8	1015.2	1022.8	0.0	1007.6	0.0	1030.6	1054.1	1078.2	0.0	1000.0	1000.0

OPA-029	992.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	963.1	992.5	0.0	0.0
OPA-	906.8	920.6	920.6	920.6	920.6	920.6	913.6	906.8	920.6	927.5	948.7	0.0	0.0	0.0	0.0
OPA-	0.0	0.0	0.0	893.8	0.0	893.8	893.8	0.0	0.0	0.0	0.0	851.5	869.4	869.4	875.4
OPA-	811.2	816.8	816.8	822.5	822.5	822.5	811.2	805.6	811.2	822.5	828.2	777.8	0.0	0.0	0.0
OPA-	680.7	685.4	690.3	690.3	685.4	685.4	685.4	675.9	675.9	680.7	695.1	709.9	720.0	725.0	730.1
OPA-	625.8	634.6	634.6	634.6	617.1	630.2	617.1	612.7	617.1	621.4	630.2	639.1	648.1	648.1	652.6
OPA-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	526.2	522.4	0.0
OPA-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	413.4	416.9	0.0
OPA-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	333.1	0.0	0.0
OPA-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	203.9	0.0	0.0

Statistical analysis of *Barbaricus bacillus* band sizes from different sites

Sites that were compared	p values
p1,2 &5,7	0.00088611
1 &3	0.02450349
3&5	0.00345415
5&7	0.00014789
2&3	6.9398E-07
1&2	0.0014114

RAPD band sizes of *Paenibacillus lautus* (PL) using primers OPA-01, OPA-02 and OPB-01

	PL-S1/1	PL-S1/3	PL-S1/6	PL-S2/1	PL-S2/3	PL-S2/6	PL-S3/1	PL-S3//3	PL-S3/6	PL-S5/1	PL-S5/3	PL-S5/6	PL-S7/1	PL-S7/3	PL-S7/6
OPA-011	1561.7	0.0	0.0	0.0	1666.3	0.0	0.0	0.0	0.0	1735.2	0.0	0.0	0.0	0.0	0.0
OPA-012	0.0	0.0	0.0	1244.6	1254.7	1254.7	0.0	1244.6	1176.0	1254.7	1265.0	1275.2	1306.6	1296.1	1285.6
OPA-013	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
OPA-014	952.5	968.1	968.1	976.0	976.0	960.3	900.0	944.8	0.0	0.0	0.0	0.0	960.3	968.1	960.3
OPA-015	0.0	0.0	0.0	800.0	800.0	811.9	0.0	0.0	0.0	800.0	0.0	0.0	836.1	836.1	836.1
OPA-016	0.0	0.0	0.0	705.5	711.1	705.5	0.0	0.0	0.0	676.4	688.1	688.1	722.3	728.0	728.0
OPA-017	561.9	570.1	570.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
OPA-018	530.0	0.0	537.8	533.9	533.9	533.9	533.9	0.0	545.7	518.6	526.2	530.0	530.0	537.8	537.8
OPA-019	478.2	482.5	482.5	486.8	491.2	486.8	0.0	0.0	0.0	465.5	469.7	473.9	0.0	0.0	0.0
OPA-010	0.0	0.0	433.5	407.2	0.0	407.2	445.2	433.5	433.5	418.3	425.8	425.8	0.0	0.0	0.0
OPA-011	383.9	0.0	353.6	359.5	0.0	359.5	365.4	390.3	0.0	371.5	374.5	374.5	0.0	0.0	0.0
OPA-012	347.8	347.8	315.2	317.8	0.0	317.8	0.0	0.0	0.0	0.0	307.5	0.0	0.0	0.0	0.0
OPA-013	0.0	0.0	0.0	267.5	269.9	269.9	0.0	292.2	269.9	267.5	258.3	0.0	0.0	0.0	274.7
OPA-014	0.0	0.0	0.0	226.3	228.3	228.3	0.0	0.0	0.0	228.3	228.3	226.3	228.3	230.3	232.3
OPA-015	178.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
OPA-016	135.2	139.8	187.0	0.0	0.0	0.0	146.2	195.6	197.8	0.0	0.0	0.0	147.9	149.6	151.2
OPB-011	0.0	0.0	2146.6	2323.1	2343.5	2282.6	0.0	2203.8	2242.9	2323.1	2343.5	2323.1	2323.1	2364.2	2364.2
OPB-012	2109.2	0.0	0.0	2165.5	2165.5	2109.2	0.0	0.0	0.0	0.0	0.0	0.0	2001.0	2018.6	0.0
OPB-013	0.0	0.0	0.0	1983.5	0.0	1931.9	0.0	0.0	0.0	0.0	1966.1	1948.9	0.0	0.0	0.0
OPB-014	1664.0	0.0	1693.5	1708.4	1754.0	1678.7	1649.5	1649.5	1649.5	1693.5	1723.5	1693.5	1738.7	1754.0	1738.7
OPB-015	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1336.1	1130.8	1150.8	1140.8	1101.4	1111.1	1111.1
OPB-016	0.0	0.0	1000.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
OPB-017	855.7	0.0	855.7	827.4	834.4	806.8	877.6	0.0	0.0	915.9	915.9	900.0	915.9	948.7	0.0
OPB-018	735.9	0.0	748.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
OPB-019	0.0	0.0	651.0	645.1	645.1	622.2	0.0	0.0	0.0	681.2	681.2	669.0	681.2	687.4	681.2
OPB-0110	639.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	639.3	627.8	500.0
OPB-0111	0.0	0.0	462.7	0.0	0.0	0.0	495.2	0.0	0.0	0.0	0.0	0.0	0.0	495.2	0.0

OPB-0112	449.4	0.0	0.0	0.0	0.0	0.0	445.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
OPB-0113	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	351.6	351.6	355.1	358.6	362.2	358.6
OPB-0114	0.0	0.0	0.0	0.0	0.0	0.0	277.8	277.8	277.8	0.0	303.0	296.7	0.0	0.0	0.0
OPB-0115	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
OPA-021	1284.3	0.0	0.0	1259.2	1259.2	1259.2	0.0	1417.7	1436.5	1242.7	1250.9	1259.2	1218.4	1242.7	1250.9
OPA-022	1075.1	1075.1	1075.1	1075.1	1061.1	1054.1	1082.2	1082.2	1075.1	1047.2	1047.2	1054.1	1111.1	1125.8	1125.8
OPA-023	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	980.4	1000.0	1000.0
OPA-024	918.0	930.1	930.1	905.9	911.9	911.9	986.9	0.0	0.0	0.0	0.0	900.0	0.0	900.0	900.0
OPA-025	886.8	0.0	0.0	889.5	879.0	881.6	873.9	879.0	876.5	897.4	894.7	0.0	894.7	884.5	863.7
OPA-026	861.1	0.0	0.0	856.1	853.5	853.5	838.6	851.0	851.0	846.0	846.0	846.0	846.0	814.3	819.1
OPA-027	720.3	713.5	713.5	720.3	713.5	706.7	762.7	0.0	0.0	0.0	0.0	0.0	741.2	755.5	748.3
OPA-028	615.6	0.0	0.0	670.7	659.3	0.0	688.1	676.4	676.4	694.0	694.0	694.0	642.5	653.6	653.6
OPA-029	0.0	0.0	0.0	615.6	610.4	0.0	0.0	0.0	0.0	642.5	642.5	642.5	0.0	0.0	0.0
OPA-0210	0.0	594.6	0.0	583.8	583.8	578.5	552.7	0.0	0.0	594.6	589.2	594.6	583.8	594.6	594.6
OPA-0211	490.4	0.0	0.0	485.7	481.0	476.3	0.0	0.0	0.0	562.9	0.0	562.9	532.9	537.8	537.8
OPA-0212	0.0	436.5	436.5	436.5	428.1	424.0	419.9	411.8	407.8	462.7	462.7	462.7	436.5	440.8	436.5
OPA-0213	357.3	0.0	391.9	0.0	0.0	0.0	387.9	0.0	0.0	0.0	0.0	411.8	376.1	342.9	339.4
OPA-0214	202.6	200.0	200.0	200.0	0.0	0.0	230.9	225.0	208.0	0.0	0.0	0.0	208.0	213.5	205.3
OPA-0215	0.0	0.0	0.0	0.0	192.8	189.3	152.1	179.3	172.8	172.8	172.8	172.8	136.4	138.9	133.9
OPA-0216	0.0	73.3	73.3	0.0	0.0	0.0	68.2	65.7	63.4	0.0	0.0	0.0	0.0	0.0	0.0

Statistical analysis of *Paenibacillus lautus* band sizes from different sites

Sites that were compared	P values
p1,2 & 5,7	0.0327
1 &3	0.9683
3&5	0.0002
5&7	0.2522
2&3	8E-06
1&2	6E-05