Diversity and functional attributes of microorganisms from stockpiled soils of coal mines in Mpumalanga Province, South Africa

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DECLARATION

I, undersigned, declare that the work contained in this dissertation is my own work and has not been previously submitted by me for a degree at another institution.

Signed: ______________________

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ABSTRACT

Coal mining in South Africa is renowned for large-scale removal of topsoil and subsoil through opencast mining. Such processes lead to an enormous amount of land degradation, and thus limit the land when mining operation ceases. The removal and stockpiling of topsoil lead to adverse effects not only on the physicochemical properties of the soil but also to the microbiological properties of the soil which greatly limit the ability of the soil to sustain plant development. Microbial properties are useful indicators of soil quality and could possibly serve as assessment criteria for successful rehabilitation of ecologically disturbed areas. The purpose of this study was to investigate the impact of soil stockpiling activities on diversity and the functional properties of microorganisms from opencast coal mines in Mpumalanga Province, South African. Samples were randomly collected from the stockpile soils of three opencast coal mines and adjacent unmined land (control) was sampled at depths of 0-20 cm ("topsoil") and >20 cm ("subsoil") in summer, winter and spring seasons. Physicochemical properties, β-glucosidase and urease activities in soils were determined using standard methods, while bacterial (16S rRNA gene) and fungal (Internal transcribed spacer 2 region) diversity were determined using culture-based methods and Polymerase Chain Reaction-denaturing gradient gel electrophoresis (PCR-DGGE). The pure bacterial and fungal isolates obtained from culture-based methods were further evaluated for their soil fertility attributes potentials to play roles in soil nutrient cycling as well as in plant growth enhancement such as phosphate solubilisation, atmospheric nitrogen fixation, and indoleacetic acid (IAA) production. The pattern of differences in the physicochemical properties of soils between unmined and stockpiled soils was not drastic across the three seasons, neither did the nutrient composition and soil physical properties clearly suggest that stockpiled soils were in poorer physicochemical condition compared to soil samples from control sites. The β-glucosidase and urease activities in stockpiled soils were mostly higher ($p<0.05$) than in unmined soils, and varied significantly ($P<0.05$) between seasons in some sites. PCR-DGGE patterns and Shannon-Wiener indices obtained revealed higher microbial diversity in unmined soils than in stockpiles soils across all seasons. Taxonomic analyses of sequences obtained (both PCR-DGGE bands and pure isolates) revealed that phyla Firmicutes (bacteria) and Ascomycota (fungi) were dominant. PCR-DGGE further revealed that *Phialocephala humicola, Mortierella* sp and *Phoma* sp were unique to Mine C. Several potential plant growth promoting microorganisms were obtained. Most of the isolates from both control and stockpiled soils had the potential to fix atmospheric nitrogen. None of the bacterial isolates from stockpiled soils produced IAA. The bacterial isolates from control soils were more efficient in phosphate solubilisation than those obtained in stockpiled soils. In general, the fungal isolates obtained from both control and stockpiled soils were more efficient in phosphate solubilisation and IAA production than bacterial isolates. The results suggest that microbial diversity, bacterial IAA production and phosphate solubilisation, and enzyme activities in soil stockpiles are affected by stockpiling operations. This may have negative implications for nutrient cycling and soil health during post-mining rehabilitation.

Keywords: bacteria, diversity, soil stockpile, enzyme activities, coal mining, nitrogen fixation and IAA production.

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ABBREVIATIONS AND ACRONMYS

Measuring units

cm	Centimetre
°C	Degree Celsius
g	Gram
g/cm^3	Gram per cubic
h	Hour(s)
<	Less than
μg^{-1}	Microgram per gram
µg/ml	Microgram per Milliliter
µl	Microlitre
ml	Millilitre
mm	Millimetre
%	Percentage
rpm	Revolutions per minute
v/v	Volume to volume

General Abbreviations

16S rDNA	Sixteen S ribosomal Deoxyribonucleic Acid
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CEC	Cation Exchange Capacity
CLPP	Community-Level Physiological Profiling
CO_2	Carbon Dioxide
DGGE	Denaturing Gradient Gel Analysis
DNA	Deoxyribonucleic Acid
Dwt	dry weight
EC	Electric Conductivity
gDNA	Genomic Deoxyribonucleic Acid
H'	Shannon-Weiner index of diversity
IAA	Indole Acetic Acid
ITS	Internal Transcribed spacer
J'	Species Evenness
MEA	Malt Extract Agar
NA	Nutrient Agar

NBIRP	National Botanical Research Institute's phosphate
NCBI	National Center for Biotechnology Information
NDP	National Development Plan
NH_3	Ammonia
OTUs	Operational Taxonomic Units
PCR	Polymerase Chain Reaction
PCR-DGGE	Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis
PDA	Potato Dextrose Agar
PFLA	Phospholipid Fatty Acid Analysis
PGPR	Plant growth Promoting Rhizobacteria
PSI	Phosphate Solubilisation Index
RDA	Redundancy Analysis
SSCP	Single-Strand Conformation Polymorphism
T-RFLP	Terminal Restriction Fragment Polymorphism
TSA	Tryptic Soy Agar

CHAPTER 1 – INTRODUCTION

1.1 Background Introduction

Coal is the world's most abundant fossil fuel and the main source of electricity generation in South Africa (Coetzee, 2016; Van Schoor & Fourie, 2014). Approximately 73% of South Africa's energy requirements are provided by coal (BMF, 2014; Mushia *et al.*, 2016). South Africa is ranked the sixth largest coal producer in the world (Hancox & Götz, 2014). Although natural gas, renewable energy sources and nuclear energy are forecast to contribute increasingly to the primary energy supply, coal will remain South Africa's major energy source in the near future, owing to its relative abundance and low cost (Jeffrey, 2005; Van Schoor & Fourie, 2014).

The majority of South Africa's reserves and mines are in the Central Basin (Mpumalanga Highveld Region), which includes the Witbank (eMalahleni), Highveld, and Ermelo coalfields (Fig. 1.1). This region accounts for a number of ecosystem services, including the provision of food through crop farming. Unfortunately, the coal deposits in this area are located below high-quality arable land; thereby posing a land use competition between coal mining and agriculture (Moolman & Fourie, 2000). With a growing energy demand, these valuable areas of land are being impacted negatively.

The 2012 State of the Environment Report indicated that coal mining practices in Mpumalanga transformed 12% of South Africa's high potential arable land, which equates to 326 022 ha (Botha, 2014). In addition to this, another 13.6% is subject to prospecting for coal in the province, equating to 439 577 ha of land that could be mined in the near future. In total, this equates to 765 559 ha of high potential agricultural land in South Africa that could be lost owing to coal mining activities (Botha, 2014). The area of arable land at risk to be mined (439 577 ha) in the near future is almost equal to the potential 500 000 ha that the National Development Plan (NDP) refers to that should be expanded for agricultural use (Botha, 2014).

It is therefore important to understand that opencast coal mining reduces the availability of agricultural land for a diversified economy, poverty alleviation and food security in South Africa. Mining companies deal with this problem by aiming for the rehabilitation of mining areas to productive agricultural land, mostly of a grazing land standard. It is incumbent upon environmental assessment professionals as well as environmental managers on mines to be fully aware of these challenges and to be in a position to be able to advise decision-makers in mining companies and authorising bodies on feasible alternatives to the *status quo*.

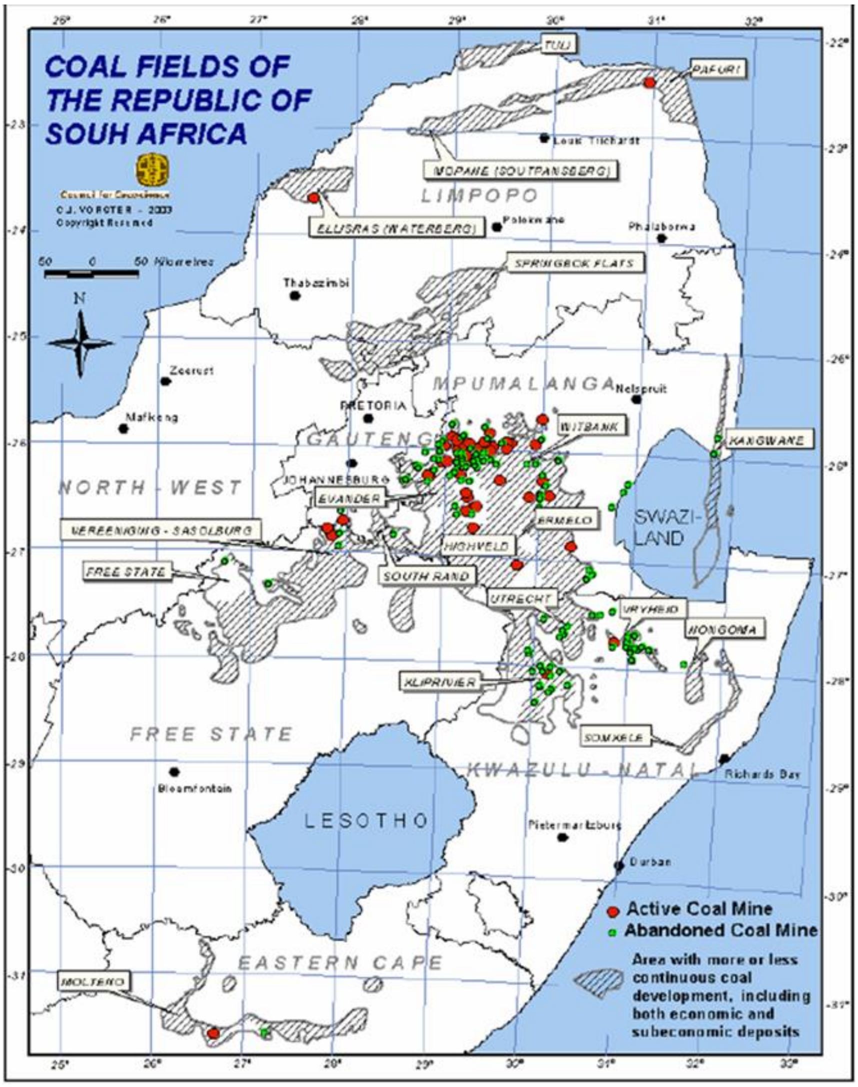


Figure 1.1: **Coalfields of South Africa (Van Schoor & Fourie, 2014).**

For soil quality assessments, a combination of the physical, chemical and biological components of the soil are important parameters. The assessment of these three soil components provides a robust insight into the health of the soil (Claassens *et al.*, 2008; Doran & Parkin, 1994). Only within the last few decades has the soil microbial diversity and activity, as well as enzymatic activity assays, been included in soil quality assessments (Claassens *et al.*, 2008; Dick *et al.*, 1996). The roles of the soil biological entities (microbial and enzyme) are important for several soil ecosystem processes, which contribute towards soil fertility. Such roles include the mobilisation of essential nutrients in the soil, the mediation of plant nutrient uptake, plant-pathogen resistance, secretion of plant-growth promoting-hormones and the formation of organic matter (Tinker, 1984; Van Der

Heijden *et al.*, 2008; Van Veen & Kuikman, 1990). Hence, the diversity and functional properties of these biological molecules have been used as soil quality indices for ascertaining nutrient cycling and availability in the soil (Tabatabai & Dick, 2002) as well as assessing the impacts of farming practices (García-Ruiz *et al.*, 2009) such as soil management (Bending *et al.*, 2004), soil tillage (Fließbach *et al.*, 2007) and fertilizer application (Mandal *et al.*, 2007).

1.2 Problem Statement

Global knowledge of soil microbial diversity and their functions are rapidly increasing (Aislabie *et al.*, 2013). However, there is a paucity of information about microbial diversity and their functions from stockpiled soils of opencast coal mines in Mpumalanga Province, South Africa. Most studies conducted on coal mines have focused on the rehabilitation process. Such studies evaluated the physicochemical properties of stockpiled soils (Wick *et al.*, 2009; Zhen *et al.*, 2015) as well as assessing the performance of those soils in the post-rehabilitation phase, either for grass establishment or for crop production (Mushia *et al.*, 2016; Rethman & Tanner, 1993). However, there is also a paucity of information about the stripping and stockpiling process, despite the fact that if these processes are carried out incorrectly, there will be problems created for later stages of the rehabilitation process.

1.3 Research Aims and Objectives

The present study aims to investigate the impact of soil stockpiling on diversity and the functional properties of microorganisms from stockpiled soils of opencast coal mines in Mpumalanga Province, South African. Specific objectives to achieve this were:

- To determine the physicochemical properties of stockpiled soil;
- To investigate the microbial diversity in stockpile soils using both culture-dependent and culture-independent (PCR-DGGE) methods;
- To determine the functional attributes of isolates and soil microbial communities, and
- To establish a correlation between the physicochemical properties and microbial species in these soils.

1.4 Dissertation Structure

Chapter 2: Literature review – This entails a literature review of the concepts of opencast coal mining and related impacts, rehabilitation practices in the Mpumalanga Highveld, as well as the importance of microorganisms in the soil environment.

Chapter 3: Materials and methods – A description of the study area is presented in this chapter. The chapter also details the methods that were used to acquire the soil: physical, chemical and microbial data. Data preparation and analytical procedures are comprehensively described.

Chapter 4: Results – This chapter offers an interpretation of all the results obtained in this study. Results include: (i) the physicochemical properties of sampled soils; (ii) microbial diversity using culture-dependent and culture-independent results; (iii) functional properties of isolates (nitrogen fixation, phosphate solubilisation and Indole acetic acid production), and functional properties of whole soil community (enzymatic assays) results. A correlation of all results using Redundancy Analysis (RDA) plots is presented.

Chapter 5: Discussion, conclusion and recommendations

A discussion of the results using a theoretical framework from literature is offered. A brief conclusion is made in terms of the findings; recommendations, and a way forward for future research is discussed.

References – Full reference list of all chapters is presented

CHAPTER 2 – LITERATURE REVIEW

2.1 Opencast coal mining

Opencast coal mining is a surface mining technique which involves the complete removal of soil, rocks and vegetation in order to access coal (Waterhouse *et al.*, 2014). Fig. 2.1 represents a graphic demonstration of the opencast coal mining process. The first step is known as the stockpiling process and it involves the stripping of the topsoil (A-horizon) and subsoil (B-Horizon/ overburden) layers by means of shovels and the removal of these materials with trucks to allocated stockpiles where they are stored until the final rehabilitation of the site (Strohmayer, 1999). In some cases, soil is directly placed on available mined-out areas. The second step involves the blasting of overburdened rock material using explosives and the removal thereof to stockpiles separate from the topsoil and subsoil piles, unless directly placed into mined-out voids. By then the first coal seam is exposed and ready to be drilled and blasted, if necessary, and then removed from the pit by trucks and shovels (Botha, 2014; WCA.s.a).

The general occurrence of coal seams in the in the Mpumalanga Highveld coalfields is of a shallow nature and large area stripping ratios are favourable (Hancox & Götz, 2014). These conditions make opencast coal mining the ideal method for the coal extraction of most of the available mineable reserves in this coalfield (Moolman & Fourie, 2000). For these reasons, a large number of opencast coal mining operations are found in this region, ranging from small contractor-based operations to large multi-dragline mines (Moolman & Fourie, 2000).

This process continues with rehabilitation processes, as shown in Fig. 2.2. At first, the interburden and overburden rock material is replaced back into the mined out voids (Botha, 2014). These spoils are then levelled and the area is prepared for the replacement of subsoil and topsoil layers. Once the soils are replaced and levelled, the area is prepared for seeding, or for other agreed end land use (Fresquez & Aldon, 1984).

The removal and storage of the soil layers involve the use of heavy equipment (Strohmayer, 1999). Mixing of the topsoil and subsoil layers creates plant establishment problems during the rehabilitation process. Stockpiled soils tend to suffer from higher bulk densities, resulting in poor aeration, decreased opportunity for root exploration, acidic pH, reduced water-holding capacity and reduced soil nutrients (Abdul-Kareem & McRae, 1984; Seybold *et al.*, 2004; Sheoran *et al.*, 2010). In addition, earthworms, as well as soil microbial populations such as bacteria, fungi and mycorrhizae, are affected (Boyer *et al.*, 2011; Harris *et al.*, 1993; Jasper *et al.*, 1987) These organisms are important in rehabilitation for enhancing plant productivity and diversity, nutrient cycling and soil structural development (Harris *et al.*, 2009).

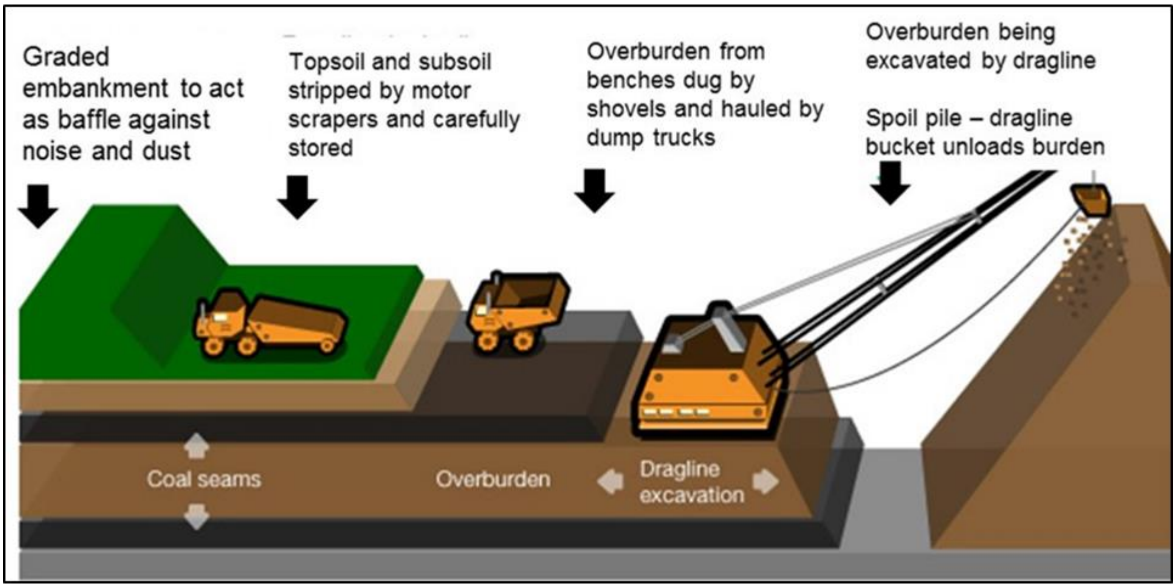


Figure 2.1: **Schematic representation of typical opencast coal mining rehabilitation methods (WCA.s.a).**

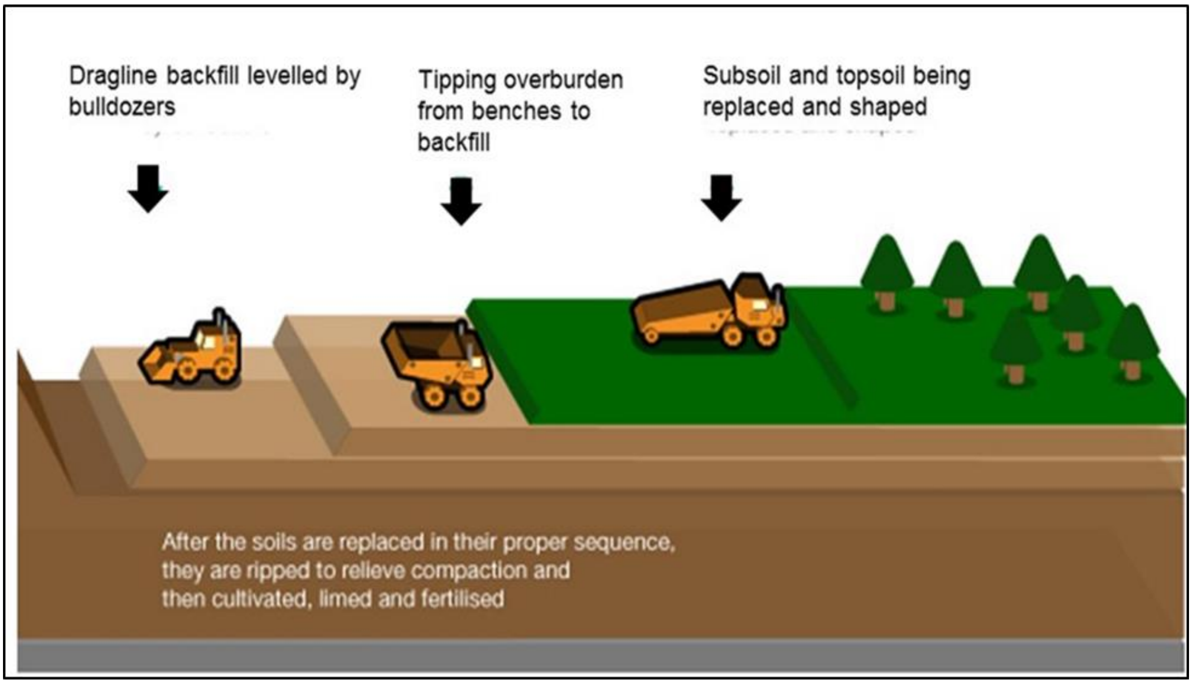


Figure 2.2: **Schematic representation of typical opencast coal mining rehabilitation methods (WCA.s.a).**

2.1.1 Environmental impacts of opencast coal mining

Opencast coal mining is associated with a number of negative environmental impacts, including the pollution of soil, water, and air (Botha, 2014; Cogho, 2012). An unavoidable impact associated with opencast coal mining is land degradation caused by the disturbance of the natural profile of the land (Ghose, 2001). Not only does it cause the natural soil layers and geological strata to be disturbed, but it also results in the disturbance of natural hydrological cycles of specific areas, as well as significant impacts on water resources (Botha, 2014). Land degradation may also lead to soil erosion, destruction of watersheds, siltation of water resources as well as the loss of a valuable resource, namely fertile soil (Ghose, 2001).

Topsoil stripping and stockpiling are essential practices during opencast coal mining, as topsoil forms a critical element for the successful restoration of mines (Strohmayer, 1999). Topsoil cannot always be placed directly onto mined-out land. Therefore, topsoil stockpiling is necessary for future use (COM & CRA, 2007; Sheoran *et al.*, 2010; Strohmayer, 1999). Poor management of topsoil stockpiles will lower the rehabilitation value of the soils (Botha, 2014; Strohmayer, 1999). This, in turn, has an impact on the post-mining land capability and land use once mining has ceased.

Soil loss is a regular occurrence at opencast coal mines, especially older mines where soil management was not a management priority (COM & CRA, 2007). In some areas, soil was not even stripped prior to mining as it was not a requirement to do so (Botha, 2014). Soil is a valuable resource, since it is the growth medium used by vegetation and for food production (Botha, 2014). Adequate soil stripping, stockpiling and management of this resource at a surface coal mine is therefore of utmost importance. Without the management thereof, the post-mining substrate might not only comprise soils (Mentis, 2006), and might not be able to support a good vegetation cover. Soil generation is a lengthy process and takes many years (Botha, 2014). Thus, inadequate management of soils will prolong or compromise the restoration process post-mining (Sheoran *et al.*, 2010; Strohmayer, 1999).

2.1.2 Rehabilitation of opencast coal mines

The South African law requires opencast coal mines to undergo rehabilitation with the end goal of producing sustainable land use (Coetzee, 2016; RSA, 2002). The general practice of rehabilitation at opencast coal mines consists of landscaping spoils, replacing topsoil on landscaped areas and then re-vegetation of those areas (Mentis, 2006). Infrastructure such as mine offices and workshop areas is usually demolished, and the area is then restored by the replacement of topsoil and the seeding thereof. Where discarded dumps are present, these sites are also covered with topsoil to attempt re-vegetation.

One of the objectives of mine rehabilitation in South Africa is to restore the land to a former agricultural capability by using pasture species which are adapted to the climatic region of the mine and are fertilizer responsive (Coetzee, 2016; Mentis, 2006). However, the rehabilitation aim of most opencast coal mining companies in the Mpumalanga Highveld is to re-establish grazing land capability potential post-mining (Botha, 2014; Coetzee, 2016). During the initial stages of mine soil revegetation, grasses are introduced to stabilize soils and to reduce erosion on replaced topsoil while additionally providing rapid methods to build soil organic matter (Coetzee, 2016). The typical seed mix used on rehabilitated coal mines in South Africa comprises annual species such as *Eragrostis tef* in combination with perennial species such as *Eragrostis curvula*, *Cynodon dactylon*, *Cenchrus celiaris*, *Digitaria eriantha* and *Medicago sativa* (Coetzee, 2016).

2.2 The Soil Environments: Physical, Chemical and Biological Properties

2.2.1 Importance of soil

A thorough comprehension of the physical, chemical and biological properties of mine soil is required before the rehabilitation process can resume, owing to the fact that mine soils are, generally inhospitable to vegetation (Smith, 2017). Soil forms the basis of life on earth and sustains environmental quality on different scales; however, if the quality of the soil ecosystems degrade it will lead to a significant decrease in the ability of the soil to maintain sufficient resources for plant communities (Smith, 2017). For ecosystems to survive, soil is needed as a vital living system to sustain, maintain and enhance plant and animal productivity, water and air quality, and plant and animal health (Doran & Zeiss, 2000). Degradation of soil health and quality dowing to anthropogenic influences is of great ecological concern.

Soil quality is one of the vital factors that influence the success of rehabilitation of land disturbed by mining.(Smith, 2017). For an adequate assessment of soil quality, a combination of physical, chemical and biological parameters is advocated (Cardoso *et al.*, 2013).This combination of parameters provides a holistic description of soil quality and embodies the soil health concept (Arias *et al.*, 2005; Doran & Parkin, 1994) which is defined by Doran and Parkin (1994) as “the capacity of soil to function, within ecosystem and land use boundaries, to sustain biological productivity, maintain environmental quality, and promote plant and animal health”.

Additionally, a sound knowledge of the physical, chemical and biological properties of soil is necessary to determine the effects from anthropogenic activities (Smith, 2017). One of the most consequential elements of soil quality is the appropriate management and rational utilisation of soil, which incorporates the importance of correct land use and environmental protection (János, 2012; Smith, 2017). Knowledge about all the processes taking place in the soil environment forms part of the important matter of sustainable development and poses quite a challenge in attempting to improve degraded systems (János, 2012). Linking ecosystem function to ecosystem biodiversity is a significant challenge, and trying to do so in soils is an even greater task (Smith, 2017). Many soil organisms do not have an explanatory role within the carbon and nitrogen cycles that occur in soils, but microbial, plant and animal diversity, and abundance in soil, appears to have various influences on ecosystem function (Smith, 2017).

2.2.2 Soil as a habitat for microorganisms

The soil is fundamental and irreplaceable; it governs plant productivity and maintains biogeochemical cycles (Jeelani *et al.*, 2017). The living biota present in soil is diverse and includes the microflora, the mesofauna as well as macrofauna that control the ecosystem functioning (Jeelani *et al.*, 2017; Nannipieri *et al.*, 2003). The presence of the living biota in soil is dependent upon the physicochemical properties of the soil (Lekhanya, 2010). Soil microbes such as bacteria, archaea and fungi play diverse and often critical roles in the ecosystem (Aislabie *et al.*, 2013) . The vast metabolic diversity of soil microbes means their activities drive or contribute to the cycling of all major elements such as carbon, nitrogen and phosphorus and this cycling affects the structure as well as the functions of soil ecosystems (Aislabie *et al.*, 2013; Jeelani *et al.*, 2017).

2.2.3 The roles of microorganisms in the soil

Microorganisms are essential parts of the living soil and of utmost importance for soil health (Nielsen *et al.*, 2002). As such they have been regarded as sensitive indicators of soil health because of the clear correlation between microbial diversity and soil health and/or quality (R. Adeleke *et al.*, 2010; Lekhanya, 2010; Nielsen *et al.*, 2002). The relationship between microbial diversity and soil functionality is important; considering the fact that 80-90% of processes in soil are mediated by microbes (Nannipieri *et al.*, 2003; Sharma *et al.*, 2014). Microorganisms play important roles in the biogeochemical cycles of the main elements (carbon, nitrogen, phosphorus, etc.) as well as the trace elements (iron, nickel, mercury, etc.), and are therefore critically involved in energy and nutrient exchanges within soil (Evans & Furlong, 2003; Jeelani *et al.*, 2017; Lekhanya, 2010).

Microorganisms are the original recyclers of nature, many of which are able to convert toxic organic compounds to harmless products, such as carbon dioxide and water (Ghosal *et al.*, 2016; Jain *et al.*, 2005). The discovery that microbes have the potential to transform and/or degrade xenobiotics, has led researchers to explore their functional diversity, especially as it relates to their potential to degrade a wide range of pollutants (Bello-Akinosho *et al.*, 2016; Jain *et al.*, 2005; Obi *et al.*, 2016). Owing to the important roles microorganisms play in soil, it is vital to understand the interrelationships between microbes and their environment by studying the structural and functional diversity of microbial communities and how they respond to natural and man-made disturbances (Lekhanya, 2010).

2.2.4 Microbial diversity

In microbiology, the term “diversity” is used to describe the qualitative variation among microbial populations (Magurran & McGill, 2011). Microbial diversity often includes the amount and distribution of genetic information within microbial communities, the diversity of bacterial and fungal species in microbial communities, and ecological diversity (Nannipieri *et al.*, 2003). Torsvik and Øvreås (2002), defined microbial diversity as the complexity and variability of microbes at different levels of biological organisations. Microbial diversity encompasses genetic variability within taxons (species), and the number (richness) as well as the relative abundance (evenness) of taxons in communities. Important aspects of diversity at the ecosystem level are the range of processes, the complexity of interactions and the number of trophic levels (Ovreas & Torsvik, 1998).

A representative estimate of microbial diversity is a prerequisite for understanding the functional activities of microorganisms in ecosystems (Garland & Mills, 1994). Microbial diversity can be divided into different levels, including genetic, taxonomic and functional diversity:

Genetic diversity is defined as the amount and distribution of genetic information in a microbial community (Johnsen *et al.*, 2001).

Taxonomic diversity is defined as the number of different bacterial types and their relative abundance present in a community (Atlas, 1984; Johnsen *et al.*, 2001) and;

Functional diversity is defined by the range of activities and carbon utilisation activities in a community (Torsvik & Øvreås, 2002).

2.2.5 The significance of studying microbial diversity

Microorganisms play important roles in the environment (Hunter-Cevera, 1998). Decomposition processes are dominated by microbial activities and are as fundamental as primary production for the long-term functioning of the ecosystem (Satyanarayana & Johri, 2005). Microbial diversity analyses are therefore essential in order to increase the knowledge of the diversity of genetic resources in a community as well as to understand the relative distribution of organisms. Microbial diversity analyses also increase the knowledge of the functional role of diversity, help to understand the regulation of biodiversity, and to understand the consequences and importance of biodiversity (to what extent the ecosystem functioning and sustainability depend on maintaining a specific level of diversity).

2.2.6 Responses of microorganisms to anthropogenic factors

Microorganisms are known to respond quickly to environmental changes than plants and animals do (Lekhanya, 2010). This is partly attributed to their faster growth rate, as compared to that of macroorganisms. A number of studies have been focused on the response of microorganisms to various stresses/disturbances, such as soil stockpiling during mining (Abdul-Kareem & McRae, 1984; Jasper *et al.*, 1987; Strohmayer, 1999), polyaromatic hydrocarbons (Bello-Akinosho *et al.*, 2016; Maila & Cloete, 2005), herbicides (El Fantroussi *et al.*, 1999) heavy metals (Giller *et al.*, 1998), as well as antibiotics (Thiele-Bruhn & Beck, 2005), farming practices such as soil management , and soil tillage (Bending *et al.*, 2004; Doran, 1980; Fließbach *et al.*, 2007; García-Ruiz *et al.*, 2009). Often after the disturbance, the microbial communities are able to recover and take advantage of the new conditions; thus illustrating the adaptability of microorganisms (Lekhanya, 2010; Shade *et al.*, 2012; Xiang *et al.*, 2014).

2.2.7 Microbial analyses techniques

Over the past 10 years, the approach to analysing soil microbial communities has changed dramatically (Hill *et al.*, 2000). Many new methods and approaches are presently available, providing soil microbiologists with better tools for the assessment of soil microbial diversity (Arias *et al.*, 2005). Some of the most important approaches for studying soil microbial communities are discussed in subsequent sections highlighting their advantages and disadvantages.

2.2.7.1 Culture-dependent methods of microbial community analysis

a) Direct plating and culturing methods

Traditionally, analysis of soil microbial diversity is assessed using selective plating and direct viable counts (Hill *et al.*, 2000). These methods are fast, inexpensive and can provide information on the active, heterotrophic component of the population. Limitations of the direct plating method include the difficulty of dislodging bacteria or spores from soil particle, growth medium selections (Tabacchioni *et al.*, 2000), growth conditions (temperature, pH, light), the inability to culture a large number of bacterial and fungal species with current techniques, and the potential for colony-

colony inhibition or of colony spread (Kirk *et al.*, 2004). In addition, plate growth favours microorganisms with fast growth rates and those fungi that produce large numbers of spores (Dix & Webster, 1995; Kirk *et al.*, 2004). All of these limitations can influence the apparent diversity of the microbial community.

Isolation of cultivable microorganisms is appropriate for functional analysis as it can give an indication of potential plant growth-promoting microorganisms in the soil (Lekhanya, 2010; Vieira & Nahas, 2005). However, a high percentage of soil microbes are non-cultivable (Vieira & Nahas, 2005). Furthermore, the main drawback for culture-dependent approaches is that they cannot reflect the total microbial diversity and, owing to this drawback, molecular tools are preferred for the study of microbial communities (Fakruddin *et al.*, 2013).

2.2.7.2 Culture-independent methods of microbial community analysis

Most soil microorganisms cannot be characterized by conventional cultivation techniques (Arias *et al.*, 2005). Approximately 80-99% of all microbial species have not yet been cultured (Arias *et al.*, 2005; Birk *et al.*, 2009). Culture-independent techniques do not rely on the cultivation of microorganisms on media in a controlled environment. Therefore, it is a more reliable way of investigating the microbial component in soil environments (Van Rensburg, 2010).

To assess microbial diversity (community structure), various molecular techniques can be used. Extracting microbial community DNA with commercially available kits constitutes the first step. Using the PCR technique, extracted DNA can be amplified using specific primers for downstream applications (Van Rensburg, 2010).

Many other culture-independent methods have been used for the investigation of soil microbial community characteristics. These techniques include community-level physiological profiling (CLPP), phospholipid fatty acid analysis (PFLA), and nucleic acid examination, such as terminal restriction fragment polymorphism (T-RFLP), denaturing gradient gel analysis (DGGE), and single-strand conformation polymorphism (SSCP) (Arias *et al.*, 2005; Hill *et al.*, 2000; Van Rensburg, 2010). A short description of the DGGE is presented below.

a) Denaturing Gradient Gel Electrophoresis

The Denaturing Gradient Gel Electrophoresis (DGGE) technique is a PCR-based fingerprinting technique (Piterina & Pembroke, 2013). DGGE separation of the PCR amplicons depends on the denaturation of double-stranded DNA in the gel containing DNA denaturants (Muyzer *et al.*, 1993). The technique exploits the difference in stability of G-C pairing, as opposed to A-T pairing. DGGE involves a mixture of different DNA fragments being electrophoresed in an acrylamide gel containing a gradient of increasing DNA denaturants (Van Rensburg, 2010). Typically, fragments richer in G-C will be more stable and will remain double-stranded until reaching higher denaturant concentrations. Double-stranded DNA fragments migrate faster through the acrylamide gel, while denatured DNA becomes larger and stop in the gel. By means of this mechanism, DNA with a differing sequence can be separated in acrylamide gel (Green *et al.*, 2010).

Bands can be excised from the DGGE gels and sequenced (Díez *et al.*, 2001). The DGGE process can thus provide diversity data as well as the identification of individual species. The technique has a variety of applications, including soil studies. Sakurai *et al.* (2007), used the technique to analyse bacterial communities in soil by targeting protease genes. Piterina and Pembroke (2013), used PCR-DGGE to analyse microbial community diversity and stability during the thermophilic stages of an ATAD wastewater sludge treatment process to monitor performance.

2.2.7.3 Soil enzymatic assays

Enzyme assay is a useful culture-independent technique to assess microbial community properties (Van Rensburg, 2010). Soil enzyme activities reflect potential activity rather than actual *in-situ* activity (Udawatta *et al.*, 2009). This is due to some important factors, which include contrasting conditions of the assay relative to the sampled site, the various enzyme sources, and the possible confounding chemical reactions that affect the measured activity (Nannipieri *et al.*, 2002).

Enzyme activities often correlate with other indicators of activity such as soil respiration, ATP content and microbial biomass (Dick, 1997). Soil enzyme activities can also provide a measure of insight into the metabolic capabilities of soil microbial communities by assessing the potential for the transformation of specific sources of energy or nutrients (Shaw & Burns, 2006). By performing this, an indication of the relative availability or limitation of particular energy or nutrient sources in the environment can be achieved (Makoi & Ndakidemi, 2008). The activity of carbon and nitrogen cycle enzymes in soil has been used to assess soil ecosystem adaptation to anthropogenic intervention and the effects of reclamation management decisions (Tate III, 2002). Soil enzymes that are useful to study include dehydrogenase, β-glucosidase, alkaline- and acid phosphatases and urease (Van Rensburg, 2010). Short descriptions of the β-glucosidase and urease are presented below.

a) β-glucosidase

β-glucosidase is an important enzyme in the carbon cycle. It is the rate-limiting enzyme in microbial degradation of cellulose to glucose (Alef & Nannipieri, 1995). β-glucosidase activity has been found to be sensitive to soil management in various studies and has been proposed as a soil quality indicator (Ndiaye *et al.*, 2000). The method used in this study is based on the colorimetric determination of the p-nitrophenol released by β-glucosidase when the soil is incubated with buffered p-nitrophenyl-β-D-glucosidase (Dick *et al.*, 1996; Van Rensburg, 2010). The *p*-nitrophenol released is extracted by filtration and determined colorimetrically (Dick *et al.*, 1996). β-glucosidase was previous determined from soil under different farming practices (Piotrowska & Koper, 2010), contaminated with heavy metals (Castaldi *et al.*, 2009; Wyszkowska *et al.*, 2010), post-mining coal discards (Claassens *et al.*, 2008) gold mine tails (Nair *et al.*, 2009) and vermicomposting soils (Nair *et al.*, 2009).

b) Urease

Urease catalyses the hydrolysis of urea to carbon dioxide (CO_2) and ammonia (NH_3), and is widely distributed in nature (Kandeler & Gerber, 1988). This process is important for plant nutrition. It is present in microbial, plant and animal cells (Alef & Nannipieri, 1995). The urease assay used in this study is based on the colorimetric determination of released ammonia after the incubation of soil with a urea solution for two hours at 37°C (Kandeler & Gerber, 1988). Wyszkowska and Wyszkowski (2010), found that petroleum pollution at a dose ranging from 2.5 to 10 cm^3/kg inhibited the activities of soil dehydrogenases, ureases and acid phosphatase. Urease activity was also determined on gold mine tailing (Nair *et al.*, 2009), post-mining rehabilitated soils and vermicomposting soils (Nair *et al.*, 2009).

CHAPTER 3 – MATERIALS AND METHODS

3.1 Study Sites and Sampling

Three opencast coal mines (designated A, B and C) located in the eMalahleni, Mpumalanga Province, South Africa were used for this study (Fig. 3.1). The exact location of the mines and names are withheld owing to a confidentiality agreement. The climate of the eMalahleni area is usually warm and moist in summer, while the winter season is usually cold and dry with frost. This area receives about 750 mm of rainfall annually, 85% of which occurs during the growing season (October to March) (Mushia *et al.*, 2016).

The soil stockpiles at the time of sampling were sparsely vegetated (~10% grass and ~2% forbs cover) and the ages of the stockpiles were a minimum of 5 years. Soil samples were randomly collected from topsoil stockpiles at depths of 0-20 cm (hereafter referred to as “topsoil”) and >20 cm (hereafter referred to as “subsoil”) by using a sterile auger during the summer, winter and spring seasons (February-September) of 2015. Unmined lands adjacent to the coal mining sites served as “controls”. However, the history of other anthropogenic activities (apart from mining) on these “control” soils could not be ascertained. Soil samples were aseptically collected in sterile bags and transported on ice to the laboratory. Samples were stored at 4°C prior to analyses. Samples for enzyme analyses were analysed within five days of collection.

3.2 Physical and Chemical Characteristics of Soil

The physicochemical properties of collected soil samples, including bulk density, pH, cation exchange capacity (CEC), electric conductivity (EC), total nitrogen, organic carbon and mineral contents (phosphorus, magnesium, zinc, copper, sodium and potassium) were analysed using standard methods (Non Affiliated Soil Analysis Working Group, 1990).

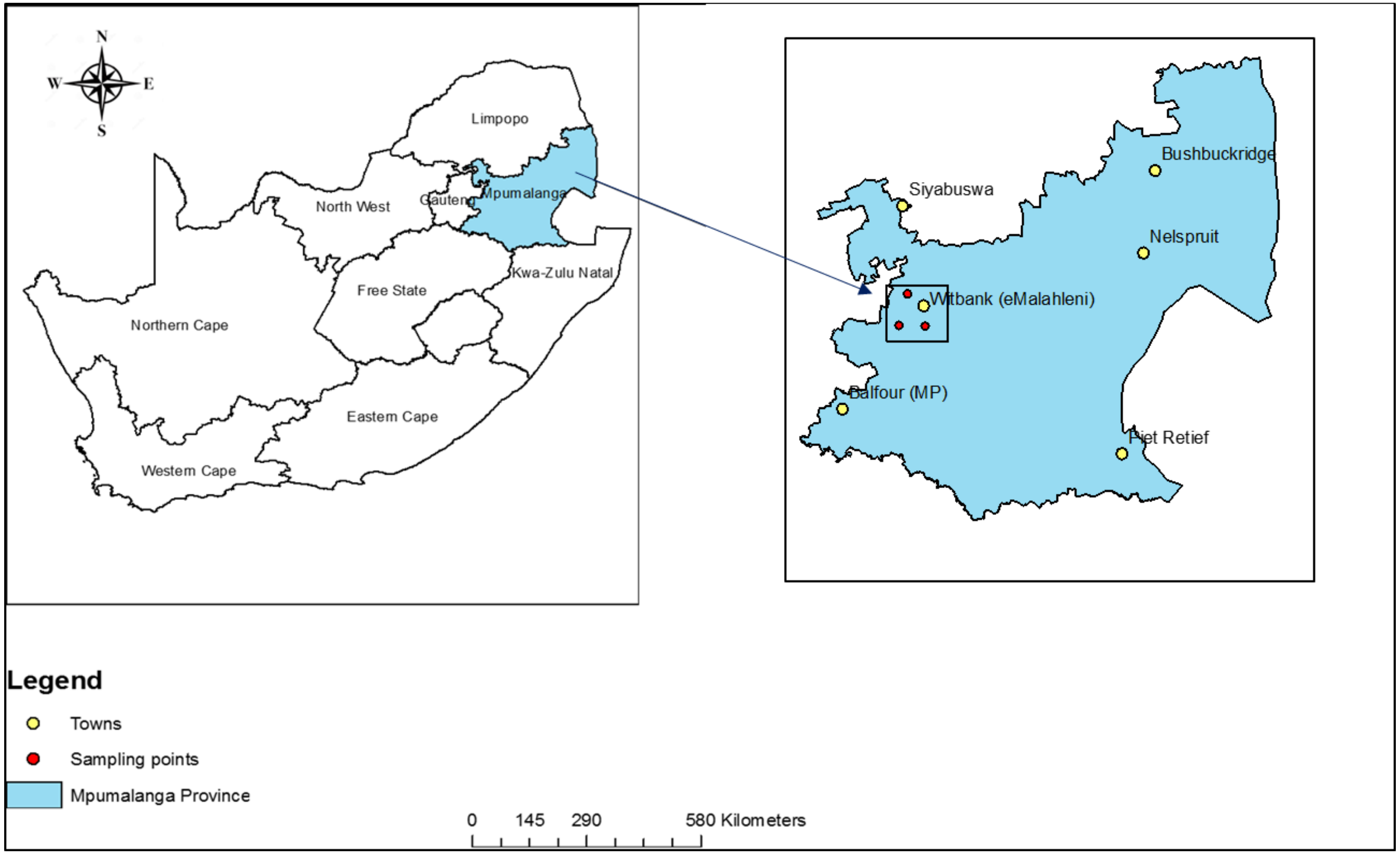


Figure 3.1: Study area located in Witbank (eMalahleni).

3.3 Microbial Diversity Analyses

3.3.1 Analyses of soil microbial diversity by culture-dependent methods

For each soil sample, 1 g of soil was suspended in 9 ml of sterile distilled water and vortexed thoroughly. From this stock solution, serial dilutions were performed to 10^{-8}. Aliquots of 100 µl from dilutions of 10^{-3} to 10^{-6} for fungi, and 10^{-4} to 10^{-7} for bacteria, were plated in triplicate on culture media (Stefani *et al*., 2015). Bacteria were plated on tryptic soy agar (TSA) and nutrient agar (NA). Fungi were plated on potato dextrose agar (PDA) and malt extract agar (MEA). Petri dishes were inoculated, inverted and incubated at 27°C for bacteria and 25°C for fungi. Following incubation, distinct colony morphotypes were picked, streaked and successively sub-cultured to obtain pure isolates. The isolates were stored in 50% glycerol at -75°C for future use.

3.3.2 Molecular identification and phylogenetic analyses of microbial isolates

For bacterial identification, the 16S rRNA gene of pure isolates was amplified using primer sets 341F (5'CCTACGGGAGGCACCAG3) and 907R (5' CCGTCAATTCCTTTGATTT3') (Muyzer *et al.*, 1993) making use of a colony PCR approach. Bacterial colonies were transferred with a sterile pipette tip into 1.5 ml microcentrifuge tubes containing 20 µl of sterile distilled water and the resulting suspension was homogenized with a vortex (Labnet International, USA). Fungal isolates were subcultured for one week in PDA before harvesting the mycelium for the isolation of genomic DNA (gDNA). All gDNA isolations were performed using ZR Bacterial and Fungal DNA extraction kit (Zymo Research Corporation, USA) according to the manufacturer's instructions. PCR conditions used for both bacterial 16S rRNA and fungal ITS2 amplifications were exactly as previously described by Ezeokoli *et al.* (2016).

3.3.3 Analysis of soil microbial community by culture-independent method

3.3.3.1 Extraction of total community DNA

Total community DNA was extracted from soil using the ZR Soil Microbe DNA extraction kit (Zymo Research, Irvine, CA, and USA) according to the manufacturer's instruction. DNA integrity and concentration were determined by agarose gel electrophoresis and fluorimetric quantification (Qubit 2.0 fluorimeter, Invitrogen, California, USA), respectively. Extracted DNA was stored at -20°C prior to analyses. All PCRs were performed in a C100^{TM} thermal cycler (Bio-Rad Laboratories, CA, USA).

3.3.3.2 Polymerase Chain Reaction-Denaturing gradient gel of soil microbial communities

For bacterial community analyses, the V3–V5 region of the 16S rRNA gene was amplified using primers 341F (5′-CCTACGGAGGCAGCAG-3′) and 907R (5′ CCGTCAATTCCTTT GAGTTT-3′) (Muyzer *et al.*, 1993). For fungal community analyses, the internal transcribed spacer 2 (ITS2) region was amplified using primers ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.*, 1990). A 40 bp GC clamp was attached to the 5′-end of all forward primers (Muyzer *et al.*, 1993). PCR conditions used for both bacterial 16S rRNA and fungal ITS2 amplifications were as previously described by Ezeokoli *et al.* (2016). PCR products of soil samples collected at similar depths during a single season were pooled in equal proportion (per volume basis) for each site.

For DGGE, 30 µl of pooled PCR amplicons were mixed with 10 µl of 6X loading dye and loaded onto a 1 mm thick 6% and 8%w/v polyacrylamide gel of denaturing gradient (40%-60%v/v urea and 40% v/v formamide) for bacteria and fungi, respectively. DGGE was performed on the Dcode™ Universal Mutation Detector System (Bio-Rad, Hercules, CA, USA) as previously described by (Ezeokoli *et al.*, 2016).

DGGE gels were subjected to densitometric analyses using the Gene Tools software version 4.03.1.0 (Syngene, Cambridge, UK) as previously described by Mashiane *et al.* (2017). A weighted similarity matrix generated based on the relative intensities of individual peaks and band positions was subjected to hierarchical clustering using the complete linkage method in R software (Team, 2013). Furthermore, the estimation of diversity indices, including the Shannon-Weiner diversity index (H’) and species evenness (J’), was computed, based on the general assumption that different species (sequences) migrate to different positions on the DGGE gel. H´ and J´ were computed in the vegan package of R software.

Dominant bands were excised from DGGE gels using a sterile scalpel. Excised bands were incubated in 20 µl of sterile PCR-grade water at 4°C overnight to elute DNA. Two microlitres of the eluted DNA were used as a template for PCR reamplification by using the same set of primers as in the PCR earlier described, excluding the 40 bp GC-rich clamp attached to the forward primers.

3.4 Sequencing and Taxonomic Assignment of Dominant DGGE Bands and Microbial Isolates

PCR amplicons of pure bacterial and fungal isolates and PCR-DGGE bands were purified and sequenced. Sequence electrophoretograms were inspected and manually edited using the BioEdit software (Hall, 1999). Edited sequences were then clustered into operational taxonomic units (OTUs) at 97% 16S rRNA gene and ITS2 sequence similarity using the mothur software (Schloss *et al.*, 2009). For taxonomic assignments, representatives of bacterial and fungal OTUs were aligned against the NCBI GenBank and UNITE ITS database (https://unite.ut.ee/analysis.php), respectively. Sequences obtained in this study are available in GenBank under the accession numbers MF001318-MF001351, KY009535-KY009570, KY985473-KY985518, KY344798-KY344916, KY344917-KY345043, KY582421-KY582432 and KX375199-KX375227

3.5 Functional Properties

3.5.1 Phosphate solubilisation assay

The ability of the isolated bacteria and fungi to solubilise insoluble inorganic phosphate was investigated on the National Botanical Research Institute's Phosphate (NBRIP) growth medium (Nautiyal, 1999). The medium contains insoluble tricalcium phosphate [$Ca_3(PO_4)_2$], as a source of phosphate. Ten microliters of a 48-hour nutrient broth culture were dispensed into wells created in the medium and incubated at 30°C (Bello-Akinosho *et al.*, 2016). A positive result for solubilisation of phosphate was characterised by a clear halo around the inoculum well after 5-7 days of incubation. The phosphate solubilisation index (PSI) was calculated using the formula below (Bello-Akinosho *et al.*, 2016).

$$PSI = (diameter\ of\ well + diameter\ of\ halozone)/(dimater\ of\ well)$$

3.5.2 Nitrogen fixation assay

Burks's nitrogen-free culture medium was used to screen isolates for atmospheric nitrogen-fixing ability. The appearance of growth within 7 days of aerobic incubation at 28°C was indicative of the isolates' potential nitrogen-fixing ability (Bello-Akinosho *et al.*, 2016).

3.5.3 Indoleacetic acid (IAA) assay

The isolates were inoculated on a 1% tryptophan culture broth and incubated at 28°C for 48 hours with continuous agitation at 130 rpm. The culture broths were subsequently centrifuged at 10 000 rpm for 10 min at 4°C. Exactly 1 ml of the supernatant was mixed with 2 ml of Salkowski's reagent. The mixture was shaken and kept at room temperature in the dark for 30 minutes. The development of a pink colouration indicated the production of IAA. Subsequent quantification of IAA was done on a spectrophotometer at a wavelength of 540 nm (Gordon & Weber, 1951). The obtained reading was used to calculate the IAA content by extrapolation from a standard curve of pure IAA.

3.5.4 Enzymatic assays

Soil enzyme activities are mostly concentrated in the topsoil (0-20 cm) region (Das & Varma, 2010). Hence, enzyme assays were performed only for topsoil samples. Samples were passed through a 2-mm sieve and oven dried at 40°C prior to the determination of β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) and urease (urea amidohydrolase, EC 3.5.1.5) activities as reported by Van Wyk *et al.* (2017).

3.5.5 Statistical analyses

Enzyme activity data were subjected to one-way analysis of variance (ANOVA) using SPSS software (v. 21, IBM Corporation, New York, USA). Significant means values were separated using the Duncan Multiple Range Test at 5%. To understand the response and/or relationship of the microbial communities to soil physicochemical conditions, the data were subjected to a redundancy analysis (RDA) performed in CANOCO 4.5 (Ter Braak & Smilauer, 2002) per soil depth.

CHAPTER 4 – RESULTS

4.1 Physical and Chemical Properties of the Soil

The physiochemical properties of the soils are summarised in Supplementary Table A1 and A2. The soils were acidic (pH 4.30 to 5.79) and had bulk density values between 1.34 to 1.62 g/cm^3. The cation exchange capacity ranged from 3.35 to 10.94 meq. 100 g^{-1}, organic carbon from 0.43 to 4.4%, total nitrogen from 0.05 to 0.59% and carbon to nitrogen ratio (C:N) from 0.01 to 42.42.

In general, the pattern of differences in the physicochemical properties of soils between unmined and stockpiled soils was not drastic across the three seasons. Neither did the nutrient composition and soil physical properties clearly suggest that stockpiled soils were in poorer physicochemical condition compared to soil samples from control sites. However, the mean C: N in Mine B and Mine C were much lower than those of Mine A and the control site (Supplementary Table S1 & Table S2). In both topsoils and subsoils, the lowest pH values were recorded in Mine C, whereas the highest bulk density value was recorded in Mine B.

4.2 Microbial Diversity using Culture-Dependent Techniques

A total of 300 bacterial and 250 fungal sequences were analysed. The bacterial and fungal sequences clustered into 10 and 8 operational taxonomic units (OTUs) respectively. The diversity and taxonomic affiliation of bacterial and fungal OTUs are presented in Table 4.1. Of the ten bacterial OTUs/species obtained, the majority (50%) belonged to phylum Firmicutes, 30% belonged to phylum Proteobacteria and the rest (20%) to the phylum Actinobacteria. The genus Bacillus was dominant (40%) and included close relatives of *Bacillus gaemokensis*, *B. zhangzhouensis, B. acidiceler* and *B. amyloliquefaciens*. The other bacterial OTUs were close relatives of *Pseudomonas granadensis, Pseudomonas turukhanskensis, Streptomyces reticuliscabiei, Paenarthrobacter nitroguajacolicus* and *Azomonas macrocytogenes* (Table 4.1 and Supplementary Fig. A1). The neighbour-joining phylogenetic tree (Supplementary Fig. A1) depicts the taxonomic affiliations of bacterial OTUs. All the OTUs were present in the topsoil samples, whereas, bacterial OTU1, OTU2, OTU3, OTU6, OTU7 and OT10 were present in subsoil samples (Table 4.1). None of the bacterial OTUs obtained were unique to a sampled site or depth (Table 4.1).

Table 4.1: Distribution and taxonomic affiliations of microbial isolates OTUs from sampled soils

*OTU no.	Closest relative	% Similarity	Site ($^{\beta}$Depth)
Bacteria			
1	*Pseudomonas granadensis*	98.72	All (T,S)
2	*Bacillus gaemokensis*	99.14	All (T,S)
3	*Bacillus acidiceler*	94.98	Control (T,S) , MA (T), MB (T,S), MC (T, S)
4	*Bacillus amyloliquefaciens*	96.17	Control(T) and MA (T)
5	*Pseudomonas turukhanskensis*	91.77	Control (T), MC (T)
6	*Azomonas macrocytogenes*	81.38	All (T,S)
7	*Lysinibacillus macrolides*	98.74	All (T,S)
8	*Streptomyces reticuliscabiei*	69.01	Control (T) and MA (T) and MC (T)
9	*Paenarthrobacter nitroguajacolicus*	99.25	Control(T) and MA (T)
10	*Bacillus zhangzhouensis*	98.56	All (T,S)
Fungi			
1	*Penicillium simplicissimum*	100	All (T,S)
2	*Fusarium oxysporum*	97.51	All (T,S)
3	*Aspergillus terreus*	97.84	All (T)
4	*Trichoderma atroviride*	95.58	Control (T), MA (S) MC (T)
5	*Chaetomium strumarium*	100	All (T,S)
6	*Talaromyces pinophilus*	100	Control (T,S), MA (T)
7	*Thielavia terricola*	99.47	All (S)
8	*Irpex lacteus*	100	MB (T), MC (T)

**Operational taxonomic units of bacterial (16S rRNA gene) and fungal (ITS units) sequences were clustered into OTUs at 97% similarity. ῳ Depth: Topsoil (T); Subsoil (S). MA, Mine A; MB, Mine B; and MC; Mine C. All: present in all mines.*

Of the eight fungal OTUs/species obtained, the majority (87.5%) belonged to phylum Ascomycota and the rest (12.5%) to the phylum Basidiomycota (Supplementary Fig. A2). The OTUs were close relatives of *Penicillium simplicissimum, Fusarium oxysporum, Aspergillus terreus, Trichoderma atroviride, Chaetomium strumarium, Talaromyces pinophilus, Thielavia terricola and Irpex lacteus* (Table 1). Across sites, fungal OTU1, OTU2, OTU3 and OTU5, were present in all sites at the topsoil throughout all seasons (Table 2). OTU7 was present in all sites at the subsoil throughout all seasons. The other OTUs were present in two or more sites. None of the OTUs were unique to any site across seasons (Table 4.1). The species belonging to this fungal phylum are indicated by the annotations in the maximum likelihood phylogenetic tree in Supplementary Fig. A2.

4.3 Microbial Diversity using Culture-Independent Techniques

The PCR-DGGE profiles of bacterial and fungal diversity in the soils are presented in Fig. 4.1. The bacterial and fungal PCR-DGGE profiles showed higher diversity in both topsoil and subsoil of control soils than in the coal mine stockpile soils (Fig. 4.1a-d). The hierarchical cluster dendrograms of bacterial diversity in topsoil reveal that the bacterial community in the topsoil of control soils was dissimilar to that of stockpile topsoils at all three seasons (Fig. 4.1b). Furthermore, there were intra-mine similarities in the seasonal bacterial diversity of topsoils for all the studied coal mines. However, the hierarchical cluster dendrograms suggest there were no clear intra-seasonal or intra-mine similarities in the bacterial communities in the subsoil (Fig. 4.1d).

Nevertheless, bacterial communities in subsoils appear to be more similar within seasons across coal (intra-seasonal similarity). In addition, the bacterial community in the subsoils of Mine A and Mine B were similar in the spring season. The summer and spring soil bacterial communities in the subsoils of the control soils were similar. Fungal communities in the topsoil of the control soils were similar across seasons (Fig. 4.1e-f) and clustered differently from those of stockpiled topsoils. In the three coal mining sites, the fungal communities in the topsoil of the spring and winter soil samples were closely similar but less similar to the fungal community in the summer (Fig. 4.1f). In contrast, fungal communities in the subsoils during the summer and winter seasons were closely similar in both subsoils of the control and for each of the three mines’ stockpiles (Fig. 4.1h). The fungal communities were more closely similar between samples collected in summer and winter at each site.

4.3.1 Microbial diversity indices of soils

The Shannon-Weiner index of diversity (H') and species evenness (J') of both bacterial and fungal communities of topsoils and subsoils are presented in Table 4.2. The highest bacterial and fungal species richness was generally observed in the topsoil and subsoil of control soils at all three sampling seasons (Table 4.2). Between seasons, bacterial and fungal species richness and diversity in topsoils were mostly highest in summer, whereas, the lowest bacterial and fungal species richness in topsoils was observed in soil samples collected during spring (Table 4.2).

In subsoils, the highest bacterial species richness and diversity (H') were observed in winter samples for both control sites and Mine B; in summer for Mine A and in spring for Mine C, whereas, the highest fungal species richness and diversity (H') were observed in the spring and winter samples respectively for the control soils; in the spring and summer subsoil samples respectively for Mine A; and in summer for Mine B and Mine C.

Table 4.2: Microbial diversity indices of soil samples

	Bacteria						Fungi					
Sample ID	**Topsoil**			**Subsoil**			**Topsoil**			**Subsoil**		
	Obs.	**H'**	**J'**	**Obs.**	**H'**	**J**	**Obs.**	**H'**	**J'**	**Obs.**	**H'**	**J'**
Summer												
Control	17	2.55	0.90	19	1.81	0.62	17	2.44	0.86	14	2.34	0.89
Mine A	7	1.78	0.91	14	1.45	0.55	15	2.10	0.78	8	1.90	0.91
Mine B	6	1.36	0.76	11	1.79	0.74	7	0.82	0.42	9	1.98	0.90
Mine C	10	2.18	0.95	8	1.86	0.90	8	1.96	0.94	8	1.92	0.92
Winter												
Control	16	2.43	0.88	21	2.45	0.80	13	2.29	0.89	16	2.49	0.90
Mine A	6	1.54	0.86	5	1.57	0.97	9	1.95	0.89	7	1.56	0.80
Mine B	5	1.34	0.83	13	1.90	0.74	10	1.06	0.46	6	1.56	0.87
Mine C	17	2.63	0.93	6	1.63	0.91	14	2.36	0.90	6	1.50	0.84
Spring												
Control	15	2.44	0.90	15	1.87	0.69	14	2.40	0.91	18	2.46	0.85
Mine A	3	0.87	0.79	13	2.44	0.95	9	2.04	0.93	10	1.85	0.80
Mine B	5	1.42	0.88	5	1.50	0.93	6	0.80	0.45	7	1.43	0.74
Mine C	13	2.29	0.89	15	2.40	0.89	9	2.05	0.93	8	1.53	0.74

Obs*: Number of bands, **H': Shannon-Weiner index and **J'**: Evenness*

The evenness values showed that bacterial populations are more evenly distributed in the topsoil than in the subsoil for most study sites across sampling seasons, whereas, the fungal species populations are more evenly distributed in the subsoil than in the topsoil for both control soils and for most of the study sites across sampling seasons.

4.3.2 Taxonomic diversity of microbial communities in soils

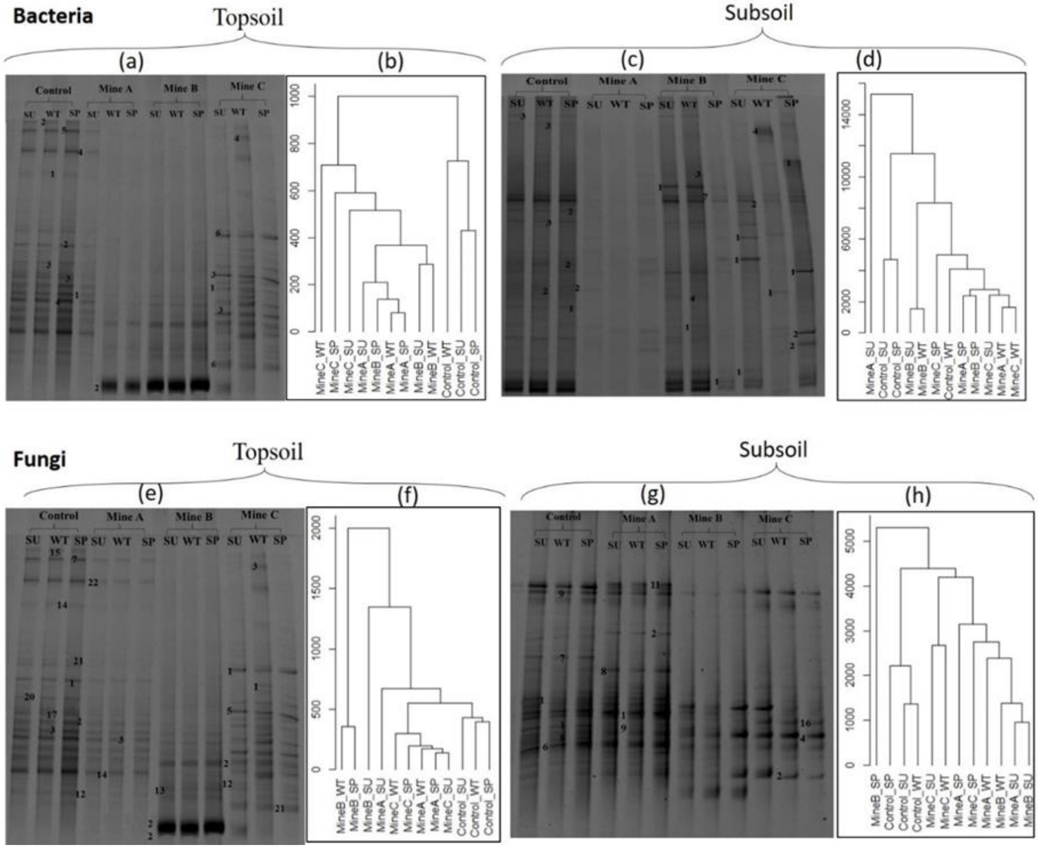
The distribution and taxonomic affiliations of OTUs in the soil samples are presented in Table 4.3. A total of 44 and 31 bands were analysed (excised and sequenced) from bacterial rRNA and fungal ITS2 PCR-DGGE profiles respectively. The bacterial and fungal sequences clustered into 7 and 22 OTUs respectively.

For all the sampled soils, bacterial OTU2 and OTU4 were present in the topsoil, but bacterial OTU7 was only present in subsoil (Fig. 4.1c). None of the bacterial OTUs obtained was unique to a sampled site or depth (Fig. 4-1a, 2b and Table 4.3). Across sites, fungal OTU2, OTU4 and OTU7 were present in all sites throughout all seasons (Table 4.3). The fungal OTU 1 and OTU3 were present in all but one site (Mine A). OTU5 was present in the control site throughout all seasons, whereas in Mine A, it was only present in winter. Similarly, OTU 6 was only present in the control sites and in Mine C throughout all seasons. None of the fungal OTUs was unique to any site across seasons (Fig. 4.1 and Table 4.3).

Amongst the 7 bacterial OTUs/species obtained, the majority (57%) belonged to phylum Firmicutes, while the rest (43%) belonged to the phylum Proteobacteria. The genus *Bacillus* was dominant (43%) and included close relatives of *Bacillus gaemokensis*, *B. zhangzhouensis* and *B. amyloliquefaciens*. The other bacterial OTUs were close relatives of *P. paralactis*, *P. matsuisoli*, *Lysinibacillus macroides*, and *Azomonas macrocytogenes* (Table 4.3 and Supplementary Fig. A3). The neighbour-joining phylogenetic tree (in Supplementary Fig. S3) depicts the taxonomic affiliations of bacterial OTUs.

Across soil depths, the fungal OTU1, OTU2, OTU3, OTU5, OTU12, OTU13, OTU14 and OTU20 were present in all topsoils (Fig. 4.1e and Table 4.3). Fungal OTU4, OTU6, OTU8, OTU16, OTU18 and OTU19 were present in all subsoil samples (Fig. 4.1g and Table 4.3). Fungal OTU9, OTU15 and OTU17 were unique to the topsoil of control soils, while OTU21 was unique to the subsoil of control sites (Table 4.3).

Fungal OTU1, OTU4, OTU6 and OTU9 were present in all the sampled soils throughout all seasons. OTU 8 was unique to Mine A while OTU16 and OTU21 were unique to Mine C. However, these OTUs were present in all seasons. The majority (86%) of the fungal OTUs obtained belonged to the phylum Ascomycota, while 9% and 5% belonged to Mucoromycota and Basidiomycota, respectively (Supplementary Fig. A4). The fungal species under each phylum are indicated by annotations in the maximum likelihood phylogenetic tree in Supplementary Fig. S4.



** SU, summer; WT, winter; SP, spring. Numbered bands/positions and numbers indicate excised bands and OTU numbers (as in Table 2, respectively.*

Figure 4.1: PCR-DGGE gel image and hierarchical cluster dendrogram of microbial communities in soils. (A-B) Bacterial 16S rRNA gene diversity in topsoils. (C-D) Bacterial 16S rRNA gene diversity in subsoils. (E-F) Fungal ITS2 gene diversity in topsoils. (G-H) Fungal ITS2 gene diversity in subsoil.

Table 4.3: Distribution and taxonomic affiliations of microbial communities OTUs in soils across sites and depth

*OTU no.	Closest relative	% Similarity	Site ($^{\omega}$ Depth)
Bacteria			
1	*Bacillus gaemokensis*	100	Control (T,S), MB (T,S) ,MC (T,S)
2	*Pseudomonas paralactis*	100	Control (T,S) , MA (T,S), MB (T,S), MC (T,S)
3	*Bacillus zhangzhouensis*	100	Control (T,S),MA (T), MB (S),MC (T,S)
4	*Bacillus amyloliquefaciens*	100	Control (T,S) , MA (T), MB (T,S), MC (T, S)
5	*Azomonas macrocytogenes*	100	Control(T) and MA (T)
6	*Lysinibacillus macroides*	100	Control (T), MC (T)
7	*Pseudomonas matsuisoli*	98.63	Control (S), MA(S), MB (S), MC (S)
Fungi			
1	*Aureobasidium pullulans*	99.76	Control (T,S), MA (T,S), MC (T)
2	*Phoma herbarum*	99.36	Control (T,S) , MA (T,S), MB (T,S), MC (T,S)
3	*Fusarium fujikuroi*	99.36	Control (T) , MA (T) , MB (T) ,MC (T)
4	*Claviceps purpurea*	99.44	Control (S) , MA (S), MB (S), MC (S)
5	*Alternaria tenuissima*	96.88	Control (T), MC (T)
6	*Deconica novae-zelandiae*	92	Control (S), MA(S), MB (S), MC (S)
7	*Austroplaca soropelta*	99.35	Control (T,S), MA (T)
8	*Umbelopsis vinacea*	85.56	MA (S)
9	*Pyrrhospora arandensis*	99.24	Control (S), MA (S), MB (S), MC (S)
10	*Alternaria petroselini*	97.6	MB (T), MC (T)
11	*Curvularia trifolii*	99.41	Control (T), MA (T)
12	*Neoscytalidium dimidiatum*	98.08	Control (T), MB(T), MC (T)
13	*Dendroclathra lignicola*	99.36	MA (T), MB (T), MC
14	*Helotiales* sp.	99.35	Control (T), MA (T), MC (T)
15	*Macrohilum eucalypti*	98.28	Control (T), MA (T)
16	*Phialocephala humicola* (*Scolecobasidium humicola)*	99.34	MC (S)
17	*Valdensinia heterodoxa*	93.33	Control (T), MC (T)
18	*Phoma* sp.	99.39	MC (S)
19	*Diaporthe foeniculina*	99.38	Control (S), MA (S)
20	*Cenangium acuum*	99.35	Control (T) , MA(T), MC (T)
21	*Mortierella* sp.	88.66	MC (T)
22	*Sordariomycetes* sp.	99.17	Control (T), MA (T)

**Operational taxonomic units at bacterial (16S rRNA gene) and fungal (ITS units) sequences were clustered into OTUs at 97% similarity. ω Depth: Topsoil (T); Subsoil (S). MA, Mine A; MB, Mine B; and MC; Mine C.*

4.3.3 Relationship between soil physicochemical properties and microbial communities

The relationship between physicochemical properties and microbial OTUs in soils are depicted by the redundancy analysis (RDA) triplots in Fig. 4.2. Based on the correlation matrix generated for the topsoil RDA (Table A3), the first species axis was positively correlated with organic carbon, total nitrogen, phosphorus (Bray 1), β-glucosidase activity and urease activity in topsoils.

However, negative correlations were observed for pH and bulk density, as indicated in Table S5. In subsoils, positive correlations were observed between the first species axis and pH, soil organic carbon, total nitrogen, phosphorus (Bray 1) and cation exchange capacity (Table A4). Contrary negative correlations were observed for Ca, Mg, k and soil value (Table A4).

Negative correlation was observed between fungal OTU15 (*Macrohilum eucalypti*), OTU7 (*Austroplaca soropelta*), OTU22 (*Cenangium acuum*) and OTU19 (*Diaporthe foeniculina*), and these appear to be positively associated with C: N. Fungal OTU13 (*Dendroclathra lignicola*) was positively correlated with organic carbon content of soils (Fig. 4.2a).

β-glucosidase activity, urease activity as well as soil nutrients (P, Na and Mg) did not directly influence microbial communities in these soils (Fig. 4.2a). On the other hand, the distribution of bacterial OTU1 (*Bacillus gaemokensis*), OTU2 (*Pseudomonas paralactis*) and OTU4 (*Bacillus amyloliquefaciens*), as well as fungal OTU2 (*Phoma herbarum*) and fungal OTU3 (*Fusarium fujikuroi*) were least influenced by any of the physicochemical properties in the topsoil (Fig. 4.2a)

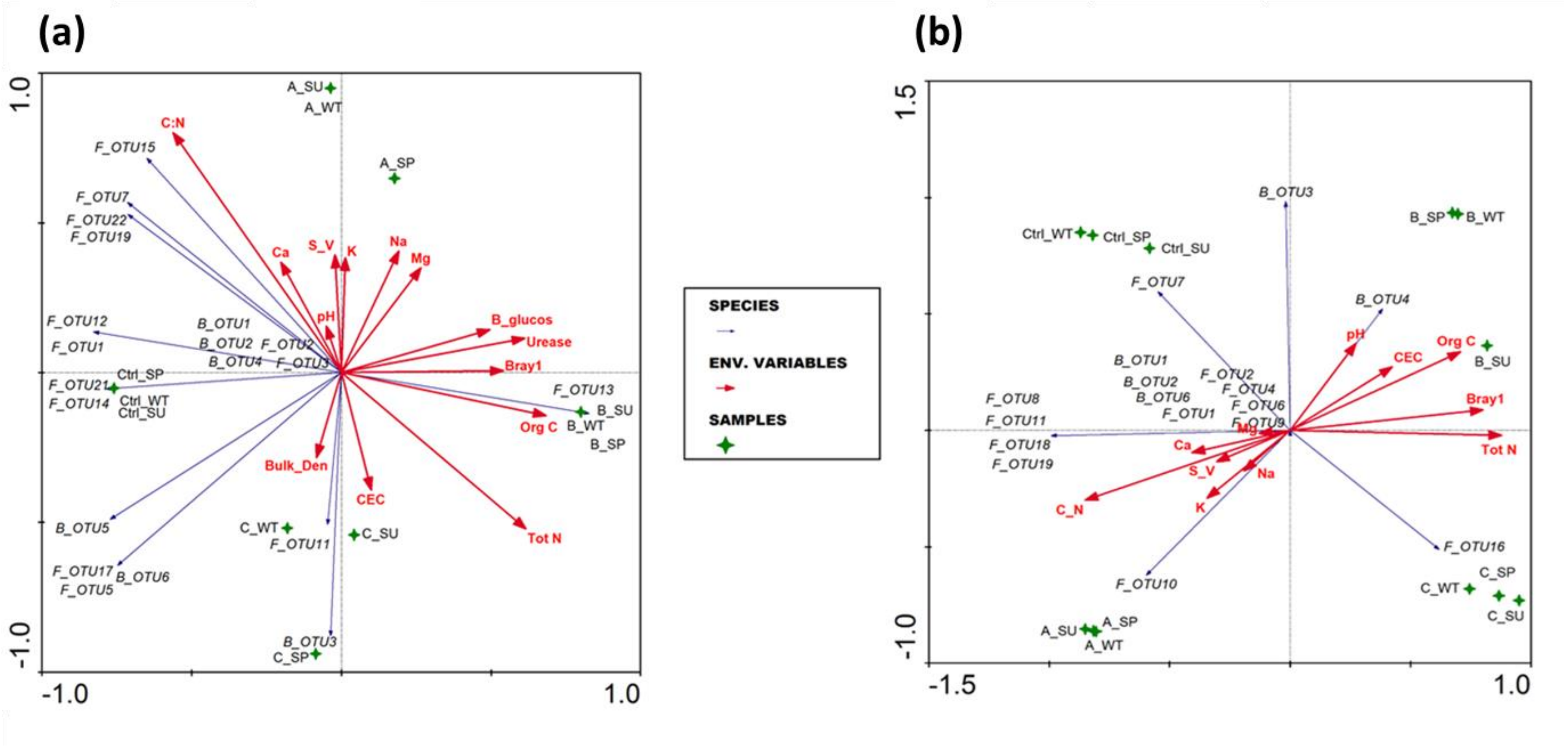


Figure 4.2: RDA triplot showing correlation between soil physicochemical properties, enzyme activities and microbial operational taxonomic units: (a) Topsoil and (b) Subsoil.

4.4 Functional Properties of Isolates

4.4.1 Nitrogen fixation

The ability of the bacterial isolates to fix atmospheric nitrogen was evaluated and the isolates divided into two groups, non-fixers or fixers. Over 60% (topsoil) and 40% (subsoil|) bacterial isolates from both stockpiled and control soils were found to be nitrogen fixers (Fig. 4.3a&b).

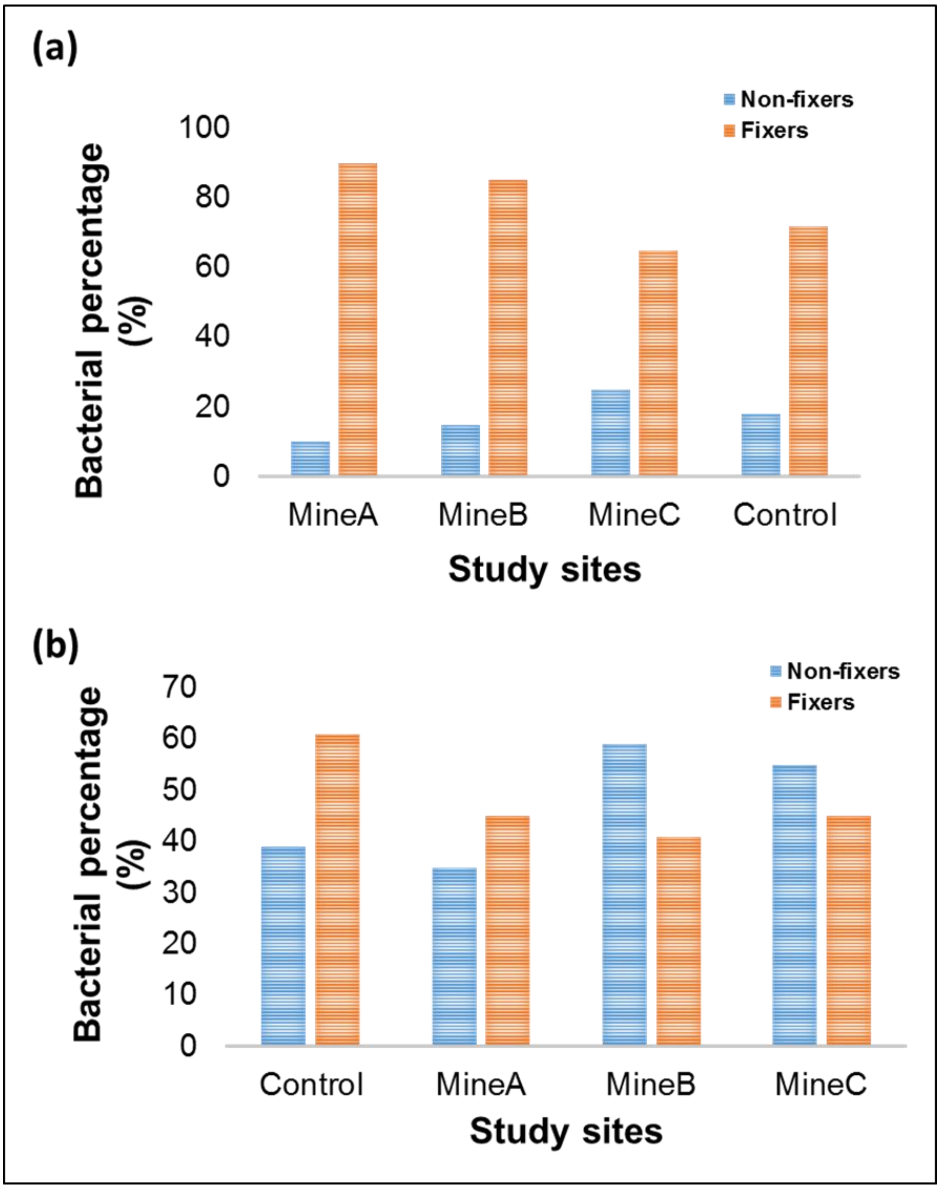


Figure 4.3: Nitrogen fixation on Burk’s media by isolates (a) Topsoil and (b) Subsoil.

4.4.2 Phosphate solubilisation

The ability of the isolates to solubilise insoluble phosphate was determined on NBRIP growth medium. A halo zone around the bacterial colonies after the incubation of plates for seven days at 30°C was an indication of phosphate solubilisation (Fig. 4.4). Based on the results, the isolates were classified into four groups depending on the halo size. The groups are non-solubilisers (0 mm), Low (1-10 mm), Intermediate (11-20 mm) and High (≥ 21 mm) solubilisers.

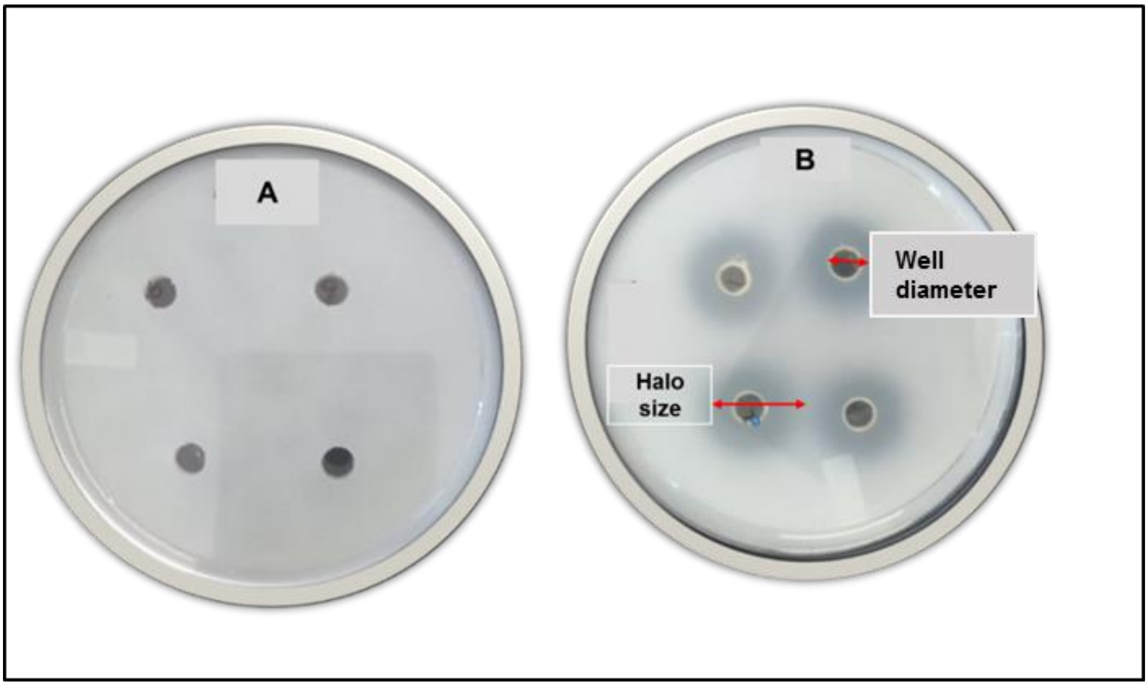


Figure 4.4: **Phosphate-solubilisation of tri-calcium phosphate on NBRIP growth media after 7 days incubation at 28°C. A-inoculum that cannot solubilise phosphate. B-inoculum that can solubilise phosphate.**

Bacterial isolates from control soils solubilised were better phosphate solubilisers than those in stockpiled soils (Fig. 4.5a & b). About 20% (topsoil) and 27% (subsoil) bacterial isolates from control soils were intermediate phosphate solubilisers whereas the bacterial isolates from stockpiled soils were non-solubilisers and low-solubilisers (Fig. 4.5a & b). Generally, the fungal isolates from both control and unmined soils were more efficient in solubilising insoluble phosphate than bacterial isolates (Fig. 4.5 & 4.6). Over 5% of fungal isolates from Control, Mine

B and Mine C were high solubilisers in the topsoil (Fig. 4.6a). Subsoil, Control and Mine A had over 15% high phosphate solubilisers (Fig. 4.6a).

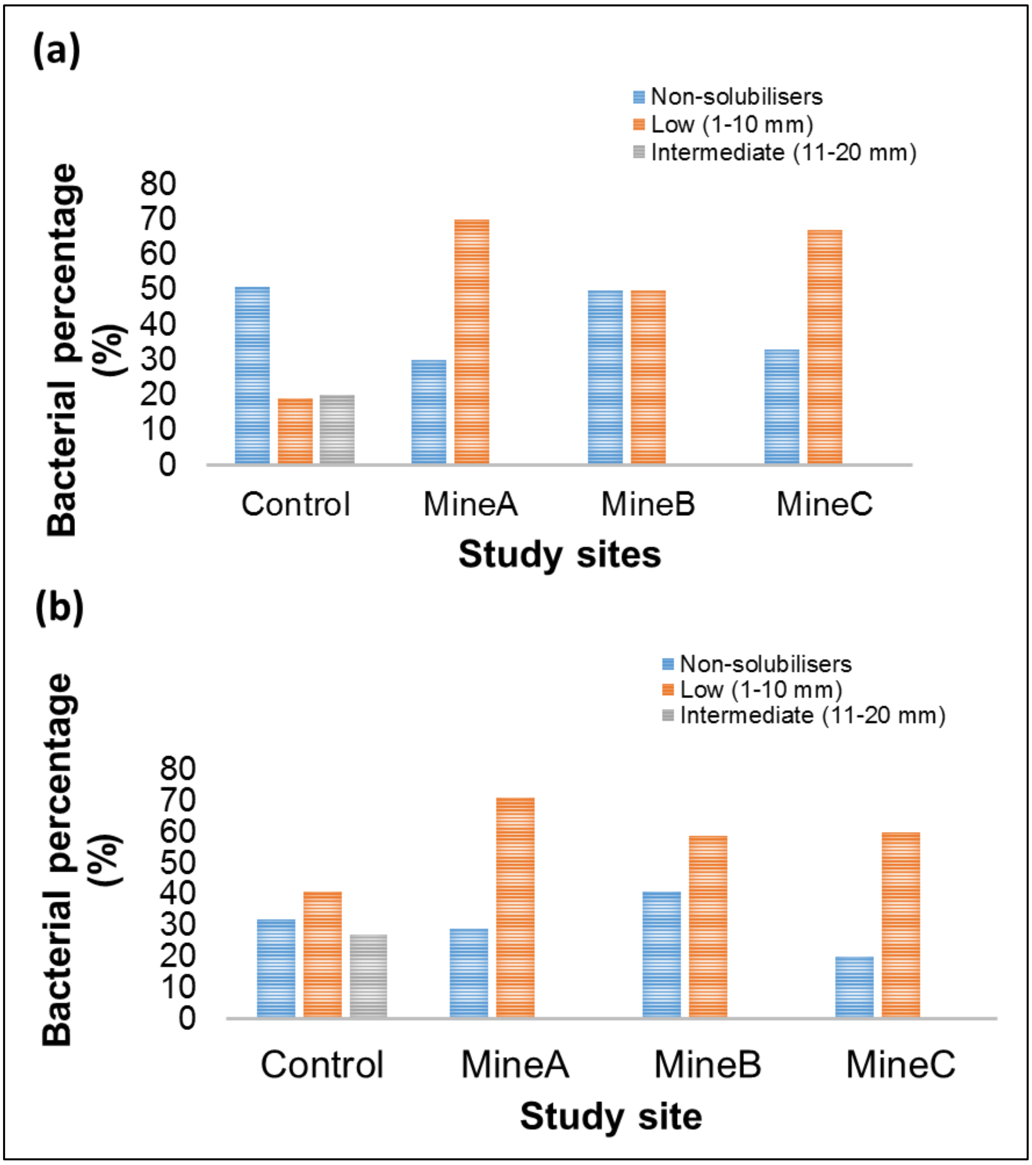


Figure 4.5: **Phosphate solubilisation by bacterial (a) Topsoil and (b) Subsoil.**

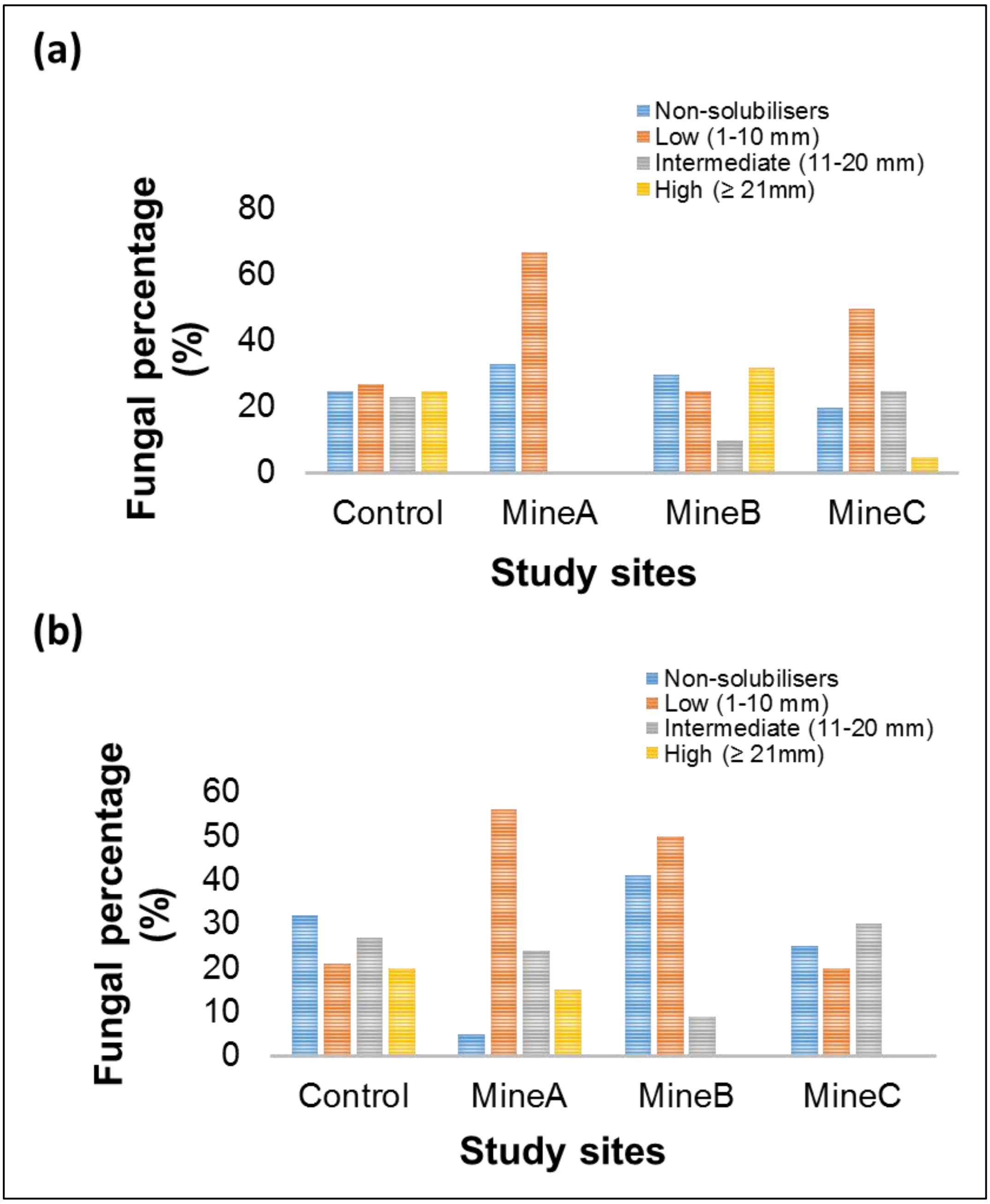


Figure 4.6: **Phosphate solubilisation by fungal isolates (a) Topsoil and (b) Subsoil.**

4.4.2.1 Indole acetic acid production

Bacterial isolates from stockpiled soils were non-IAA producers because there was no pink colour development after the addition of Salkowski's reagent (Fig. 4.7a & b). Over 46% of bacterial isolates from control soils produced IAA (Fig. 4.7a & b). The IAA concentration of bacterial isolates from control soils ranged from 0.425-0.66 µg/ml.

The fungal IAA concentration ranged from 0.091-0.66 µg/ml and 0.001-0.60 µg/ml in control soils and stockpiled soils respectively. Approximately 75% and 68% fungal isolates from topsoil and subsoils of control soils produced IAA, respectively. In addition, over 59% of isolates from stockpiled soils in both topsoil and subsoil produced IAA (Fig. 4.8a & b).

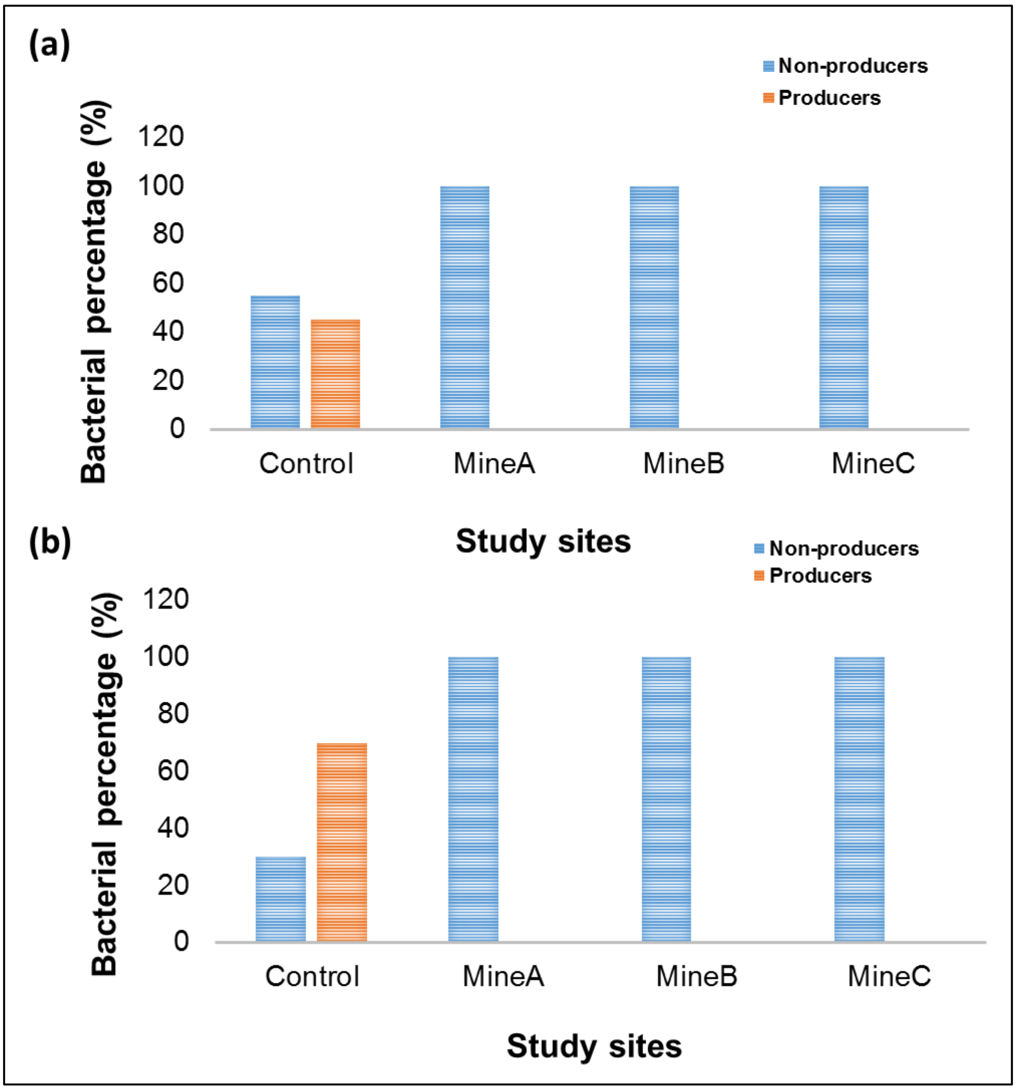


Figure 4.7: Indole acetic acid production by bacterial isolates (a) Topsoil and (b) Subsoil.

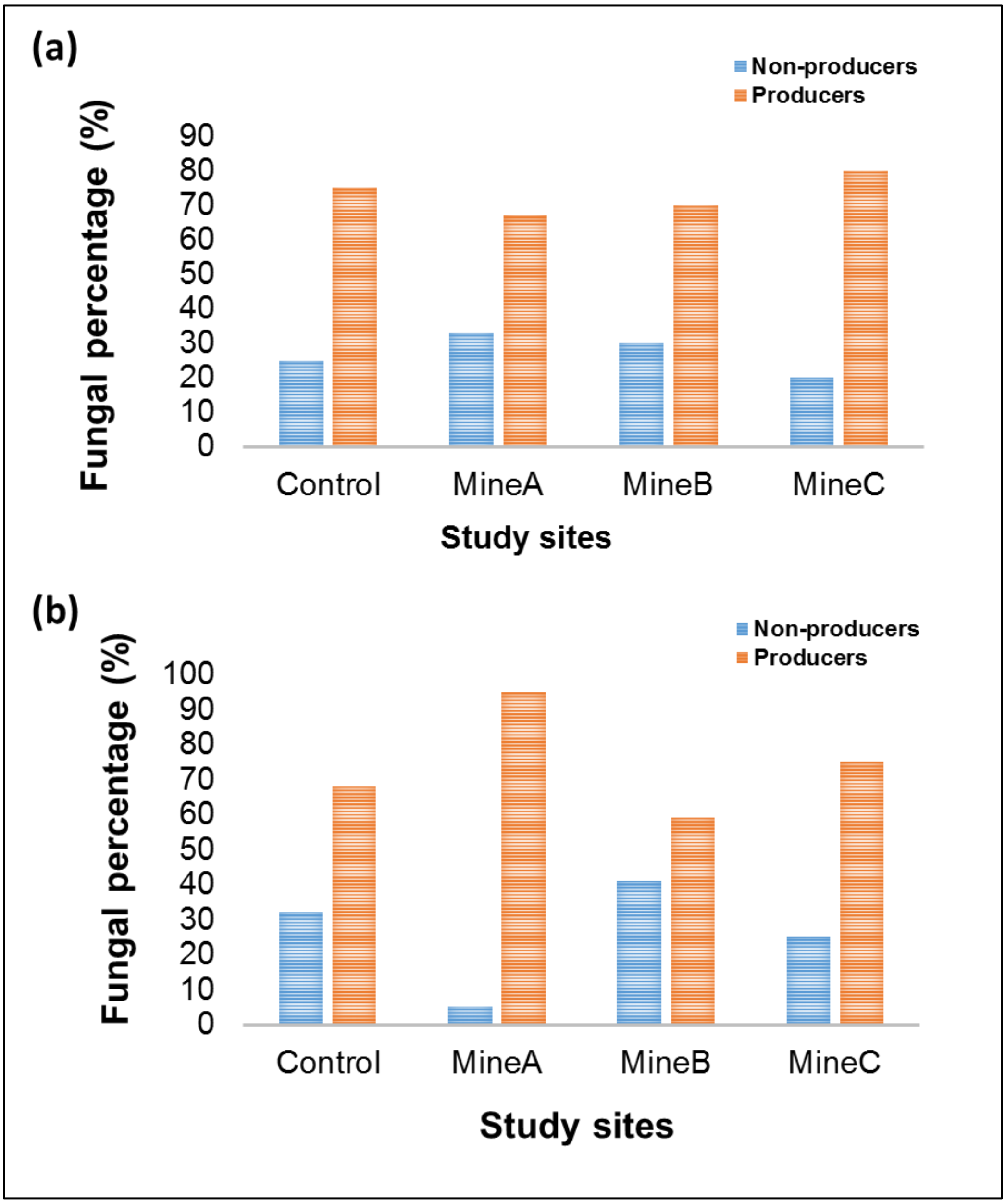


Figure 4.8: Indole acetic acid production fungal isolates (a) Topsoil and (b) Subsoil.

4.5 β-glucosidase and Urease Activities from Sampled Soils

The β-glucosidase and urease activities of both stockpile and control (unmined) soils are presented in Fig. 4.9a and b. β-glucosidase activity was generally higher in stockpile soils than in control soils (Fig. 4.9a). β-glucosidase activities significantly differed ($P<0.05$) among seasonal samples collected from each study site, except in Mine C, where there was no significant difference ($P>0.05$) among seasonal samples (Fig. 4.9a).

The β-glucosidase activity was higher in Mine B and Mine C during summer, but higher in Mine A during winter. In contrast, control soils had the highest β-glucosidase activity during spring. The mean β-glucosidase activity during summer and winter was significantly different ($P<0.05$) between sites (Table A5), whereas in spring, there was no significant difference in β-glucosidase activities among all sites (Table A5).

Urease activity was generally higher in stockpile soils than in control soils during the summer and winter seasons (Fig. 4.9b). However, in spring, urease activity was higher in the control soils than in both Mine A and Mine C (Fig. 4.9b). Differences between urease activities in soils across seasons in Mine B and Mine C were not significant ($P>0.05$). On the other hand, differences between urease activities in soils across seasons were significant ($P<0.05$) in the control site and Mine A. Similar to β-glucosidase activity, urease activity did not significantly differ ($P>0.05$) between study sites in spring (Table S4), whereas, during summer, urease activity in Mine B and Mine C were significantly higher than in the control soils. However, during winter, urease activities in stockpiled soils of Mine A were significantly higher than all other urease activities in sampled soils (Table A6). Overall, standard deviations from the mean β-glucosidase and urease activities were higher in stockpiled soils when compared to control soils.

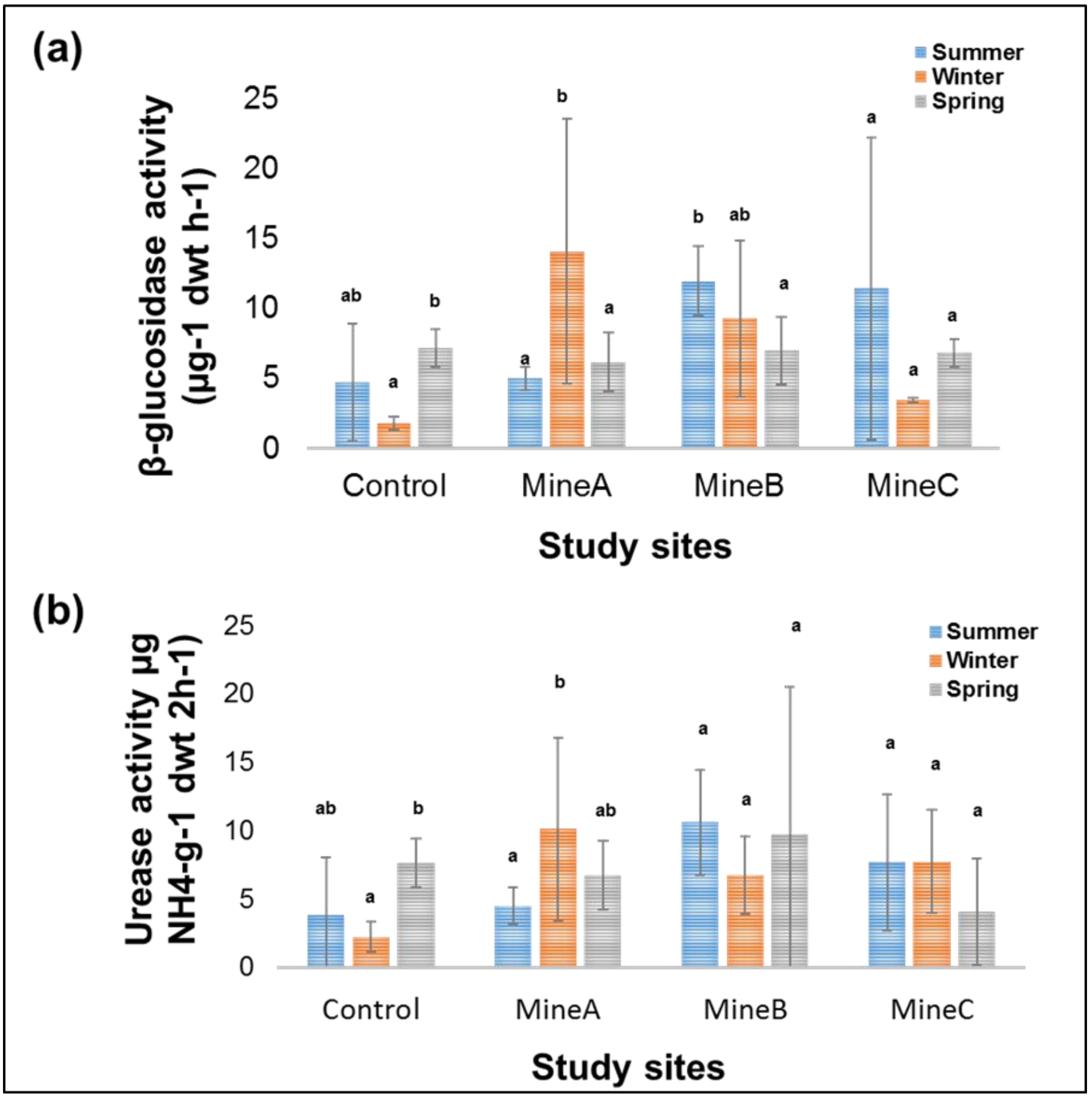


Figure 4.9: **Enzymatic activities from sampled soils (a) β-glucosidase and (b) urease activities. Bars with different letters are significantly different ($p < 0.05$). Error bars are standard deviations from means.**

CHAPTER 5 – DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Reliable interpretation of parameters that express the status of soil health and quality can only be achieved if physical, chemical and biological properties of the soil are considered. This approach was adopted for the present study where correlations were established for the physicochemical and biological properties of the soil.

5.1.1 Physicochemical properties of soils

Differences between physicochemical properties of stockpiled topsoils and unmined sites (Fig A1& A2) did not suggest that stockpiling operations impaired topsoil quality in all mining sites studied. However, Cruz-Ruíz *et al.* (2016) found that gravimetric water content, water holding capacity, bulk density, available phosphorus, total nitrogen and soil organic carbon were greatly influenced by pumice mining operations. Similarly, Ghose (2004) reported that the quality of topsoil dumps during the opencast coal mining process altered drastically and that the alteration of soil quality in the topsoil increased with the duration of storage. The observation of no difference between control sites and soil stockpiles in the current study equally reflects the quality of the control soils, although the history of disturbance (other than mining activities) of the control sites could not be ascertained, the high bulk density, low CEC, organic carbon, total nitrogen and acidic pH in control soils and stockpiled soils reflect the low soil quality and fertility. High bulk density in stockpiled soils may be attributed to compaction caused by heavy machinery, such as those used during the stripping and stockpiling process (Ghose, 2004; Shrestha *et al.*, 2005). The high bulk density would pose restrictions on the growth of deep-rooted plants during post-mining reclamation and/or revegetation (Ghose, 2004; Mensah, 2015; Shrestha *et al.*, 2005). Furthermore, the acidic pH of the topsoil stockpiles is below the optimum (pH 6.5 – 7.5) for good arable soils. The acidity of the soils could be due to the leaching of basic cations from the soils by water runoff during rainfall. Under such acidic conditions, H^+ toxicity, aluminium and manganese toxicity, and the unavailability of molybdenum (Mo) become principal limitations for plant growth (Cregan, 1981; Iqbal, 2012). Also, the low carbon and nitrogen levels in the soils may result in a gradual decrease in the degradability of plant materials by microbes and possible nutrient limitations or stress in stockpiled soils (Batjes, 1996).

5.1.2 Microbial diversity using culture-dependent and culture-independent methods

Diversity indices are useful approaches to estimate the diversity of microbial communities (Qing *et al.*, 2007). The Shannon-Weiner index of diversity and PCR-DGGE showed that unmined soils had more bacterial and fungal diversity when compared to stockpiled soils. Previous studies reported on the fact that heavy metal contamination on mine soils reduces soil bacterial diversity (Qing *et al.*, 2007) and arbuscular mycorrhizal fungal spores (Del Val *et al.*, 1999; Xie *et al.*, 2016). The microbial diversity in control and stockpiled soils varied between seasons. The variation of microbial communities in response to environmental conditions such as temperature and moisture are well documented (Baldrian *et al.*, 2010; Habekost *et al.*, 2008; López-Mondéjar *et al.*, 2015; Wang *et al.*, 2016). For example, López-Mondéjar *et al.* (2015) reported that the spring and summer bacterial community of temperate deciduous soils significantly differed from those of the autumn and winter seasons. Distinct changes in soil microbial community composition across seasons have also been reported in a grassland plant diversity gradient (Habekost *et al.*, 2008) and across two typical zonal vegetation types in Wuyi mountains, northern China (Wang *et al.*, 2010). The changes in microbial diversity across seasons may be due to the variations in the supply of nutrients owing to certain seasonal (environmental) factors such as temperature and moisture (Fekete *et al.*, 2012). These factors influence the allocation of photosynthates to soil by the roots of primary producers, the input of fresh litter, and above- or below ground biomass production (López-Mondéjar *et al.*, 2015; Wang *et al.*, 2016).

We observed phylum Firmicutes in both culture-dependent and culture-independent studies to be dominant. This observation contradicts findings of most soil bacterial diversity studies that observed that the phylum Proteobacteria to be the most abundant bacterial phyla in the soil environment (Chen *et al.*, 2013; Kolton *et al.*, 2011; López-Mondéjar *et al.*, 2015; Schadt *et al.* (2003); Tait *et al.*, 2007). However, Firmicutes have also been reported to be dominant in high-salinity desert soils (Van Horn *et al.*, 2014). The fungal phylum Ascomycota was dominant in all the sampled soils (culture-dependent and culture-independent). This observation is similar to that reported by Schadt *et al.* (2003) in their study of fungal lineages associated with tundra soils. It was hypothesized that species belonging to the phyla Firmicutes and Ascomycota are more tolerant to the high bulk density, acidic conditions, nutrient limiting and high compaction levels in the stockpile soils.

At the genus level, dominance of *Bacillus* spp. in these soils shows that species of *Bacillus* are well adapted to the prevailing conditions of the soils, and most likely play predominant roles in the mineralization of nutrients in these soils. Previous studies have indicated that *Bacillus* spp. contributes to the mineralization of plant-derived materials (Mandic-Mulec & Prosser, 2011), humus (Prescott *et al.*, 2002), pesticides (Garbeva *et al.*, 2003; Prescott *et al.*, 2002) and hydrocarbons (Garbeva *et al.*, 2003) in the soil. Interestingly, some of the bacterial species detected in this study have been previously associated with mining soils (Karličić *et al.*, 2017). For example, *Bacillus amyloliquefaciens* has been shown to colonize roots efficiently and to overcome the antibacterial action of some plant root exudates (Reva *et al.*, 2004). Various *Bacillus amyloliquefaciens* isolates have also been shown to promote salt tolerance in plants (Bochow *et al.*, 2001), to increase the yield of tomato (Gül *et al.*, 2008) and to enhance the root/shoot biomass of oilseed rape, wheat, and maize seedlings (El-Daim *et al.*, 2014; Idriss *et al.*, 2002; Talboys *et al.*, 2014). To the best of our knowledge, the functional roles of other *Bacillus* spp., including *Bacillus gaemokensis* and *Bacillus zhangzhouensis* are largely unknown. *Pseudomonas paralactis* have been reported to enhance plant growth and to modify essential oil content in *Leptospermum scoparium* (tea tree), to solubilise tricalcium-phosphate and produce siderophores *in vitro* (Wicaksono *et al.*, 2017). *Pseudomonas turukhanskensis* was previously isolated from oil-contaminated soils, and can produce urease and β-galactosidase (Korshunova *et al.*, 2016). In addition, some *Pseudomonas* spp*.* have been reported to possess heavy metal detoxification mechanisms for arsenic, ferric, and manganese. Given that mining soils are usually rich in heavy metals, the presence of such metal detoxifying *Pseudomonas* spp. in the coal-mining area may have ecological significance, especially towards the detoxification of metal contaminated mine soil (Keil *et al.*, 2011). Similarly, *Azomonas macrocytogenes* has been reported as being an aerobic, non-symbiotic, nitrogen-fixing organism (New & Tchan, 1982; Page & Collinson, 1987). Nitrogen-fixing microorganisms are widespread in the soil and are involved in one of the most important parts of the nitrogen cycle which, together with photosynthesis, ensures the productivity of the biosphere (Kizilova *et al.*, 2012).

Interestingly, most of the fungal species detected in the soils (control and stockpiles) are known plant pathogens. For example, *Fusarium fujikuroi* is the causal agent of Bakanae disease of rice (Carter *et al.*, 2008). *Claviceps purpurea* is a ubiquitous pathogen of cereals and grasses, causing Ergot disease, which results in substitution of grains by sclerotia (Tudzynski & Scheffer, 2004). *Alternaria tenuissima* causes the occurrence of leaf spot on *Aloe barbadensis* in Greece (Vakalounakis *et al.*, 2016). *Alternaria petroselini* was reported as the causal agent of leaf blight of *Foeniculum vulgare* Mill (Fennel) in Spain (Bassimba *et al.*, 2012). *Curvularia trifolii* causes leaf spot on *Trifolium alexandrinum (Berseem Clover)* (Khadka, 2016).

However, *Aureobasidium pullulans* has a high importance in biotechnology for the production of different enzymes, siderophores and pullulan (Chi *et al.*, 2009). Furthermore, *A. pullulans* is used in the biological control of plant diseases, especially storage diseases (Ferreira-Pinto *et al.*, 2006). *Phoma herbarum* is an endophytic fungus and has proteolytic activities (Kharwar *et al.*, 2014), antibacterial activity against *Shigella flexnii*, *Shigella boydii*, *S. enteritidis*, *Salmonella paratyphi* and *P.aeruginosa* (Kharwar *et al.*, 2014), antifungal activity against fungal pathogens and antioxidant activity (Kharwar *et al.*, 2014).

Additionally, *Mortierella sp*. can solubilise rock phosphate (Habte & Osorio, 2012), degrade 4-dichlorophenol (DCP) (Nakagawa *et al.*, 2006) and produce microbial oils for the generation of biodiesel (Kumar *et al.*, 2011). Hence, these soils may serve as a source for the bioprospecting of such industrially important fungal strains. *Phialocephala humicola* (*Scolecobasidium humicola)* is a common dark septate entophytic fungal. This fungus was found to grow endophytically in the roots of tomato seedlings*. S. humicola* species have shown the ability to increase plant biomass (Mahmoud & Narisawa, 2013)*. Sordariomycetes sp* was previously reported as an endophytic fungus, which has antimicrobial and anticancer properties (Jinfeng *et al.*, 2017).

On the other hand, very little is known about the functional roles of *Deconica novae-zelandiae*, *Austroplaca soropelta*, *Umbelopsis vinacea, Pyrrhospora arandensis, Alternaria petroselini*, and *Canangium acuum* in the soil. It is therefore unclear if these species are functionally important to the sustainability of the soil ecosystem.

5.1.3 Functional attributes of bacterial and fungal isolates

The isolates were tested for their ability to fix nitrogen, solubilise phosphates and produce (IAA) auxins. Most of the bacterial isolates obtained in both control and stockpiled soils showed the potential to fix nitrogen. There was no clear distinction between the bacterial isolates from both stockpiled and control soils in their ability to fix atmospheric nitrogen. The nitrogen fixers obtained from stockpiled soils can potentially amend low nitrogen soils during post-mining land reclamation (White *et al.*, 1985). The stockpiling process did not affect the ability of the bacterial isolates to fix atmospheric nitrogen.

Phosphate solubilisation data revealed that the bacterial isolates from both topsoil and subsoil in control soils were more efficient in phosphate solubilisation than those obtained in soil stockpiles (Fig. 4.5). Perhaps the acidic conditions, high bulk density, nutrient limiting and high compaction levels observed in the stockpiled soils affect the solubilisation abilities of bacterial phosphate. It was also observed that the fungal isolates were more efficient in solubilising phosphate than bacterial isolates (Fig. 4.5 and 4.6). It was previously reported that phosphate solubilising fungi produce more acids than bacteria and consequently exhibit greater phosphate solubilising activity (Adeleke *et al.*, 2010; Sharma *et al.*, 2013; Venkateswarlu *et al.*, 1984). The phosphate solubilisers obtained can be used in solubilisation of phosphatases and in fixing soil phosphorus during post-mining land rehabilitation, as a result, yielding higher crops.

None of the bacterial isolates from stockpiled soils produced IAA (Fig. 4.7). However, most of the fungal isolates in both control and stockpiled soils produced IAA. Perhaps the soil stockpiling process affects the mechanisms of IAA production in soil bacteria. IAA is the most effective growth hormone in plants, so the fungal isolates that could produce IAA from stockpiled soils could play an important role of host plant during post-mining land rehabilitation (Banerjee *et al.*, 2010).

5.1.4 Enzymatic activities

Studies on enzymes activities in the soil are important as they indicate the potential of the soil to support biochemical processes, which are essential for the maintenance of soil fertility (Maior *et al.*, 2011). β-glucosidase and urease activity in the soil are important owing to their roles in the carbon and nitrogen cycles respectively. The urease activity and β-glucosidase activities from stockpiled soils were higher than those of unmined soils. This observation is in contrast to a previous study conducted by Nair *et al.* (2009) on enzyme activities in mine soil (MT) (gold mine tailings). MT was compared with control mine soils (CM) (collected 5 km away from mine dumps where plants showed normal growth), fertile garden soil (GS) from Bangalore and vermicompost (VC) (Nair *et al.*, 2009). In their study, Nair *et al.* (2009) found that soil enzyme activities from mine soil were very low compared to those found in fertile soils (VC>GS>CM). However, high β-glucosidase activity in the stockpiled soils could signal carbon deficiency, while higher urease activity signals nitrogen deficiency (Makoi & Ndakidemi, 2008; Ndakidemi, 2006; Sinsabaugh & Moorhead, 1994). An inverse relationship between β-glucosidase activity and carbon availability, as well as between urease activity and nitrogen availability can be attributed to increased extracellular secretion of these enzymes by soil microbes as an aggressive nutrient scavenging response strategy under nutrient-limiting conditions. In a bid to counter low nitrogen availability, microorganisms secrete urease as an alternative pathway to obtain nitrogen by metabolising urea to yield ammonia and carbamate. On the other hand, microorganisms secrete β-glucosidase to metabolise high molecular carbon precursors (such as cellulose) as carbon and energy source (Nair *et al.*, 2009).

The β-glucosidase and urease activities significantly differed between seasonal samples (winter and summer), but there was no variation in spring. Seasonal changes in soil enzyme activity have been reported in other studies (Fekete *et al.*, 2012; Wang *et al.*, 2016). However, Boerner *et al.* (2005), found that seasonal variations in enzyme activity and organic carbon in the soil of a burned and unburned hardwood forest did not significantly differ across seasons (Boerner *et al.*, 2005). These seasonal changes may be attributed to changes in nutrient availability (Kotroczó *et al.*, 2014), and in the changing inputs of leaf litter (Boerner *et al.*, 2005; Kang *et al.*, 2009). Note that the high deviations from the mean enzyme activities observed in stockpiled soils suggest heterogeneity of these soils and/or the influence of other confounding variables such as the ages of the different stockpiles sampled intra-mining-site. Further studies on the effect of stockpile ages on enzyme activities will help to provide clarifications on the observed intra-site enzyme variations.

5.2 Conclusion

Soil stockpiling processes during opencast coal mining involve the removal of the topsoil layer and any other layer necessary to get coal. For a meaningful post-mining reclamation of mined sites, the topsoil layer is required to be removed and stockpiled separately from other overburden material stockpiles. The period of time in which these stockpiles are kept prior to land reclamation and the uncontrolled removal and replacement of these soil layers with heavy equipment are factors which could significantly impair the quality of soil from an agricultural-use standpoint. Such factors are likely to affect important soil – physical, chemical and biological properties, which will eventually lead to soil infertility. The impact of soil stockpiling on diversity and functional properties of microorganisms of soil stockpiles based in three opencast coal mines located in the Mpumalanga Province, South Africa was therefore investigated. The patterns in the physicochemical properties between unmined and stockpiled soils did not clearly suggest that stockpiled soils were in poorer conditions compared to soil samples from control sites. However, the microbial diversity, enzymatic activities, bacterial IAA production and phosphate solubilisation suggest that soil stockpiling operations alter soil health. Such alteration in soil health parameters may have consequences on key functional groups, which play important roles in biological processes critical for soil nutrient cycling in these soils. More importantly, such alteration in soil quality may have implications for post-mining reclamation/revegetation as well as impairing land use capability.

5.3 Recommendations

Best soil stockpiling practices which would guarantee the optimum preservation of topsoil quality parameters are recommended. Also, the need has been emphasised for deciphering the molecular mechanisms of adaptation and resistance of microbes in mine sites. Understanding their role in remediation, and subsequent harnessing of their potential will pave the way for future rehabilitation strategies for mined sites.

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ANNEXURE A: TABLES

Table A1: Physical and chemical properties of topsoil across seasons

		Exch. Cations (cmol(+)/kg)											
	Season	**Na**	**K**	**Ca**	**Mg**	**S-V**	**CEC (meq/100 g)**	**pH**	**Org C (%)**	**Tot N**	**C:N**	**P (Bray 1)**	**Bulk density (g/cm^3)**
Control	Summer	0.02±0.00	0.19±0.08	1.18±1.62	0.55±0.20	2.94±1.62	8.52±6.70	5.79±1.06	1.16±0.26	0.05±0.00	22.20±4.92	9.84±8.13	1.62±0.09
	Winter	0.03±0.00	0.27±0.09	1.36±0.53	0.66±0.40	2.32±0.41	4.80±1.32	5.31±0.45	1.71±0.56	0.08±0.02	22.40±2.36	10.71±6.34	1.34±0.19
	Spring	0.10±0.04	0.83±0.19	3.60±2.21	1.21±0.52	5.77±2.78	3.81±1.81	4.77±0.35	1.14±0.72	0.05±0.03	24.14±1.26	4.97±4.67	1.53±0.07
Mine A	Summer	0.05±0.01	0.20±0.06	0.74±0.01	0.44±0.05	1.47±0.20	4.16±2.11	4.64±0.24	1.34±0.86	0.05±0.03	27.60±3.10	26.60±26.15	1.47±0.12
	Winter	0.03±.00	0.19±0.04	1.13±0.10	0.49±0.17	1.84±1.60	5.71±1.13	5.37±0.58	1.44±0.00	0.04±0.01	36.60±5.09	14.15±11.84	1.47±0.10
	Spring	0.20±0.08	3.71±0.91	5.15±2.50	2.52±1.01	11.58±4.20	3.49±1.41	4.50±1.00	1.01±0.23	0.04±0.00	22.49±1.74	8.87±1.84	1.38±0.22
Mine B	Summer	0.03±0.01	0.15±0.07	1.18±0.40	0.95±0.44	2.61±1.00	6.21±1.59	5.38±0.27	2.18±0.56	0.48±0.05	0.02±0.00	26.62±2.27	1.80±0.02
	Winter	0.05±0.02	0.15±0.03	1.25±0.30	1.23±0.43	2.80±0.76	8.84±3.20	5.40±0.29	4.10±0.78	0.34±0.09	0.01±0.01	25.05±4.04	1.36±0 .22
	Spring	0.10±0.04	0.26±0.25	1.10±0.04	1.04±0.02	2.30±0.38	4.90±0.342	4.90±1.48	4.40±0.31	0.52±0.31	0.02±0.01	25.37±5.47	1.22±0.09
Mine C	Summer	0.02±0.01	0.18±0.03	1.17±0.03	0.17±0.02	0.42±0.06	3.35±1.11	4.71±0.11	3.80±0.93	0.40±0.06	0.01±0.00	35.00±2.10	1.60±0.19
	Winter	0.02±0.01	0.13±0.11	0.69±0.03	0.36±0.19	0.95±0.49	10.36±8.50	4.42±0.18	0.97±0.32	0.23±0.18	7.66±4.75	7.66±4.75	1.51±0.02
	Spring	0.07±0.01	0.07±0.02	0.54±0.06	0.39±0.04	1.102±1.02	3.63±0.41	4.32±0.08	0.96±0.74	0.53±0.18	0.04±0.01	14.40±3.51	1.76±0.22

Table A2: Physical and chemical properties of subsoil across seasons

		Exch. Cations (cmol(+)/kg)										
	Season	**Na**	**K**	**Ca**	**Mg**	**S-V**	**CEC (meq/100 g)**	**pH**	**Org C (%)**	**Tot N (%)**	**C:N**	**P (Bray 1)**
Control	Summer	0.02±0.01	0.17±0.06	0.82±0.19	0.54±0.34	1.55±0.44	8.06±6.00	4.82±0.25	0.46±0.04	0.04±0.00	12.90±1.08	0.71±0.39
	Winter	0.04±0.01	0.15±0.06	0.53±0.37	0.40±0.24	1.11±0.61	3.19±1.68	4.82±0.57	0.42±0.09	0.04±0.01	11.27±1.11	1.11±0.28
	Spring	0.07±0.03	0.25±0.11	1.97±0.75	0.66±0.37	2.96±1.12	3.79±1.72	4.67±0.35	0.43±0.09	0.02±0.01	22.33±2.070	0.65±0.24
Mine A	Summer	0.03±0.01	0.18±0.08	0.66±0.10	0.44±0.074	1.34±0.22	2.39±1.18	4.71±0.29	0.50±0.17	0.02±0.00	24.09±3.79	10.41±1.60
	Winter	0.03±0.01	0.09±0.04	1.28±1.50	0.52±0.03	1.92±1.77	4.25±1.76	5.51±0.86	0.78±0.59	0.02±0.01	42.42±12.11	3.17±1.22
	Spring	0.08±0.03	2.89±0.17	3.98±0.70	3.27±2.00	10.61±2.79	3.73±0.65	4.42±0.27	0.75±0.39	0.03±0.02	22.90±0.33	3.38±2.75
Mine B	Summer	0.13±0.09	0.12±0.07	1.28±0.44	1.30±0.63	2.90±1.18	7.16±2.79	5.50±0.22	3.00±1.00	0.30±0.10	0.01±0.002	23.38±7.07
	Winter	0.03±0.88	0.13±0.02	1.07±0.154	1.50±1.07	2.83±0.39	9.13±1.93	5.78±0.49	2.53±0.16	0.31±0.04	0.01±0.002	29.12±0.91
	Spring	0.07±0.05	0.14±0.03	1.07±0.31	1.07±0.37	2.35±0.12	5.14±2.40	5.21±0.15	2.51±3.32	0.40±0.13	0.02±0.006	26.31±6.41
Mine C	Summer	0.03±0.01	0.06±0.02	0.19±0.04	0.19±0.02	0.47±0.06	2.79±0.66	4.93±0.13	1.03±0.20	0.41±0.19	0.01±0.004	32.1±5.50
	Winter	0.02±0.01	0.10±0.08	0.62±0.08	0.29±0.23	1.03±0.23	10.94±6.92	4.77±0.13	0.95±0.22	0.14±0.07	0.03±0.002	5.46±2.50
	Spring	0.05±0.00	0.07±0.03	0.57±0.06	0.46±0.43	1.19±1.10	3.91±0.89	4.30±0.19	1.21±0.94	0.59±0.25	0.04±0.007	15.20±4.33

Table A3: Correlation matrix for influence of soil properties on microbial species in topsoil

	SPEC AX1	SPEC AX2	SPEC AX3	SPEC AX4	ENVI AX1	ENVI AX2	ENVI AX3	ENVI AX4
SPEC AX1	1.0000							
SPEC AX2	0.0000	1.0000						
SPEC AX3	0.0000	0.0000	1.0000					
SPEC AX4	0.0000	0.0000	0.0000	1.0000				
ENVI AX1	1.0000	0.0000	0.0000	0.0000	1.0000			
ENVI AX2	0.0000	1.0000	0.0000	0.0000	0.0000	1.0000		
ENVI AX3	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000	1.0000	
ENVI AX4	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000	1.0000
Na	0.1921	0.4055	0.0599	-0.1594	0.1921	0.4055	0.0599	-0.1594
K	0.0132	0.3831	0.2646	-0.1806	0.0132	0.3831	0.2646	-0.1806
Ca	-0.2023	0.3683	-0.1047	-0.0489	-0.2023	0.3683	-0.1047	-0.0489
Mg	0.2662	0.3493	-0.1359	-0.0753	0.2662	0.3493	-0.1359	-0.0753
S_V	-0.0208	0.3924	-0.0062	-0.1144	-0.0208	0.3924	-0.0062	-0.1144
CEC	0.0991	-0.3919	0.0061	0.7552	0.0991	-0.3919	0.0061	0.7552
pH	-0.0502	0.1556	-0.6724	0.1796	-0.0502	0.1556	-0.6724	0.1796
Org C	0.6841	-0.1432	-0.4733	-0.0993	0.6841	-0.1432	-0.4733	-0.0993
Tot N	0.6166	-0.5211	-0.0678	-0.4618	0.6166	-0.5211	-0.0678	-0.4618
C:PN	-0.5616	0.8007	-0.0196	0.1408	-0.5616	0.8007	-0.0196	0.1408
Bray1	0.5410	0.0059	-0.0558	-0.4289	0.5410	0.0059	-0.0558	-0.4289
BD	-0.0840	-0.2828	0.1630	-0.3661	-0.0840	-0.2828	0.1630	-0.3661
BG	0.4960	0.1432	0.0945	0.0497	0.4960	0.1432	0.0945	0.0497
Urease	0.6128	0.1151	-0.0491	-0.2755	0.6128	0.1151	-0.0491	-0.2755

Table A4: **Correlation matrix for influence of soil properties on microbial species in topsoil**

	SPEC AX1	SPEC AX2	SPEC AX3	SPEC AX4	ENVI AX1	ENVI AX2	ENVI AX3	ENVI AX4
SPEC AX1	1.0000							
SPEC AX2	-0.0041	1.0000						
SPEC AX3	-0.0414	0.0100	1.0000					
SPEC AX4	-0.0864	0.0209	0.2098	1.0000				
ENVI AX1	0.9914	0.0000	0.0000	0.0000	1.0000			
ENVI AX2	0.0000	0.9995	0.0000	0.0000	0.0000	1.0000		
ENVI AX3	0.0000	0.0000	0.9484	0.0000	0.0000	0.0000	1.0000	
ENVI AX4	0.0000	0.0000	0.0000	0.7498	0.0000	0.0000	0.0000	1.0000
Na	-0.1908	-0.1739	-0.4122	0.0595	-0.1924	-0.1740	-0.4347	0.0793
K	-0.3419	-0.2901	-0.2610	-0.0221	-0.3448	-0.2903	-0.2752	-0.0294
Ca	-0.4044	-0.0946	-0.3445	0.0775	-0.4079	-0.0947	-0.3633	0.1034
Mg	-0.1287	-0.0120	-0.5388	0.0567	-0.1298	-0.0120	-0.5682	0.0757
S_V	-0.3029	-0.1338	-0.3979	0.0429	-0.3055	-0.1338	-0.4195	0.0575
CEC	0.4200	0.2724	-0.0091	0.0334	0.4237	0.2725	-0.0096	0.0446
pH	0.2700	0.3728	-0.5424	0.0984	0.2723	0.3730	-0.5719	0.1312
Org C	0.7004	0.3376	-0.5297	0.1937	0.7065	0.3378	-0.5585	0.2583
Tot N	0.8697	-0.0207	-0.0080	-0.1105	0.8772	-0.0208	-0.0084	-0.1474
C_N	-0.8442	-0.3001	-0.1911	-0.0037	-0.8515	-0.3002	-0.2015	-0.0050
Bray1	0.7942	0.0879	-0.3613	-0.0815	0.8010	0.0879	-0.3810	-0.1088

Table A5: β-glucosidase activities in soils by seasons

	Summer	Winter	Spring
Control	4.72±4.18^{a}	1.81±0.49^{a}	7.17±1.34^{a}
Mine A	5.01±0.83^{a}	14.11±9.47^{b}	6.19±2.10^{a}
Mine B	11.96±2.51^{b}	9.3±5.57^{ab}	6.70±2.43^{a}
Mine C	11.44±10.83^{b}	3.47±0.14^{a}	6.86±0.99^{a}

Table A6: Urease activities in soils by seasons

	Summer	Winter	Spring
Control	3.8±4.25^{a}	2.21±1.09^{a}	7.62±1.78^{a}
Mine A	4.48±1.34^{a}	10.10±6.69^{b}	6.76±2.51^{a}
Mine B	10.58±3.84^{b}	6.71±2.82^{ab}	9.74±10.77^{a}
Mine C	7.68±4.97^{b}	7.72±3.74^{b}	4.09±3.88^{a}

ANNEXURE B: FIGURES

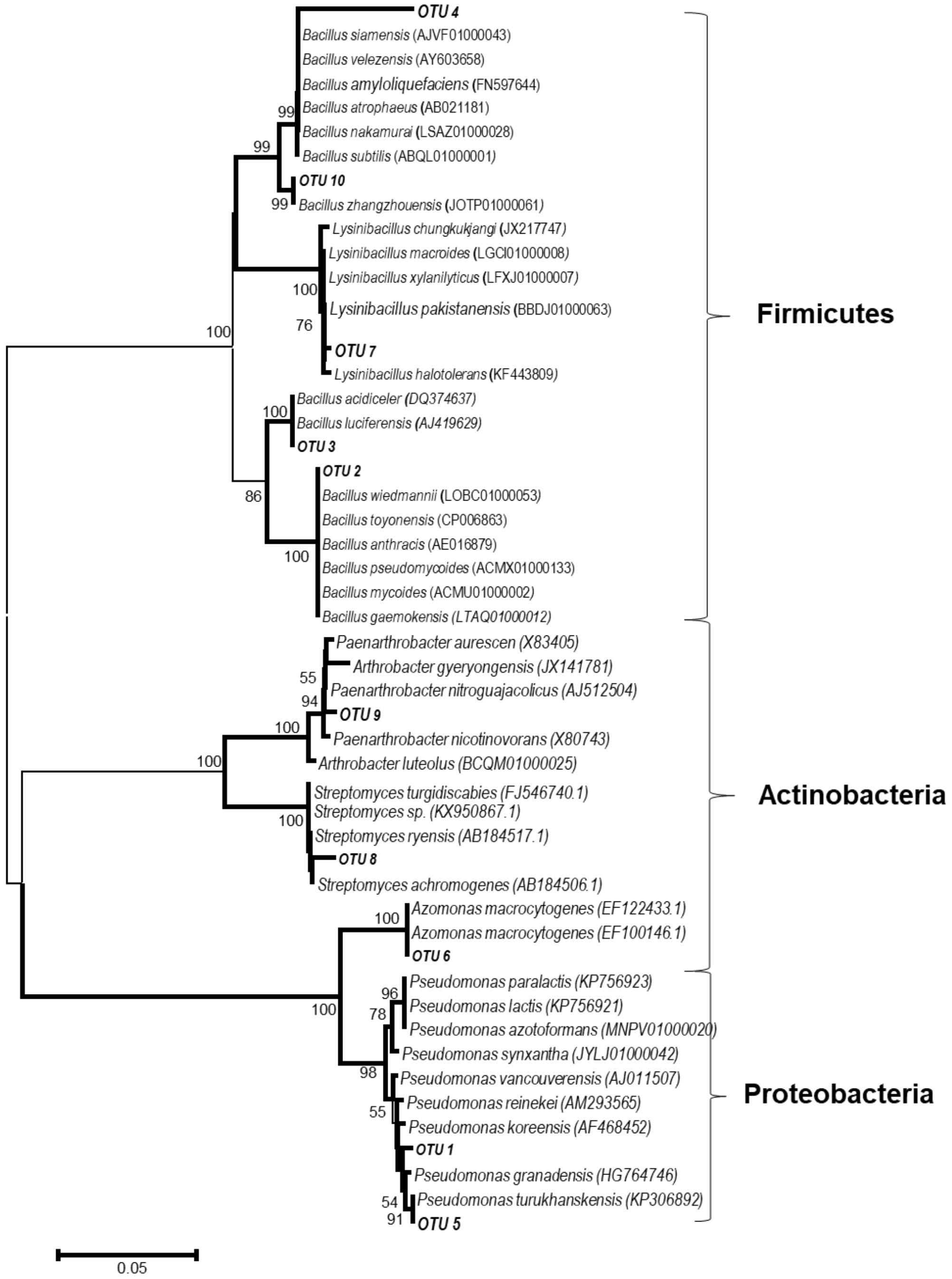


Figure A1: **Unrooted neighbour-joining tree of bacteria isolates in stockpile and unmined soils. Bootstrap support for branches less than 50% were excluded. Phylogenetic tree was constructed using MEGA7 software.**

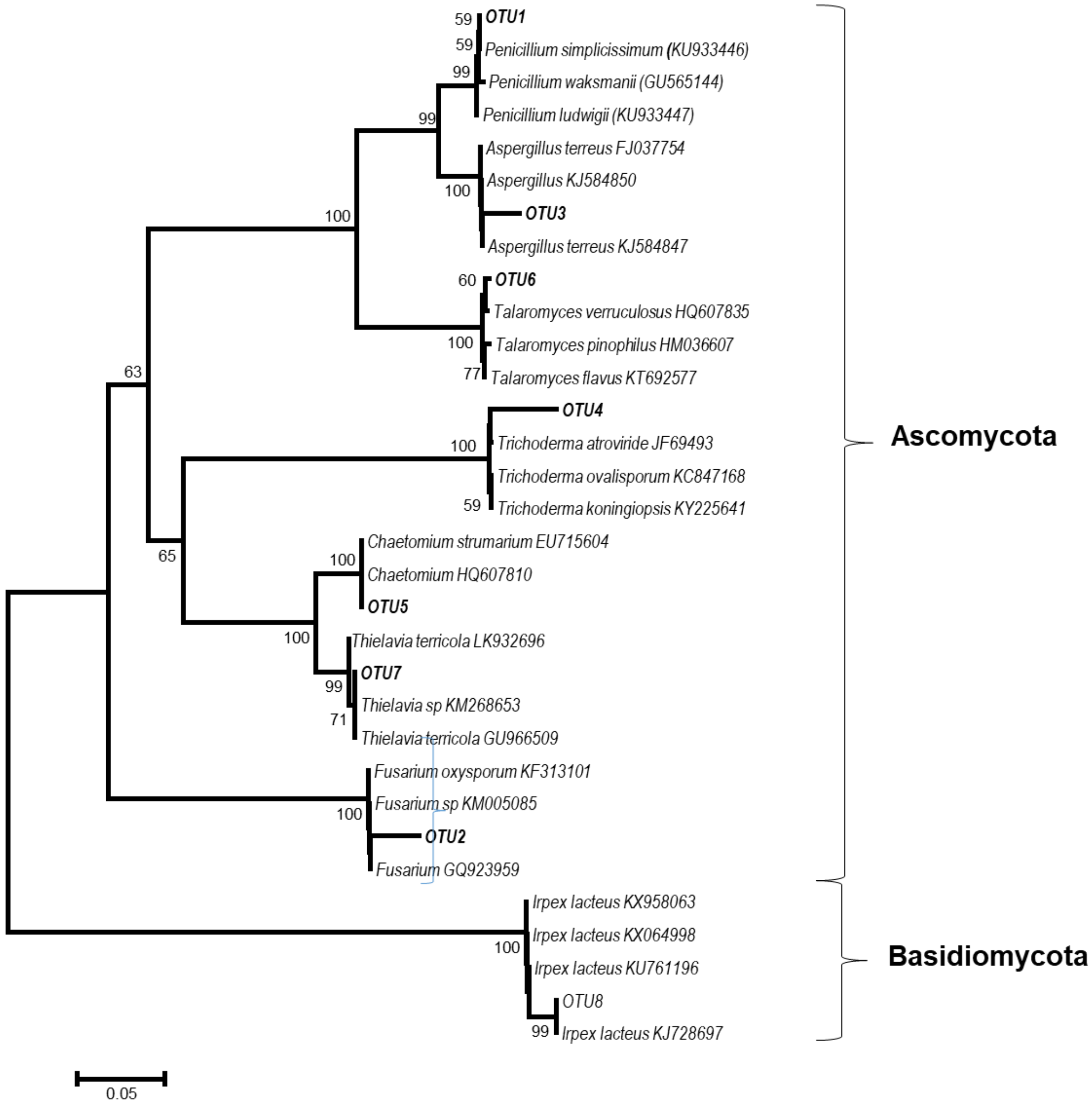


Figure A2: **Unrooted neighbour-joining phylogenetic tree of fungal isolates in stockpile and unmined soils. Bootstrap support for branches less than 50% were excluded. Phylogenetic tree was constructed using MEGA7 software.**

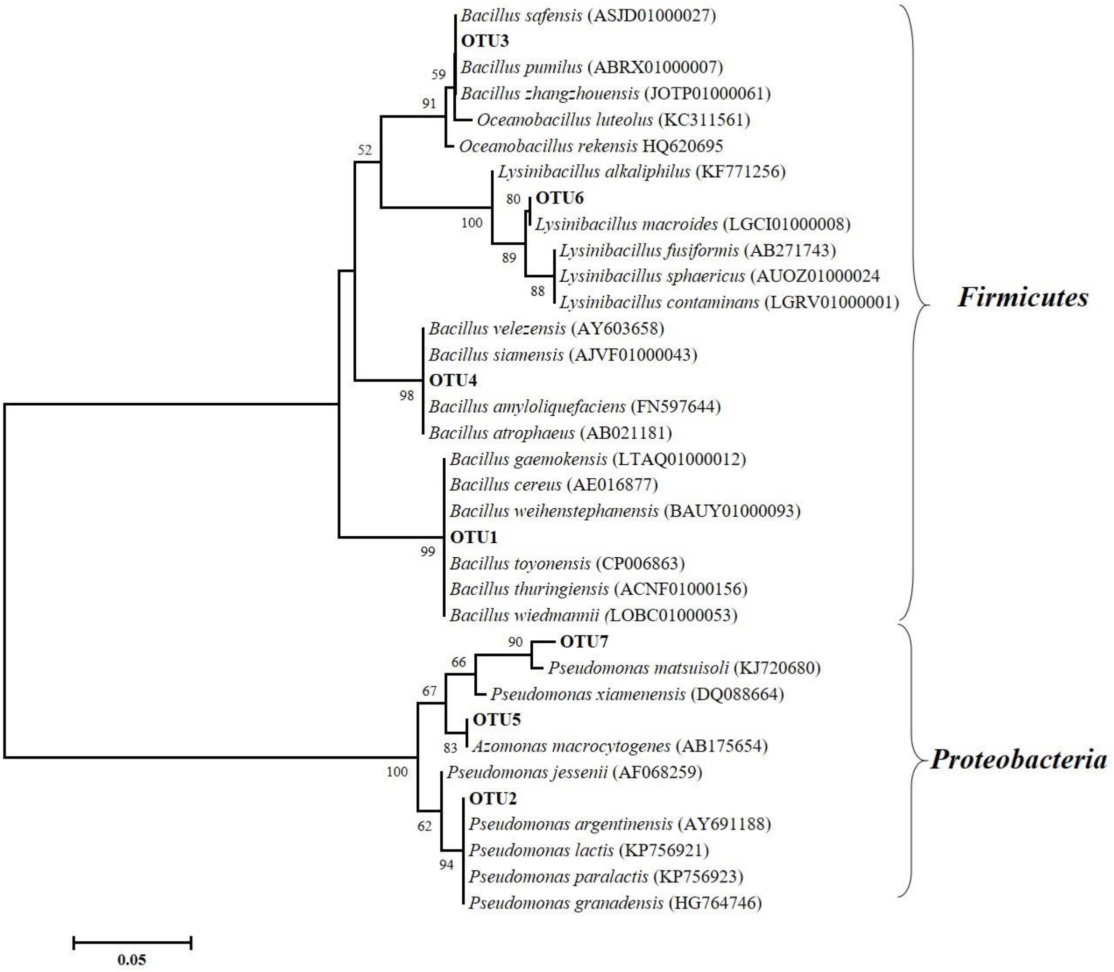


Figure A3: Unrooted Neighbour-joining tree of PCR-DGGE bacteria in stockpile and unmined soils. Bootstrap support for branches less than 50% were excluded. Phylogenetic tree was constructed using MEGA7 software.

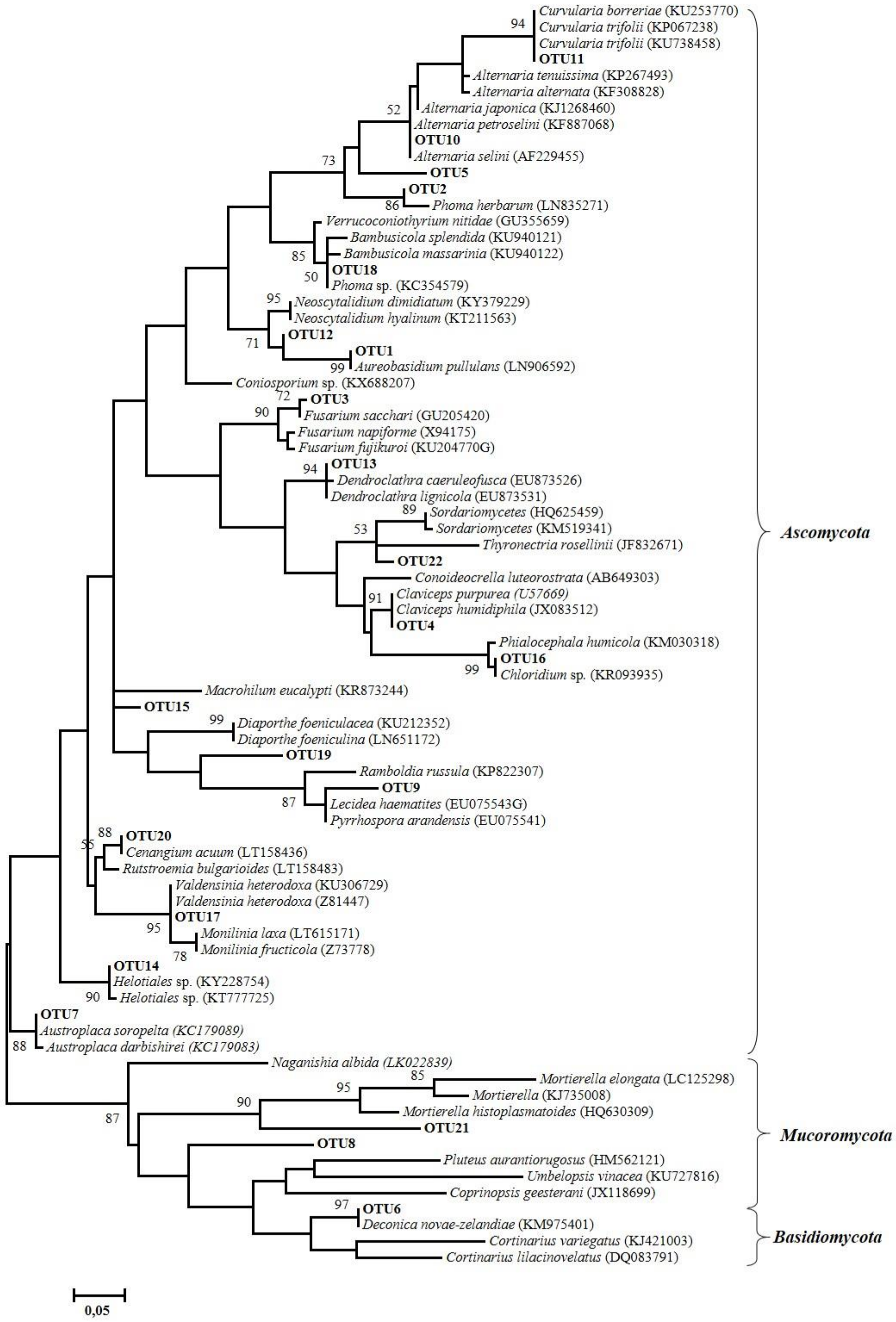
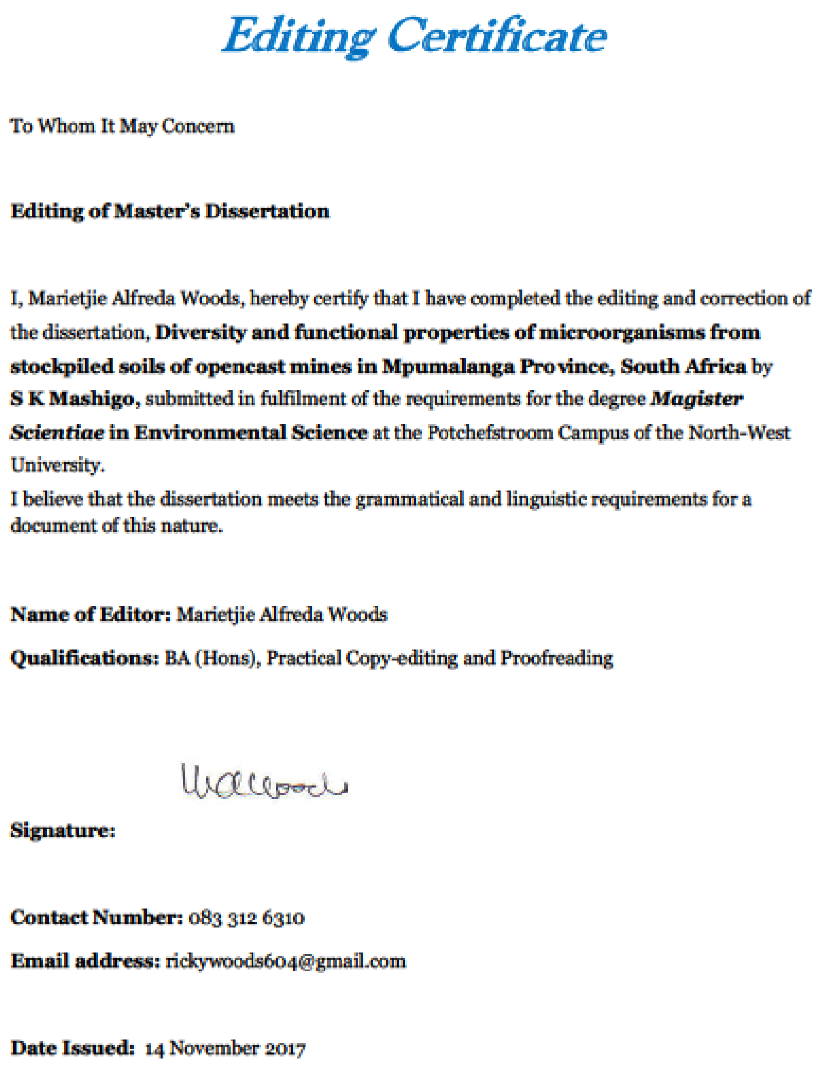


Figure A4: **Unrooted neighbour-joining phylogenetic tree of PCR-DGGE fungi in stockpile and unmined soils. Bootstrap support for branches less than 50% were excluded. Phylogenetic tree was constructed using MEGA7 software.**

ANNEXURE C: SUPORTING DOCUMENTS

ANNEXURE A1: CONFIRMATION OF EDITING OF THE DISSERTATION



Editing Certificate

To Whom It May Concern

Editing of Master's Dissertation

I, Marietjie Alfreda Woods, hereby certify that I have completed the editing and correction of the dissertation, **Diversity and functional properties of microorganisms from stockpiled soils of opencast mines in Mpumalanga Province, South Africa** by **S K Mashigo,** submitted in fulfilment of the requirements for the degree ***Magister Scientiae* in Environmental Science** at the Potchefstroom Campus of the North-West University.

I believe that the dissertation meets the grammatical and linguistic requirements for a document of this nature.

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Mashigo S.K., Ezeokoli, T.O., Adeleke, R.A., Bezuidenhout, C.C & Paterson, G. 2017. Enzyme activities and microbial community dynamics in soil stockpiles of South African open cast coal mines. *Water, Air and Soil Pollution*. (Submitted).

Conference outputs:

Adeleke, R.A., Mashigo, S.K., Ezeokoli, O.T & Bezuidenhout C.C. 2017. Enzyme activities and microbial communities of coal mine topsoil stockpiles: A soil quality assessment. International Phytotechnologies Conference (IPC) 2017; 25-29 September, Montreal, Canada. Oral Presentation Code: S7C-1-28-AM.

Mashigo, S.K., Adeleke, R.A., Ezeokoli O.T & Bezuidenhout C.C. 2017. Enzyme activities and microbial communities of coal mine soil stockpiles. Federation of European Microbiologists Societies (FEMS) 2017 Congress. 9-13 July, Valencia, Spain. Poster No. 657.

Mashigo, S.K., Adeleke, R.A & Bezuidenhout, C.C. 2016. Diversity and functional properties of bacteria from opencast coal mines stockpile soils. (PDP) Conference, Pretoria, South Africa. Oral presentation.

Mashigo, S.K., Adeleke, R.A & Bezuidenhout, C.C. 2016. Stockpiled soils from opencast coalmines: Impacts on microbiological properties. South African Society for Microbiology (SASM) Conference, Durban, South Africa. Poster presentation.